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A Structural Study of Ecto-mycorrhizas Formed in Seedlings of *Eucalyptus camaldulensis* Dehnh.

KHALED MISBAHUZZAMAN AND KEVIN INGLEBY†
Institute of Forestry and Environmental Sciences, Chittagong University,Chittagong 4331, Bangladesh
†*Centre for Ecology and Hydrology, Bush Estate, Penicuik, Midlothian EH26 0QB, Edinburgh, UK*
Corresponding author's e-mail: kmzaman_for@yahoo.com

ABSTRACT

Seedlings of *Eucalyptus camaldulensis* Dehnh. were inoculated with six isolates of *Pisolithus tinctorius* (Pers.) Coker and Couch (K55, PTE, PT3, PT7 & PT8 & Vietnam isolate) and grown under four nutrient regimes for 18 weeks in a glasshouse under controlled environment. Roots were assessed for colonisation by the fungi. Sections of colonised root samples were prepared for the study of structures produced by ectomycorrhiza (EM) and, where observed, photographs of EM structures were taken using a camera mounted on compound microscope. Colonisation by isolates PTE, PT3, PT7 and PT8 were <1% of the root samples studied and EM formation on roots was inconspicuous; therefore, structures produced by only K55 and Vietnam isolates where colonisation was >10% were studied in detail. Structures produced by the two isolates did not appear to be typical of a normal symbiosis when compared with mycorrhizal structures of *E. globulus* seedlings inoculated with *P. tinctorius* K55 isolate.

Key Words: *Eucalyptus camaldulensis; E. globulus; Pisolithus tinctorius;* ectomycorrhiza (EM); EM structure

INTRODUCTION

Morphological aspects of ectomycorrhizal (EM) roots have been studied in a number of plant-fungus combinations and recently reviewed (Smith & Read, 1997). Morphogenesis of EM follows a complex sequence. The precolonisation stage involves hyphal growth and branching close to the host followed by adhesion of hyphal branches to the root surface (Tagu & Martin, 1996). Major morphogenetic events for the fungal symbiont in a normal EM are: (1) the aggregation of hyphae to produce a tissue-like structure (the mantle) and (2) a labyrinthine growth of hyphae between the epidermal or cortical cells to produce the Hartig net (Bonfante *et al*., 1998). In a normal EM development, the initial changes in the host plant include: (1) stimulation of lateral root formation by fungal auxins, (2) radial elongation of epidermal cells and (3) arrest of cell divisions once the mantle has enclosed the root (Peterson & Bonfante, 1994). Fully developed EM formation in a normal symbiosis between *Pisolithus tinctorius* and *Eucalyptus* spp. (*E. globulus* & *E. grandis*) has been described by Horan *et al*. (1988) as one having a dense mantle, a blunt apex, a reduced root cap and meristem, vascular differentiation close to the apex and radially elongated epidermal cells with a Hartig net of intercellular hyphae. In a few studies, the extent of mantle and Hartig net development in EM has been found to be dependent on environmental conditions, for example, temperature (Marx *et al*., 1970) and pH (Metzler & Oberwinkler, 1987), plant genotype and maturity (Tonkin *et al*., 1989), the ecotypes of a given fungal species (Malajczuk *et al*., 1990) and fungal variants (Wong *et al*., 1989). High N concentration is reported to affect Hartig net development in EM (Brunner & Scheidegger, 1994).

In this study, root samples of EM inoculated *E. camaldulensis* seedlings were assessed in order to determine whether mycorrhiza formation and structures present were typical of a normal symbiosis. The objective of this investigation, therefore, was to describe formation and development of the EM structures found in *E. camaldulensis* roots using light microscopy. EM structures of the roots of *Eucalyptus globulus* Labill. seedlings inoculated with *P. tinctorius* K55 were also studied so that EM structures in *E. camaldulensis* may be compared with those of *E. globulus*.

MATERIALS AND METHODS

Seedlings of *E. camaldulensis* at the age of 4 weeks were inoculated with various isolates of *P. tinctorius* (K55, PTE, PT3, PT7, PT8 & Vietnam isolate) and were grown in 5 replicate blocks under four nutrient treatments (Ingestad's 2.5 mg L^{-1} phosphorus, P, 5.0 mg L^{-1} P, 10 mg L^{-1} P & 20 mg L^{-1} P) (Ingestad, 1971) under glasshouse conditions at the Centre for Ecology and Hydrology, Edinburgh, UK (Misbahuzzaman, 1999). Root samples were removed in October 1997 from seedlings harvested at the end of 18 weeks. However, root samples from only one inoculation treatment, that is, *Pisolithus tinctorius* isolate K55 which resulted in >25% colonisation (other isolates of *P. tinctorius* did not result in significant colonisation; <1% of the root tips being colonised) were considered for study of EM

structures. Therefore, four treatment combinations out of one inoculation treatment and four nutrient treatments were obtained thereby giving 20 samples from 5 blocks. *E. globulus* seedlings inoculated with one of the *P. tinctorius* isolates used to inoculate *E. camaldulensis*, that is, isolate K55 resulted in >70% colonisation in 1993 in the same glasshouse under similar conditions but at lower nutrient regimes (Mason *et al*., 1999). Therefore, seedlings of *E. globulus* were inoculated with *P tinctorius* K55 isolate only and grown at two nutrient regimes (Ingestad's 2.5 mg L^{-1} P, 5.0 mg L^{-1} P) (Ingestad, 1971), thereby giving two nutrient combinations. Roots were sampled at random from pots in five replicate blocks, that is, a total of 10 samples were studied. *E. globulus* seedlings inoculated with *P. tinctorius* K55 isolate resulted in 35-40% colonisation of the root tips (Misbahuzzaman, 1999). As another isolate of *P. tinctorius* (isolated from under a *E. camaldulensis* plantation in Vietnam) was obtained later in 1997, two replicate blocks of *E. camaldulensis* seedlings inoculated with this isolate and grown in the four nutrient regimes (Ingestad's 2.5 mg L^{-1} P, 5.0 mg L^{-1} P, 10 mg L^{-1} P, & 20 mg L^{-1} P) were also set up one week after the beginning of the main experiment. The seedlings were harvested one week later than the main experiment so that they were of the same age as those in the main experiment (that is, 18 weeks) at the time of harvest. Roots were sampled at random from pots in two replicate blocks (that is, a total of 8 pots). It is to be noted here that, *E. camaldulensis* seedlings inoculated with this isolate resulted in only 5-10% colonisation of the root tips (Misbahuzzaman, 1999). Root samples from *E. globulus* seedlings inoculated with *P. tinctorius* K55 and from *E. camaldulensis* seedlings inoculated with *P. tinctorius* Vietnam isolate were incorporated in this study for comparison with those from *E. camaldulensis* seedlings inoculated with *P. tinctorius* K55. All samples were preserved in 2% glutaraldehyde and stored at 4°C.

Processing for microscopy. Longitudinal and cross sections of root tips were removed using a base sledge microtome with a Peltier cooled freezing stage (Mectron Instruments Ltd.). This technique was useful because it is a rapid method of preparing sections for diagnostic purposes and it enables the observation of fats, lipids, and other tissue components (Culling, 1974). The sections were mounted on slides in PVLG under coverslips (without squashing them) for observation under a compound microscope.

Microscopy and imaging. All root samples were first observed using a Wild M5 stereo dissecting microscope (x5 to x50 magnifications). Root squashes and sections were observed using an Olympus BH2 compound microscope (x125 to x500 magnifications). Materials were observed under normal brightfield illumination or Differential Interference Contrast (DIC). Images for EM were recorded with a 35 mm automatic photo system camera mounted on the compound microscope.

RESULTS

Although the nutrient treatments had significant effects on the extent of colonisation on seedlings of *E. camaldulensis* (Misbahuzzaman & Wilson, 2002), they did not appear to have any effect on the kind of EM structures produced in the study.

EM colonisation of *E. camaldulensis* only occurred with isolate *P. tinctorius* K55. Initial observation of this mycorrhiza under the stereo microscope indicated that many of the mycorrhizal root tips were not covered by a fungal mantle (Plate 1c). In contrast, mycorrhiza formed by the isolate *P. tinctorius* K55 with *E. globulus* seedlings (set up at the same time) were found to be fully enveloped by a fungal mantle (Plate 1a). This absence of a mantle on some *E. camaldulensis* mycorrhiza was confirmed by examining longitudinal sections of such roots under a compound microscope (Plate 1d) and comparing them with longitudinal sections of *E. globulus* mycorrhiza (Plate 1b).

Further examination of longitudinal and cross sections of mycorrhizal root tips showed that EM colonisation in *E. globulus* seedlings led to an occurrence of a typical ectomycorrhiza with the presence of a mantle, a fully developed paraepidermal Hartig net and elongated epidermal cells (Plate 1b & Plate 2e,f). It should also be noted that the labyrinthine branching of the Hartig net could be observed where a plan view of the Hartig net was seen (Plate 2e, f). EM colonisation in *E. camaldulensis* seedlings was characterised by a mantle, but with a poorly developed Hartig net, little elongation of the epidermal cells and the presence of many globules or vacuoles in the epidermal cells (Plate 1d & Plate 2a,b).

Longitudinal and cross sections were also examined of *E. camaldulensis* seedlings inoculated with *P. tinctorius* Vietnam isolate. Unlike *E. camaldulensis-P. tinctorius* K55 mycorrhizas, fewer root tips were found to have grown out of the fungal mantle. However, like *E. camaldulensis-P. tinctorius* K55 mycorrhizas, these mycorrhizas also showed a poorly developed Hartig net, little elongation of epidermal cells and the presence of many globules in the epidermal cells (Plate 2c,d).

DISCUSSION

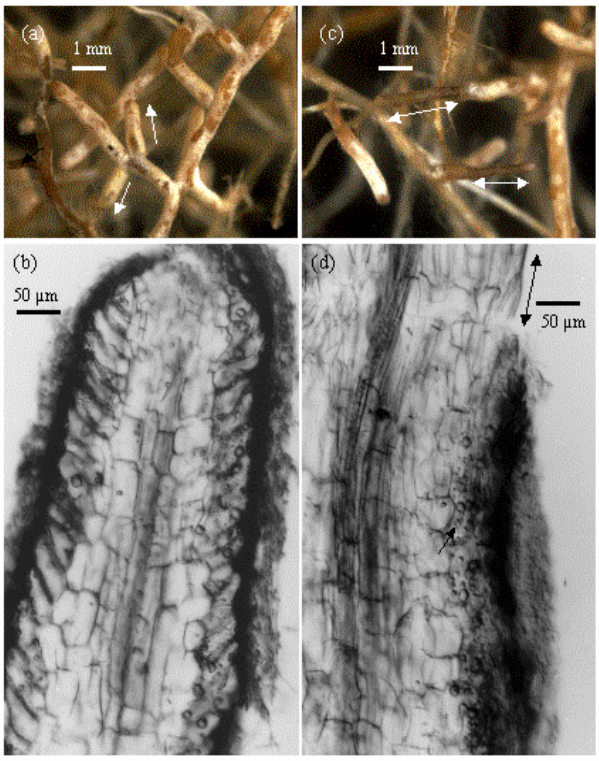
When an EM fungus develops a mantle, the hyphae stop growing in bundles or as isolated hyphae, and are organised into a more complicated structure (Bonfante-Fasolo & Scannerini, 1992). However, the structure and morphology of the mantle is largely determined by the fungal species independent of host species (Godbout & Fortin, 1985; Ingleby *et al*., 1990). Generally, mantles formed by *P. tinctorius* are poorly differentiated with loose hyphae forming the outer mantle which gradually become compacted in the inner mantle (Rose *et al*., 1981; Massicotte *et al*., 1988; Weiss, 1992). Burgess *et al*. (1996) observed a thickened mantle divided into two layers in their study of

compatible *E. grandis-P. tinctorius* mycorrhizas. In an earlier study, Burgess *et al*. (1994) found that less aggressive *P. tinctorius* colonisers of *Eucalyptus* tended to form a superficial association with a loose mantle. Loose mantles were also observed by Jones *et al*. (1998) in their study on *Laccaria bicolor-E. coccifera* mycorrhiza. In this study, differences in mantle thickness or the presence of layering were not observed on mycorrhiza of *E. camaldulensis* or *E. globulus*, due mainly to the difficulty of determining thickness or layering in the loosely structured mantles formed by *P. tinctorius*. Wong and Fortin (1990) observed hyphal colonisation of root surfaces with incompatible partners and suggested that hyphal envelopes may simply be the result of growth on root exudates without requiring fungus-root attachment, but they still considered hyphal envelopes as specific to EM development. In most samples of this study, *E. camaldulensis-P. tinctorius* K55 association was found to have produced hyphal envelopes but with very poor internal structures such as Hartig net. Structurally, therefore, these hyphal envelopes were specific to EM formation but lacked full Hartig net development. This phenomenon has been explained by Martin and Hilbert (1991) who suggested that the array of signals (morphological, biochemical & molecular) between host root and fungal isolate may be inadequate for sustained EM development. Dell *et al*. (1994) suggested that mantle formation may proceed in the absence of compatible recognition signals. Therefore, mantle formation in the associations between *E. camaldulensis* and *P. tinctorius* isolate K55 and between *E. camaldulensis* and *P tinctorius* Vietnam isolate in this study was far from that required for effective EM development.

In the study of *E. camaldulensis-P. tinctorius* K55 mycorrhiza, root apices of some mycorrhizas were found to be growing out from the mantle. A similar phenomenon was observed by Massicotte *et al*. (1999) and Martins *et al*. (1996) in their studies on *Paxillus involutus-Alnus glutinosa* and *Laccaria laccata-Castanea sativa* mycorrhizas respectively. Massicotte *et al*. (1999) called these roots 'transient ectomycorrhiza' and suggested that their presence indicated a certain degree of incompatibility.

In the process of a typical Hartig net formation, hyphae are oriented transversely to the root axis and branch irregularly forming a labyrinthine pattern (Kottke & Oberwinkler, 1986). The epidermal cells of *Eucalyptus* show a rapid response to the presence of EM fungus in the form of a considerable radial elongation (Smith & Read, 1997). Horan *et al*. (1988) proposed that the Hartig net only develops after the fungus has altered the development of epidermal cell walls following contact near the root tip. Hartig net formation in *Eucalyptus-P. tinctorius* mycorrhiza is described as paraepidermal, with hyphae partially enclosing the epidermal cells (Massicotte *et al*., 1987). In this study, *E. globulus* mycorrhiza was found to have possessed a typical paraepidermal labyrinthine Hartig net formation with a radial elongation of epidermal cells,

Plate 1. Comparison of EM of *E. camaldulensis* and *E. globulus* formed with *P. tinctorius* isolate K55: (a) low power view of *E. globulus* mycorrhiza with mantle enveloping root tips, and (b) a longitudinal section of one *E. globulus* root tip showing mantle and Hartig net development and some globules in epidermal cells; and (c) low power view of *E. camaldulensis* mycorrhizas showing root tips which have grown out from the mantle (↔), and (d) a longitudinal section of one *E. camaldulensis* root tip showing absence of mantle towards root tip (↔), poor development of Hartig net and profusion of globules in epidermal cells (→)



whereas *E. camaldulensis* mycorrhiza showed little Hartig net development with very poor radial elongation in epidermal cells. The process of Hartig net development requires the modification of cell walls leading to wall loosening thus enabling its mechanical penetration which is facilitated by fungal IAA signals (Gea *et al.*, 1994). It seems therefore that the Hartig net development is tightly controlled in the symbiosis whereas mantle formation, as observed in the *E. camaldulensis-P. tinctorius* mycorrhiza in this study, may have proceeded in the absence of compatible recognition signals (Dell *et al*., 1994), which resulted in little Hartig net formation and poor elongation of epidermal cells.

Even with structurally compatible associations between *Eucalyptus* and different isolates of *P. tinctorius*, the speed of colonisation initiation may reflect differences in the host-fungus recognition process (Tonkin *et al.*, 1989). Dell *et al*. (1994) suggested that sometimes the specificity in

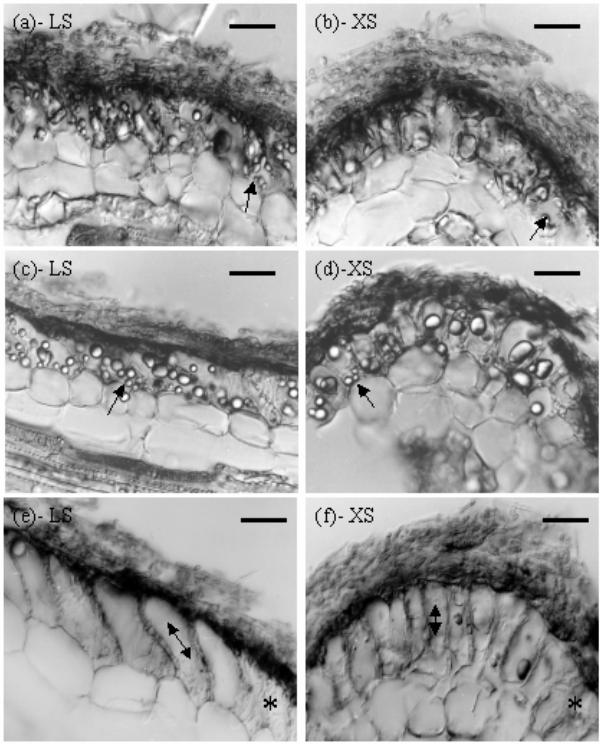
the *Pisolithus-Eucalyptus* system may appear to be related to the rate of development rather than the extent of differentiation of EM structures. In some samples of *Eucalyptus* mycorrhiza observed in these experiments, Hartig net development appeared to be more pronounced than others. Therefore, the rate of development of EM might explain why development of Hartig net in those samples was more pronounced than others.

In a study of EM formation on micropropagated *Eucalyptus* plantlets and seedlings, Tonkin *et al*. (1989) found that one of the two *P. tinctorius* isolates formed EM only on clonal lines from mature trees while the other isolate formed EM on seedlings as well as clonal lines of juvenile and mature trees. This observation suggests that the developmental maturity of host material can also influence compatibility. Therefore, it could be possible that the isolates used here might have had higher affinity for older *E. camaldulensis* seedlings. However, even after 24 weeks of growth, *E. camaldulensis* seedlings inoculated with *P. tinctorius* isolate K55 formed incomplete mycorrhiza with loose mantles, little Hartig net formation and poor elongation of epidermal cells (Misbahuzzaman, 1999). *E. camaldulensis* seedlings also responded similarly to the *P. tinctorius* Vietnam isolate after 18 weeks of growth. Whereas, *E. globulus* seedlings were found to be colonised as early as 12 weeks by that isolate that resulted in a complete EM development (Misbahuzzaman, 1999).

Tonkin *et al.* (1989) also found that the *P. tinctorius* isolate which only formed mycorrhiza on clonal lines of mature trees showed a build-up of phenolics in the root epidermal cells of juvenile clones, which is thought to be a sign of incompatibility (Molina *et al*., 1992). Deposition of phenolic compounds in plant cell walls and vacuoles frequently indicates an incompatible interaction between EM fungi and host roots (Ling-Lee *et al*., 1975; Nylund & Unestam, 1982; Malajczuk *et al*., 1984; Duddridge, 1986; Horan *et al*., 1988; Massicotte *et al*., 1999). Thus a higher accumulation of phenolics occurring immediately below the hyphal mantle and in vacuoles of the epidermal cells in mycorrhizal *E. camaldulensis* roots in the experiments of this study might also indicate an incompatible association. In spite of the deposition of phenolics in epidermal cell walls and vacuoles, some hyphae were able to penetrate between epidermal cells to form a limited Hartig net which is in agreement with the observations of Massicotte *et al*. (1999) in the *Paxillus involutus-Alnus glutinosa* mycorrhiza. However, the Hartig net found in *E. camaldulensis* mycorrhiza was never very extensive and rarely showed the labyrinthine branching observed in *E. globulus* mycorrhizas. These observations are in agreement with those of Molina (1981) who noted similar symptoms in mycorrhizas of *Paxillus involutus* and a number of *Alnus* species.

The environmental conditions such as temperature or pH did not seem to have been responsible for incomplete EM development. Temperature was well above 20°C. Similar temperature has been found to be suitable for EM

Plate 2. (a) Longitudinal section and (b) cross section of *E. camaldulensis* roots inoculated with *P. tinctorius* strain K55-both sections showing profusion of globules in epidermal cells and poor elongation of epidermal cells (→) (c) longitudinal section and (d) cross section of *E. camaldulensis* roots inoculated with *P. tinctorius* Vietnam isolate- both sections showing profusion of globules in epidermal cells and poor elongation of epidermal cells (→) (e) longitudinal section and (f) cross section of *E. globulus* seedlings inoculated with *P. tinctorius* isolate K55-both sections showing few globules in epidermal cells and well-developed Hartig net with elongation of epidermal cells (↔) and plan view of labyrinthine branching (*). Scale bars=25µm.



formation in *Eucalyptus* by *P. tinctorius* in several studies (for example, in the studies of Bougher & Malajczuk, 1990; Burgess *et al*., 1994; Mason *et al*., 1999). The pH in growth medium in these experiments ranged between 5.0-5.5. Similar pH has been reported to be suitable for growth of most EM fungi (Smith & Read, 1997). Although availability of light controls EM formation, it is unclear whether the light intensity used in the experiments (400-800 µmol photons m^{-2} s^{-1}) was responsible for the formation of incomplete EM structures. Choice of fungal species used in these experiments being inappropriate also can be discounted as *P. tinctorius* has been widely used as an inoculant for nursery seedlings of *Eucalyptus* (see Brundrett *et al*., 1996 & references therein). Moreover, both the

isolates used here were of *Eucalyptus* origin (*E. globulus* & *E. camaldulensis*). *P. tinctorius* isolates of pine origin have been reported to have formed incompatible EM in *E. grandis* in the study of Burgess *et al.* (1994). Therefore, host specificity of the isolates might not have been the reason for an incompatible EM formation in *E. camaldulensis* in this study.

In summary, the occurrence of very limited epidermal cell elongation or an absence of it in most samples, a poor Hartig net development, and presence of phenolics-filled vacuoles or globules in epidermal cells tend to confirm the proposition that the EM associations formed by *E. camaldulensis* and *P. tinctorius* isolates in these studies were incompatible. The possible reasons for the incompatibilty could have been the developmental maturity of the host or a lack of aggressiveness on the part of the fungal isolates used to form EM in *E. camaldulensis*. Alternatively, the growth conditions under which the experiments were undertaken might have been influential.

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