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In vitro ectomycorrhiza formation between *Abies firma* and *Pisolithus tinctorius*

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Abstract The first in vitro aseptic synthesis of *Abies firma* Sieb. et Zucc. with *Pisolithus tinctorius* (Pers.) Coker & Couch is reported. Techniques were improved for the aseptic synthesis of ectomycorrhizas of *A. firma*, a slow-growing species in vitro, and *Pisolithus tinctorius* using a novel culture medium and both sterilized and re-rooted seedlings. After 2–3 months incubation, ectomycorrhizas were formed by both methods. The mycorrhizas possessed a mantle and a highly branched nonseptate Hartig net mycelium colonizing the intercellular spaces within the host cortex, features characteristic of ectomycorrhizas. These techniques will prove useful for addressing physiological and biochemical questions on the interactions of microbes with roots of whole plants.

Key words Japanese fir · Momi fir · Ectomycorrhizas · In vitro culture · Kotsubutake

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Introduction

The genus *Abies* (Pinaceae) is widely distributed throughout Europe, northern Africa, northern and central Asia, and North America and includes over 40 species (Record and Hess 1943), all of which form obligate mycorrhizal associations (Meyer 1973). Consequently, each *Abies* species has great ecological importance within natural forests. *Abies firma* Sieb. et Zucc. (Japanese fir, momi fir) is a species endemic to the warmer parts of Japan, specifically Kyushu, Shikoku and Honshu south of Iwate prefecture (Iwatsuki et al. 1995). It forms natural coniferous forests with *Tsuga sieboldii* Carr. on rich and mesic soils and is a species of commercial importance in Japan. Its timber is used for construction, packing crates, paper pulp and particularly coffin construction because of its pure white color (Japan Forest Technical Association 1964). In the few studies of naturally occurring ectomycorrhizas of *Abies*, species other than *A. firma* have been shown to host a number of naturally occurring ectomycorrhizas (Trappe 1962, 1977; Alvarez and Cobb 1977; Acsai and Largent 1983; Harley and Harley 1987; Pillukat and Agerer 1992; Kernaghan et al. 1997). In Japan, more than 40 species of fungi representing 14 genera have been reported to form ectomycorrhizal associations with *A. firma* (Masui 1926; Nara et al. 1992; Matsuda and Hijii 1998); however, *P. tinctorius* is not among them. Although *Abies* are obligately mycorrhizal (Meyer 1973), studies of their mycorrhizal symbionts are less abundant than for other coniferous genera (Trappe 1962) and recent focus on mycorrhizas as tools for agricultural and forestry practice has not rectified this situation.

Pisolithus tinctorius, a broad host-range fungus (Malajczuk et al. 1982, 1990; Carroll 1992), has previously been recorded in mycorrhizal association with many host tree species (Trappe 1962; Harley and Harley 1987), and has been inoculated in nursery plots onto some *Abies* species (Castellano and Trappe 1991). *Pi-*

solithus tinctorius is easy to culture in the laboratory in a variety of solid and liquid media, where it has easily detectable yellow-gold mycelium (Marx 1980), and is ecologically adaptive to adverse soil conditions and biologically hostile environments (Metzler and Metzler 1992). Trees inoculated with *P. tinctorius* have been shown to survive drought, toxic mine spoils and high temperatures (Trappe 1977). Consequently, this species is considered highly relevant for reforestation and many American lumber companies regularly inoculate tree seedlings with this fungus to promote reforestation (Metzler and Metzler 1992).

Fir forest decline occurs in both Asia (Donaubauer 1993) and central Europe (Freer-Smith 1996) and is intensifying (Kandler 1993), rendering *Abies* more susceptible to attack from biotic agents (Donaubauer 1993). Firs are one of the most sensitive trees reacting to environmental change (Požgaj et al. 1996) and decline may be the result of a number of diverse factors such as drought and other climatic variations, ill-applied forestry management practices, low potassium levels and air pollution (Freer-Smith 1996). With the current remarkable decline of *Pinus densiflora* Sieb. et Zucc. forests in Japan due to the pinewood nematode *Bursaphelenchus xylophilus* (Steiner et Buhrer) Nickle, the Japanese forest industry can ill-afford such a decline in a further commercially important timber species.

Accordingly, to better understand the benefits and stress management capabilities of the ectomycorrhizal association between *P. tinctorius* and *A. firma* and to examine the physiological and biochemical changes associated with or controlling ectomycorrhizal development, simple, reproducible and easily manipulated model systems were established. In this report, we describe two such in vitro systems for ectomycorrhiza synthesis between *A. firma* and *P. tinctorius*, which may be applied to any number of mycorrhizal fungus-host associations.

Materials and methods

Fungal culture

Pisolithus tinctorius (deposited as strain Pt2 in the culture collection of the Laboratory of Forest Botany, The University of Tokyo) was isolated from mycorrhizal fruitbodies growing on *Pinus luchuensis* Mayr roots on Hahajima Island, one of the Ogasawara Islands, southwest of Tokyo, in November 1996. Tissue blocks were aseptically excised from the fruit body and cultured on modified Melin-Norkrans (MMN) agar medium (Marx 1969). The resultant pure mycelial isolate was maintained on MMN in darkness at 25 ± 2 °C.

Preparation of plant material

Seeds of *A. firma* were collected in a warm-temperate natural forest (University Forest at Chiba, The University of Tokyo), air-dried and stored in a polyethylene bag in darkness at 4 °C until use. They were sown in vermiculite following immersion in 1/2000 Benlate (Dupont Co. Ltd., USA) for 1 day and germinated at room temperature under diffused fluorescent illumination. Once germinated, the seedlings were surface-sterilized in 70% ethanol for 1 min and then in sodium hypochlorite containing 1% (w/v) active chlorine for 10 min. Following three rinses in sterile deionized water, they were soaked in 0.05% (w/v) mercuric chloride for 6 min and finally rinsed four times in sterile deionized water.

Ten sterilized seedlings were selected for ectomycorrhiza synthesis by the culture plate method and a further 10 sterilized seedlings were selected for ectomycorrhiza synthesis by the Agripots culture pot method.

Because the effects of harsh sterilizing reagents on the delicate radicle were unknown, sterilized seedlings were selected for re-rooting treatment and ectomycorrhizal synthesis by the Agripots culture pot method. The radicles of 20 sterilized seedlings were aseptically removed and discarded and the cut ends of the seedlings were immersed in 0.1% aqueous naphthaleneacetic acid for 1 min to stimulate rooting. The seedlings were then transferred to 50% SH containing 0.32% Gelrite (Schenk and Hildebrandt 1972) supplemented with 0.3% activated charcoal (Duclos and Fortin 1983) in 120 × 24-mm glass test tubes and incubated. After 4 weeks, three or four lateral roots had developed on each seedling. Ten of the re-rooted seedlings were then selected for ectomycorrhizal synthesis by the Agripots culture pot method. Because of the three-dimensional distribution of the induced roots, re-rooted seedlings were not considered for ectomycorrhizal synthesis by the culture plate method. All incubations were carried out with 3000 lux diffuse fluorescent light at 25 ± 2 °C with a 16-h photoperiod.

Culture plate method for the aseptic synthesis of ectomycorrhizas using sterilized seedlings

A modified Chilvers' paper sandwich method (Chilvers et al. 1986) was used for ectomycorrhizal synthesis (Fig. 1). Rectangular clear plastic culture plates (200 × 90 × 10 mm) were filled with 80 ml Fungus-Host (FH) medium which was modified from SH medium and contained: KNO_3, 2500 mg; $NH_4H_2PO_4$, 300 mg; $MgSO_4 \cdot 7H_2O$, 400 mg; $CaCl_2 \cdot 2H_2O$, 200 mg; $FeSO_4 \cdot 7H_2O$, 15 mg; Na_2EDTA, 20 mg; H_3BO_3, 0.5 mg; $ZnSO_4 \cdot 7H_2O$, 0.1 mg; $MnSO_4 \cdot H_2O$, 1 mg; $Na_2MoO_4 \cdot 2H_2O$, 0.01 mg; KI, 0.1 mg; $CuSO_4 \cdot 5H_2O$, 0.02 mg; $CoCl_2 \cdot 6H_2O$, 0.01 mg; myo-inositol, 100 mg; thiamine HCl, 5 mg; nicotinic acid, 5 mg; pyridoxine HCl, 0.5 mg; glucose, 1 g; activated charcoal, 0.3 g; agar, 15 g; distilled

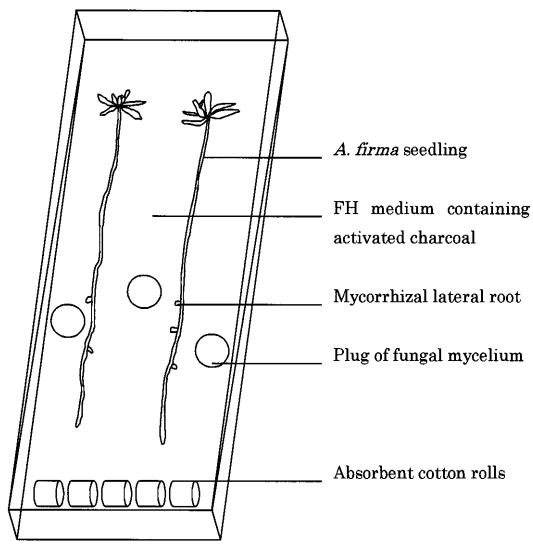

Fig. 1 Culture plate method for in vitro aseptic synthesis of *Abies firma*/*Pisolithus tinctorius* ectomycorrhizas

H_2O, 1000 ml. The pH was adjusted to 5.6 with 1 N NaOH prior to autoclaving (121 °C, 20 min). For each culture plate, two sterilized seedlings were laid directly on the agar surface and covered with a sheet of autoclaved Advantec No. 2 filter paper (Toyo Roshi Kaisha Ltd.) to maintain root surface moisture. The plates were then incubated for 8 weeks, after which time the main roots had grown to a length of 15 cm, but no lateral roots had formed. The cover paper was then aseptically removed and discarded and two 6-mm-diameter plugs of *P. tinctorius* mycelium were placed on the medium, adjacent to the root tip. Sterile cotton rolls (10 × 5 mm) were placed along the bottom edge of the plates to absorb water condensed during subsequent incubation. Prior to incubation, the plates were sealed with Parafilm (American Can Company, Detroit) and the lower portion of the plates, containing both the developing host root system and ectomycorrhizal fungus, was covered with aluminum foil.

Culture pot method for the aseptic synthesis of ectomycorrhizas using re-rooted seedlings

Bases of culture pots were filled with a growth substrate consisting of 100 ml vermiculite and 40 ml FH liquid medium (Fig. 2). The pots were re-assembled and autoclaved. Two 6-mm-diameter plugs of *P. tinctorius* mycelium were then placed on the substrate surface and the pots were incubated. After 3 weeks, when the fungal mycelium had colonized the substrate, the fungal mycelium and substrate were mixed, then sterilized seedlings and re-rooted seedlings were introduced aseptically into the substrate. A 12-mm-diameter hole was first made in the substrate with a sterile cork borer. The seedlings were then placed inside a 12-mm-diameter sterile cork borer which was inserted into the hole and then slowly withdrawn, leaving the seedling in the hole. The medium was gently replaced around the root system and lightly tamped down. The bases of the pots were wrapped in aluminum foil and the dual cultures were incubated.

Light microscopy

Both mycorrhizal and control roots were removed from culture plates and segments 1–2 mm in length were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) containing 1% acrolein for 2 h under vacuum at room temperature. The samples were washed twice in 0.1 M sodium cacodylate buffer and were then postfixed for 90 min in 2% OsO_4 in 0.1 M sodium cacodylate buffer (pH 7.2) at room temperature, washed in three changes of distilled water and dehydrated in an ascending acetone series in 20% increments followed by three changes of 100% propylene oxide. The root segments were subsequently infiltrated with Spurr's resin (Spurr 1969) prior to polymerization at 70 °C for 12 h. Sections of 4–6 μm thickness were cut with glass knives and gently heat fixed to glass microscope slides. Sections were bleached in 1% hydrogen peroxide for 45 min, washed in tap water and then stained with 0.05% toluidine blue O in 1% sodium tetraborate (Roland and Vian 1991) for 3 min. Following three tap water washes, the sections were destained in tap water for 20 min, air-dried, mounted in DPX (Fluka BioChemika) and examined with an Olympus BH2 microscope.

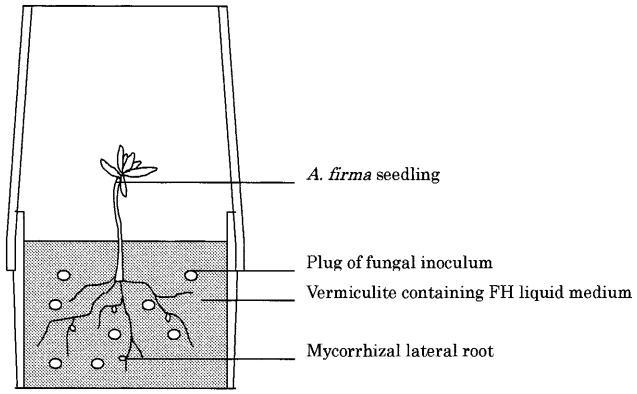

Fig. 2 Culture pot method for in vitro aseptic synthesis of *A. firma*/*P. tinctorius* ectomycorrhizas

Results

Mycorrhizal development in culture plates

After 8 weeks incubation in culture plates, *A. firma* seedlings grew a single main root approximately 15 cm in length. First-order lateral roots of one seedling were produced after 3 weeks incubation following inoculation with *P. tinctorius*, and they were confined to the medium surface. The remaining seedlings, four of which succumbed to contamination, produced neither first- nor second-order lateral roots (Table 1).

The mycorrhizas, which formed on the only seedling to produce lateral roots, were straight and unramified (Fig. 3a). The mantle did not ensheath single lateral roots entirely, the proximal ends of the lateral roots were devoid of ensheathing hyphae and the mother root remained unsheathed. The plectenchymatous mantle possessed a loosely woven outer surface and many yellow emanating hyphae. The mycorrhizal roots, approximately 2 mm in length, were golden-yellow in color and concolorous from the distal to proximal end. Rhizomorphs were not observed, but hyphae emanating from the mantle surface formed loose hyphal fans at a distance from the mycorrhizal root.

Mycorrhizal development in culture pots

After 12 weeks incubation, both sterilized seedlings and re-rooted seedlings were gently removed from the

Table 1 Comparison of two in vitro synthesis methods (10 seedlings per treatment) for *Abies firma/Pisolithus tinctorius* mycorrhizas (*FLR* first-order lateral root, *N* not applied, *RRS* re-rooted seedling, *SLR* second-order lateral root, *SS* sterilized seedling)

	Contamination		Lateral root formation				Mycorrhizas	
			FLR		SLR			
	SS	RRS	SS	RRS	SS	RRS	SS	RRS
Culture plate	4/10	N	1/10	N	0/10	N	1/10	N
Culture pot	0/10	0/10	4/10	10/10	0/10	3/10	1/10	3/10

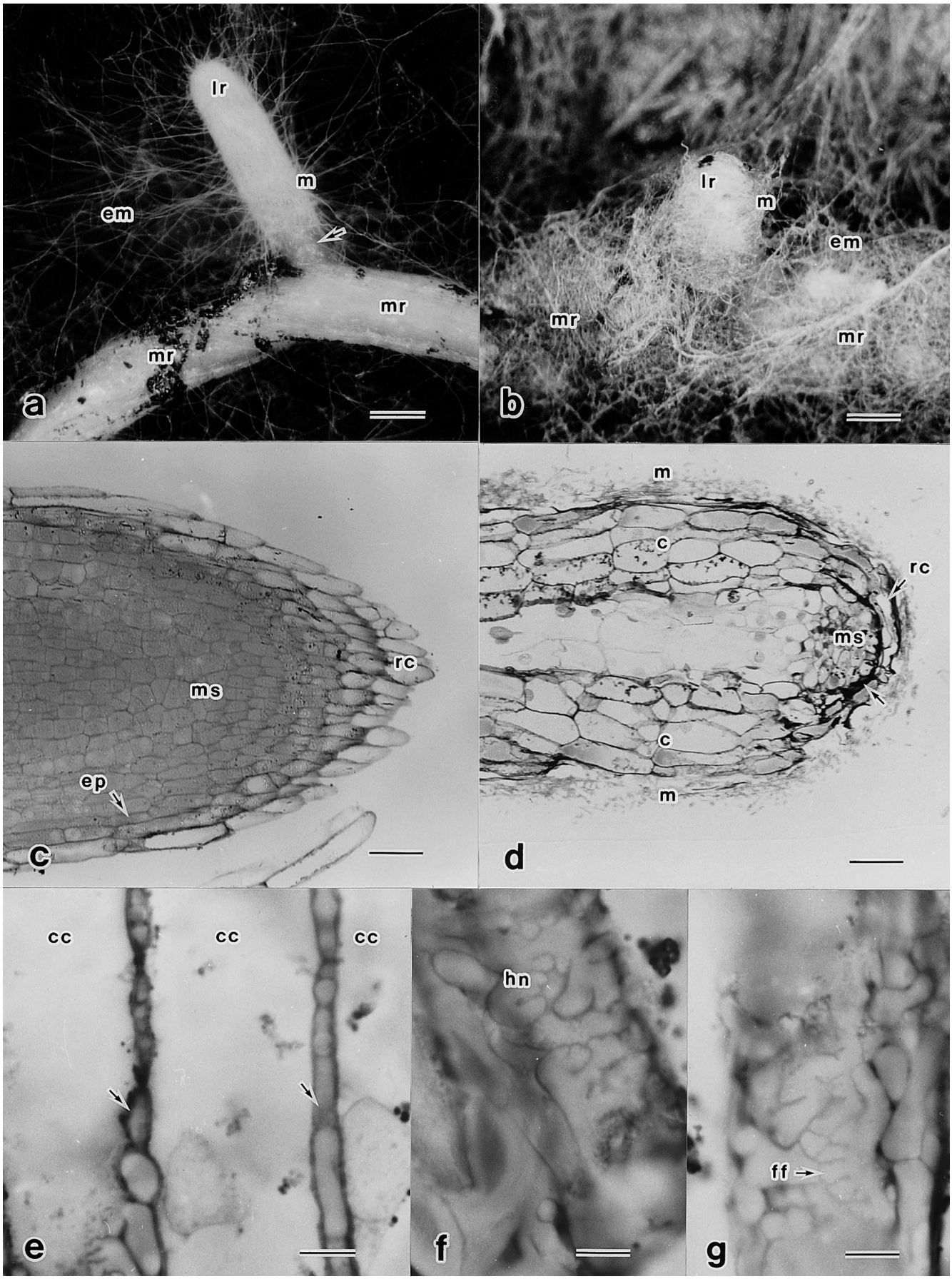
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Fig. 3a-g *Abies firma*/*P. tinctorius* ectomycorrhizas formed by in vitro aseptic synthesis. **a** *Abies firma*/*P. tinctorius* mycorrhizal first-order lateral root formed in a culture plate after 18 weeks incubation. The lateral root (*lr*) is colonized along its length by hyphae forming a conspicuous mantle (*m*) from which copious extraradicle hyphae emanate (*em*). The mantle is distinguishable from unsheathed lateral root (*arrow*) and is absent from the main root (*mr*); *bar* 0.5 mm. **b** *Abies firma*/*P. tinctorius* mycorrhizal first-order lateral root formed within a culture pot after 12 weeks incubation. The lateral root (*lr*) is ensheathed in a loose mantle, which also extends to the main root (*mr*); *bar* 0.35 mm. **c** Light micrograph of an uninoculated *A. firma* first-order lateral root in longitudinal section, grown by the culture plate method. The extensive root cap (*rc*) overlies the meristematic region (*ms*) and also extends to cover the juvenile epidermis (*ep*); *bar* 60 μm. **d-g** Light micrographs of longitudinal sections of *A. firma*/*P. tinctorius* mycorrhizas formed by the in vitro culture plate method. **d** The mycorrhizal lateral root tip possesses a loose mantle (*m*) and a reduced root cap (*rc*). A zone of collapsed cells and dark-staining areas between the mantle and cortex (*c*) characterizes the epidermal area. The upper limit of the meristem (*ms*) is defined by a zone of darkly staining material (*arrow*); *bar* 60 μm. **e** Intercellular spaces between cortical cells (*cc*) are colonized by fungal hyphae (*arrows*); *bar* 7 μm. **f** The multibranched, nonseptate Hartig net mycelium (*hn*); *bar* 7 μm. **g** The distinctive multilobed fan-shaped structure (*ff*) of the Hartig net; *bar* 7 μm

medium and their root systems were observed. All 10 re-rooted seedlings developed main roots bearing first-order lateral roots. Of these, three re-rooted seedlings formed multiple main roots bearing short second-order lateral roots, which often developed short, straight, unramified mycorrhizas (Fig. 3b, Table 1). The second-order mycorrhizal lateral roots were approximately 1 mm in length, straight and unramified. The mantle ensheathed both the second-order lateral root and the mother root. Emanating yellow hyphae, which formed distinct rhizomorphs and loose hyphal fans, obscured the plectenchymatous mantle surface. The mantle was golden-yellow in color and concolorous from the distal to the proximal end. No re-rooted seedling dual cultures were affected by contamination.

Of the 10 sterilized seedlings to develop long main roots, four seedlings developed first-order lateral roots, of which one developed mycorrhizas. The mycorrhizal morphology was identical to that of mycorrhizas formed on second-order laterals of re-rooted seedlings in culture pots. No sterilized seedling dual cultures were affected by contamination (Table 1).

Light microscopy

In longitudinal cross section of an uninoculated control *A. firma* root tip grown by the culture plate method (Fig. 3c), the meristematic area was overlaid with an extensive root cap, which extended to cover the juvenile epidermal layer. In contrast, mycorrhizal root tips (Fig. 3d) selected from each culture system, possessed a reduced root cap and the tip was ensheathed in a distinct, loose mantle. Individual epidermal cells were indistinguishable, but the epidermal area, an indistinct layer between the mantle and root cortex, was characterized by many darkly staining inclusions and collapsed root cap cells. The upper limit of the meristematic region was delineated by darkly staining inclusions. At higher magnification, intercellular spaces within the root cortex were seen to be colonized by fungal hyphae (Fig. 3e) which remained extracellular and did not invade the cortical cells. The highly branched, intracortical, intercellular hyphae were nonseptate (Fig. 3f) and formed fan-shaped lobed structures (Fig. 3g). No intracellular penetration of host tissue by the fungus was observed.

Discussion

Abies spp. are slow-growing in vitro, seedlings often fail to thrive and root development is frequently retarded (Saravitz and Blazich 1996). Both the artificial methods presented here promoted good seedling growth and mycorrhization of two symbionts, which has not been recorded to occur naturally. In the plate culture method, three modifications to the paper-sandwich technique of Chilvers et al. (1986) were made. Firstly, MMN medium was replaced with FH medium, itself derived from SH medium of Schenk and Hildebrandt (1972), a popular medium for tissue culture of conifers (Webb et al. 1988; Martinez-Pulido et al. 1994). Secondly, activated charcoal was added to the medium to improve root growth and to absorb toxic substances which may be excreted by the root (Fridborg et al. 1978; Duclos and Fortin 1983). Finally, the seedlings were applied directly to the medium surface to allow full contact between roots and the medium. Following establishment of healthy seedlings, fungal inocula were applied directly to the medium surface and the symbionts remained observable at all times throughout their interaction. While fewer lateral roots were formed by the culture plate method, all lateral roots supported a mycorrhizal association. However, root growth was restricted to the medium surface, limiting the potential number of lateral roots. Furthermore, this technique is susceptible to contamination, which affected a number of seedlings. The culture pot method used a vermiculite substrate which allowed more natural root development and the formation of more first-order laterals and some second-order laterals (data not shown). Re-rooted seedlings grown in culture pots responded better than sterilized seedlings grown in both plates and pots, and a higher rate of mycorrhization of seedlings in the culture pots was observed. The pot method was simpler overall, easier to manipulate and less time consuming than the plate method. However, the physical properties of the medium do not allow observation of mycorrhizal development.

The mycorrhizas produced by both culture systems exhibited structures typical of ectomycorrhizas. From the ensheathing mantle, hyphae penetrated and colo-

nized the host root cortical intercellular spaces. They underwent changes in their growth morphology to form a highly branched, rarely septate Hartig net, which increases the contact surface area between the two symbionts and facilitates nutrient transfer (Kottke and Oberwinkler 1986). The presence of these characteristic features has previously been used to define the establishment of ectomycorrhizal associations between *P. tinctorius* and other conifer species in vitro (Fortin et al. 1980; Piché et al. 1982, 1983; Tam 1994). Their presence in the mycorrhizal roots artificially synthesized by the methods described here indicates that *P. tinctorius* forms an ectomycorrhizal association with host *A. firma* roots under the culture conditions employed. Tam (1994) reported intracellular invasion of host cortical cells, which is considered an artifact of in vitro synthesis even between naturally occurring symbionts (Wang et al. 1997) attributable to host plant stress (Duddridge and Read 1984; Tam 1994), high exogenous glucose concentrations and/or physiological imbalances (Duddridge and Read 1984). Furthermore, *P. tinctorius* has been implicated in the stimulation of dichotomous root branching (Piché et al. 1982) and dichotomous *P. tinctorius* mycorrhizas form on host pines in vitro (Fortin et al. 1980; Piché and Fortin 1982; Piché et al. 1982; Tam 1994). The mycorrhizal roots formed in our in vitro systems retained the naturally unbranched or limited dichotomously branched state of *Abies* roots (Wilcox 1954) and dichotomous mycorrhizas did not develop. While the failure of *P. tinctorius* to stimulate dichotomy in *A. firma* roots in vitro requires further investigation, the absence of intracellular invasion indicates that our systems provide favorable conditions for compatible interactions.

Pisolithus tinctorius mycorrhizas have been synthesized on roots of both *Pinus* spp. (Fortin et al. 1980; Sohn 1981; Piché et al. 1982, 1983; Kasuya et al. 1992; Tam 1994) and *Eucalyptus* spp. (Mullette 1976; Malajczuk et al. 1982; Chilvers et al. 1986; Burgess et al. 1994) in vitro and in nursery plots on roots of some *Abies* spp. (Castellano and Trappe 1991). Artificially synthesized *P. tinctorius* mycorrhizas are generally considered to increase growth of eucalypts and pines in plantations (Burgess et al. 1994) although host responses vary (Trappe 1977). This may in part explain the low rate of mycorrhization by different conifers (Castellano and Trappe 1991) and poor performances observed by some ectomycorrhizal trees following outplanting (Castellano and Trappe 1991; Lee and Koo 1992), despite significant growth stimulation of the seedlings in sterile nursery soil (Marx 1980; Lee and Koo 1983). The failure of outplanted seedlings to thrive in the natural environment may be due to the poor competitive ability of *P. tinctorius* against other soil organisms (Marx et al. 1984; Lee and Koo 1992).

The artificial culture techniques described here are currently being applied to study the developmental physiology and biochemistry of in vitro *Abies firma*/*P. tinctorius* mycorrhization to select candidate fungal strains (Trappe 1977; Lee and Koo 1983) with the characteristics required for successful forestry application (Marx and Cordell 1989) and to monitor host responses for future outplanting trials. The ability of mycorrhizal seedlings to manage host tree stresses has been recognized (Marx and Cordell 1989). The successful mycorrhization of commercially important timber crops such as *Abies* spp, which are very sensitive to environmental fluctuations (Požgaj et al. 1996), with tolerance-bestowing fungi such as *P. tinctorius* will enhance their chances of withstanding increasing and seemingly irreversible environmental and climatic variations.

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References

Acsai J, Largent DL (1983) Ectomycorrhizae of selected conifers growing in sites which support dense growth of bracken fern. Mycotaxon 16:509–518

Alvarez IF, Cobb FW Jr (1977) Mycorrhizae of *Abies concolor* in California. Can J Bot 55:1345–1350

Burgess T, Dell B, Malajczuk N (1994) Variation in mycorrhizal development and growth stimulation by 20 *Pisolithus* isolates inoculated on to *Eucalyptus grandis* W. Hill ex Maiden. New Phytol 127:731–739

Carroll GC (1992) Fungal mutualism. In: Carroll GC, Wicklow DT (eds) The fungal community – its organization and role in the ecosystem, 2nd edn. Dekker, New York, pp 327–354

Castellano MA, Trappe JM (1991) *Pisolithus tinctorius* fails to improve plantation performance of inoculated conifers in southwestern Oregon. New For 5:349–358

Chilvers GA, Douglass PA, Lapeyrie FF (1986) A paper-sandwich technique for rapid synthesis of ectomycorrhizas. New Phytol 103:397–402

Donaubauer E (1993) On the decline of fir (*Abies densa* Griff.) in Bhutan. In: Huettl RF, Mueller-Dombois D (eds) Forest decline in the Atlantic and Pacific regions. Springer, Berlin Heidelberg pp 332–337

Duclos JL, Fortin JA (1983) Effect of glucose and active charcoal on in vitro synthesis of ericoid mycorrhiza with *Vaccinium* spp. New Phytol 94:95–102

Duddridge JA, Read DJ (1984) The development and ultrastructure of ectomycorrhizas. II. Ectomycorrhizal development on pine in vitro. New Phytol 96:575–582

Fortin AJ, Piché Y, Lalonde M (1980) Technique for the observation of early morphological changes during ectomycorrhiza formation. Can J Bot 58:361–365

Freer-Smith PH (1996) Forest growth and decline: what is the role of air pollutants? In: Yunus M, Iqbal M (eds) Plant response to air pollution. Wiley, Chichester, pp 437–447

Fridborg G, Pedersén M, Landström L, Eriksson T (1978) The effect of activated charcoal on tissue cultures: adsorption of metabolites inhibiting morphogenesis. Physiol Plant 43:104–106

Harley JL, Harley EL (1987) A check-list of mycorrhiza in the British flora. New Phytol 105:1–102

Iwatsuki K, Yamazaki T, Boufford DE, Ohba H (1995) Flora of Japan, vol I. Pteridophyta and Gymnospermae. Kodansha, Tokyo

Japan Forest Technical Association (1964) Illustrated important forest trees of Japan. Chikyu Shuppan, Tokyo

Kandler O (1993) Development of the recent episode of Tannensterben (fir decline) in Eastern Bavaria and the Bavarian Alps. In: Huettl RF, Mueller-Dombois D (eds) Forest decline in the Atlantic and Pacific regions. Springer, Berlin Heidelberg New York, pp 216–226

Kasuya MCM, Muchovej RMC, Bellei MM, Borges AC (1992) In vitro ectomycorrhizal formation in six varieties of pine. For Ecol Manage 47:127–134

Kernaghan G, Currah RS, Bayer RJ (1997) Russulaceous ectomycorrhizae of *Abies lasiocarpa* and *Picea engelmannii*. Can J Bot 75:1843–1850

Kottke I, Oberwinkler F (1986) Mycorrhiza of forest trees – structure and function. Trees 1:1–24

Lee KJ, Koo CD (1983) Inoculation of pines in a nursery with *Pisolithus tinctorius* and *Thelephora terrestris* in Korea. In: Atkinson D, Bhat KKS, Coutts MP, Mason PA, Read DJ (eds) Tree root systems and their mycorrhizas. (Developments in plant and soil sciences, vol 7). Nijhoff/Junk, The Hague, pp 325–329

Lee KJ, Koo CD (1992) Results of ectomycorrhizal inoculation of pine species with *Pisolithus tinctorius* and *Thelephora terrestris* in Korea. In: Read DJ, Lewis DH, Fitter AH, Alexander IJ (eds) Mycorrhizas in ecosystems. Cambridge University Press, Cambridge, pp 388–389

Malajczuk N, Molina R, Trappe JM (1982) Ectomycorrhiza formation in *Eucalyptus*. I. Pure culture synthesis, host specificity and mycorrhizal compatibility with *Pinus radiata*. New Phytol 91:467–482

Malajczuk N, Lapeyrie F, Garbaye J (1990) Infectivity of pine and eucalypt isolates of *Pisolithus tinctorius* on roots of *Eucalyptus urophylla* in vitro. I. Mycorrhizal formation in model systems. New Phytol 114:627–631

Martinez-Pulido C, Harry IS, Thorpe TA (1994) Effect of various bud induction treatments on elongation and rooting of adventitious shoots of Canary Island pine (*Pinus canariensis*). Plant Cell Tissue Organ Cult 39:225–230

Marx DH (1969) The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. I. Antagonism of mycorrhizal fungi to root pathogenic fungi and soil bacteria. Phytopathology 59:153–163

Marx DH (1980) Ectomycorrhizal fungus inoculations: a tool for improving forestation practices. In: Mikola P (ed) Tropical mycorrhiza research. Clarendon, Oxford, pp 13–71

Marx DH, Cordell CE (1989) The use of specific mycorrhizas to improve artificial forestation practices. In: Whipps JM, Lumsden RD (eds) Biotechnology of fungi for improving plant growth. Cambridge University Press, Cambridge, pp 1–25

Marx DH, Cordell CE, Kenney DS, Mexal JG, Artman JD, Riffle JW, Molina RJ (1984) Commercial vegetative inoculum of *Pisolithus tinctorius* and inoculation techniques for development of ectomycorrhizae on bare-root tree seedlings. For Sci Mono 25:1–101

Masui K (1926) A study of the mycorrhiza of *Abies firma*, S. et Z., with special reference to its mycorrhizal fungus, *Cantharellus floccosus*, SCHW. Mem Coll Sci Kyoto Imp Univ Ser B 2:15–84

Matsuda Y, Hijii N (1998) Spatiotemporal distribution of fruitbodies of ectomycorrhizal fungi in an *Abies firma* forest. Mycorrhiza 8:131–138

Metzler S, Metzler V (1992) Texas mushrooms – a field guide. University of Texas Press, Austin

Meyer FH (1973) Mycorrhizae in native and man-made forests. In: Marks GC, Kozlowski TT (eds) Ectomycorrhizae, their ecology and physiology. Academic, New York, pp 79–101

Mullette KJ (1976) Studies of eucalypt mycorrhizas. I. A method of mycorrhiza induction in *Eucalyptus gummifera* (Gaertn. & Hochr.) by *Pisolithus tinctorius* (Pers.) Coker & Couch. Aust J Bot 24:193–200

Nara K, Hogetsu T, Suzuki K (1992) Spatial distribution of ectomycorrhizae and their morphological features in a plantation of *Abies firma*. (in Japanese) Bull Tokyo Univ For 87:195–204

Piché Y, Fortin JA (1982) Development of mycorrhizae, extramatrical mycelium and sclerotia on *Pinus strobus* seedlings. New Phytol 91:211–220

Piché Y, Fortin JA, Peterson RL, Posluszny U (1982) Ontogeny of dichotomizing apices in mycorrhizal short roots of *Pinus strobus*. Can J Bot 60:1523–1528

Piché Y, Peterson RL, Howarth MJ, Fortin JA (1983) A structural study of the interaction between the ectomycorrhizal fungus *Pisolithus tinctorius* and *Pinus strobus* roots. Can J Bot 61:1185–1193

Pillukat A, Agerer R (1992) Studien an Ektomykorrhizen. XL. Vergleichende Untersuchungen zur baumbezogenen Variabilität der Ektomykorrhizen von *Russula ochroleuca*. Z Mykol 58:211–242

Požgaj A, Iqbal M, Kucera LJ (1996) Development, structure and properties of wood from trees affected by air pollution. In: Yunus M, Iqbal M (eds) Plant response to air pollution. Wiley, Chichester, pp 395–424

Record SJ, Hess RW (1943) Pinaceae. In: Timbers of the new world. Yale University Press, New Haven, Conn, pp 11–24

Roland JC, Vian B (1991) General preparation and staining of thin sections. In: Hall JL, Hawes C (eds) Electron microscopy of plant cells. Academic, London, pp 1–66

Saravitz CH, Blazich FA (1996) *Abies fraseri* (Pursh) Poir. (Fraser Fir). In: Bajaj YPS (ed) Biotechnology in agriculture and forestry. Vol 35. Springer, Berlin Heidelberg, pp 345–358

Schenk RU, Hildebrandt AC (1972) Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Can J Bot 50:199–204

Sohn RF (1981) *Pisolithus tinctorius* forms long ectomycorrhizae and alters root development in seedlings of *Pinus resinosa*. Can J Bot 59:2129–2134

Spurr AR (1969) A low-viscosity epoxy resin embedding medium for electron microscopy. J Ultrastruct Res 26:31–34

Tam PCF (1994) Mycorrhizal associations in *Pinus massoniana* Lamb. and *Pinus elliottii* Engel. inoculated with *Pisolithus tinctorius*. Mycorrhiza 4:255–263

Trappe JM (1962) Fungus associates of ectotrophic mycorrhizae. Bot Rev 28:538–606

Trappe JM (1977) Selection of fungi for ectomycorrhizal inoculation in nurseries. Annu Rev Phytopathol 15:203–222

Wang Y, Hall IR, Evans LA (1997) Ectomycorrhizal fungi with edible fruiting bodies. 1. *Tricholoma matsutake* and related fungi. Econ Bot 51:311–327

Webb DT, Flinn BS, Georgis W (1988) Micropropagation of eastern white pine (*Pinus strobus* L.). Can J For Res 18:1570–1580

Wilcox H (1954) Primary organization of active and dormant roots of noble fir, *Abies procera*. Am J Bot 41:812–821