Mycoscience 41: 395–399, 2000

Short Communication

Aseptic ectomycorrhizal synthesis between *Abies firma* and *Cenococcum geophilum* in artificial culture

Lu-Min Vaario[1)]*, Warwick M. Gill[1)], Megumi Tanaka[1)], Yuji Ide[2)] and Kazuo Suzuki[1)]

[1)] Graduate School of Agricultural and Life Sciences, The University of Tokyo, Yayoi 1–1–1, Bunkyo-ku, Tokyo 113–8657, Japan
[2)] The University Forests, Faculty of Agriculture, The University of Tokyo, Yayoi 1–1–1, Bunkyo-ku, Tokyo 113–8657, Japan

Accepted for publication 24 May 2000

A simple in vitro system is described for the synthesis of *Abies firma-Cenococcum geophilum* ectomycorrhizas. Sterilized *A. firma* seedlings on both MMN and FH media were inoculated with hyphal discs from actively growing margins of *C. geophilum* colonies. Typical ectomycorrhizas formed on seedlings on FH medium after 3 mo of incubation. By light microscopy, the synthesized mycorrhizas were seen to possess a thin mantle from which emanated extraradicle hyphae and highly branched, rarely septate intracortical Hartig net mycelium, characteristic ectomycorrhizal features. This is the first report of aseptic ectomycorrhization of *A. firma* seedlings by *C. geophilum*. This model system will facilitate detailed studies on ectomycorrhizal development of *Abies* species.

Key Words——*Abies firma*; *Cenococcum geophilum*; ectomycorrhizas; in vitro synthesis.

Abies firma Sieb. et Zucc. (Japanese fir; momi fir) is a tree species endemic to the warmer parts of Japan, forming natural coniferous forests with *Tsuga sieboldii* Carr (Ishizuka, 1974). *Abies* spp. are sensitive to environmental stresses such as drought, low potassium levels and air pollution (Freer-Smith, 1996), and they are considered indicators of sound forest vegetation in the warm temperate zone of Japan. All *Abies* spp. are regarded as obligately mycorrhizal (Meyer, 1973), but studies of *Abies* ectomycorrhizas are less abundant than for other coniferous host species (Trappe, 1962). Within Japan, a number of naturally occurring ectomycorrhizal fungi on *Abies firma* have been recorded (Masui, 1926; Nara et al., 1992; Matsuda and Hijii, 1999), and *C. geophilum* has recently been described (Matsuda and Hijii, 1999).

Cenococcum geophilum Fr. has frequently been described in ectomycorrhizal association with economically important tree families such as Myrtaceae, Salicaceae and Pinaceae (Trappe, 1962; Heslin and Douglas, 1986; Danielson and Pruden, 1989). It grows well at high salt concentrations and in soils ranging in pH from 3.4 to 7.5 (Trappe, 1977). It is also adapted to droughty environments and extremes of hot and cold, under which conditions it is able to form functional ectomycorrhizas. Consequently, *C. geophilum* has been reported to enhance a seedling's ability to withstand drought stress (Mexal and Reid, 1973). The controlled in vitro mycorrhization of *Abies firma* seedlings by *C. geophilum* may therefore assist in reforestation by increasing the tolerance of seedlings to unfavorable environmental fluctuations and protecting against the causal agent of *Abies* forest decline. Furthermore, *C. geophilum* is considered an excellent model for the isolation and regeneration of protoplasts from ascomycetous ectomycorrhizal fungi (Stülten et al., 1995). Such a fungus, suited to both experimental and practical applications, is a prime candidate for experimentation in controlled in vitro ectomycorrhizal syntheses.

Abies spp. are difficult to grow in vitro (Saravitz and Blazich, 1996). Consequently, studies on mycorrhizal development, function and molecular biology within the genus are scarce. However, a previous report on the establishment of an in vitro synthesis system between *Abies firma* and *Pisolithus tinctorius* (Vaario et al., 1999a) has helped to address this imbalance. The objective of this study was to establish a simple, easily manipulated model system for the in vitro synthesis of ectomycorrhizas between *A. firma* and *C. geophilum* by optimizing existing culture media. This system will be applied to examine the physiological and biochemical changes that are associated with, or which control ectomycorrhizal development.

Cenococcum geophilum, (deposited in the culture collection of the Laboratory of Forest Botany, The University of Tokyo as strain FBCg1) was isolated from a sclerotium recovered from soil within a natural forest in the University Forest in Chichibu, The University of Tokyo, in July 1990. It was maintained at 25±2°C in darkness

* Corresponding author. E-mail: lumin@uf.a.u-tokyo.ac.jp

on modified Melin-Norkrans medium (MMN; Marx, 1969) containing 10 g/l glucose instead of sucrose, solidified with 1.5% agar.

Seeds of *A. firma* were collected from a warm-temperate natural forest in the University Forest in Chiba, The University of Tokyo. They were 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 d and germinated at room temperature under diffused fluorescent illumination. Once germinated, the seedlings were surface-sterilized in 70% ethanol for 1 min, 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. Twenty sterilized seedlings were selected for subsequent ectomycorrhiza syntheses.

The more favourable medium for *C. geophilum* culture was determined by comparing growth on MMN and Fungus-Host (FH) medium, which was modified from SH medium (Schenk and Hildebrandt, 1972). FH medium contains: KNO_3, 2500 mg; $(NH_4)H_2PO_4$, 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; activated charcoal, 0.3 g; distilled H_2O, 1000 ml. Both media contained 10 g/l glucose and the pH was adjusted to 5.6 with 1 N NaOH prior to autoclaving. The increase in colony diam was measured on solid media (containing 1.5% agar), and the colony biomass was measured from liquid cultures on the same media.

One 6-mm diam plug, cut from the actively growing margin of a *C. geophilum* colony on MMN, was placed on to five agar plates (9-cm diam Petri dishes) each of MMN and FH solid media. Similarly, 6-mm diam mycelial plugs were inoculated into 20 ml liquid media of the same composition in 100-ml Erlenmeyer flasks and incubated in stationary culture. All inoculated flasks were incubated in darkness at 25±2°C.

To determine the optimal medium, the diameter of fungal colonies growing on each solid medium was measured weekly in two directions, perpendicular to each other. The weekly growth was expressed as a mean of the colony diameters of all replicates. After 47 d of incubation, the fungal colonies growing in liquid media were recovered on filter paper and dried at 100°C for 90 min. The colony biomass from each medium was expressed as a mean of all replicates. From this comparison, the better medium was determined and used in the following ectomycorrhizal synthesis protocol, based on a modified Chilvers' (Chilvers et al., 1986) paper sandwich method (Vaario et al., 1999a). Rectangular clear plastic culture plates (200×90×10 mm) were filled with 80 ml of MMN or FH agar medium, both containing 1 g/l glucose. The pH was adjusted to 5.6 with 1 N NaOH prior to autoclaving. 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. Two 6-mm diam plugs of *C. geophilum* mycelium were placed on the medium, adjacent to the taproot tip. Sterile cotton rolls (10×5 mm) were placed along the inside bottom edge of the plates to absorb water condensed during subsequent incubation. The plates were sealed with Parafilm (American Can Company, Detroit), the lower portions of the plates, containing both the developing host root system and ectomycorrhizal fungus, were covered with aluminum foil, and the plates incubated in an incubator with 3000 lux diffuse fluorescent light with a 16-h photoperiod at 25±2°C.

Putative mycorrhizal roots were removed from culture plates, and segments of 1–2 mm 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 with three times distilled water and dehydrated in an ascending acetone series (from 20% to 100%) in 20% increments followed by three changes of 100% propylene oxide. The root segments were subsequently infiltrated with Spurr's resin (Spurr, 1969) and polymerized at 70°C for 12 h. Sections of 4–6 μm thickness were cut with glass knives, gently heat-fixed to glass microscope slides, and stained with 0.1% toluidine blue O in 1% sodium tetraborate (Feder and O'Brien, 1968) for 10 min. Following three tap-water washes, the sections were destained in tap-water for 20 min, air-dried, mounted in DPX (Fluka BioChemika) beneath a coverslip, and examined with an Olympus BH2 microscope.

After 7 wk of incubation on solid media, a difference was noted in colony diam of *C. geophilum* incubated on MMN medium and FH medium (Fig. 1). The radial exten-

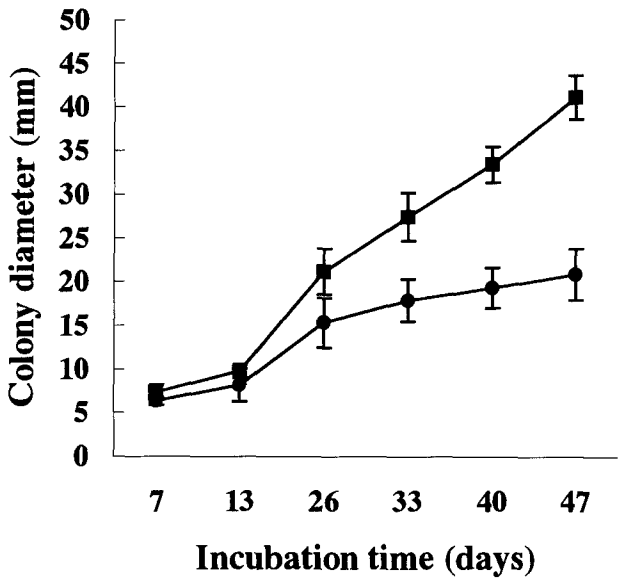

Fig. 1. Colony growth rate of *C. geophilum* on MMN and FH media. Mean values of 5 replicates±SD. (—●— MMN; —■— FH)

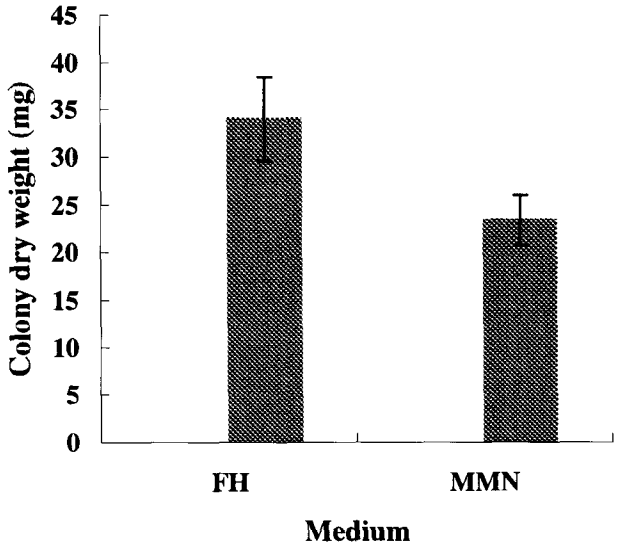

Fig. 2. Colony dry weight of *C. geophilum* on FH and MMN media after 47 d of incubation. Mean values of 5 replicates ± SD.

sion rate of fungal colonies incubated on FH medium was almost two times greater than that of colonies incubated on MMN medium. There was a significant difference between the two media in colony dry weight according to the Tukey test ($p < 0.05$), of which the better results were obtained on FH medium (Fig. 2). Other mycelial characteristics were affected by growth on different media. On MMN, *C. geophilum* grew slowly and formed concentric rings with the hyphae penetrating the medium and growing beneath the surface. The actively growing colony margin was composed of thick dense hyphae. FH medium resulted not only in more rapid radial growth but also in progressively smoother fungal layers, resulting in a thin and homogeneous mycelial mat upon the medium surface.

Ten wk after inoculation, short lateral roots were induced from the taproots, some of which came into contact with hyphae arising from the fungal inocula. Lateral root formation occurred in all 10 seedlings incubated on FH medium and in 6 of the 10 seedlings growing on MMN (Table 1). A network of black extraradicle hyphae formed around the short lateral roots bases, and the roots stopped elongating and failed to form root hairs. Twelve wk after inoculation, the fungus had completely enveloped some of the lateral roots and had formed black mycorrhizas with a rough mantle surface from which black, kinked, extraradicle hyphae emanated (Fig. 3a). Six of the 10 seedlings formed black mycorrhizas on FH medium. However, on MMN, *C. geophilum* grew slowly and failed to contact the lateral roots, and after 16 wk of incubation only one lateral root had formed a mycorrhiza (Table 1).

Abies firma-C. geophilum mycorrhizas synthesized on FH medium consisted of a thin, loose, discontinuous fungal mantle which failed to completely ensheath the host root (Fig. 3b). Mantle hyphae gave rise to profuse

Table 1. Comparison of aseptic *A. firma-C. geophilum* ectomycorrhizal synthesis on MMN and FH media 16 wk after inoculation.

Media	Lateral root formation	Mycorrhization
MMN	60% (6/10)	10% (1/10)
FH	100% (10/10)	60% (6/10)

intracortical hyphae, which invaded and colonized the epidermal and cortical intercellular spaces, appearing to separate and isolate host cells (Fig. 3c). A fungal continuum was formed from the extraradicle rhizosphere via the mantle to the host endodermis. Hartig net hyphae invaded the host cortex and enveloped the host cells in a uniseriate or multiseriate layer of fungal cells, but did not penetrate them. The Hartig net formed a typical highly branched labyrinthine 'palmetti' structure (Fig. 3d), which maximizes available cell surface area and optimizes cell-to-cell nutrient transfers (Smith and Read, 1997).

The long-term goal of our studies is to better understand the physiological and biochemical events that control and affect ectomycorrhization of fir trees. To realize this goal, establishment of a simple, rapid and convenient mycorrhiza synthesis system is necessary. Initially, two common media were tested to find a substrate suitable for both symbionts. *Abies* spp. are slow-growing in vitro, seedlings often fail to thrive, and root development is frequently retarded (Saravitz and Blazich, 1996). Furthermore, the growth rate of *C. geophilum* on MMN has been shown to be insufficient for it to match that of host seedlings (Kasuya et al., 1992). However, Stülten et al. (1995) achieved mycorrhization between *Picea abies* and *C. geophilum*, inoculated by mycelial suspension, on MMN in aseptic conditions. In our investigation, MMN was found to be inappropriate for mycorrhizal formation as few lateral roots formed on *A. firma* and the host seedlings outgrew *C. geophilum*. Consequently, the chance of interaction between lateral roots and hyphae was low. Furthermore, not only was the biomass production of *C. geophilum* on MMN low (Job and Aragno, 1992), but the growth form was concentrated and the fungus did not form smooth layers. The concentrated growth form is considered unsuitable for mycorrhiza formation (Ineichen and Wiemken, 1992), resulting in only part of the colony making good contact with the host root system. Fungus-Host medium, modified from SH medium, a popular medium for tissue culture of conifers (Webb et al., 1988; Martinez-Pulido et al., 1994), has previously been demonstrated to be suitable for *A. firma* growth in vitro by promoting development of lateral roots and increasing root growth rate (Vaario et al., 1999b). Here, FH medium successfully improved the biomass production and growth rate, altered the growth form of *C. geophilum*, and also improved the synthesis rate of *A. firma-C. geophilum* mycorrhizas.

Mycorrhizas synthesized by this culture system exhibited characteristics typical of ectomycorrhizas. The thin discontinuous mantle supports hyphae, which pene-

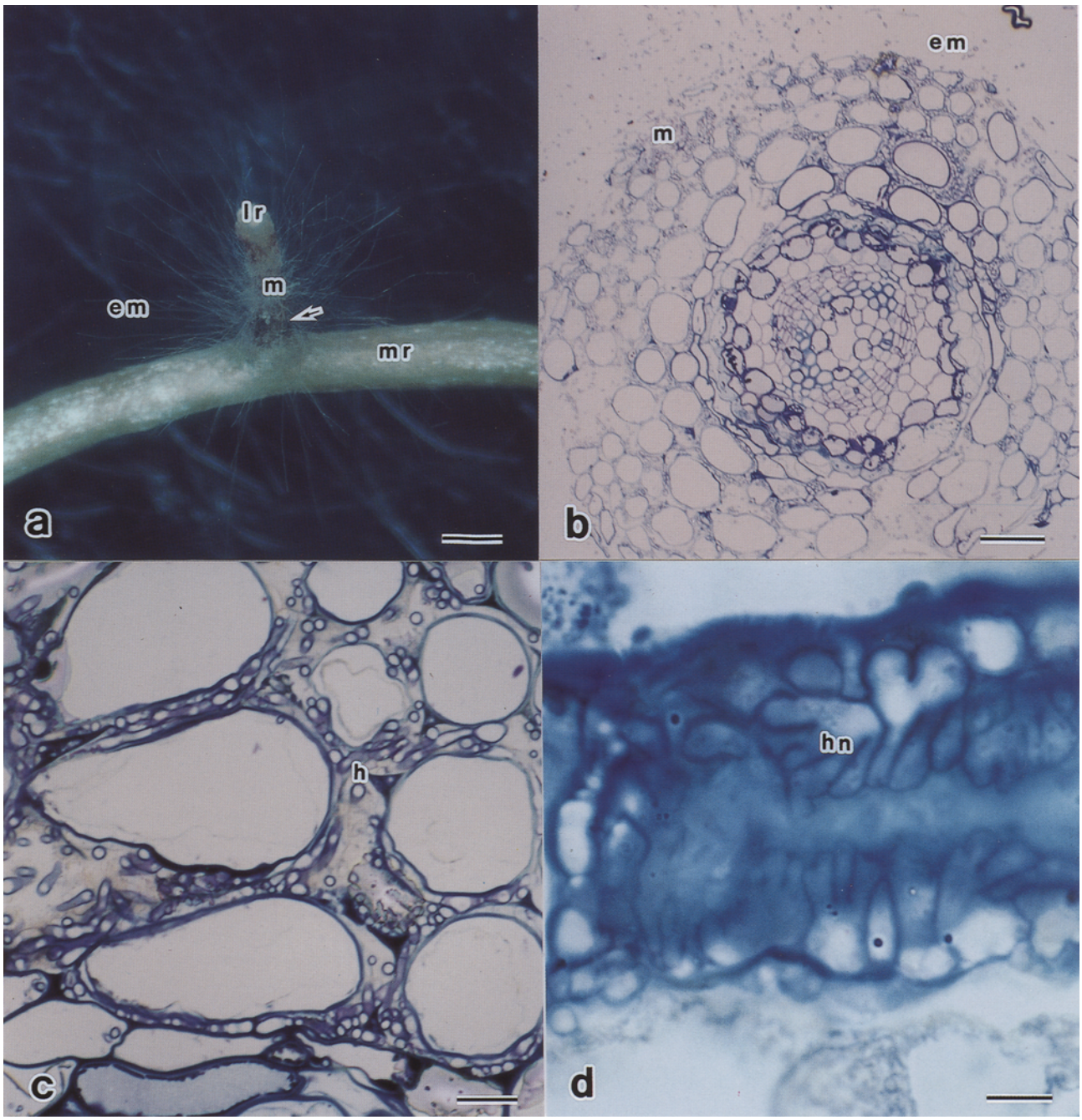

Fig. 3. *Abies firma* root colonization by *C. geophilum* in vitro. a) External mycorrhiza morphology. *Abies firma-C. geophilum* mycorrhizal root formed on FH medium after 12 wk of incubation. The lateral root (lr) is colonized along its length by hyphae forming a sparse mantle (m), from which copious extraradicle hyphae emanate (em). The mantle is distinguishable from unsheathed lateral root (arrow) and is absent from the main root (mr). Bar = 1 mm. b) Light micrograph of an inoculated *A. firma* lateral root in transverse cross section demonstrating the sparse mantle (m) and extraradicle mycelium (em). Bar = 60 μm. c) In transverse section, the intercellular spaces of the mycorrhiza cortex are colonized by copious hyphae (h) which appear to separate the host cells. Bar = 30 μm. d) In longitudinal section, the multibranched, nonseptate fan-shaped Hartig net 'palmetti' (hn) are observed. Bar = 12 μm.

trate and colonize the host root cortical intercellular spaces. These invasive hyphae undergo change in their growth morphology to form a highly branched, rarely septate labyrinthine Hartig net. The modified hyphae envelop the host root cortical cells (Kottke and Oberwinkler, 1986; Gianinazzi-Pearson and Smith, 1993), increasing the contact surface area between the two symbionts and maximizing nutrient transfer (Kottke and Ober-

winkler, 1986). The presence of these defining features in the mycorrhizal roots examined indicates that *C. geophilum* forms an ectomycorrhizal association with host *A. firma* roots under the culture conditions employed.

Mycorrhization increases the tolerance of host seedlings to drought and other environmental stresses, increasing the rate of successful reforestation of damaged or unfavorable sites. The clearly defined in vitro system described here will ensure that sufficient inoculum can be produced in the laboratory to be applied to practical forestry situations. Furthermore, by using this system, it will be possible to follow the morphological, physiological, molecular and biochemical changes which occur during development of *A. firma-C. geophilum* mycorrhizas and in many other host-fungus ectomycorrhizal associations. Such widespread applicability will ensure that this synthesis protocol will continue to be used for further in vitro ectomycorrhizal studies.

Acknowledgements——This research was supported by a grant from the Bio-oriented Technology Research Advancement Institution (BRAIN). The authors wish to thank Dr. F. Lapeyrie (INRA, France) for useful discussions.

Literature cited

Chilvers, G. A., Douglass, P. A. and Lapeyrie, F. F. 1986. A paper-sandwich technique for rapid synthesis of ectomycorrhizas. New Phytol. **103**: 397–402.

Danielson, R. M. and Pruden, M. 1989. The ectomycorhizal status of urban spruce. Mycologia **81**: 335–341.

Feder, N. and O'Brien, T. P. 1968. Plant microtechnique: some principles and new methods. Am. J. Bot. **55**: 123–142.

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

Gianinazzi-Pearson, V. and Smith, S. E. 1993. Physiology of mycorrhizal mycelia. In: Mycorrhiza Synthesis. Advances in Plant Pathology Vol. 9, (ed. by Tommerup, I. C.), pp. 55–82. Academic Press, London.

Heslin, M. C. and Douglas, G. C. 1986. Synthesis of poplar mycorrhizas. Trans. Br. Mycol. Soc. **86**: 117–122.

Ishizuka, T. 1974. Forest vegetation zones. In: The flora and vegetation of Japan, (ed. by Numata, M.), pp. 87–124. Tokyo.

Ineichen, K. and Wiemken, V. 1992. Changes in the fungus-specific, soluble-carbohydrate pool during rapid and synchronous ectomycorrhiza formation of *Picea abies* with *Pisolithus tinctorius*. Mycorrhiza **2**: 1–7.

Job, D. and Aragno, M. 1992. Nutritional growth requirements for submerged cultures of the ectomycorrhizal fungi *Cenococcum geophilum* Fr. Crypt. Mycol. **13**: 79–85.

Kasuya, M. C. M., Muchovej, R. M. C., Bellei, M. M. and Borges, A. C. 1992. *In vitro* ectomycorrhizal formation in six varieties of pine. For. Ecol. Manage. **47**: 127–134.

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

Martinez-Pulido, C., Harry, I. S. and Thorpe, T. A. 1994. Effect of various bud induction treatments on elongation and rooting of adventitious shoots of Canary Island pine (*Pinus canariensis*). Plant Cell Tiss. Org. Cult. **39**: 225–230.

Marx, D. H. 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.

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. and Hijii, N. 1999. Ectomycorrhizal morphotypes of naturally grown *Abies firma* seedlings. Mycoscience **40**: 217–226.

Mexal, J. and Reid, C. P. P. 1973. The growth of selected mycorrhizal fungi in response to induced water stress. Can. J. Bot. **51**: 1579–1588.

Meyer, F. H. 1973. Mycorrhizae in native and man-made forests. In: Ectomycorrhizae, their ecology and physiology, (ed. by Marks, G. C. and Kozlowski, T. T.), pp. 79–101. Academic Press, New York.

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

Saravitz, C. H. and Blazich, F. A. 1996. *Abies fraseri* (Pursh) Poir. (Fraser Fir). In: Biotechnology in agriculture and forestry, (ed. by Bajaj, Y. P. S.), pp. 345–358. Springer-Verlag, Berlin, Heidelberg.

Schenk, R. U. and Hildebrandt, A. C. 1972. Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Can. J. Bot. **50**: 199–204.

Smith, S. E. and Read, D. J. 1997. Mycorrhizal symbiosis, pp. 197–200. 2nd ed. London: Academic Press Ltd.

Spurr, A. R. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. **26**: 31–34.

Stülten, C. H., Kong, F. X. and Hampp, R. 1995. Isolation and regeneration of protoplasts from the ectomycorrhizal ascomycete *Cenococcum geophilum* Fr. Mycorrhiza **5**: 259–266.

Trappe, J. M. 1962. Fungus associates of ectotrophic mycorrhizae. Bot. Rev. **28**: 538–606.

Trappe, J. M. 1977. Selection of fungi for ectomycorrhizal inoculation in nurseries. Ann. Rev. Phytopathol. **15**: 203–222.

Vaario, L.-M., Tanaka, M., Ide, Y., Gill, W. M. and Suzuki, K. 1999a. *In vitro* ectomycorrhiza formation between *Abies firma* and *Pisolithus tinctorius*. Mycorrhiza **9**: 177–183.

Vaario, L.-M., Tanaka, M. and Ide, Y. 1999b. *In vitro* plantlet regeneration of *Abies firma* from germinated seedlings. Bull. Tokyo Univ. For. **102**: 103–111.

Webb, D. T., Flinn, B. S. and Georgis, W. 1988. Micropropagation of eastern white pine (*Pinus strobus* L.). Can. J. For. Res. **18**: 1570–1580.