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***In vitro* Ectomycorrhiza Formation on Two Larch Species of Seedlings with Six Different Fungal Species**

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Abstract

The ability of ectomycorrhizal formation in two species of larch seedling (*Larix kaempferi* Sarg. and its F1 larch; *Larix kaempferi* × *Larix gmelinii* Rupr.) with six species of ectomycorrhizal fungi (*Russula emetica*, *Tricholoma saponaceum*, *Lactarius hatsudake*, *Suillus grevillei*, *Suillus laricinus* and *Cenococcum geophilum*) was tested using an *in vitro* synthesis technique. All six fungal isolates formed ectomycorrhizae association on roots of Japanese larch and F1 hybrid larch after ten weeks inoculation. The degrees of infection were depending on the ability of fungal growth on the artificial vermiculite-peat substrate and the fungal affinity to the hosts. The results suggest that the two larch species tested have the ability to form ectomycorrhizae with diverse ectomycorrhizal fungal species under this artificial culture condition.

Key words: ectomycorrhizal infection, Japanese larch and F1 larch, petri dish method, *in vitro* synthesis

Introduction

In temperate and boreal forest ecosystems, most tree species from ectomycorrhizal association with diverse species of fungi in the Ascomycetes and Basidiomycetes. A number of studies have shown the importance of mycorrhizal fungi for the survival and growth of trees in forest ecosystem (Brundrett *et al.* 1996, Marx 1969, Browing and Whitney 1993). Mycorrhizae can enhance nutrient, water absorption by their mycelial networks, and protect against pathogens (Smith and Read 1997). Ectomycorrhizae can be found on about 90% of the trees in temperate and boreal forests (Le Tacon *et al.* 1992). Conifers and many hardwoods are strongly depending on mycorrhizae for their growth and survival. In some forests under infertile condition, the growth of conifer seedlings often restricted for essential mineral nutrient in soil unless mycorrhizae are developed in their root system (Trofymow and van den Driessche 1991). Therefore, ectomycorrhizal association is necessary for successful establishment of newly planted seedlings in afforestation and reforestation sites. However, the influences of ectomycorrhizae on plant growth and nutrient uptake vary according to species of ectomycorrhizal fungi (Jonsson *et al.* 2001).

Although some studies (Zhou *et al.* 1999, 2000, 2001a, 2001b, 2002, Yang *et al.* 1998) have shown that larch species are mostly associated with *Suillus* spp. and *Cenococcum geophilum* in natural condition, little data is still available describing the ectomycorrhizal development and growth of larch seedlings artificially inoculated with different ectomycorrhizal fungi. Larch is an introduced tree species in Hokkaido region from

Nagano prefecture, central Japan and becomes an important tree species dominated on disturbed sites (Koike *et al.* 2000; Wang *et al.* 2001). In Japan, there were not many studies have been carried out on mycorrhizal symbiosis associated with larch forests grown on soil originated from volcanic eruption. Recently, Yang *et al.* (1998) reported that Japanese larch (*Larix kaempferi*) might be associated with 3 or 4 types of mycorrhizal fungi depending on elevations. This plant is an obligatory ectomycorrhizal species, requiring mycorrhizal association for its survival and growth in the disturbed field. Japanese larch, due to its tolerant to cold moist climate and relatively higher production rate as timbers, had been reforested intensively throughout deforestation zones and on bare ground in northern Japan. However, many plantations of Japanese larch had not been established well, which is partly due to biological stresses (disease, grazed by bole etc.) and to poor nutrient condition (Koike *et al.* 2000). Its hybrid larch was a cross between a Dahurican larch (*L. gmelinii*) as a mother tree from the Kurils and the Japanese larch as a father tree is also planted in this region. Hybrid larch are often more tolerant to environmental stresses, although Japanese larch has higher growth rate as compared with Dahurican larch or its hybrid larch (Koike *et al.* 2000). Therefore, we included hybrid larch in this study to evaluate any differences in relation to the ability of mycorrhizal formation.

Numerous *in vitro* systems of mycorrhizal synthesis have been developed and examined the ability of fungi to form ectomycorrhizae (Fortin *et al.* 1983, Chilvers *et al.* 1986, Duddridge 1986, Kottke *et al.* 1987, Wong

and Fortin 1988, Kasuya *et al.* 1992, Wu *et al.* 1999, Vaario *et al.* 1999, Guerin-Laguette *et al.* 2000, Rincon 2001). In this study, we employed the modified Petri dish technique followed by the procedures described by Duddridge (1986). This method provides seedlings with a natural shoot-root compartmentation by exposing the shoots to the atmosphere. Some studies (Straatsma *et al.* 1986, Rupp and Mudge 1985, Read and Armstrong 1972) showed that the selective enclosure the roots might be particularly important because certain stages of ectomycorrhizae development are influenced by gaseous factors. Moreover, Duddridge (1986b) reported the availability of an external source of carbon to the fungus upsets the balance of the symbiosis in favor of the fungal partner, causing changes in the host cell wall and at the host-fungus interface. The rate and degree of colonization of the root surface is also influenced by the presence of carbohydrate in the synthesis medium. Based on the Duddridge's method (1986 a, b), we also examined the effects of small amount of external carbon sources on the development of mycorrhizae.

Because of the low degree of host specificity of most ectomycorrhizal fungi, we introduced six different kinds of fungi to this trial. The purposes of this study were to examine the infective ability of different species isolates of ectomycorrhizal formation with Japanese larch and its hybrid larch seedlings by pure culture synthesis technique under laboratory condition. The present study is an initial effort to improve our current knowledge of ectomycorrhizal fungi to associate with Japanese larch and its hybrid larch in order to develop further research.

Materials and Methods

Culture Media

During the ectomycorrhizal formation, sugar is added to the medium of Yang and Wilcox (1984); glucose (10 g·l⁻¹) is added or absent to the medium was discussed by Duddridge (1986a, 1986b). Based on Duddridge's idea and our previous work (data unpublished), we added lower amount of glucose (2 g·l⁻¹) in the media. In this petri dish method, we used vermiculite: peat substrate that allowed natural root development, formation of laterals. The substrate often used for seedling growth and artificial inoculation of containerized seedlings (Quoreshi and Timmer, 1998). Three different types of modified MMN solutions (Marx 1969) were used for the purpose of ectomycorrhizal fungal isolation, fungal inoculum culture and seedling culture. The original MMN media were slightly modified to fit our study objectives. The three modified solutions contained:

- (a) CaCl₂, 0.05 g; NaCl, 0.025 g; KH₂PO₄, 0.5 g; (NH₄)₂HPO₄, 0.25 g; MgSO₄ · 7H₂O, 0.15 g; FeCl₃ (1%), 1.2 ml; Thiamine HCl, 100 mg; Malt extract, 3 g; Glucose, 10 g; stock solution of Micronutrient * (see below), 1 ml; deionized water 1000 ml; plus 15 g agar in case of agar media. The pH of the media was adjusted to 5.5 before autoclave.
- (b) CaCl₂, 0.05 g; NaCl, 0.025 g; KH₂PO₄, 0.25 g; (NH₄)₂HPO₄, 0.125 g; MgSO₄ · 7H₂O, 0.15 g;

FeCl₃ (1%), 1.2 ml; Thiamine HCl, 100 mg; Glucose, 2 g; solution of Micronutrient * (see below), 1 ml; and deionized water 1000 ml. The pH of the media was adjusted to 5.5 before autoclave.

- (c) CaCl₂, 0.05 g; NaCl, 0.025 g; KH₂PO₄, 0.5 g; (NH₄)₂HPO₄, 0.25 g; MgSO₄ · 7H₂O, 0.15 g; FeCl₃ (1%), 1.2 ml; Thiamine HCl, 100 mg; Malt extract, 1.5 g; Glucose, 4.5 g; stock solution of Micronutrient * (see below), 1 ml; Agar, 15 g; streptomycin, 50 mg; chlortetracycline, 5 mg; deionized water 1000 ml; and Agar, 15 g. The pH of the media was adjusted to 5.0 before autoclave.

* One liter micronutrient stock solutions contained: H₃BO₃ (2.86 g), MnCl₂ (1.81 g), ZnSO₄ (0.22 g), CuSO₄ (0.08 g) and Na₂MoO₄ (0.02 g).

Plant materials

Before germination, the seeds of Japanese larch (*Larix kaempferi*) originated from Kuriyama at the central Hokkaido and its F1 hybrid larch (*Larix kaempferi* × *Larix gmelinii*) produced in the Uryu Experiment Forest were soaked in distilled water at 4°C for 10 days. Then the seeds were surface sterilized by shaking them for 20 min in 30% H₂O₂ and rinsed them 4-5 times with sterile deionized water. Germination was carried out aseptically on sterilized vermiculite-peat-sand media (vermiculite: peat: black sand = 2:1:2). The seed were incubated in a growth chamber maintained at 20°C under photosynthetic photon flux density of 150 μmol m⁻² s⁻¹ diffuse fluorescent light (Toshiba, Tokyo) with a 16-h photoperiod until the seedlings are ready for inoculation.

Fungal materials

Six different species of ectomycorrhizal fungi were tested for synthesis of ectomycorrhizae with larch seedlings. The fungal isolates (*Russula emitica* (Schaeff.: Fr.) S. F. Gray (strain: 995), *Tricholoma saponaceum* (Fr.) Kummer (strain: 920), and *Lactarius hatsudake* (strain: 641)) were obtained from Biological Environment Institute, KANSO Co., Ltd. Japan. The isolates were collected from mixed pine forest in Honsu Island, Japan. The strains of *Suillus grevillei* (Klotz.) Sing. (strain: SG-1), and *Suillus laricinus* (Bk.) O.Kuntz (strain: SL-1) were stock culture of Laboratory of Forest Resource Biology, Faculty of Agriculture, Hokkaido University. Both the species were collected from Mt. Komagatake (Mori town, Hokkaido) and isolated from fruit body tissues. *Cenococcum geophilum* Fr. was isolated (medium c) from roots of *Picea glehnii* (Fr. Schm.) collected from a mixed larch and spruce stand located at the experimental forests of Forestry and Forest Products Research Institute (FFPRI), Sapporo, Japan. The isolated fungal culture and the culture obtained from other laboratory were maintained on modified MMN agar medium (a) and periodically subculture for further inoculation study.

Mycorrhizal synthesis

A potting substrate 2100 ml (containing vermiculite and peat moss (6:1), moisten with 1000 ml modified MMN nutrient solution (b) containing glucose (2%), were autoclaved at 121°C, 35 min. An electric cutter (Ultrasonic cutter SUW 30, Suzuki, co. Ltd., Osaka, Japan) was used to cut a slit into the side of petri dishes and their covers to make a space for seedling insertion. The petri dishes were aseptically filled with about 40 ml of autoclaved substrate. Before inoculation, the six ectomycorrhizal isolates were cultured on agar plates containing MMN nutrient solution (a) for three to four weeks at 25°C in the dark. Three small plugs of (about 5-mm square each) actively growing mycelia from the margin of 3-week-old fungal cultures were transferred to petri dishes containing the substrate. The petri dishes were sealed with Parafilm and incubated about four weeks at 25°C in the dark.

During incubation, the fungal colonies were allowed to grow on the media and permeate maximum areas of the substrate (as shown in Fig. 1 I). Six replicate plates of each fungal species were incubated for this experiment.

The germinated seedlings (2-week-old after germination) were surface sterilized with H₂O₂ (1%, for 10 min), rinsed three to four times with sterilized deionized water. This measure was taken to ensure that seedlings were free of contamination at inoculation. An aseptic individual seedling was transplanted per plate aseptically and the root system was carefully placed on surface of the growing colonies so that most laterals are in close contact with fungal mycelia (Fig. 1 II). The seedling, including roots, was positioned horizontally. Three milliliters of MMN nutrient solution (b) was added to the substrate at this point. The shoot remains out side of the petri dish through the slit and the root remains in aseptic condition. The petri dish was sealed with parafilm and aluminum foil to keep the roots and fungus in darkness. The petri dishes were placed vertically in a plastic basket (Fig. 2) and arranged in the growth cabinet (at 20°C under photosynthetic photon flux density of 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$ diffuse fluorescent light with a 16-h photoperiod) at the Experimental Nursery of Hokkaido University Forests. Cultures were monitored monthly and sterilized deionized water was added twice during culture period.

Harvest

Ten weeks after inoculation (Fig. 1 III, IV), estimation of ectomycorrhizae formation and seedling growth were measured. Root systems of seedlings were photographed for the evidence of ectomycorrhizal formation and the seedlings were removed from culture plates. The seedlings were separated into root and shoot components and the roots carefully washed with water free of substrate. Ectomycorrhizal roots were excised and kept aside for further anatomical studies. The seedling shoots were oven dried (60°C, 48h).

Evaluation of ectomycorrhiza

All root segments selected were soaked in distilled water and observed under a stereomicroscope at 20-80x

magnifications for ectomycorrhizal evaluation. Ectomycorrhizal tips were confirmed by the color, form and size of ectomycorrhizae, the presence emanating hyphae and web of mycelia on the root surface, the presence of rhizomorphs, the absence of root hairs using a stereomicroscope and a compound light microscope.

Anatomical analyses

Preparation of roots for microscopic study was similar to the procedure described by Burn *et al.* (1995), Peterson (1991), and Murakami *et al.* (1999). Root segments of each type were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for overnight at room temperature and washed three times for 15 min each in the same buffer. After washing, the root samples were dehydrated in a graded ethanol series (25%, 50% 75%, 90% and 100%). They were then gradually embedded with epoxy resin at room temperature and then the resin was polymerized. The sections (1 μm thick) were cut with a glass knife on an ultramicrotome (Ultracut J; Austria). The sections were stained with a 1% solution of safranin or a 1% solution of safranin and gentian violet. They were observed under a light microscope (BHS-2; Olympus, Japan).

Fresh mycorrhizas were also used for microscopic study as described by Ursic and Peterson (1997). A fresh root segment was placed on a glass slide, and hand sections were made using sharp steel blade, stained with 1% (w/v) of aqueous toluidine blue and with 0.1% (w/v) of cotton blue in 10% (v/v) lactophenol/water and observed. At least 10 tips of each type were sectioned for anatomical study. Transverse sections and vertical sections were observed to verify the presence of mantle hyphae and Hartig net.

Results and discussion

All fungal species tested apparently formed typical ectomycorrhizae with both Japanese larch and its F1 larch under the present test conditions (Fig. 3). Mycelial spread and ectomycorrhizal formation with well developed rhizomorphs and emanating hyphae were shown in Figure 1 and 3. The short ectomycorrhizal roots of Japanese larch with *Suillus grevillei* were completely enveloped with cottony hyphate growing on the mantle (Fig. 3 a-c). Among the two *Suillus* spp., *S. grevillei* had faster growth (infection rate 100% of total root tips) and spread better on the artificial media than *Suillus laricinus* (infection rate 92% of total root tips). The relatively high infection rates of two *suillus* species demonstrated that they were suitable species for developing ectomycorrhizal association with Japanese larch and its hybrid larch. It may due to they were isolated from larch forest and they have closer relationship with the larch. A thick black and dark brown straight emanating hyphae are the characterized of *Cenococcum geophilum* (Agerer 1994). *C. geophilum* only colonized near the inoculum plugs (Fig. 3 f, g), which may due to *C. geophilum* be a slow growing fungus. Microscopic examinations of the root system of all seedlings showed that the most laterals that are in contact with growing

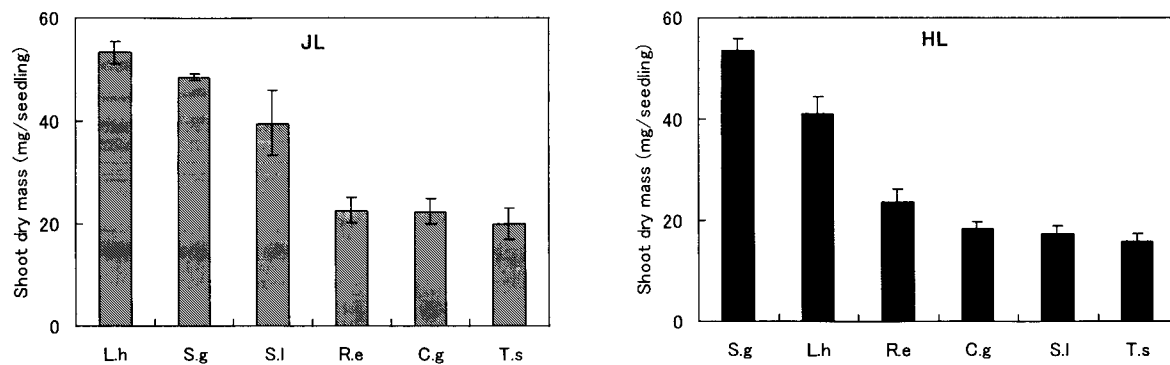


Fig. 5. Shoot biomass of Japanese Larch (JL) and its hybrid larch (HL) seedlings inoculated with six different ectomycorrhizal fungi. The abbreviation Sg, Lh, Re, Cg, Sl, and TS stands for *Suillus grevillei*, *Lactarius hatsudake*, *Russula emitica*, *Cenococcum geophilum*, *Suillus laricin*, and *Tricholoma saponaceum*, respectively.

colony are completely enveloped by the fungal hyphae (Fig. 3). Mantle colors of the each mycorrhizae were similar to colony in pure culture in most of the cases. Infection status observed after morphological and anatomical observation showed the evidences of successful ectomycorrhizal formation in all species (Figs. 3 and 4). Apparently, the best infection based on visual estimation occurred with *S. grevillei* (Fig. 3 a-e) with two plant species followed by *S. laricin* (Fig. 3 l) and *T. saponaceum* (infection rate 77% of total root tips)(Fig. 3 h, j). Other species of isolates, such as *L. hatsudake* (Fig.3 m), *R. emitica* (Fig.3 k), and *C. geophilum* (Fig.3 f, g) showed relatively slower growth in this culture condition. The infection rate of *L. hatsudake*, *R. emitica*, and *C. geophilum* were 63%, 48% and 41% of total root tips, respectively. However, successful mycorrhizae formation was observed with both plant species with these three species. *L. hatsudake* produced smooth ectomycorrhiza (Fig.3 m).

After several attempts of anatomical studies provided the evidence of ectomycorrhizae formation with *L. hatsudake* (Fig. 4 A, G, H). Microscopic examination revealed the presence of mantle some times in two layers, an inner layer consisting well stained and compact hyphae, and an outer region with loose and lightly stained external mycelia (Fig. 4). These preliminary results indicate the six fungi able to colonize the roots of Japanese larch and F1 hybrid larch at the end of this experiment, once hyphae had contacted the root surface.

Figure 5 shows the shoot biomass accumulation of both larch seedlings at the end of culture period. Although growth in all seedlings was not satisfactory as we expected after 10 weeks, probably due to cultivation system and N limitation. However, the shoot growth showed some variations depending on the fungal species inoculated. The reduced biomass growth compared to expected 10-week growth for larch probably attributed to exhaustion of nutrients in this culture system since both plant and fungal growth required substantial amount of N. Nevertheless, we do

not have clear explanation of these differences that inoculation affected seedling growth, partly because of small amount of samples and lack of non-inoculated control seedlings. Unfortunately, we could not provide the root biomass because of loss in samples during mycorrhizal evaluations.

This study demonstrated that *S. grevillei*, *S. laricin*, *R. emitica*, *T. saponaceum*, *C. geophilum* and *L. hatsudake* can develop mycorrhizal association on roots of Japanese larch and F1 hybrid larch under present artificial culture conditions. The fungal species varied in both spreading on vermiculite-peat media and the ability to form ectomycorrhizae. We could get the initial step for studying nutritional association in larch-fungus based on Duddridge's method (1986 a, b).

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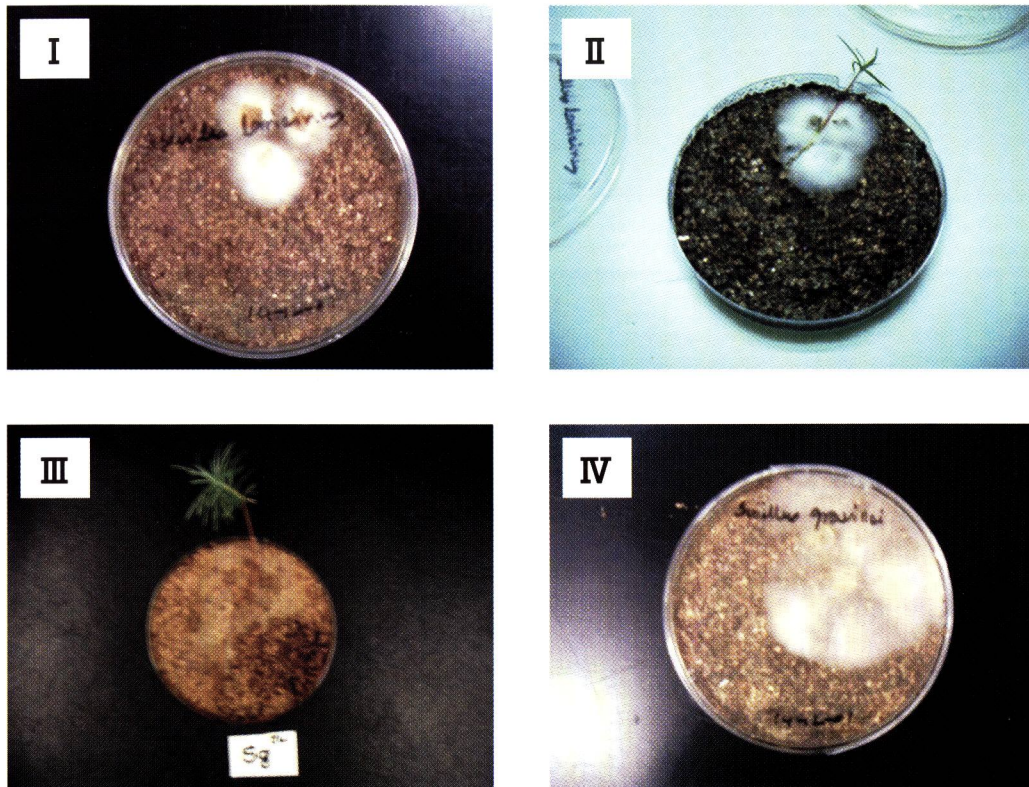


Fig. 1. Macroscopic view of fungal inoculum growth on vermiculite: peat media in petri dishes and inoculated larch seedlings. I and II *Suillus laricinus* grew on vermiculite: peat medium one month and transferred seedlings to petri dish. III and IV Harvest seedlings growing with *Suillus grevillei* after ten weeks inoculation.



Fig. 2. Aseptic synthesis of ectomycorrhizae. Tree seedlings growing in petri dishes placed in plastic box inside a growth cabinet.

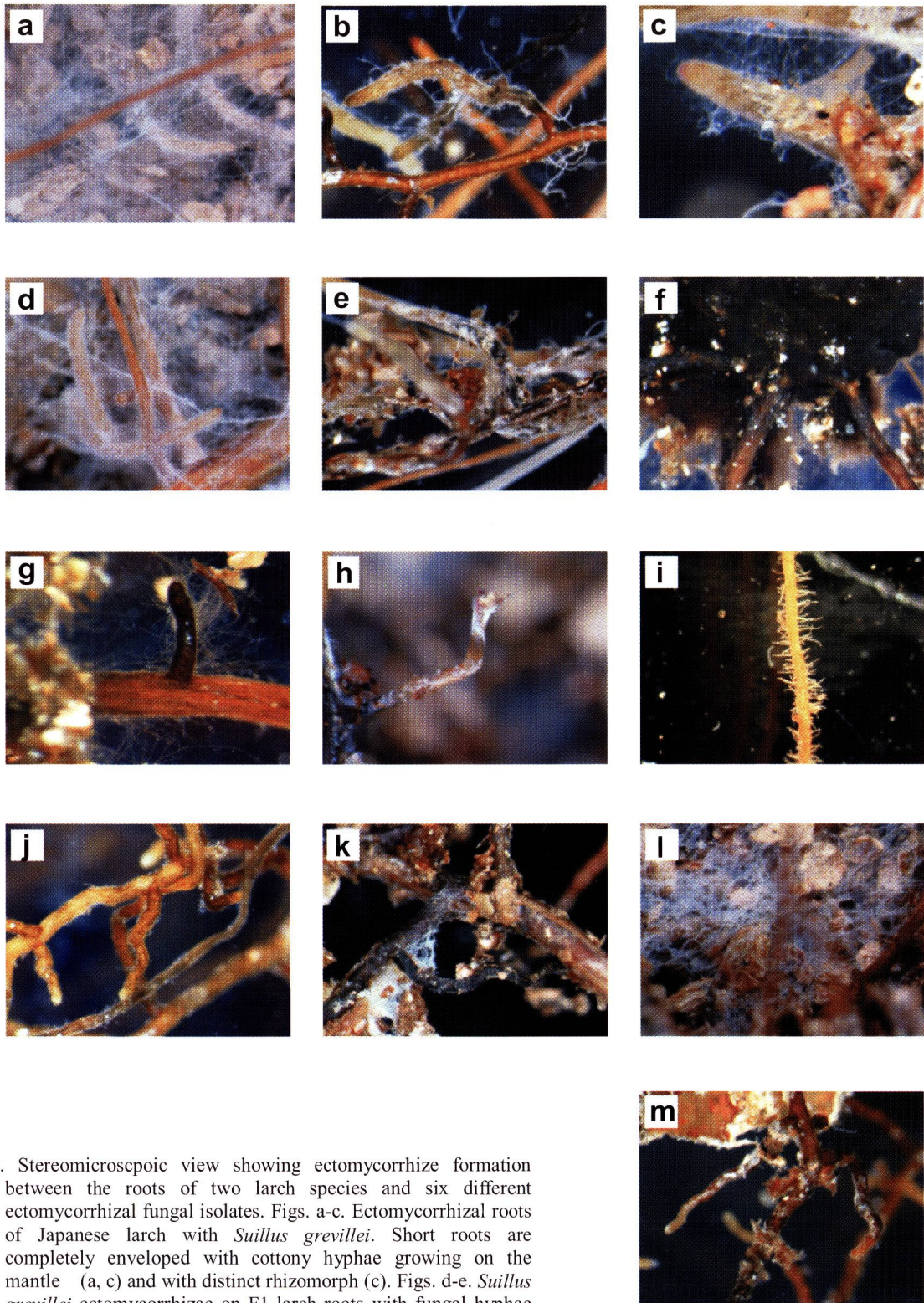


Fig. 3. Stereomicroscopic view showing ectomycorrhize formation between the roots of two larch species and six different ectomycorrhizal fungal isolates. Figs. a-c. Ectomycorrhizal roots of Japanese larch with *Suillus grevillei*. Short roots are completely enveloped with cottony hyphae growing on the mantle (a, c) and with distinct rhizomorph (c). Figs. d-e. *Suillus grevillei* ectomycorrhizae on F1 larch roots with fungal hyphae and rhizomorphs.

Figs. f-g. Compact *Cenococcum geophilum* fungal colony growing on the substrate (f) and formed ectomycorrhizae showing characteristic black emanating hyphae (g). Fig. h. A single root segment colonized by *Tricholoma saponaceum* on Japanese larch. Fig. i. A non-mycorrhizal root segment F1 larch with abundant root hairs. Figs. j-m. Ectomycorrhizal roots with attached fungal hyphae between *Tricholoma saponaceum* and F1 larch (j), *Russula emitica* and Japanese larch (k), *Suillus laricinus* and F1 larch (l), and *Lactarius hatsudake* and Japanese larch (m).

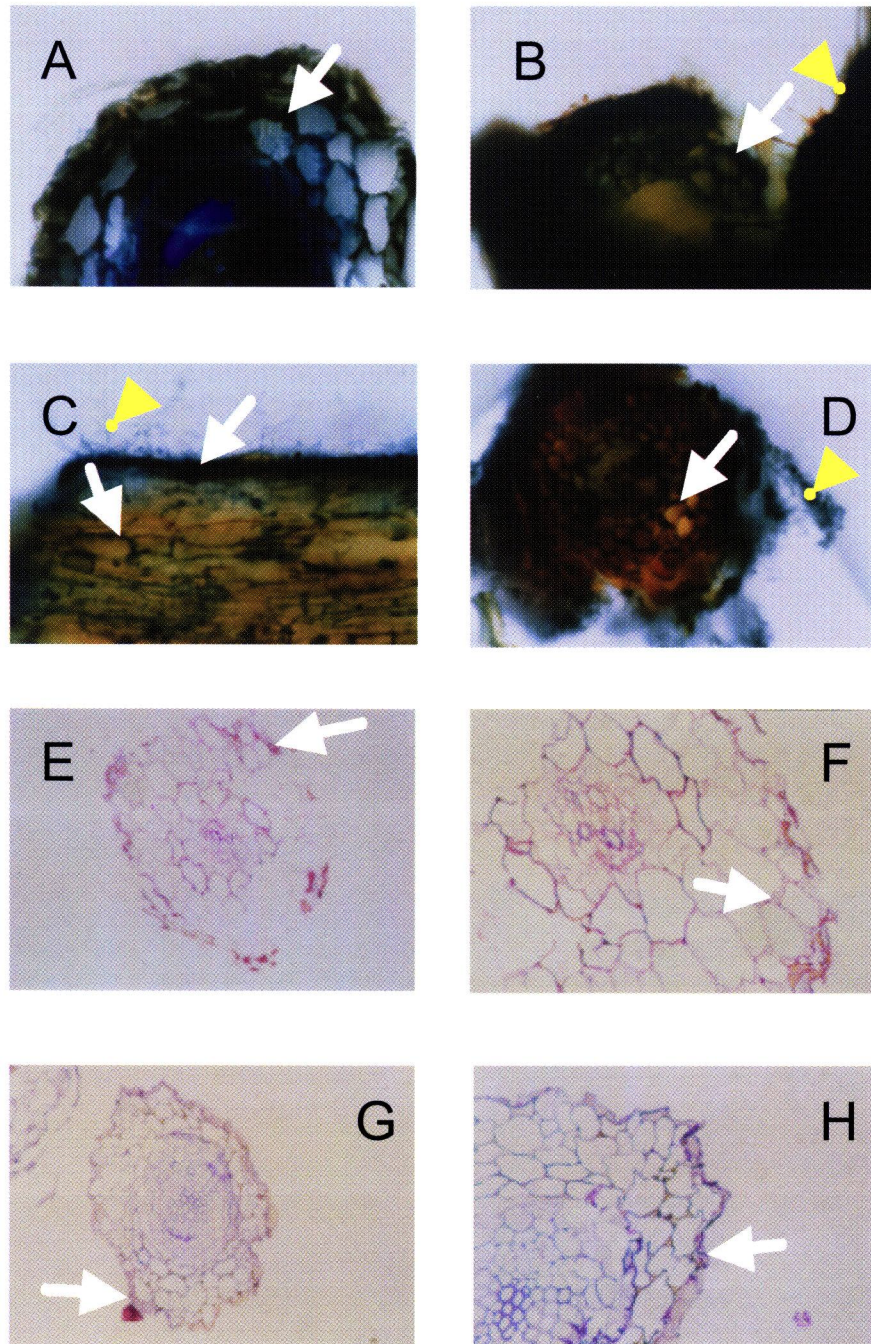


Fig. 4. Light microscopic view showing anatomical features of ectomycorrhizae formation. Fig. A. Transverse section (100x) of short roots from inoculated *Lactarius hatsudake* and Japanese larch, showing the dark color outer mantle and the arrow indicates Hartig net hyphae. Fig. B. Showing a thick mantle (100x) of *Cenococcum geophilum* with distinct Hartig net (arrow) and partially separated mantle (yellow arrowhead) with septate mycelia. Fig. C. Longitudinal section of roots from *Suillus grevillei* and Japanese larch mycorrhizae (100x) showing hyphal growth from the mantle (yellow arrowhead), a well developed mantle surface, and Hartig net formation. Fig. D. *Tricholoma saponaceum* - F1 larch, showing a broken part of mantle surface (yellow arrowhead) and formation of Hartig net (arrow). Figs. E-H. Transverse section taken by using glasscutter from roots of *Suillus grevillei* - Japanese larch (E, F) and *Lactarius hatsudake* F1 larch (G, H) ectomycorrhizae, showing the penetration by the Hartig net and thin mantle surface (arrows).