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ORIGINAL PAPER

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Cultivation of mushrooms of edible ectomycorrhizal fungi associated with *Pinus densiflora* by in vitro mycorrhizal synthesis

II. Morphology of mycorrhizas in open-pot soil

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Abstract The morphology and anatomy of ectomycorrhizas of edible mushroom fungi in association with *Pinus densiflora* seedlings are described. These include species of *Lyophyllum*, *Tricholoma*, *Suillus*, *Rhizopogon*, and *Lactarius*. Almost all mycorrhizas synthesized in vitro could be acclimatized in open-pot soil conditions after 8–9 months. Although mycorrhizal anatomy was almost identical under in vitro and open-pot culture conditions, external morphology, such as the development of rhizomorphs and hydrophobic aerial hyphae, differed between the two conditions in some fungal species. Fully developed, mature mycorrhizas of different fungal species could be distinguished as ectomycorrhizal morphotypes, which could also be distinguished by PCR-RFLP analysis of their rDNA.

Keywords Edible mushrooms · Ectomycorrhizas · Cultivation · In vitro synthesis · Basidiocarp

Introduction

Morphological descriptions of ectomycorrhizas are critical for understanding the mycorrhizal association. The inner tissues of ectomycorrhizas, i.e. the Hartig net structure, are important for the intercellular symbiosis of the mycorrhiza. In particular, it is the key characteristic determining plant–fungus specificity (Smith and Read 1997). On the other hand, outer tissue structures, e.g. fungal sheath, rhizomorph, and extraradical mycelium, are largely determined by the causal fungi and are stable within a fungal species (Godbout and Fortin 1985; Agerer 1991). Thus, the plan view of ectomycorrhizas is important for identifying fungi at the species or higher taxonomic levels (Agerer 1995). Comprehensive morphological description of ectomycorrhizas, including both outer and inner structures, has been conducted exclusively for naturally formed mycorrhizas under field conditions (Agerer 1987–1998; Ingleby et al. 1990; Goodman et al. 1996–1998; Agerer et al. 1996–1998).

Two-member culture systems of mycorrhizas, i.e. in vitro mycorrhizal synthesis, have been used in various physiological, biochemical, and nutritional studies of ectomycorrhizas (Varma 1998). They have also been used in the study of mycorrhizal morphogenesis (Massicotte et al. 1990; Ditengou and Lapeyrie 2000). To understand the relationship between structure and function of ectomycorrhizas in nature, it is necessary to compare natural mycorrhizas to those synthesized in vitro. Massicotte et al. (1994, 1999) and Molina and Trappe (1994) studied mycorrhizas of 24 species within the genus *Rhizopogon* in association with various Pinaceae hosts using in vitro syntheses and open-pot cultures. Overall, the specificity of mycorrhization was consistent regardless of the method used and followed that in the natural field condition, i.e. the ecological specificity (Massicotte et al. 1994; Molina and Trappe 1994). Furthermore, mycorrhizal morphology was comparable between species, which also reflects the taxonomic relationship of the sporocarp (Massicotte et al. 1994, 1999; Molina and Trappe 1994).

We previously reported basidiocarp and primordium formation of edible, ectomycorrhizal fungi (*Lyophyllum*, *Tricholoma*, *Suillus*, *Rhizopogon*, *Lactarius*) in mycorrhizal association with *Pinus densiflora* Sieb. et Zucc. seedlings in open-pot soil cultures following acclimatization of in vitro mycorrhizal syntheses (Yamada et al., unpublished data). Morphological descriptions of these mycorrhizas are required for both cultivation studies of

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edible mycorrhizal fungi and taxonomic studies of ectomycorrhizal morphotyping. Comparison of the morphology of mycorrhizas synthesized in vitro and those formed in open-pot cultures provide information about the mycorrhizal morphogenesis of these fungi, as has been suggested for other mycorrhizas (Massicotte et al. 1994, 1999; Molina and Trappe 1994; Agerer 1995).

Materials and methods

Mycorrhiza preparation

Mycorrhizas used in the present study were either synthesized in vitro or produced in open-pot culture conditions in association with *P. densiflora* seedlings (Yamada et al. unpublished data). These fungi were *Lyophyllum semitale* (Fr.) Kuhn., *Lyophyllum shimeji* (Kawam.) Hong, *Tricholoma flavovirens* (Pers.: Fr.) Lund., *T. portentosum* (Fr.) Quél., *T. saponaceum* (Fr.) Kummer, *Suillus granulatus* (L.: Fr.) O. Kunze, *S. luteus* (L.: Fr.) S.F. Gray, *S. bovinus* (L. :Fr.) O. Kunze, *Rhizopogon rubescens* Tul., *Lactarius hatsudake* Tanaka, and *Lactarius akahatsu* Tanaka.

Morphological and anatomical observation of ectomycorrhizas

The external morphology of mycorrhizas formed in vitro and in open-pot soil was examined and photographed under a dissecting microscope (Olympus BH11). Further microscopic observations were made using a Leica DMRBE and differential interference and fluorescent microscopic investigations were conducted (Yamada et al. 1999a). To confirm ectomycorrhiza formation, transverse sections of each fungal species were prepared and examined microscopically. Descriptions of mycorrhiza macromorphology followed Goodman et al. (1996–1998). The terms of Ingleby et al. (1990) were used for descriptions of fungal sheath texture. Other morphological and anatomical descriptions followed Agerer (1987–1998).

PCR-RFLP analysis of mycorrhizas

DNA analyses of mycorrhizas were carried out (1) to ensure that mycorrhizas forming in open-pot soil were identical with the inoculated fungus and (2) to characterize the mycorrhizas of the tested fungi. The targeted DNA was the internal transcribed spacer region (ITS) of the rDNA, which was analyzed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) according to Gardes and Bruns (1993). Cultured mycelium from MNC agar (Yamada et al. unpublished data) and 10–20 mycorrhizal tips were washed with distilled water and stored at –80°C until used in PCR-RFLP analyses.

DNA extraction: fungal DNA was extracted from a 1×1-cm piece of the mycelium grown on MNC agar according to Gardes and Bruns (1993). For mycorrhizas, ca. 10 tips were used. Extracted DNA was dissolved in 0.1× TE buffer (1 mM Tris-HCl, 0.1 M EDTA, pH 8.0). An aliquot of the extracted DNA was diluted 100-fold with sterile, double-distilled water and 1 µl of the diluted DNA solution was used as template DNA in the reaction mixture (50 µl) for PCR.

PCR amplification: we employed the primer pair ITS1F/ITS4B, since these primers specifically function with basidiomycetes (Gardes and Bruns 1993). The samples were run in a Perkin-Elmer DNA Thermocycler (model 9700) according to the following cycling parameters: initial denaturation for 3 min at 95°C, followed by 30 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 55°C, and extension for 90 s at 72°C. The cycling was ended by an extension phase for 10 min at 72°C. Negative controls (no DNA template) were run for each experiment to check for DNA contamination of the reagents. The amplified PCR products were separated by electrophoresis for 1 h at 100 V (Mupid-2, Advance Inc.) on 10×6×0.5-cm gels of 3% NuSieve 3:1 agarose (FMC BioProducts Inc.) in 0.5× Tris-borate buffer (44.5 mM Tris, 44.5 mM borate, 1 mM EDTA, pH 8.0) and stained with 1 µg/ml ethidium bromide for 20 min. When only one DNA band was present in a PCR product, the product was used for RFLP analysis after estimation of its length.

RFLP analysis: aliquots (2 µl) of PCR product solution were digested separately with *Alu*I, *Hinf*I, *Rsa*I *Hae*III, and *Taq*I according to the manufacturer's recommendations (TaKaRa Biochemicals Inc.). The fragments were separated by electrophoresis for 45 min–4 h on 3–4% agarose gels. A 100-bp ladder marker (100–1,000 bp; Superladder Low, GenSura Laboratories Inc.) was used to determine the sizes of fragments larger than 500 bp and a 20-bp ladder marker (20–1,000 bp) was used for fragments smaller than 500 bp. Each PCR product was digested at least twice with each enzyme to confirm the stability of the digestion pattern and the precision of fragment sizes. The gels were stained with ethidium bromide, photographed under ultraviolet light and scanned using a flat-bed scanner at a resolution of 300 ppi. The scanned picture was expanded using Adobe Photoshop (Adobe Systems Inc.) to aid in calculating the fragment sizes. The fragment sizes were determined following a weight curve based on the pattern of co-electrophoresed markers. The maximal resolution of the calculated fragment sizes by this method was 5 bp.

Results

Morphological and anatomical description of ectomycorrhizas

Mycorrhizas synthesized in vitro and developed in open-pot soil were examined microscopically to determine whether interspecific characteristics corresponded to a particular mycorrhizal morphotype. As mycorrhizal morphology within a species/isolate may vary with substrate and other external environmental conditions, the descriptions reported were based exclusively on mycorrhizas

Figs. 1–4 Micrographs of mycorrhizas showing external morphology (Figs. 1, 5, 8, 11, 15, 18, 22, 23, 27, 31, 35, 36, 41). Differential interference (Nomarski) micrographs are shown in Figs. 2–4, 6, 7, 9, 10, 12–14, 16, 17,19–21, 24–26, 28–30, 32–34, 37–40, 42–44; *bars* 20 µm (*C* cortical cell, *En* endodermal cell, *Ep* epidermal cell, *H* Hartig net hyphae, *N* nucleus, *S* fungal sheath) ▶

Figs. 1–4 *Lyophyllum semitale* mycorrhizas

Fig. 2 Surface view of the fungal sheath showing hyphae with clamp connections (*arrow*)

Fig. 3 Transverse section of the fungal sheath showing interhyphal gelatinous material

Fig. 4 Labyrinthine Hartig net hyphae at the innermost area of the root cortex

Figs. 5–7 *Lyophyllum shimeji*

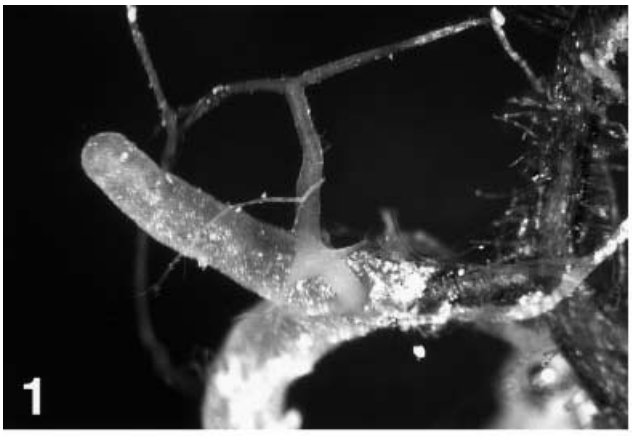
Fig. 5 Mycorrhizas

Fig. 6 Surface view of the fungal sheath

Fig. 7 Transverse section of a mycorrhizal root with one- to two-layered Hartig net hyphae at the root cortex

Figs. 8–10 *Tricholoma flavovirens*

Fig. 8 Mycorrhizas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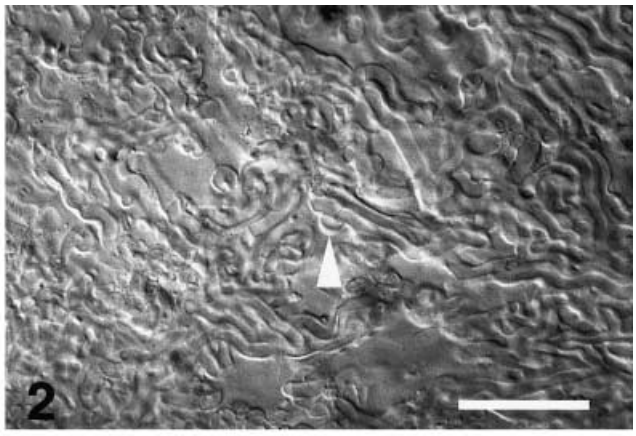
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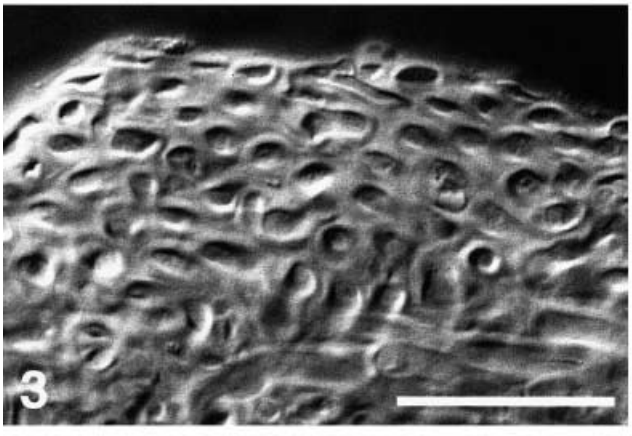
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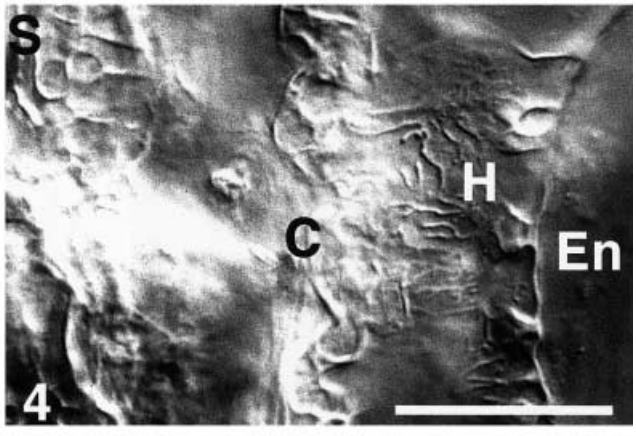
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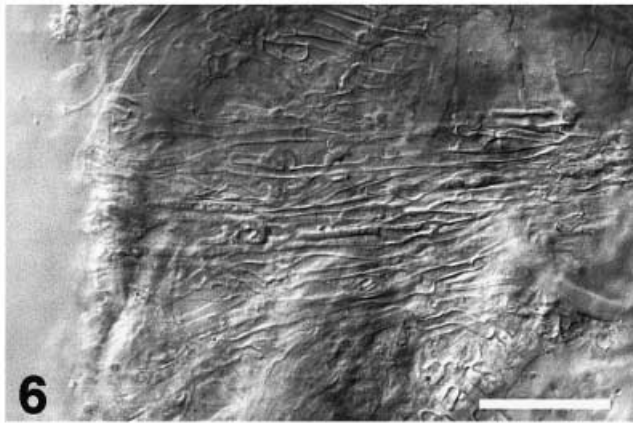
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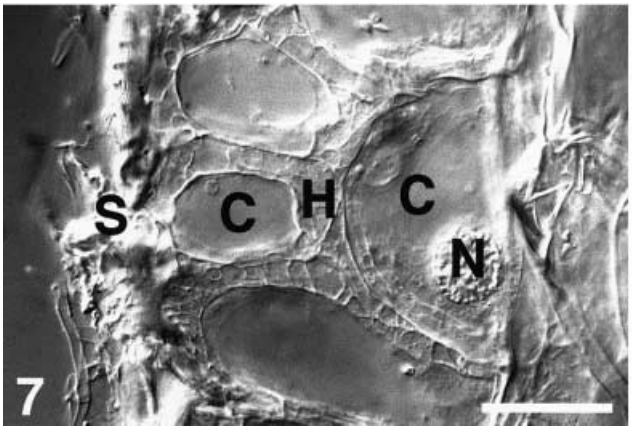
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developed in open-pot soil. Mycorrhizal root systems were mostly dichotomously branched and mycorrhizal tips were 0.4–0.8 mm in diameter and 0.4–3.0 mm in the length of the branched end.

Lyophyllum semitale

Macromorphology: mycorrhizal tips with distinct fungal sheath and rhizomorphs attached at restricted points, developing up to 0.3 mm in width (Fig. 1); mycorrhiza surface white or semi-transparent, smooth, and matte or sometimes shiny; no color change observed when the fungal sheath was cut; characteristics mostly the same for mycorrhizas synthesized in vitro.

Micromorphology and anatomy: few emanating hyphae connecting with the fungal sheath straight, 1.5–4 µm in diameter with clamp connections; rhizomorphs constructed from thick-walled hyphae, up to 5 µm in diameter, some of which had clamp connections, types B and C in texture; surface layer of fungal sheath net synenchymatous, with intercellular gelatinous material, constructed hyphae 2–8 µm in diameter (Figs. 2, 3); inner layer of fungal sheath mostly the same as the surface layer but relatively straight in hyphal arrangement; fungal sheath 15–50 µm thick; Hartig net developed continuously at the cortex of roots, hyphae 0.7–2 µm in diameter (Fig. 4); cortical cells sometimes brownish and opaque in the cytoplasmic region.

Lyophyllum shimeji

Macromorphology: mycorrhizal tips with distinct fungal sheath and rhizomorphs attached as hyphal fans but not conspicuously developed (Fig. 5); mycorrhiza surface white, felty or smooth, shiny or reflective; no color change observed when the fungal sheath was cut; mycorrhizas synthesized in vitro had a thin fungal sheath; ectomycorrhiza formation confirmed by microscope examination of transverse section.

Micromorphology and anatomy: emanating hyphae connecting with the fungal sheath present straight, 1–2 µm in diameter, with clamp connections; rhizomorphs type A in texture; surface layer of fungal sheath net prosenchymatous, constructed hyphae 1–3 µm in diameter (Fig. 6); inner layer net synenchymatous, constructed hyphae 1–3 µm; fungal sheath relatively thin, 5–10 µm; Hartig net developed continuously at the cortex of the root, hyphae 1.5–3 µm in diameter (Fig. 7).

Tricholoma flavovirens

Macromorphology: mycorrhizal tips with distinct fungal sheath and rhizomorphs attached at flat angles and sometimes developed (Fig. 8); mycorrhizal surface white, felty, reflective; no color reaction when the fungal sheath was cut; characteristics mostly the same for mycorrhizas synthesized in vitro but external mycelia less abundant under in vitro conditions.

Micromorphology and anatomy: emanating hyphae connecting with the fungal sheath developed, straight, 2–5 µm in diameter, without clamp connections; rhizomorphs type B in texture; surface layer of fungal sheath net prosenchyma, constructed hyphae 2–8 µm in diameter with occasional fine reddish-brown crystals; middle and inner layers net synenchyma with brilliant indefinite plate-like crystals (Fig. 9); fungal sheath 25–40 µm thick; Hartig net developed continuously at the cortex of roots, hyphae 1.5–3 µm in diameter (Fig. 10).

Tricholoma portentosum

Macromorphology: mycorrhizal tips with distinct fungal sheath and rhizomorphs attached at flat angles and sometimes developed (Fig. 11); mycorrhizal surface white, felty, reflective; no color change when the fungal sheath was cut; characteristics mostly the same for mycorrhizas synthesized in vitro, but the external mycelium less abundant under in vitro conditions.

Micromorphology and anatomy: emanating hyphae connecting with fungal sheath, straight, 1.5–3 µm in diameter with few clamp connections (Figs. 12, 13); rhizomorphs types B and C in texture, central hyphae up to 4.5 µm in diameter; surface layer of fungal sheath net prosenchyma to net synenchyma, constructed hyphae 1.5–3.5 µm in diameter; inner layer net synenchyma, constructed hyphae 2–3.5 µm in diameter, with amorphous, brilliant, polarized material (Fig. 12); fungal sheath 5–20 µm thick; Hartig net developed continuously at the cortex of roots, hyphae 1.5–3 µm in diameter (Fig. 14).

Tricholoma saponaceum

Macromorphology: mycorrhizal tips with distinct fungal sheath and rhizomorphs attached at flat angles, up to ca.

Fig. 9 Surface view of the fungal sheath ornamented with crystals (*arrows*) ▶

Fig. 10 Labyrinthine Hartig net hyphae at the root cortex

Figs. 11–14 *Tricholoma portentosum*

Fig. 11 Mycorrhizas

Fig. 12 Surface view of the fungal sheath ornamented with crystals

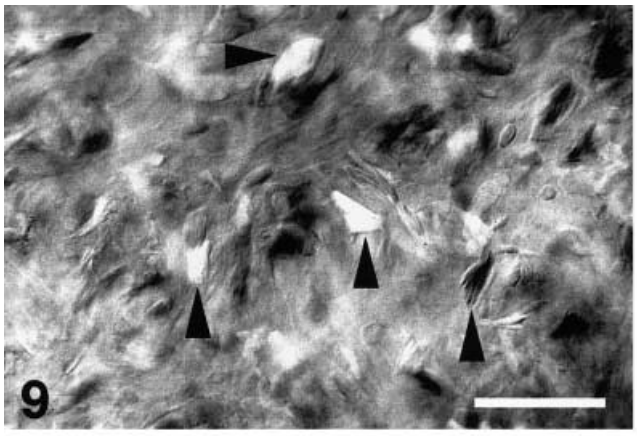
Fig. 13 Rhizomorphs with a clamp connection on a single hyphae (*arrow*)

Fig. 14 Transverse section of a mycorrhizal root with Hartig net hyphae at the root cortex

Figs. 15–17 *Tricholoma saponaceum*

Fig. 15 Mycorrhizas

Fig. 16 Inner layer of the fungal sheath with a star-like arrangement of brownish mycelium (*arrows*)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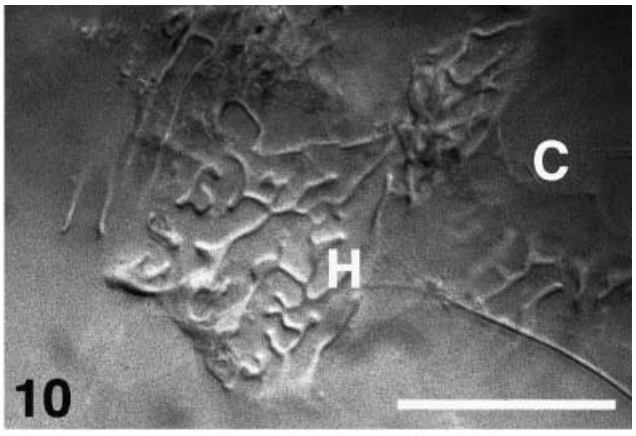
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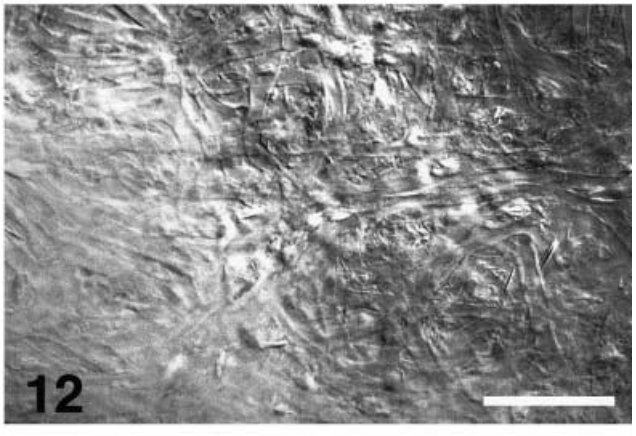
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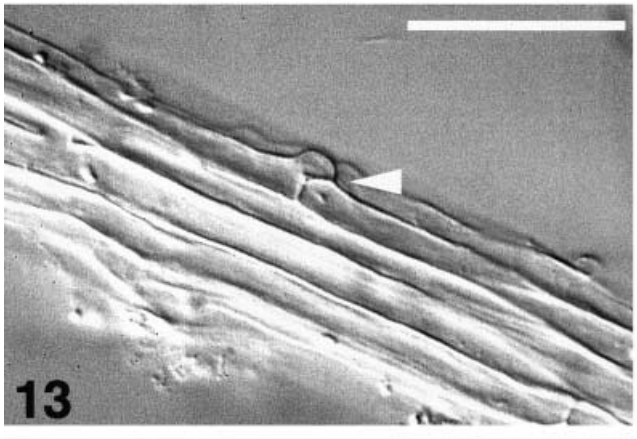
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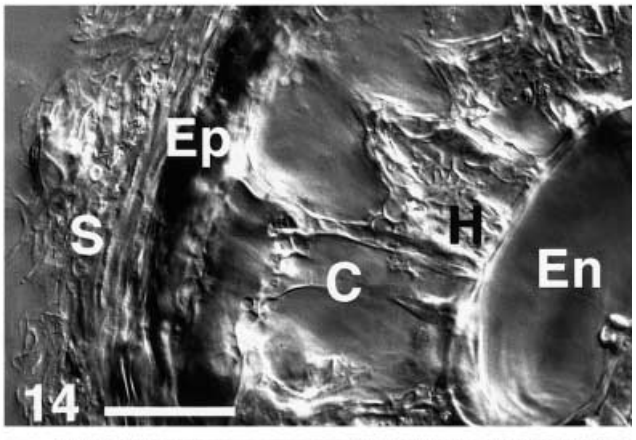
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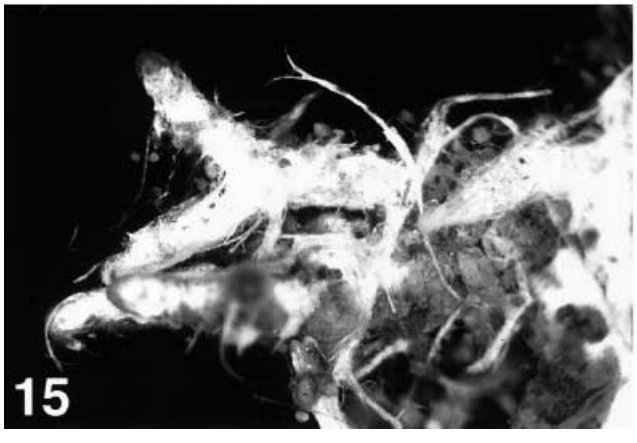
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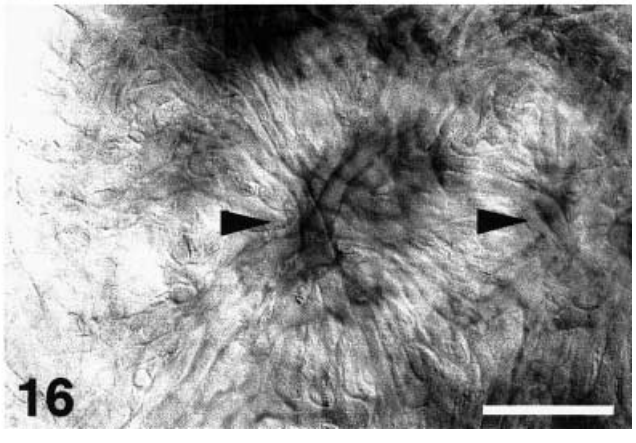
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0.5 mm in diameter (Fig. 15); mycorrhizal surface white, felty, reflective; no color change when fungal sheath was cut; characteristics mostly the same for mycorrhizas synthesized in vitro, but external mycelium less abundant under in vitro conditions.

Micromorphology and anatomy: emanating hyphae connecting with fungal sheath, straight, 2–11 µm in diameter with clamp connections; rhizomorphs types C, E, and F in texture; surface layer of fungal sheath net prosenchyma to net synenchyma, constructed hyphae 2–10 µm in diameter; middle layer net synenchyma; inner layer net synenchyma with a star-like arrangement, brown coloring of the hyphal cell wall in the center (Figs. 16, 17); fungal sheath 20–40 µm thick; Hartig net developed continuously at the cortex of root, hyphae 2–4 µm in diameter (Fig. 17).

Suillus granulatus

Macromorphology: mycorrhizal tips with distinct fungal sheath and rhizomorphs attached at restricted points, up to 0.2 mm in width, well branched with undifferentiated mycelium and external mycelium (Fig. 18); external mycelium white or yellowish-white, pale brown in older parts, felty or smooth, matte or shiny; surface of mycorrhizas sometimes sticky; no color change when fungal sheath was cut; white mycelium turned pinkish and yellowish and brownish mycelium turned reddish-brown in 10% KOH; characteristics mostly the same for mycorrhizas synthesized in vitro, but development of external mycelium less abundant under in vitro conditions.

Micromorphology and anatomy: emanating hyphae connecting with fungal sheath, straight, 1–4 µm in diameter with no clamp connections; rhizomorphs types C and F in texture, central thick hyphae with thick cell walls, reaching 10.5 µm in diameter; clamp connections rarely observed on single hyphae in the rhizomorphs, especially on the anastomosing hyphae; surface layer of fungal sheath net prosenchyma to net synenchyma, constructed hyphae 2–4 µm in diameter with intercellular crystals and oily droplets (Figs. 19, 20); crystals fine, linear in shape, parallel to the hyphal arrangement; oily droplets brownish, turning dark purple in 10% KOH; crystals and oily droplets also present on surfaces of external hyphae and rhizomorphs; inner layer of fungal sheath net synenchyma, constructed hyphae 2–4 µm in diameter; fungal sheath 20–50 µm thick; Hartig net developed continuously at the cortex of roots, hyphae 1–3 µm in diameter (Fig. 21).

Suillus luteus

Macromorphology: mycorrhizal tips with distinct fungal sheath and rhizomorphs attached at restricted points, developing up to 0.2 mm in width, well branched with undifferentiated mycelium; mycorrhizal root system formed a coralloid cluster (Fig. 22); carbonization sometimes observed on mycorrhizal tips (Fig. 23); exterior white or yellowish white, pale brown in older parts, felty or smooth, matte or shiny; surface of mycorrhizas sometimes sticky; no color change when fungal sheath was cut; color reaction to 10% KOH negligible; characteristics mostly the same for mycorrhizas synthesized in vitro, but development of external mycelium less abundant under in vitro conditions.

Micromorphology and anatomy: emanating hyphae connecting with fungal sheath, straight, 1.5–2.5 µm in diameter with no clamp connections; rhizomorphs types C and F in texture, central thick hyphae with thick cell walls reaching 20 µm in diameter; clamp connections rarely observed on single hyphae in rhizomorphs, especially on anastomosing hyphae: surface layer of fungal sheath net prosenchyma, constructed hyphae 2–4 µm in diameter containing intercellular crystals and oily droplets; crystals indefinite, plate-like, occurring randomly against hyphal arrangement (Fig. 24); oily droplets brownish, turning dark purple in 10% KOH; crystals and oily droplets also present on surfaces of external hyphae and rhizomorphs; middle layer of fungal sheath net synenchyma, constructed hyphae 4–12 µm in diameter; innermost layer of fungal sheath irregular synenchyma, constructed hyphae 4–12 µm in diameter (Fig. 25); fungal sheaths 30–60 µm thick; Hartig net developed continuously at the cortex of roots, hyphae 1–3 µm in diameter (Fig. 26).

Suillus bovinus

Macromorphology: mycorrhizal tips with distinct fungal sheath and rhizomorphs attached at flat angles or at restricted points, developed up to 0.3 mm in width, well branched with undifferentiated mycelium and external

Fig. 17 Transverse section of a fungal sheath with brownish mycelium (*arrows*) presenting a star-like arrangement in plane view (see Fig. 16) ▶

Figs. 18–21 *Suillus granulatus*

Fig. 18 Mycorrhizas

Fig. 19 Surface view of the fungal sheath with brownish, oily droplets (*arrows*)

Fig. 20 Surface view of the fungal sheath ornamented with fine crystals oriented along the hyphae

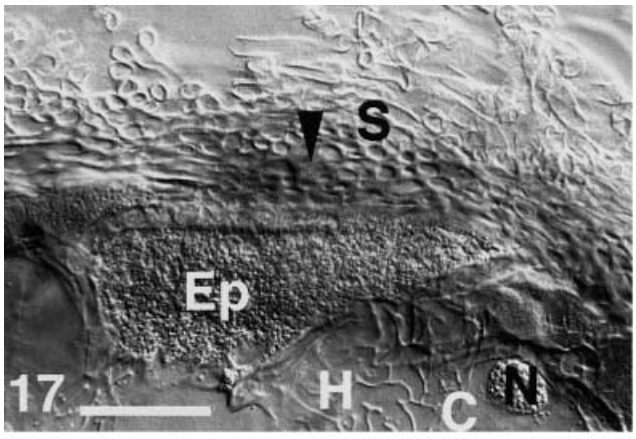
Fig. 21 Transverse section of a mycorrhizal root with Hartig net hyphae at the root cortex

Figs. 22–26 *Suillus luteus*

Fig. 22 Mycorrhizas

Fig. 23 Transverse section of some mycorrhizal root tips showing carbonization at the root cortex (*arrows*)

Fig. 24 Surface view of the fungal sheath with plate-shaped crystals oriented at random against the hyphae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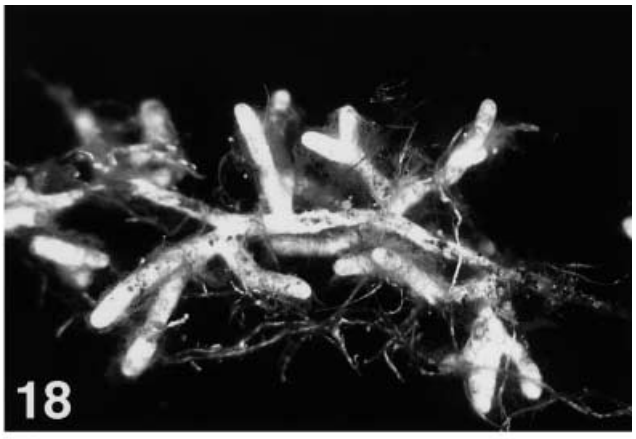
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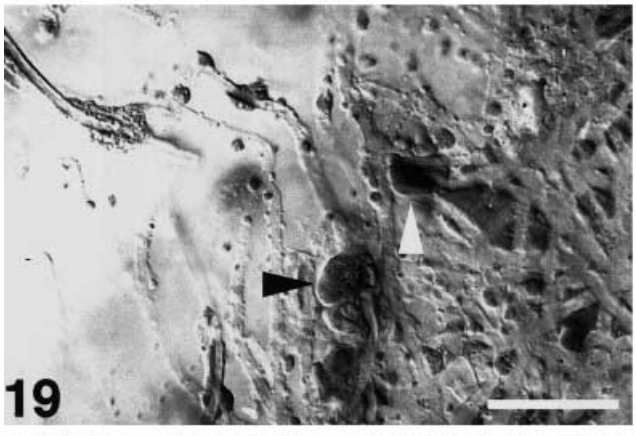
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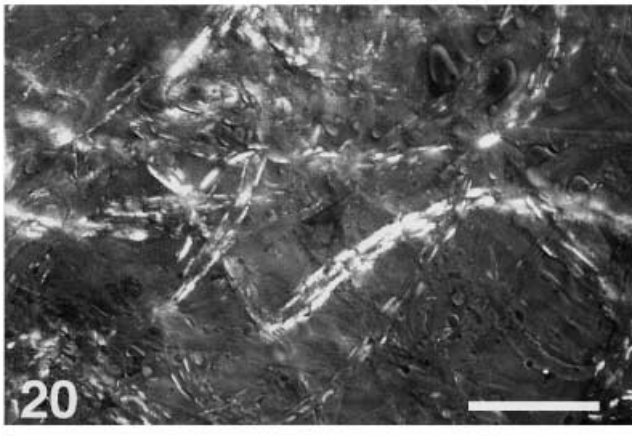
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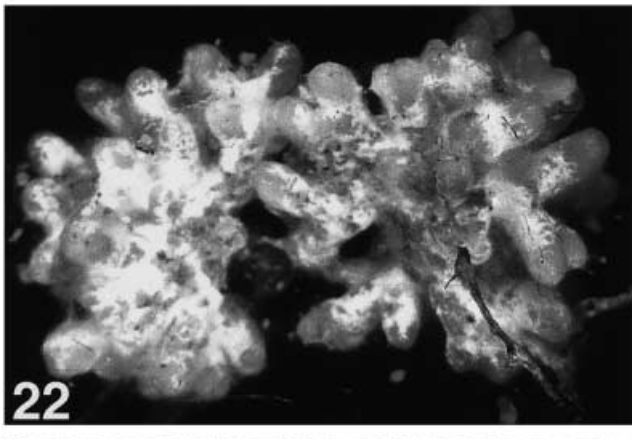
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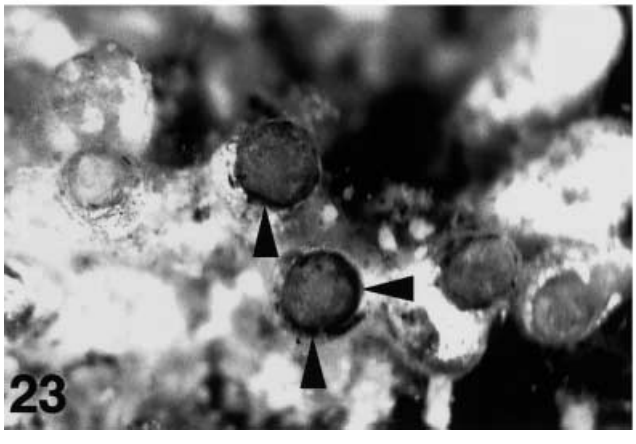
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mycelium (Fig. 27); external mycelium white or yellowish-white, pale brownish in older parts, felty or smooth, matte or shiny; surface of mycorrhizas sometimes sticky; no color change when fungal sheath was cut; white mycelium turned reddish-brown, pale brown sectors turned dark brown in 10% KOH; characteristics mostly the same for mycorrhizas synthesized in vitro, but development of external mycelium less abundant under in vitro conditions.

Micromorphology and anatomy: emanating hyphae connecting with fungal sheath, well developed and straight, 2–4 µm in diameter with no clamp connections; rhizomorphs types D and E in texture, central thick hyphae of rhizomorphs reaching up to 8 µm in diameter; surface layer of fungal sheath net prosenchyma, constructed hyphae 2–5 µm in diameter with intercellular crystals and oily droplets (Figs. 28, 29); crystals indefinite, plate-like, or petaloid, occurring randomly against hyphal arrangement; oily droplets brownish, turning dark purple in 10% KOH; crystals and oily droplets also present on surfaces of external hyphae and rhizomorphs; innermost layer of fungal sheath irregular synenchyma, constructed hyphae 2–6 µm in diameter; fungal sheaths 20–80 µm thick; Hartig net developed continuously at cortex of root, hyphae 1.5–3 µm in diameter (Fig. 30).

Rhizopogon rubescens

Macromorphology: mycorrhizal tips with distinct fungal sheath and rhizomorphs attached at restricted points, up to 0.2 mm in width and well branched, with undifferentiated mycelium and external mycelium (Fig. 31); carbonization rarely observed on mycorrhizal tips; mycelium white or yellowish-white, pale brown in older parts, felty or smooth, matte or shiny; surface of mycorrhizas sometimes sticky; no color change observed when fungal sheath was cut; mycelium turning purplish-brown in 10% KOH; characteristics mostly the same for mycorrhizas synthesized in vitro, but development of external mycelium less abundant under in vitro conditions.

Micromorphology and anatomy: emanating hyphae connecting with fungal sheath, developed, straight, 1.5–4.5 µm in diameter, without clamp connections; rhizomorphs types C and F in texture, central thick hyphae with thick cell walls reaching 10 µm in diameter; clamp connections rare on single hyphae in rhizomorphs; surface layer of fungal sheath net prosenchyma, constructed hyphae 2–4 µm in diameter with crystals and intercellular mucilaginous material (Figs. 32, 33); crystals indefinite or plate-like, sometimes present at high density, occurring randomly against hyphal arrangement; mucilaginous material deposited around hyphae, forming outermost surface of sheath, but neither a distinct oily drop or a vertical layer; mucilaginous material transparent or brownish, turning dark purple in 10% KOH; both crystals and mucilaginous material also present on surfaces of external hyphae and rhizomorphs; innermost layer of fungal sheath irregular synenchyma, constructed hyphae 2–5 µm in diameter; fungal sheaths 30–50 µm thick; Hartig net developed continuously at cortex of roots, hyphae 1–3 µm in diameter (Fig. 34).

Lactarius akahatsu

Macromorphology: mycorrhizal tips with distinct fungal sheath and rhizomorphs attached at restricted points, developed up to 0.2 mm in width (Figs. 35, 36); mycelium pale ocher or pale orange-ocher, smooth, matte or sometimes shiny; fungal sheath turning green when cut; characteristics mostly the same for mycorrhizas synthesized in vitro.

Micromorphology and anatomy: few emanating hyphae connecting with fungal sheath, straight, 1.5–2.5 µm in diameter without clamp connections; rhizomorphs types C and E in texture, central thick hyphae with thick cell walls reaching 9 µm in diameter; surface hyphae of rhizomorphs with thick cell walls up to 1.5 µm diameter, irregularly branched (Figs. 37, 38); surface layer of fungal sheath net prosenchyma or net synenchyma, constructed hyphae 1.5–3 µm in diameter, embedded in gelatinous material (Fig. 37); middle layer of fungal sheath net synenchyma, constructed hyphae 2–4 µm in diameter; laticiferous hyphae present in this layer, 2–6 µm in diameter, turning black in sulfovaniline (Fig. 39); inner layer net synenchyma or irregular synenchyma, 3–10 µm in diameter; fungal sheaths 20–50 µm thick; Hartig net developed continuously at cortex of roots, hyphae 2.5–4.5 µm in diameter (Fig. 40).

Lactarius hatsudake

Macromorphology: mycorrhizal tips with distinct fungal sheath, connected rhizomorphs attached at restricted

Fig. 25 Inner layer of the fungal sheath ▶

Fig. 26 Transverse section of a mycorrhizal root with Hartig net hyphae at the root cortex

Figs. 27–30 *Suillus bovinus*

Fig. 27 Mycorrhizas

Fig. 28 Surface view of the fungal sheath with brownish, oily droplets (*arrows*)

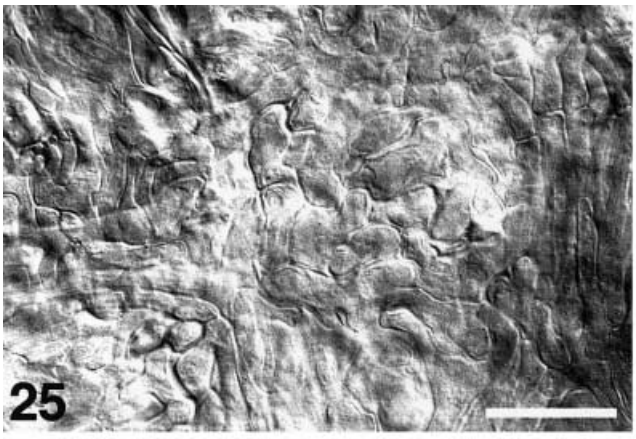
Fig. 29 Surface view of the fungal sheath with plate-shaped crystals oriented at random against the hyphae

Fig. 30 Transverse section of a mycorrhizal root with Hartig net hyphae at the root cortex

Figs. 31–34 *Rhizopogon rubescens*

Fig. 31 Mycorrhizas

Fig. 32 Surface view of the fungal sheath with brownish extraradical mycelium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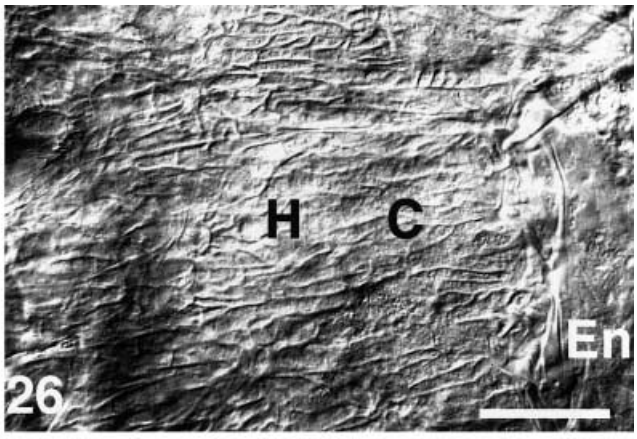
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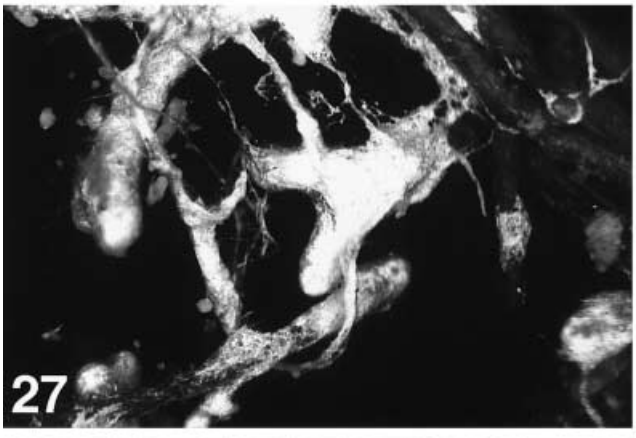
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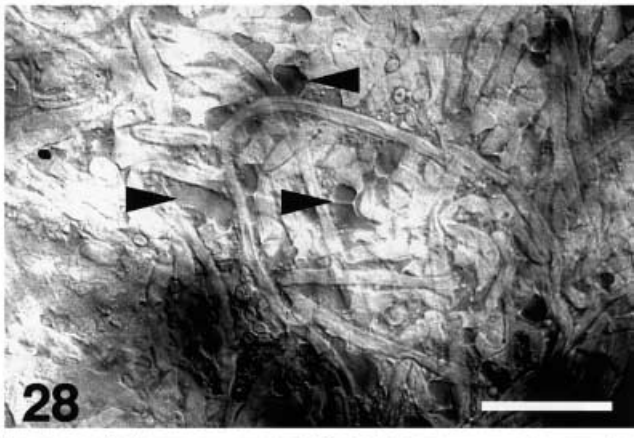
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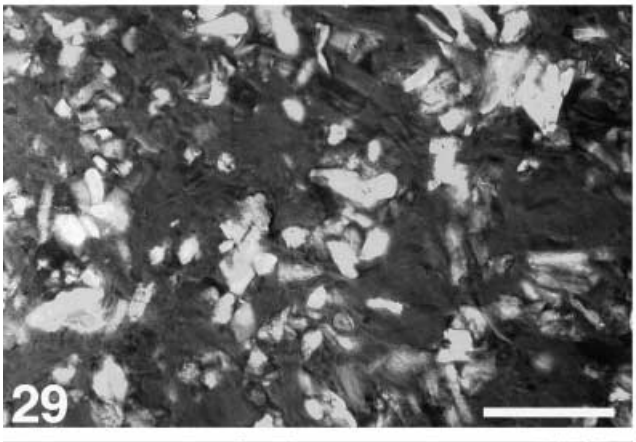
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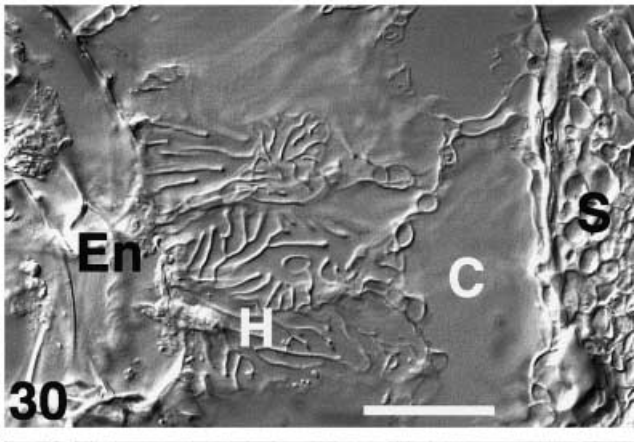
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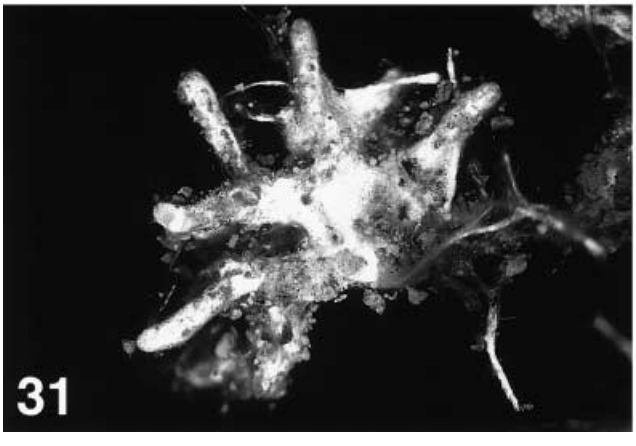
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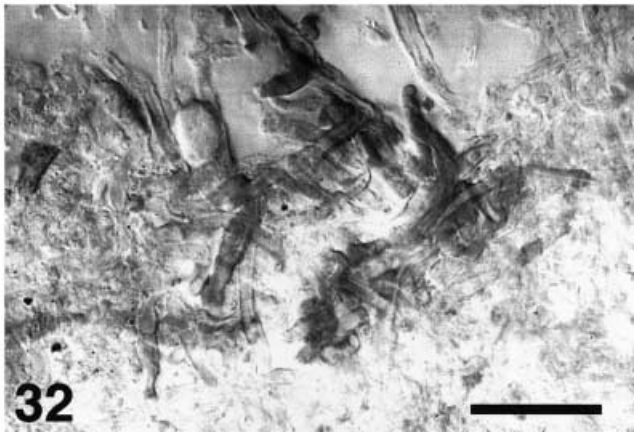
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Table 1 Comparison of the morphological characteristics of mycorrhizas (– absent, + present, ++ well developed, * red-brown crystals)

Family	Species	Macroscopic					Microscopic		
		External color	Coralloid branching of mycorrhizas	Rhizomorph development	External texture	Carbonization of root cortex	Clamp connections	Crystals	Laticiferous hyphae
(Tricholomataceae)									
	Lyophyllum semitale	Opaque	–	++	Reflective	–	+	–	–
	L. shimeji	White, opaque	–	+	Felty	–	+	–	–
	Tricholoma saponaceum	White	–	++	Felty	–	+	–	–
	T. flavovirens	White	–	++	Felty	–	–	Plate-like	–
	T. portentosum	White	–	++	Felty	–	–	(+)*	–
(Boletaceae)									
	Suillus granulatus	White, pale brown	–	++	Felty, sticky	–	–	Needle-like	–
	S. luteus	White, pale brown	+	++	Felty, sticky	+	–	Plate-like	–
	S. bovinus	White, pale brown	–	++	Felty, sticky	–	–	Plate-like	–
	Rhizopogon rubescens	White, pale brown	–	++	Felty, sticky	+	–	Plate-like	–
(Russulaceae)									
	Lactarius akahatsu	Ocher, yellow ocher	–	++	Reflective	–	–	–	+
	L. hatsudake	Ocher, pale orange	–	+	Reflective	–	–	–	+

points and developed up to 0.1 mm in width (Fig. 41); mycelium pale yellow-ocher or pale ocher, smooth, matte or sometimes shiny; fungal sheath turning green when cut; characteristics mostly the same for mycorrhizas synthesized in vitro, but rhizomorphs only slightly developed under in vitro conditions.

Micromorphology and anatomy: few emanating hyphae connecting with fungal sheath, straight, 1.5–3 µm in diameter without clamp connections; rhizomorphs types A and C in texture, central thick hyphae of rhizomorphs with thick cell walls and up to 4 µm in diameter; surface layer of fungal sheath net synenchyma, constructed hyphae embedded in gelatinous material, 1.5–3 µm in diameter (Fig. 42); middle layer of fungal sheath net synenchyma, constructed hyphae 2–4 µm in diameter; laticiferous hyphae present in this layer, 2–8 µm in diameter, turning black in sulfovaniline (Fig. 43); innermost layer net synenchyma and irregular synenchyma, constructed hyphae 2.5–10 µm in diameter; thickness of fungal sheaths 10–30 µm; Hartig net developed continuously at cortex of roots, hyphae 2–5 µm in diameter (Fig. 44).

These morphotypes were also compared within a genus. The macroscopic and microscopic characters are summarized in Table 1.

PCR-RFLP analysis of ectomycorrhizal fungi

All tested fungal materials produced a single PCR product (single band), ranging in size from 850 to 925 bp (Table 2). The RFLP patterns for harvested mycorrhizas and the inoculated mycelium were the same. In contrast, the digestion patterns of the different fungal species differed. No intraspecific variation was observed in the compared species, *T. flavovirens*, *R. rubescens*, and *Lactarius akahatsu*. Furthermore, there was no sequence polymorphism within any PCR product: the sum of the fragment sizes was the same or slightly smaller than the size of the original PCR product.

Fig. 33 Surface view of the fungal sheath with plate-shaped crystals oriented at random against the hyphae ▶

Fig. 34 Labyrinthine Hartig net hyphae at the root cortex

Figs. 35–40 *Lactarius akahatsu*

Fig. 35 Mycorrhizas

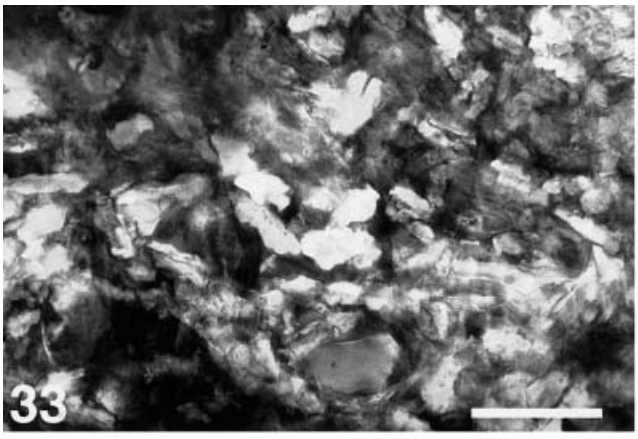
Fig. 36 Rhizomorphs (*arrows*) developed along the long root with mycorrhizal lateral root tips

Fig. 37 Surface view of the fungal sheath

Fig. 38 Surface view of the rhizomorph

Fig. 39 Laticiferous hyphae (*arrows*) in the fungal sheath

Fig. 40 Transverse section of a mycorrhizal root with Hartig net hyphae at the root cortex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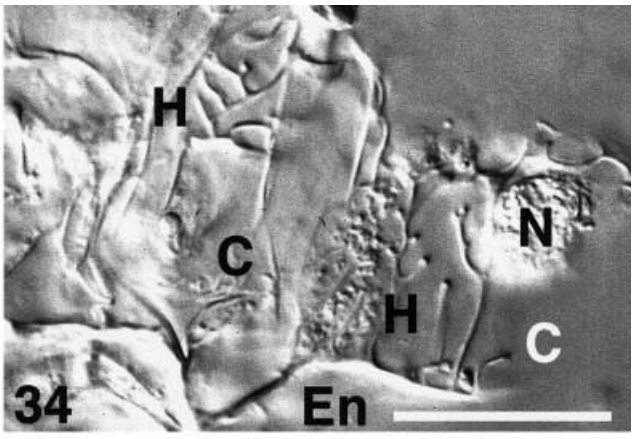
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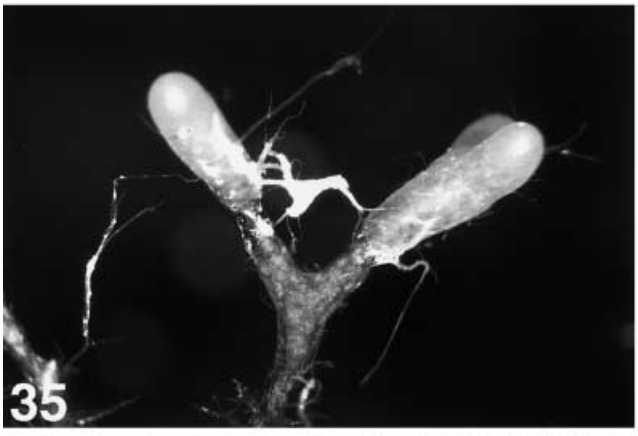
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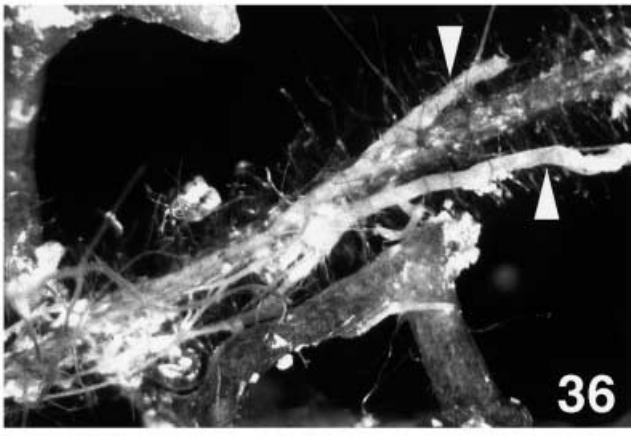
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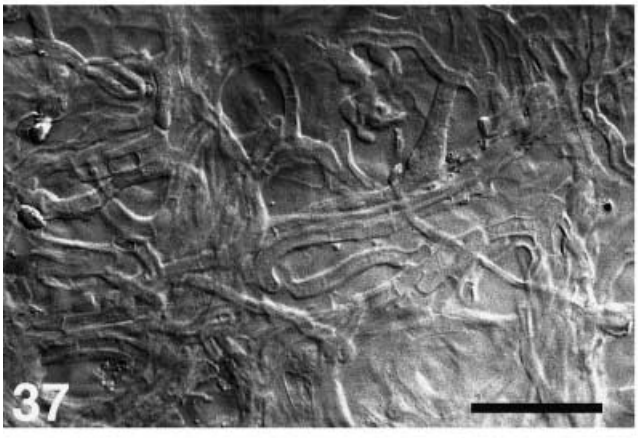
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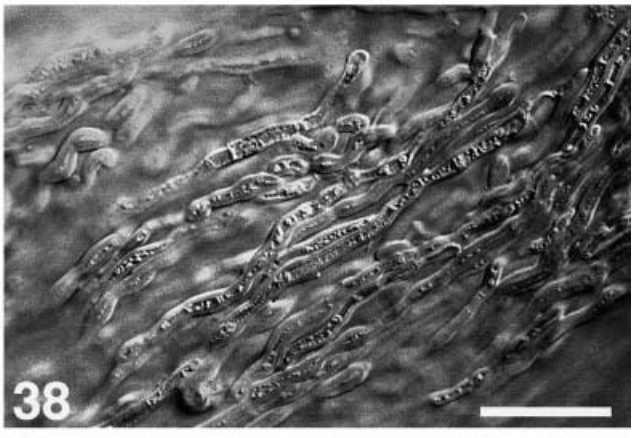
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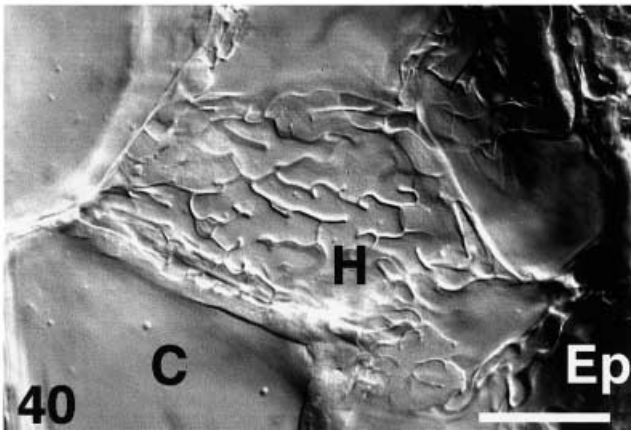
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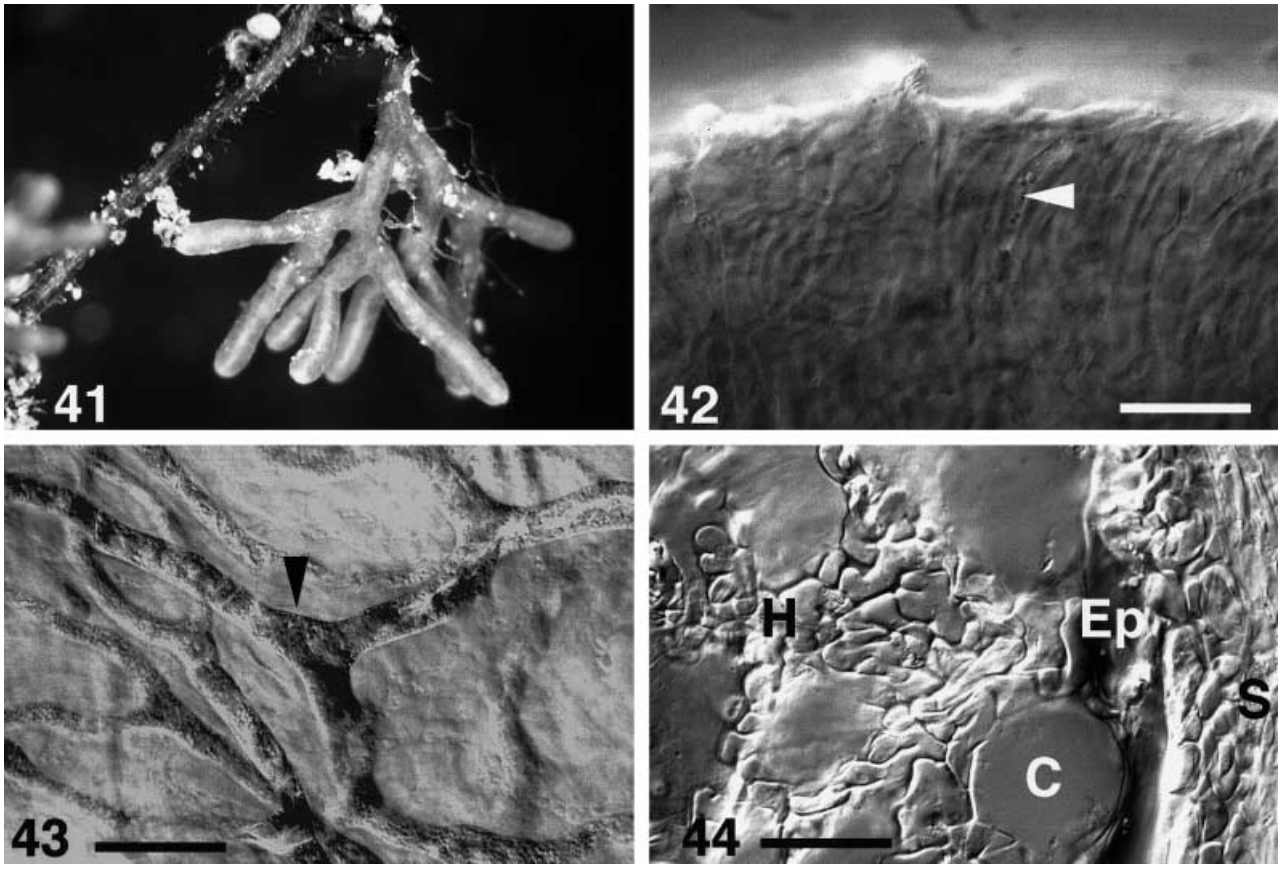
Table 2 RFLP patterns of the internal transcribed spacer (ITS) regions of the 11 fungal species tested

Species	Size of DNA (bp)			
	PCR product	Restriction enzyme		
		Hinf I	*Rsa* I	*Hae* III
Lyophyllum semitale	860	380, 340 125	850	515, 280, 60
L. shimeji	870	385, 350, 125	860	590, 280
Tricholoma flavovirens	910	390, 265, 125, 120	890	840, 60
T. portentosum	905	405, 350, 125	380, 250, 225	505, 315, 60
T. saponaceum	875	400, 345, 125	635, 225	875
Suillus granulatus	850	225, 205, 130, 80, 75, 75	830	450, 235, 110, 55
S. luteus	855	225, 210, 145, 80, 75, 75	835	445, 245, 110, 55
S. bovinus	870	235, 230, 145, 110, 80	845	700, 115, 55
Rhizopogon rubescens	895	250, 245, 130, 115, 80	870	800, 95
Lactarius hatsudake	895	365, 210, 120, 100, 90	815, 75	495, 330, 60
L. akahatsu	925	310, 205, 180, 120, 100	840, 75	515, 340, 60



Figs. 41–44 *Lactarius hatsudake*

Fig. 41 Mycorrhizas

Fig. 42 Surface view of the gelatinous fungal sheath with laticiferous hyphae (*arrow*)

Fig. 43 Laticiferous hyphae (*arrows*) in the fungal sheath

Fig. 44 Transverse section of a mycorrhizal root with Hartig net hyphae at the root cortex

Discussion

Mycorrhizas formed in open-pot soil had the characteristics of their respective fungal genera (Table 1). They had well-developed fungal sheaths and extraradical mycelium, including rhizomorphs, as well as the typical mycorrhizal anatomy of natural mycorrhizas of *P. densiflora* (Masui 1927; Yamada and Katsuya 1996; Yamada et al. 1999a). They were thus comparable to naturally formed mycorrhizas reported from other plants (Agerer 1987–1998; Agerer et al. 1996–1998; Goodman et al. 1996–1998).

Only rarely have *Lyophyllum* mycorrhizas been described microscopically (Kawai 1997; Agerer 1998; Agerer and Beenken 1998). Mycorrhizas between *Lyophyllum decastes* and *Quercus robur* L. have a well-developed fungal sheath (Agerer 1998; Agerer and Beenken 1998), similar to *Lyophyllum shimeji* (Fig. 5). Although the two mycorrhizas share some macroscopic characteristics, they are clearly different: only *Lyophyllum decastes* mycorrhizas develop well-differentiated rhizomorphs and have thin, straight crystals forming on the fungal sheath and rhizomorphs (Agerer 1998; Agerer and Beenken 1998). Kawai (1997) reported mycorrhizas between *Lyophyllum shimeji* and *P. densiflora* as having multi-layered thick Hartig net mycelium between root cortical cells. This anatomy must be an artifact, as suggested already by Agerer and Beenken (1998); the synthetic system used by Kawai contained a large amount of inoculated mycelium with grained oat substrate and nutrients. Our results consistently showed a thin Hartig net mycelium both in vitro and in open-pot soil conditions (Fig. 7). *Lyophyllum semitale* was quite different from the other *Lyophyllum* mycorrhizas, having a fungal sheath which was opaque and matte due to well-developed gelatinous material, similar to that reported for *Lactarius* spp. (Agerer 1995). The shape and attachment of the rhizomorphs was also unique and different to that of previously described *Lyophyllum* mycorrhizas (Agerer 1998; Agerer and Beenken 1998).

Tricholoma mycorrhizas in the present study had a well-developed external mycelium, i.e. a thick fungal sheath and rhizomorphs, similar to other *Tricholoma* mycorrhizas on various host plants (Agerer 1987a, b; Brand 1992; Agerer and Waller 1993; Nezzar-Hocine et al. 1998). However, they differed from *T. matsutake* on *P. densiflora* (Yamada et al. 1999a, b; Guerin-Laguette et al. 2000) and *T. magnivelare* (Peck) Redhead on *P. contorta* Dougl. var. *latifolia* Engelm. (Lefevre and Müller 1998), which had a thin, sometimes discontinuous fungal sheath. We also observed new characteristics of *Tricholoma* mycorrhizas: the two *Tricholoma* species tested had distinct crystals or crystal-like polarizing materials on and in the fungal sheath (Figs. 9, 12). Furthermore, *T. saponaceum* mycorrhizas had a unique fungal sheath inner layer, in which the central region of the star-like, radiating array of the mycelium consisted only of deposited brown-colored sections of the cell walls. Such a patchy deposition of pigments in the mycelium has been described previously for the middle layer of the fungal sheath of the unidentified mycorrhiza *Pinirhiza rufomaculata* on *Pinus sylvestris* L. (Wollecke et al. 1998a, b). The available information about *Tricholoma* mycorrhizas is indicative of a complex taxonomy, i.e. the mycorrhizas have diverse morphological and anatomical variation, as presented in this study and elsewhere, and unique DNA patterns in the retroelement encoding motif of retrotransposons (Murata et al. 1999; Murata and Yamada 1999, 2000). One of the primary points of morphology is the variation in thickness of the fungal sheath between sections and within the section Geinea of this genus (Singer 1986). There is also variation in the interface of the symbiotic cells, i.e. various types of fungal infection structures within the root cortical cells (Brand 1992; Yamada et al. 1999a) and a host reaction defined as carbonization (Agerer and Waller 1993; Lefevre and Müller 1998; Yamada et al. 1999a). Fungi exhibiting the latter character are limited to the section Geinea, although not all species within the section exhibit carbonization (Agerer 1987b). Furthermore, *T. portentosum* had distinct clamp connections on single hyphae of its rhizomorphs, which does not match the definition of the basidiocarp of the section *Tricholoma*, which lacks clamp connections (Singer 1986). Thus, it is difficult both to generalize on the complex taxonomic aspects of the mycorrhiza of *Tricholoma* and to address this fungal taxon in the course of a simple cultivation experiment (Yamada et al. 1999a, b).

Suilloid fungi, i.e. *Suillus* and *Rhizopogon*, had similar mycorrhizal morphologies (Table 1). Mycorrhizal associations of suilloid fungi are relatively well studied from the point of view of basic biology and forestry (Molina and Trappe 1994; Castellano 1996; Molina et al. 1997; Massicotte et al. 1994, 1999). Most of the morphological and anatomical characteristics observed in the present study were identical with the common characteristics of Suilloid fungi previously reported, i.e. a well-developed rhizomorph, hyphae turning brownish with age, crystals, and sometimes coralloid mycorrhizal branching patterns (Agerer 1987–1998; Molina and Trappe 1994; Massicotte et al. 1994, 1999; Agerer et al. 1996–1998; Goodman et al. 1996–1998). Crystals were observed commonly on mycorrhizas both in vitro and in open-pot soil, suggesting that calcium oxalate formation (Cromack et al. 1979; Lapeyrie et al. 1987) continues throughout the developmental of the mycorrhizas. A unique characteristic was observed in the cases of *S. luteus* and *R. rubescens*, namely carbonization of several mycorrhizal tips. This has been reported previously for mycorrhizas of fungi in the section Geinea of *Tricholoma* (Agerer and Waller 1993; Lefevre and Müller 1998; Yamada et al. 1999a) and for fungi of the Thelephoraceae (Agerer 1987–1998). The carbonization appears to be a unique host reaction resembling a necrotic reaction of the plant against a parasite infection. Further biochemical analysis is necessary to analyze the interaction of root cortical cells and the Hartig net.

Two *Lactarius* mycorrhizas had distinct laticiferous hyphae (containing latex) (Figs. 39, 43). These same mycorrhizas had other common morphological characteristics of mycorrhizas in this genus, especially those belonging to the section Dapetes, i.e. a green discoloration when injured (Singer 1986; Agerer 1987–1998, 1995; Guerin-Laguette 1998). Mycorrhizas of *Lactarius hatsudake* and *Lactarius akahatsu* could be distinguished from each other by their microscopic characteristics, but their macroscopic characteristics were almost identical, except for the rhizomorphs. Although both species had rhizomorphs, those of *Lactarius akahatsu* developed further and had differentiated surface-layer

hyphae with thick cell walls. This has not been described before for this genus (Agerer 1995). Due to this similarity in anatomy of the two mycorrhizas, cross-contamination could be a serious problem when they are grown in adjacent nursery beds. It was possible to distinguish these two species clearly using PCR-RFLP analyses of ITS regions within the rDNA (Table 2). This is, thus, a desirable criterion for characterizing mycorrhizal morphotypes (Goodman 1996–1998; Agerer 1996–1999). Yamada and Katsuya (1996) described two *Lactarius* mycorrhizal morphotypes on *P. densiflora*, one of which (type 38; Yamada and Katsuya 1996) was similar to *Lactarius hatsudake*. *Lactarius deliciosus*, which is taxonomically allied to *Lactarius akahatsu* and a popular edible mushroom in Europe, shows morphologically and anatomically similar mycorrhizas (Guerin-Laguette 1998).

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