Mycorrhiza (1995) 5:357–370

ORIGINAL PAPER

Cathy L. Cripps · O. K. Miller, Jr.

Ectomycorrhizae formed in vitro by quaking aspen: including *Inocybe lacera* and *Amanita pantherina*

Abstract Ectomycorrhizae formed in synthesis tubes by aspen (*Populus tremuloides*) seedlings and each of seven fungal isolates are described. Isolates of *Amanita muscaria* v. *formosa, A. pantherina, Inocybe lacera,* and *Paxillus vernalis,* from sporocarps collected in aspen stands in southwestern Montana, developed mantles and Hartig nets on aspen roots, as did the broad-host-range fungi *Cenococcum geophilum* and *Pisolithus tinctorius* from the VPI culture collection. *Chalciporus piperatus* failed to form mycorrhizae, and *Piloderma croceum* formed a mantle, but no Hartig net. The first syntheses of *I. lacera* and *A. pantherina* with aspen are reported.

Key words *Amanita* · Aspen · Ectomycorrhizae *Inocybe lacera* · Mycorrhizae · *Populus tremuloides*

Introduction

Aspen (*Populus tremuloides* Michx.) is found in many diverse habitats (Mueggler 1985) throughout its wide range in North America. This adaptability may, in part, be attributable to its association with so many ectomycorrhizal fungi. Forty-six species of mycorrhizal fungi were collected on three aspen-covered sites in Montana and Idaho (Cripps and Miller 1993, 1994), and 29 species were found with aspen in Quebec and Ontario (Godbout and Fortin 1985). Proof that certain fungi are mycorrhizal with aspen by in vitro syntheses has accumulated slowly, as aspen is not easily grown aseptically. Aspen seeds are small, prone to desiccation, and lose viability rapidly, and cloning is labor intensive. Aspen seeds have been sterilized with mercury (Melin 1923), minicuttings surface sterilized (Heslin and Douglas 1986; Pirazzi et al. 1989), and frozen aspen seeds germinated on nutrient-enriched white sand for 30 days and placed in growth pouches (Godbout and Fortin 1985). The pouch method gives a rapid assessment of the ability to form mycorrhizae (29 of 54 fungal isolates tested positively in Godbout and Fortin's study), but is not amenable to long-term physiological studies and investigations such as that of Cripps (1992) in which the growth responses of aspen seedlings to inoculation with nine fungal isolates were investigated. Using sterilized seedlings in synthesis tubes to determine if fungi are mycorrhizal with a specific host is more time consuming than the pouch method, but is a viable alternative if a lack of humidity in the growth chamber or other considerations preclude using the pouch method. It is also of interest to discover whether mycorrhizal morphology is dependent on method and/or the amount of sugar present.

A method for the sterilization of aspen seed for tube synthesis is described, and seven isolates, four from aspen stands in Montana and three broad-host-range fungi, were grown with aspen seedlings; the resulting mycorrhizae are described according to Agerer (1990).

C. L. Cripps (✉) · O. K. Miller, Jr.
Biology Department, Virginia Polytechnic Institute and State University (VPI), Blacksburg, VA 24061-0406, USA

Materials and methods

Fungal isolates

Fungal isolates and their respective origins are listed in Table 1. Reference cultures are located in the Virginia Tech Culture Collection (VT) and reference sporocarps in the Virginia Tech Massey Herbarium.

Tissue was removed from sporocarps according to the sterile technique described by Molina and Palmer (1982) and grown on Hagem's medium as modified by Van Cotter (1987, unpublished work): 4 g malt extract, 1 g yeast extract, 5 g D-glucose, 0.5 g NH_4Cl, 0.5 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 ml $FeCl_3$ (1% aqueous), 100 µl biotin (0.5 mg biotin/ml aqueous), and 100 µl thiamine-HCl (1 mg thiamine/ml aqueous) added to 1000 ml of distilled H_2O. Agar 11 g/l was added to solidify the medium. Cultures were incubated in the dark at 20° C for a minimum of 1 month, and used to inoculate 250-ml flasks containing 75 ml of liquid Van Cotter's modified Hagem's (no agar).

Table 1 Isolates from the Virginia Tech Culture Collection (VT) tested for the cabability of forming mycorrhizae with *Populus tremuloides*

Isolate number	Fungal species	Mantle	Hartig net	Host, origin and date
VT 2238	*Amanita muscaria* var. *formusa*	+	+	*Populus tremuloides*, Park Co., Mont., 1990
VT 2239	*Amanita pantherina* var. *pantherina*	+	+	*Populus tremuloides*, Park Co., Mont., 1990
VT 1009	*Cenococcum geophilum*	+	+	Loblolly pine, Ga., 1978
VT 2240	*Chalciporus piperatus*	−	−	*Populus tremuloides*, Park Co., Mont., 1990
VT 2241	*Inocybe lacera*	+	+	*Populus tremuloides*, Silverbow Co., Mont., 1990
VT 2242	*Paxillus vernalis*	+	+	*Populus tremuloides*, Silverbow Co., Mont., 1990
VT 987	*Piloderma croceum*	+	−	Unknown
VT 1398	*Pisolithus tinctorius*	+	+	Unknown

Seed sterilization and germination

Fresh aspen seeds were stored in a dry place at 0–5° C. Seeds were gently agitated in a 15% Chlorox solution for 15 min, and rinsed three times (10 min each) in double-distilled H_2O. Two drops of the detergent Tween were added to the first two solutions to reduce surface tension. Seeds were placed in petri dishes containing Van Cotter's modified Hagem's made with 11 g/l agar, and illuminated in a growth chamber under incandescent and fluorescent lights for 16 h per day followed by 8 h dark. Uncontaminated seedlings were planted in synthesis tubes 23 days later.

Tube method of mycorrhizal synthesis

Molina's tube method of synthesis (1979) was followed using 10 ml peat, 90 ml vermiculite, and 70 ml Van Cotter's modified Hagem's modified (without agar) for each 200-ml synthesis tube. Liquid cultures of fungi were homogenized in a sterile Waring blender for 5 s, and 5 ml of the slurry added to each synthesis tube. The mycelium of *Amanita muscaria* v. *formosa* (Pers. per Fr.) Bert, *A. pantherina* (DC. per Fr.) Krombh, *Inocybe lacera* (Fr.:Fr.) Kummer, and *Paxillus vernalis* Watling were not blended as this was found to inhibit growth. The substrate part of the tube was encased in aluminum foil. After the fungal mycelium had colonized the peat-vermiculite medium for 2 weeks, sterile seedlings were introduced, and the tubes placed in a growth chamber.

Tubes were periodically checked for mycorrhization, and seedlings harvested after 3–9 months. Roots were gently washed with deionized water; morphological descriptions and chemical tests were performed on fresh material. Roots were preserved in FAA, dehydrated in a series of alcohols, and embedded in paraffin as follows: 50% EtOH/20% H_2O/30% tertbutyl alcohol (TBA), 2 h; 50% EtOH/10% H_2O/40% TBA , 2 h; 50% EtOH/50% TBA, 2 h; 25% EtOH/75% TBA, 16 h; 100% TBA, 2 h; fresh 100% TBA, 16 h; fresh 100% TBA, 16 h; 50% TBA/50% paraffin oil, 24 h; 100% paraffin oil, 24 h, 50% paraffin oil/50% paraplast (melted), 24 h; 100% paraplast (melted), 24 h; and fresh 100% paraplast (melted), 24 h.

Embedded mycorrhizae were sliced with a microtome into 5-μm sections and stained with safranin O and fast green in a manner similar to that cited by Wilcox (1982) and described by Johansen (1940): xylene, 5 min; fresh xylene, 5 min; fresh xylene, 5 min; 100% ETOH, 5 min; 100% EtOH, 5 min; 95% ETOH, 5 min; safranin O solution (4 g of safranin O in 200 ml ethoxyethanol, plus 100 ml 95% ethanol, 8 ml formalin and 4 g sodium acetate and stored under the fume hood), 30 min; 95% ETOH, a few seconds; fresh 95% EtOH, a few seconds; fast green solution (37.5 ml absolute ethanol, 37.5 ml clove oil, 25 ml ethoxyethanol, 500 mg fast green powder), 10 s; fresh 95% EtOH, a few seconds; 50% clove oil/25% absolute EtOH/25% xylene, 5 min; 50% xylene/50% EtOH, 5 min; xylene, 15 min; fresh xylene, 20 min.

Mycorrhizal descriptions

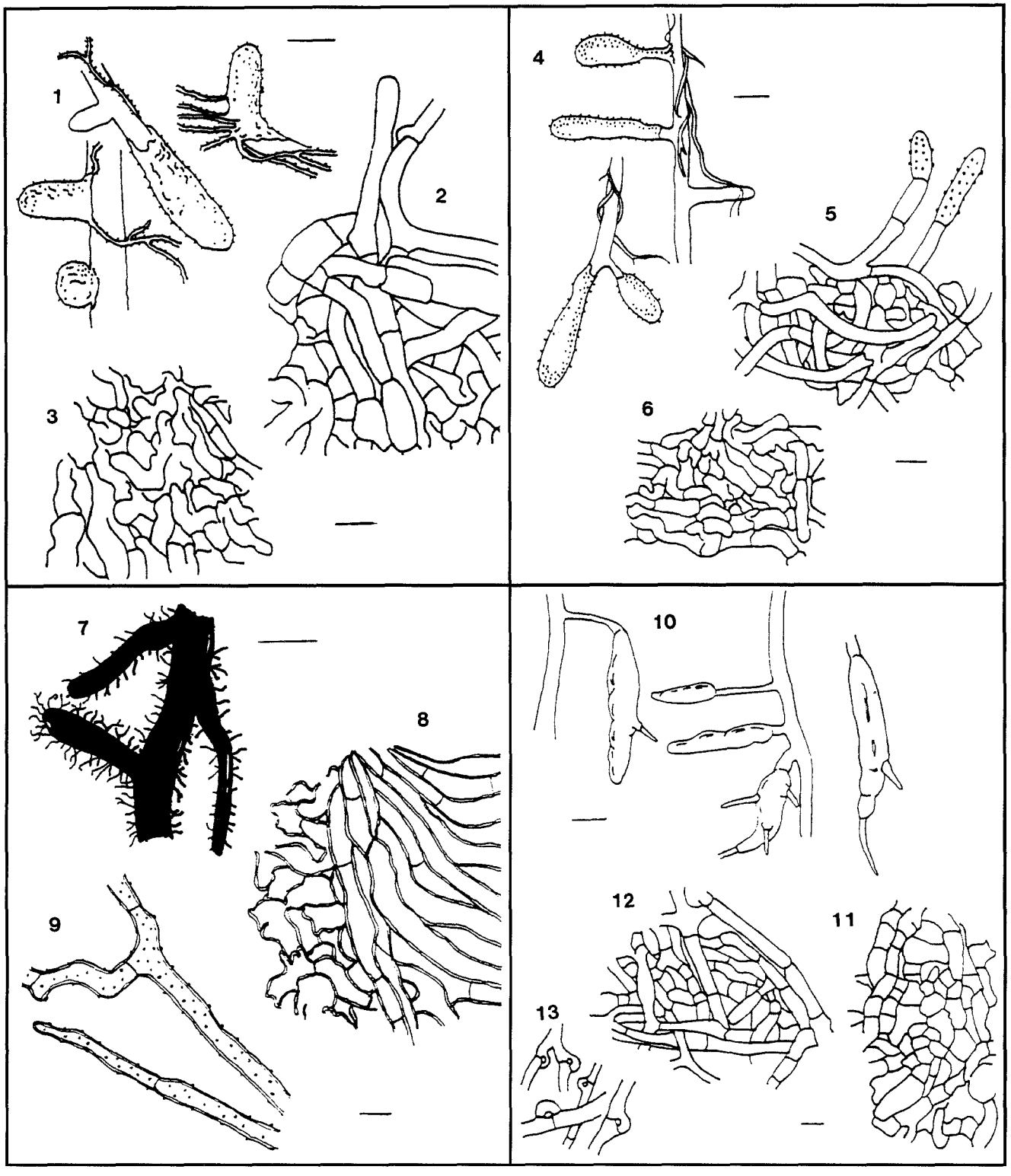
Descriptions of mycorrhizae follow the checklists of Agerer (1990). The only deviation is that our study defines ECt as average tangential length of epidermal cells, and ECq as average ratio between tangential length and radial width of epidermal cells (to determine radial elongation). These measurements are listed by Agerer as CCt and CCq for the cortical cells of conifers. Godbout and Fortin (1985) used the reciprocal value of these ratios.

Results

A majority of the seedlings were in good condition at the end of the study except for those inoculated with *I. lacera.* The latter were stunted and most of the leaves were black. There was some blackening in the leaf tips of seedlings inoculated with *Cenococcum geophilum, Pisolithus tinctorius, Piloderma croceum, Chalciporus piperatus,* and *Paxillus vernalis.* The leaves of seedlings inoculated with *A. pantherina* and *A. muscaria* and uninoculated controls were entirely green.

All fungal cultures grew well, except for *I. lacera.* This culture was associated with a yeast: pockets of the yeast were apparent, scant mycelium was observed, and trees were stunted in most tubes. A plug of *I. lacera* mycelium was added to an established 6-month-old seedling; this procedure produced copious clamped mycelium in one tube, and mycorrhizae 7 months later.

All isolates formed a mantle and a Hartig net, except for *Piloderma croceum* Erikss. and Hjortst., which formed a mantle but no net, and *Chalciporus piperatus* (Bull. ex Fr.), which formed neither (Table 1). *Pisolithus tinctorius* formed mycorrhizae with aspen seedlings in less than a month: other isolates took a minimum of 2 months to form mantles and Hartig nets by this method.



Figs. 1–13 *Populus tremuloides* mycorrhizae. *Upper bars* 1 mm, unless otherwise noted; *all lower bars* 5 μm. **Figs. 1–3** *Amanita muscaria* v. *formosa.* **Fig. 1** Morphology. **Fig. 2** Outer mantle. **Fig. 3** Inner mantle. **Figs. 4–6** *Amanita pantherina.* **Fig. 4** Morphology. **Fig. 5** Outer mantle. **Fig. 6** Inner mantle. **Figs. 7–9** *Cenococcum geophilum.* **Fig. 7** Morphology. **Fig. 8** Mantle. **Fig. 9** Emanating hyphae. **Figs. 10–13** *Inocybe lacera.* **Fig. 10** Morphology. *Upper bar* 0.5 mm. **Fig. 11** Inner mantle. **Fig. 12** Outer mantle. **Fig. 13** Extramatricular hyphae

Amanita muscaria var. *formosa* (Pers. per Fr.) Bertillon in DeChambre 1866

Synopsis

Mycorrhizae white, silvery (in water), pubescent, appearing swollen; pubescence due to the smooth-walled, septate, cystidium-like end cells protruding from the

mantle and on margins of hyphal cords; clamps in localized areas of outer mantle and hyphal cords.

Morphology (Figs. 1, 24)

Mycorrhizal system unramified (simple) or irregularly pinnate with straight to slightly bent unramified ends; tips commonly clavate to swollen; length of unramified systems 2–3 mm and ramified systems up to 8 mm; diameter of unramified ends 0.4–0.8 mm; mantle distinct, no epidermal cells visible, but mantle often covers only tip half of the short root; mantle white, silvery in water from trapped air, pubescent due to short emanating hyphae dispersed over entire surface; rhizomorphs (hyphal cords) sparingly branched and hairy, present at base of mantle or along main roots; rhizomorph diameter 0.10–0.25 mm and mantle attachment restricted.

Anatomical characteristics of mantle

Outer surface of mantle (Fig. 2). Plectenchymatous, interwoven net to occasionally parallel hyphae; cells hyphal-like, or rectangular, a few swollen (terminal or intercalary); cell length varies greatly, 10–45 μm; cell diameter 2.5–3.5 mm, swollen cells up to 7 μm (rarely 17 μm); cell walls thin, smooth and hyaline; clamps rare, localized.

Inner surface of mantle (Fig. 3). Transitional between plectenchymatous and pseudoparenchymatous, no pattern apparent; irregular cells 3–5 μm in diameter and 10–22 μm in length; cell walls thin, smooth, and hyaline; no clamps.

Tip of mycorrhizae. Same as above.

Rhizomorphs (hyphal cords). Common, white, sparsely branching; cystidium-like cells on margins similar to emanating hyphae on mantle; up to 50 μm in diameter; hyphae loosely woven, anastomosing to parallel, undifferentiated; cells 3–3.5 μm in diameter, up to 40 μm in length; cell walls thin, smooth and hyaline; connection with mantle restricted; clamps rare to common in localized areas.

Emanating hyphae. Abundant cystidium-like end cells over mantle surface and on rhizomorph margins; 2–3 septa, tip bluntly rounded, 3–3.5 mm in diameter and up to 36 μm (60 μm) long; unramified, rarely branched; cell walls thin and smooth.

Cystidia. None (see emanating hyphae).

Clamps. Rare on mantle, rare in rhizomorphs.

Anastomoses. Peg to peg.

Anatomical characteristics, cross section (Fig. 31)

Mantle. Pseudoparenchymatous, 55–95 μm thick, homogeneous throughout; cells 3–9(14) μm tangentially, 2–4 μm radially; cell walls thin.

Residues of calyptra cells. Present.

Tannin cells. None present.

Epideramal cells. One row with Hartig net, square to rectangular, tangentially 14–28 μm, radially 25–40 μm; ECt = 17.8 μm, ECq = 0.5.

Hartig net. Periepidermal, one (two) row(s) of round to square fungal cells surround epidermal cells; fungal cells 2–5 μm in diameter.

Anatomical characteristics, longitudinal section

Mantle. Cells tangentially 3–12 μm, radially 2–3(4) μm; cell walls thin; homogeneous throughout.

Very tip. Mantle 45–65 μm thick, similar to rest of mantle.

Residues of calyptra cells. Present.

Tannin cells. None present.

Epidermal cells. Rectangular, arranged obliquely; tangentially 14–30 μm, radially 23–60 μm; ECt = 20.7 μm, ECq = 0.5.

Hartig net. Palmetti type, strongly developed; lobes 3–5 μm in diameter.

Color reaction in different reagents

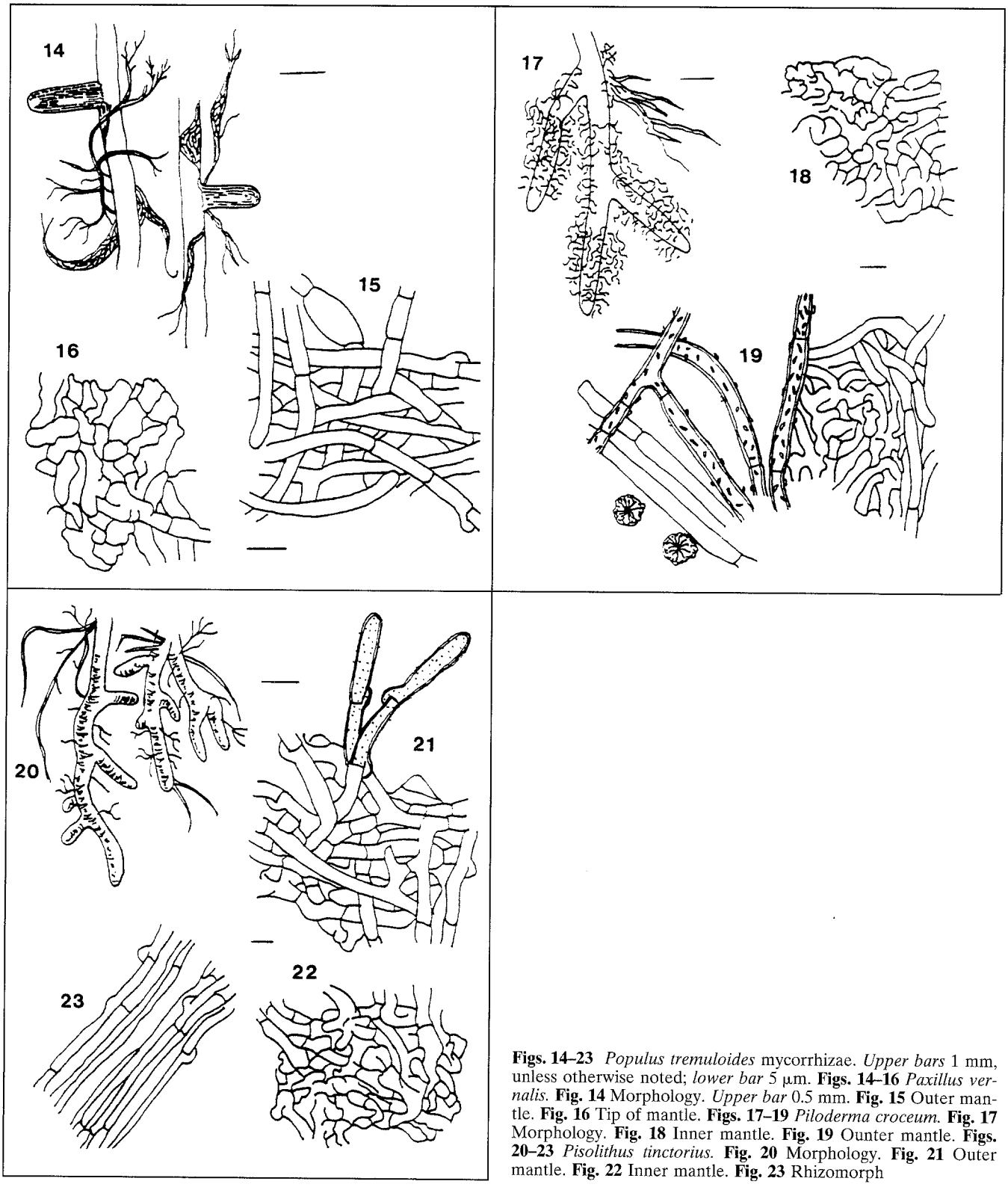
Brilliant cresyl blue, −; cotton blue lactic acid, cell contents blue and granular; ethanol 70%, −; $FeSO_4$, −; KOH 15%, −; lactic acid, −; Melzer's, −; phenol, −; sulfo-vanillin, −; Sudan IV, −; Sudan Black, −.

Autofluorescence of whole mycorrhizae

At 254 nm, −; 366 nm, −.

Material studied

Isolate VT 2238.



Figs. 14–23 *Populus tremuloides* mycorrhizae. *Upper bars* 1 mm, unless otherwise noted; *lower bar* 5 μm. **Figs. 14–16** *Paxillus vernalis.* **Fig. 14** Morphology. *Upper bar* 0.5 mm. **Fig. 15** Outer mantle. **Fig. 16** Tip of mantle. **Figs. 17–19** *Piloderma croceum.* **Fig. 17** Morphology. **Fig. 18** Inner mantle. **Fig. 19** Ounter mantle. **Figs. 20–23** *Pisolithus tinctorius.* **Fig. 20** Morphology. **Fig. 21** Outer mantle. **Fig. 22** Inner mantle. **Fig. 23** Rhizomorph

Amanita pantherina var. *panterina* (DC. per Fr.) Krombh. 1836

Synopsis

Mycorrhizae white, silvery (in water), pubescent, appearing swollen towards tip; pubescence from septate cystidium-like cells with slightly roughened walls (or dense cytoplasm) which emanate from the mantle, and hyphal cords; no clamps present.

Morphology (Figs. 4, 25)

Mycorrhizal system unramified, with swollen tip and constricted base or irregularly pinnate with bent to

slightly tortuous ends; length of ramified system up to 10 mm; unramified ends up to 8 mm with a diameter of 0.6–0.8 mm; diameter of axis 0.5–0.8 mm; mantle surface pubescent out of water, appearing smoother and silvery in water due to trapped air, all parts white; rhizomorphs (thin hyphal cords) white, sparingly branched, sparsely hairy, present particularly along the main roots or rarely emanating from the base of the mycorrhizae.

Anatomical characteristics of mantle

Outer surface of mantle (Fig. 5). Plectenchymatous, a coarse loose net of branching hyphae 3–4(5) μm in diameter, cells 9–35 μm long; cell walls thin and smooth; cells hyaline; no clamps present.

Inner surface of mantle (Fig. 6). Transitional between plectenchymatous and pseudoparenchymatous, rarely branching coarse net, more densely interwoven than mantle surface; irregular cells 5–25 μm in diameter, thin-walled, hyaline; 15–20 cells in a 20-μm square; no clamps present.

Tip of mycorrhizae. Same as above.

Rhizomorphs (hyphal cords). White, up to 25 μm in diameter and undifferentiated (or rarely with larger vessel-like hypha in center), sparsely hairy margins; hyphae 3–4 μm in diameter; walls thin, hyaline, smooth except on distinctive emanating hyphae which appear rough, incrusted, or with dense cytoplasm; no clamps.

Emanating hyphae. Distinctive cystidium-like end cells, abundant on mantle surface giving it a pubescent texture, also present on rhizomorph margins; bluntly rounded, 2–3 septa; walls appear roughened or incrusted (or with dense cytoplasm); 3–4 μm in diameter, up to 35 μm long; no clamps present.

Cystidia. None (see emanating hyphae).

Clamps. None.

Anatomical characteristics, cross section (Fig. 32)

Mantle. Pseudoparenchymatous, (10)56–70 μm thick; cells of outermost layer tangentially 7–15 μm, radially 2–4 μm; innermost layer of cells tangentially 2–6 μm, radially 2–4 μm, cell walls thin throughout.

Residues of calyptra cells. Present.

Tannin cells. None present.

Epidermal cells. One row with Hartig net, square to rectangular, tangentially (12)18–27 μm, radially (14)18–25 μm; ECt = 19.6 μm, ECq = 1.0.

Hartig net. Periepidermal, one to two rows of fungal cells completely surround root cells; fungal cells round in cross section, rectangular in longitudinal section; thickness of Hartig net 3–5 μm.

Anatomical characteristics, longitudinal section

Mantle. Outermost cells tangentially 3–12 μm, radially 2–5(8) μm; innermost cells 4–8(10) μm tangentially, (2)3–7 μm radially; cell walls thin throughout.

Very tip. Mantle 80–100 μm thick, pseudoparenchymatous with irregularly shaped cells; cell size similar to rest of mantle.

Residue of calyptra cells. Present.

Tannin cells. None present.

Epidermal cells. Rectangular, arranged obliquely; tangentially 15–20 μm, radially (20)25–45 μm; ECt-=18.3 μM, ECq = 0.6.

Hartig net. Palmetti type, lobes 2–3 μm in diameter.

Color reaction in different reagents

Cotton blue lactic acid, highlights ornamentation on emanating end cells; brilliant cresyl blue, −; Melzer's, −; ethanol 70%, −; $FeSO_4$, −; KOH 15%, −; lactic acid, phenol, −; sulfo-vanillin, −; NH_4OH, −.

Autofluorescence of whole mycorrhizae

At 254 nm, −; 366 nm, −.

Material studied

Isolate VT 2239.

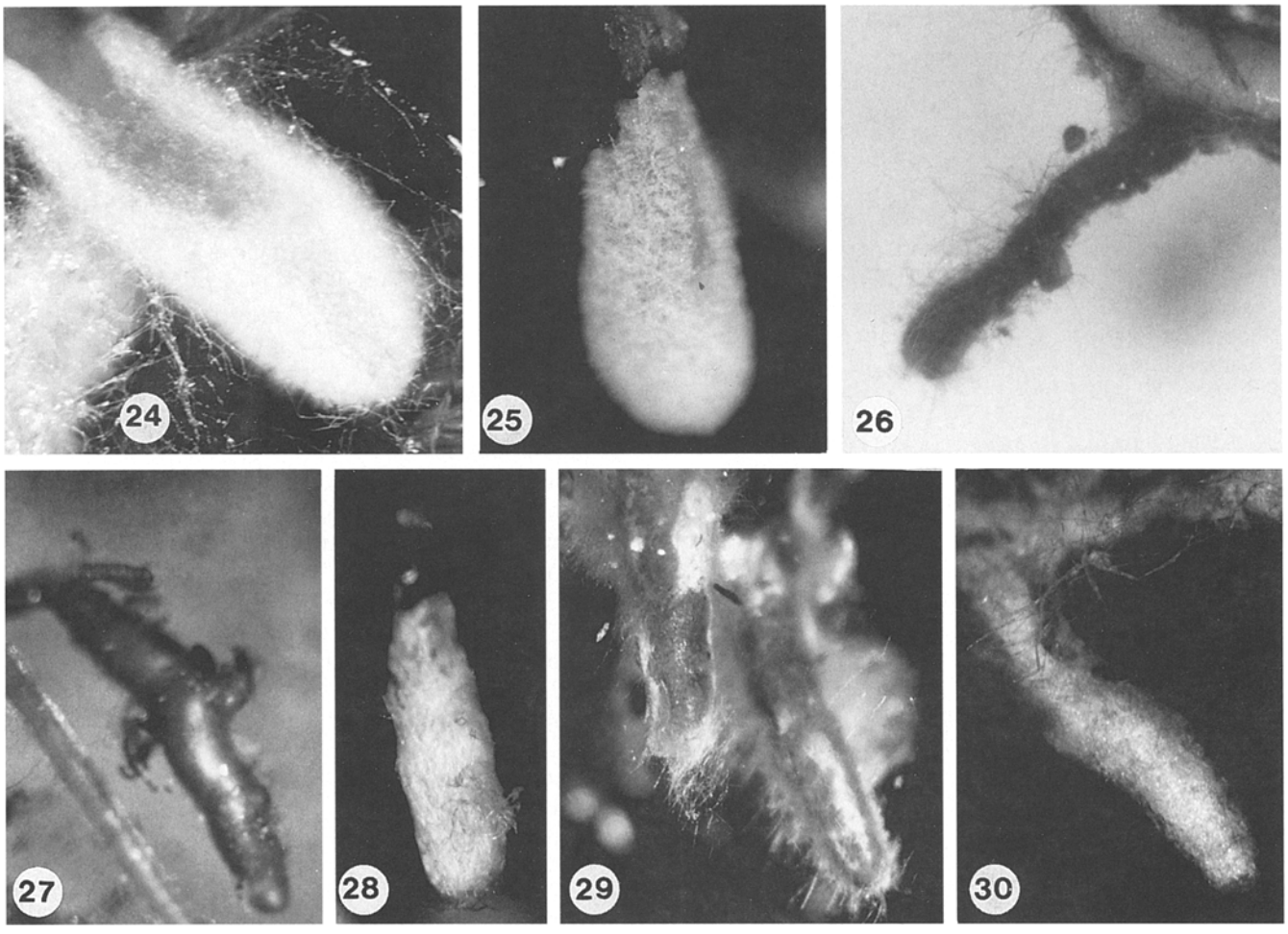
Cenococcum geophilium Fr.

Synopsis

Black mycorrhizae with rough mantle surface; kinked, black, individual hyphae emanating from surface; rectangular cells of mantle form a star pattern, but epidermoidal cells present in some areas.

Morphology (Figs. 7, 26)

Mycorrhizal system irregularly ramified, monopodial with bent to slightly tortuous ends; length of ramified



Figs. 24–30 Morphology of *Populus tremuloides* mycorrhizae. **Fig. 24** *Amanita muscaria* v. *formosa.* ×70. **Fig. 25** *Amanita pantherina.* ×60. **Fig. 26** *Cenococcum geophilum.* ×20. **Fig. 27** *Inocybe lacera.* ×30. **Fig. 28** *Paxillus vernalis.* ×50. **Fig. 29** *Piloderma croceum.* ×50. **Fig. 30** *Pisolithus tinctorius.* ×40

system, up to 10 mm; length of unramified ends 7 mm, diameter 0.3–0.5 mm; diameter of axes, 0.5 mm; few mantles well formed, epidermal cells as well as root hairs often visible; copious black mycelium present along main roots; mantle surface loosely woolly, covered by emanating bristle-like, kinky hyphae; no rhizomorphs or sclerotia present.

Anatomical characteristics of mantle

Outer surface of mantle (Fig. 8). Pseudoparenchymatous, stellate pattern (an epidermoidal pattern is also present; it is not readily apparent if these cells are internal, adjacent to the stellar pattern, or part of the Hartig net; they are described below as the inner mantle surface); cells comprising the stellate pattern are rectangular, 5–50 μm long, 4–5 μm wide, thick walled; 6–10 cells in a 20×20 μm square.

Inner surface of mantle (Fig. 8). Pseudoparenchymatous; consisting of epidermoidal type cells which are thick walled, 5–15 μm in diameter; 12–15 cells in a 20×20 μm square.

Tip of mycorrhizae. Not examined.

Rhizomorphs. None present.

Emanating hyphae (Fig. 9). Regularly septate, up to 1 mm in length, 4–5 μm wide, occasionally ramified; cell walls very thick, up to 0.5 μm, rough; distal ends rounded or tapering.

Cystidia. None.

Clamps. None.

Anatomical characteristics, cross section (Fig. 33)

Mantle. Loosely organized, hyphal-like or round in cross-section, 2–10 μm thick; cells jammed between epidermal cells giving exterior of cross-section an eroded appearance; mantle cells tangentially 2–14 μm, radially 2-4 μm; cell walls thickened.

Residues of calyptra cells. Present.

Tannin cells. None present.

Epidermal cells. One row with Hartig net, irregularly shaped and isodiametric, tangentially 11–18 μm, radially (7)12–25 μm; ECt = 13.8 μm, ECq = 0.9.

Hartig net. Periepidermal, round to epidermoidally shaped cells surrounding each epidermal cell, having a beaded appearance; cells 2–4 μm across. Palmetti-type cells in plan view look epidermoidal en masse, lobes 2–4 μm in diameter.

Material studied

Isolate VT 1009.

Inocybe lacera (Fr.:Fr.) Kummer

Synopsis

Unramified smooth, shiny, light brown, tuber-like (tapering, often lumpy) mycorrhizae. Outer mantle net-like with 90° branching, short rectangular cells on inner mantle. Medallion clamps on extramatricular hyphae.

Morphology (Figs. 10, 27)

Mycorrhizal system unramified, up to 4 mm in length, 0.5 mm in diameter; unramified ends somewhat flexuous, swollen in the middle tapering towards the distal end, often tuber-like with branching roots protruding from mantle sides; a distinct mantle surface present, epidermal cells not visible except for protruding roots; mantle surface smooth, pale yellow-brown or light brown; no rhizomorphs or emanating hyphae, only extramatricular hyphae.

Anatomical characteristics of mantle

Outer surface of mantle (Fig. 12). Plectenchymatous; extreme outer cells hyphal-like, often short and branching at 90° angles producing an interwoven to net-like arrangement; cells colorless, 14–55 μm in length, 3–5(7) μm in diameter, wider cells to 7 μm; cell walls smooth and thin; medallion clamps rare in outer layer, but extremely common in extramatricular hyphae.

Inner surface of mantle (Fig. 11). Pseudoparenchymatous; cells colorless, squarish to rectangular, 5–10 μm long and 3–5(8) μm in diameter producing no apparent pattern, with oily refractive contents; clamps absent.

Rhizomorphs. None.

Emanating hyphae. None.

Cystidia. None.

Clamps. In extramatricular hyphae.

Anastomoses. Frequent, tip to peg.

Remarks. Extramatricular hyphae are highly septate, frequently branched at 90° angles, medallion clamps common (Fig. 13).

Anatomical characteristics, cross section (Fig. 34)

Mantle. Pseudoparenchymatous, cells narrower towards exterior, (5)18–46 μm thick, outer hyphae 2–3 μm × 15–40 μm, inner hyphae 2–4 μm × 5–15 μm, some septa thickened, cell walls appear thickened in a few areas on inner mantle, but these may be collapsed calyptra cells.

Residues of calyptra cells. Present.

Tannin cells. None present.

Epidermal cells. One row of epidermal cells, tangentially 11–20 μm, radially 35–48 μm; ECt = 12 μm, ECq = 0.4.

Hartig net. Paraepidermal, 1(2) row of irregularly shaped fungal cells, 2.5–6 μm across between epidermal cells, no Palmetti lobes.

Anatomical characteristics, longitudinal section

Mantle. Pseudoparenchymatous, hyphae of outer layer radially 1.5–3 μm, tangentially 5–10 μm, some septa thickened, hyphae of inner layer tangentially 5–20 μm, radially 2–4 μm.

Residues of calyptra cells. Present.

Tannin cells. None present.

Epidermal cells. One row of rectangular, obliquely arranged cells, tangential 12–16 μm, radially 40–55 μm; ECt = 13.3 μm, ECq = 0.3.

Hartig net in surface view. No Palmetti-type cells, only irregular cells 5 μm × 6 μm.

Color reaction in different reagents

Cotton blue, refractive cell contents (oil globules) apparent in some hyphae; other tests not performed due to lack of fresh material.

Material studied

Isolate VT 2241.

Paxillus vernalis Watling

Synopsis

Fibrous, silvery, light brown mycorrhizae of uniform width, with rhizomorphs and anastomosing hyphal wefts; scattered mantle cells with yellow refractive contents, clamps frequent in rhizomorphs and hyphal wefts.

Morphology (Fig. 14, 28)

Mycorrhizal system unramified with straight to rarely bent ends; length of unramified ends up to 6 mm; diameter 0.17–0.25 mm, strikingly uniform up to the bluntly rounded tips; mantle surface distinct but not always complete, epidermal cells visible in small areas especially at tip; mantle surface fibrous, silvery due to trapped air; hyphal strands oriented parallel to root axis; all parts light brown; hyphal wefts repeatedly ramified and hairy, swelling in water like balloons from trapped air; no sclerotia present.

Anatomical characteristics of mantle

Outer surface of mantle (Fig. 15). Plectenchymatous, of densely interwoven hyphae 3.5–5.0 μm in diameter (cells occasionally swollen to 7 μm), up to 30 μm in length; cell walls thin, smooth, hyaline; cell contents pale yellow in water; scattered intercalary and end cells with yellow refractive contents; clamps common, more frequent in hyphal wefts.

Inner surface of mantle. More pseudoparenchymatous, similar to tip of mycorrhizae.

Tip of mycorrhizae (Fig. 16). Transitional between plecenchymatous and pseudoparenchymatous, some cells square; cells 4.5–5.5 μm wide and up to 13 μm long; cell walls thin, contents highly refractive; 10–15 cells in a 20 μm × 20 μm square; no clamps.

Rhizomorphs and emanating hyphae. Sporadically abundant extramatricular hyphae 3–4 μm in diameter, also loosely interwoven hyphal wefts up to 55 μm in diameter, resembling rhizomorphs under dissecting scope; clamps abundant.

Cystidia. None.

Clamps. Common in hyphal wefts, rarer in outer mantle, none in tip.

Anatomical characteristics, cross section

Mantle (Fig. 35). Plectenchymatous, interspersed with round hyphal cross sections; inner layer tightly woven, surface layer loosely woven; mantle 16–40 μm thick; outermost hyphae tangentially 3–40 μm, radially 3–4 μm; innermost hyphae tangentially 3–12 μm, radially 2–3 μm; cell walls thin; numerous cells with yellow refractive contents; clamps present in outer mantle.

Residues of calyptra cells. Present.

Tannin cells. None present.

Epidermal cells. One row of square to rectangular cells; tangentially 13–20 μm, radially (17)22–30 μm; ECt = 17.5 μm, ECq = 0.7.

Hartig net. Paraepidermal, 1–2 rows of fungal cells found between epidermal cells; Hartig net cells round in cross-section and rectangular longitudinally; thickness of Hartig net 2–3 μm.

Anatomical characteristics, longitudinal section

Mantle. Plectenchymatous, tightly interwoven on inner surface to loosely interwoven on outer surface; hyphae of outermost layer tangentially 3–25 μm, radially 2–4 μm; innermost hyphae tangentially 3–25 μm, radially 2–4 μm; tip of mantle 23–48 μm thick.

Residues of calyptra cells. Present.

Tannin cells. None present.

Epidermal cells. One row, rectangular, arranged obliquely; tangentially 12–14 μm, radially 27–40 μm; ECt = 12.2 μm, ECq = 0.4.

Hartig net in surface view. No Palmetti-type lobes apparent, Hartig net of hyphal-like cells.

Color reaction in different reagents

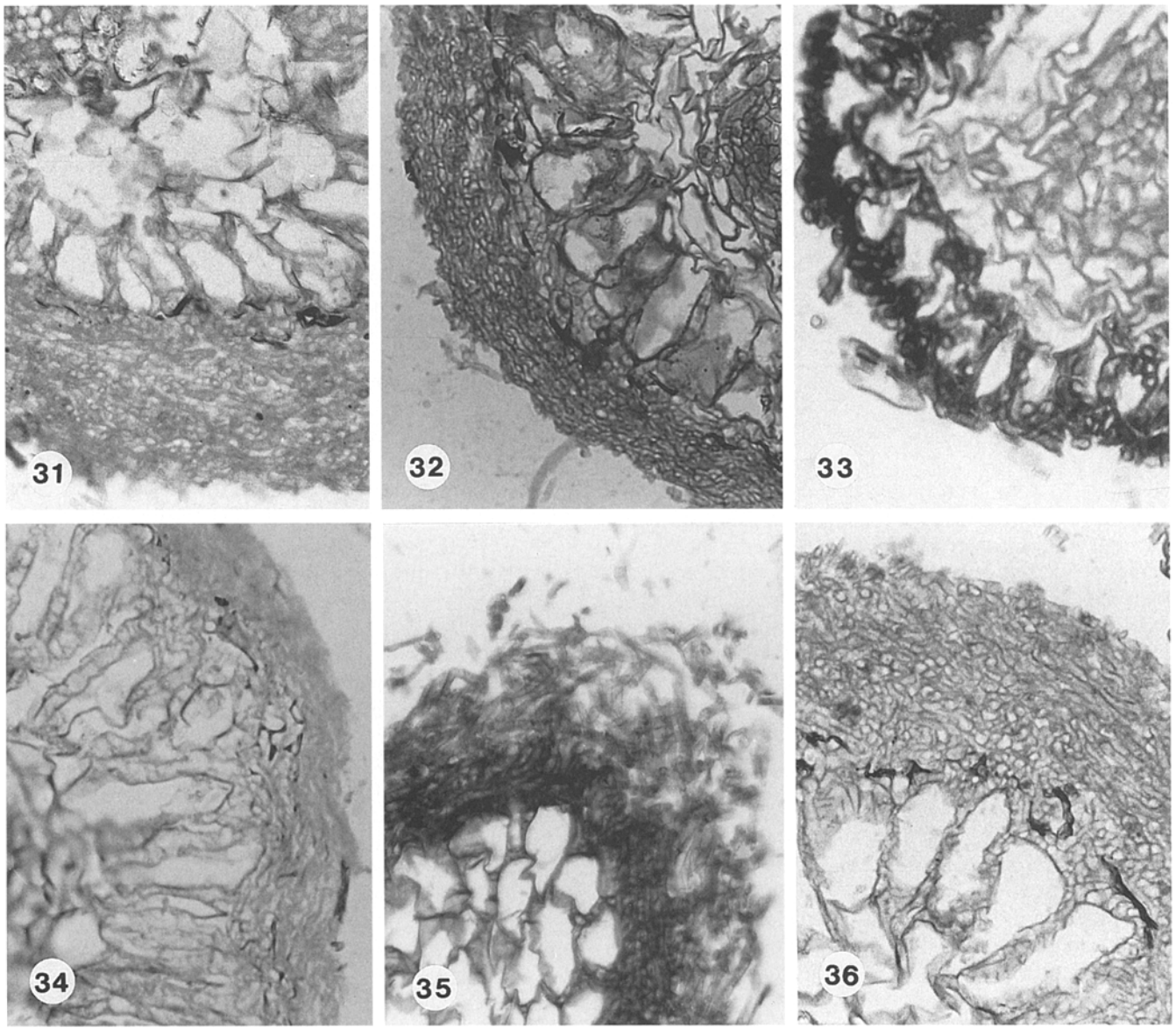
Lactic acid, highlights cells with yellow refractive contents; brilliant cresyl blue, cell contents forest green; cotton blue lactic acid, −; ethanol, −; $FeSO_4$, −; KOH 15%, −; Melzer's, −; phenol, −; sulfo-vanillin, −; Sudan Black, −.

Autofluorescence of whole mycorrhizae

At 254 nm, −; 366 nm, −.

Material studied

Isolate VT 2242.



Figs. 31–36 Cross-sections of *Populus tremuloides* mycorrhizae showing Hartig net. **Fig. 31** *Amanita muscaria* v. *formosa.* ×250. **Fig. 32** *Amanita pantherina.* ×250. **Fig. 33** *Cenococcum geophilum.* ×400. **Fig. 34** *Inocybe lacera.* ×250. **Fig. 35** *Paxillus vernalis.* ×250. **Fig. 36** *Pisolithus tinctorius.* ×250

Piloderma croceum Erikss. & Hjortst. 1981

Synopsis

Bright yellow mycorrhizae, with thin cottony to woolly mantle; yellow oleiferous-type cells in mantle, emanating hyphae thick-walled, encrusted with needle-shaped ornamentation which dissolves in KOH.

Morphology (Figs. 17, 29)

Mycorrhizal system irregularly pinnate; length of ramified system up to 15 mm; unramified ends with maximum length of 9 mm, diameter of axis up to 1 mm; mantle surface not distinct; epidermal cells visible especially at tip, silvery in restricted areas, densely cottony to woolly; all parts bright yellow; no rhizomorphs; copious extramatricular hyphae from mantle and along main roots, up to 1 mm long.

Anatomical characteristics of mantle

Outer surface of mantle (Fig. 19). Plectenchymatous to transitional between plectenchymatous and pseudoparenchymatous, no pattern although hyphae occasionally parallel; irregular cells 4–20 μm in length, 2–3 μm in diameter; cell walls thin, smooth.

Inner surface of mantle (Fig. 18). Transitional between plectenchymatous and pseudoparenchymatous; cells ir-

regular to epidermoidal, 5–14 μm in diameter; cell walls thin; 25–35 cells in a 20 μm × 20 μm square.

Tip of mycorrhizae. Same as above.

Emanating hyphae (Fig. 19). Extramatricular hyphae up to 90 μm long, 2–2.5 μm in diameter, dichotomously ramified; cell walls thickened and incrusted with needle-shaped ornamentation which dissolves in KOH; occasional hyphal strands with slightly thickened, smooth cell walls, 1.5–2 μm in diameter.

Oleiferous hyphae. Intercalary or end cells with yellow refractive contents.

Anastomoses. Peg to peg.

Clamps. None.

Anatomical characteristics, cross section

Mantle. Some 8–23 μm thick, cells hyphal-like, round in cross section, tangentially 2–14 μm, radially 2–3 μm; cell walls thin except for emanating hyphae.

Residues of calyptra cells. Present.

Tannin cells. None present.

Hartig net. None present.

Color reaction in different reagents

Brilliant cresyl blue, highlights ornamentation; cotton blue lactic acid, −; ethanol, −; $FeSO_4$, −; guaiac, −; KOH, dissolves needle-like ornamentation; Melzer's, dextrinoid; phenol, −; sulfo-vanillin, −; Sudan Black, −; Sudan IV, −.

Autofluorescence of whole mycorrhizae

At 254 nm, −; 366 nm, −.

Material studied

Isolate VT 987.

Pisolithus tinctorius (Pers.) Coker & Couch

Synopsis

Mycorrhizae light brown, silvery in water, somewhat smooth to very sparsely woolly with thick mantle, occasional emanating hyphae with thick, rough walls; rhizomorphs differentiated, with abundant clamps; with brilliant cresyl blue, mantle cells green, emanating hyphae reddish-violet.

Morphology (Figs. 20, 30)

Mycorrhizal system monopodially pinnate or irregularly pinnate, rarely monopodially pyramidal, often narrowing slightly at base; ramified system up to 20 mm long; axis, 0.6–0.7 mm in diameter; unramified ends up to 7 mm long, 0.4–0.6 mm in diameter, ends straight or bent; mantle thick, well developed, epidermal cells never visible; mantle surface silvery, somewhat smooth to loosely sparsely woolly; all parts yellow-brown to light brown, rarely with dark brown areas, no senescent mycorrhizae present; rhizomorphs frequent, occurring near axes, rarely on tips, mantle connection restricted, ramified, sparsely hairy margins; emanating hyphae common; no sclerotia present.

Anatomical characteristics of mantle

Outer surface of mantle (Fig. 21). Plectenchymatous, a loose net of hyphal-like cells; cells 8–37 μm long, 3.5–5(7) μm wide; cell walls somewhat thickened, smooth; emanating hyphae with thick rough walls and rounded ends; clamps common.

Inner surface of mantle (Fig. 22). Transitional between plectenchymatous and pseudoparenchymatous; cells irregular, 5–20 μm long, 3.7–4.5 μm wide; 10–12 cells in a 20 × 20 μm square; cell walls thinner than those of outer surface; no clamps.

Tip of mycorrhizae. Same as above, but more densely interwoven.

Rhizomorphs (Fig. 23). Typically 6–9 μm, but up to 24 μm in diameter, differentiated with thicker hypha in center; occasionally ramified with a few hairs on margin, restricted mantle connection; vessel-like hyphae septate, up to 5 μm in diameter, 70 μm in length, unramified with thickened cell walls; remaining hyphae septate, 3–3.5 μm in diameter, up to 50 μm long, cell walls thickened and smooth; clamps abundant.

Emanating hyphae. Septate, 3.5–4.5 μm in diameter, up to 70 μm long, rarely ramified with rounded distal ends; cell walls thick, rough (finely granular); clamps frequent.

Cystidia. None.

Clamps. Present in outer mantle, emanating hyphae, frequent in rhizomorphs.

Anatomical characteristics, cross section

Mantle (Fig. 36). Pseudoparenchymatous, 35–75 μm thick; outer cells more hyphal-like, tangentially cells 3–20 μm, radially 2–4 μm; innermost layer of cells tangentially 3–25 μm, radially 2–4 μm; cell walls somewhat thickened.

Residues of calyptra cells. Present.

Tannin cells. None present.

Epidermal cells. One row of epidermal cells with Hartig net; cells square to rectangular, tangentially 14–23(30) μm, radially 23–70(80) μm; ECt = 19.5 μm, ECq = 0.4.

Hartig net. Well developed, periepidermal or deeper, 3–5 μm thick.

Anatomical characteristics, longitudinal section

Mantle. Outermost cells 3–23 μm tangentially, 3–5 μm radially; innermost cells 3–15 μm tangentially, 3–5 μm radially; cell walls somewhat thickened.

Very tip. Mantle 45–70 μm thick, similar to rest of mantle.

Residues of calytra cells. Present.

Tannin cells. None present.

Epidermal cells. Rectangular, arranged obliquely, tangentially (14)18–32 μm, radially 46–70 μm; ECt = 21.6 μm, ECq = 0.3.

Hartig net in surface view. Palmetti type, well developed, numerous lobes 2–5 μm in diameter.

Color reaction in different reagents

Brilliant cresyl blue, cells light green and emanating hyphae reddish-violet; cotton blue lactic acid, −; $FeSO_4$, −; guaiac, −; KOH 15%, −; lactic acid, −; Melzer's phenol, −; sulfo-vanillin, reddish-brown; Sudan Black, −; Sudan IV, −.

Autofluorescence of whole mycorrhizae

At 254 nm, −; 366 nm, −.

Material studied

Isolate VT 1398.

Discussion

Mycorrhizae formed from *A. muscaria* mycelium and *Populus tremuloides* seedlings in synthesis tubes are similar to those developed in pouches (Godbout and Fortin 1985), except clamps were present in ours in localized areas: their synthesis lasted 25 days, ours 3 months. Clamps were not noted in mycorrhizae of *A. muscaria* and *Betula pendula* Roth (Ingleby et al. 1990), and *Pinus patula* Schiede and Deppe (Mohan et al. 1993), and the latter are bipodial (a characteristic of pine mycorrhizae), but these mycorrhizae are otherwise similar to ours. Regardless of host or synthesis method, septate cystidium-like end cells are a consistent character for *A. muscaria* mycorrhizae. These cells are more obvious on fresh material, than on stained sections.

This is the first mycorrhizal synthesis of *A. pantherina* to our knowledge. *A. pantherina* and aspen mycorrhizae are similar to those of our *A. muscaria* mycorrhizae, except clamps were present in the latter. Similarly, clamps are present in basidiocarps of *A. muscaria,* but not those of *A. pantherina* (Thiers 1982). Septate, cystidium-like end cells, similar to those found in the mantles of *A. muscaria* mycorrhizae are present in *A. pantherina* mycorrhizae, but appear to have somewhat roughened walls or very dense cytoplasm in the latter. The septate cystidium-like end cells observed in *A. muscaria* and *A. pantherina* may be a typical feature of these Amanitas. It will be interesting to discover if a generic pattern unfolds as additional *Amanita* mycorrhizae are described.

Mycorrhizae formed in tubes by *Cenococcum geophilum* and aspen had a morphology similar to those synthesized in pouches (Godbout and Fortin 1985), although the star pattern characteristic of *Cenococcum* mantle cells (Lihnell 1942) was not described for pouch mycorrhizae. Our mycorrhizae were unique in forming not only a star pattern, but additionally a layer of epidermoidally shaped cells in some areas, which might be interpreted as an early developmental stage, an internal mantle layer, or the Hartig net.

This is the first synthesis of *I. lacera* and aspen. Our mycorrhizae were similar to those formed by *I. lacera* on *Betula pendula* (Ingleby et al. 1990), except no young white stage was noted in ours which were light brown to yellow-brown, like naturally occurring mycorrhizae of *I. lacera* and *Pinus radiata* (Chu-chou and Grace 1983). Schramm (1966) suggests naturally occurring *I. lacera* mycorrhizae are basically colorless with a waxy mat surface, but are tinted by plant material showing through. This may explain why our mycorrhizae occasionally appeared gray in artificial light. Some thick septa in the outer mantle of *I. lacera* and birch mycorrhizae were reported to stain blue in lactophenol cotton blue (Ingleby et al. 1990). This staining reaction was not tested by us; however, thick hyphal septa were present in the outer mantle of our synthesized mycorrhizae. Medallion clamps typical of Inocybes were not

present in the synthesized mantle, but were common in the extramatricular hyphae which are easily lost in processing. Our synthesized mycorrhizae were similar to *I. lacera* mycorrhizae which occur naturally with aspen on our research site. Inocybes are notoriously difficult to grow (Chu-chou and Grace 1981; Kuyper 1986). Ours was grown on modified Hagem's and modified Melin-Norkrans (Marx 1969) with minimal agar (11 g/l) as thicker agar obstructs mycelial growth. Only a few attempts at culturing *I. lacera* were successful; the mycelium is slow growing, and occurred with a yeast which may be an associate (not a contaminant), although the rate of mycelial growth increased in liquid culture, especially with dilute media. The mycelium grew well in only a few synthesis tubes, and formed mycorrhizae with one 6-month-old established aspen seedling 7 months after inoculation.

Mycorrhizae of *Paxillus vernalia* (Watling 1969) and aspen were similar to those of *Paxillus involutus* (Batsch: Fr.)Fr. and aspen (Godbout and Fortin 1985), alder (Miller et al. 1991), birch (Ingleby et al. 1990), and spruce (Agerer 1990). Our synthesized mycorrhizae were typically light brown, but naturally occurring cream-colored mycorrhizae of *Paxillus vernalis* which bruise brown and eventually turn completely brown have been observed under aspen where the *Paxillus vernalis* culture originated. The color of the natural mycorrhizae better fits descriptions for *Paxillus involutus* mycorrhizae. The rhizomorphs of the *Paxillus vernalis* mycorrhizae occurring naturally on our study site also had more-developed rhizomorphs with a descending ramification from large, smooth main axes to smaller, hairy side branches. Intermittent mantle cells with yellow refractive contents, a distinctive character, were present in our in vitro mycorrhizae and have been described for alder mycorrhizae (Miller et al. 1991). Unlike other synthesized mycorrhizae, ours did not form sclerotia.

Chalciporus piperatus does not readily form mycorrhizae in culture (Chu-chou 1979; Godbout and Fortin 1985) and our results support this; the mycelium grew well but did not form mycorrhizae.

Pisolithus tinctorius formed mantles in less than a month, compared to several months for other fungi under these conditions, and was the only fungus to completely colonize the root system, i.e. 90% compared to 15% or less for others (Cripps 1992). Mycorrhizae synthesized in tubes were similar to those formed in pouches (Godbout and Fortin 1985) except that sclerotia and large mantle cells (9–12 μm) were absent in our study.

Our synthesized mycorrhizae may be too young to display full mantle and rhizomorph development, and may not be totally comparable to natural mycorrhizae. We found the synthesized and natural mycorrhizae of *I. lacera* to be similar, but the synthesized mycorrhizae of *Paxillus vernalis* lacked the full rhizomorph and sclerotial development of *Paxillus* mycorrhizae found on our site (unpublished data).

Of the fungal species tested, *Paxillus vernalis* and *Pisolithus tinctorius* have potential as commercial inoculants for aspen seedlings because they grow well in culture. However, *Pisolithus tinctorius* has only been recorded with *Populus tremuloides* once (Schramm 1966), and is rare or absent in Montana and Idaho, the location of our study sites, and may be considered an exotic in these areas. *Paxillus vernalis* is a common associate of aspen in Montana and Idaho (Cripps 1992).

Acknowledgements We thank Dr. R. Treu for sharing mycorrhizal methods, Dr. J. G. Palmer for sharing knowledge, Dr. I. Brunner for reprints, the Virginia Museum of Natural History for logistical support, and the Biology Department of Virginia Tech for financial support during this project.

References

Agerer R (1990) Color atlas of ectomycorrhizae. Einhorn, Schwäbisch Gmünd

Chu-chou M (1979) Mycorrhizal fungi of *Pinus radiata* in New Zealand. Soil Biol Biochem 11:557–62

Chu-chou M, Grace LJ (1981) Mycorrhizal fungi of *Pseudotsuga menziesii* in the North Island of New Zealand. Soil Biol Biochem 13:247–249

Chu-chou M, Grace Li (1983) Characterization and identification of mycorrhizas of radiata pine in New Zealand. Aust For Res 13:121–132

Cripps CL (1992) Aspen mycorrhizae: ecology, syntheses, and growth studies. M Sc thesis, Virginia Polytechnic Institute and State University, Blacksburg, Va

Cripps CL, Miller OK Jr (1993) Ectomycorrhizal fungi associated with aspen on three sites in the north-central Rocky Mountains. Can J Bot 71:1414–1420

Cripps CL, Miller OK Jr (1994) A new *Cortinarius* from a mature aspen stand in Montana. Mycotaxon 50:315–321

Godbout C, Fortin JA (1985) Synthesized ectomycorrhizae of aspen: fungal genus level of structural characterization. Can J Bot 63:252–262

Heslin MC, Douglas GC (1986) Synthesis of poplar mycorrhizas. Trans Br Mycol Soc 86:117–122

Ingleby K, Mason PA, Last FT, Fleming LV (1990) Identification of ectomycorrhizas. (ITE Research Publication No 5) HMSO, London

Johansen DA (1940) Plant microtechnique. McGraw-Hill, New York London

Kuyper TW (1986) A revision of the genus *Inocybe* in Europe. Persoonia 3:[Suppl] 1–247

Lihnell D (1942) *Cenococcus graniforme* als Mykorrhizabildner von Waldbäumen. Symb Bot Ups 5:1–19

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

Melin E (1923) Experimentalen Untersuchungen über die Birken und Espenmykorrhizen und ihre Pilzsymbionten. Svensk Bot Tidskr 17:479–520

Miller SL, Koo CD, Molina R (1991) Characterization of red alder ectomycorrhizae: a preface to monitoring below-ground ecological responses. Can J Bot 69:516–531

Mohan V, Natarajan K, Ingleby K (1993) Anatomical studies on ectomycorrhizas. II. The ectomycorrhizas produced by *Amanita muscaria, Laccaria laccata* and *Suillus brevipes* on *Pinus patula*. Mycorrhiza 3:43–49

Molina R (1979) Pure culture synthesis and host specificity of red alder mycorrhizae. Can J Bot 57:1223–1228

Molina-R, Palmer JG (1982) Isolation, maintenance, and pure culture manipulation of ectomycorrhizal fungi. In: Schenk NC

(ed) Methods and principles of mycorrhizal research. American Phytopathological Society, St Paul, Minn, pp 115–130
Mueggler WF (1985) Vegetation associations. In: DeByle V, Winokur RP (eds) Aspen: ecology and management in the western United States. (US Forestry Service General Technical Report RM-119) Rocky Mountain Forest and Range Experiment Station, Fort Collins, Colo, pp 45–55
Pirazzi R, Anselmi N, Giorcelli A (1989) Micorrizazione artificiale in piante di pioppo. Micol Veget Medit 4:43–56
Schramm JR (1966) Plant colonization studies on black wastes from anthracite mining in Pennsylvania. Trans Am Philos Soc 56:1–194
Thiers HD (1982) The Agaricales (gilled fungi) of California. Mad River, Eureka, Calif
Watling R (1969) New fungi from Michigan. Notes R Bot Gard Edinburgh 29:59–66
Wilcox HE (1982) Morphology and development of ecto- and ectendomycorrhizae. In: Schenk NC (ed) Methods and principles of mycorrhizal research. American Phytopathological Society, St Paul, Minn, pp 103–113