# The ectomycorrhizae of *Russula acrifolia*: an anatomical and ultrastructural treatise[1]

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Summary. The ectomycorrhizae of *Russula acrifolia* on *Picea abies* are studied in detail anatomically and ultrastructurally. They are well characterized by a gelatinous mantle, by knob bearing cystidia on the mantle and by knob bearing rhizomorphal hyphae. The knobs are formed solitary, in pairs or even in groups of three and can easily break off. The shape of the knob bearing elements of mantle, ectomycorrhizal rhizomorphs, fruitbody rhizomorphs, primordium, fruitbody stipe, hymenium, and cuticle is compared; a modification of the shapes is obvious and possible roles of the cystidia are discussed. Intrahyphal hyphae were found in emanill as in mantle hyphae. Hyphal inclusions identified in ultrathin sections were analysed by energy loss spectroscopy (EELS), electron spectroscopic imaging (ESI) and by PATAg test, a polysaccharid-staining method for electron microscopy. The identifed elements are discussed in connection with the role of ectomycorrhizae in nutrient storage and transport.

KEY WORDS: Anatomy, cuticle, cystidia, element analysis (EELS, ESI), ectomycorrhizae, hymenium, hyphal inclusions, intrahyphal hyphae, *Picea abies*, primordium, rhizomorphs, *Russula acrifolia*, ultrastructure.

## Introduction

The characteristics of ectomycorrhizae can contribute to the knowledge of genera, of sections and of species; even families and orders of fungi can have common features with respect to these symbiotic structures (Agerer 1994b). But it is disappointing that only from few species the ectomycorrhizae are known. The most informative characteristics are the organization of the mantles as seen

[1] Considered as part LIII of the series "Studies on ectymycorrhizae". Part LII (Agerer 1994a).

in plan views of the outer surface, of rhizomorphs and of cystidia, if the latter are formed at all (Agerer & al. 1989, Agerer 1994b).

Several types of ectomycorrhizal mantles (Agerer 1987–1993, 1991) are known to occur in the genus Russula (Agerer 1994b): a) Mantles with net like arranged hyphae bearing prominent cystidia (Type D), b) with a coarse hyphal net (Type H), c) with irregularly shaped cells, some staining dark in sulfo-vanillin (Type N), d) with angular cells bearing heaps of roundish cells (Type K), and e) mantles with angular cells bearing heaps of flattened cells (Type O).

The rhizomorphs are mostly undifferentiated or unknown (Agerer 1994b), and cystidia often belong to the flask-shaped type with an apical knob (Type D: Agerer 1987–1993, 1991, 1994b). In low magnification all ectomycorrhizae of the genus *Russula* appear smooth (Agerer & al. 1989, Agerer 1994b, Dominik 1969, Gobout & Fortin 1985).

The present contribution concentrates on *Russula acrifolia*, a member of Section Compactae (Singer 1986). To date no species of this relationship has been characterized satisfactorily.

## Material and Methods

The ectomycorrhizae were carefully excavated beneath the fruitbody, cleaned in a water bath under a dissecting microscope and their identity was proved by tracing rhizomorphs from the ectomycorrhizae to the stipe base of the fruitbody and by microscopical comparison of the respective hyphae (Agerer 1991). Fresh samples were studied in water regarding all features which were expected to change during preservation in the fixative FAA (Agerer 1991):

a) colour of the ectomycorrhizae was characterized in water using day light quality lamps, colour charts were omitted because they have not proven to be useful (Agerer 1987–1993),
b) colour of hyphae and the features of incrustations, and
c) chemical reactions. All other characteristics were studied using fixed material. Mantle and rhizomorph preparations were studied with Normarski's interference contrast and lactic acid was used as medium; for studies of sections ectomycorrhizae were embedded in historesin, cut with a microtome and observed in normal phase contrast, or cut with a cryotome (if the resin did not penetrate the mycorrhizae completely), observed in Normarski interference contrast and lactic acid as medium (Agerer 1991).

Methods to characterize ectomycorrhizae have been comprehensively explained by Agerer (1986a, 1987–1993, 1991), and a glossary of terms has been published (Agerer 1987–1993). Description of the current ectomycorrhizae will follow previous descriptions.

The voucher specimen is deposited in M (Botanische Staatssammlung München) as fixed material (FAA), together with colour slides and microscope slides (Agerer 1991).

For electron microscopy, freshly isolated ectomycorrhizae and rhizomorphs were fixed with glutaraldehyde-OsO4 and embedded in Spurr's resin (Spurr 1969). For ultrastructure investigations, ultrathin cross-, longitudinal and tangential sections, mounted on Formvar coated grids, were stained with uranyl acetate solution 2%, w/v, pH 4.6) and subsequently with lead citrate (Reynolds 1963). They were examined in a conventional TEM, ZEISS EM 109 (Oberkochen, Germany), or in a Zeiss CEM 902 A supplied with an electron energy spectrometer and a digital image analysis system. For ESI (electron spectometric imaging) and EELS (electron energy loss spectroscopy) investigations on unstained ultrathin sections (30 to 40 nm), cut with a diamond knife in a ultramicrotrome, model Ultracut (Reichert, Vienna), mounted on uncovered 700 mesh grids (Science Services, Munich), the mentioned Zeiss CEM 902 A microscope was used; elemental mapping was done by comuter-assisted image processing (Bauer 1987; Egle & al. 1984; Kottke 1991; Ottensmeyer & Andrew 1980; Probst & Bauer 1987).

For detecting polysaccharide-containing inclusions in thin sections, we applied the PATAg (periodic acid-thiocarbohydracide (TCH)-Ag proteinate) test or Thiery reaction (Thiery 1967) according to the protocol described in detail by Gianinazzi & Gianinazzi-Pearson (1992). The test is often applied for detecting vicinal (vic-) glycol groups (1–4 polysaccharides). Thin sections of glutaraldehyde OsO4-fixed samples were mounted on gold grids and incubated as indicated in the protocol used on a droplet of periodic acid for specific oxidation of vic-glycols into aldehydes; subsequently, the sections were treated with TCH and silver proteinate for the visualization of aldehydes in the microscope as electron opaque silver grains. In negative control samples, the treatment of the ultrathin sections with periodic acid and/or TCH was omitted.

## Results

Reference specimen: Deutschland, Bayern, Vorderer Bayerischer Wald, Lkr. Regensburg, nahe Forstmühle, im Waldbezirk Rabenzipfl, unter *Picea abies* leg. et det. R. Agerer, 3.10.1992. Fruitbody and ectomycorrhizae in Herb. RA 11777 (in M). Only the reference specimen was examined.

Anatomy of the ectomycorrhizae of *Russula acrifolia* Romagn.

## Morphological characters (Fig. 1a):

Mycorrhizal systems monopodially-pyramidal to monpodially-pinnate, concentrated in mineral soil layer; mycorrhizal ends straight or slightly tortuous, up to 3.9(–5.2) mm long and 0.33–0.45 mm in diameter; axes 0.45–0.51 mm thick; surface of unramified ends silvery, more or less smooth, but covered by soil particles, very tips free of soil debris; unramified ends whitish, due to enclosed air between cystidia, if air displaced by water then mycorrhizae ochre due to the colour of the root, very tips ochre, older parts and older mycorrhizae gray and infrequently covered with soil-particles. Rhizomorphs infrequent, originating in the middle part or at the basis of the system, connection with mantle rather restricted, but mostly obscured by soil debris, up to at least 150 µm in diameter, in cross-section roundish, densely covered by soil particles, rather fragile, white or slightly brownish, several hyphae emanating from the margin. Emanating hyphae of the mantle discernable, covered by soil particles.

Anatomical characters in plan views (Fig. 2): Outer surface of mantle (Figs. 1b, c) loosely plectenchymatous, strongly gelatinous (Agerer 1987–1993, 1991, 1994b: mantle Type C/D), no pattern discernable, hyphae forming frequent ramifications and simple anastomoses resulting in thin, straight, strongly netted, loose, cystidia bearing hyphal systems, hyphae 1.5–2.5(–3.5) µm in diameter, ungelatinized parts of hyphal walls thin, hyphae slightly brownish membranaceous, smooth, septa often with a round central globule. – Middle layers of mantle (Fig. 1d) with irregularly net-like, in part considerably inflated hyphae, but often also oriented in parallel rather dense bundles, gelatinous matrix not so distinct as in outer layers, hyphae (3–)4–6.5(–9) µm in diameter, walls thin, colourless to slightly ochre membranaceous. – Inner surface of mantle (Fig. 1e) densely plectenchymatous, hyphae mostly with very short cells, in part hyphae arranged in small bundles and enclosing nests of short cells, globular thickenings of septa very distinct, hyphae 4–8 µm in diameter, walls up to 0.5 µm thick but mostly caused by adjoining hyphal walls, gelatinous matrix occasionally discernable, hyphae colourless. – Surface of very tip (Fig. 2a) plectenchymatous, hyphae gelatinous, no pattern discernable, weakly differentiated, without typical cystidia, but with undifferentiated hyphal ends similar to young cystidia, hyphae 2–3 µm in diameter, walls thin, smooth, slightly ochre. Rhizomorphs (Figs. 2c–f; Pl. 1d): Hyphae strongly interwoven, slightly gelatinous, thinner rhizomorphs and those of fruitbody basis undifferentiated (Type B: Agerer 1987–1993, 1991, 1994b), thicker rhizomorphs with some irregularly distributed (Type E: Agerer 1987–1993, 1991, 1994b), over shorter distance considerably inflated hyphae, often with an enlarged septal pore, but obviously without dissolvation of septa; thick hyphae 4–8(–10) µm

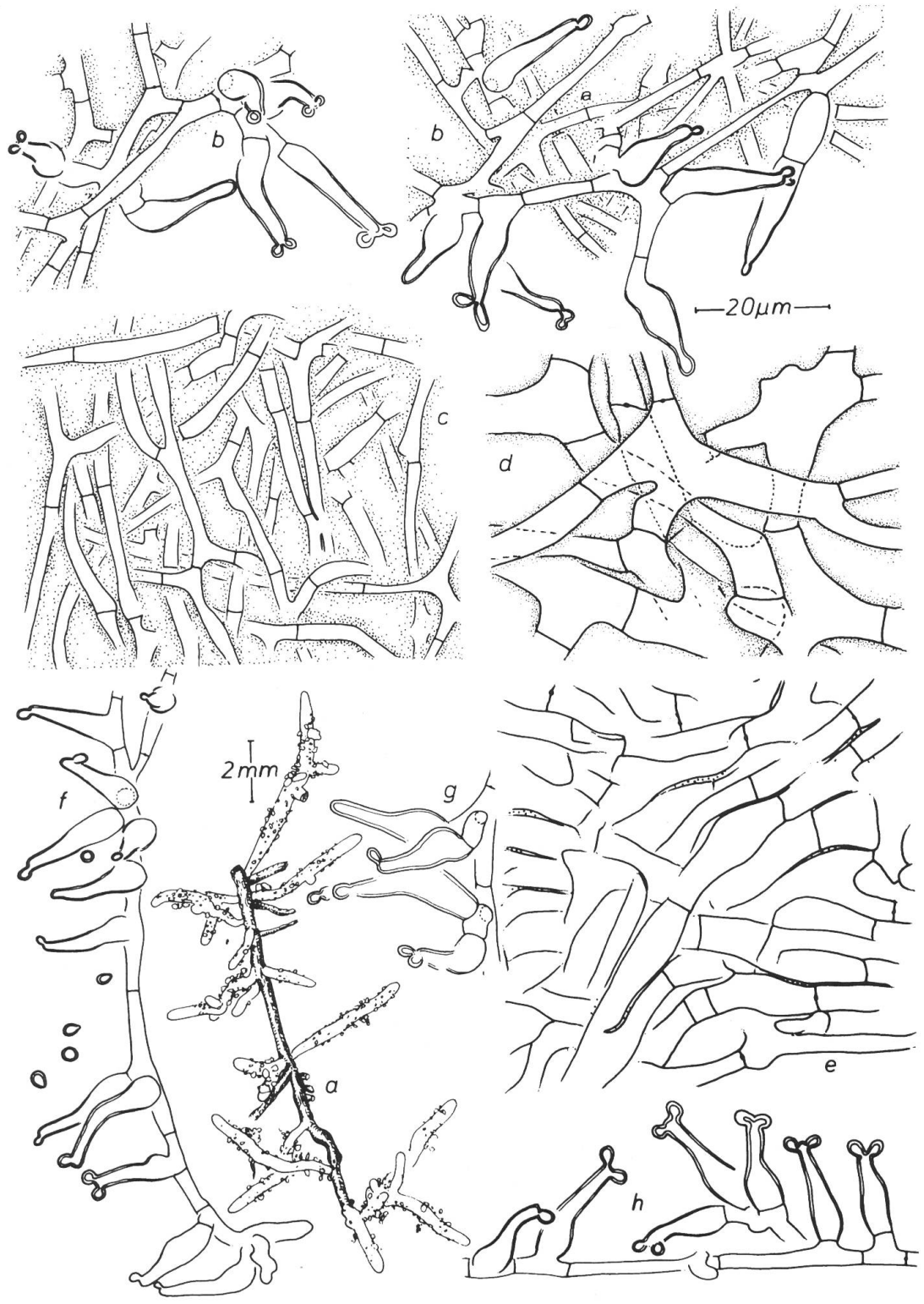



*Fig. 1.* Russula acrifolia × Picea abies. – *a. Habit. – b–e. Plan views: b. Mantle surface with cystidia, hyphae embedded in a gelatinous matrix. – c. Outer layer of mantle, hyphae embedded in a gelatinous matrix. – d. Middle layer of mantle, hyphal diameter enlarged, matrix gelatinous. – e. Inner surface of mantle, gelatinous matrix still discernable. – f. Mantle hypha with several cystidia in different stages of development, some free cystidial knobs. – g–h. Some cystidia. (All Figs. from RA 11777).*

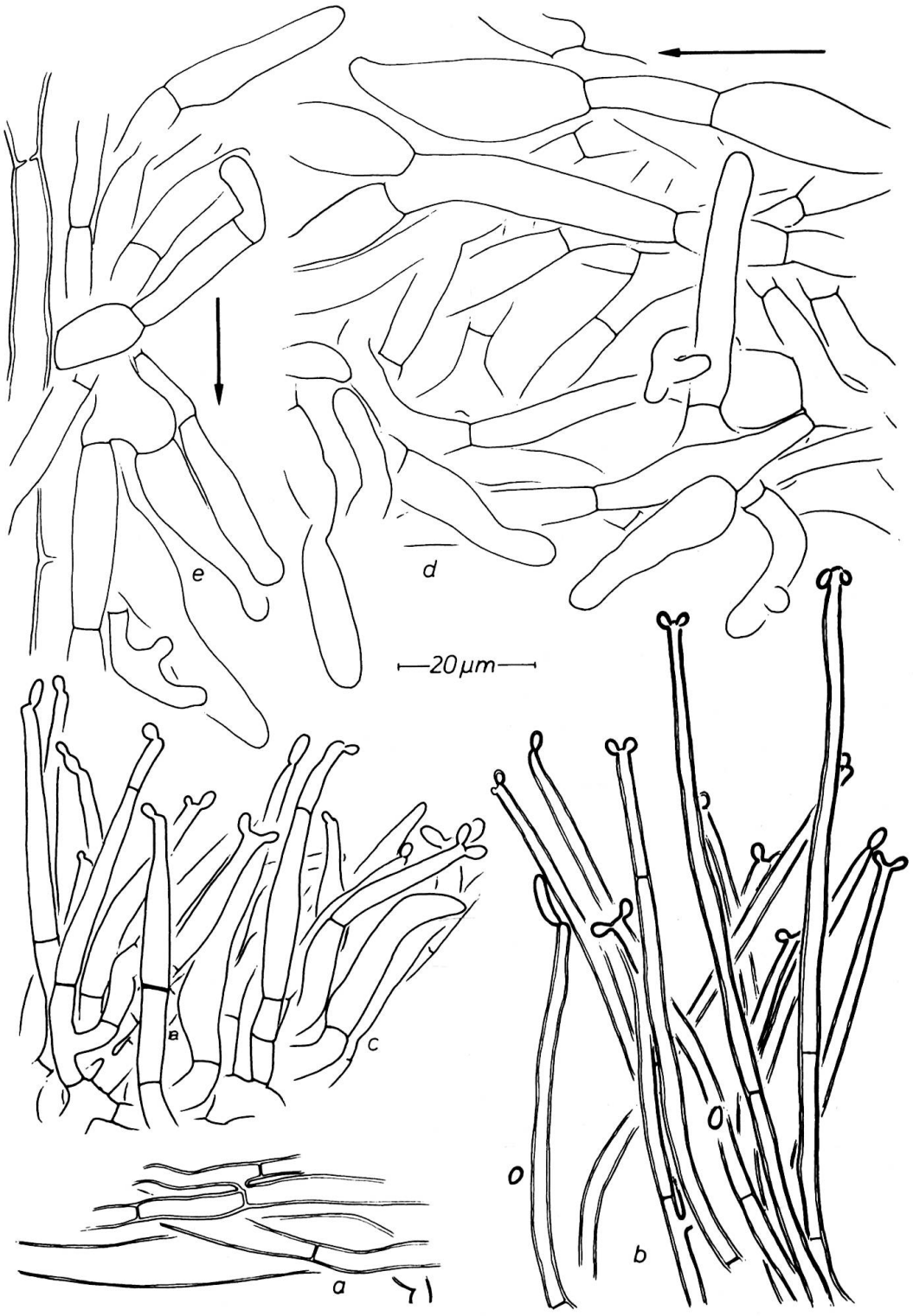



*Fig. 3.* Russula acrifolia. – *a. Detail of a fruitbody rhizomorph with a somewhat inflated hypha (infreqently occurring). – b. Knob bearing, cystidium-like hypha of fruitbody rhizomorph; two knobs with delimiting septa (arrowheads). – c. cystidia at primordium basis. – d. Fruitbody stipe, plan view of the surface at the upper quarter of the stipe, cystidia lacking. – e. Fruitbody stipe, section through the margin, at the upper quarter of the stipe, cystidia lacking. (Arrows indicate upper direction of the stipe; all Figs. from RA 11777, herbarium material).*

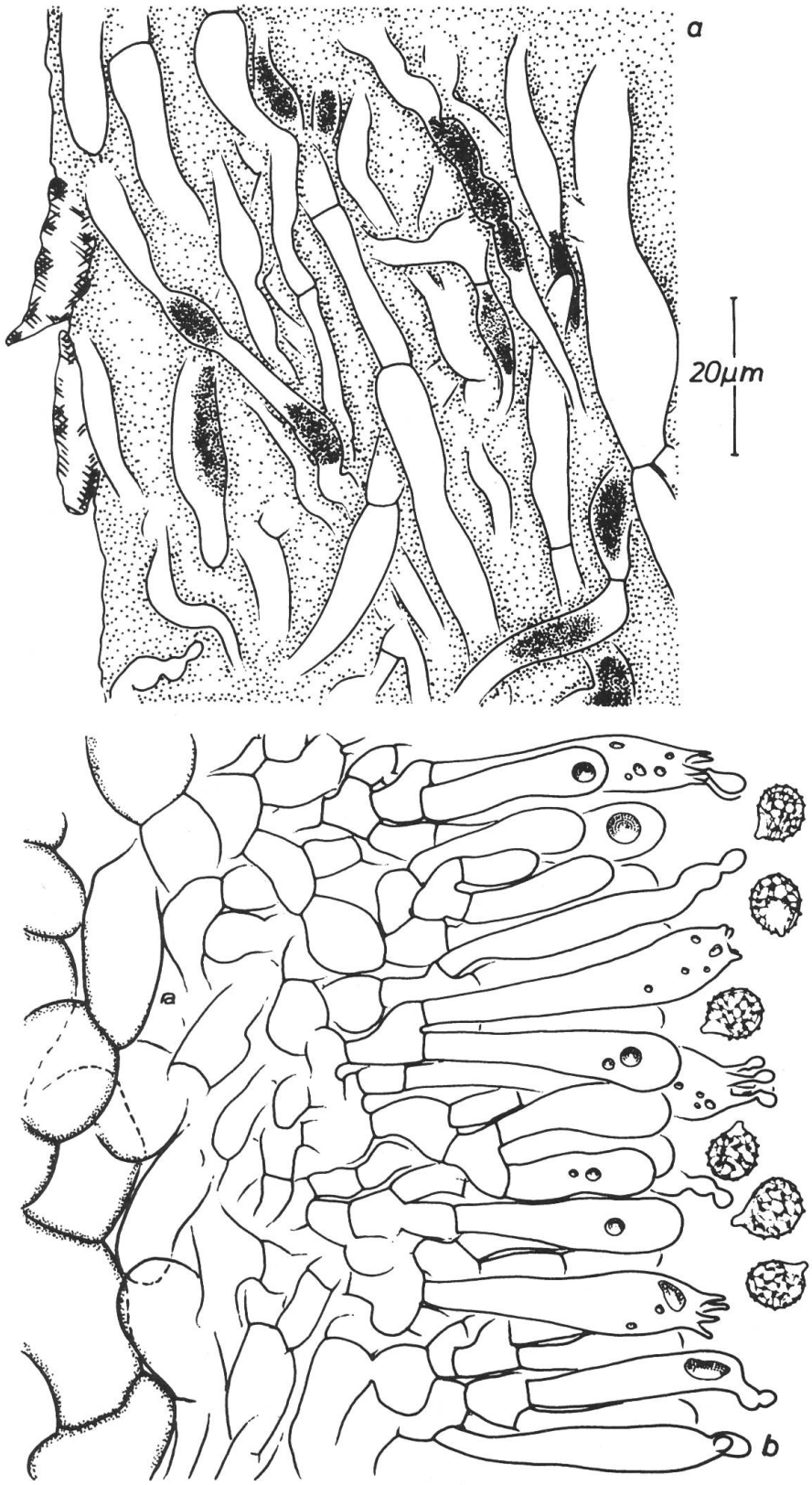
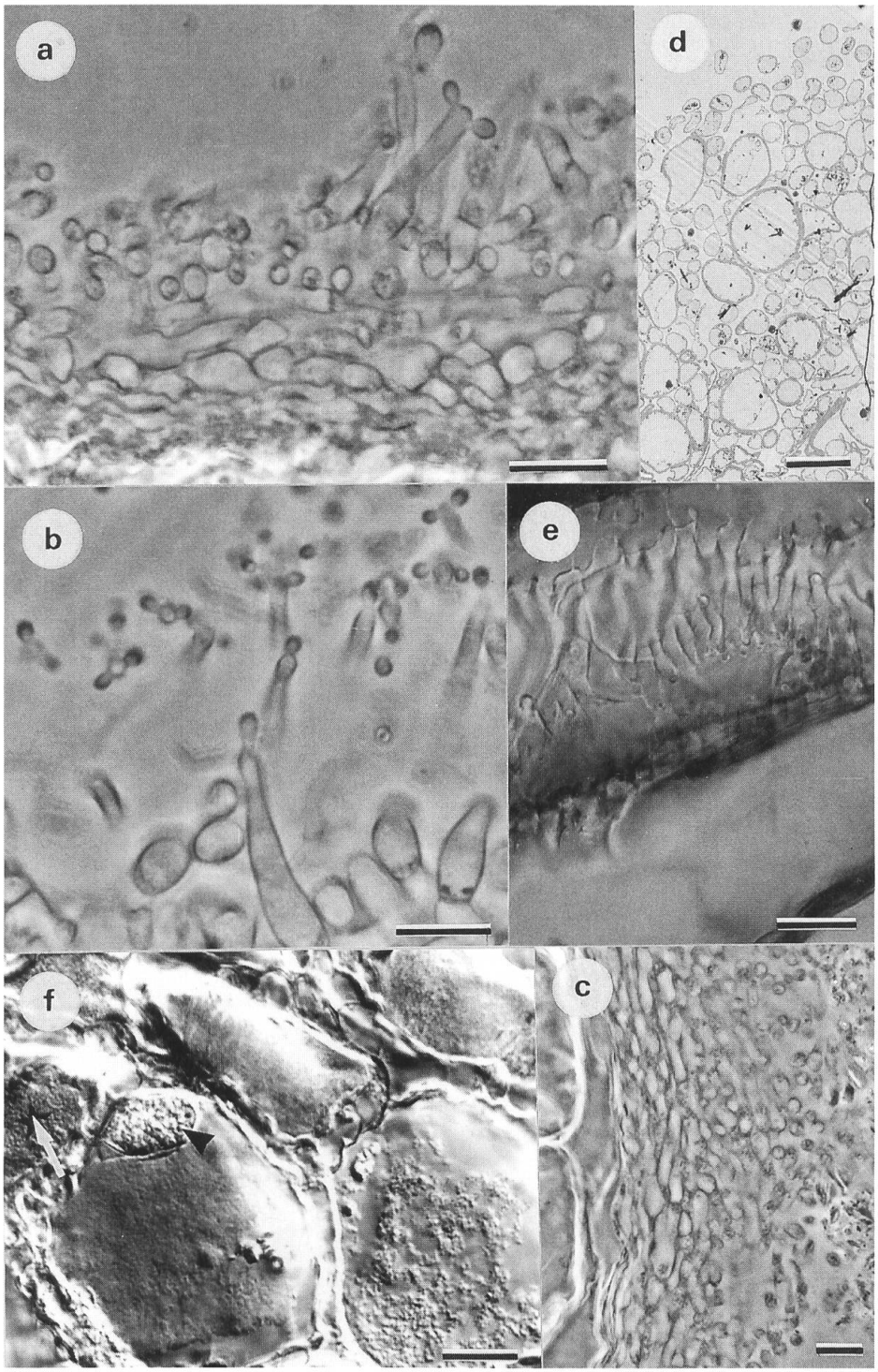



*Fig. 4.* Russula acrifolia. – *a. Radial section through the pileipellis, surface (left), gelatinous matrix, a beaded cystidium-like hypha (serial knobs (?, left below), and some hyphae with brownish contents. – b. Radial section through the hymenium with cystidia possessing serial knobs, subhymenium and part of the central gill trama with somewhat thick-walled, slightly brownish, inflated cells. (All Figs. from RA 11777, herbarium material).*

in diameter, walls up to 0.5 µm, sometimes with intrahyphal hyphae; remaining hyphae 2.5–4 µm in diameter, outer hyphae forming cystidia-like hyphal ends (see below), septa simple, in variable distance, walls 0.5–1 µm thick, smooth, colourless, anastomoses simple.

Emanating hyphae (Fig. 2b): Mostly irregularly shaped, rarely straight, 3–3.5 µm in diameter, walls more than 0.5 µm thick, but thinner than 1 µm, smooth, colourless, sometimes with intrahyphal hyphae, septa simple and variable in distance, frequently with central globular thickenings, hyphal ends with thinner walls, sometimes tortuous and distinctly ramified, often associated with soil debris.

Cystidia (Figs. 1b, f–h, 2a, c–d, g, 3a–b, 4; Pl. 1a, b; 2b): Cystidia of mantle (Figs. 1b, f–h 2g; Pl. 1a, b, 2b) flask-shaped with 1–2(–3) apical knobs, single knobs consistently oriented obliquely, very infrequent with two or even more knobs in a single row, cystidia slightly thick-walled (up to 0.5 µm), smooth, colourless, without special contents, 13–21(–26) µm long, (4–)4.5–5.5(–6) µm diameter at the basis, neck (1.5–)2–2.5(–3) µm in diameter, with (0–)1–2 knobs, knobs (1.5–)2–2.5(–3.5) × (1–)1.5–2(–2.5) µm, knob with asymmetrically thick walls (up to 1 µm), isthmus often very thin, knobs apparently only exceptionally separated by a wall from the body of the cystidium, but observation of the connection zone very difficult; knobs occasionally breaking off. – Cystidia of mycorrhizal rhizomorphs (Figs. 2c–d) not flask-shaped, similar to normal hyphae, (23.5–)27.5–40(–47.5) µm long (from the last septum to the apex), (2.5–)3–4(–4.5) µm at the thickest position, (2–)2,5(–3) µm at the distal region, walls ca. 0.5 µm thick; apically with (0–)1–2(–3) knobs; if with three knobs they are all directly originating from the apex of the cystidium, in this case apex very slighty enlarged; knobs 2.5–4×1.5–2.5 µm, with asymetrically thickened walls (apex of knob with thicker wall), up to 1 µm thick; isthmus between knob and cystidium often very thin, perhaps sometimes with an occlusion by wall material; knobs occasionally breaking off. – Cystidia of fruitbody rhizomorphs (Fig. 3b) with the same features as those of mycorrhizae, but straighter than those of mycorrhizal rhizomorphs. – Cystidia of the primordium (Fig. 3) flask-shaped with apical knobs, longer than cystidia of mycorrhizal mantle, 25–50(–60) µm long, 3.5–6 µm thick at the thickest position, at the neck 2–2.5 µm in diameter, with 1–2 apical knobs, walls only slightly thickened, as is true for the knobs, knobs 2.5–3.5(–4)×1.5–2(–2.5) µm, not separated by a septum from thecystidial body, asymmetrically arranged, knobs with constrictions at their middle part (= serial knobs) more frequent than on mantle and rhizomorphs. – Cystidia of lamellae (Fig. 4b) inclusive of knobs thin-walled, knobs asymmetrically arranged, mostly solitary but frequently with a constriction (= serial knobs). – Typical cystidia on stipe lacking (Figs. 3d–e); cystidia infrequent in the cuticle, with serial knobs (Fig. 4a).




*Pl. 1.* Russula acrifolia × Picea abies. – *a. Cross-section, mantle from cystidia (one with two knobs) to close to tannin cells. – b. Tangential section, mantle with cystidia, in the upper background some cystidia with knob bearing apical parts. – c. Longitudinal section, mantle of the very tip of the mycorrhiza. – d. Rhizomorph, part of a cross-section, some thicker hyphae distributed over the whole section. – e. Longitudinal section, part of a cortical cell with Hartig net, above Hartig net in plan view. – f. Cross-section, Hartig net around cortical cells with a nucleus (arrowhead), part of a tannin cell (asterisk). Bar = 10 µm. (Figs. a–c in phase contrast, Figs. d–e in transmitted light, Fig. f in Normarski's interference contrast; all Figs. from RA 11777).*

### Anatomical characters, cross-section (Pl. 1a, f; 2a):

Mantle (Pl. 1a; 2a) plectenchymatous, gelatinous, (20–)25–40(–45) µm thick, composed of three different layers, innermost layer 5–10 µm thick, hyphal measure tangential 3–10(–15) µm, radial 3–4(–5) µm, walls mostly thin, middle layer (5–)10–15 µm thick, hyphal measure tangential 5–15(–20) µm, radial 2–3 µm, distinctly gelatinous, outer layer 15–20 µm thick, composed of cystidia. Residues of calyptra cells close to root surface, difficult to discern from compressed tannin cells.

Tannin cells (Pl. 1f; 2a) tangentially to radially oval, arranged in 1–2 rows; tangentially (11–)23–50(–80) µm long, radially (7–)15–25(–33) µm broad; TCt (= average tangential length of tannin cells) = 33.5 µm; TCq (average ratios between tangential length and radial width of tannin cells) = 2.1; Hartig net 3–5 (7) µm thick, composed of 1 (2) rows of hyphal cells, hyphal cells in section oval or roundish.

Cortical cells (Pl. 1f; 2a) radially to more frequently tangentially oval, tangentially (15–)25–45(–60) µm long, radially (12–)22–45(–50) µm broad; CCt (= average tangential length of cortical cells) = 37 µm, CCq (= average ratios between tangential length and radial width of cortical cells) = 1.2. Hartig net 3 cell layers deep (tannin cells not included) and reaching the endodermis; Hartig net 2–3(–3.5) µm thick, composed of one row of hyphal cells, hyphal cells in section approximately cylindric.

### Anatomical characters, longitudinal section (Pl. 1c, e):

Mantle (Pl. 1c) corresponding to cross-section in structure and measurement. Mantle of mycorrhizal tip (30–)40(–50) µm thick, outer layers very loosely woven, with gelatinous matrix, middle parts of mantle more distinctly gelatinous, hyphae roundish to oval, hyphal measurement tangential 5–10(–20) µm, radial (2–)3–4 µm, cystidia not differentiated.

Tannin cells irregularly slender oval, arranged in parallel to root surface or slightly obliquely, tangentially 40–100(–105) µm long, radially (3–)6–17 µm broad. TCt = 73 µm, TCq = 7.1. Cortical cells (Pl. 1e) slightly irregularly oval to cylindric, oriented in parallel to root surface or slightly obliquely, tangentially (17–)45–105(–115) µm, long, radially (13–)16–30 µm broad; CCt = 73 µm, CCq = 3.3. Hartig net in plan view (Pl. 1e) of palmetti type, weakly ramified, lobes (2–)3–4 µm broad, palmetti hyphae with septa.

## Colour reaction in different reagents

Mantle and rhizomorph preparation: Aniline: weakly reddish to slightly ochre; cotton blue: walls slightly blue; formol: no reaction (= n. r.); guaiac: blue; iron-sulfate: slightly greenish; KOH 15%: more intensively ochre; lactic acid: n. r.; Melzer's reagent: n. r.; phenol: n. r.; safranin: red; sulfo-vanillin: walls distinctly gray, content of some cystidia homogeneously gray.

## Autofluorescence

Cross-sections of mycorrhizae: UV-filter 340–380 nm: greenish-blue, cystidia and inner mantle layers more intensive than middle parts of mantle; blue-filter 450–490 nm: yellow (zonation like above); green-filter 530–560 nm: red (zonation like above).

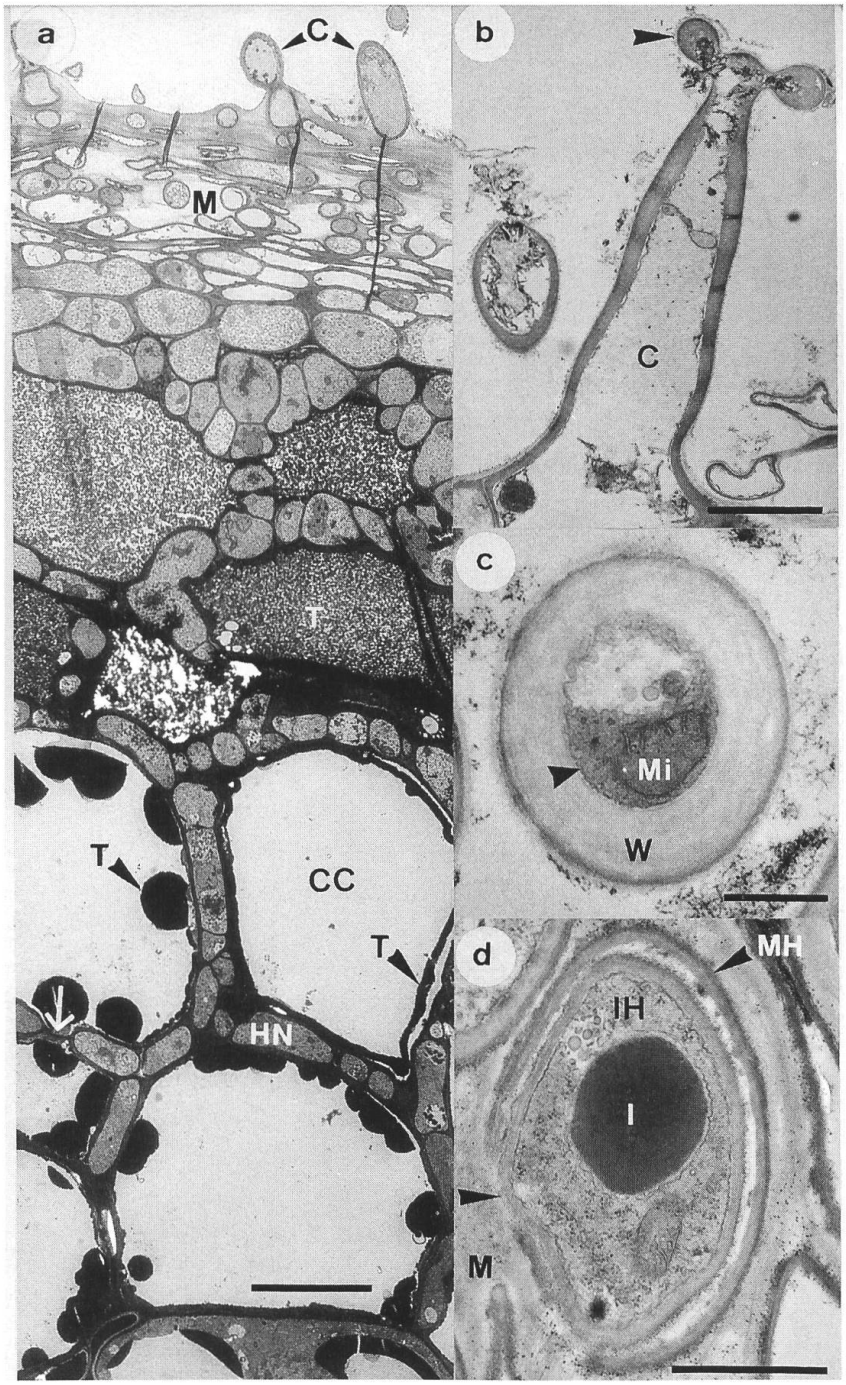
## Staining of nuclei (Figs. 2g–h)

The cells posses two nuclei. Best observable in cystidia: dikaryon with few exceptions at the basis of the cystidial body, 2×1.5–2 µm, round or elliptic, mostly very close together; knobs of cystidia without nuclei, only once a liberated knob was found with a structure reminiscent of a nucleus due to its shape and staining behaviour (Fig. 2h). Ultrastructure of ectomycorrhizae and elemental analysis of hyphal inclusions.

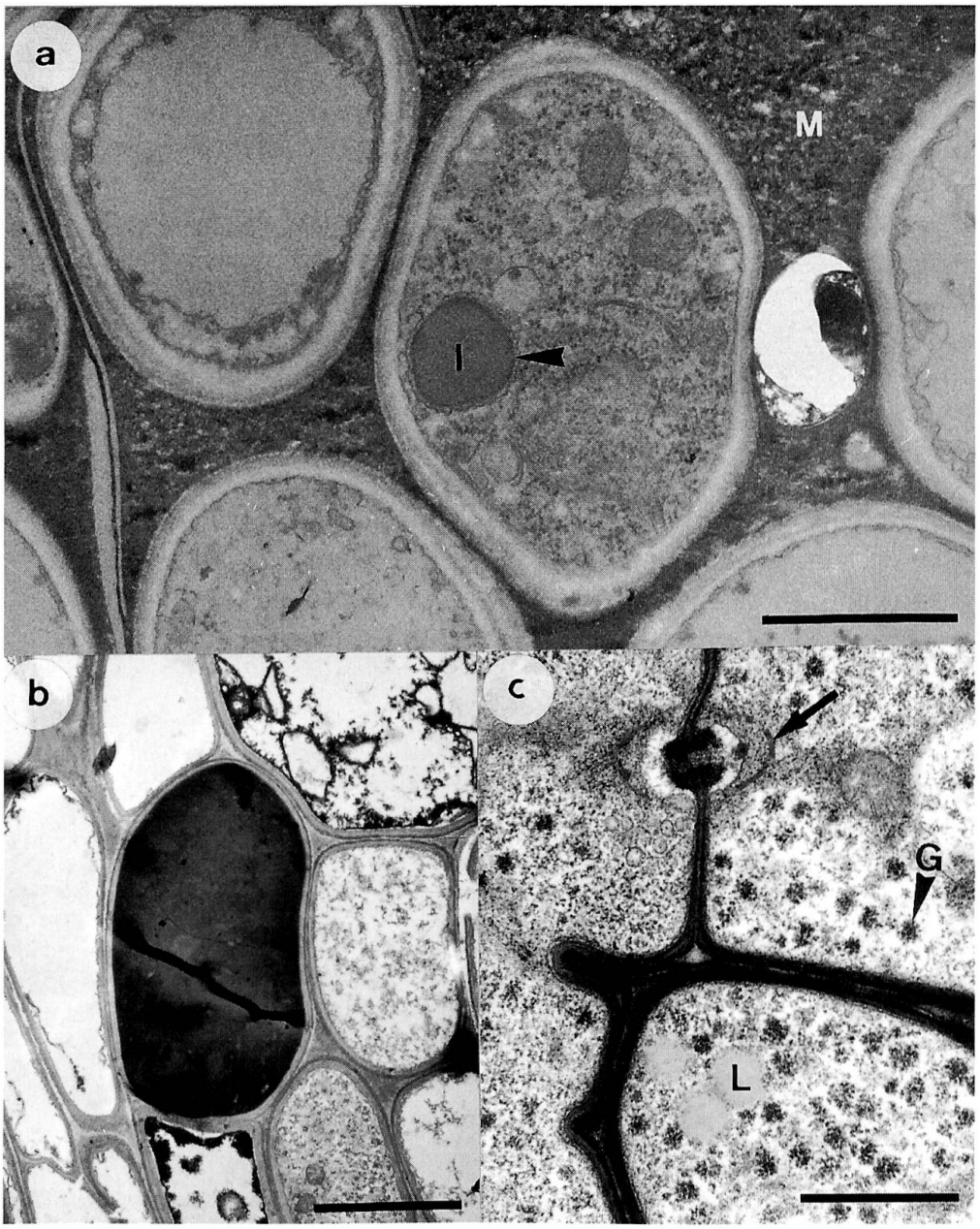
The depicted cross-section (Pl. 2a) through a *R. acrifolia* ectomycorrhiza presents an overview of the characteristic compact hyphal mantle, which is differentiated into distinct layers, and the Hartig net. In the surface layer, the hyphae are embedded in a granular, electron-dense interhyphal matrix material. In this layer, knobs possessing cystidia (Pl. 2b) were found. The knob shown in Pl. 2c, consists of a thickened wall and a membrane structure on its inner side. Cristae of the visualized mitochondrium, embedded in cytoplasmic content, are recognizable. In middle layer the matrix material becomes electron-transparent, and the interhyphal space decreases and the hyphae appeared often partially collapsed. Hyphae in the inner layer are continuous with those of the Hartig net, often showing a rounded appearance in cross-section. These hyphae are larger than hyphae in the outer mantle layers. In the outer mantle layer the hyphae were usually devoid of cytoplasmic contents, whereas the hyphae of the inner mantle layer are characterized by an increase of cytoplasmic organells.

Using electron microscopy, intrahyphal hyphae could be found in rhizomorphal hyphae and in mantle hyphae (Pl. 2d). In this micrograph the intrahyphal hypha seems to penetrate through a gap of the cell wall of the "moth-

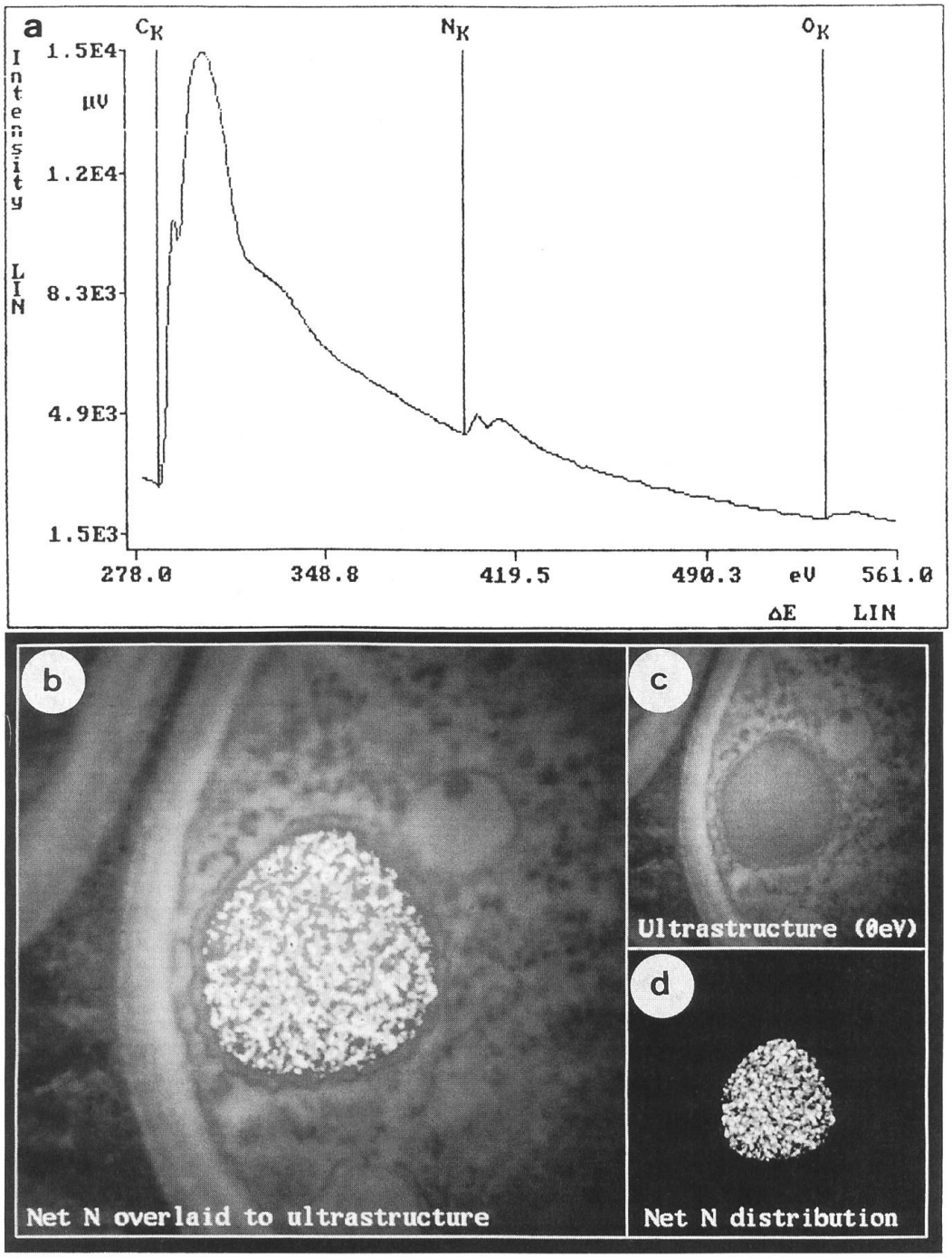

*Fig. 2.* Russula acrifolia × Picea abies. – *a. Some young cystidia at the very tip of the ectomycorrhiza. – b. Emanating hyphae originating from the mantle (left), with intrahyphal hyphae, and some distal ends connected to soil particles. – c. Surface of a mycorrhizal rhizomorph with knob producing hyphae.– d. Optical section through a young mycorrhizal rhizomorph with a somewhat enlarged central hypha, connected to a knob bearing cystidium-like hypha. – e. Optical section through a thick mycorrhizal rhizomorph with thick, inflated hyphae. – f. Some thick hyphae of a mycorrhizal rhizomorph, one with a intrahyphal hypha. – g. Some cystidia after staining of nuclei, a pair of nuclei mostly arranged basally. – h. Free knob with nuclear-like stained contents. (All Figs. from RA 11777).*




*Pl. 2.* Russula acrifolia × Picea abies: *Ultrathin sections. – a. The hyphal mantle containing interhyphal matrix (M) is differentiated into distinct layers. Cystidia (C); cortical cell (CC); Hartig net (HN); plasmodesmata between cortical cells (arrow); tannin (T). Bar = 3 µm. – b. Cystidium (C; longitudinal section) revealing two knobs (arrowhead). Bar = 3 µm. – c. Cross-section through a knob of a cystidium. The cytoplasmic content of this knob is surrounded by the thickened wall (W) and a membrane structure (arrowhead). The structure of the visualized mitochondrium (Mi) seems to be well preserved. Bar = 0.5 µm. – d. Ultrathin section through a "mother" hypha (MH), containing an intrahyphal hypha (IH) the intrahyphal hypha seems to penetrate through a gap in the cell wall of the "mother" hypha (arrowhead). Nitrogen-containing inclusion (I; for explanation see results); intrahyphal matrix (M). Bar = 1 µm. (All Figs. from RA 11777).*




*Pl. 3.* Russula acrifolia × Picea abies: *Ultrathin sections, inclusions in mantle hyphae. – a. Nitrogen containing inclusion (I) surrounded by a membrane structure (arrowhead). The EEL spectrum and the ESI of this inclusion are shown in Pl. 4. Interhyphal matrix (M). Bar = 1 µm. – b. Hypha filled with nitrogen-containing electron dense inclusion material (for details see results). Bar = 2 µm. – c. Lipid inclusions (L) and glycogen particles (G). The glycogen particles appeared as electron-opaque complex due to their PATAg-positive reaction. Doliporus with perforated parenthesome (arrow). Bar = 1 µm. (All Figs. from RA 11777).*




*Pl. 4.* Russula acrifolia × Picea abies: *Elemental analysis of the membrane-surrounded hyphal inclusions shown in Pl. 3a. EEL spectrum presenting theCk, Nk and Ok edges. – 3b–d. Distribution of nitrogen is demonstrated by ESI. – b. This micrograph is composed of the ultrastructure image (c) and the superposed net nitrogen distribution (d). The net nitrogen distribution image (d) was computer calculated from the edge et E = 410 eV and the background image at E = 390 eV. (All Figs. from RA 11777).*

er" hypha. From the cytoplasm of the "mother" hypha only a thin layer of degenerated cytoplasmic remnants could be observed. In contrast, the intrahyphal hypha is rich in cytoplasmic content.

In cross-section of ectomycorrhiza, usually one row of hyphae could be observed among the tannin and cortical cells of the root (Pl. 1e, f; Pl. 2a). The cortical cells contained electron-dense deposits, described as tannin deposits (Edwards & Gessner 1984) or polyphenol deposits (Kottke & Oberwinkler 1987). As described for most ectomycorrhizae, pits with plasmodesmata between the cortical cells were present and not separated by the Hartig net (arrow in Pl. 2a). Only little interhyphal matrix material was observed between hyphae of the Hartig net. In these hyphae with a dense cytoplasmic appearance, more than two nuclei could often be observed indicating a coenocytic organization of the Hartig net.

Cell inclusions of unknown chemical composition were observed in hyphae (Pl. 3) and intrahyphal hyphae (Pl. 2d). Membrane surrounded inclusions as shown in Pl. 3a were examined by EEL (= electron energy loss) spectroscopy. The EEL spectrum obtained presents the $C_k$, $N_k$ and $O_k$ edges (Pl. 4a). The distribution of nitrogen was illustrated by ESI (= electron spectroscopic imaging). Important computer micrographs, selected from the series obtained with the computer program used, are shown in Pl. 4b–d; illustrating simultaneously the ultrastructure and the nitrogen distribution (Pl. 4b) results by overlaying the net nitrogen distribution image (Pl. 4d) to the ultrathin image (Pl. 4c). Similar membrane-surrounded inclusions as shown in Pl. 2d and 3a were frequently observed in rhizomorphs and occasionally in ectomycorrhizal mantles examined, and similar EEL spectra and nitrogen distribution micrographs were obtained. Serial ultrathin sections showed that these inclusions were completely surrounded by a membrane. Inclusion material, filling the whole hypha (Pl. 3b) was rarely observed in mantle hyphae. It is worth to note that for the latter type of inclusion a similar spectrum as shown in Pl. 4a presenting a $N_k$ edge was obtained.

Inclusions shown it Pl. 3c were also examined by EEL spectroscopy and only the presence of carbon and oxygen was observed. With the PATAg test applied to such ultrathin sections, polysaccharide inclusions were detected as electron-opaque complexes. These inclusions represent most probably glycogen particles. By applying the PATAg test to inclusions (L) in Pl. 3c, no cross-reaction could be observed.

## Discussion

### Ectomycorrhizae of *Russula* sect. Compactae

*Russula adusta* (Pers.) Fr. ectomycorrhizae were synthesized by Pachlewski & Pachlewska (1974) with *Pinus silvestris* in a totally artificial agar medium. They described the mycorrhizae as being smooth and they showed a cross-section with thick mantle; cystidia were not depicted. Ogawa (1981) observed ectomycorrhizae of *R. delica* Fr. isolated from soil and noted only a thick mantle with "ornamental hyphae" and rhizomorphs. *Russula densifolia* Secr. was verified as an ectomycorrhiza former of *Abies alba* (Colla 1931), and described in more detail by Ceruti & al. (1988) in combination with *Fagus sylvatica*. They describe cystidia of 16–30×3.5–9 µm without any further details. The mantle consists of two different layers; and an additional external layer is formed by the cystidia. This fits to our findings in *R. acrifolia*. Linnemann (1971) depicts an incompletely formed ectomycorrhiza of *R. nigricans* (Bull.) Fr. in synthesis experiments with *Pseudotsuga menziesii*.

### Ectomycorrhizae of other *Russula* species

Ectomycorrhizae of only a few *Russula* species have been described comprehensively by Agerer (1994b) to enable a more detailled comparison to those of *R. acrifolia*. Only those articles are considered in the following.

Actually, *Russula* ectomycorrhizae can best be subdivided with respect to the organization of the mantles in plan views and regarding their lack or presence of special types of cystidia (Agerer 1994b, Agerer & al. 1989). The following species are known to form flask-shaped cystidia with an apical knob: *Russula aeruginea* Lindl. (Taylor & Alexander 1989), *R. grisea* (Pers.: Secr.) Fr. (Ceruti & Bussetti 1962), *R. illota* Romagn. (Brand 1991), *R. paludosa* Britz. (Peyronel 1963) and *R. xerampelina* (Schaeff.: Secr.) Fr. (Agerer 1986b). *Russula paludosa* forms in addition ramified cystidia (Peyronel 1963), *R. grisea* in addition flask-shaped cystidia with a hair-like prolongation (Ceruti & Busseti 1962). *Russula violascens* (Secr.) Sacc. seems to have only fusiform cystidia without an apical knob (Luppi & Gautero 1967).

*Russula acrifolia*, thus groups together with *R. aeruginea*, *R. illota*, and *R. xerampelina*, all having exclusively the characteristic cystidia with an apical knob-like protrusion. But the shape of their cystidia is different.

*Russula aeruginea* forms cystidia (2.5–5 µm diameter at the base, 1–2 µm at the neck, their length is not reported) in synthesis experiments with *Picea sitchensis*, but they bear only a single and apparently asymmetrically arranged,

spherical to ellipsoidal, obviously thin-walled, knob-like projection at the apex (Taylor & Alexander 1989). *Russula illota* produces, as *R. aeruginea*, cystidia with a single knob (Brand 1991). The thick-walled knobs break off easily and thus exude latex-like contents through the formed pore. The cystidia are longer (15–40 µm) and thicker at their bases (5–8 µm). Normal hyphae can form apical knobs, too. The mantle structure of *R. acrifolia*, *R. aeruginea*, and *R. illota* resemble one another. *Russula acrifolia*, however, seems to possess a more distinctly gelatinous matrix.

*Russula xerampelina* forms cystidia only occasionally (Agerer 1986b), they are slender and subulate with an abruptly tapering apex; the mantle surface is built of a coarse net of hyphae.

All other *Russula* ectomycorrhizae known up to now are devoid of cystidia: *Russula fellea* Fr. (Brand & Agerer 1988, Brand 1991), *R. emetica* Fr. var. *sylvestris* Sing. (Brand 1991), *R. firmula* J. Schaeff. (Treu 1990), *R. laricina* Vel. (Treu 1990), *R. mairei* Sing. (Brand 1991), *R. nana* Killermann (Brand 1991), *R. ochroleuca* (Pers.) Fr. (Agerer 1986b, Brand 1991, Gronbach 1988, Haug & Pritsch 1992, Pillukat & Agerer 1992).

Rhizomorphs of *Russula*-species are mostly undifferentiated (Type A: Agerer 1987–1993, 1991, 1994b), and known from *R. illota* (Brand 1991), *R. emetica* (Brand 1991), *R. mairei* (Brand 1991), *R. ochroleuca* (Pillukat & Agerer 1992) or differentiated (Type E: Agerer 1987–1993, 1991, 1994b) like in *R. acrifolia*, and also known in *R. delica* (Ogawa 1981), *R. ochroleuca* (Agerer 1986b), and *R. xerampelina* (Agerer 1986b).

## Ectomycorrhizal organization and subgeneric relationships

As the knowledge of *Russula* species is still very scarce with respect to their ectomycorrhizal organization is not possible to draw far-reaching conclusions regarding infrageneric positions of the species with the aid of mycorrhizal features. But since in the better known genus *Lactarius* some coincindence between mycorrhizal organization and affiliation to sections could be shown (*Lactarius* sect. Dapetes, *Lactarius* sect. Plinthogali; Agerer & al. 1989, Agerer 1994b), at least a compilation of present day knowlegde may be presented for *Russula*.

Subsections (Singer 1986): Subc = Subcompactinae, Xeram = Xerampelinae, Foet = Foetentinae, Fell = Felleinae, Russ = Russula, Poly = Polychromae, Uren = Urentes, Inte = Integrae, Firm = Firmiores.

Species: acri = *R. acrifolia*, aeru = *R. aeruginea*, xeram = *R. xerampelina*, illo = *R. illota*, ochro = *R. ochroleuca*, fell = *R. fellea*, mair = *R. mairei*, lari = *R. laricina*, firm = *R. firmula*, gris = *R. grisea*, nana = *R. nana*, emet = *R. emetica* var. *sylvestris*.

Although only a very limited number of species has been investigated regarding their ectomycorrhizae to date, it can be concluded at least, that mycorrhizal features can promote the knowledge of species and might contribute to the delimitation of subgeneric entities within the genus *Russula*.

## Ultrastructure

It becomes more and more evident that function of ectomycorrhizae with respect to tree nutrition and tree growth is highly dependent on the fungal species involved (Agerer 1993). It is also known that features of mantles which are involved in transport and storage function of nutrients as well in root protection against pathogens, are exclusively determined by the fungus (Agerer 1994b). Therefore, the advantage of studying the ultrastructure and elemental composition by EELS (electron energy loss spectroscopy) and ESI (electron spectroscopic imaging) of identified ectomycorrhizae is obvious.

Our ultrastructural investigations showed that *Russula* acrifolia forms a compact mantle revealing features described for other ectomycorrhizae (Haug & Oberwinkler 1987, Haug & Pritsch 1992). In the hyphal mantle, a great amount of interhyphal matrix was observed. The occurrence of both electron-transparent and electron-dense matrix material was shown by Haug & Oberwinkler (1987) and discussed by Agerer (1991, 1993). Strullu (1985) considered mantles and Hartig net with electron-dense matrix material as typical for Ascomycetes, with electron-transparent as typical of Basidiomycetes. But Berndt & al. (1990) indicated that this feature is age dependent in ascomycetous ectomycorrhizae. *Russula acrifolia* as a basidiomycete showed both types of matrix material. Gelatinous matrix material is considered as commonly produced by fungi and can function in anchorage fungal structures of parasites (Onyle & al. 1982), or as suitable aid to protect against fast and complete desiccation (Oberwinkler 1985). Both interpretations might fit to the tasks of the ectomycorrhizal mantle. As concluded by Strullu (1979), the ageing of hyphal mantles occur probably from the outer to the inner part. Further remarks on the ontogeny of hyphal mantles are given by Haug (1987).

The occurrence of intrahyphal hyphae in ectomycorrhizal rhizomorphs was reviewed, and their possible role as resistant structures and repair mechanism was discussed (Agerer 1994b).

However, no report is available on their occurrence in ectomycorrhizal mantles. In the present work, the occurrence of intrahyphal hyphae in mantle hyphae could be demonstrated only by electron microscopy. Degenerated cytoplasm between the cell walls of the intrahyphal hypha and the 'mother' hypha (Pl. 2d) was described for other fungi and its role as initial source of nutrients for the growing intrahyphal hypha was discussed (Chan & Stephen 1967,

Lim & al. 1983). Some hints could be found that the central globular thickenings of the hyphal septa (Figs. 1e; 2a), which can be seen frequently, correspond ultrastructurally to an electron-dense globule around the dolipore, but further studies are necessary for a conclusive interpretation.

A comprehensive report on the cellular structure of the Hartig net of in vitro grown ectomycorrhizae of *Amanita muscaria* (Pers.: Fr.) Hook. was given by Kottke & Oberwinkler (1987). The formation of interhyphal matrix in the Hartig net seems to differ with the species of ectomycorrhiza. Observations that pits between cortical cells remain intact during Hartig net formation revealing their symplastic continuity were made for many ectomycorrhizae (Kottke & Oberwinkler 1987, Nylund 1980, Warmbrodt & Eschrich 1985). Our observation that more than two nuclei can occur in hyphae of the Hartig net is in agreement with the interpretation of Kottke & Oberwinkler (1987) of a coenocytic organization of the Hartig net. However, the lobed appearance of the nuclei described by these authors for the in vitro grown ectomycorrhiza of *Amanita muscaria* could not be detected in our natural ectomycorrhiza of *Russula acrifolia*.

In a previous report (Franz & Acker 1993), an introduction on the elemental analysis of inclusions in mantle hyphae of 20 different ectomycorrhizae of spruce was presented; by applying the EEL spectroscopy to membrance-surrounded inclusions, similar as shown in Pl. 3a, the presence of nitrogen could be detected. This was concluded from a spectrum profile revealing a nitrogen edge, similar as shown in Pl. 4a; these observations were confirmed by ESI (Pl. 4b–d). EELS was also applied to inclusion material filling the whole hypha (Pl. 3b), and a similar spectrum as shown in Pl. 4a was obtained. We assume that both the membrane-surrounded inclusions (Pl. 3a) and the free inclusion material (Pl. 3b) represent protein accumulations. As discussed in a previous paper (Franz & Acker 1994) this interpretation is in accordance with the statement of Finlay (1992) that uptaken nitrogen is incorporated in proteins. Further studies are necessary to clarify the role of hyphal inclusions in nitrogen storage, transport or in fungal pathway.

Polyphosphate granules, as shown by Kottke (1991) and discussed as filter mechanism for toxic substances in mycorrhizal hyphae by Thurnau & al. (1993), could not be detected in our material.

The positive staining results obtained with PATAg test (Pl. 3c) demonstrate the occurrence of polysaccharide containing inclusions, most probably glycogen particles, described as a common storage product in fungal hyphae (Becket & al., 1974). From our negative staining results obtained by applying PATAg test to similar inclusions as indicated (Pl. 3c, L), they can be interpreted as lipid inclusions.

## Some thoughts about the function of *Russula* cystidia

An excretion function is ascertained for hymenial cystidia of several fungi, especially in those cases where substances are deposited outside the cystidium, e. g. *Baeospora myosura* (Clemencon 1972), *Inocybe* spp. (Waterkeyn & al. 1992), *Phanerochaete chrysosporium* (Setcliff 1979). An excretion function is also assumed only based on their ultrastructure (Gull & Newsam 1975). But cystidia are also assumed to have a function in exudation (Chiu & Moore 1990a), in evaporation of moisture or volatile substances, in acting as an air trap (Smith 1966), or, as buttresses, in holding the gills apart as in certain *Coprinus* species (Smith 1966), or *Volvariella bombycina* (Chiu & Moore 1990a). Chiu & Moore (1990b) suggest for *Coprinus cinereus* that, cystidia in combination with their cystidial counterparts, act as tension elements holding adjacent hymenia together. The cystidia of *Russula* and *Lactarius* fruitbodies are regarded as prolongations of the laticifers into the hymenium or epicutis or at least as homologous organs (Singer 1986). In *Russula acrifolia* the hymenial cystidia originate from subhymenial hyphae without any connection to hyphae with oily contents (see Fig. 4b) which is in contrast to *R. emetica*, where the cystidia are terminations of laticifers (Oberwinkler 1977). A definite function of the hymenial cystidia of *Russula* is still unkown. For gloeocystidia of resupinate fungi Overholtz (1929) suggested that they might have a protective function if their contents were unpalatable to animals (Talbot 1954).

Ectomycorrhizal cystidia of *Russula* species, however, suggest a possible excretion function as a defense mechanism against grazing soil invertebrates (Brand 1991, Edwards & Gessner 1984). Although Edwards & Gessner (1984) were unable to observe a breaking off of the apical knobs, nor any free knobs of the cystidia of a *Russula* ectomycorrhiza, Brand (1991) demonstrated that apical knobs of the ectomycorrhizal cystidia of *Russula illota* broke off, with a subsequent exudation of oily substances. Cytoplasmatical features of knob bearing hyphae on rhizomorphs of *Russula acrifolia* do not reveal any secretion function, because no oily contents could be discovered (Figs. 2c–d, 3b; cf. Brand 1991).

A second function of *Russula* cystidia may be considered. Again, cystidia of belowground structures serve as a device for an additional hypothesis. In *R. acrifolia* ectomycorrhizae and rhizomorphs, the apical knobs break easily off, and are often arranged in pairs, sometimes also in groups of three on normal hyphae as well as on cystidia. If more than two knobs are formed, the apical part of the knob bearing hypha can be slightly inflated (Fig. 2c). This is in a restricted sense reminiscent of conidiophores of different fungi related to Russulaceae (*Bondarzewia montana, Heterobasidion annosum:* Stalpers 1974) and occur also in other genera (Kendrick & Watling 1979, Stalpers 1974). But

extremely infrequently, delimiting septa between knobs and hyphae or cystidial bodies can be found (Fig. 3b), and denticles are lacking. In addition, staining revealed only pairs of nuclei, which mostly were laying very close together in the basis of the cystidia, and only in one case a free knob could be found where the staining behaviour of the contents were reminiscent of nuclei (Fig. 2h). TEM studies, however, revealed no nuclei in the apical knobs, whereas mitochondria could be found (Pl. 2c). Hence, a suggested function of the knobs as conidia possibly disseminated by soil animals must still remain a working hypothesis. But it would be in agreement with the occurrence of conidia of *Heterobasidion annosum*, which occasionally are formed in bark or soil cavities and are assumed as being disseminated by insects (Hodges 1969).

There is an evident change in the structure of the cystidia starting from the ecomycorrhizal mantle (Figs. 1b, f–h; 2g), over the rhizomorphs (Figs. 2c–d; 3b), to the primordium (Fig. 3c), and lastly to the hymenium (Fig. 4b; cf. Romagnesi 1967) and cap cuticle (Fig. 4a; cf. Romagnesi 1967, Schwöbel 1974).

The cystidia of mycorrhizae, rhizomorphs, and even of primordia of *Russula acrifolia* are well adapted to soil conditions as well as to a possible function in this environment, due to their thick walls and easily breaking off knobs. It can be suggested that they function as defensive structure against grazing soil invertebrates or/and as cells to produce propagules. But to date, no special function is apparent for the hymenial cystidia. They are neither suitable as buttresses (Chiu & Moore 1990a) nor as tension elements (Chiu & Moore 1990b) because of their small dimensions. An excretion function might be assumed (Smith 1966), and an extrusion of oily contents after grazing of the hymenium seems possible, but both has not been proven yet. Presently, it is tempting to hypothesize that the cystidia of *Russula acrifolia* have their primary function in the soil, and that they have lost their previous function within the hymenium.

In summary, the belowground knob bearing cystidia and hyphae of *Russula* species might have or might have had a secretory protecting function and/or a function in asexual dissemination in the soil. Further studies have to corroborate or disprove these hypotheses.

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