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A morphological study of the mycorrhiza of *Entoloma clypeatum* f. *hybridum* on *Rosa multiflora*

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Mycorrhizas of *Entoloma clypeatum* f. *hybridum* on *Rosa multiflora* in the field in Japan were studied by stereo, light and electron microscopy. In most mycorrhizas, the root cap, meristem, and apical region of the cortex disappeared, but in a few mycorrhizas, these tissues remained. Fungal hyphae of the mycorrhizas invaded root tissues and branched palmately. Hyphae in contact with cortical cells were larger than those far from the root cells and contained many mitochondria, cisternae of endoplasmic reticulum and transitional vesicles. Invading hyphae were undulate in the apical part of the mycorrhiza, and some of them lacked distinct organelles. Electron-dense granules accumulated in the root cells adjacent to the fungal hyphae. Both the remnants of the plant cells and the fungal hyphae were included in the amorphous materials on the tip of the stele. These observations suggest the destructive infection by fungal hyphae of the root cells and their collapse near the tip of the stele.

Key Words——*Entoloma clypeatum* f. *hybridum*; mycorrhiza; *Rosa multiflora*; ultrastructure.

The genus *Entoloma* (Fr.) Kumm. belongs to Agaricales (Basidiomycetes) and consists of saprotrophic and biotrophic (mycorrhizal) species, of which some of the latter are known to be associated with the Rosaceous plants (Becker, 1956; Trappe, 1962; Andruszewska and Dominik, 1971; Noordeloos, 1981; Harley and Smith, 1983; Molina et al., 1992; Agerer and Waller, 1993). While several authors have reported that Rosaceous plants formed ectomycorrhizas with species of the genus *Entoloma* (Becker, 1956; Trappe, 1962; Andruszewska and Dominik, 1971), light microscopic observation of mycorrhizas formed by *Entoloma saepium* (Noulet & Dass.) Richon & Roze. on *Rosa* sp. and *Prunus domestica* L. revealed that the fungus "invades and almost completely destroys root meristem and young root cells" (Agerer and Waller, 1993), indicating that their mycorrhizal relationship is not ectomycorrhizal. The ultrastructural features of this type of mycorrhiza have not yet been reported, and ultrastructural studies are expected to advance our understanding of this mycorrhizal relationship. In this report, we examine the ultrastructure, in addition to light microscopic structure, of mycorrhizas formed by *E. clypeatum* (L.) Kumm. f. *hybridum* (Romag.) Noordel. on *Rosa multiflora* Thunb. to understand cellular interaction in mycorrhizal association of Entolomatoid fungi and Rosaceous plants from the morphological point of view.

Materials and Methods

The sampling site was beside the Uji River, Kyoto (34°55′ N, 135°47′ E), where *R. multiflora* grew mixed with *Salix* spp. Based on the methods of Agerer (1986), mycorrhizas were collected as follows. On 14 April 1994 and 1995, three cubic cores of 10 cm edge were excavated from soil under fruit-bodies of *E. clypeatum* f. *hybridum*. Mycorrhizal fungi in the soil cores were identified by following rhizomorphal connections with the fruit-bodies. The mycorrhizal samples were washed out from soil particles in the soak water with fine forceps and brushes.

The mycorrhizas were vacuum-infiltrated for 1 h with a fixative consisting of 5% glutaraldehyde in 1/15 M Sørensen's phosphate buffer (pH 7.4), then treated with freshly prepared fixative at 4°C for 24 h. They were then washed with the same buffer, and postfixed with 1% osmium tetroxide in the same buffer at 4°C for 24 h. After washing with distilled water, the specimens were stained *en bloc* in 1% uranyl acetate at 4°C for 2 h, then dehydrated through a graded acetone series (20–100%) at room temperature. Samples were then embedded in Spurr's Resin (Spurr, 1969), which was allowed to polymerize at 60°C for 24 h. Semi-thin sections (1–1.5 μm) were cut with a Reichert ultramicrotome, stained with 0.05% toluidine blue in 0.1% boric acid, and observed with a light microscope. Some descriptive terms were based on Agerer (1987–1993). Ultra-thin sections (50–150 nm) were also prepared with the same ultramicrotome and mounted on copper grids. The sections were then stained with lead citrate as described by Reynolds (1963) and examined with a Hitachi H-7000 transmission electron microscope. Areas of mitochondria and hyphae were measured in 10 hyphal sections randomly selected from three mycorrhizas, and

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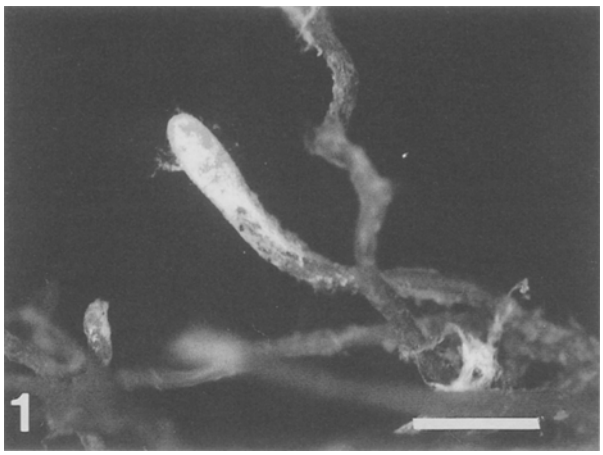


Fig. 1. Mycorrhizas of *Entoloma clypeatum* f. *hybridum* on *Rosa multiflora*. Bar=1 mm.

the area of mitochondria was expressed as a percentage of hyphal area.

Results

Stereomicroscopic observations The mycorrhizas (Fig. 1) of *E. clypeatum* f. *hybridum* on *R. multiflora* were unramified, up to 3 mm long, 1–2 mm in diam, and silvery white with felt-like texture. Tips of the mycorrhizas were cylindrical to clavate.

Light microscopic observations In the longitudinal sections of most mycorrhizas, the cortex remained in the basal part, whereas root cells had disappeared and were replaced by the fungal hyphae in the apical part (Fig. 2,*). Outer cortical cells in the basal part contained dark granules (Fig. 3, arrows) and were more flattened than inner cortical cells. Epidermal cells were not observed under the hyphal sheath. The fungal hyphae in this part partially invaded between outer flattened cortical cells (Fig. 3, arrowheads), but they did not penetrate between inner cortical cells. These observations suggest that typical Hartig nets are not formed.

Hyphal sheath outside of the cortex was divided into two layers according to hyphal thickness and arrangement (Fig. 3). The outer layer (Fig. 3, OL) was a loose plectenchymatous tissue composed of hyphae of 2–5 μm in diam. The inner layer (Fig. 3, IL) was a pseudoparenchymatous tissue composed of hyphae of 7–14 μm in diam that were approximately perpendicularly oriented to the root tissues. Remnants of flattened cortical cells (Fig. 3, double arrows) were involved in the inner layer.

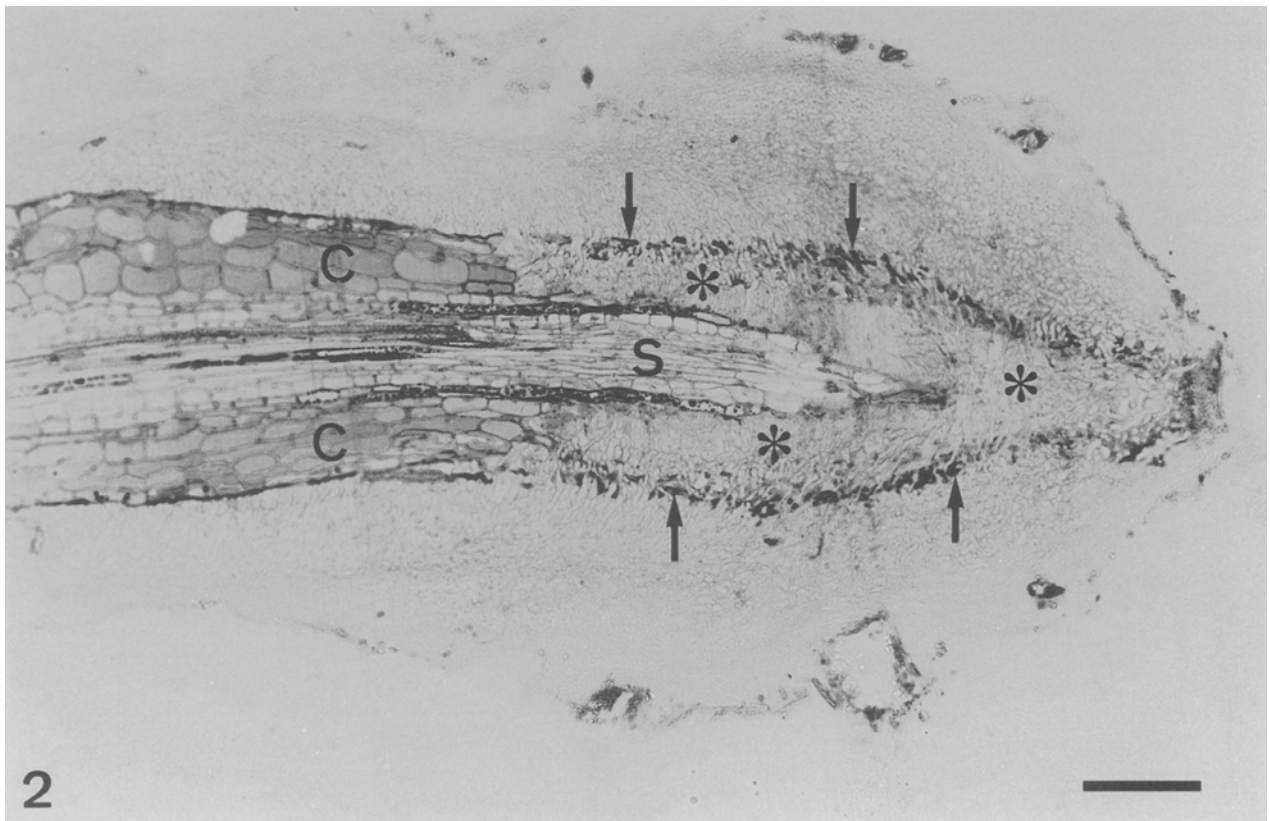
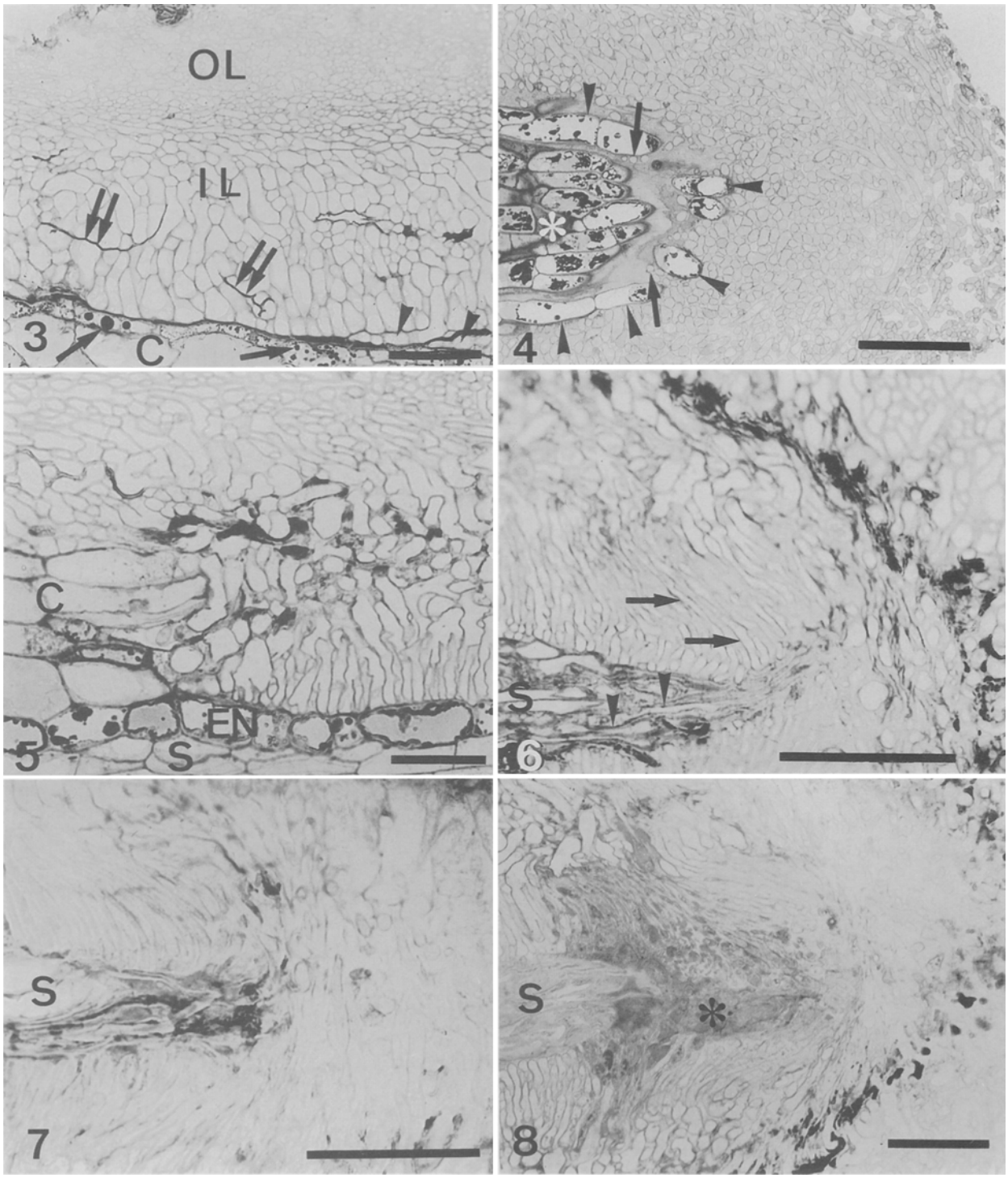
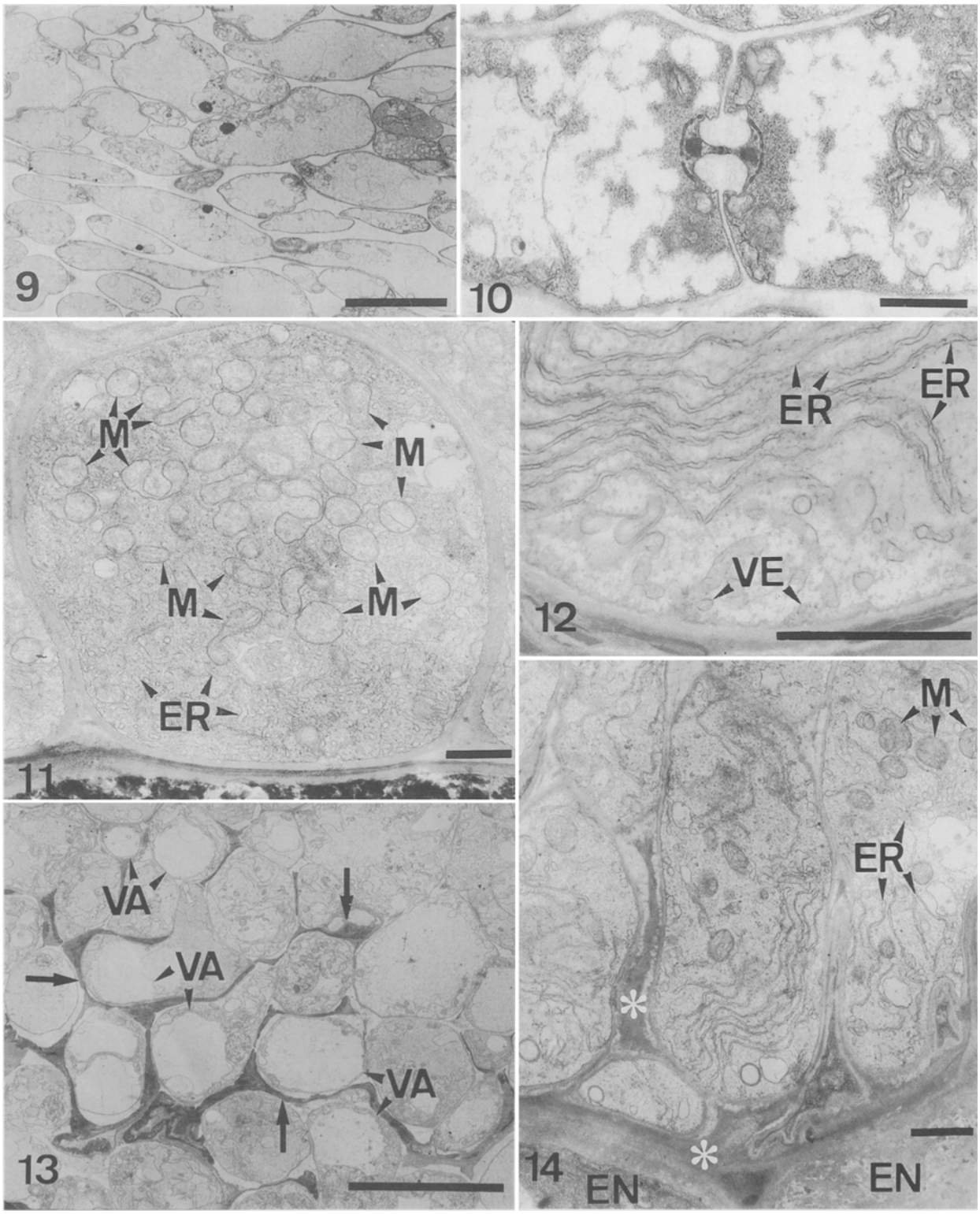


Fig. 2. Typical mycorrhiza showing disappearance of root cap and apical meristem. Arrows indicate dark amorphous materials in the apical part of a mycorrhiza. Asterisks (*) indicate the region in which apical root cells had disappeared and been replaced by fungal hyphae. Bar=100 μm.

Abbreviations: C, cortex; EN, endodermis; ER, cisternae of endoplasmic reticulum; M, mitochondria; S, stele; VA, vacuoles; VE, vesicles.



Figs. 3–8. Light micrographs of longitudinal sections of mycorrhizas. 3. Basal cortex and two layers of the hyphal sheath outside of cortex: the inner layer (IL) and the outer layer (OL). Arrows indicate dark granules in vacuoles of cortical cells. Arrowheads indicate hyphae between flattened cortical cells. Double arrows indicate remnants of flattened cortical cells in the inner layer of the fungal sheath. 4. Apical part of a mycorrhiza in which root cap and apical meristem remain. Arrows indicate the fungal hyphae between root cap cells. Arrowheads indicate root cap cells. An asterisk (*) indicates apical meristem. 5. Apical part of a mycorrhiza in which apical root cells are replaced by fungal hyphae. 6. Apical part of a mycorrhiza in which fungal hyphae become undulate near the tip of the stele. Arrows indicate undulating hyphae. Arrowheads indicate compressed stelar cells. 7. Apical part of a mycorrhiza in which the fungal hyphae are undulate and sparsely distributed. 8. Apical part of a mycorrhiza with sparse, undulate fungal hyphae, in which amorphous materials deposit on the tip of the stele. An asterisk (*) indicates amorphous materials. Bars = 50 μm.



Figs. 9–14. Electron micrographs of fungal hyphae in various parts of the mycorrhiza. 9. Hyphae in the outer layer of the hyphal sheath outside of the cortex. Bar=10 μm. 10. Dolipore septum observed in the hyphae of the outer layer of the hyphal sheath. Bar=1 μm. 11. Hyphae close to a cortical cell. Bar=1 μm. 12. Marginal part of a hypha close to a cortical cell. Bar=1 μm. 13. Hyphae and dark amorphous materials in the apical part of a mycorrhiza. Arrows indicate dark amorphous materials. Bar=1 μm. 14. Palmate hyphal branches near the endodermis close to the stelar cells. Asterisks (*) indicate electron-dense materials. Bar=1 μm.

Morphology of the apical part differed among mycorrhizal samples (Figs. 4–8). In a few mycorrhizas, the root cap and apical meristematic cells remained (Fig. 4, arrowheads, *). Dark granules were deposited in these root cells. The fungal hyphae covered the root tip and penetrated between the root cap cells (Fig. 4, arrows).

In most mycorrhizal tips, however, apical root cells had disappeared and were replaced by the fungal hyphae (Figs. 2, 5–8). Dark amorphous materials (Fig. 2, arrows), probably remnants of the various root cells, were cylindrically arranged to indicate the shape of the root in the apical part. Invading hyphae in the apical part were palmately branched (Fig. 5). Tips of their branches were 2–4 μm in diam, radially elongated, and perpendicularly attached to the endodermis (Fig. 5). Morphology of apical hyphal branches and stelar cells varied among the mycorrhizal tips (Figs. 6–8). Apical hyphal branches were undulated (Fig. 6, arrows) and apical stelar cells were compressed (Fig. 6, arrowheads) in some mycorrhizas. In other mycorrhizas, hyphae near the tip of the stele were sparsely arranged, and apical stelar cells were compressed (Fig. 7). The region between stelar cells and undulate hyphae was stained well by toluidine blue in other mycorrhizas (Fig. 8, *).

Transmission electron microscopic observations

Hyphae Large vacuoles, a small number of mitochondria and several vesicles were seen in the hyphae of the outer layer of the hyphal sheath (Fig. 3, OL and Fig. 9). Mitochondria occupied 6% of the hyphal area in sections. Dolipore septa were observed in the hyphae (Fig. 10), which confirm that the mycorrhizal fungus belongs to basidiomycetes. Dolipore septa were rarely observed without hyphae of the outer layer of the hyphal sheath.

In the inner layer of the hyphal sheath (Fig. 3, IL), hyphae contained large amounts of mitochondria, cisternae of endoplasmic reticulum and small vesicles (Figs. 11, 12). The mitochondria were spherical, ellipsoidal or alley-like in shape, contained many cristae, and occupied 15% of the hyphal area in sections (Fig. 11, M). The cisternae of endoplasmic reticulum were often stratified (Fig. 12, ER). Many vesicles were observed near cisternae of endoplasmic reticulum and plasma membrane (Fig. 12, VE).

In the apical part of the mycorrhiza, dark amorphous materials were observed between the hyphae (Fig. 2, arrows). These were composed of electron-dense materials (Fig. 13, arrows). Hyphae near these materials were highly vacuolated (Fig. 13, VA) compared with those attached to cortical cells (Fig. 11).

Large vacuoles were not observed in the branching hyphae adjacent to endodermal cells (Fig. 14). Electron-dense materials were deposited in the interface between hyphal branches and endodermal cells (Fig. 14, *).

The undulate hyphal branches (Fig. 6) near the endodermis close to the apical stelar cells contained spherical bodies (Fig. 15, arrows) that appeared to contain lipids, and electron-dense granular bodies (Fig. 15, arrowheads). The sparsely arranged hyphae (Fig. 7) ahead of the stele tip did not contain obvious organelles (Fig. 16). Interhyphal fibrillar materials were observed between the undulate hyphae (Figs. 15, 16, *). Various materials with different electron densities were deposited near the tip of the stele (Fig. 17, *). Similar materials were included in the sparsely arranged hyphae (Fig. 16).

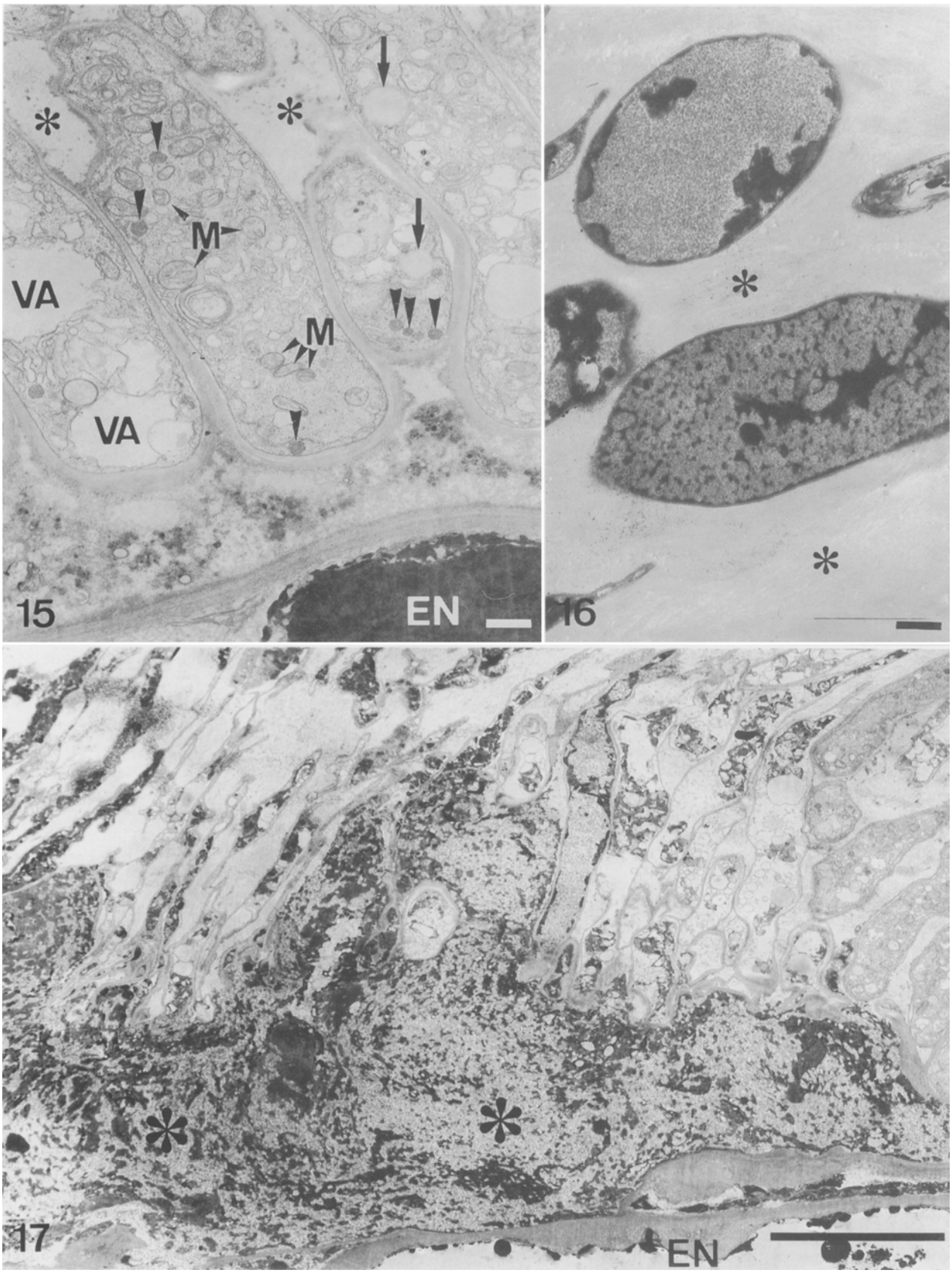
Root cells Root cap cells, apical meristematic cells (Fig. 18), cortical cells (Fig. 19) and endodermal cells (Fig. 20) near the fungal hyphae deposited electron-dense granular materials in vacuoles (arrows in each figure). A few layers of flattened cortical cells (Fig. 3) near the hyphae contained only electron-dense materials (Fig. 19, arrowheads). Some cortical cells had partly broken down walls and were filled with hyphae (Fig. 21, *).

Discussion

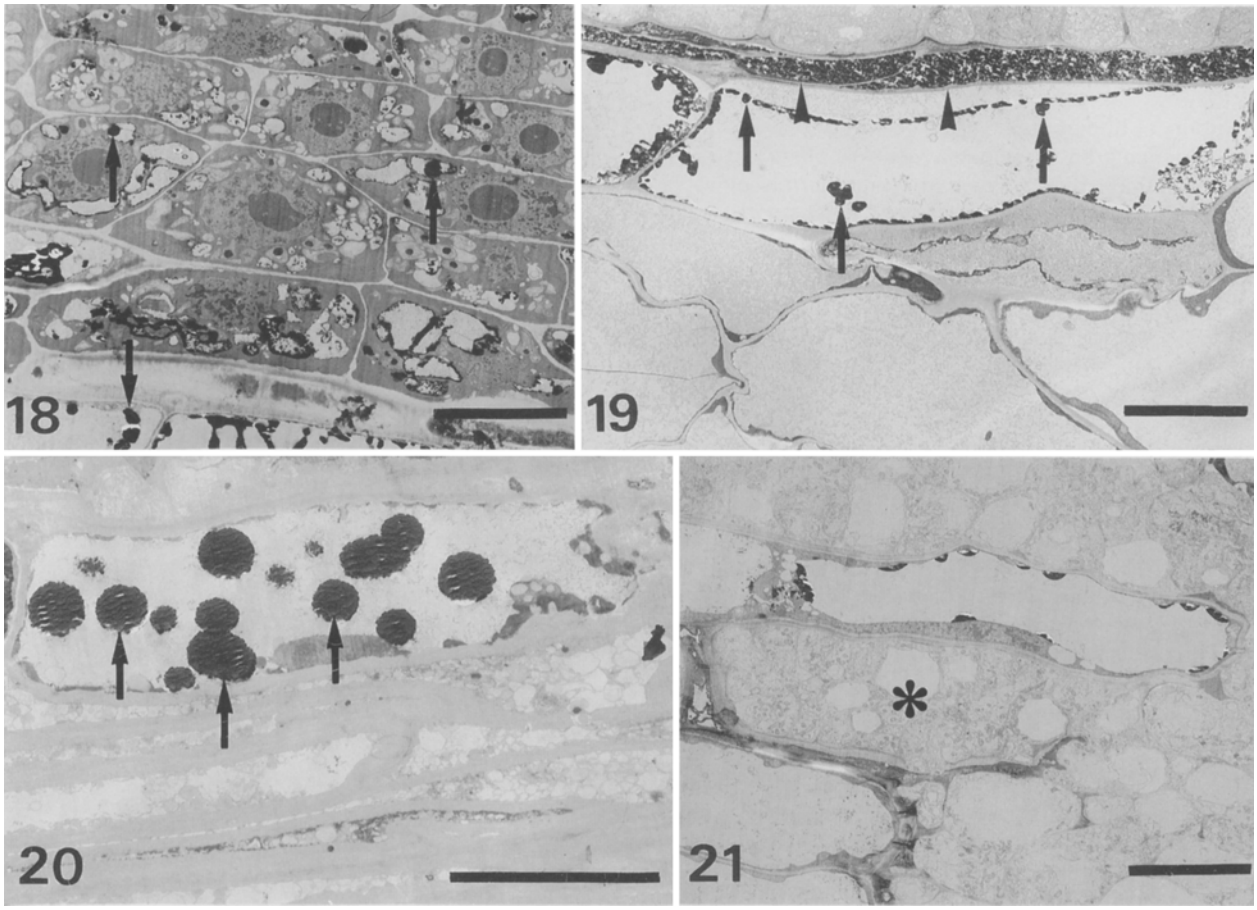
Morphology of the mycorrhiza of Entolomatoid fungi and Rosaceous plants Fungal sheath-like structures were formed on root tips, but apical root cells had completely disappeared in most mycorrhizas examined. These morphological features are not ectomycorrhizal but are similar to those of the mycorrhizas of *E. saepium* on *Rosa* sp. and *P. domestica*, previously reported by Agerer and Waller (1993). Haug et al. (1991) described that apical cortex was destroyed but apical meristem remained in tuberculate mycorrhiza of *Castanopsis* and *Engelhardtia*. In the present study, we found apical meristematic cells in a few exceptional mycorrhizas. However, these cells contained dark granules in vacuoles, which are expected to be destroyed, and then to be replaced by the fungal hyphae. The disappearance of apical meristematic cells seems to be characteristic of the development of the mycorrhiza of Entolomatoid fungi and Rosaceous plants. The collapse of apical meristematic cells may cause the duration of the mycorrhizal relationship between *E. clypeatum* f. *hybridum* and *R. multiflora* to be short.

Morphological variations in the apical parts of the mycorrhizas suggest that mycorrhizas develop through the following stages. At the beginning of mycorrhizal formation, hyphae of *E. clypeatum* f. *hybridum* cover root tips of *R. multiflora*. The fungal hyphae penetrate between apical root cap, meristematic and cortical cells, and then these root cells collapse. The fungal hyphae invade root tissues from the apical part. When hyphae reach the central stele of the root tissues, they become undulate and sparsely arranged. In the final stage, amorphous materials deposit at the tip of the stele.

Morphological features of the fungal hyphae Morphological variations of the fungal hyphae in the mycorrhiza are correlated with the distribution of organelles in the hyphae. Hyphae of the inner layer of the sheath were thicker than those of the outer layer. Mitochondria occupy a larger percentage area of hyphae in the inner layer of the sheath than in the outer layer. Moreover, large vacuoles were observed in the hyphae of the outer layer of the sheath and the hyphae near dark amorphous materials, but they were not observed in the hyphae of the inner layer of the sheath in the basal part and branching hyphae. These results revealed that the intracellular organization of the hyphae composed of the fungal sheath varies with proximity to the root cells. Similar results on



Figs. 15–17



Figs. 18–21. Electron micrographs of the root cells in various parts of the mycorrhiza. Arrows indicate electron-dense granules in vacuoles. 18. Apical meristematic cells adjacent to fungal hyphae. 19. Cortical cells near the fungal hyphae. Arrowheads indicate flattened cortical cells. 20. Endodermal cells. 21. A broken cortical cell occupied by fungal hyphae. An asterisk indicates the broken cortical cell. Bars = 10 μm.

the fungal sheath were also obtained from morphological studies of ectomycorrhizas (Strullu, 1979; Kottke and Oberwinkler, 1986; Bonfante-Fasolo and Scannerini, 1992).

Morphological features of hyphae near cortical cells may reflect the fungal nutritional behaviour toward root cells. Large amounts of mitochondria are supposed to produce energy actively from chemical fuels, which may be derived from the root cells. Furthermore, large amounts of cisternae of endoplasmic reticulum and transitional vesicles may contribute to the synthesis of various materials, their release extracellularly, and the expansion of hyphal tips to cortical cells.

Reaction of root cells Light and electron microscopy revealed the accumulation of dense granular materials in vacuoles of root cap cells, meristematic cells, cortical cells and endodermal cells adjacent to the fungal hyphae. Similar accumulation in the cells of the different root regions suggests that these root cells react similarly to the destructive fungal infection. Morphological studies on ectomycorrhizal symbiosis have demonstrated the presence of electron-dense deposits in the cortical cells, and these deposits were histochemically identified as phenolic compounds (Ling-Lee et al., 1977). Deposits in vacuoles of *Rosa* root cells may also contain phenolic compounds like ectomycorrhizal roots.

Collapse of fungal hyphae near the tip of the stele Amorphous structures composed of materials with various electron densities accumulated on the tips of steles. Sparsely arranged hyphae ahead of the tips contained similar materials and no organelles. These observations suggest that hyphae collapse outside of the stele, and then invasion of mycorrhizal hyphae is blocked. Agerer and Waller (1993) indicated the presence of root cell rem-

Figs. 15–17. Electron micrographs of fungal hyphae in apical parts of mycorrhiza. 15. Undulate hyphal branches near the tip of a stele. Arrows indicate spherical bodies that seemed to contain lipids. Arrowheads indicate electron-dense granular bodies. Asterisks (*) indicate interhyphal fibrillar materials. Bar = 1 μm. 16. Collapsed hyphae ahead of the tip of a stele. Asterisks (*) indicate interhyphal fibrillar materials. Bar = 1 μm. 17. Amorphous materials between undulate hyphae and stelar cells. Asterisks (*) indicate amorphous materials. Bar = 10 μm.

nants in that region. However, the present study showed that the amorphous structures were composed of the remnants of not only plant cells but also fungal hyphae.

Spherical bodies that appeared to contain lipid, and electron-dense granular bodies were only observed in undulate branching hyphae near the endodermis close to the apical stelar cells. Hyphae near the tip of the stele did not contain organelles. These results suggest changes in the structure and the function of hyphae around the tip of the stele as they collapse. Undulating fungal hyphae may accumulate lipid materials into spherical bodies and translocate them to active parts before collapsing.

Hyphae collapse within the cortical cells in arbascular, ericoid, arbutoid and orchid mycorrhiza, and E-strain ectoendomycorrhiza (Scannerini and Bonfante-Fasolo, 1983; Fusconi and Bonfante-fasolo, 1984; Piché et al., 1984; Uetake et al., 1992). In the present study, we found that hyphae undulate and collapse outside of root tissues. Fungal sheath formation of this type of mycorrhiza resembles that of ectomycorrhiza, but it differs in the disappearance of apical meristematic cells, as previously mentioned. Further morphological studies on mycorrhizas of diverse plant and fungal species should provide clues for understanding the unknown mycorrhizal relationship found in this study.

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