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Mycorrhiza

Ectomycorrhizal types and endobacteria associated with ectomycorrhizas of *Morchella elata* (Fr.) Boudier with *Picea abies* (L.) Karst

F. Buscot

Institut für Bodenbiologie, Bundesforschungsanstalt für Landwirtschaft, Bundesallee 50, D-38116 Braunschweig, Germany

Abstract. Subterranean morel sclerotia connected with ascomata of *Morchella elata* (Fr.) Boudier were found to surround 155 ectomycorrhizal root tips of *Picea abies* (L.) Karst, belonging to seven different types. Based upon anatomical and cytological studies, three ectomycorrhizal types could be attributed to types already described, whereas four types appeared to be undescribed. The nature of the association between the morel and the mycorrhizal types was dependent on the type and was not related to their vitality. In particular, morel ectomycorrhizas formed secondarily and exclusively by succeeding to primary mycorrhizas of a heterobasidiomycete. In addition to this triple association, an endobacterium was observed growing within the Hartig net of this heterobasidiomycete mycorrhiza. The significance of this complex of associations for the formation of ectomycorrhizas by the morel is discussed.

Key words: *Morchella elata* – Mycorrhizal succession – Endobacteria

Introduction

Mycorrhizal symbioses are often considered to be pure associations between single mycobionts and fine rootlets. However, more and more reports on field material mention the presence of additional microorganisms upon or within mycorrhizas. Supplementary parasitic or presaprophytic fungi are often observed in ageing ectomycorrhizas (EM) (Berndt et al. 1990). The supplementary fungi can also be secondary mycobionts which succeed to a primary mycorrhizal fungus. Succession of vesicular-arbuscular mycorrhizas (VAM) to EM has been observed on eucalypt and obtained under controlled culture conditions (Horan et al. 1988; Bougarda et al. 1990). Additional fungi are also observed in young and active EM (Brand 1992). Similarly, bacteria are often associated with EM, for example in *Tuber* mycorrhizas (Boutekrabt and Pargney 1991). The helper role of such bacteria for mycorrhizal synthesis has been demonstrated (Duponnois and Garbaye 1990) and they are now inoculated together with selected EM in order to improve the growth of seedlings in tree nurseries (Duponnois and Garbaye 1991; Garbaye et al. 1992).

The case of the morel is a good example of multiple associations in EM. In recently disturbed soils with a reduced competition pressure, the fungus grows and fructifies saprophytically. In contrast, in stabilized forest soils with high competition for nutrients, the morel mycelium contracts complex associations with tree roots (Buscot 1992a). Under such conditions, Buscot and Roux (1987) observed that voluminous, subterranean morel sclerotia ensheathing sectors of mature tree roots were connected with the ascomata. Buscot (1989) showed that the ascomata develop at the expense of these sclerotia. In the association of the late fructifying *Morchella esculenta* with *Picea abies* the relationship with the mature roots appeared to be presaprotrophic and morel EM were found on fine rootlets derived from the ensheathed mature roots (Buscot and Kottke 1990). Furthermore, ageing EM of an unidentified heterobasidiomycete in the vicinity of the morel EM were invaded by morel hyphae, suggesting a possible succession process. Investigations on the early fructifying *M. elata* confirmed this hypothesis (Buscot 1992b). In its association with spruce, this morel formed subterranean sclerotia not only around mature roots but also around clusters of different EM. Among these EM, the morel formed secondary EM exclusively when succeeding the heterobasidiomycete mentioned above. Cultures under controlled conditions with spruce seedlings confirmed the capacity of the morel to contract a presaprophytic association with short-lived tissues of mature roots and a symbiotic association with absorbing rootlets (Buscot 1992c). The presaprophytic association with mature roots was enhanced by plants mycorrhizal with other mycobionts. Additionally, bacteria appeared to help the mycorrhizal synthesis with the morel.

The aim of this paper is to characterize some of the

primary EM to which morels associate in nature and to describe the presence of endobacteria together with morel hyphae within ageing sectors of the heterobasidiomycete EM to which the morel is able to succeed as mycobiont.

Materials and methods

Ascocarps of *M. elata* (Fr.) Boudier were found at the 'emergence stage' (see Buscot 1989) in a 60-year-old forest of *Picea abies* (L.) Karst. In Herrenberg, Germany at the end of February 1990. The soil surrounding the base of the ascocarps to a depth of 20 cm was collected and washed gently with tap water to reveal the fungal-root associations. These associations were dissected under a stereophotomicroscope (Zeiss SV 8) and photographed with Kodak EPT Ektachrome (ISO 160) tungsten film.

The sampled rootlets were fixed in 2% glutaraldehyde in a 0.2 M cacodylate buffer (pH 7.2), postfixed in 1% osmium tetroxide in the same buffer and embedded in ERL resin (Spur 1969). Semithin sections (0.5 μm) were cut on glass knives with a Reichert ultramicrotome, and stained with toluidine blue. Ultrathin sections were cut on a diamond knife, contrasted with uranyl acetate and lead citrate following standard procedures, and mounted on formvar slotted copper grids. Transmission electron microscopy was performed with a Zeiss EM 109.

The description terminology of the mycorrhizas comes from Agerer (1991a) and Haug and Oberwinkler (1987) for the macroscopic and microscopic characteristics, respectively. The colours of the hyphal mantle, emanating hyphae and rhizomorphs were characterized with the help of the colour identification chart of the Flora of British Fungi 1969 (Her Majesty's Stationery Office, Edinburgh, Scotland) and are marked with an asterisk in the text. Several colours in this chart are only named by a letter. In such cases, the effective colour corresponding to a letter is given in parenthesis, for example: H* (ochre).

At least five mycorrhizas of each type were cut for the microscopic observations. Vitality was estimated on the basis of cytological appearance, which was finally correlated with colour.

Results

Mycorrhizal forms associated with the morel

The subterranean sclerotial structures connected with the nonmature ascomata of *M. elata* were compact and voluminous (see Buscot 1989). They surrounded not only sectors of mature roots of *Picea abies* (0.3–2 cm in diameter), but also formed tubercule-like sclerotia 5–10 cm^3 in volume around fine and nonsuberized root sectors. Such sclerotia completely ensheathed clusters of EM (Fig. 1), and 155 mycorrhizal root tips could be isolated within such sclerotia. They were all adult or senescent, suggesting that the surrounding morel sclerotia had developed after their formation. Seven different types could be recognized within these EM.

Type A (51 specimens)

Macroscopic characteristics. Monopodial-pinnate, straight mycorrhiza without rhizomorphs but with a few straight, G* (medium-pale ochre) emanating hyphae; hyphal mantle rust* to fuscous-black* when ageing, compact with a smooth surface.

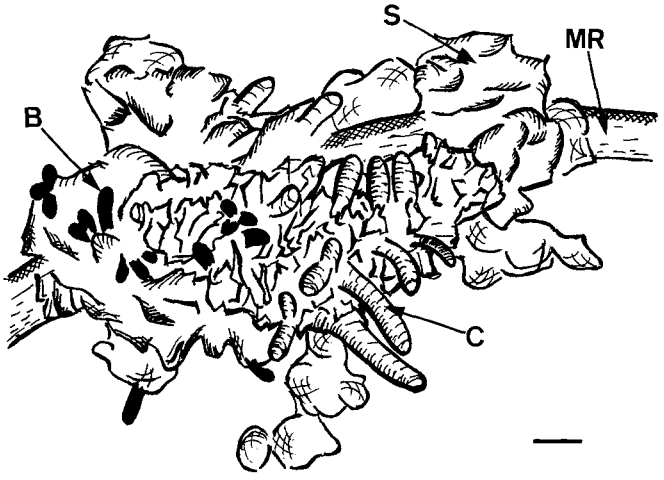


Fig. 1. Subterranean sclerotia (*S*) of *Morchella elata* surrounding mature roots (*MR*) and ectomycorrhizas of *Picea abies*. Partial dissection of the association revealed two types of mycorrhizas, (*B* and *C*); *bar* = 2 mm

Microscopic characteristics (Fig. 2a, b). Mycorrhiza surrounded by a compact and opaque matrix (2–3 μm thick); hyphal mantle 6–10 μm thick, polygon synenchyma with wide lumen hyphae (3–5 μm) embedded in a thin and opaque matrix in the outer part, gradually turning to a compact prosenchyma (hyphae diameter, 2–4 μm) without matrix in the inner part; hyphae without clamps; Hartig net developed around four layers of cortical cells; intracellular hyphae in cortical cells.

Ultrastructural characteristics. Hyphae of the Hartig net regularly septate with one or two big nuclei (1.5 μm in diameter) per cell; simple intercellular pores with round Woronin bodies (Fig. 2b): ascomycete.

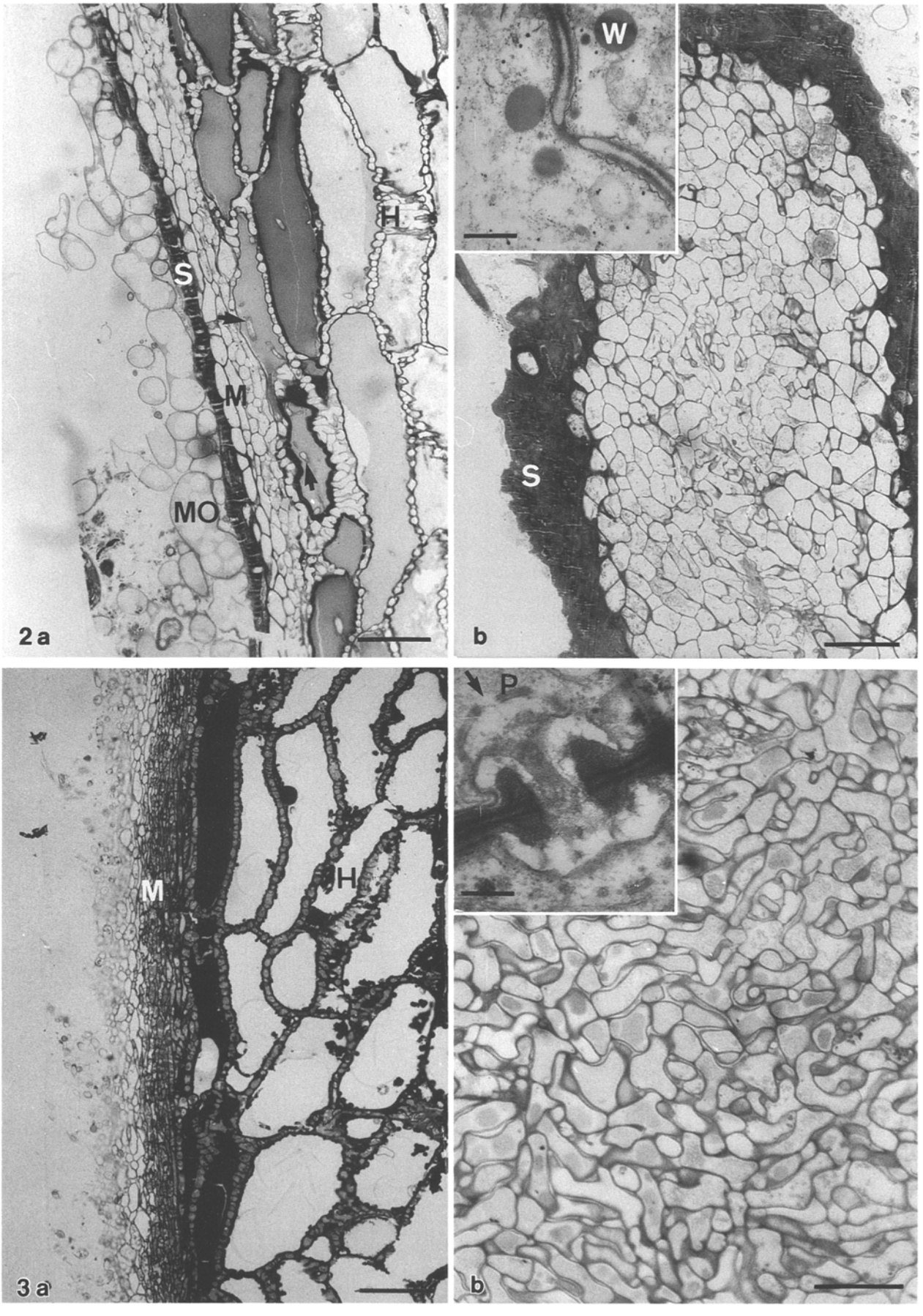
Vitality. Vital to senecent.

Type B (11 specimens)

Macroscopic characteristics. Monopodial-pinnate, straight mycorrhiza without rhizomorphs but with a few black, emanating hyphae; hyphal mantle olivaceous-black*, slightly grainy.

Fig. 2. a Longitudinal section of type A with adhering morel hyphae (*MO*), external matrix (*S*), hyphal mantle (*M*) and Hartig net (*H*) with hyphae penetrating into cortical cells (*arrows*); *bar* = 10 μm. **b** Tangential section through the hyphal mantle of type A; *inset* top left, septal pore of the mycobiont with Woronin bodies (*W*); *bars* = 10 and 0.5 μm, respectively

Fig. 3. a Longitudinal section of type C with hyphal mantle (*M*) and Hartig net (*H*); *bar* = 10 μm. **b** Tangential section through the median layer of the hyphal mantle of type C; *inset* top left, dolipore of the mycobiont with perforated (*arrows*) parenthesomes (*P*); *bars* = 10 and 0.2 μm, respectively


S
H
M
MO
2 a
W
S
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M
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3 a
P
b

Microscopic characteristics. Hyphal mantle 6–10 μm thick, loose prosenchyma with large diameter hyphae (1.5–3 μm) embedded in an opaque matrix in the outer part, compact prosenchyma with Y-shaped, regularly septate hyphae (0.5–1 μm in diameter) in a thin and opaque matrix in the inner part; hyphae without clamps; Hartig net developed up to the central cylinder.

Ultrastructural characteristics. Coenocytic Hartig net (diameter of the nuclei 0.2–0.3 μm) with few hyphal septa; dolipores with perforated parenthesomes: homobasidiomycete, *Piceirhiza obscura* (Gronbach 1988; Agerer 1991a).

Vitality. Vital to senescent.

Type C (43 specimens)

Macroscopic characteristics. Monopodial-pinnate, straight mycorrhiza without rhizomorphs and emanating hyphae; hyphal mantle whitish transparent (cortex visible), black when ageing; smooth surface.

Microscopic characteristics (Fig. 3a, b). Hyphal mantle 12–16 μm thick, loose prosenchyma of dead, regularly septate hyphae (0.3–1 μm in diameter) in the outer part, gradually turning to a puzzle synenchyma of active hyphae with wide lumen (1.5–5 μm in diameter) in the inner part; no matrix; hyphae without clamps; Hartig net developed up to the central cylinder; several intracellular hyphae in cortical cells.

Ultrastructural characteristics. Coenocytic Hartig net with few hyphal septa; dolipores with perforated parenthesomes (Fig. 3b): homobasidiomycete.

Vitality. Vital to senescent.

Type D (15 specimens)

Macroscopic characteristics. Unramified, club-shaped mycorrhiza, constricted at the base, without rhizomorphs and emanating hyphae; F* (pale ochre) at the tip to H* (ochre) in the proximal part; reticulate surface.

Microscopic characteristics (Fig. 4a, b). Hyphal mantle 20–30 μm thick, extremely loose prosenchyma in the outer part, thin (4–6 μm) compact prosenchyma with regularly septate hyphae (1 μm in diameter) in the inner part; no matrix; hyphae without clamps; Hartig net developed around three layers of cortical cells.

Ultrastructural characteristics. Coenocytic Hartig net with few hyphal septa; dolipores with perforated parenthesomes (Fig. 4b): homobasidiomycete, resembling *Piceirhiza gelatinosa* except of the absence of matrix material (Agerer 1991a; Haug and Oberwinkler 1987).

Vitality. Vital to senescent.

Type E (13 specimens)

Macroscopic characteristics. Unramified, straight mycorrhiza without rhizomorphs but with many straight emanating C* (cream) hyphae; hyphal mantle C*, woolly surface.

Microscopic characteristics (Fig. 5a, b). Hyphal mantle 6–12 μm thick, irregular synenchyma with wide lumina, dead hyphae (2–5 μm) in the outer part, irregular synenchyma with active hyphae (0.8–2.5 μm in diameter) in the inner part; no matrix; hyphae without clamps; Hartig net surrounding two to three layers of cortical cells; several intracellular hyphae in cortical cells.

Ultrastructural characteristics. Coenocytic Hartig net with few hyphal septa; dolipores with perforated parenthesomes (Fig. 5b); homobasidiomycete.

Vitality. Senescent.

Type F (13 specimens)

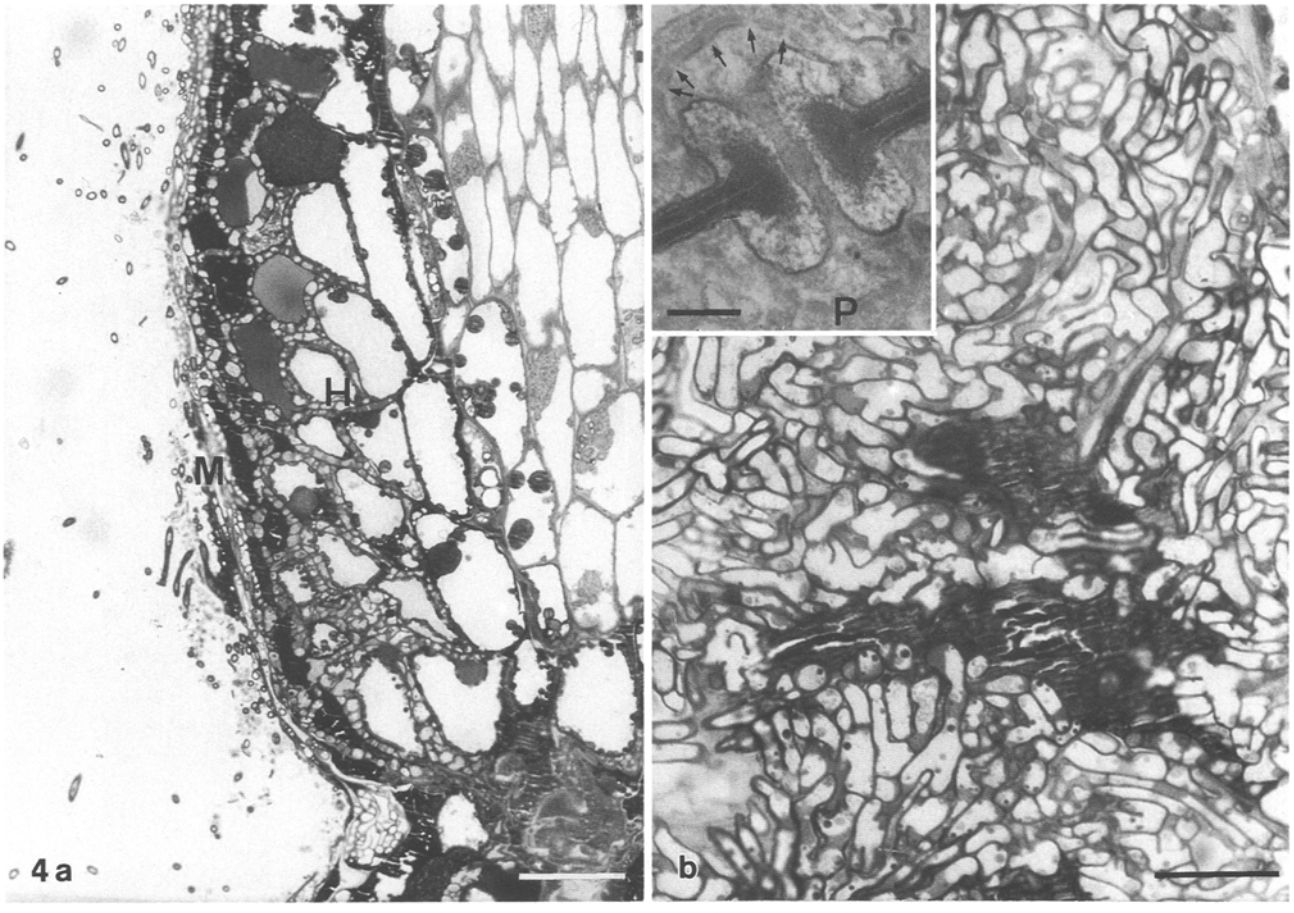
Macroscopic characteristics. Monopodial-pinnate, straight mycorrhiza; ensheathed to smooth, brick* rhizomorphs with restricted point of connection onto the mantle; hyphal mantle brick* to dark brick*, smooth surface.

Microscopic characteristics (Fig. 6a, b). Hyphal mantle thin (0–4 μm thick) and inconspicuous, loose prosenchyma in the outer part; compact prosenchyma with regularly septate, Y-shaped hyphae (0.6–1 μm in diameter) in the inner part; no matrix; hyphae without clamps; Hartig net developed up to the central cylinder.

Ultrastructural characteristics. Coenocytic Hartig net, dolipores with continuous parenthesomes: heterobasidiomycete already described by Buscot and Kottke

Fig. 4. a Longitudinal section of type D with hyphal mantle (*M*) and Hartig net (*H*); *bar* = 10 μm. **b** Tangential section through the hyphal mantle of type D; *inset* top left, dolipore of the mycobiont with perforated (*arrows*) parenthesomes (*P*); *bars* = 10 and 0.2 μm, respectively

Fig. 5. a Longitudinal section of type E with hyphal mantle (*M*) and Hartig net (*H*) with hyphae penetrating into the cortical cells (*large arrows*); *bar* = 10 μm. **b** Tangential section through the median layer of the hyphal mantle of type E; *inset* top left, dolipore of the mycobiont with perforated (*small arrows*) parenthesomes (*P*); *bars* = 10 and 0.2 μm, respectively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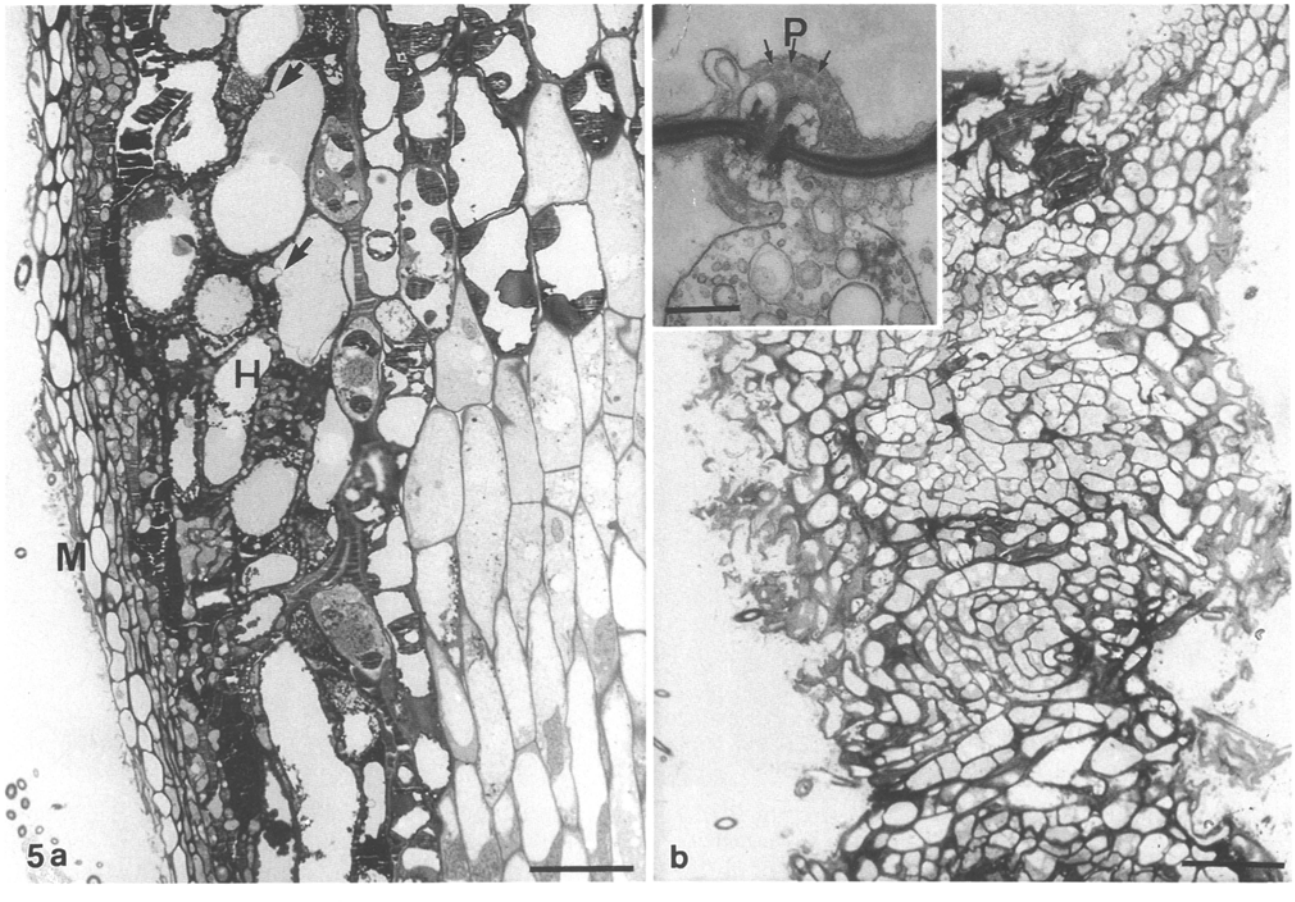
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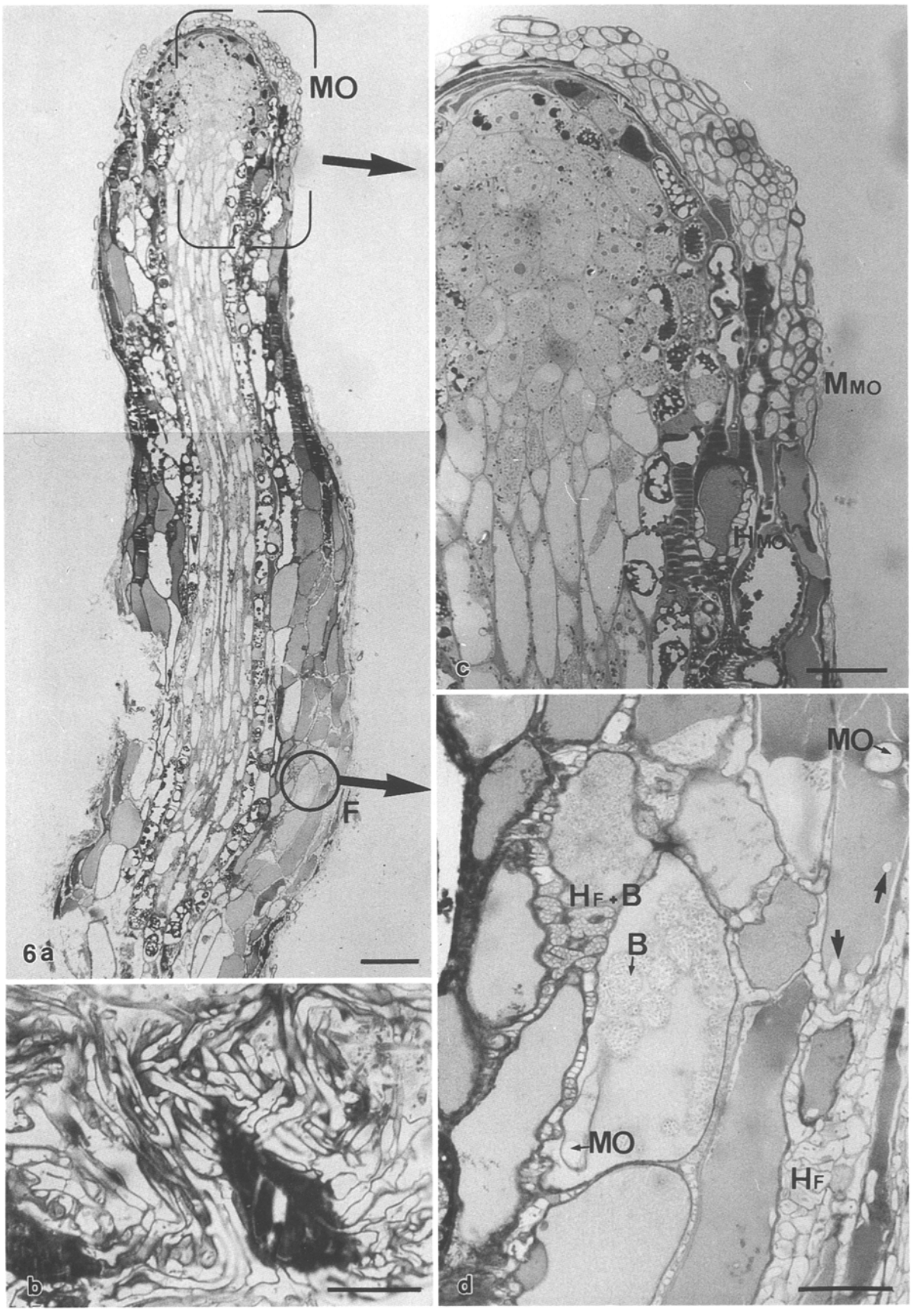

Fig. 6. a Longitudinal section of a secondary mycorrhiza of *Morchella elata* (*MO*) on type F (*F*); *bar* = 20 μm. **b** Tangential section in the hyphal mantle of type F; *bar* = 5 μm. **c** Detail view of the hyphal mantle (M_{mo}) and the Hartig net (H_{mo}) of the morel mycorrhiza; *bar* = 10 μm. **d** Detail view of the Hartig net (H_F) of type F invaded by endobacteria (*B*) and morel hyphae (*MO*); *bar* = 5 μm

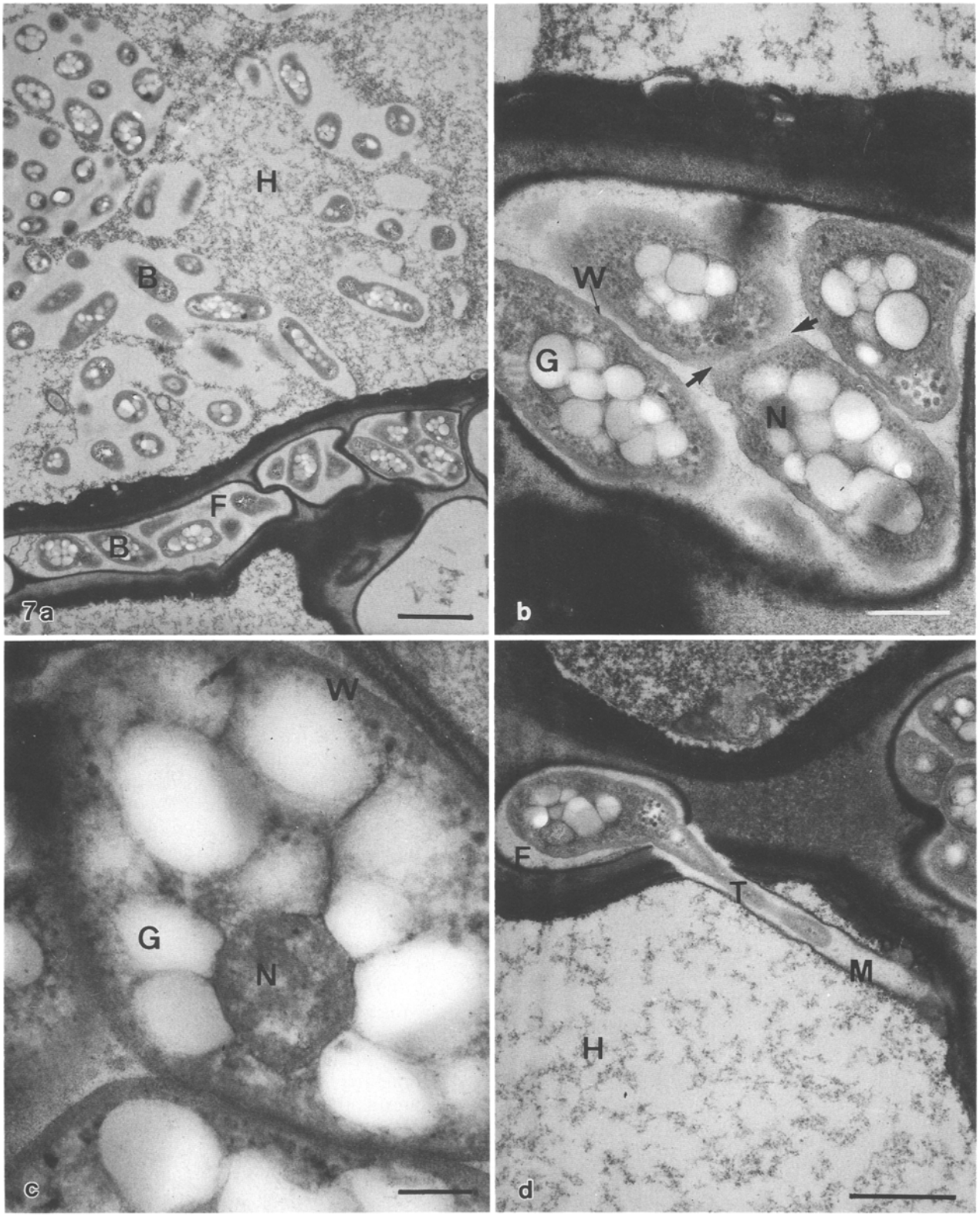

Fig. 7. a Endobacteria (*B*) within the Hartig net of type F: *P* and *F*, plant and fungal cells, respectively; *bar* = 2 μm. **b**, **c** Detail views of the endobacteria. *W*, Wall; *G*, poly-β-hydroxybutyrate globuli; *N*, nuclear zone; *arrows*, bacterial division; *bars* = 0.5 and 0.1 μm, respectively. **d** Penetration of an endobacteria from a fungal (*F*) into a plant (*P*) cell. *T*, Penetration tube; *M*, membrane of the plant cell; *bar* = 1 μm

(1990), except the colour also corresponding to type 8 of Haug and Oberwinkler (1987), which was recently named *Piceirhiza terrea* by Haug and Pritsch (1992).

Vitality. All specimens senescent and secondarily mycorrhizal with the morel (Fig. 6a–d).

Type G (9 specimens)

Macroscopic characteristics. Monopodial-pinnate mycorrhizas without rhizomorphs but with short woolly emanating hyphae; Hyphal mantle F* (pale ochre), short woolly, densely covered with mineral particles: sandy mycorrhiza.

Microscopic characteristics. Hyphal mantle 10–15 μm thick, medium compact prosenchyma including soil mineral particles and formed by long septate hyphae (0.4–0.6 μm in diameter) in the outer part, compact prosenchyma with long septate hyphae (0.9–1.2 μm in diameter) in the inner part; Hartig net around three layers of cortical cells; no matrix; observation of possible clamps on the emanating hyphae impossible (reduced size and no septa), hyphae of the mantle and the Hartig net without clamps: this type corresponds to *Piceirhiza harenosa* (Berg 1989) except for a slight difference in the colour and the impossibility of observing whether or not the emanating hyphae have clamps.

Ultrastructural characteristics. Not observed.

Vitality. Senescent.

Relationships of the mycorrhizal types with the morel and with other microorganisms

The type of interactions of the EM with the surrounding morel sclerotia appeared independent of their vitality but to be type specific. Three kinds of relationships were observed: (*1*) With type A, the morel mycelium was attached to the external matrix layer and in some cases tended to form a kind of secondary, concentric, hyphal mantle. However, no penetration of morel hyphae into the EM of type A was observed (Fig. 2a), even when these were senescent. (*2*) Adhesion of morel hyphae was very reduced around EM of forms B, C, D, E and G from which the surrounding morel sclerotia were easy to detach during the dissection. No penetration of morel hyphae was observed within the primary EM of these forms (Figs. 3a, 4a, 5a). (*3*) Morel hyphae penetrated into the ageing proximal part of the EM of type F and formed secondary EM with all EM of this type (Fig. 6a, c, d).

A few other unidentified fungi were observed in the Hartig net of ageing mycorrhizas in particular of forms E, F and G, and intercellulary growing bacteria were found within the hyphal mantle of forms E and G (not shown). More remarkable was the presence of endobacteria in the ageing proximal sectors of EM of type F, in which morel hyphae were also observed (Fig. 6a, and d). These bacteria totally filled many ageing hyphae of the Hartig net and were also observed within cortical cells (Figs. 6d, 7a). Within fungal cells, they were pleomorphic to rod shaped and their size varied from 0.5 to 2 μm. They were limited by a mostly regular and amorphous wall approximately 50 nm thick and included an electron-dense nuclear zone (0.25 μm in diameter) in a granular cytoplasm with large electron-transparent globules (0.1–0.2 μm in diameter) looking like poly-β-hydroxybutyrate globules currently observed in bacteria (Fig. 7b, c). Stages of division were observed (Fig. 7b). Within the cortical cells, the endobacteria were located in clear zones which were not limited by any membrane system (Fig. 7a). Their rod shape was more constant and their cell wall appeared slightly more amorphous than when growing in hyphae. At some sites on the Hartig net, intrahyphal endobacteria perforated the cell walls of both the mycobiont and its plant partner, and invaginated the plant plasmalemma into the cortical cell, forming a kind of haustorium (Fig. 7d). Such seldomly observed haustoria looked like tubes (2–3 μm long, 0.4 μm wide). No perforation of the plant plasma membrane was observed but it can be assumed that this had happened as almost all bacteria in the plant cells had no surrounding membrane.

Discussion

According to Agerer (1991b), the characteristics of the hyphal mantle and of the attached structures are of crucial importance for determination of EM. Nevertheless, this part of the EM is directly exposed to external environmental factors, i.e. type of soil and rhizospheric microflora. Such exogenous factors are prone to modify, sometimes drastically, the morphology of EM (Gronbach 1988). This is the case for EM of *Laccaria laccata* and *Picea abies*, the hyphal mantle and Hartig net of which are totally modified when exposed to the root pathogen *Cylindrocarpon destructans* (Buscot et al. 1992). In the present case, EM wholly ensheathed by thick morel sclerotia were investigated. The impossibility of knowing to which extent their morphological characteristics (colour, presence and density of emanating hyphae and rhizomorphs, presence of matrix material, thickness and structure of the hyphal mantle) had been modified by the surrounding morel mycelium was the major problem for their description and final identification. Additionally, these EM were mature and often senescent.

From the seven forms characterized, four correspond to or have major characteristics in common with already described types. The differences are mostly slight differences in the colour or a reduced density and size of emanating hyphae which may be due to the surrounding morel sclerotia:

1. Except for a lower density of emanating hyphae, type B corresponds to *Piceirhiza obscura* described by

Gronbach (1988) and almost certainly belongs to this type.

2. Type F had already been observed in the vicinity of EM of *M. esculenta* (Buscot and Kottke 1990). Except for the brick* colour of the hyphal mantle, this type is similar to type 8 of Haug and Oberwinkler (1987), the hyphal mantle of which was translucid. A more recent description of type 8, now termed *Piceirhiza terrea*, mentions an ochre-brown colour (Haug and Pritsch 1992). Although these authors did not mention the presence of rhizomorphs observed in type F, this type probably corresponds to *P. terrea.*

3. The characteristics of type G are similar to those of the sandy mycorrhiza *Piceirhiza harenosa* described by Berg (1989). Nevertheless, in type G, the reduced size of the nonseptate, emanating hyphae obscured the question of whether or not they would have clamps were they not surrounded by the morel mycelium and, therefore, more developed.

4. The case of type D is more debatable. Except for the absence of a distinct matrix in the outer hyphal mantle, this type corresponds to *Piceirhiza gelatinosa* described by Agerer (1991a) and Haug and Oberwinkler (1987). However, these authors consider the matrix to be a key characteristic of *P. gelatinosa.* It is not possible to decide whether or not the surrounding morel sclerotia could have impeded the formation of this matrix. For this reason, it cannot be confirmed that the two forms are identical.

On the contrary, forms A, C and E are very different from any type already described in the identification literature, and they can be considered as new, even if several of their characteristics could be atypical due to the presence of surrounding morel hyphae. Among these types is the ascomycete of type A. Because of its dark colour, the relatively reduced diameter of its hyphae and especially the reduced number and the large diameter of its nuclei, this ascomycete cannot be the morel. All identified ascomycete EM are discomycetes (Miller 1984). If this is the case for type A, the reduced number of nuclei per cell (1–2) would plead for an inoperculate or for a primitive operculate species (Berthet 1964).

These different types of EM exhibited three kinds of relationship with the surrounding morel sclerotia. The morel exclusively formed EM in succession to the ageing type F. Because this type had the most additional microorganisms, it could be hypothesized that the morel takes advantage of its apparent low resistance. However, three facts speak for a specific recognition and succession: (*1*) the morel didn't form EM in succession to any other types, although many were also invaded by additional microorganisms when ageing (for example, forms E and G); (*2*) all EM of type F were secondarily mycorrhizal with the morel; (*3*) this is the second report of morel EM in association with type F. It must be noted that, all EM of type F described so far were senescent in spring (Buscot and Kottke 1990). Whether the morel provokes this ageing or whether this type normally ages in spring even when not associated to the morel remains unclear. Another particular kind of relationship was observed exclusively with type A. This was a tight contact without mixing of the hyphae even in ageing mycorrhizas. The role of the matrix surrounding type A cannot be determined. It could be a kind of aposition layer allowing exchanges between the two associated ascomycetes, as well as a barrier preventing penetration of other microorganisms. No visible sign of recognition was observed between the morel sclerotia and EM forms B, C, D, E, and G. Nevertheless, the fact that EM of forms B, C and D were vital, despite their low density of emanating hyphae, suggests that exchanges with the surrounding hyphae of the morel could have existed. In conclusion, it appears that the kinds of interactions between the morel and primary formed EM were diverse and probably specific. These specific modifications of the normal behaviour of mycorrhizal fungi when associated have been observed by other authors. In the three-way associations of *Chroogomphus helveticus* ssp. *tatrensis* with *Rhizopogon vulgaris* on *Picea abies*, of *C. helveticus* ssp. *helveticus* with *Suillus plorans* or *S. sibericus* on *Pinus cembra*, and of *C. rutilus* with *Suillus bovinus*, *S. collintus* or *S. variegatus* on *Pinus sylvestris* the normally ectomycorrhizal *Chroogomphus* species forms haustoria within the cortical cells of the *Suillus* or *Rhizopogon* EM (Agerer 1990, 1991b). Brand (1991) also described a specific three-way association of the endophytic ascomycete *Leucoscypha leucotricha*, which regularly forms haustoria in the cortex of *Lactarius* EM on beech.

Among the microorganisms associated with type F was the endobacterium observed together with morel hyphae in proximal, ageing parts of the mycorrhizas. There are only a few reports of the association of endobacteria with mycorrhizas. McDonald and Chandler (1981) observed bacteria-like organelles in VAM similar to the findings of other authors in ecto- or ectendomycorrhizas. However, the endobacteria observed here do not resemble these bacteria-like inclusions, but are more like the "epiparasitic bacteria" also observed by these authors in the external layers of spores of *Glomus caledonius* and free bacteria of the rhizosphere of clover (Forster et al. 1983). The presence of endobacteria together with morel hyphae within ageing EM of type F is noteworthy because the morel forms secondary EM exclusively with this type and also because mycorrhization trials with the morel revealed the helping role of so far undetermined bacteria. Whether only the primary EM of type F or also the endobacteria allow the morel to form EM cannot be decided on the basis of field observations. But the fidelity of the morel to certain microorganisms in nature and the specificity of some interactions with them indicates a necessity to supplement the field observations by experimental investigations with these specific microorganisms.

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