Light and electron microscopy of the host–fungus interaction in the achlorophyllous gametophyte of *Botrychium lunaria*

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The host–fungus interaction between the achlorophyllous gametophyte of *Botrychium lunaria* and its fungal endophyte was studied by means of light and electron microscopy. Aseptate hyphae with a multilayered cell wall formed intracellular coils. The interface consisted of a thick layer of fibrillar matrix material, an electron-translucent zone, and the host plasmalemma. Several vesicles that show different stages of development and degeneration occurred within one host cell. Degenerating vesicles were encased by large amounts of an electron-translucent material. Arbuscules were not observed. The fungus did not infect the young sporophyte but degenerated within intact gametophyte cells.

Key words: *Botrychium lunaria*, gametophyte, mycorrhiza, ultrastructure.

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À l'aide de la microscopie photonique et électronique, les auteurs ont étudié l'interaction hôte–champignon entre le gamétophyte du *Botrychium lunaria* et un endophyte fongique. Des hyphes aseptés munis d'une paroi cellulaire à plusieurs couches forment des pelotons intracellulaires. L'interface est constituée par une épaisse couche de matériel matriciel fibrillaire, une zone transparente aux électrons, et la plasmalemme de l'hôte. On retrouve à l'intérieur d'une même cellule, plusieurs vésicules montrant différents stades de développement et de dégénérescence. Les vésicules en dégénérescence sont enveloppées par de grandes quantités de matériel transparent aux électrons. Aucune arbuscule n'a été observée. Le champignon ne colonise pas le jeune sporophyte, mais dégénère à l'intérieur de cellules intactes du gamétophyte.

Mots clés : *Botrychium lunaria*, gamétophyte, mycorhize, ultrastructure.

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Introduction

In the Ophioglossaceae, gametophytes are achlorophyllous. They are regularly associated with endophytic fungi, and in axenic culture they only grow on media containing sucrose (Whittier 1972, 1981, 1983; Gifford and Brandon 1978; Whittier and Peterson 1984). Transfer of carbohydrates from the endophytic fungi to their hosts is very probable. Knowledge about the host–fungus interaction within these gametophytes is limited to several light-microscope studies (Jeffrey 1897; Lang 1902; Bruchmann 1904, 1906; Campbell 1907, 1908; Burgeff 1938; Nishida 1956; Boullard 1963; Mesler 1975, 1976). These authors described an aseptate endophytic fungus as forming intracellular coils and masses of irregular swollen vesicles containing large amounts of lipids. Bruchmann (1906) listed these characteristics as well for the endophytic fungus of *Botrychium lunaria* (L.) Sw. gametophytes. The only description of arbuscules within a gametophyte of the Ophioglossaceae is that of Nishida (1956). He considered the host–fungus interaction within the gametophyte of *Botrychium virginianum* Sw. to be vesicular–arbuscular (VA). Harley and Smith (1983) also grouped the mycorrhiza-like association within the gametophyte of *B. lunaria* with VA mycorrhiza and suggested an "epiparasitism" on a chlorophyllous host.

In Pteridophytes, achlorophyllous mycothalli are also known in the Psilotaceae (Lawson 1918; Bierhorst 1953; Peterson et al. 1981), Lycopodiaceae (Bruchmann 1898, 1910; Lang 1899; Holloway 1920, 1935; Burgeff 1938; Bruce 1979; Schmid and Oberwinkler 1993), Schizaeaceae (Bierhorst 1966), and Stromatopteridaceae (Bierhorst 1968). Photosynthetic, surface-living gametophytes are associated with fungi in some species of the Lycopodiaceae (Treub 1884; Goebel 1887; Holloway 1920; Duckett and Ligrone 1992), in the Marattiaceae (Campbell 1908; Bower 1923), in *Schizaea* (Bower 1923), and in the Gleicheniaceae (Campbell 1908).

Duckett and Ligrone (1992) reported on the ultrastructure of the host–fungus interaction in the chlorophyllous gametophyte of *Lycopodium cernuum* L. The only electron-microscope studies on the host–fungus interaction within achlorophyllous gametophytes are those on *Psilotum nudum* (L.) Beauv. (Peterson et al. 1981) and *Lycopodium clavatum* L. (Schmid and Oberwinkler 1993). In Ophioglossaceae there is no information on the ultrastructure of the mycorrhiza-like associations within gametophytes.

Materials and methods

In September 1991 and July 1992 a total of seven gametophytes were found within soil taken from the top of Mount Iseler near Oberjoch, Germany (1890 m above sea level). Sporophytes of *B. lunaria* of different sizes were abundant at this location. Soil that contained numerous roots of alpine plants was carefully spread and studied with a stereomicroscope. Gametophytes found within the soil were fixed at once in 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2).

For transmission electron microscopy (TEM) three gametophytes were postfixed with osmium tetroxide (1% OsO_4 in 0.1 M cacodylate buffer, pH 7.2, 1 h) and en bloc contrasted with uranyl acetate (1%, 1 h). Spurr's (1969) ERL was used as embedding medium. Four infiltration steps were carried out: ERL–acetone in the proportions 1:2, 1:1, and 2:1 each for 30 min, followed by ERL for 3 days at 23°C. Semithin sections (0.5 μm) were stained with neofuchsin – crystal violet (Morgenstern 1969). Ultrathin sections (80–120 nm) were contrasted with lead citrate.

For light microscopy, four gametophytes were cleared with 2.5% KOH for 15 min and stained with acidic glycerol – trypan blue (0.05%) (Koske and Gemma 1989).

Results

Gametophytes have a white color, a spherical shape (Fig. 1), and their diameter ranges from 0.1 to 0.3 mm. A few rhizoids of different length project from the surface. Some of them are

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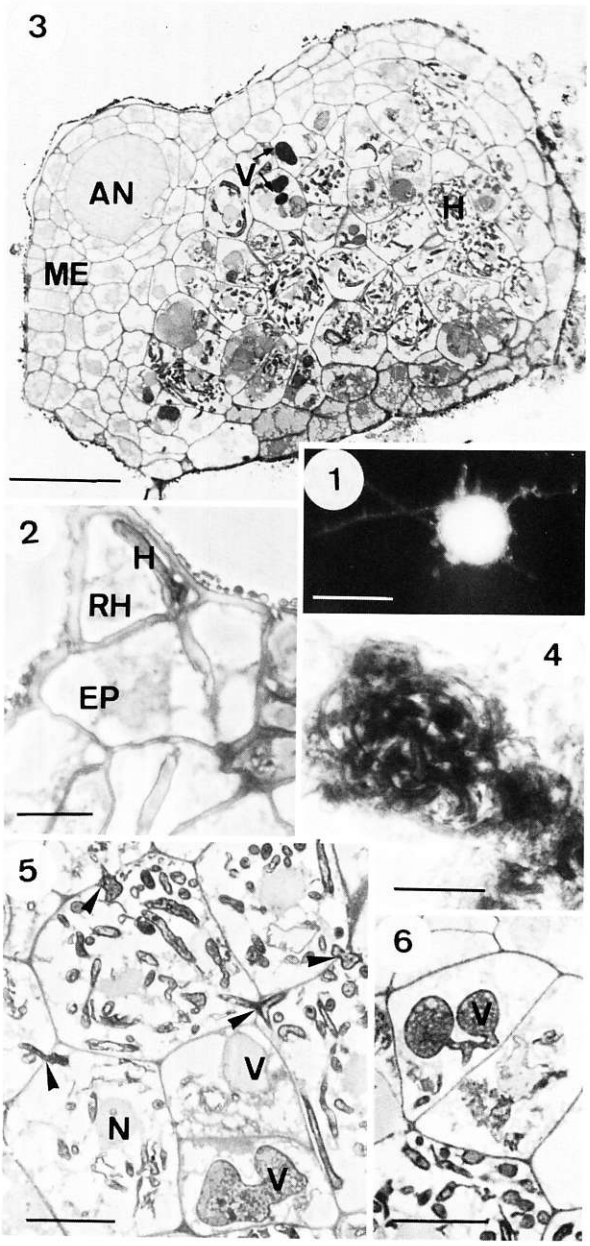
transversed by the fungus that penetrates the rhizoid base (Fig. 2). Figure 3 gives an overview of the distribution of the fungal endophyte within a mature gametophyte that already has developed an antheridium. Fungal hyphae grow in the inner gametophyte cells. Most of the epidermal cells, the antheridium, and the meristematic tissue are not infected. The fungus forms irregular hyphal coils (Fig. 4) that may fill the entire host cell. Hyphae transversing walls between adjacent host cells are abundant (Fig. 5). Vesicles occur throughout the infected zone (Fig. 3). However, host cells filled with dense hyphal coils are free of vesicles. Vesicles are roundish but may also be of an irregular shape (Fig. 5), and often several vesicles occur within one host cell (Figs. 3 and 6). They may arise from branches of the same hypha (Fig. 6).

Host cells with the coiled aseptate hyphae have several vacuoles (Fig. 7). In the infected host cells, organelles such as mitochondria, endoplasmatic reticulum (ER), and plastids with starch grains are abundant (Fig. 8). Hyphae forming intracellular coils range from 1.5 to 3 μm in diameter. Hyphal walls are mostly multilayered and vary in thickness between 0.2 and 0.5 μm (Fig. 8). Fungal cytoplasm is vacuolate, showing mitochondria, ER, and lipid-like droplets (Figs. 7, 8, 10, and 11). Figure 9 depicts a hypha within the outer epidermal wall of the gametophyte. During host-wall penetration hyphae do not reduce their diameter (Fig. 10). The interfacial region, which may show irregular outgrowths (Fig. 11), consists of the host plasmalemma, an electron-translucent zone, and a thick layer of fibrillar matrix material that is continuous with the host cell wall and morphologically similar (Figs. 8 and 10). Within the same host cell, collapsed fungal hyphae were regularly observed next to intact ones (Figs. 7 and 8).

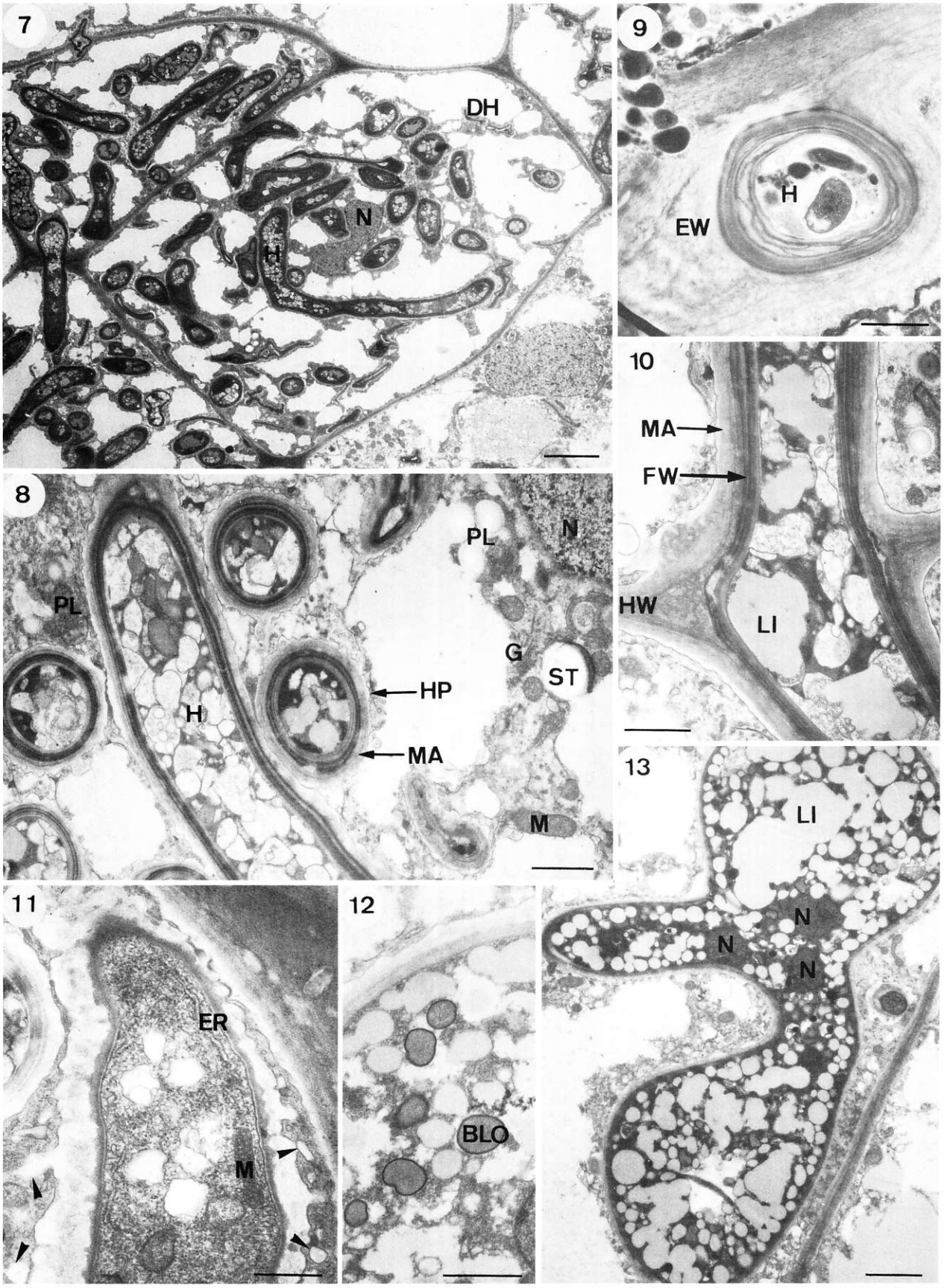
Vesicles reach about 30 μm in diameter and like the coiled hyphae they are surrounded by a thick layer of matrix material (Figs. 12 and 15). Figure 13 shows two young vesicular swellings that have arisen from the same hypha. Several nuclei and large amounts of lipid-like droplets are present within this structure. Bacterium-like organelles of an irregular coccal shape, about 0.7 μm in diameter, are often observed within young vesicles (Fig. 12). Vesicle walls regularly consist of just one layer. Vesicles are often filled with lipid-like material (Fig. 14), and their wall sometimes appears to be partially degenerated (Fig. 15) and may show infoldings (Fig. 16). Matrix material adjacent to the vesicle wall is present (Fig. 16), and between this and the host plasmalemma is a layer that is less osmiophilic than matrix material surrounding intact hyphae and vesicles (Fig. 16). Collapsed vesicles are enclosed within this encasement, which may also contain remnants of lipid-like material (Figs. 14 and 17). The whole structure is surrounded by the host plasmalemma. The same host cell often contains degenerated vesicles, collapsing vesicles, and swollen hyphae (Fig. 14). Figure 18 shows a vesicle with a ruptured wall within a host cell that is filled with large amounts of lipid-like material and vesicles that have degenerated. Plastid envelopes within such host cells may be associated with lipid-like droplets and ER (Fig. 19). Starch grains, plastoglobuli, and irregular membrane profiles occur inside the plastids.

Uninfected host cells do not differ very much from those containing fungal hyphae, except that plastids with starch grains are more abundant than in infected cells (Fig. 20). Within a gametophyte bearing a young sporophyte the host cells are still intact (Fig. 22), and all fungal structures have collapsed. Fungal hyphae were not found in the young sporophyte (Fig. 21).



ABBREVIATIONS: AN, antheridium; BLO, bacterium-like organelle; DH, degenerated hypha; DV, degenerated vesicle; EN, encasement; EP, epidermal cell; ER, endoplasmatic reticulum; EW, epidermal wall; FW, fungal wall; G, Golgi apparatus; H, hypha; HP, host plasmalemma; HW, host wall, LI, lipid-like material; M, mitochondrion; ME, meristematic tissue; MA, matrix material; N, nucleus; PG, plastoglobuli; PL, plastid; PM, membranous profiles; RH, rhizoid; SP, sporophyte; ST, starch; V, vesicle; VW, vesicle wall.

FIGS. 1–6. Light micrographs of *Botrychium lunaria* gametophytes. Fig. 1. Gametophyte with rhizoids. ×125. Scale bar = 0.1 mm. Fig. 2. Hypha, penetrating a rhizoid base. ×2000. Scale bar = 5 μm. Fig. 3. Longitudinal section of a mature gametophyte showing the distribution of the fungal infection. ×310. Scale bar = 50 μm. Fig. 4. Coiled hyphae. ×2000. Scale bar = 5 μm. Fig. 5.Longitudinal section with intracellular hyphae and an irregular lobed vesicle. Arrowheads point to hyphae transversing host cell walls. ×1000. Scale bar = 10 μm. Fig. 6. Two vesicles arising from a branched hypha. ×1330. Scale bar = 10 μm.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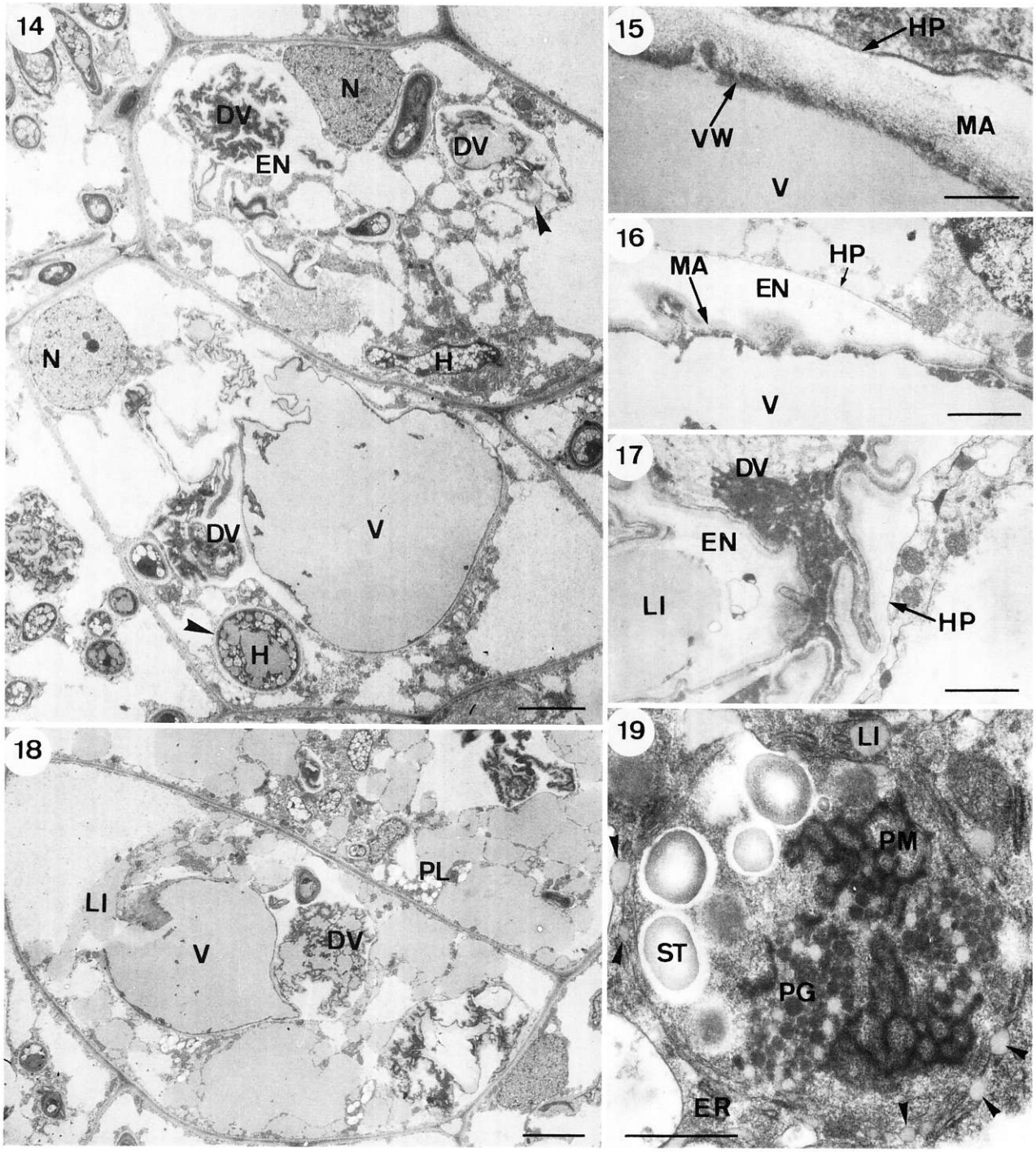
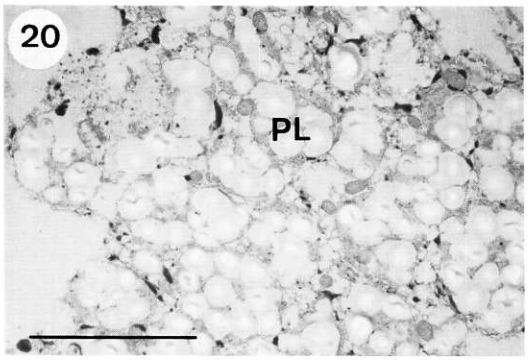
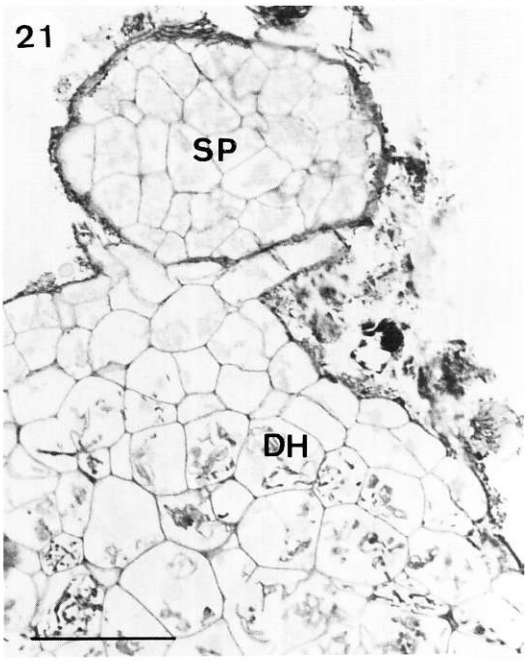
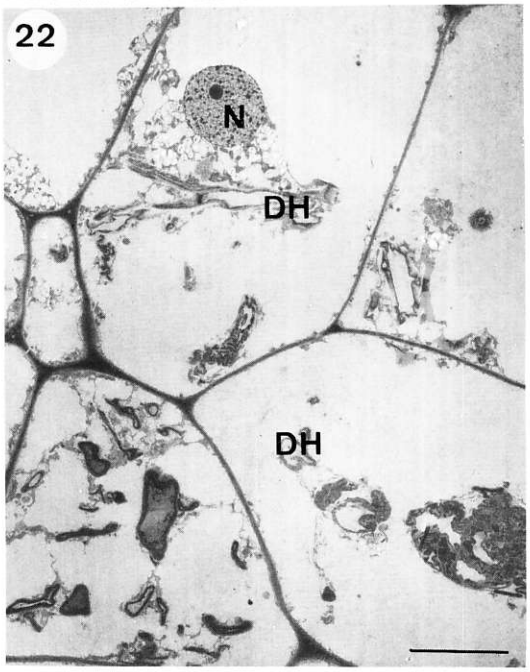


FIGS. 14–19. Ultrastructure of endophytic fungus in *Botrychium lunaria* gametophytes. Fig. 14. Vesicles, material from degenerated vesicles, and a swollen hypha (arrowhead) within the host cell at the bottom. Note the completely encased degenerating vesicle at the top (arrowhead). ×2200. Scale bar = 5 μm. Fig. 15. Dissolving wall of a mature vesicle and the adjacent interface. × 62 500. Scale bar = 0.2 μm. Fig. 16. Mature vesicle with wall infolds. Note the encasement that is less osmiophilic than matrix material. × 12 000. Scale bar = 1 μm. Fig. 17. Encased degenerated vesicle. The encasement contains lipid-like material. × 12 000. Scale bar = 1 μm. Fig. 18. Vesicle with a ruptured vesicle wall within a host cell containing large amounts of lipid-like material. ×2000. Scale bar = 5 μm. Fig. 19. Plastid with starch, plastoglobuli, and prolamellar material. Note the lipid-like droplets (arrowheads) associated with the plastid envelope. × 37 500. Scale bar = 0.5 μm.

FIGS. 7–13. Ultrastructure of endophytic fungus in *Botrychium lunaria* gametophytes. Fig. 7. Intracellular hyphal coils. Hyphae in close proximity to the host nucleus. × 11 000. Scale bar = 5 μm. Fig. 8. Intracellular hyphae with multilayered walls surrounded by a thick layer of matrix material and the host plasmalemma. ×2000. Scale bar = 1 μm. Fig. 9. Hypha within the outer epidermal wall. ×2000. Scale bar = 1 μm. Fig. 10. Hypha, crossing the wall between two adjacent host cells. Matrix material is continuous with the host cell wall. × 12 000. Scale bar = 1 μm. Fig. 11. Hypha surrounded by matrix material that shows irregular outgrowths (arrowheads). × 12 000. Scale bar = 1 μm. Fig. 12. Part of a young vesicular swelling with bacterium-like organelles. × 14 000. Scale bar = 1 μm. Fig. 13. Two multinucleated vesicular swellings arising from a hypha. ×5000. Scale bar = 2 μm.







FIGS. 20–22. Uninfected host cell; gametophyte with a sporophyte. Fig. 20. Uninfected host cell with numerous plastids. ×4000. Scale bar = 5 μm. Fig. 21. Gametophyte, bearing a sporophyte ×700. Scale bar = 25 μm. Fig. 22. Degenerated fungal material within intact host cells next to the gametophyte–sporophyte junction ×1375. Scale bar = 10 μm.

Discussion

The morphology and distribution of the endophytic fungus within the gametophyte of *B. lunaria* is similar to that reported by Bruchmann (1906). The fungus may enter the host tissue by transversing rhizoids as known in Hepatics (Ligrone and Lopes 1989) and in the gametophyte of *L. clavatum* (Schmid and Oberwinkler 1993). Also, as reported by Bruchmann (1906), hyphae may invade the gametophyte through the epidermal wall. Comparable to VA mycorrhizal fungi, the aseptate fungus within the *Botrychium* gametophyte forms hyphal coils and vesicular swellings (Kinden and Brown 1975; Scannerini and Bonfante-Fasolo 1983; Bonfante-Fasolo 1984). These two kinds of fungal structures occur within different host cells, as pointed out by Lang (1902), Campbell (1907), and Burgeff (1938) for the host–fungus interaction in the gametophyte of *Ophioglossum pendulum*, and by Campbell (1908) for the gametophyte of *B. virginianum*. In contrast with VA mycorrhiza within roots, where hyphal coils appear in the outer cortical cells, gametophyte host cells have coils or vesicles irregularly mingled throughout the infected zone. As already stated by Bruchmann (1906), the young sporophyte is not infected by the gametophyte fungus that dies off within intact gametophyte cells.

Arbuscules are lacking in the gametophyte of *B. lunaria*. The only description of arbuscules within a gametophyte of Ophioglossaceae was by Nishida (1956) for *B. virginianum*, but Nishida's micrographs are not convincing. In his report on the host–fungus interaction within the sporophyte and the gametophyte of *O. pendulum*, Burgeff (1938) called fungal haustoria with several vesicular swellings Sternarbuskel, but he clearly did not mean arbuscules with many ramifications as they occur within VA mycorrhiza.

The ultrastructure of the fungus is similar to that of VA mycorrhizal fungi. Irregular coccal bacterium-like organelles are also reported for several VA mycorrhizal fungi (MacDonald and Chandler 1981; MacDonald et al. 1982; Ligrone 1988; Ligrone and Lopes 1989) and for the fungal endophyte of the gametophyte of *L. clavatum* (Schmid and Oberwinkler 1993). As in VA mycorrhiza, coiled hyphae usually have a thick wall with a lamellar structure (Bonfante-Fasolo and Fontana 1985) and are surrounded by a thick layer of matrix material (Scannerini and Bonfante-Fasolo 1983). The interface sometimes shows irregular outgrowths reminiscent of those of the fungal pegs in the monotropoid mycorrhiza (Duddridge and Read 1982; Robertson and Robertson 1982). In comparison with uninfected cells, plastids containing starch are less abundant in infected cells, but starch does not disappear completely as stated by Bruchmann (1906).

Irregularly lobed vesicles as they occur within the gametophyte of *B. lunaria* are also known in Acaulosporaceae (Morton and Benny 1990). Vesicle walls always consist of just one layer. Vesicles of VA mycorrhizas are often described as having a trilaminate wall when they are mature and thus may differ from those described here (Kinden and Brown 1975; Holley and Peterson 1979; Scannerini and Bonfante-Fasolo 1983; Bonfante-Fasolo 1984). Within the *Botrychium* gametophyte the wall of mature vesicles is very thin and seems to degenerate. The encasement of degenerating vesicles is much thicker than that described for degenerated arbuscules (Cox and Sanders 1974). Lipid-like material appears within the encasement, probably indicating its resorption by the host cell. Some host cells contain large amounts of lipid-like material. The direct release of lipid-like material into the host cell was only observed when vesicle walls and the surrounding matrix layer were rup-

tured. However, vesicle degeneration by increasing infoldings of the vesicle walls and the surrounding matrix material within an encasement was observed more often, and the rupture of vesicle walls might be artificially induced. Unfortunately there is no detailed report on vesicle degeneration in VA mycorrhiza. Since swollen hyphae, mature vesicles, and degenerated vesicles occur together within the same host cell, the repeated development of vesicles can be assumed. This is not described for any VA mycorrhiza and is considered to be a characteristic feature of the described interaction.

The host–fungus interaction in the gametophyte of *B. lunaria* may be similar to that found by Peterson, et al. (1981) in the gametophyte of *P. nudum*. However, in *P. nudum* vesicle development is not as conspicuous as in the gametophyte of *B. lunaria*. Peterson et al. suggested the host–fungus interaction within the *Psilotum* gametophyte, corresponding to the evolutionary primitiveness of *Psilotum*, "to be not as well developed as vesicular–arbuscular associations." However, it must be additionally taken into account that in contrast with VA mycorrhiza of chlorophyllous hosts, the transfer of carbohydrates is from the fungus to the host within the achlorophyllous gametophytes. The different function might be related to the different morphology observed.

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