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Ultrastructure of germination and mucilage production in *Halosphaeria appendiculata* (Halosphaeriaceae)

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The germination of ascospores of the marine fungus *Halosphaeria appendiculata* was investigated with transmission electron microscopy. Prior to germination, settled ascospores became surrounded by a fibro-granular layer. Small, membrane-bounded vesicles and larger electron-dense membrane-bounded vesicles aggregated at the site of germ tube formation where the plasmalemma adjacent to the aggregation was convoluted. The vesicles appeared to fuse with the plasmalemma, releasing their contents. Enzymatic digestion of the spore wall probably occurred at the time of germ tube emergence. After the nucleus had migrated into the newly formed germ tube, a septum was formed to delimit the germ tube from the ascospore. The growing germ tube can be divided into 3 morphological regions, namely the apical, sub-apical and vacuolated regions, and is typical of other fungi. A mucilaginous sheath was associated with the older mycelium. The germ tube displaced the polar appendage, and the ascospore, germ tube and appendage were enclosed in a mucilaginous sheath. In ascospores which subtended old germ tubes, the nucleus and lipid body became irregular in shape and the cytoplasm was more vacuolated. Microbody-like structures remained associated with the lipid throughout development, and were present in old ascospores.

Key Words——ascospores; germ tube; *Halosphaeria*; marine fungi; ultrastructure.

Transmission electron microscope (TEM) studies of marine fungi have concentrated on ascospore ontogeny and are essentially limited to the structure of the mature ascospore and their appendages (Lutley and Wilson, 1972b; Johnson, 1980, 1982; Manimohan et al., 1993; Yusoff et al., 1994; Read et al., 1995). Read et al. (1995) have also examined the ascus structure, but there are few ultrastructural studies of spore germination in marine fungi. Onyile et al. (1982) have illustrated the hyphopodium of *Buergenerula spartinae* Kohlm. & Gessner at the ultrastructural level and Lutley and Wilson (1972a) have illustrated the germination process in *Ceriosporopsis halima* Linder. The only report of the germination process in marine fungi at the ultrastructural level is that of Lutley and Wilson (1972a).

Investigation into fungal spore morphology and germination at the TEM level have been carried out by many workers (Beakes, 1980; Hoch and Staples, 1983; Hemmes and Stasz, 1984; Dyke and Mims, 1991), although few of these studies are concerned with ascospore germination. Beckett et al. (1974) demonstrated spore germination in *Daldinia concentrica* (Bolton: Fr.) Ces. & De Not., while Lowry and Sussman (1968) have illustrated changes during germination of ascospores of *Neurospora tetrasperma* Shear & B. O. Dodge.

Detailed ultrastructural information on the cytoplasm, organisation of the ascospore, hyphal structure and mucilage production during germination is not available for marine fungi. Hyde et al. (1994) have illustrated the ultrastructure of ungerminated ascospores of *Halosphaeria appendiculata* Linder, and Hyde et al. (1986) illustrated mucilage production and mucilage mediated attachment by germ tubes of several marine fungi at the SEM level. In this paper, the germination process, including mucilage mediated attachment in *H. appendiculata* is illustrated at the TEM level.

Materials and Methods

A suspension of *H. appendiculata* ascospores was prepared by extracting mature ascomata contents and placing in distilled water. The suspension was settled on to sea water corn meal agar or wood slivers and spores were allowed to germinate at 29°C. Blocks (0.5 cm^3) of agar or wood slivers with attached germinating ascospores were removed and fixed in 4% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, adjusted to an osmolality, determined by the depression of freezing point, equal to that of sea water, by the addition of sucrose, for 4 h at 25°C. Fixed material was washed in 4 changes of the same buffer, with decreasing sucrose concentrations to zero. Material was post fixed in 2% (w/v) osmium tetroxide in 0.1 M sodium cacodylate buffer for 16 h at 4°C. Fixed material was allowed to attain room temperature, washed in distilled water and then dehydrated in a graded ethanol series (15 min in each of 10% steps starting at 10%). Absolute ethanol was sequentially replaced by acetone, the material

infiltrated gradually with an Epon-Araldite mixture, flat embedded and polymerized at 60°C for 3 d.

Embedded ascospores at the required stages of development were removed from the flat blocks and reorientated on to blank stubs. Thin sections were cut on a LKB III Ultratome using glass knives, mounted on parlodion-coated, carbon-stabilized grids, stained for 30 min in lead citrate and examined in a JEOL 100S transmission electron microscope at 60 kV.

Results

On settlement to a substratum ascospores of *H. appendiculata* germinated and produced one or more germ tubes. However, prior to germ tube production, the ascospores became surrounded by a layer, 0.3–0.7 μm thick, of fibro-granular material, except at the mid-septum and polar regions (Fig. 1). At this early stage in germination the cytoplasmic organisation of the ascospore was similar to that of ungerminated spores (see Hyde et al., 1994), apart from irregularities in the outline of the lipid body, an increase in the number of vacuoles and increased convolution of the plasmalemma.

Germ tube initials formed predominantly at the polar regions 6–96 h after settlement. It was usual for one germ tube to develop from each ascospore, although bipolar germination was occasionally observed. Small, membrane-bounded vesicles, 0.05–0.1 μm in diam, and larger electron dense membrane-bounded vesicles, 0.2–0.35 μm in diam, aggregated at the site of germ tube formation (Figs. 2, 3). The plasmalemma adjacent to the aggregation became convoluted, and vesicles appeared to fuse with the plasmalemma, releasing their contents on to the ascospore wall (Fig. 3). Lomasome-like structures (LS, Fig. 3) and endoplasmic reticulum (ER, Fig. 2) were associated with the region of germ tube initiation, whereas mitochondria, ribosomes, and glycogen were absent (Fig. 2).

Penetration of the ascospore wall by the germ tube, was concurrent with reduction of the thickness of the mesosporium and the formation of an endosporium; no rupture of the ascospore wall layers was observed (Figs. 4, 5). An electron-dense layer was deposited immediately external to the convoluted plasmalemma of the germ tube initial (Figs. 4, 5). Growth of the germ tube initial was associated with accumulation of cytoplasmic vesicles, multivesicular bodies and lomasome-like structures within the cytoplasm of the germ tube, and a bulge in the adjacent ascospore wall (Figs. 4, 5).

The young germ tube was enrobed in a mucilaginous sheath which was continuous with the ascospore sheath (Fig. 11). The germ tube wall was 2-layered and originated endogenously from the ascospore (Fig. 7). Elongation of the germ tube was accompanied by the migration of organelles, including vesicles, mitochondria, ribosomes, endoplasmic reticulum and to a lesser extent glycogen rosettes, towards the tip (Figs. 6, 7, 9, 11). Lomasome-like structures remained associated with the base of the germ tube in the region of eventual septum formation (Figs. 9, 10).

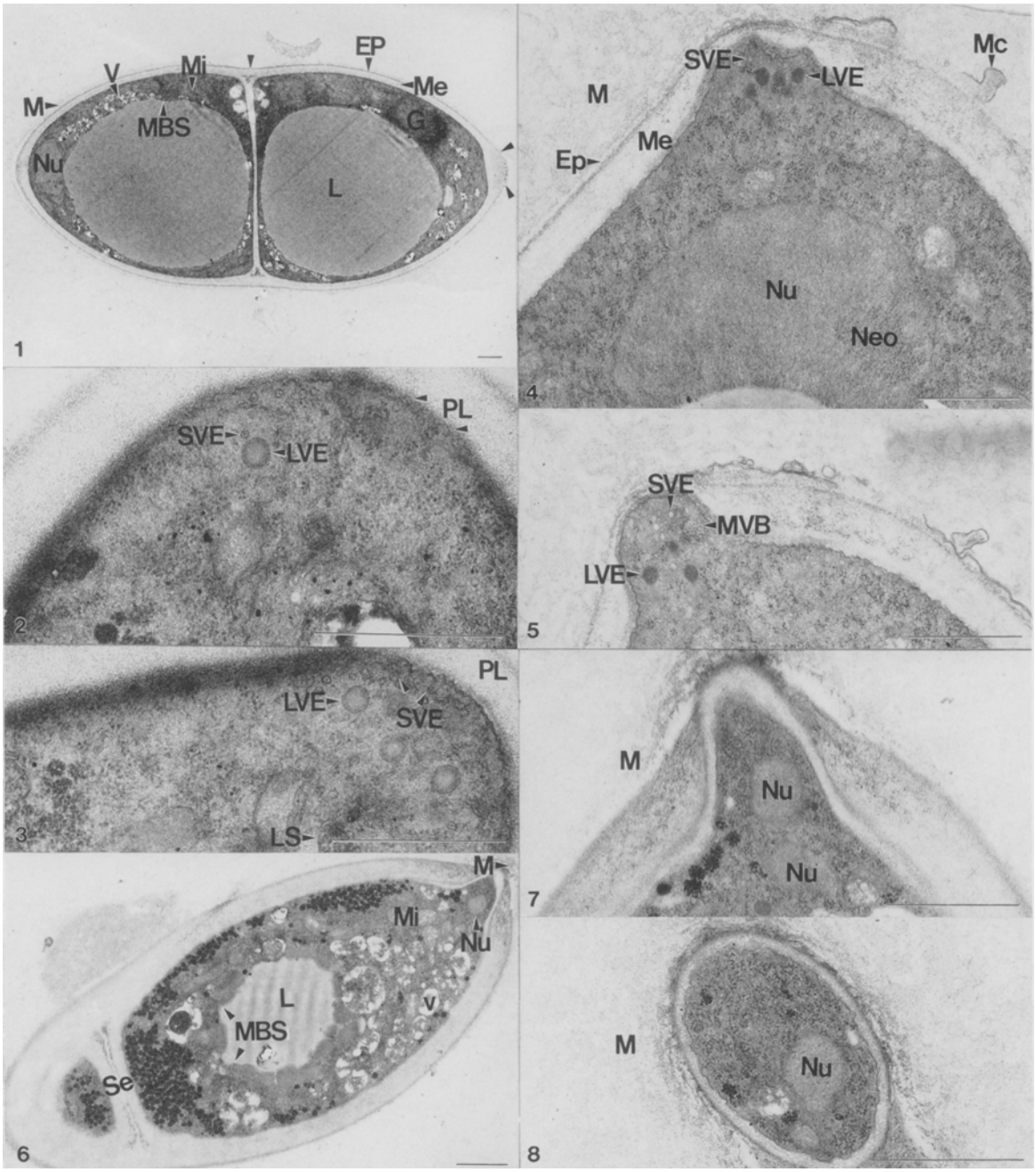
Following germ tube formation the nucleus within the subtending cell of the ascospore divided and one daughter nucleus migrated into the developing germ tube (Figs. 6–8). Continued increase in the length of the germ tube was concomitant with the formation of vacuoles containing electron-dense material at their base. They also occurred in the cytoplasm around the lipid body within the ascospore (Fig. 11).

After the nucleus had migrated into the germ tube a septum was formed to delimit the germ tube from the ascospore (Figs. 12, 13, 15). Formation of the cross wall was associated with the presence of lomasome-like bodies (Figs. 9, 10). The lipid body in the ascospore was at this stage less sphaerical, had a reduced electron density, but was still surrounded by microbody-like structures (Fig. 15). No morphological difference was detected in the microbody-like structures at different stages of germination (Figs. 1, 15, 29–31). The mucilaginous material around the ascospore became thicker with age (0.3–0.5 μm) and was continuous over the germ tube (Figs. 14, 15).

The cross wall delimiting the germ tube tapered towards the central pore and was surrounded by a convoluted plasmalemma which was continuous through the pore (Figs. 9, 12). The region immediately each side of the pore was devoid of ribosomes and contained several electron-dense membrane-bounded Woronin bodies (Fig. 12). The septum extended towards the germ tube, which indicated cytoplasmic pressure from within the ascospore (Figs. 13, 15). The endogenously formed germ tube wall layer continued to be deposited within the ascospore to form the endosporium (Figs. 13, 15).

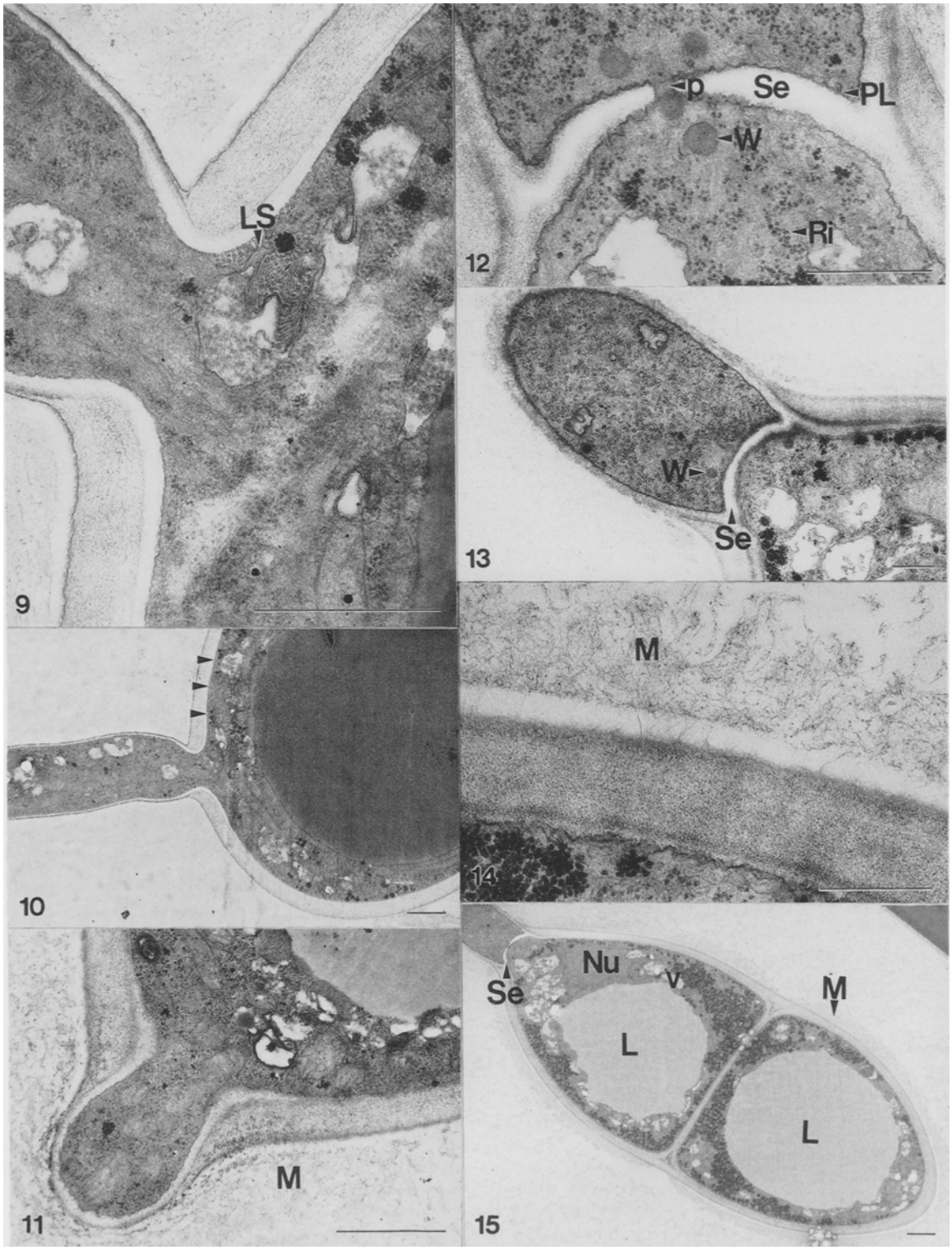
The germ tube can be divided into 3 morphological regions, namely the apical, subapical and vacuolated regions. The apical region (Figs. 16–20) incorporated many small membrane-bounded vesicles (0.02–0.03 μm in diam) and fewer but larger electron-dense membrane-bounded vesicles (0.1–0.2 μm in diam); other organelles and ribosomes were absent from this region. The plasmalemma of this apical region was highly convoluted and showed continuity with some of the smaller vesicles and associations with lomasome-like structures (Figs. 16–20), although the latter were never observed at the extreme tip.

The subapical region occurred immediately behind the germ tube tip and extended for several micrometres. Unlike the apical region, which either lacked or possessed a very thin mucilaginous sheath, the subapical region was bounded by a discrete sheath which increased in thickness with increased distance from the tip (Fig. 20). The plasmalemma was still convoluted, although to a lesser extent than at the tip (Figs. 21, 22). In a single section, lomasome-like structures lined the plasmalemma (Figs. 22, 32). Vacuoles containing various membranous inclusions were present throughout the cytoplasm, although there was a tendency for these to occur close to the germ tube wall. Mitochondria with plate-like cristae, endoplasmic reticulum, ribosomes and a small number of vacuoles comprised the rest of the cytoplasm (Fig. 21).

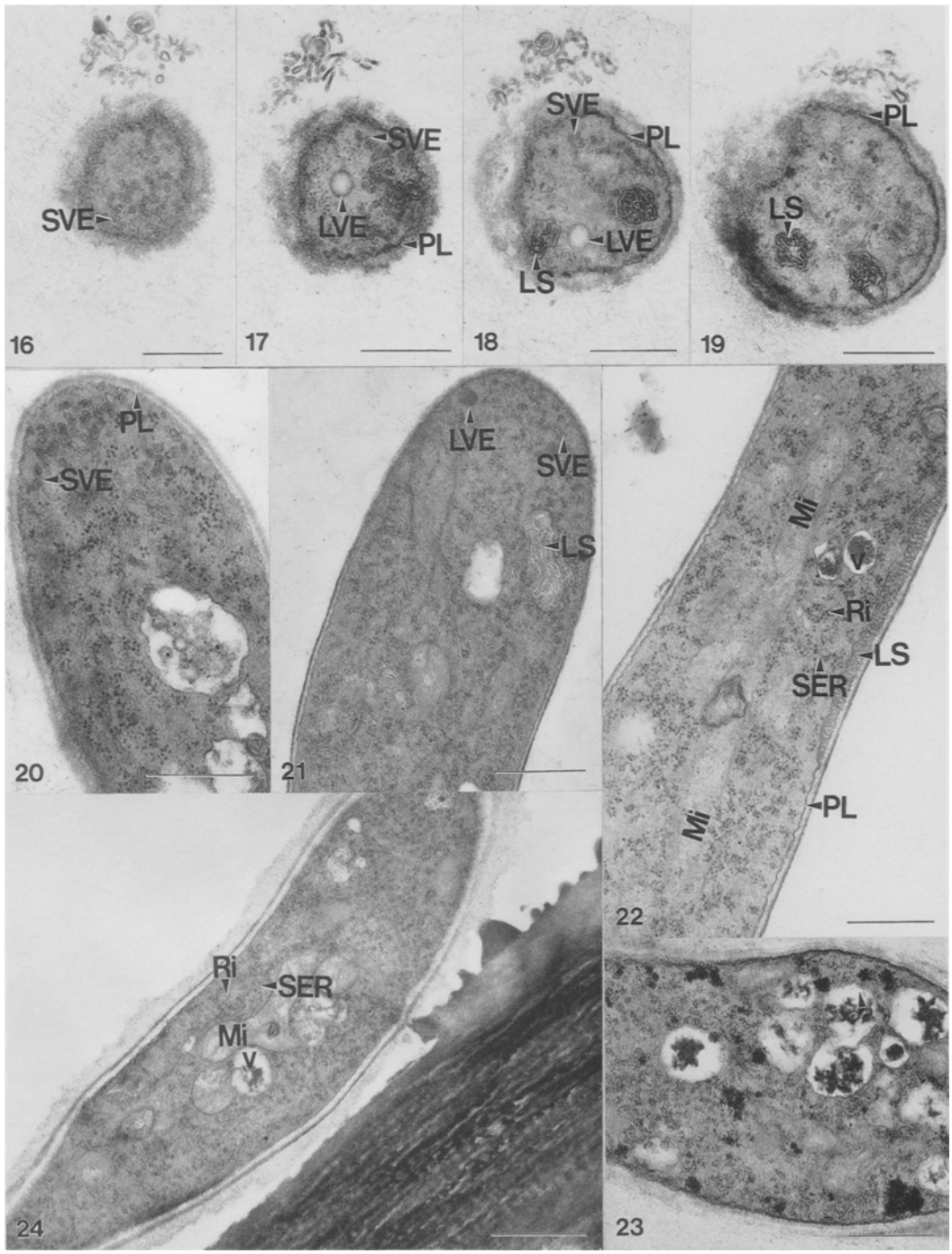


Figs. 1–8. Transmission electron micrographs of ascospores of *Halosphaeria appendiculata*.

1. A longitudinal section of an ascospore immediately prior to germination. A layer of mucilage (M) surrounds the ascospore, except at the thicker-walled polar and septal region (arrowed). A large lipid globule (L) occupies each cell and is surrounded by disc-shaped microbody-like structures (MBS). A nucleus (Nu), mitochondria (Mi), vacuoles (V) and glycogen rosettes (G) can be seen in the cytoplasm. The cell wall is two-layered comprising a mesosporium (Me) and an episporium (Ep). 2, 3. Skipped serial sections of a germinating ascospore showing the germ tube initial. Large membrane-bounded, electron-dense vesicles (LVE) and smaller membrane-bounded, less electron-dense vesicles (SVE) aggregate at the region of germ tube formation. This region lacks ribosomes and other organelles and the plasmalemma (PL) is highly convoluted (arrowed). 4, 5. Longitudinal sections through polar regions of ascospores and germ tube initials. Small membrane-bounded vesicles (SVE) are aggregated close to or fused with the convoluted plasmalemma while the larger vesicles (LVE) are more proximal. External to the convoluted plasmalemma is a layer of amorphous electron-dense material (arrowed) and the mesosporium (Me) in the region is thin or absent. Multivesicular bodies (MVB) are also present in the germ tube initial (5). Regions of the membrane complex (Mc) and mucilage (M) occur outside the episporium (Ep), while the nucleus (Nu) and nucleolus (Neo) can be seen within the cytoplasm. 6–8. Skipped oblique longitudinal sections through ascospore and base of developing germ tube. Note the septum (Se), lipid globule (L), microbody-like structures (MBS), mitochondria (Mi) and vacuoles (V) in 6. The nucleus (Nu) has divided and the daughter nucleus is migrating into the germ tube. Note the mucilage surrounding the young germ tube (M). Scale bars = 1 μm.

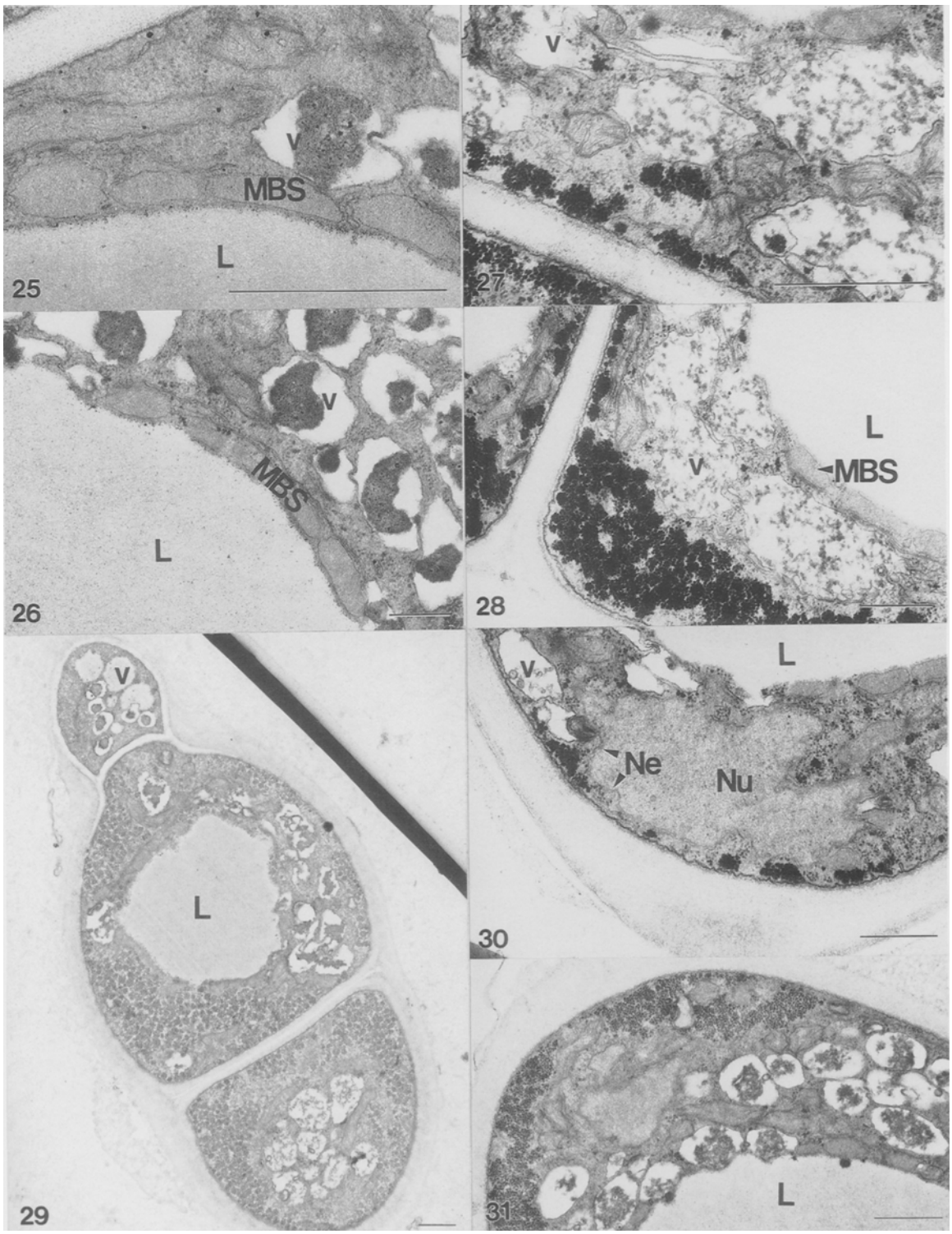


Figs. 9–15. Transmission electron micrographs of germinating ascospores of *Halosphaeria appendiculata*.
9. Base of germ tube. Lomasome-like structures (LS) occur adjacent to or fused with the plasmalemma in the region of wall formation. 10. Germ tube formed sub-polarly. Note that the inner, electron-transparent wall layer of the germ tube is formed endogenously within the ascospore (arrowed). 11. Oblique longitudinal section of young germ tube surrounded by mucilage (M). 12–15. Longitudinal section of germinated ascospore showing the septum (Se) at the base of the germ tube. The germ tube has a pore (P) with associated Woronin bodies (W). The cross wall is bowed towards the germ tube and tapers towards the pore through which the plasmalemma (PL) is continuous. Cytoplasm adjacent to the pore lacks ribosomes (Ri). Both the lipid body (L) and the nucleus (Nu) of the ascospore have a convoluted outline and the cells are vacuolated (V). Note the mucilaginous sheath (M) around the ascospore in 15, which can be seen at higher magnification in 14. Scale bars = 1 μm.



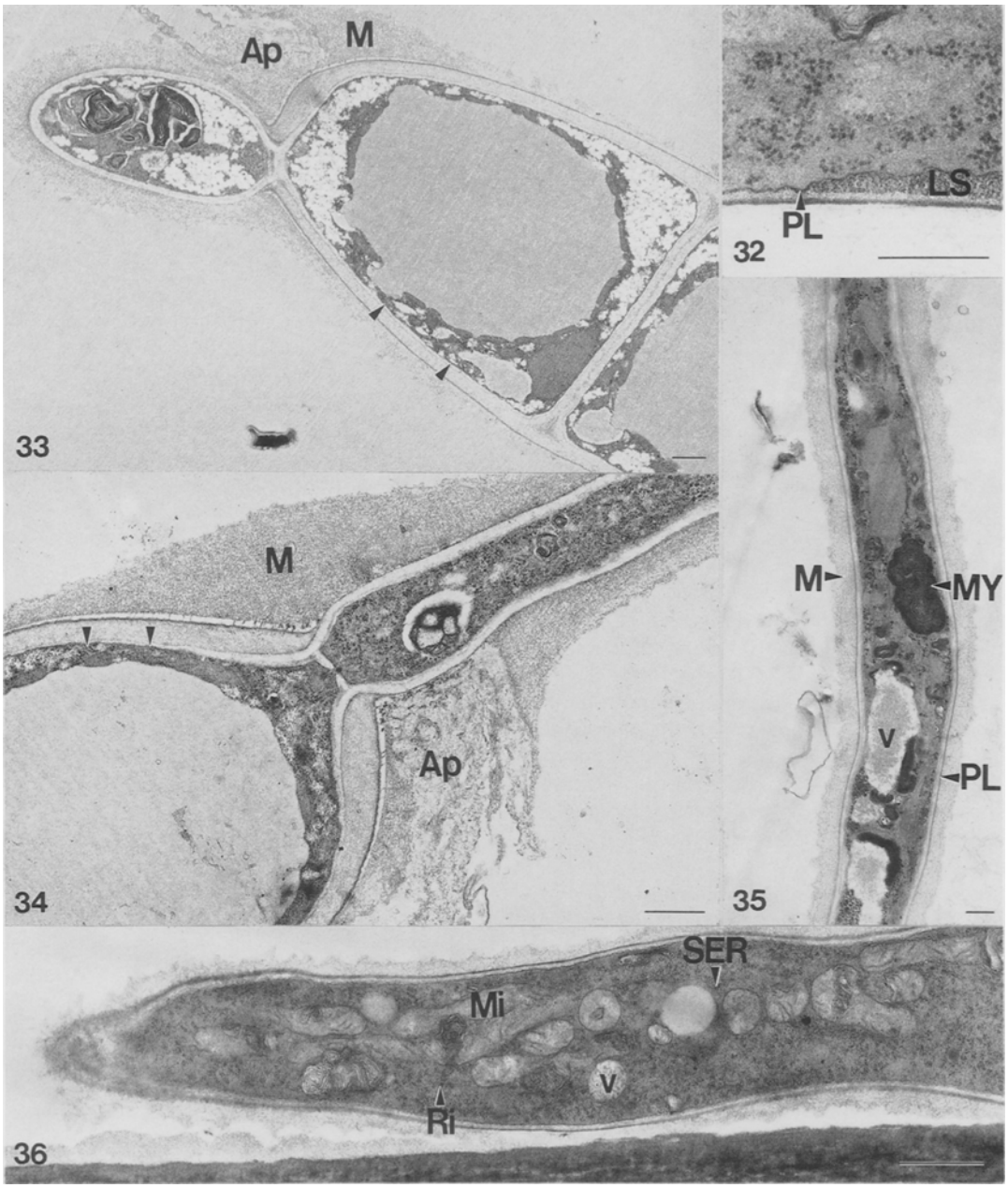
Figs. 16–24. Transmission electron micrographs of the germ tube of *Halosphaeria appendiculata*.

16–19. Skipped serial oblique transverse sections through tip of germ tube. Large electron-dense membrane-bounded vesicles (LVE) and smaller less electron-dense membrane-bounded vesicles (SVE) are associated the germ tip. Only the smaller vesicles occur at the extreme tip (16) which lacks ribosomes and other inclusions. Lomasome-like structures (LS) are associated with the plasmalemma (PL) proximal to the tip (17-19) and the plasmalemma is convoluted. 20, 21. Skipped longitudinal sections through the tip of a germ tube. Small membrane-bounded vesicles (SVE) are aggregated at the extreme tip (20), where ribosomes and other organelles are lacking. The plasmalemma at the tip of the germ tube is more convoluted than that further back. Lomasome-like structures (LS) and large electron-dense membrane-bounded vesicles (LVE) occur immediately behind the tip region. 22, 23. Longitudinal section of sub-apical region of germ tube. The wall of the germ tube is two-layered and the cytoplasm has a high density of ribosomes (Ri), mitochondria (Mi), endoplasmic reticulum (SER) and vacuoles with electron dense contents (V). The plasmalemma (PL) is convoluted and associated with extensive lomasome-like bodies (LS). 24. Longitudinal section of sub-apical region of germ tube on wood veneers. The wall of the germ tube is two-layered and the cytoplasm has a high density of ribosomes (Ri), mitochondria (Mi), endoplasmic reticulum (SER) and vacuoles with electron dense contents (V). The mucilage sheath around the germ tube is more pronounced than that of spores germinating on agar. Scale bars = 0.5 μm.



Figs. 25–31. Transmission electron micrographs of ascospores of *Halosphaeria appendiculata* with well developed germ tubes. The cytoplasm is more vacuolated (V) than in ungerminated spores, but in other respects they are similar. However, the nuclear envelope (Ne) is convoluted. Note the microbody-like structures (MBS) which are associated with the lipid body (L). Scale bars = 1 μm.

The cytoplasmic organisation of the subapical regions of germ tubes developing on wood veneers (Fig. 24) was similar to that of germ tubes grown on sea water corn meal agar. The mucilaginous sheath was, however, more compacted and this may have been due to its inability to diffuse into the woody substratum. Only



Figs. 32–36. Transmission electron micrographs of germinated ascospores and germ tubes of *Halosphaeria appendiculata*. 32. Higher magnification of subapical region of germ tube as in Fig. 22. The plasmalemma (PL) is convoluted and associated with extensive lomasome-like bodies (LS). 33, 34. Longitudinal sections through an old ascospore and base of an old germ tube. The mucilaginous sheath (M) around the ascospore is continuous with and morphologically similar to the hyphal sheath. Growth of the germ tube has displaced the polar appendage (Ap) and the endogenously produced germ tube wall has formed an endosporium (arrowed). 35. Longitudinal section of vegetative hyphae. The wall has a discrete, mucilaginous sheath (M). The plasmalemma (PL) is smooth and large vacuoles (V) and myelin bodies (MY) occur in the cytoplasm. 36. Longitudinal section of vacuolated hyphae on wood veneer. The wall of the germ tube is two-layered and the cytoplasm has a high density of ribosomes (Ri), mitochondria (Mi), endoplasmic reticulum (SER) and vacuoles with electron dense contents (V). The mucilage sheath around the germ tube is more pronounced than that of spores germinating on agar. Scale bars = 1 μm.

a single nucleus was present in the subapical region. In no sections was an archontosome observed, although membrane-bounded structures resembling microbodies were occasionally seen in the perinuclear region.

The vacuolated region of the germ tube embodied many membrane-bounded autolytic vesicles, lipid bodies and glycogen rosettes, as well as mitochondria, ribosomes, lomasome-like bodies and endoplasmic reticulum (Figs. 23, 35). The plasmalemma was convoluted (Fig. 23), but in older mycelium it was smooth (Fig. 35). A

mucilaginous sheath was associated with older mycelium (Figs. 35, 36). The germ tube displaced the polar appendage and the ascospore, germ tube and appendage was enrobed with a mucilaginous sheath (Figs. 33, 34).

In ascospores which subtended old germ tubes, the nucleus and lipid body became irregular in shape and the cytoplasm was more vacuolated (Figs. 25–31). The microbody-like structures remained associated with the lipid throughout development, and were present in germinated ascospores (Figs. 25–31).

Discussion

Prior to germ tube development, mucilage is secreted from the ascospores of many marine fungi and forms a sheath (Hyde et al., 1986). This has been shown in *Corollospora maritima* Werderm. and *Halosphaeriopsis mediosetigera* (Cribb & Cribb) T. W. Johnson at the SEM level (Rees, 1982; Rees and Jones, 1984) and *H. appendiculata* at the SEM and TEM level (Hyde et al., 1986). The sheath is thought to be adhesive and to aid attachment of the ascospore to the substrate prior to germination (Rees, 1982; Rees and Jones, 1984; Hyde et al., 1986; Jones, 1994). Germ tube formation usually occurs from the polar region of the ascospore, although occasionally they may arise laterally. The presence of vesicles associated with the plasmalemma of germ tube initials, the probable concurrent digestion of the mesosporium, and deposition of the germ tube wall augurs for the vesicular transport mechanism for enzymes and wall material to the sites of germ tube growth and emergence.

There are few studies on the early stages of germ tube formation in the ascomycetes. However, in *Colletotrichum lagerarium* (Pass.) Ellis & Halst. and *Chalara* sp. a new wall layer is laid down, and germ tube production is by polarized growth of the new wall layer through a rupture in the original wall layers of the germinating conidium (Akai and Ishida, 1967; Hawes, 1980). This has also been noted in ascospores of *D. concentrica* (Beckett et al., 1974) and basidiospores of *Fomes fomentarius* (L.:Fr.) Fr. (Tsuneda and Kennedy, 1978). Germination by enzymatic breakdown of the spore wall, and concurrent formation of the endogenous wall layer, as probably occurs in *H. appendiculata*, have also been noted for teliospores of the sugar cane smut, *Ustilago scitaminea* Syd. (Paine and Hess, 1984) and *Tilletia* sp. (Hess, 1973) and a number of other fungi (Border and Trinci, 1970). Attached spores of *Drechmeria coniospora* (Drechsler) W. Gams & Jansson initially germinate, presumably by enzymatic breakdown of the spore wall, to produce an appressorium. Further enzymatic breakdown by the penetration tube allows entry through the nematode cuticle (Dijksterhuis et al., 1990). It is not clear if these two processes are essentially different, as both involve *de novo* formation of a vegetative wall under the spore wall. Rupture of the spore wall occurs in one group (Hawker and Abbott, 1963; Bartnicki-Garcia, 1969; Hemmes and Stasz, 1984), while enzymatic breakdown of the spore wall was observed in *H. appendiculata*.

The germ tube tip in *H. appendiculata* is typical of other fungal species as growth is by apical extension (Bracker, 1967; Dyke and Mims, 1991). The young hypha consists of 3 regions: apical, subapical and a region of vacuolation. The apical region is characterised by an accumulation of numerous vesicles to the exclusion of other organelles and ribosomes. Morphologically discrete Golgi bodies or Dictyosomes have not been observed and it is hypothesised that the vesicular system is responsible for the synthesis of the wall material as proposed by Bracker (1967). Such vesicles are generally hypothesised to be integrally involved in growth and cell wall formation in fungi (Grove et al., 1971; Cole and Samson, 1979). The hypothesis that vesicles release their contents into the apical region is supported by the highly convoluted plasmalemma and its continuity with vesicles in the region of hyphal growth. These vesicular secretions are likely to be wall materials, or wall precursors, that comprise the developing apical wall (Bracker, 1967). In *H. appendiculata*, larger membrane-bounded vesicles were found to be associated with the cytoplasm in the apical region. These however, have not been clearly associated with the plasmalemma and their function has not been determined, whereas, the smaller, less electron-dense membrane-bounded vesicles are in continuity with the convoluted plasmalemma at the apex.

Lomasome-like structures, were also associated with the subapical region of the germ tube, and although they may be artifacts, are common features of non-phycomycetous fungi (Garrison and Boyd, 1977; Hess, 1981). One of the several suggested functions of lomasomes is in wall formation (Bracker, 1967; Beckett et al., 1974). These lomasome-like structures were not found at the extreme tip, but were commonly associated with the regions of secondary wall development in the post apical region. Elongated mitochondria, ribosomes, nuclei and endoplasmic reticulum occurred in the subapical region of the germ tube. These structures were probably involved in the production of materials, required for germ tube elongation, and were transported in packaged vesicles to the apical tip. Similar mechanisms of wall elaboration have been observed in the Basidiomycota (Olah and Reisinger, 1981) and the Deuteromycota (Mangenot and Reisinger, 1976).

A mucilaginous sheath was observed around the germ tube of *H. appendiculata*. Such sheaths are common and have been previously noted in marine and terrestrial fungi (Robb et al., 1973; Hohl and Suter, 1976; Hau and Rush, 1982; Onyile et al., 1982; Dyke and Mims, 1991; Jones, 1994). The origin of the sheath in these fungi is uncertain, although Hohl and Suter (1976) have implicated cytoplasmic vesicles in the production of mucilage by *Phytophthora infestans* (Mont.) De Bary and Robb et al. (1973) have suggested that provacuolar bodies may be involved in the secretion of an extracellular matrix. The origin of the sheath in *H. appendiculata* has not been established. A convoluted plasmalemma was present along the entire length of the germ tube, but vesicles were not found to be associated with the cell wall, except at the tip. Lomasome-like structures were as-

sociated with the wall of the developing germ tube, except at the extreme tip and they cannot be excluded as the origin of mucilage, although their role in secondary wall formation has also been suggested.

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