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Ultrastructural observations on *Nimbospora bipolaris* (Halosphaeriaceae, Ascomycetes)

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SUMMARY

The ultrastructure of the ascus, ascospores and appendages of *Nimbospora bipolaris* were examined at the transmission electron microscope level. The mature ascus wall comprises a single narrow electron-opaque layer which deliquesces and lacks an apical elaboration. Ascospore walls comprise a bipartite mesosporium, a thinner electron-opaque episporium and an outer mucilaginous sheath. Before release from the ascus the mucilaginous sheath is compact and folded. The fibrillar, trailing ascospore appendages are arranged in eight equidistant tufts around the circumference of the septum, originate from the mesosporium and emerge through pores in the episporium. In early stages of development the appendages are retained within the mucilaginous sheath but upon release from the ascus the sheath expands and the appendages pierce the sheath and radiate out to form their characteristic appearance. Ascospore ontogeny in *N. bipolaris* is compared with other appendaged members of the Halosphaeriaceae.

1. INTRODUCTION

Nimbospora effusa Koch (type species) was described from driftwood collected on a sandy beach in Thailand (Koch 1982). The diagnostic feature of *N. effusa* is the ascospore which is hyaline, bicelled and enveloped by a sheath-like appendage from which a tuft of mucilaginous material forms a trailing appendage attached at the septum. The sheath does not appear to be constricted at the septum in *N. effusa*. Hyde & Jones (1985) have shown that the sheath and trailing fibrillar appendage are sticky and help to adhere the ascospores to substrata. Hyde & Jones (1985) and Kohlmeyer (1985) have described two further species: *Nimbospora bipolaris* Hyde et Jones and *N. octonae* Kohlm. respectively. These differ from *N. effusa* in both the number of fibrillar appendages emanating from the sheath and in the possession of a constriction of the sheath at the central septum. In *N. bipolaris* there are eight tufts of appendages arranged regularly around the septum where the sheath is constricted (Kohlmeyer & Volkmann–Kohlmeyer 1987). Conversely, *N. octonae* lacks tufts of fibrillar appendages, instead the sheath protrudes at several points.

This study was undertaken to determine the origin of the sheath and appendages in *Nimbospora bipolaris* and also to illustrate the way in which the appendages are attached to the ascospore. This is of particular interest as *Nimbospora* is the only genus within the Halosphaeriaceae with a sheath that envelopes the mature ascospore.

2. MATERIALS AND METHODS

Nimbospora bipolaris was obtained from pieces of marine driftwood which had been collected from Taiwan, Republic of China and incubated in the laboratory. Material for transmission electron microscopy was fixed with 4% (by volume) glutaraldehyde in 0.1 M sodium phosphate buffer for 4 h, washed and then post-fixed with 20 g l^{-1} aqueous osmium tetroxide overnight. Ruthenium red was added to both glutaraldehyde and osmium tetroxide solutions to give a final concentration of 0.2 g l^{-1} (Luft 1971). The material was washed, dehydrated through an ethanol series into acetone and embedded in Mollenhauer's resin (Mollenhauer 1964). Ultrathin sections were stained with lead citrate, post-stained with uranyl acetate and examined at 80 kV in a JEOL 100S transmission electron microscope.

3. RESULTS

(*a*) *Asci*

The asci deliquesced at maturity and as a result the number of intact asci examined was small. Asci were eight-spored, clavate, thin-walled and without any apical apparatus or elaboration. Examination of an ascus containing immature, non-septate ascospores showed that the ascus wall comprised a single, 25–30 nm thick, electron-opaque layer (figures 1–5). Large numbers of glycogen rosettes were present within the asci, particularly at the base of the ascus/ascogenous cell.

(*b*) *Immature ascospores*

The walls of immature, non-septate ascospores consisted of a 20–30 nm thick episporium (figures 2–8) although in the central region of some ascospores the presence of a 25–35 nm thick amorphous electron-

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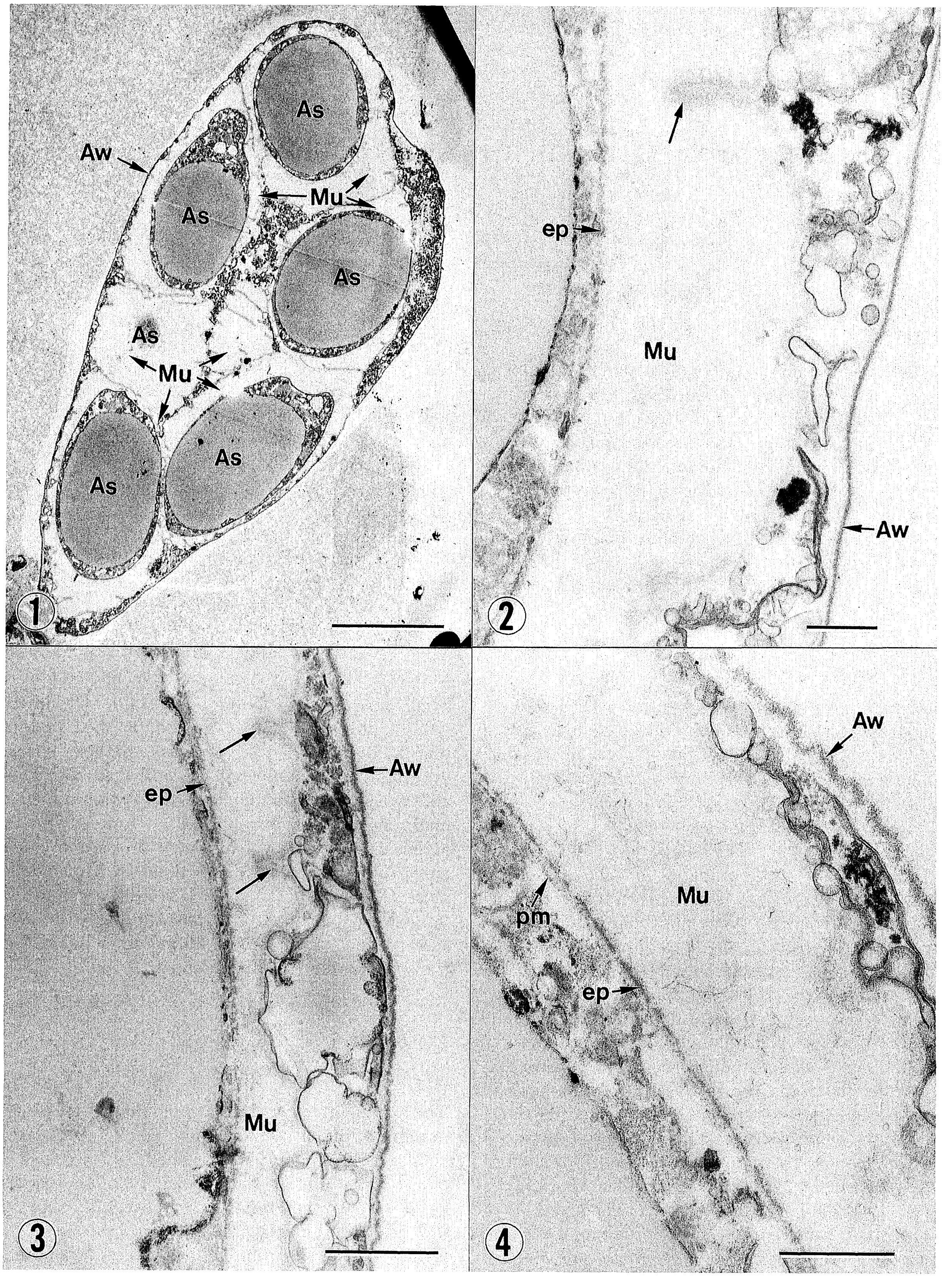
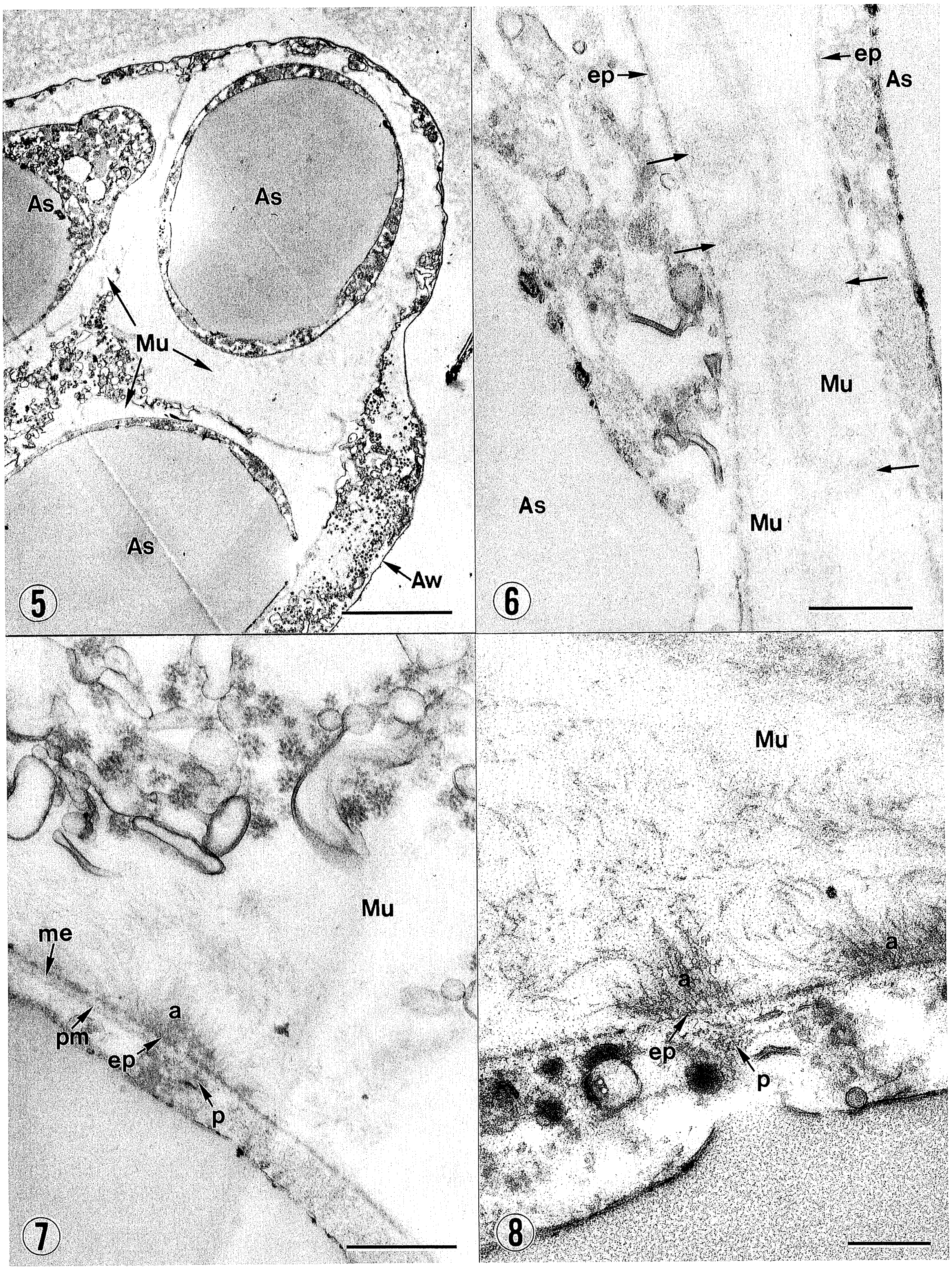


Figures 1–4. Ascospores within asci.

Figure 1. Ascus showing six of the eight ascospores (As). Note the thin ascus wall (Aw) and the ascospores surrounded by a mucilaginous sheath (Mu).

Figures 2–4. Ascus and immature ascospore wall layers. The ascus wall (Aw) is thin and in figure 4 has begun to deliquesce. The ascospores possess only a discontinuous episporium (ep) and a plasma membrane (pm in figure 4). The ascospores are surrounded by a mucilaginous sheath (Mu) which is folded (arrowed). Bar lines: figure 1, 10 μm; figures 2 and 4, 500 nm; figure 3, 1 μm.

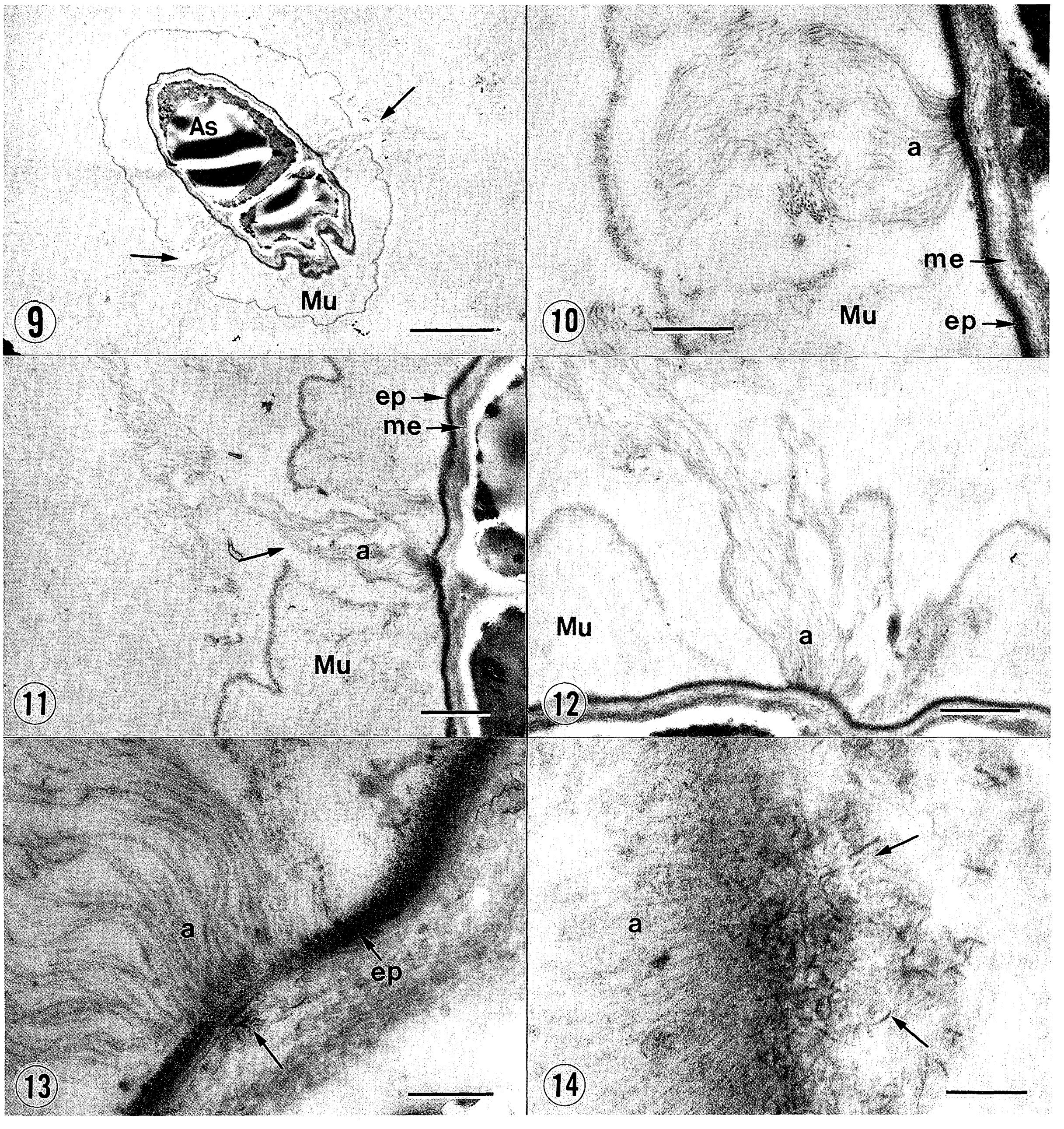


Figures 5–8. Ascospores within asci.

Figure 5. Ascospores (As) are surrounded by a mucilaginous sheath (Mu) within the ascus wall (AW).

Figure 6. Two adjacent ascospores (As) within an ascus. The episporium (ep) is discontinuous and the mucilaginous sheath (Mu) is folded (arrowed).

Figures 7 and 8. Skipped serial sections through origins of the fibrillar appendages (a). Note that the appendages are contained within the mucilaginous sheath (Mu) and the presence of appendage precursor material (p) within the ascospore. The discontinuous episporium (ep) and plasma membrane (pm) are separated (figure 7) by a thin electron-lucent region, the mesosporial initial (me). Bar lines: figure 5, 5 µm; figure 6, 1 µm; figure 7, 500 nm; figure 8, 250 nm.



Figures 9–14. Released ascospores.

Figure 9. Whole ascospore (As) surrounded by a mucilaginous sheath (Mu). Fibrillar trailing appendages (arrowed) attached around the septum penetrate through the sheath.

Figure 10. Fibrillar appendage (a) within the mucilaginous sheath (Mu). Note the electron-opaque episporium (ep) and the mesosporium (me).

Figures 11 and 12. A mature released ascospore with fibrillar appendages (a) and a mucilaginous sheath (Mu). The mesosporium is bipartite with an inner electron-lucent region and an outer more electron-dense region, the episporium (ep) is electron-opaque.

Figures 13 and 14. Origin of the fibrillar appendage (a) with appendage precursor material (arrowed) present within the ascospore wall. The fibrillar appendage is secreted through pores in the episporium (ep). Bar lines: figure 9, 5 μm; figure 10, 500 nm; figures 11 and 12, 1 μm; figure 13, 200 nm; figure 14, 100 nm.

lucent region inside the episporium (figure 7) indicated that the mesosporium had been initiated. The contents of the immature ascospores comprised a large central oil guttule which restricted the cytoplasm to the periphery of the cell (figures 1 and 5). At this stage in ontogeny the ascospores already possessed a well developed, up to 1.5 μm thick, mucilaginous sheath (figures 1–6) in which numerous invaginations were present (figures 2, 3 and 6). In some regions the mucilaginous sheath appeared to be in contact with the ascospore plasma membrane through pores in the episporium (figures 2, 4 and 6). Around the equator of

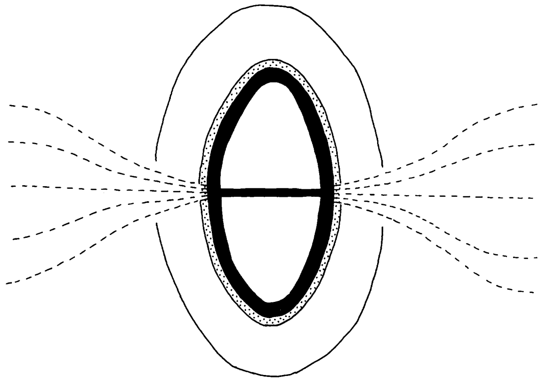


Figure 15. Diagram of the mature ascospore of *Nimbospora bipolaris* showing the relationship of ascospore wall layers and origin of the tufts of equatorial appendages. ▬ = mesosporium; ▒ = episporium; ⊐ = exosporium; --- = appendages.

the ascospore the cytoplasm and adjacent mesosporium contained slightly fibrillar appendage precursor material and the episporium contained pores through which the electron-dense fibrillar appendage material appeared to be secreted (figures 7 and 8). At this stage the appendages, although small, appeared similar to their final form, however, they were confined within the mucilaginous sheath. At the base of the appendage the individual fibrils were 10 nm thick and closely aggregated, whereas towards the periphery of the appendage, near to the outer margin of the mucilaginous sheath, the fibrils were dispersed (figures 7 and 8).

(*c*) *Mature ascospores*

Mature released ascospores were bicelled and surrounded by an extensive, up to 4 µm thick, mucilaginous sheath (figure 9). The cell wall was thicker than in earlier stages and consisted of a 375 nm thick, bipartite mesosporium with an inner electron-lucent region and an outer, 75 nm thick, more electron-dense region. Released ascospores did not section well, possibly owing to poor resin impregnation caused by the thick cell wall and mucilaginous sheath. However, the cell contents included a large central oil guttule.

Appendages (figures 10–14) were present as discrete tufts around the equator of the ascospore, attached through the episporium to the mesosporium adjacent to the septum. The long 10–15 nm thick fibrils of the mature appendages penetrated through the mucilaginous sheath and radiated out into the surrounding medium (figures 11–13). Basally the appendage fibrils were associated with pores in the episporium through which they emerged from the mesosporium (figures 13 and 14).

The mucilaginous sheath comprised two regions, an inner 2.5–4.0 µm thick electron-lucent region and an outer, 80 nm thick, electron-dense region. Where the appendages were contained within the sheath the outer electron-dense region remained intact (figure 10). However, in more mature ascospores the appendages break through the sheath and the outer region is ruptured (figure 11).

4. DISCUSSION

Examination of different stages in ascospore ontogeny of other genera within the Halosphaeriaceae indicate that the episporium forms first then the exosporium and finally the mesosporium (Johnson 1980). This also appears to be the case in *Nimbospora bipolaris* where the episporium is laid down first and is surrounded by a mucilaginous sheath at an early stage in development. The sheath may be considered to be exosporial in origin. The invaginations of the sheath observed in ascospores within asci indicates that the sheath is folded into a compact form before release.

The secondary, fibrillar appendages present around the equator of the ascospore are formed as outgrowths of the mesosporium (figure 15). Once the ascospores are released from the ascus the fibrillar appendages break through the mucilaginous sheath adjacent to the central septum. Thus the 'invaginations' in the mucilaginous sheath observed at the light microscope level result from rupture rather than invagination of the sheath.

In *Nimbospora* species two types of appendage are present, each different in structure and ontogeny. Within the Halosphaeriaceae only *Nimbospora* spp. produce a sheath-like appendage which persists at maturity. In species of *Corollospora*, *Halosphaeriopsis*, *Ocostaspora*, and *Lanspora* there is an exosporial sheath but in all these genera it fragments in various ways to form characteristic appendages (Hyde & Jones 1986; Jones & Moss 1980; Jones *et al.* 1983*a*,*b*).

Sheaths surrounding ascospores are quite common in ascospores of bitunicate marine and terrestrial Ascomycotina. These sheaths may be relatively simple as in *Leptosphaeria* spp. and *Phaeosphaeria* spp. (Shoemaker 1984; Shoemaker & Babcock 1989; Hyde & Jones 1989; Hyde *et al.* 1986; Leuchtmann & Newell 1991), *Pleospora* spp. (Hyde & Jones 1989; Kohlmeyer & Volkmann-Kohlmeyer 1991*a*), *Trematosphaeria striatispora* Hyde (Hyde 1988; Hyde & Jones 1989), and *Massarina* spp. (Bose 1961; Hyde 1989; Kohlmeyer & Volkmann-Kohlmeyer 1991*b*). However, they are more elaborate in *Pleospora gaudefroyi* Patouillard where the sheath forms four lobes (Kohlmeyer 1962; Hyde *et al.* 1986) and in *Paraliomyces lentiferus* Kohlm. where there is a disc-like appendage (Kohlmeyer 1959) which is external to a mucilaginous sheath (Read *et al.* 1992), whereas in some species of *Massarina* there are both polar appendages and a sheath (K. D. Hyde & E. B. G. Jones, unpublished observations). Little is known of the structure and ontogeny of these sheaths. The mucilaginous sheath of *Pleospora gaudefroyi* is elaborate forming cornute appendages when mounted in sea water and viewed with the light microscope (Kohlmeyer 1962). However, ultrastructurally, there was little elaboration of the sheath with only electron-opaque bodies similar to those found in the melanized wall of the ascospore (Yusoff 1991).

In *Paraliomyces lentiferus* the sheath appeared to be exosporial while the disc-like lateral appendage arose from either the episporium or, more likely, the mesosporium. The appendage can be traced back to aggregates of material within the sheath which are connected to the perforated episporium by condensed fibrillar material (Read *et al.* 1992). Thus the origin and sub-structure of the sheath and appendage in *N. bipolaris* is quite distinct from those observed in *Pleospora gaudefroyi* but with some similarities to those of *Paraliomyces lentiferus*.

Initial observations might indicate that the fibrillar appendages in *N. bipolaris* are similar to those of species of *Nereiospora, Nautosphaeria* and *Torpedospora*, all of which arise as outgrowths of the mesosporium. However, in *Nereiospora comata* (Kohlm.) Jones, Johnson et Moss the appendages are rod-like with an outer electron-dense region and an electron-lucent core (Jones *et al.* 1983*a*), *N. comata* also lacks the exosporial sheath present in *Nimbospora* spp. In *Nautosphaeria cristaminuta* Jones the appendages are amorphous while in *Torpedospora radiata* Meyers the appendages are fibrillar, but form discrete appendages at one pole of the spore (Johnson 1980). Furthermore, the appendages of *T. radiata* do not disperse easily into individual fibrils upon immersion in sea water as do those of *N. bipolaris*.

Nimbospora, on the basis of light microscope observations and ascospore appendage ontogeny, is a well delineated genus, differing from all other genera of the Halosphaeriaceae. Although the genera assigned to the Halosphaeriaceae have a variety of appendage morphologies, none has a mucilaginous sheath as found in *Nimbospora* species.

It is interesting to speculate why some ascospores of the marine Loculoascomycetes have mucilaginous sheaths and lack the diverse morphologies of the Halosphaeriaceae. An explanation may be that members of the Halosphaeriaceae are primarily found on substrata that are submerged in the sea, where the asci deliquesce early and the ascospores are released passively. The marine Loculoascomycetes are primarily intertidal, where the ascospores are actively discharged. In these forms the presence of elaborately shaped appendages may impede ascospore discharge. The presence of a sheath which is composed of highly compacted mucilage offers no resistance to discharge. Following ascospore discharge the sheath absorbs water from the substratum or from the surrounding sea once the tide returns. The sheath swells and becomes adhesive. Similarly, the appendages of *Nimbospora* species are initially compact, but become hydrated in water, swell, then become sticky and adhere ascospores to the surfaces they contact.

The type of appendage formation described here for *Nimbospora bipolaris* does not fall readily into any of the groups thus far outlined by Jones & Moss (1987). It most closely resembles group 5, namely, appendages formed by a combination of wall outgrowth and elaboration of the outer wall layer of the ascospore. In species of *Ceriosporopsis* and *Marinospora* the appendages arise from the mesosporium while the exosporium may form polar tube-like structures or partially rupture or fragment. In *Nimbospora bipolaris* the exosporial sheath remains intact and is not ornamented. There is no obvious evolutionary link between *Nimbospora* and any other genus assigned to the Halosphaeriaceae.

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REFERENCES

Bose, S.K. 1961 Studies on *Massarina* Sacc. and related genera. *Phytopath. Z.* **41**, 151–213.

Hyde, K.D. 1988 Studies on the tropical marine fungi of Brunei. *Bot. J. Linn. Soc.* **98**, 135–151.

Hyde, K.D. 1989 Intertidal fungi from the mangrove fern, *Acrostichum speciosum*, including *Massarina acrostichi* sp. nov. *Mycol. Res.* **93**, 435–438.

Hyde, K.D. & Jones, E.B.G. 1985 Marine fungi from Seychelles I. *Nimbospora effusa* and *Nimbospora bipolaris* sp. nov. from driftwood. *Can. J. Bot.* **63**, 2989–2992.

Hyde, K.D. & Jones, E.B.G. 1986 Marine fungi from Seychelles II. *Lanspora coronata* gen. et sp. nov. from driftwood. *Can. J. Bot.* **64**, 1581–1585.

Hyde, K.D. & Jones, E.B.G. 1989 Observations on ascospore morphology in marine fungi and their attachment to surfaces. *Bot. Mar.* **32**, 205–218.

Hyde, K.D., Jones, E.B.G. & Moss, S.T. 1986 How do fungal spores attach to surfaces? In *Biodeterioration*, vol. 6. (ed. S. Barry, D. R. Houghton, G. C. Llewellyn and C. E. O'Rear), pp. 284–289. Commonwealth Agricultural Bureau, International Mycological Institute, and The Biodeterioration Society, London.

Johnson, R.G. 1980 Ultrastructure of ascospore appendages of marine ascomycetes. *Bot. Mar.* **23**, 501–527.

Jones, E.B.G., Johnson, R.G. & Moss, S.T. 1983*a* Taxonomic studies of the Halosphaeriaceae: *Corollospora* Werdermann. *Bot. J. Linn. Soc.* **87**, 193–212.

Jones, E.B.G., Johnson, R.G. & Moss, S.T. 1983*b* *Ocostaspora apilongissima* gen. et sp. nov. A new marine pyrenomycete from wood. *Bot. Mar.* **26**, 353–360.

Jones, E.B.G. & Moss, S.T. 1980 Further observations on the taxonomy of the Halosphaeriaceae. *Bot. Mar.* **23**, 483–500.

Jones, E.B.G. & Moss, S.T. 1987 Key and notes of the Halosphaeriaceae examined at the ultrastructural level. *Systema Ascomycetum* **6**, 179–200.

Koch, J. 1982 Some lignicolous marine fungi from Sri Lanka. *Nord. J. Bot.* **2**, 163–169.

Kohlmeyer, J. 1959 Neufunde holzbesiedelnder Meerespilze. *Nova Hedwigia* **1**, 77–99.

Kohlmeyer, J. 1962 Über *Pleospora gaudefroyi* Patouillard. *Willdenowia* **3**, 315–324.

Kohlmeyer, J. 1985 *Nimbospora octonae*, a new marine ascomycete from Hawaii. *Can. J. Bot.* **63**, 1122–1125.

Kohlmeyer, J. & Volkmann-Kohlmeyer, B. 1987 Marine fungi from Aldabra, the Galapagos, and other tropical islands. *Can. J. Bot.* **65**, 571–582.

Kohlmeyer, J. & Volkmann-Kohlmeyer, B. 1991*a* Illustrated key to the filamentous higher marine fungi. *Bot. Mar.* **34**, 1–61.

Kohlmeyer, J. & Volkmann-Kohlmeyer, B. 1991*b* Marine fungi of Queensland, Australia. *Aust. J. Mar. Freshwater Res.* **42**, 91–99.

Leuchtmann, A. & Newell, S.Y. 1991 *Phaeosphaeria spartinicola*, a new species on *Spartina*. *Mycotaxon* **41**, 1–7.

Luft, J.H. 1971 Ruthenium red and ruthenium violet. I. Chemistry, purification, methods for use for electron microcopy, and mechanism of action. *Anat. Rec.* **171**, 347–368.

Mollenhauer, H.H. 1964 Plastic embedding mixtures for use in electron microscopy. *Stain Technol.* **39**, 111–114.

Read, S.J., Hsieh, S.-Y., Jones, E.B.G., Moss, S.T. & Chang, H.S. 1992 *Paraliomyces lentiferus*: an ultrastructural study of a little known marine ascomycete. *Can. J. Bot.* **70**, 2223–2232.

Shoemaker, R.A. 1984 Canadian and some extralimital *Leptosphaeria* species. *Can. J. Bot.* **62**, 2688–2729.

Shoemaker, R.A. & Babcock, C.E. 1989 *Phaeosphaeria*. *Can. J. Bot.* **67**, 1500–1599.

Yusoff, M. 1991 Ultrastructural studies of ascospore appendage ontogeny in selected genera of the Halosphaeriaceae and the Pleosporaceae (Ascomycotina). Ph.D. Thesis, CNAA, Portsmouth Polytechnic.

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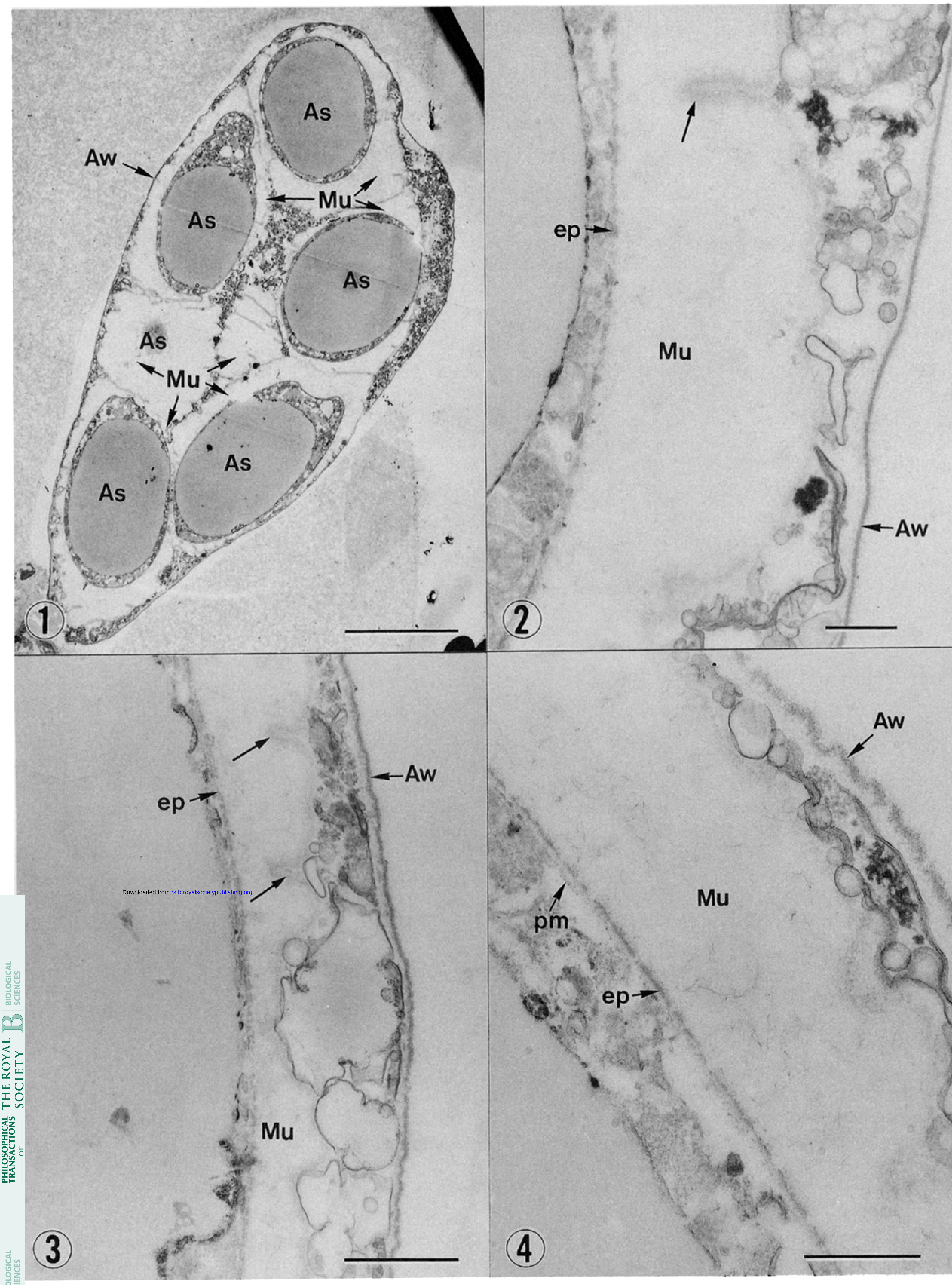

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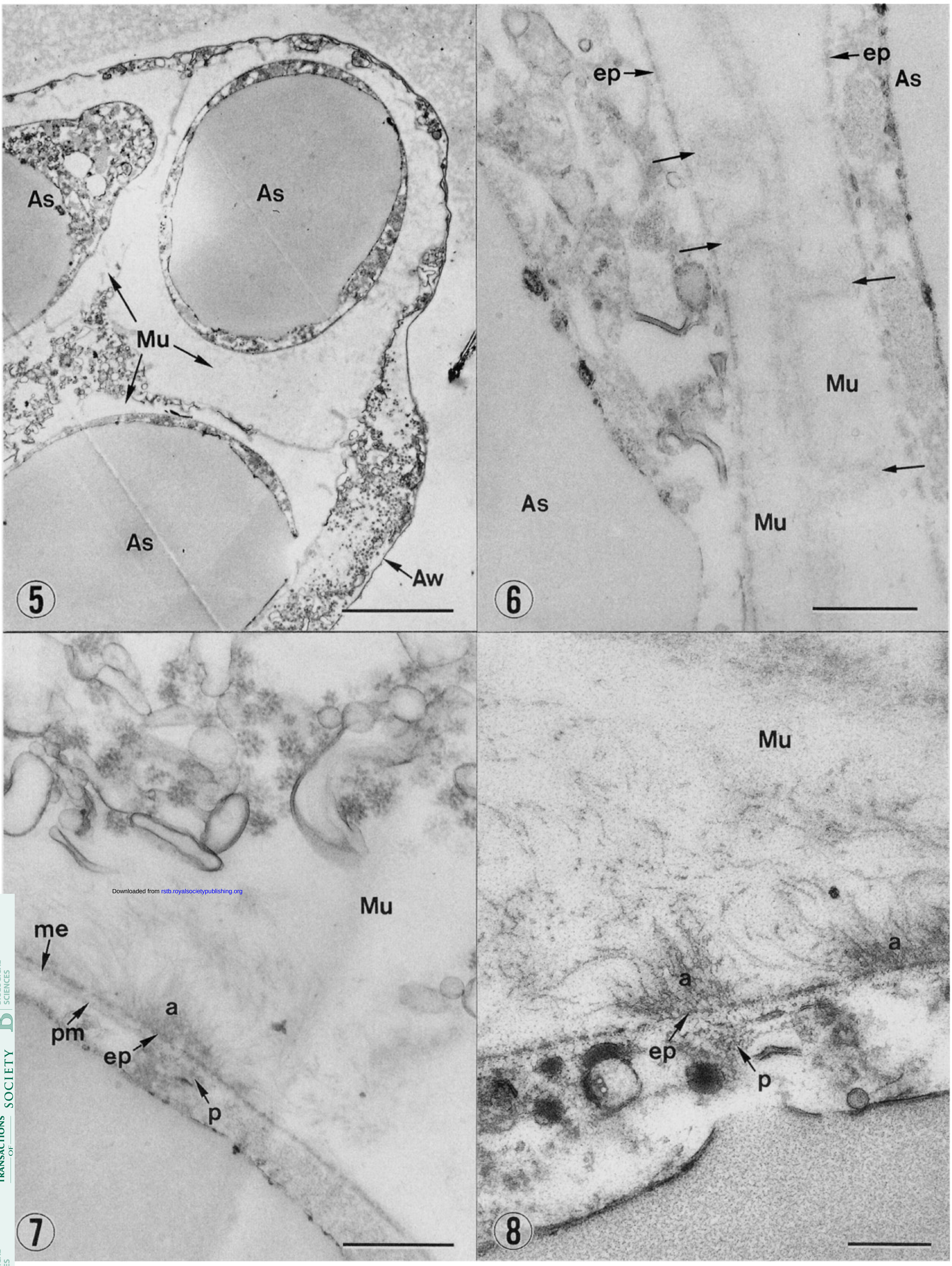

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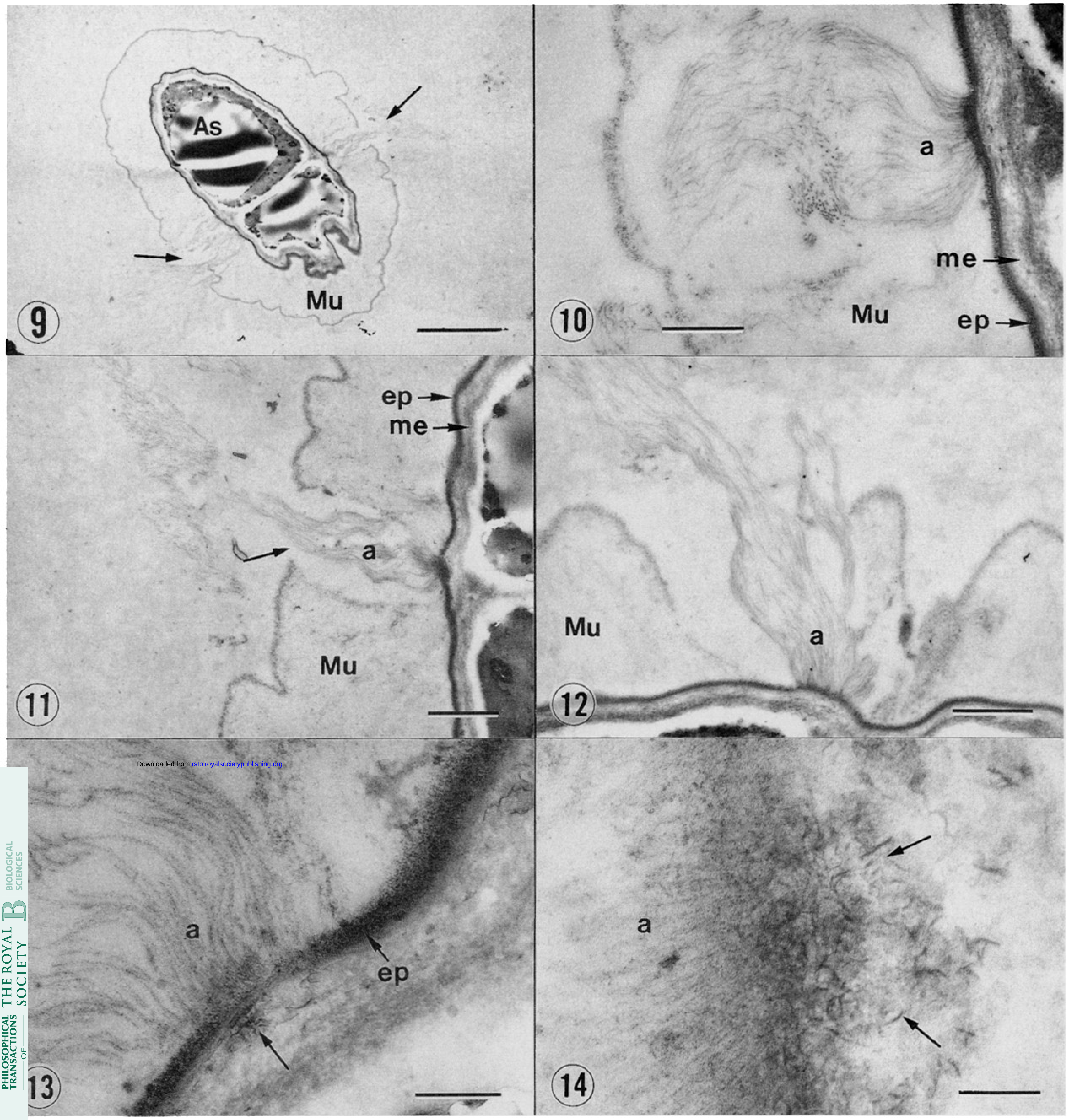

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