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LIGHT AND ELECTRON MICROSCOPIC STUDIES OF THE ASCUS TOP IN ASCOZONUS WOOLHOPENSIS

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(With Plates 15–18 and two Text-figures)

The structure of the top of the ascus in live *Ascozonus woolhopensis* has been studied by phase-contrast and interference-contrast microscopy, and by ordinary light microscopy after glutaraldehyde-OsO_4-fixation. New information was obtained from stained 0.5 μm-sections of asci embedded in epoxy resin. Electron micrographs have been made of median sections of asci that were first fixed in 1.5% sodium permanganate and postfixed with osmium tetroxide.

Light and electron microscopy have given concordant information on the organization of the top of the ascus in *Ascozonus*. In the ascoplasma no structures of an apical apparatus have been found. After meiosis the wall of the ascus consists of a broad, electron-transparent inner layer and a thin, electron-dense outer layer. The structure of the ring and the conical top of the ascus wall becomes more complicated. At the time of ascospore discharge the thick inner layer locally disintegrates in the apex just under a more resistant apical disk in the outer layer.

In the taxonomy of the Ascomycetes the organization of the ascus, especially the structure of the ascus wall and the ascus top, have proven to be of great importance (Boudier, 1879; Chadefaud, 1942, 1973; Luttrell, 1951). Even within the Pezizales the characters of the ascus play a major role in the distinction of families and genera.

The asci of Pezizales were considered to be of the 'unitunicate' type (Luttrell, 1951). Investigations in the last decennium have demonstrated, however, that in several species of Pezizales the walls of the mature asci consist of two layers (Gäumann, 1964; Delay, 1966, Kimbrough, 1966; van Brummelen, 1967; Schrantz, 1970). In general these two layers closely adhere and can not be separated. Only where the ascus is fractured or damaged sometimes a thin, more brittle, firm outer layer and a thicker, soft, inner layer are recognizable in the ascus wall. A relatively high grade of independence of both layers can still be found in some species of *Thelebolus* Tode.

The ascus dehiscence in most Pezizales occurs by means of the rupture of an apical operculum. The presence or absence of an operculum in the top of the ascus is used since Boudier (1879) as the main character to subdivide the unitunicate Discomycetes into two groups: the operculate Discomycetes (or Pezizales) and the inoperculate Discomycetes (or Helotiales).

In a few genera of Ascomycetes, usually incorporated in the Pezizales, an operculum is lacking and different structures exist in the top of the ascus. Such a deviating type of ascus dehiscence is found in the genus *Ascozonus* (Renny) E. C. Hansen. The special position in the Pezizales and the ease to grow its species in culture made *Ascozonus* an attractive object for this study.

Ascozonus is characterized by asci opening by a transversal slit down to a conspicuous subapical ring, giving the ascus top a bilabiate appearance. The first species of *Ascozonus* was described more than a century ago by the Crouan brothers under the name *Ascobolus leveillei* Crouan (Crouan & Crouan 1867, 57, pl. suppl.). Boudier (1869) described *Peziza cunicularia* Boud., which might be conspecific with the species of the Crouans. He noted that the ring in the ascus wall is not identical with the opercular commissure of the typical operculum but constitutes a small ridge at the inner side of the wall.

Several other species of this genus were described by Renny (1872, 1873, 1874) in *Ascobolus* section *Ascozonus* Renny. He also gave a description 'of the formation of the zonal stripe upon the ascus'. The accompanying illustrations of asci, however, strongly suggest images observable during plasmolysis of the ascoplasm.

More recently Kimbrough (1966) in a study of selected genera of the 'Pseudoascoboleae' paid special attention to the light microscopic structure of asci. In *Ascozonus* he described a three-layered ascus wall. The outermost layer appears as a thin hyaline membrane. The second or middle layer stains differentially in Congo red and shows an abrupt thickening near the apex of the ascus, which forms the prominent ring. This middle layer of the wall continues beyond the ring almost to the tip, there leaving an opening 10–12 μm wide. The inner layer, finally, stains in acid fuchsin but not in Congo red, and extends to the full length of the ascus. At the tip this layer bulges through the opening of the middle layer and forms the nippled end. In later studies Kimbrough (1970, 115; 1972, 398) described the ascus wall as two-layered and the ring as part of the outer layer.

Materials and methods

In the present study a strain of *Ascozonus woolhopensis* (Berk. & Br. apud Renny) E. C. Hansen was used. This fungus was isolated from rat dung obtained from the border of Schelde river near Antwerp, Belgium. Oatmeal agar enriched with horse dung decoct was used for the production of apothecia. Since *Ascozonus*-species prove to be psychrophilic in their phase of fructification, the strain was cultured at 12 °C. Periods of 8 hours of light with an illumination intensity of about 5000 lux were alternated with periods of 16 hours of darkness. After 7 days apothecia with mature asci had developed. From the 5th day on small pieces of agar with ripening apothecia were fixed for purposes of light and electron microscopy.

Living isolated asci or bundles of gently squashed asci were observed in a drop of water or in a weakly hypotonic solution of glucose in distilled water. The slides were

examined with Zernike's phase-contrast and Zeiss Nomarski's interference-contrast optics. Within a few minutes the protoplasts of asci became disturbed and observations had to be continued with a new slide of freshly prepared asci.

For light microscopy asci were stained with a wide variety of dyes of which Congo red, acid fuchsin, trypan blue, methyl blue, and methylene blue gave satisfactory results.

Sections of material embedded in epoxy resin, cut with glass knives to a thickness of 0.5 μm, proved to be of great value for observations with the light microscope. This material was fixed in glutaraldehyde and osmium tetroxide, and subsequently embedded according to the methods described below for electron microscopy. Among the methods available for staining sections of this kind, those based upon the use of methylviolet, methylene blue, and toluidine blue proved to be useful. Especially 0.1–1.0% toluidine blue in an 1% aqueous solution of sodium tetraborate (borax) (Trump *& al.*, 1961) produced a clear differentiation of the components of the walls, displaying blue orthochromasia, violet β-metachromasia, and red or pink γ-metachromasia.

For electron microscopy, small blocks of agar with ripening apothecia on their upper surface were cut from the plates and excess agar was trimmed off.

One part of this material was fixed for 2 hours in about 5 ml of 1–1.5% $KMnO_4$ in distilled water, to which 1 drop of Invadine (Geigy) was added in order to reduce the surface tension of the fixing liquid. Another part of the material was fixed for 4 hours in 3–6.5% glutaraldehyde buffered at pH 7.2 with 0.2 M cacodylate at 4 °C. The latter material was post-fixed for 1 hour in 1% buffered OsO_4 at 4 °C. Fixed material was either dehydrated in an aceton series and embedded in Vestopal, or dehydrated in an ethanol series and embedded in Epon 812 (Luft, 1961).

During fixation and impregnation, the material was evacuated several times to draw all the air from the tissues. During dehydration the material was stained for 5 minutes in a solution of 1% uranyl acetate. Longitudinal median sections of asci in different stages were cut with glass knives on an LKB Ultrotome III, occasionally stained on the grids with various combinations of Reynolds' lead citrate, uranyl acetate, and barium permanganate.

As asci are relatively large objects, single-hole grids were used to collect the sections for electron microscopy.

A Philips EM 300 electron microscope was used for the electron micrographs.

Measurements taken from electron micrographs are indicated in nanometers.

Results

Observations with the light microscope

Very young asci are broadly clavate with a broad base and a rounded top. The wall is of uniform thickness. After meiosis the shape of the asci becomes more slender-

clavate with a flattened top. In *A. woolhopensis* usually 64 navicular ascospores are formed. During the ripening of the ascospores the top of the ascus becomes conical in shape, while its volume increases considerably. A short distance under the tip a light-refractive thickening in the shape of a ring is formed on the inner side of the ascus wall. At this time two layers can be distinguished in the lateral and the apical regions of the ascus wall: a thin rather rigid outer layer which stains red with Congo red and bluish violet with toluidine blue, and a thicker rather soft inner layer not tinted in Congo red and staining reddish violet with toluidine blue. The inner layer also shows affinity to methyl blue, trypan blue, and acid fuchsin.

The differentiation of the subapical ring is initiated shortly after meiosis in the ascus. The first indication of the ring is visible as a slight thickening of the wall of the truncate young asci, and is well observed in longitudinal, 0.5 μm thick sections. The

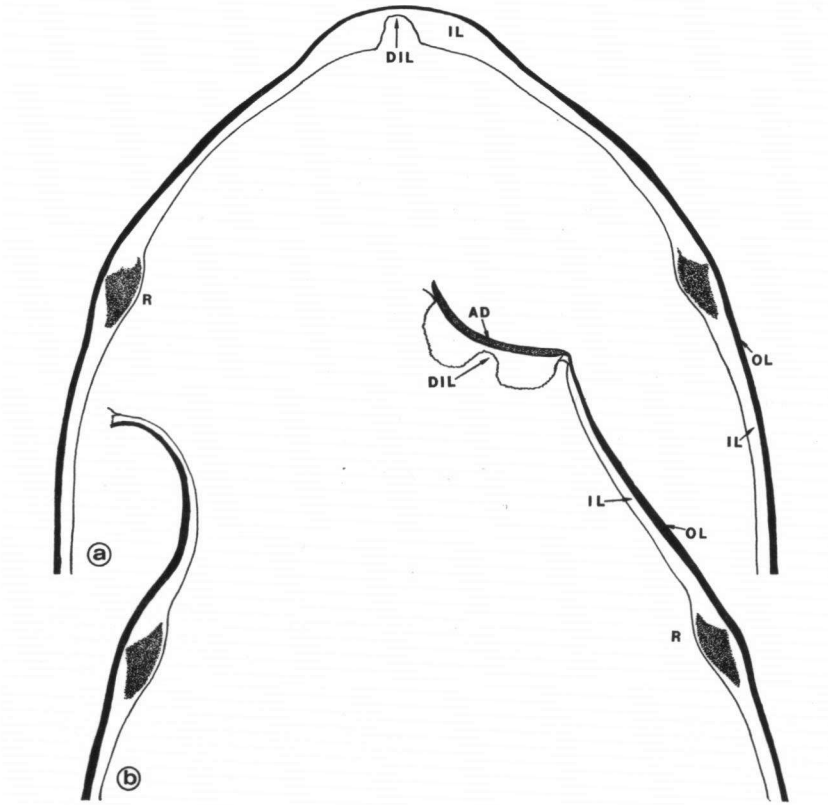

Fig. 1. *Ascozonus woolhopensis*, diagrammatic sections of ascus tops, as seen with light microscopy. — a. Almost mature ascus. — b. Ascus after spore discharge. — For abbreviations used see p. 31.

body of the young ring is about semicircular in section and stains differentially blue with toluidine blue (Pl. 15C, D).

During the formation of the ring changes take place in the top of the ascus, whereas the lateral ascus wall under the ring remains almost unchanged. In a mature ascus the ring has a thickness of 1.3–1.5 μm and reaches a diameter of 13–17 μm. The zone which stains blue with toluidine blue becomes more or less triangular in section and is free from the outer layer of the ascus wall.

In the conical top, the outer wall becomes increasingly thinner towards the tip, except for a small apical disk up to 0.3 μm thick and about 2–3 μm across. This disk is rather rigid and stains intensely in most of the stains used (Pl. 15A, B). The inner layer on the other hand thickens towards the tip, reaching a thickness of up to 1.8 μm. Shortly before ascospore discharge the central part of the inner layer breaks down locally just underneath the apical disk, thus seriously weakening the wall in the tip (Pl. 15E, F). Soon the outer layer ruptures just at the margin of the apical disk. From this slightly excentric spot extending down to the ring there appears a fissure splitting the ascus top into two halves. The mass of 64 ascospores are forcibly discharged through this tear in a single jet.

In a mature apothecium often collapsed empty asci can be found which clearly show the apical disk, sometimes even with the remnants of the inner wall layer adhering to it (Pl. 15A, G–K).

Investigations with phase-contrast and interference-contrast optics made it clear that the asci of *Ascozonus* are devoid of a 'tractus', an 'entonnoir', and a 'chambre oculaire' as described by Chadefaud (1942, 1960, 1973) for other genera.

No part of the wall stains with iodine-containing reagents.

Electron microscopy

Of the different methods of fixation and embedding used in this study, the $KMnO_4$-OsO_4-fixation followed by Epon-embedding proved to be most suited to produce images with sufficient contrast in the walls of asci. If not stated otherwise, the observations are based on such material.

The walls of the croziers and the young asci up to the moment meiosis begins are of rather uniform thickness (approximately 210 to 250 nm) and do not show a layered structure (Pl. 16A, C). After meiosis the lateral as well as the apical regions of the ascus wall become stratified by differentiation inside the wall. Then the ascus wall is composed of an inner, thick, electron-transparent layer and an outer, thin, electron-dense layer. In the lateral region of the ascus wall the inner layer reaches a thickness of approximately 440 to 540 nm and the outer layer 140 to 240 nm (Pl. 16B, D, Pl. 17B–D).

During differentiation of the ring, no structures in the ascoplasm can be observed that indicate an increased activity at this place. Adjacent to the inner side of the ascus wall the plasma membrane or the ascoplasmalemma is visible. Especially in the top of the ascus the shape of this membrane is often denticulate or irregular

(Pls. 17B, D, 18A, B). In the last phase of ripening of the ascospores the epiplasm disappears almost completely from the sporogenous part of the ascus. In well-fixed material the plasma membrane remains visible till ascospore discharge. Even in asci that have just released their spores, remnants of the plasma membrane are often found (Pls. 17A, 18C, D).

From its beginning the ring is manifest as an rather electron-transparent thickening on the inside of the ascus wall. During the maturation of the ascus, changes take place in its top. The ring reaches a thickness of 1000 to 1300 nm. On its innermost side a more electron-dense layer 50 to 70 nm thick can be distinguished. This layer cannot be followed in the ascus wall above and below the ring (Pl. 18A). In the ring an electron-transparent central part is found which is not very sharply delimited. The electron-dense outer layer of the ascus wall is seen to remain at some distance of the ring proper and can be followed right to the top as an undeflected zone. In the ring this layer measures about 130 nm, in the top it decreases to less than 80 nm. The electron-transparent inner wall layer measures 270 to 390 nm just above the ring and thickens towards the tip where it may reach a thickness of more than 1800 nm (Pls. 17B–D, 18A, B). In some preparations, with favourable staining, a weak stratification with 3 or 4 strata becomes visible in the thickest part. At maturity the central part of this layer breaks down locally, thus weakening the tip (Pl. 17B).

From the ring upwards in the outer half of the wall an electron-dense zone is seen underneath the outer layer. This middle layer is only found in the top of the ascus,

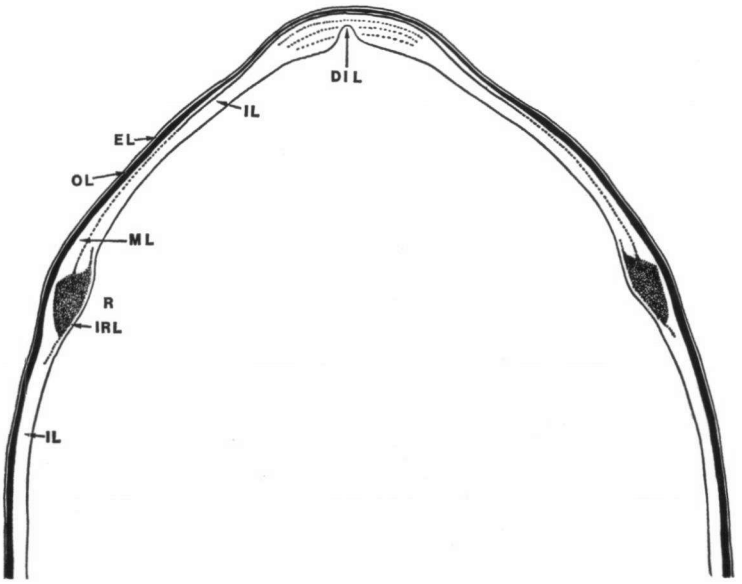

Fig. 2. *Ascozonus woolhopensis*, diagrammatic section of an almost mature ascus top, as seen with electron microscopy. — For abbreviations used see p. 31.

tapering from a thickness of 200 to 240 nm just above the ring to about 60 nm at the apex. The delimitation of the middle layer from the outer layer diminishes gradually (Pl. 17B–C).

In the central part of the extreme tip of the ascus, as part of the outer, and probably also middle, layer a disk-shaped zone, 2100 to 3030 nm across, appears. This apical disk is evident only in median sections of emptied asci as a 110 to 270 nm thick, electron-transparent structure. The fracture in the ascus top initiating the spore discharge usually arises exactly at the margin of this apical disk (Pls. 17A, 18C, D).

In sufficiently contrasted sections an electron-transparent layer with a thickness of 50 to 130 nm is frequently found completely surrounding the young and the ripening asci. This extra-ascan layer becomes evident by the staining of the surrounding hymenial mucus (Pls. 16A, D, 17B–D, 18A, B, D).

Discussion

Although no extensive cultural experiments have been carried out, our experience with cultures of *Ascozonus woolhopensis* and some other members of this genus clearly indicates that species of *Ascozonus* are psychrophilic in the phase of fructification. Fruit-bodies are produced abundantly at temperatures from 4° to 12 °C. At higher temperatures fructification is far less or absent. This may explain the fact that these fungi are only found in winter. The same phenomenon has been described by Wicklow & Malloch (1971) in the genus *Thelebolus* Tode (incl. *Rhyparobius* Boud.) and by Bergman & Shanor (1957) in *Streptotheca psychrophila* Bergm. apud Bergm. & Shanor, which is a representative of *Thelebolus* (cf. Kimbrough & Korf, 1967).

Comparison of the results of light and electron microscopy gives concordant data (Figs. 1 and 2). Using both methods, a layered lateral region of the ascus wall is observed which becomes more complex in the ring and the top. It is here that electron microscopy reveals more details. On the other hand, in young and mature asci the apical disk is only visible after staining with light microscopy.

The ring is not homogeneous and does not originate as a part of the outer layer of the ascus wall, as stated by Kimbrough (1970, 1972). It is differentiated within the ascus wall free from the outer layer, which can be followed through the region of the ring without interruption. The inner layer of the ascus wall is not continuous at the level of the ring. The structure of this layer is locally changed during the development of the ring.

Also the assumption of Vuillemin (1887) that the ring is a simple jellification of the ascus wall cannot be maintained.

The apical disk in the wall of the ascus has not been noticed by Kimbrough, but it is certainly identical with the very small operculum described by Vuillemin. At the moment of ascospore discharge, both in *Ascozonus woolhopensis* and in other species of this genus, the ascus wall is disrupted at the margin of the apical disk, immediately followed by bilabiate splitting of the wall in the ascus top. In no case a mechanism of

spore discharge was observed like described by Vuillemin for a 32-spored species of *Ascozonus*[1] in which the apical disk functions as a very small operculum, disclosing a narrow aperture through which the ascospores are projected one by one.

In the terminology of Chadefaud (1942, 1969, 1973) the inner layer of the ascus wall is called endoascus, the outer layer exoascus, while the thin extra-ascal layer surrounding the ascus is probably identical with his ectoascus. What Chadefaud calls "film interne de l'endoascus" is probably the same as the plasma membrane of the epiplasm.

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[1] Described as *Streptotheca boudieri* Vuill.

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EXPLANATION OF PLATES 15–18

ABBREVIATIONS USED IN PLATES AND TEXT-FIGURES. — AD, apical disk; AW, ascus wall; DIL, desintegration of inner layer of the ascus wall; E, epiplasm; EL, extra-ascan layer; ER, endoplasmatic reticulum; G, glycogen; IL, inner layer of the ascus wall; IRL, inner electron-dense layer of the ring; M, mitochondrion; ML, middle layer of the ascus top; N, nucleus; OL, outer layer of the ascus wall; P, paraphysis; PM, plasma membrane; R, ring; S, ascospore; SP, sporoplasma.

PLATE 15

Figs. A–K. *Ascozonus woolhopensis*, photomicrographs of asci (Figs. A, B. from squash-mounts; Figs. C–K. semi-thin median sections of asci fixed in glutaraldehyde and OsO_4 and embedded in Epon): Fig. A. empty ascus stained with Congo red; Fig. B. top of a mature ascus filled with spores, stained with Congo red; Fig. C. distal portion of ascus shortly before sporogenesis, stained with toluidine blue; Fig. D. id. stained with methyl violet; Figs. E, F. distal portion of almost mature asci, showing desintegration of the inner layer of the ascus wall in the tip, stained with methylene blue; Figs. G, J, K. apices of collapsed asci showing the apical disk, stained with toluidine blue; Fig. H. id. stained with methylene blue.

The scale markers in Plate 15 equal aproximately 10 μm.

PLATE 16

Figs. A–D. *Ascozonus woolhopensis*, electron micrographs of developing asci: Fig. A. median section of distal portion of a diploid ascus showing an undifferentiated ascus wall, fixed in 1.5% $KMnO_4$ and stained with uranyl acetate, lead citrate, and barium permanganate; Fig. B. longitudinal section of the distal portion of an ascus at an early multi-nucleate stage with initial development of the ring, fixed in 6.5% glutaraldehyde and 1% OsO_4 and stained with uranyl acetate; Fig. C. transverse section of the lateral wall of a diploid ascus, fixed in 1.5% $KMnO_4$ and stained with uranyl acetate; Fig. D. median section of the distal portion of an ascus shortly before ascospore-delimitation, showing incipient differentiation of ascus wall, fixed in 1.5% $KMnO_4$ and stained with uranyl acetate.

The scale marker equals approximately 1 μm.

PLATE 17

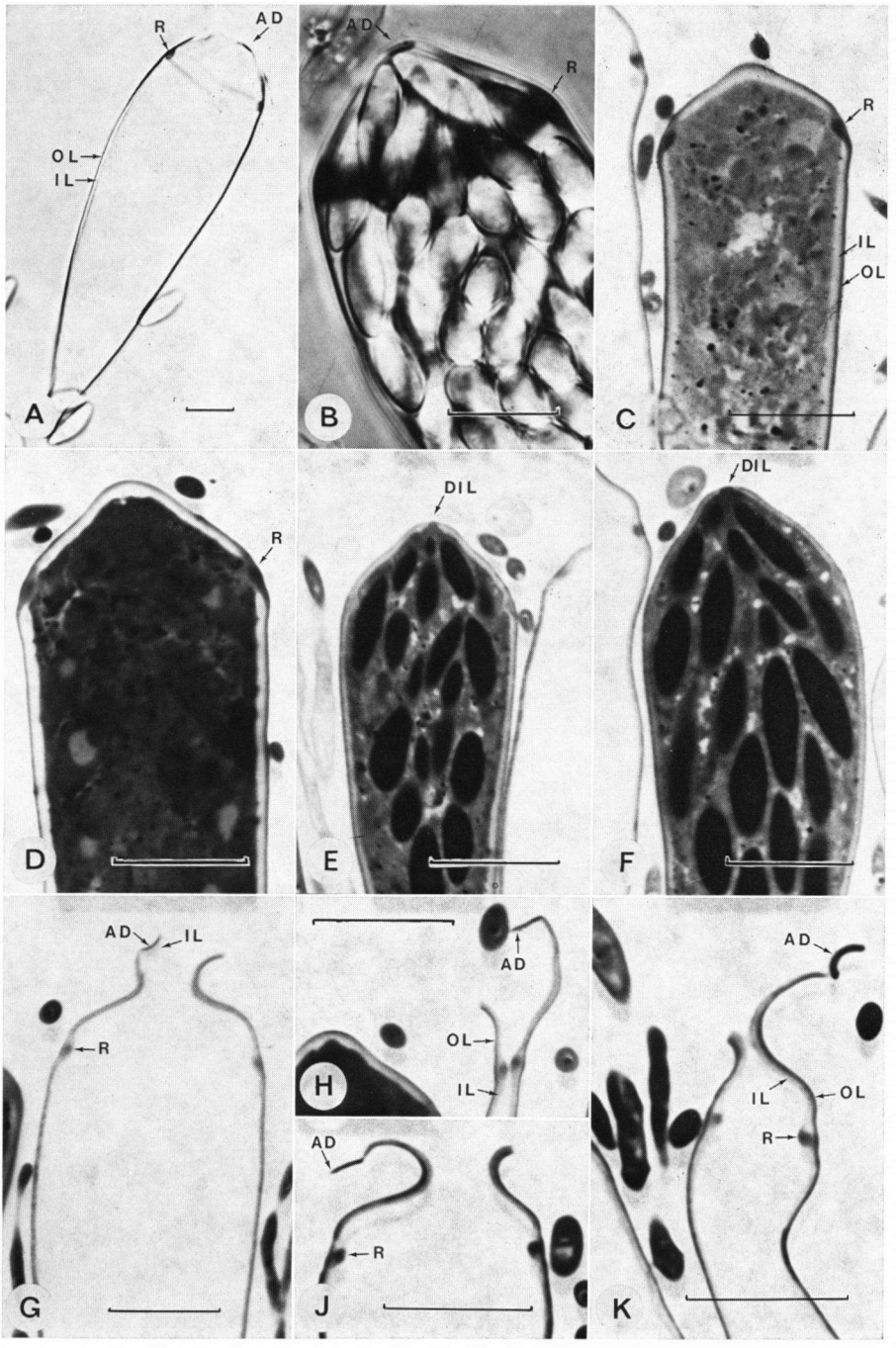
Figs. A–D. *Ascozonus woolhopensis*, electron micrographs of ripening and collapsed asci: Fig. A. median section through extreme apex of a collapsed ascus, showing 'lid' with apical disk, fixed in 1.5% $KMnO_4$ and stained with uranyl acetate, lead citrate and barium permanganate; Fig. B. median section of apical portion of an almost mature ascus, fixed in 1.5% $KMnO_4$ and stained with uranyl acetate; Fig. C. id. fixed in 1.5% $KMnO_4$, embedded in Vestopal, and stained with uranyl acetate and lead citrate; Fig. D. median section of apex of ripening ascus, fixed in 1.5% $KMnO_4$ and stained with uranyl acetate, lead citrate, and barium permanganate.

The scale marker equals approximately 1 μm.

PLATE 18

Figs. A–D. *Ascozonus woolhopensis*, electron micrographs of ripening and collapsed asci (all fixed in 1.5% $KMnO_4$, embedded in Epon, and stained with uranyl acetate, lead citrate, and barium permanganate): Fig. A. transverse section of ascus wall near the ring in a ripening ascus; Fig. B. median section of ascus wall in the extreme tip of a ripening ascus; Figs. C, D. median sections through extreme apices of collapsed asci.

The scale marker equals approximately 1 μm.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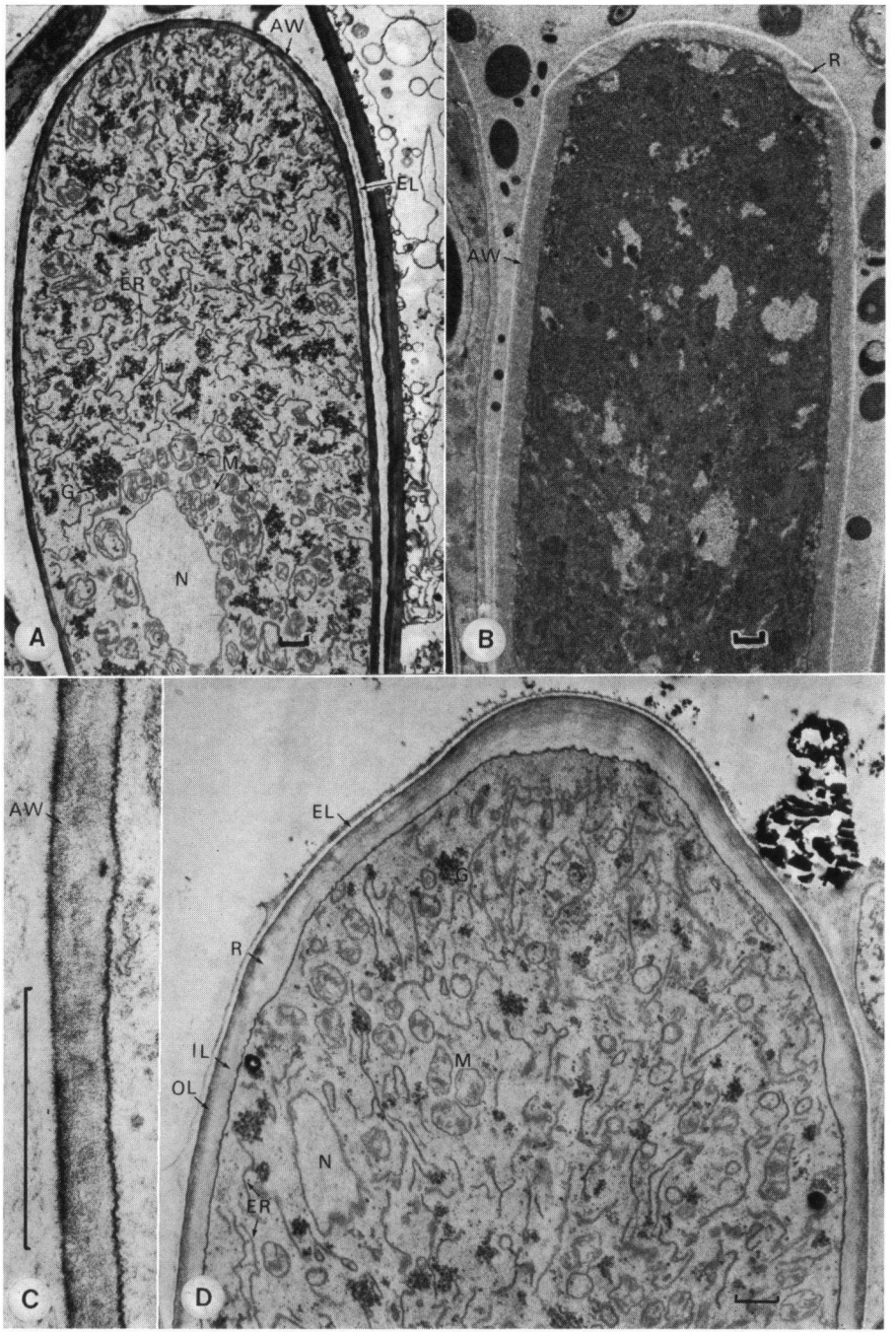
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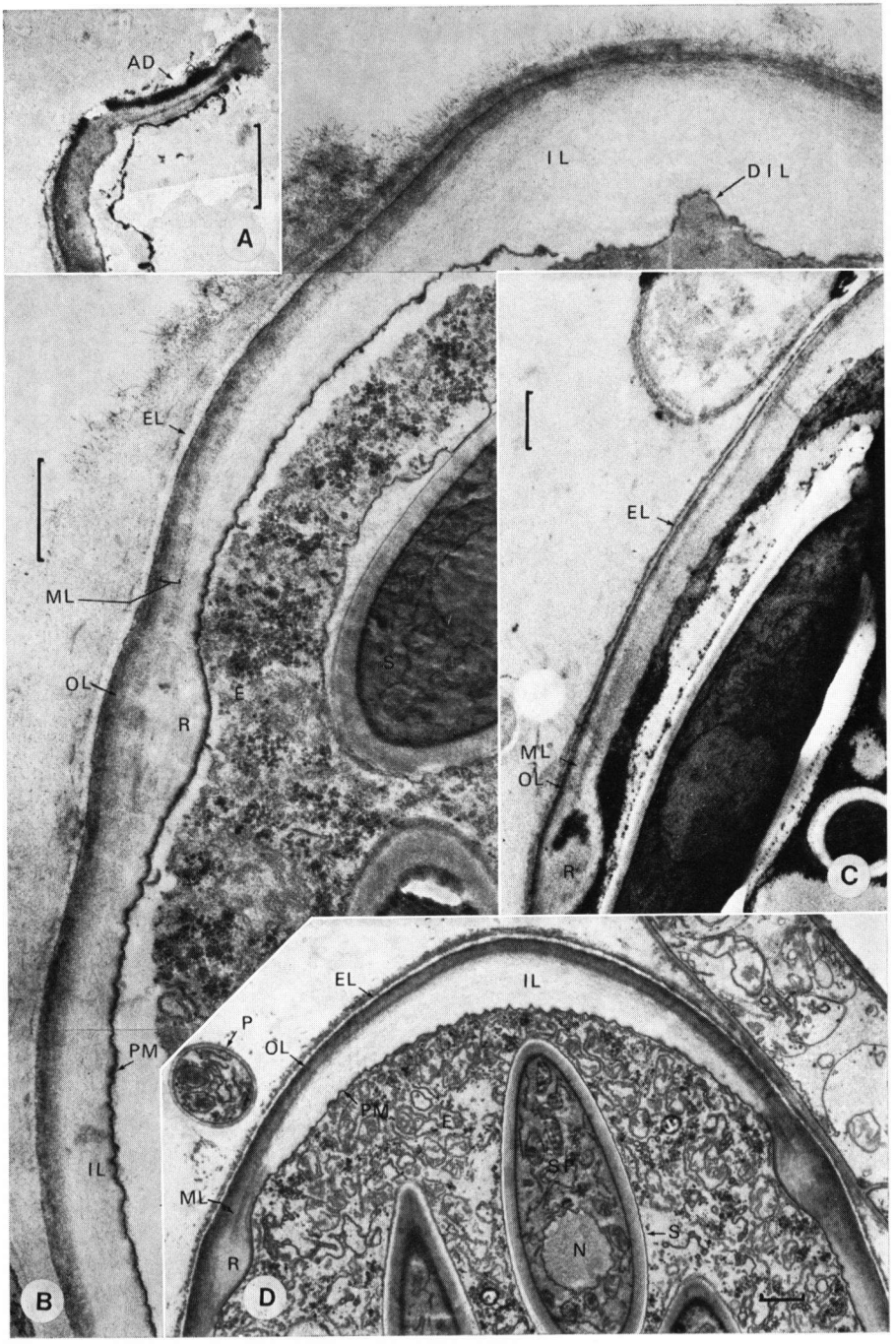
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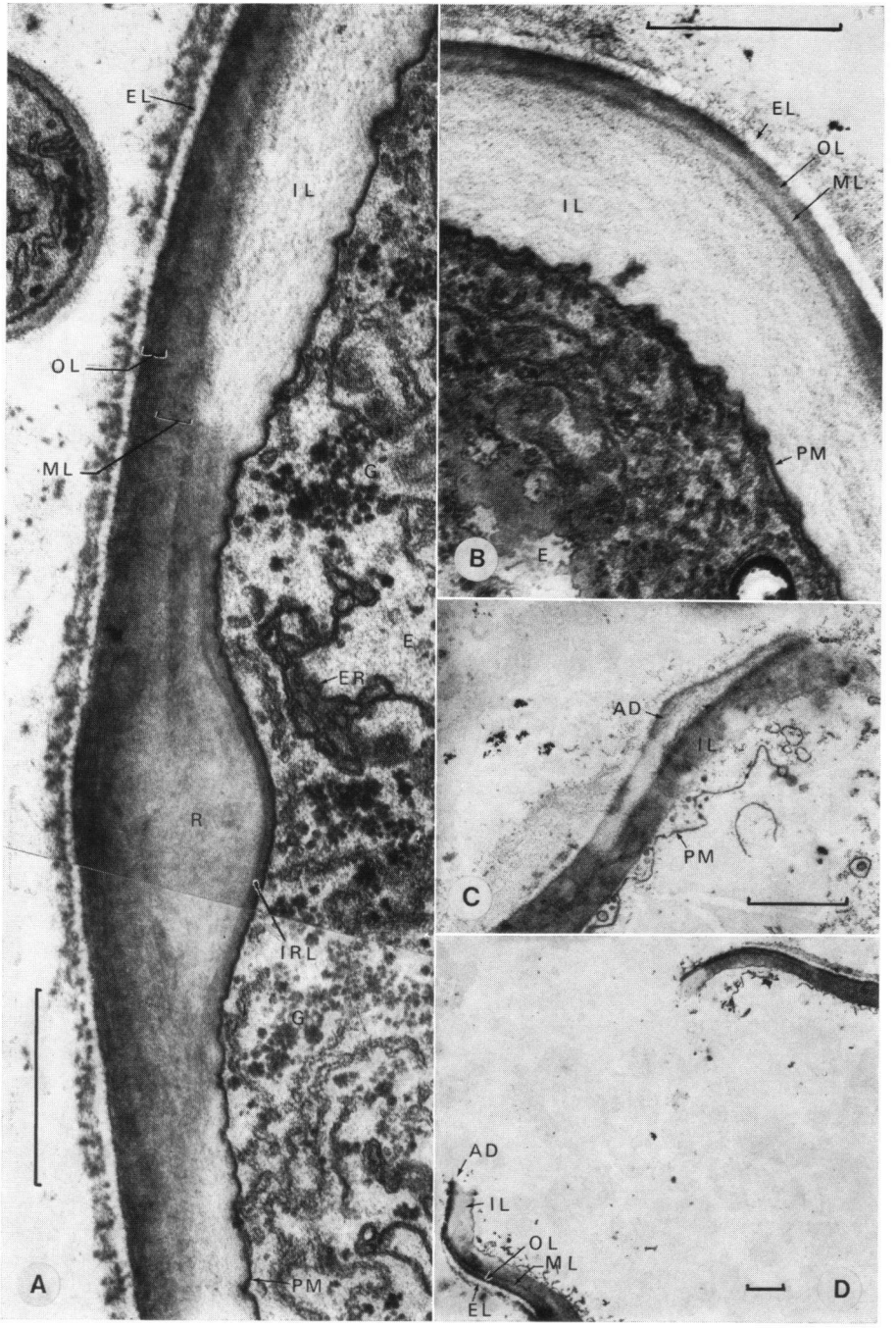
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