

ULTRASTRUCTURE OF THE ASCOSPORE WALL IN PEZIZALES (ASCOMYCETES)—I

**Ascodesmis microscopica (Crouan) Seaver and *A. nigricans*
van Tiegh.**

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(With Plates 28-38)

The development of wall layers and ornamentation of ascospores is studied with the electron microscope in *Ascodesmis microscopica* (Crouan) Seaver and *A. nigricans* van Tiegh. In both species a primary and a secondary wall are formed in succession. The primary wall differentiates into two layers, an inner endospore and an outer episporium. The secondary wall gives rise to patterns of ornamentation and is composed of membranous structures in a homogeneous matrix; the epiplasm seems to be involved in its formation. The reported differences in the development of ascospore ornamentation between these two species must be denied. Only the final patterns of ornamentation are different.

INTRODUCTION

Hitherto it has proved difficult to classify the Pezizales. In the majority of the 'classic' works the families have been delimited by the gross morphology of apothecia and asci, including the blueing of the ascus wall in iodine. Characteristics for the recognition of genera and species have been the morphology of the spores and the number of spores per ascus. In more recent works new criteria have been added, e.g. the ontogeny of the apothecia especially of the exipulum, the opening mechanism in the ascus top, the morphology of the sterile parts of the fruit bodies, and several cytochemical and cytological characteristics. This has led to an increase in the number of families and re-classification of the genera within these families.

It must be emphasized that in all the studies dealing with the classification of Pezizales the morphology of the ascospores has proved to be very important. All the typical features of the spores such as shape, contents and ornamentation have been taken into consideration. The spores are one-celled, symmetrically bipolar, rather large, mostly without pigmentation, but in a number of genera with ornamentation. Of further taxonomic value is the occurrence of oil droplets (Boudier, 1885, 1907; Nannfeldt, 1937, 1938; Le Gal, 1953; Dennis, 1960, 1968) and 'deBary bubbles' (Ingold, 1956; Dodge, 1957; Kimbrough & Korf, 1967). On the basis of data given by Kimbrough & Korf, Eckblad (1968) concludes that the presence or absence of 'deBary bubbles' may be related to the thickness of the spore wall.

For many years Le Gal has been studying the pigmentation and ornamentation of ascospores of the Pezizales with the light microscope. In her earlier work she described the ornamentation patterns. She incorporated the results of her studies into her taxonomy. In her later work she investigated the development of the ornamentation patterns and tried to determine their chemical composition. This is described in her 'Recherches sur les ornements sporales des Discomycètes operculés' (1947). In the first part of this work she gives a detailed description of the ornamentation patterns of the ascospores in a large number of genera. This is followed by a close examination of the development of these patterns. On the basis of her investigations she suggests some modifications in Boudier's classification. In a separate study (Le Gal, 1949) she gives the results of her investigations on *Ascodesmis microscopica* (Crouan) Seaver and *A. nigricans* van Tiegh.

The work of Le Gal is regarded as a valuable contribution to the study of the Pezizales (Kimbrough, 1970). According to her there may be considerable variety in the mode of development of the ornamentation patterns, which can be very complicated.

However, the results so far obtained from electron microscopy indicate fewer complications than Le Gal thinks likely. It is well known that use of the light microscope demands considerable prudence and it is not impossible that Le Gal was misled by misinterpretations. Since a good description of the development of the ornamentation patterns of ascospores is important in the classification of the Pezizales and because of discrepancies in the observations published to date, new information obtained through electron microscopy is presented and the results compared with those of Le Gal and others. For this purpose their work is here briefly summarized.

LE GAL'S LIGHT MICROSCOPY

A fairly thick primary spore wall surrounding the developing ascospores is found in all species. On the outer surface of this wall ornamentation patterns are formed. With the aid of microscopical stains, like cotton blue and naphthalene blue, their chemical composition is determined. On the basis of this chemical composition and the way the ornamentation patterns develop she gives the following division.

- I. The ornamentation is simple, consists of callose and pectine formations, and is of sporal origin.

In *Trichophaea paludosa* (Boud.) Boud. the ornamentation arises directly on the primary wall and contains callose and pectine.

In all the other species of this group an 'assise sous-périssporique' arises on the primary wall. This is surrounded by a 'pellicule membranaire', called 'tunique externe' if it arises before ornamentation and 'coque interpérissporique' if it arises simultaneously. The ornamentation appears on the primary wall, on the inner side of the 'assise sous-périssporique'. A 'périsspore' may be present on the outside.

1. A 'périspore' is lacking. The ornamentation contains callose and pectine. It breaks through its covering layers in *Lamprospora cec'hqueraultii* (Crouan) Boud., *L. cec'hqueraultii* (Crouan) Boud. var. *macracantha* Boud. and *Ascodesmis microscopica* (Crouan) Seaver [non sensu Seaver]. This does not occur in *Lamprospora ascoboloides* Seaver, *L. areolata* Seaver, *L. miniata* (Crouan) Boud.,¹ *L. dictydiola* Boud., *L. polytrichi* (Schum. per Fr.) Le Gal, *Boudiera areolata* Cooke & Phill. apud Cooke, *B. echinulata* Seaver and *Plicaria persoonii* (Crouan) Boud.²

Around the mature spores of *Ascodesmis microscopica* the 'pellicule membranaire' remains. Obrist (1961) calls this layer a perisporium.

2. A 'périspore' is formed.
 - a. The ornamentation does not contain callose and pectine. It penetrates its covering layers in *Cookeina sulcipes* (Berk.) O. K., *C. tricholoma* (Mont.) O. K. and *C. insititia* (Berk. & Curt.) O. K.³ It remains within these layers in *Phillipsia dochmia* (Berk. & Curt. apud Berk.) Seaver,⁴ *P. dominicensis* Berk., *Wynnea americana* Thaxt., *Sarcosoma sarasini* (P. Henn.) Boed.,⁵ and *Urnula platensis* Speg.⁶
 - b. The ornamentation contains callose and pectine. The 'périspore' disappears in *Ciliaria asperior* (Nyl.) Boud.,⁷ *Galactinia succosa* (Berk.) Sacc.,⁸ *G. badia* (Pers. per Mérat) Boud.,⁹ *Aleuria umbrina* (Boud. apud Cooke) Gill,¹⁰ and *Plicaria trachycarpa* (Curr.) Boud.¹¹ The 'périspore' remains in *Rhizina inflata* Schaeff. per P. Karst.,¹² *Gyromitra gigas* (Krombh.) Quél.,¹³ *Discina perlata* (Fr. per Fr.) Fr., *Aleuria apiculata* (Cooke) Boud.,¹⁴ and *A. reperta* (Boud.) Boud.¹⁵

II. The ornamentation is complex, contains callose and pectine and sometimes cytoplasmic elements; it is of sporal origin.

1. The 'périspore' encloses epiplasmic vacuoles, which are then called 'masses globuleuses'. The ornamentation appears on the 'assise sous-périsporique'

¹ = *Lamprospora crouani* (Cooke) Seaver, probably identical with *L. miniata* De Not.

² = This belongs to *Pulpuria* P. Karst.

³ = *Boedijnopeziza insititia* (Berk. & Curt) Ito & Imai.

⁴ = *Aurophora dochmia* (Berk. & Curt. apud Berk.) Rifai.

⁵ = *Plectania campylospora* (Berk.) Nannf. apud Korf.

⁶ = *Plectania platensis* (Speg.) Rifai.

⁷ = *Scutellinia asperior* (Nyl.) Dennis.

⁸ = *Peziza succosa* Berk.

⁹ = *Peziza badia* Pers. per Mérat.

¹⁰ = *Peziza echinospora* P. Karst.

¹¹ = *Peziza trachycarpa* Curr.

¹² = *Rhizina undulata* Fr. per Pers.

¹³ = *Neogyromitra gigas* (Krombh.) Imai.

¹⁴ = *Peziza apiculata* Cooke.

¹⁵ = *Peziza reperta* (Boud.) Moser, not validly published.

and its covering layer. At a later stage both 'périspore' and 'masses globuleuses' disappear. A pattern of this kind is found in *Melastiza miniata* (Fckl.) Boud.,¹⁶ *Peziza aurantia* Pers. per Hook.,¹⁷ and *Ciliaria pseudotrechispora* (Schroet.) Boud.¹⁸

2. The 'périspore' contains 'masses globuleuses' that originate from the spore itself. The ornamentation appears on the 'assise sous-périsporique' and its covering layer. In *Peziza bicucullata* Boud.¹⁹ and *Ascodesmis nigricans* van Tiegh. both 'périspore' and 'masses globuleuses' disappear. In *Melastiza chateri* (W. G. Smith) Boud., sensu Grelet only the 'périspore' disappears, while the 'masses globuleuses' remain.

III. The ornamentation is of vacuolar origin.

In the epiplasmic vacuoles a pigment is present that precipitates on the spore wall as small granules. This type of ornamentation is found in *Ascobolus* Pers. per Hook. and *Saccobolus* Boud.

IV. The ornamentation is false.

In *Helvella* L. per St.-Amans the ornamentation consists of 'formations sous-épisporique'.

ELECTRON MICROSCOPY

The electron microscopy of ascospores started with studies on yeasts. Hashimoto & al. (1958), Marquardt (1963) and Hagedorn (1964) studied *Saccharomyces*; Conti & Naylor (1960) *Schizosaccharomyces*; and Thyagarajan & al. (1962) *Rhodotorula*. Since then more recent studies have been made by Bandoni & al. (1967) on *Hansenula* and by Lynn & Magee (1970) on *Saccharomyces*.

Most of the other studies on ascospores deal with the operculate Discomycetes. The most important of these are the studies on *Pyronema* by Reeves (1967), Griffith (1968) and Hung & Wells (1971); on *Saccobolus kerverni* by Carroll (1966, 1967, 1969); on *Ascobolus immersus* by Delay (1966); on *Ascobolus stercorarius* by Wells (1972); on *Ascobolus viridulus* by Oso (1969); on *Ascodesmis sphaerospora* by Moore (1963) and Carroll (1966), on *Ascodesmis nigricans* by Bracker & Williams (1966); and on *Pustularia cupularis* by Schrantz (1966, 1967).

Of the inoperculate Discomycetes only *Dasyscyphus* has been studied (Moore & McAlear, 1962).

Research was done on the other ascospores by Lowry & Sussman (1958) on *Neurospora crassa*, by Beckett (1966) and Beckett & al. (1968) on *Podospora anserina*,

¹⁶ = *Melastiza chateri* (W. G. Smith) Boud.

¹⁷ = *Aleuria aurantia* (Pers. per Hook.) Fckl.

¹⁸ = *Scutellinia pseudotrechispora* (Schroet.) Le Gal.

¹⁹ = *Aleuria bicucullata* (Boud.) Boud.

by Greenhalgh & Evans (1968) on *Hypoxylon fragiforme* and by Furtado & Olive (1970) on *Sordaria fimicola*.

The origin of the ascospores was one of the first problems to be adequately solved with the electron microscope. In nearly all studies the ascospores appear to be delimited by two unit membranes that separate a certain amount of plasma from the ascoplasm surrounding each nucleus. Although the origin of the two unit membranes is also interesting and still far from being solved it does not concern this study and will therefore not be discussed.

In all the foregoing studies the formation of wall layers between the two unit membranes is evident. This formation seems to be universal in the Ascomycetes. Since not all the statements of the authors about the position of the wall layers are clear their terminology is somewhat confusing.

Although Lynn & Magee (1970) observed two differently structured layers in the first wall, a thick electron-transparent inner layer (the endospore) and a rather thin electron-dense outer layer (the episore), most authors stated at the start the first wall consists only of electron-transparent material, while the episore at the outer edge of the endospore appears only when a certain stage of spore development has been reached. The episore was described as either multi-layered (Marquardt, 1963; Delay, 1966; Wells, 1972) or granular (Furtado & Olive, 1970).

According to Hashimoto & al. (1960), the sporoplasmalemma originates from the inner delimiting membrane, the generally accepted view. In the more recent publications the outer delimiting membrane is often called an investing membrane (Reeves, 1967; Carroll, 1969; Furtado & Olive, 1970).

In many species of Ascomycetes an extra wall develops between the first wall and the investing membrane after the first wall has formed. Reeves (1967) described this extra wall as a spore matrix; Carroll (1969), Furtado & Olive (1970) and Wells (1972) called it a perispore; Delay (1966) used the term perispore sac; and Moore (1963) spoke of a secondary wall. Oso (1969) reported the development of a mucilaginous layer outside the investing membrane.

The appearance of this extra wall varies considerably. In *Pyronema*, Reeves (1967) observed a smooth electron-transparent layer; in *Saccobolus*, Carroll (1969) reported a mucilaginous layer in which pigment of vacuolar origin could be found at a later stage; in *Ascobolus* this was seen by Delay (1966) and Wells (1972); in *Sordaria fimicola*, Furtado & Olive (1970) detected a fine fibrillar layer; the extra wall in *Ascodesmis sphaerospora* was described by Carroll (1966) as consisting of fine fibrillar material and by Moore (1963) as reticulate, electron-dense material.

Some authors (Oso, 1969; Furtado & Olive, 1970) stated that the episore belongs to this extra wall.

ASCODESMIS MICROSCOPICA AND A. NIGRICANS

The aim of this study is an investigation of the ultrastructure of the ascospore wall in the Pezizales. The study begins with two of the species of the genus *Ascodesmis*, *A.*

microscopica (Crouan) Seaver [non sensu Seaver] and *A. nigricans* van Tiegh. These two species are of special significance because in Le Gal's observations they represent very different types of wall formation. From a practical point of view they are especially suitable as they can be easily obtained and grown in culture.

MATERIALS AND METHODS

Material of *Ascodesmis microscopica* (Crouan) Seaver was provided by Dr. M. J. Richardson, Department of Agriculture and Fisheries for Scotland, Edinburgh, Scotland; *Ascodesmis nigricans* van Tiegh. was received as strain CBS 389.68 from the Centraal Bureau voor Schimmelcultures, Baarn, Holland.

The cultures were grown on agar containing an extract of horse dung and oatmeal; *Ascodesmis microscopica* for 2 days at 12° and 3–4 days at 20°; *Ascodesmis nigricans* for 2 days at 12° and 3 days at 20°. They were grown in conditioned growth-chambers, a 16 hour light period alternating with an 8 hour dark period.

As soon as ripe ascospores were found against the lid of the petri-dish the species were fixed. Small blocks of agar with apothecia were then cut from the plates and placed in the fixative. Several different types of fixatives were applied.

Apothecia were fixed for 2, 3 or 4 hours at room temperature in 1% KMnO_4 in distilled water, to which one drop of Invadine (Geigy) was added; or for 16 hours at 4°C in 1% OsO_4 in veronal-acetate buffer; or for 30 minutes at 20°C in 15% KMnO_4 in distilled water and after several washings in distilled water postfixed for 1 hour at room temperature in 1% OsO_4 in veronal-acetate buffer. Moreover other material was fixed for 4 hours at 4°C in 3.25% glutaraldehyde in cacodylate buffer and after several washings in cacodylate buffer postfixed for 2 hours at 4°C in 1% OsO_4 , again in cacodylate buffer; each buffer had a pH of 7.3.

The KMnO_4 -fixed material was washed in distilled water and dehydrated at room temperature in ethanol; the OsO_4 -fixed material and the glutaraldehyde- OsO_4 -fixed material was washed at 4°C in the same buffer as that used in the fixatives, and dehydrated at 4°C in ethanol. After dehydration it was stained for 5 minutes at room temperature in 1% uranylacetate in 96% ethanol. The material was then transferred to Epon via 100% ethanol, propylene oxide and mixtures of propylene oxide and Epon. Polymerisation lasted 36 hours at 60°C.

Special care was taken to ensure that all air had been drawn from between the asci during both the fixation and the impregnation with Epon.

The Epon components were used at a rate of 6.1 g. Epikote 812, 1.9 g. dodecenyl succinic anhydride, 3.3 g. methyl nadic anhydride and 0.15 g. 2,4,6-tri(dimethylaminomethyl)phenol.

Sections were cut with glass knives on an LKB Ultratome III, occasionally stained with various combinations of uranyl acetate and Reynolds' lead citrate, and examined in a Philips EM 300 electron microscope.

OBSERVATIONS

ASCI AND SPORES BEFORE THE DEVELOPMENT OF A SPORE WALL

It was frequently difficult to detect the early stages of development before the delimitation of the ascospores in the ascusplasm had taken place but these can be disregarded as irrelevant to this study. A description of the development of the ascus starts therefore when delimitation has become apparent. The description applies to both *Ascodesmis microscopica* and *A. nigricans*.

The double membrane bounding the spores consists of two unit membranes with an intermediate electron-transparent space; each of the membranes measures about 10 nm in thickness, the interspace being about 6 nm thick (Pl. 28A, B). The young ascospores are shaped irregularly, many of them somewhat ellipsoidal or even stellate (Pl. 28A, B). The distribution of the spores in the ascoplasm seems to be at random.

By the time delimitation of the spores has taken place the sporoplasm will have become separated from the ascoplasm; the remaining part is often called the epiplasm. From then on the development of the sporoplasm and the epiplasm each proceeds characteristically.

The epiplasm

Mitochondria of different sizes are visible (Pl. 28A); they may vary from circular to elongated. When fixed in permanganate the inside of the mitochondria appears to be an electron-transparent matrix; in the centre of this matrix a light spot can often be found. The mitochondria are surrounded by a double membrane whose inner layer is convoluted to give a number of long cristae. The cristae are often arranged in groups. They extend in various directions within the mitochondria. Fixed in glutaraldehyde-OsO₄ the mitochondria have the same appearance, but the matrix seems to be completely homogeneous.

Irregularly shaped and sometimes branched tubular structures with a diameter of about 35 nm, and small vesicular structures of similar dimensions are scattered among the mitochondria; these structures constitute the endoplasmic reticulum. Globular vesicles about 250–300 nm in diameter are also visible. When fixed in permanganate these structures are easily perceived. They are membrane-bounded and have an electron-transparent content (Pl. 28A–C). In material fixed in OsO₄ or in glutaraldehyde-OsO₄ the endoplasmic reticulum has a membranous structure. Here larger vesicles with an electron-transparent content are also visible. It is difficult to determine whether or not these larger vesicles can be compared with those found in the permanganate-fixed material. Although the dimensions are about the same the vesicles are not globular; they are more irregular in shape; sometimes one of them is invaginated by another, which then seems to become electron-dense.

Ribosomes are visible only in material fixed in OsO₄ or glutaraldehyde-OsO₄. They appear as numerous electron-dense granules about 15–20 nm in diameter distributed regularly over the epiplasm but they can also be seen to line the endo-

plasmic membranes. In material fixed in permanganate the ribosomes are lost. The ascoplasmalemma is about 10 nm thick and is visible as a unit membrane surrounding the epiplasm. In the types of fixative used, it is slightly undulating and not closely pressed to the ascus wall. Lomasomes are not found. Occasionally some connections seem to exist between the plasmalemma and the other membranous and vacuolar epiplasmic structures. Possibly the outer delimiting membrane is involved as well. In all the types of fixative used dictyosomes of the Golgi-apparatus are absent.

In material fixed in permanganate rather large areas of glycogen particles are visible throughout the epiplasm. These particles are electron-dense and about 15–30 nm in diameter (Pl. 28A–C). When fixed in glutaraldehyde-OsO₄ they are less electron-dense; when fixed only in OsO₄ they are visible as electron-transparent spots. Oil vacuoles and other storage vacuoles or special inclusions are not present.

If the material is fixed in permanganate several large areas of electron-transparent material can be detected adjacent to the spores. In most of these areas scattered electron-dense spots about 15–25 nm in diameter (probably glycogen) and tiny electron-transparent vesicles about 45 nm in diameter are revealed (Pl. 28A–C). Although the boundaries of these areas may be formed partly by tubular structures (Pl. 28C) they cannot be regarded as organized structures. They disappear during the maturing of the spores. In other fixatives they are not visible.

The sporoplasm

Owing to the way in which the spores originate it is evident that at an early stage no differences between the epiplasm and the sporoplasm can be expected. All the organelles described for the epiplasm are found in the sporoplasm as well (Pl. 28A, B).

A large nucleus is also present in the sporoplasm. This nucleus is spherical, with minor irregularities in outline, and bounded by a nuclear envelope. This envelope consists of two unit membranes, each about 8 nm thick, separated by a perinuclear space of about 10–30 nm. It has pores about 30–80 nm in diameter through which the nucleoplasm is in contact with the surrounding plasm. When fixed in permanganate the inside of the nucleus appears electron-transparent and homogeneous, sometimes slightly granular (Pl. 28A). After fixation in glutaraldehyde-OsO₄ it is possible to detect many fairly electron-dense granules scattered throughout the nucleoplasm and one large and spherical electron-dense spot consisting of clusters of these granules. The large spot is supposed to be the nucleolus and the granules are possibly ribosomal particles. Evidence for the presence of chromatin has not been obtained.

Connections between the sporoplasmic organelles may be present.

THE DEVELOPMENT OF THE FIRST SPORE WALL

In both *Ascodesmis microscopica* and *A. nigricans* an electron-transparent wall develops between the two unit membranes surrounding a young ascospore (Pls. 28C; 29A, B). During this development the rate of growth around the spore wall is not always the same.

The appearance of the organelles in epiplasm and sporoplasm does not change very much at this stage except that in the sporoplasm the mitochondria seem to enlarge, while the number of glycogen granules lessens. In the epiplasm the number and size of the vacuoles increases (Pls. 29A, B; 30A-C; 31A, B; 32C). During the development of the spore wall the spores remain irregularly shaped but when the spore wall is completed the spores become more spherical. The inner delimiting unit membrane, now inside the spore wall, becomes the sporoplasmalemma; here small lomasomes can be found. The complete spore wall, called here primary spore wall, has a homogeneous structure and shows no signs of ornamentation. It is 300-500 nm thick when fixed in permanganate or osmium and about 250 nm thick when fixed in glutaraldehyde-OsO₄.

Up to this point *Ascodesmis microscopica* and *A. nigricans* have developed similarly, the slightly different ornamentation patterns found in the mature ascospores arising only during the formation of new wall layers.

THE DEVELOPMENT OF AN EXTRA SPORE WALL

Once the primary wall is formed an extra wall begins to develop. The outer delimiting spore membrane separates from the primary wall. The space thus formed is almost immediately filled with new wall material. In *Ascodesmis microscopica* the separation of the investing membrane starts at a few places only and becomes obvious before further separation spreads all over the spore, the investing membrane itself remaining fairly 'straight' (Pl. 30A-C). In *Ascodesmis nigricans* the separation starts all over the spore before becoming evident in several places; meanwhile the investing membrane will have become very irregular and undulating (Pl. 31A, B). More liftings occur between the existing ones; growing together may also be found.

The first formations of new wall material are slightly granular and have the same appearance and electron density as the epiplasm and sporoplasm (Pls. 30A-C; 31A, B). At a later stage it is possible to detect a difference, the inner part of the wall material becoming more electron-dense than the outer part (Pl. 33A, B). This difference is particularly apparent in material fixed in glutaraldehyde-OsO₄ (Pls. 32C; 34A). In permanganate-fixed material it is most clearly revealed by post-staining with lead and uranyl salts (Pl. 32A, B).

Later stages indicate that the electron-dense inner-wall material is an accumulation of outer-wall material embedded in a homogeneous matrix. It consists of circular and tubular membranous structures with a diameter of about 10 nm (Pls. 34A, C; 35A-C; 36A-D; 37A, B; 38A-C). If osmium is used in the fixative the inmost layers of these structures are directed perpendicular to the primary spore wall; the outer layers are more irregularly arranged (Pls. 32C; 34A; 36A). The continual addition of new wall material results in the formation of a distinct ornamentation pattern all over the spore. Where new wall material is formed the investing membrane is often indistinct and sometimes even interrupted.

The epiplasm undergoes considerable changes during the development of the

new wall and the maturing of the spores. As mentioned above, the organelles are distributed throughout the epiplasm at an early stage of development. When the primary wall is completed and the formation of the new wall begins the organelles in the epiplasm accumulate outside the investing membrane (Pls. 33A, B; 36B, C; 37A, B; 38A, B) and inside the ascoplasmalemma. Between these organelles the amount of glycogen granules greatly increases (Pls. 32A, B; 33A, B; 37A, B; 38A, C). The vesicles enlarge and they become especially apparent just outside the investing membrane; as vacuoles they fill up the gaps between the developing ornamentation (Pls. 33A, B; 36B, C; 37A, B; 38A, B). Since these vacuoles develop at the same time as the ornamentation pattern it is not clear whether their place of origin is the cause or merely the result of this pattern. It is also possible that the vacuoles and the ornamentation pattern develop independently.

The vacuoles surrounding the spores (Pl. 36) fuse until each of the spores is surrounded by a single large vacuole. In turn these large vacuoles also fuse until they have formed a vacuole that fills up the major part of the ascus. At this stage most of the epiplasm, including the glycogen, will have disappeared. It is found only in a thin layer just inside the ascus wall. What happens to the investing membrane is uncertain. It is not possible to detect whether it adheres to the spore wall or disappears with the epiplasm.

The sporoplasm darkens slightly but remains intact. Only the glycogen disappears. Possibly a small enlargement of the mitochondria occurs.

THE DIFFERENTIATION IN THE FIRST SPORE WALL

During the development of the extra spore wall differentiation in the first spore wall takes place. This differentiation is most clearly visible in material fixed in permanganate.

It would seem as though material from the inner part of the wall moves to the outer edge and there condenses into a broad band (Pl. 33B). At a later stage the outer edge appears to have differentiated into two layers (Pls. 33A; 35A, C), the outer layer becoming electron-dense. As the spores mature still more layers become visible between the existing ones (Pls. 37A, B; 38A, B). The whole complex of layers is called the episporium. It is about 30–40 nm thick. The remaining inner part of the first wall is called the endospore. The process is identical in *Ascodesmis microscopica* and *A. nigricans*.

THE ORNAMENTATION PATTERNS

The maturing spores develop an ornamentation pattern differing in *Ascodesmis microscopica* and *A. nigricans*.

In *Ascodesmis microscopica* new wall material is added in all directions. This results in a complete network all over the spore. The mature spores are brown, $12\text{--}14 \times 9.5\text{--}12 \mu\text{m}$ without ornamentation, $13\text{--}15 \times 10.5\text{--}13 \mu\text{m}$ with ornamentation.

In *Ascodesmis nigricans* new wall material is added more irregularly. Individual

spines are formed that grow especially at the tips. Here they can branch and join together. The mature spores are brown, $9.5-12 \times 8-10 \mu\text{m}$ without ornamentation, $10.5-13 \times 9-11 \mu\text{m}$ with ornamentation.

DISCUSSION

Since my methods are not essentially different from those described in other studies it is not surprising that the same general ultrastructure has been attributed to *Ascodesmis microscopica* and *A. nigricans* as to all the other ascomycetes investigated thus far. It must be emphasized that the cell walls of most fungi have low permeability thereby inhibiting penetration of fixatives and embedding materials. This means that good overall fixation and impregnation is not easily accomplished and good pictures are difficult to obtain.

All the typical fungal organelles are found and their structure answers to the usual descriptions. Hawker (1965) and Bracker (1967) state that in fungi the endoplasmic reticulum consists of a sparse and irregular internal membranous system that varies greatly in form and extent according to the physiological condition of the cells. In *Ascodesmis microscopica* and *A. nigricans* this internal membranous system is also irregular. It is rather extensive and its appearance depends on the type of fixative used. In permanganate it is tubular and vesicular; in glutaraldehyde- OsO_4 it is membranous and associated with ribosomes, like in the cells of higher organisms.

It is not clear whether the mitochondrial shape is dependent on an organism and its developmental stage or on unknown external influences (see also Hawker, 1965 and Bracker, 1967). The plane of sectioning must also be taken into account. Compared with the mitochondria of higher organisms the fungal mitochondria are thought to be less rigid and more irregular in the outline and shape of their internal foldings (Moore & McAlear, 1963). It is difficult to determine whether or not the internal electron-transparent zones visible after a permanganate fixation are correlated with the appearance of DNA as stated by Delay (1966).

Although in the asci of *Ascodesmis microscopica* and *A. nigricans* lomasomes are found only in the sporoplasmalemma they are probably rather common in fungi. Moore & McAlear (1961) point out that lomasomes are restricted to the fungi. They are supposed to have a function in wall formation (Moore & McAlear, 1961; Wilsenach & Kessel, 1965; Carroll, 1966, 1969). A close resemblance to the plasmalemmasomes of bacteria and Actinomycetes is evident and the same function is ascribed to these organelles. Ryter (1969) points out that the choice of fixative determines the appearance of the plasmalemmasomes. This could explain the differences.

The occurrence of dictyosomes in fungi is often discussed. Most investigators assume that the real dictyosomes found in higher organisms are not present in fungi; their absence in *Ascodesmis microscopica* and *A. nigricans* confirms this.

It is obvious that the large electron-transparent areas found in the epiplasm just

after spore delimitation must be regarded as normal endoplasmic areas without or with very few organelles and glycogen particles. It is not impossible that they arise during the delimitation of the spores and that redistribution of the organelles causes them to disappear.

The observations on the development of spores confirm not only the generally accepted hypothesis that in the Ascomycetes the spores are delimited by two unit membranes but also that the formation of wall layers between the two unit membranes is very common. In both *Ascodesmis microscopica* and *A. nigricans* it has become evident that there are two different stages in the process of wall formation. In the first stage a wall layer develops between the two delimiting membranes and in the second stage an extra wall layer is formed between the first layer and the outer delimiting membrane. These two layers differ in appearance and perhaps even in their formation. Therefore I will term them primary and secondary walls. All wall material formed between the primary wall and the outer delimiting membrane, whatever its appearance or further differentiation, is considered to belong to the secondary wall. The primary wall must be seen as a common wall, always present and showing little differentiation. The secondary wall should be regarded as an extra wall layer, varying in appearance and giving rise to the ornamentation patterns of ascospores. In yeasts the secondary wall is absent.

It must be emphasized that most of the wall layer terminological problems arise from the formation of the episporium at the outer edge of the primary wall during the development of the secondary wall. Most investigators fail to clearly point out the exact position of this episporium. Oso (1969) and Furtado & Olive (1970) stated that the episporium is part of the secondary wall. From the way the episporium develops in *Ascodesmis microscopica* and *A. nigricans* I assume that in these species the episporium is the outer layer of the primary wall. The remaining inner layer of the primary wall is the endospore.

The development of the episporium is not often discussed. It is highly improbable that Marquardt's description (1963) of it is correct. He assumes that the episporium originates through the deposition of extra membranes and other cytoplasmic material via 'canals' in the epiplasm on the outer delimiting membrane. This theory must be rejected because it has become evident that all wall material is formed between the two delimiting membranes, thus both the primary and the secondary wall material. This does not exclude all activity of the epiplasm during the formation of the episporium, as supposed by Lynn & Magee (1970); they think that the episporium consists of fats originating in the epiplasm. As I have been unable to discover any addition of new wall material in *Ascodesmis microscopica* and *A. nigricans* but merely differentiation of the primary wall into an episporium and an endospore I underline Delay's view (1966) that the episporium and the endospore differentiate in situ from material already present in the primary spore wall.

The thickness of the mature primary wall appears to be dependent on the type of fixative used. Fixed in glutaraldehyde-OsO₄ the wall is seen to be only half as thick as it is when fixed in permanganate or only in OsO₄. This phenomenon may

be caused by a swelling of the wall material. It is also mentioned by Delay (1966), who says that a certain artificial swelling of the wall material should not be overlooked.

Many suggestions have been made about the origin of the spore walls. Wilsenach & Kessel (1965) and Carroll (1966, 1969) assume that lomasomes play a role. It is not likely that the formation of the primary spore wall in *Ascodesmis microscopica* and *A. nigricans* is due only to them since they are small and found only incidentally in the inner delimiting membrane.

Some investigators think that the endoplasmic reticulum is important to the development of the spore walls, either in the epiplasm (Marquardt, 1963; Delay, 1966) or in the sporoplasm (Moore, 1963, 1965; Reeves, 1967; Lynn & Magee, 1970). Moore (1963) states that at places where a secondary wall arises in *Ascodesmis sphaerospora* an endoplasmic reticulum is present just inside the sporoplasmalemma, acting as a mould on which the secondary wall is formed. Bandoni & al. (1967) state that complexes of endoplasmic reticulum in the sporoplasm can be abundant even when a secondary wall is absent. As already mentioned, Le Gal (1949) assumes that in *Ascodesmis microscopica* and *A. nigricans* the secondary wall is of sporal origin.

In the present study no evidence is found for a special arrangement of organelles in the sporoplasm or transport of wall material via the primary wall. Therefore it is assumed that the formation of the secondary wall is due to the activity of the epiplasm. Here an accumulation of organelles along the developing spore wall can be detected at these stages. This accords with Delay's view (1966) that the epiplasm may play a dominant part in the formation of the secondary wall; she surmises that in *Ascobolus immersus* gelification of the epiplasm is not impossible.

The way in which the epiplasm is involved possibly determines the appearance of the secondary wall. In the species of the genera *Ascobolus* and *Saccobolus* studied so far the secondary wall is made up of a mucilaginous layer called the perispore or the perisporal sac; this is used as a matrix for the deposition of vacuolar material giving rise to ornamentation patterns. In *Ascodesmis microscopica* and *A. nigricans* the formation of the secondary wall appears to be quite different. Here the entire secondary wall consists of material used for the formation of ornamentation patterns. Supported by light microscopy and staining techniques Le Gal (1949) concluded that this material consists of callose and pectine. My studies have shown that the structure of the secondary wall is formed by the deposition of real membranous fragments in a homogeneous matrix.

As already mentioned, the exact behaviour of the investing membrane is still uncertain. It is not impossible that the membranous layer remaining around the mature ascospores of *Ascodesmis microscopica* (Le Gal, 1949; Obrist, 1961) is identical with this investing membrane. This could mean that in *Ascodesmis microscopica* the investing membrane adheres to the ornamentation, while in *Ascodesmis nigricans* it disappears with the epiplasm. This hypothesis must be considered with some reserve.

It should be stressed that the results of the present study only partly agree with Le Gal's observations on *Ascodesmis microscopica* and *A. nigricans* (1949). Hence no

differences have been found in the development of the secondary spore wall between these species; in *Ascodesmis microscopica* no evidence has been obtained for the existence of an 'assise sous-périsporique' and a 'pellicule membranaire'; in *Ascodesmis nigricans* the 'masses globuleuses' and the 'périspore' are not present. A sporal origin of the secondary wall could not be confirmed.

ACKNOWLEDGEMENTS

The author is greatly indebted to Professor Dr. C. Kalkman, Director of the Rijksherbarium (Leiden), for his interest in and stimulation of the present study and to Dr. J. van Brummelen, mycologist at the Rijksherbarium, for his valuable criticism. She wishes to acknowledge the skilled technical assistance of Miss B. A. D. Dam and Mrs. F. P. Boers-van der Sluijs. She also wishes to thank Miss D. Ohler and Mrs. E. Helmer van Maanen Ph. D. for suggestions about the manuscript. Gratitude is expressed to Professor Dr. W. K. H. Karstens of the Botanical Laboratory (Leiden) for making use of the electron microscope possible. This study could not have been carried out without the financial support of the Foundation for Fundamental Biological Research (BION).

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PLATE 28

FIGS. A, B. *Ascodesmis nigricans*, fixed in 1% KMnO_4 and stained with uranyl acetate: Fig. A. young ascospore just after delimitation x 9,900; Fig. B. id. detail area of electron transparent material, x 17,200.

FIG. C. *Ascodesmis microscopica*, area of electron transparent material, fixed in 1% KMnO_4 and stained with uranyl acetate, x 16,500.

AP, ascoplasmalemma; AW, ascus wall; DAM, double ascospore-delimiting membrane; E, epiplasm; ER, endoplasmic reticulum; ETA, area of electron transparent material; G, glycogen; IAM, inner ascospore-delimiting membrane; M, mitochondrion; N. nucleus; NE, nuclear envelope; OAM, outer ascospore-delimiting membrane; S, sporoplasm; V, vesicle.

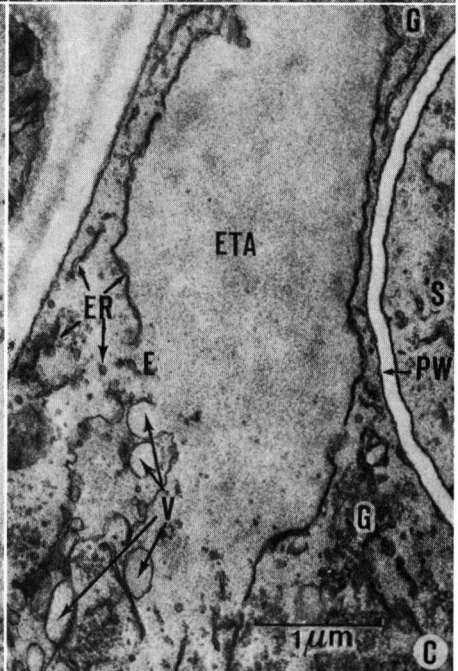
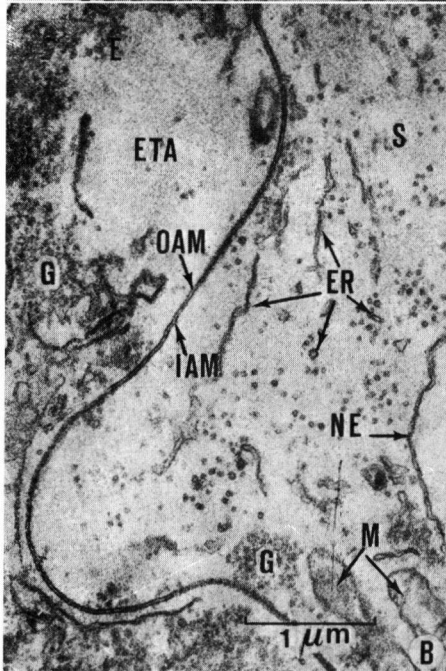
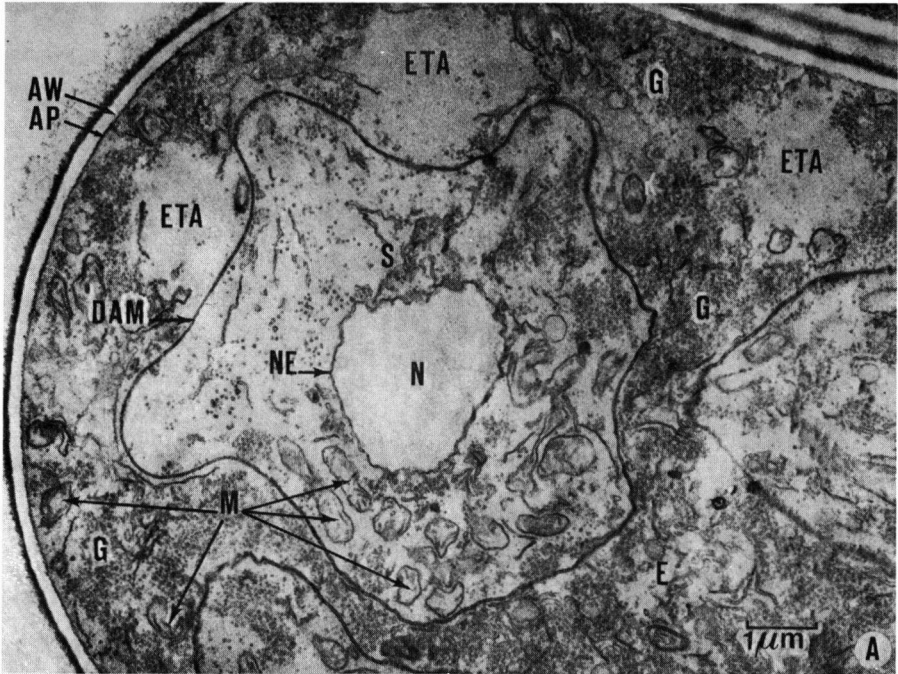


PLATE 29

FIGS. A, B. *Ascodesmis microscopica*, young ascospore with developing primary wall: Fig. A. fixed in 1% KMnO_4 and stained with uranyl acetate, x 16,500; Fig. B. fixed in 0.5% KMnO_4 and stained with uranyl acetate and lead citrate, x 20,800.

AP, ascoplasmalemma; AW, ascus wall; E, epiplasm; ER, endoplasmic reticulum; G, glycogen; M, mitochondrion; N, nucleus; NE, nuclear envelope; PW, primary wall; S, sporoplasm.

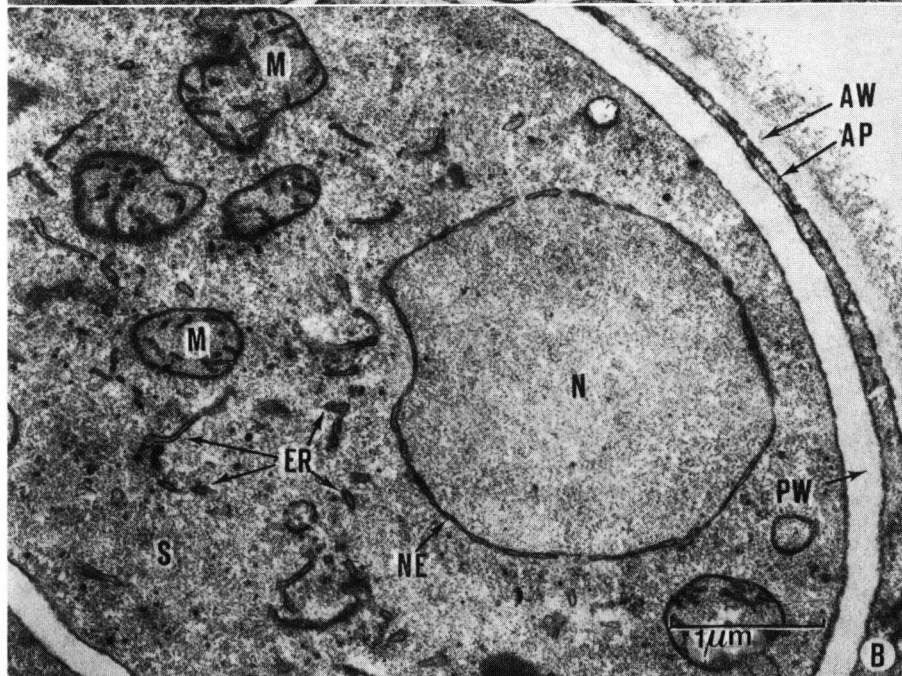
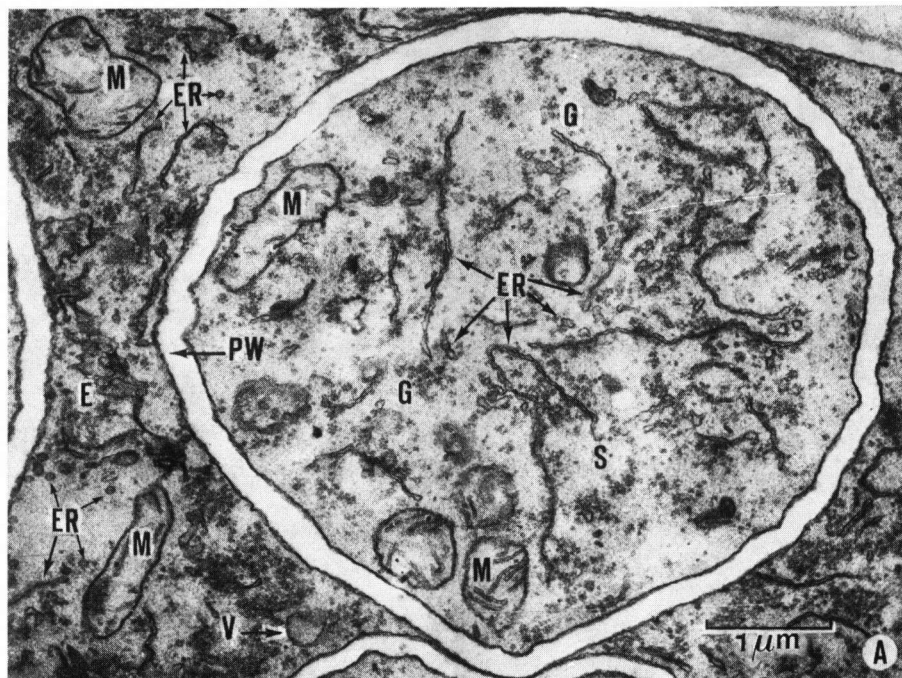


PLATE 30

FIGS. A-C. *Ascodesmis microscopica*, beginning of secondary wall formation, fixed in 1.5% KMnO_4 and stained with uranyl acetate; Fig. A. x 29,700; Fig. B, C. x 18,100.

E, epiplasm; ER, endoplasmic reticulum; G, glycogen; IM, investing membrane; M, mitochondrion; N, nucleus; NE, nuclear envelope; PW, primary wall; S, sporoplasm; SP, sporoplasmalemma; SW, secondary wall; V, vesicle.

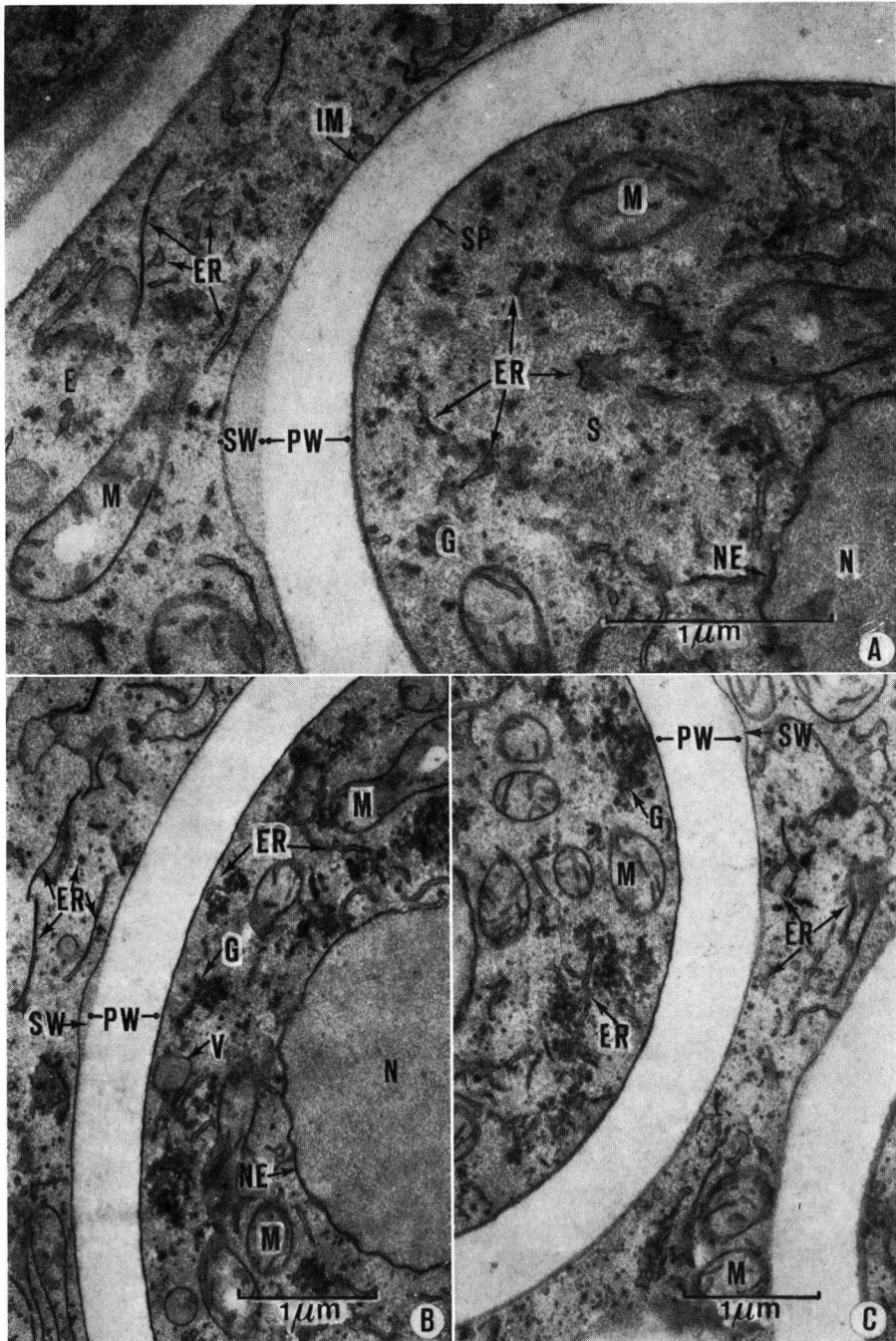


PLATE 31

FIGS. A, B. *Ascodesmis nigricans*, beginning of secondary wall formation, fixed in 1% KMnO_4 and stained with uranyl acetate, $\times 46,200$.

ER, endoplasmic reticulum; G, glycogen; IM, investing membrane; M, mitochondrion; PW, primary wall; SP, sporoplasmalemma; SW, secondary wall; Va, vacuole.

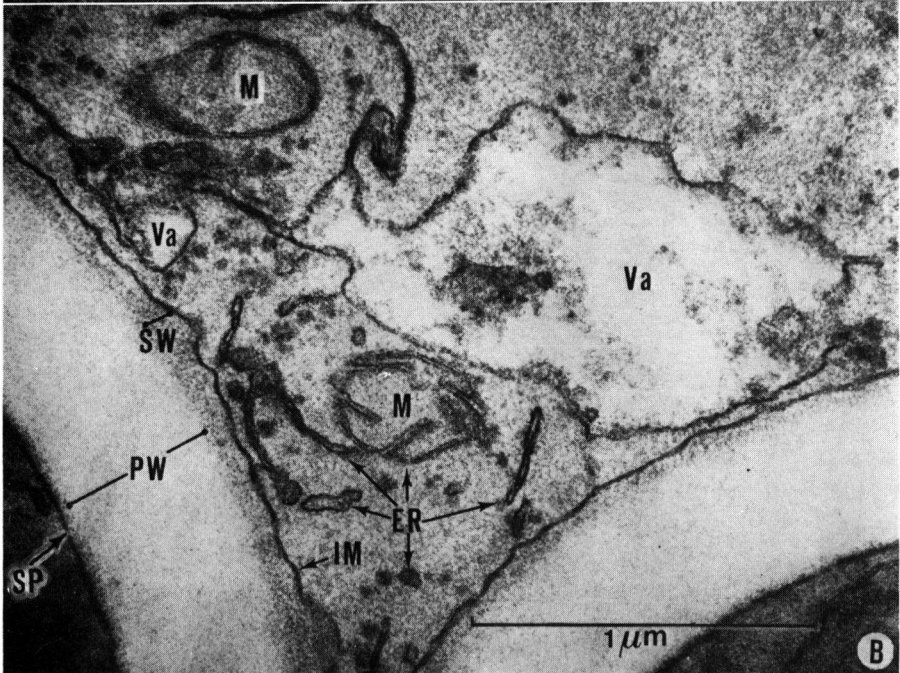
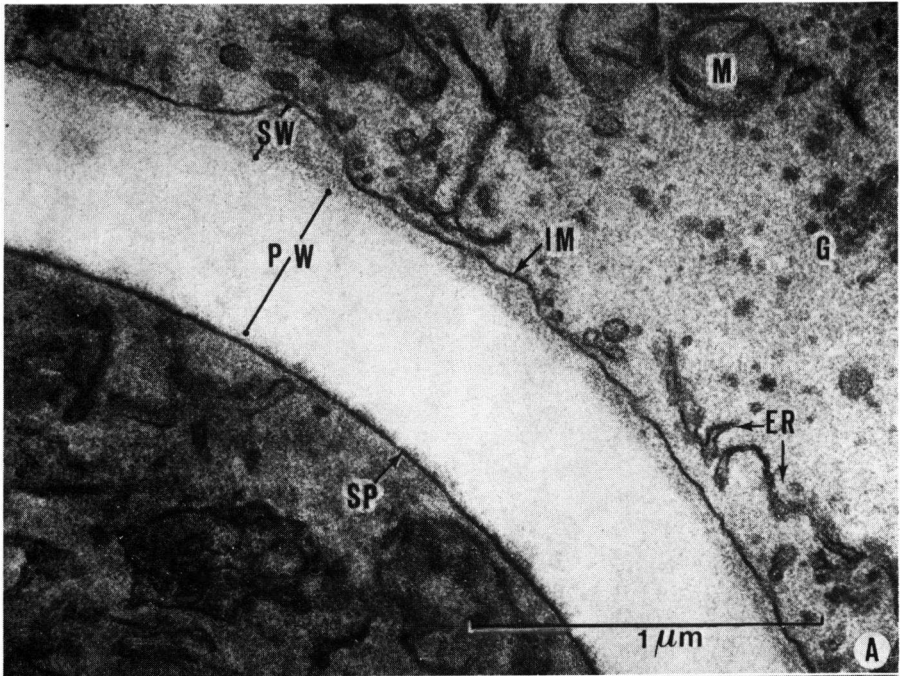


PLATE 32

FIGS. A–C. *Ascodesmis microscopica*, development of the secondary wall: Fig. A. fixed in 1.5% KMnO_4 and stained with uranyl acetate, $\times 9,900$; Fig. B. also showing a difference between the inner and outer part, fixed in 0.5% KMnO_4 and stained with uranyl acetate and lead citrate, $\times 29,700$; Fig. C. id. fixed in 3.25% glutaraldehyde and 1% OsO_4 , $\times 31,100$.

ER, endoplasmic reticulum; G, glycogen; IM, investing membrane; M, mitochondrion; N, nucleus; NE, nuclear envelope; PW, primary wall; SP, sporoplasmalemma; SW, secondary wall; V, vesicle.

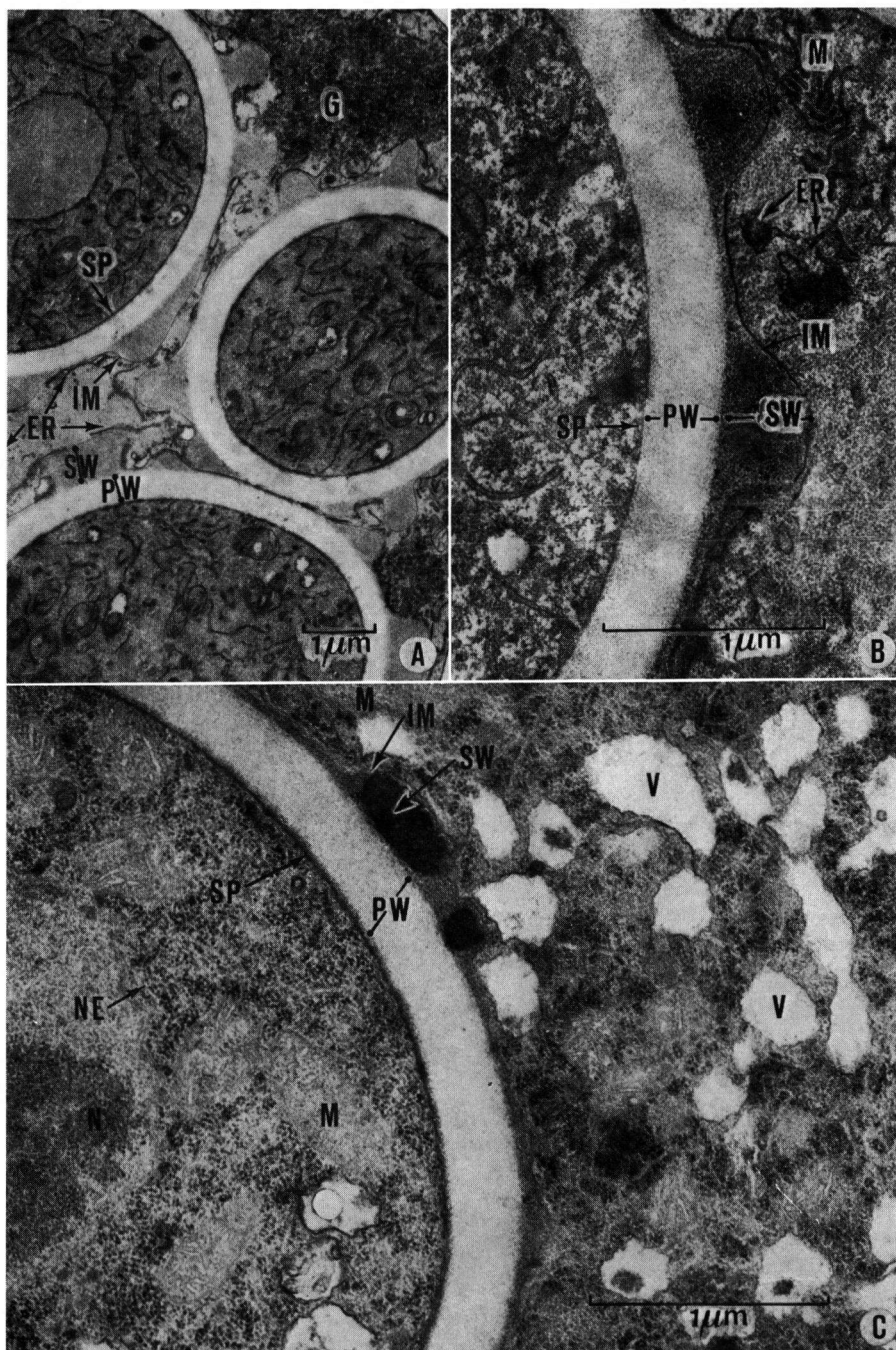


PLATE 33

FIGS. A, B. *Ascodesmis nigricans*, differentiation of the primary wall, and development of the secondary wall showing a difference between the inner and outer part, fixed in 1% KMnO_4 and stained with uranyl acetate: Fig. A. $\times 29,700$; Fig. B. $\times 57,700$.

ER, endoplasmic reticulum; G, glycogen; IM, investing membrane; M, mitochondrion; PW, primary wall; SW, secondary wall; T, tonoplast; Va, vacuole.

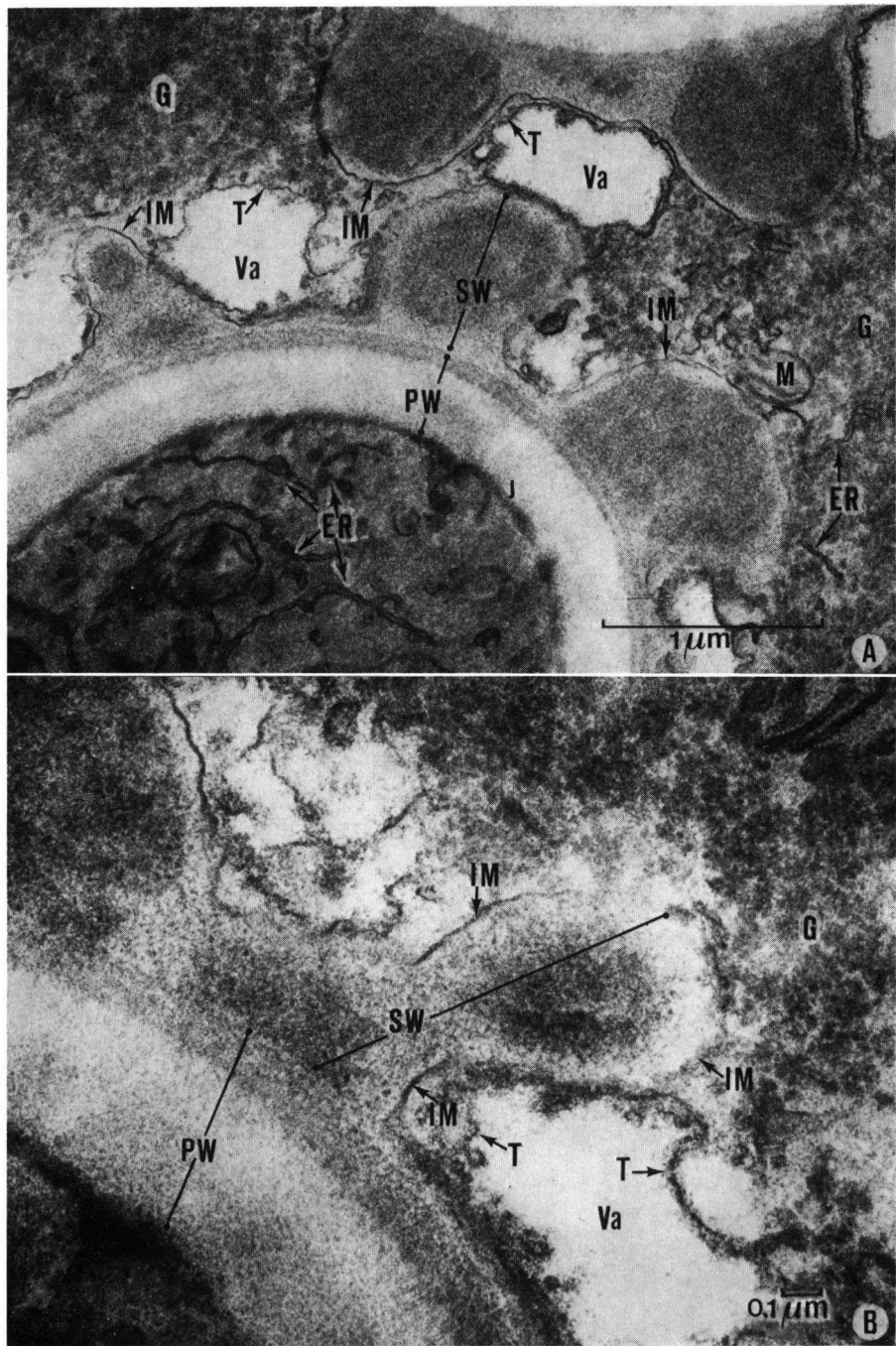


PLATE 34

FIGS. A–C. *Ascodesmis microscopica*, development of the secondary wall showing a difference between the inner and outer part: Fig. A. fixed in 3.25% glutaraldehyde and 1% OsO_4 and stained with uranyl acetate and lead citrate, $\times 32,400$; Fig. B. also showing the internal structure, fixed in 0.5% KMnO_4 and stained with uranyl acetate, $\times 53,900$; Fig. C. id. stained with uranyl acetate and lead citrate, $\times 50,300$.

IM, investing membrane; ISW, inner secondary wall; M, mitochondrion; OSW, outer secondary wall; PW, primary wall; SP, sporoplasmalemma; V, vesicle.

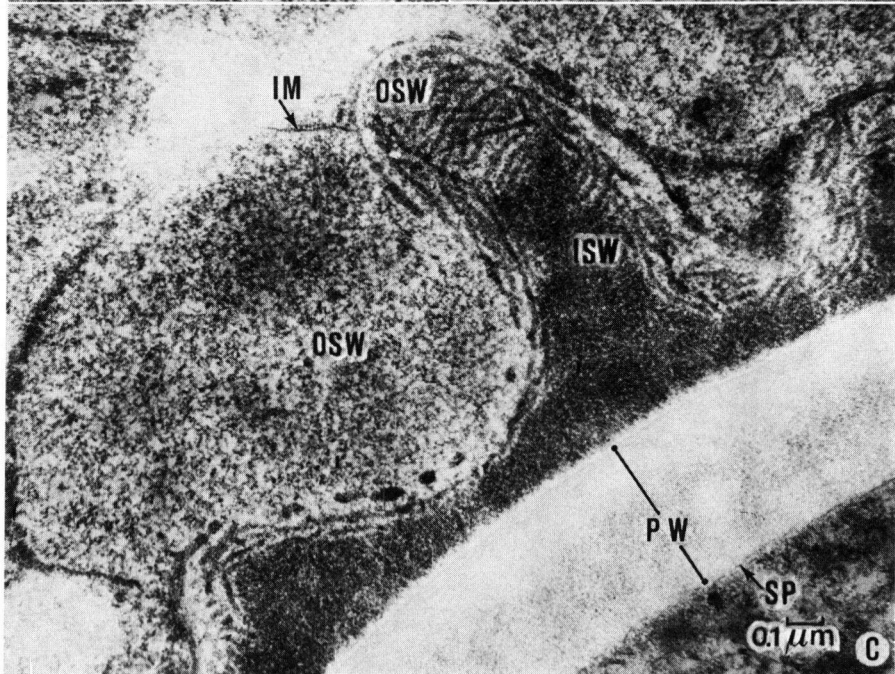
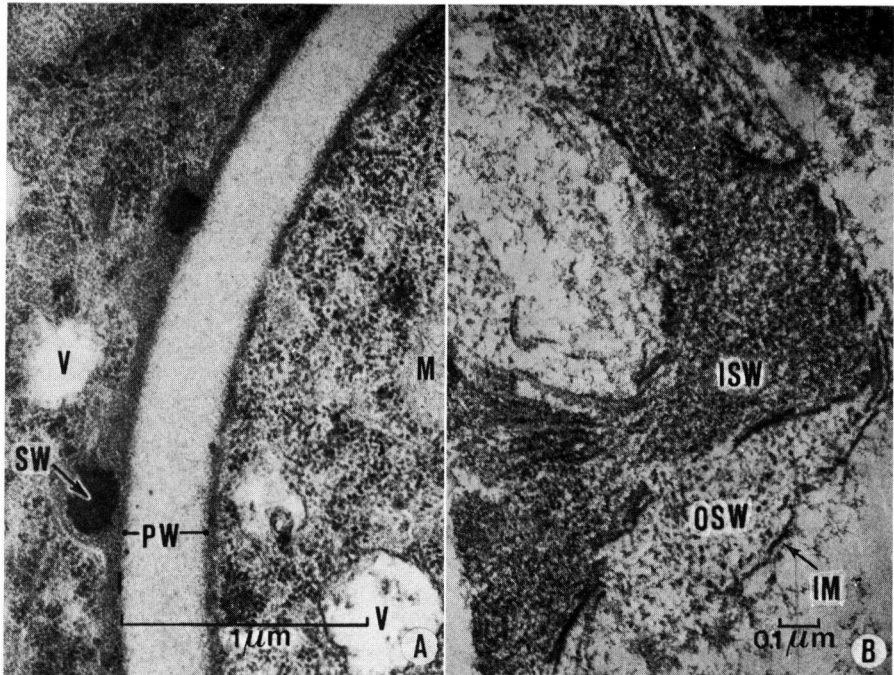


PLATE 35

FIGS. A–C. *Ascodesmis microscopica*, development of the secondary wall showing the internal structure of the inner and outer part, fixed in 0.5% KMnO_4 , $\times 57,700$: Fig. A. also showing the differentiation of the primary wall, stained with uranyl acetate and lead citrate; Fig. B. stained with uranyl acetate; Fig. C. also showing the differentiation of the primary wall, stained with uranyl acetate and lead citrate.

ER, endoplasmic reticulum; IM, investing membrane; ISW, inner secondary wall; OSW, outer secondary wall; PW, primary wall; SP, sporoplasmalemma.

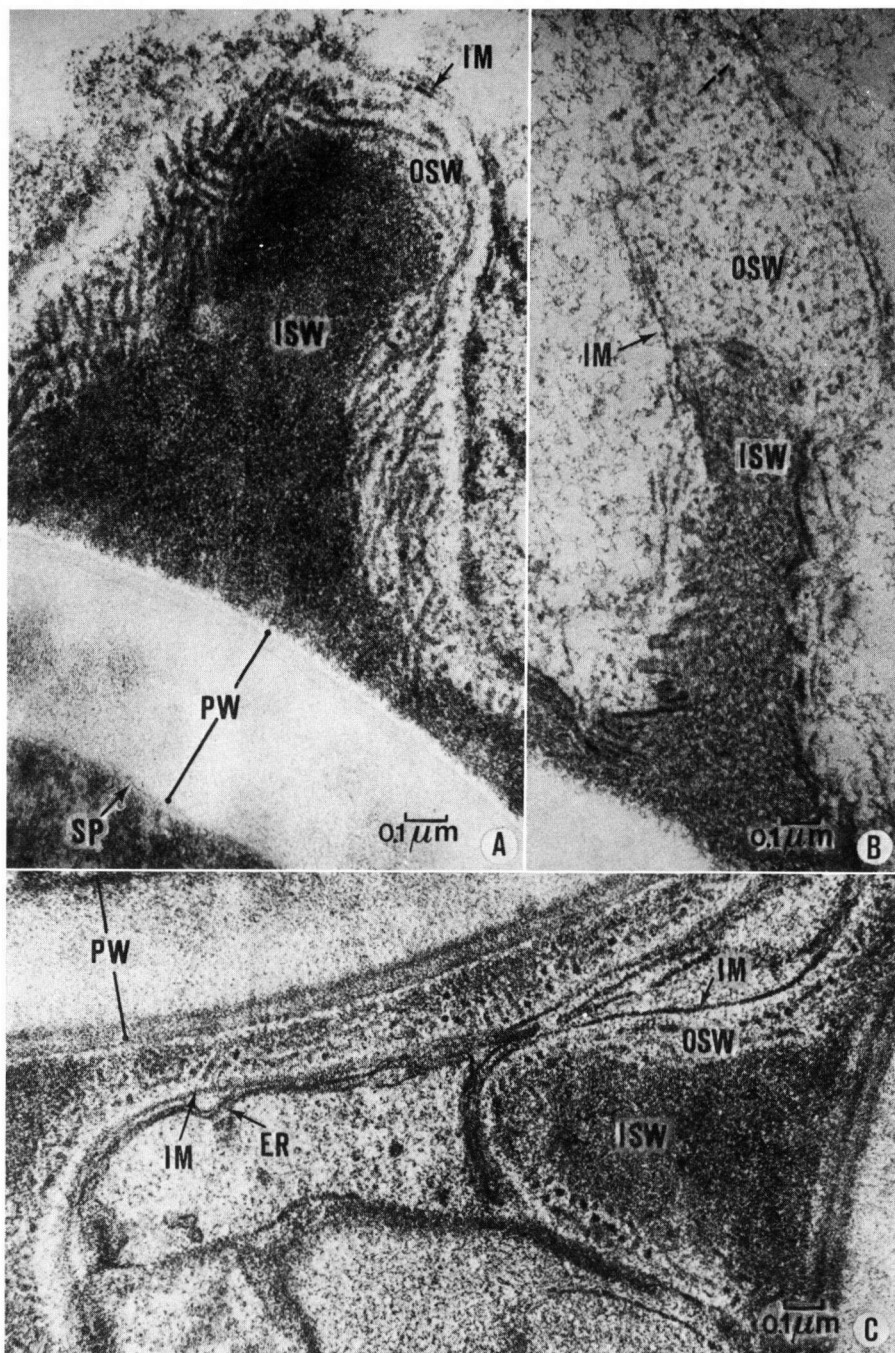


PLATE 36

FIGS. A-D. *Ascodesmis microscopica*: Fig. A. secondary wall showing the orientation of the internal structure, fixed in 1% OsO₄ and stained with uranyl acetate, x 55,000; Fig. B. development of the vacuoles, fixed in 1.5% KMnO₄ and 1% OsO₄ and stained with uranyl acetate and lead citrate, x 70,500; Fig. C. id. fixed in 0.5% KMnO₄, x 108,000; Fig. D. id. fixed in 1.5% KMnO₄ and stained with uranyl acetate, x 29,700.

ER, endoplasmic reticulum; G, glycogen; IM, investing membrane; ISW, inner secondary wall; OSW, outer secondary wall; SW, secondary wall; T, tonoplast; Va, vacuole.

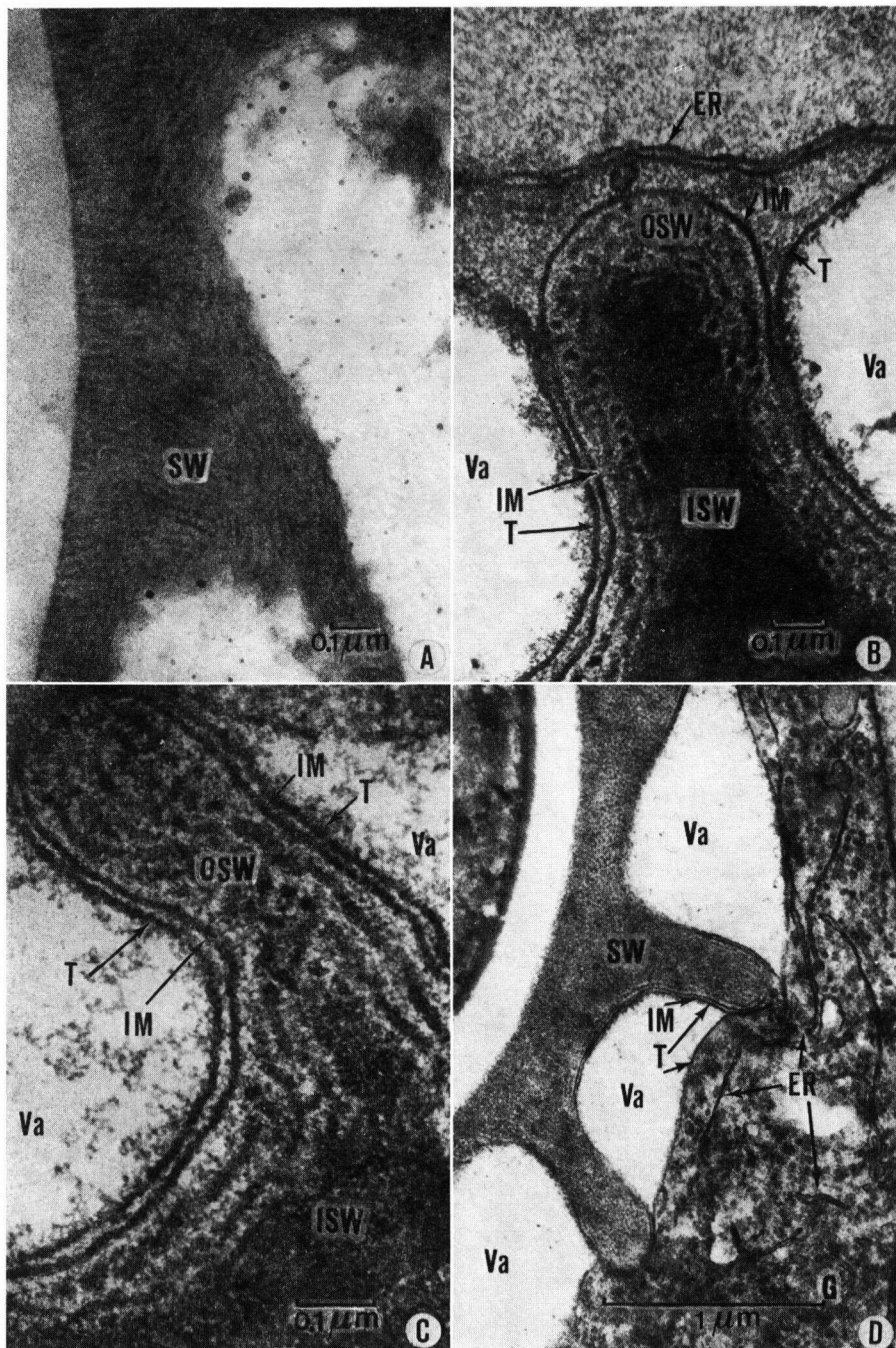


PLATE 37

FIGS. A, B. *Ascodesmis nigricans*, differentiation of the primary wall, and development of the secondary wall showing the internal structure of the inner and outer part, fixed in 1% KMnO_4 and stained with uranyl acetate: Fig. A. $\times 53,200$; Fig. B. $\times 36,300$.

En, endospore; Ep, episporium; ER, endoplasmic reticulum; G, glycogen; IM, investing membrane; ISW, inner secondary wall; M, mitochondrion; OSW, outer secondary wall; PW, primary wall; SP, sporoplasmalemma; T, tonoplast; Va, vacuole.

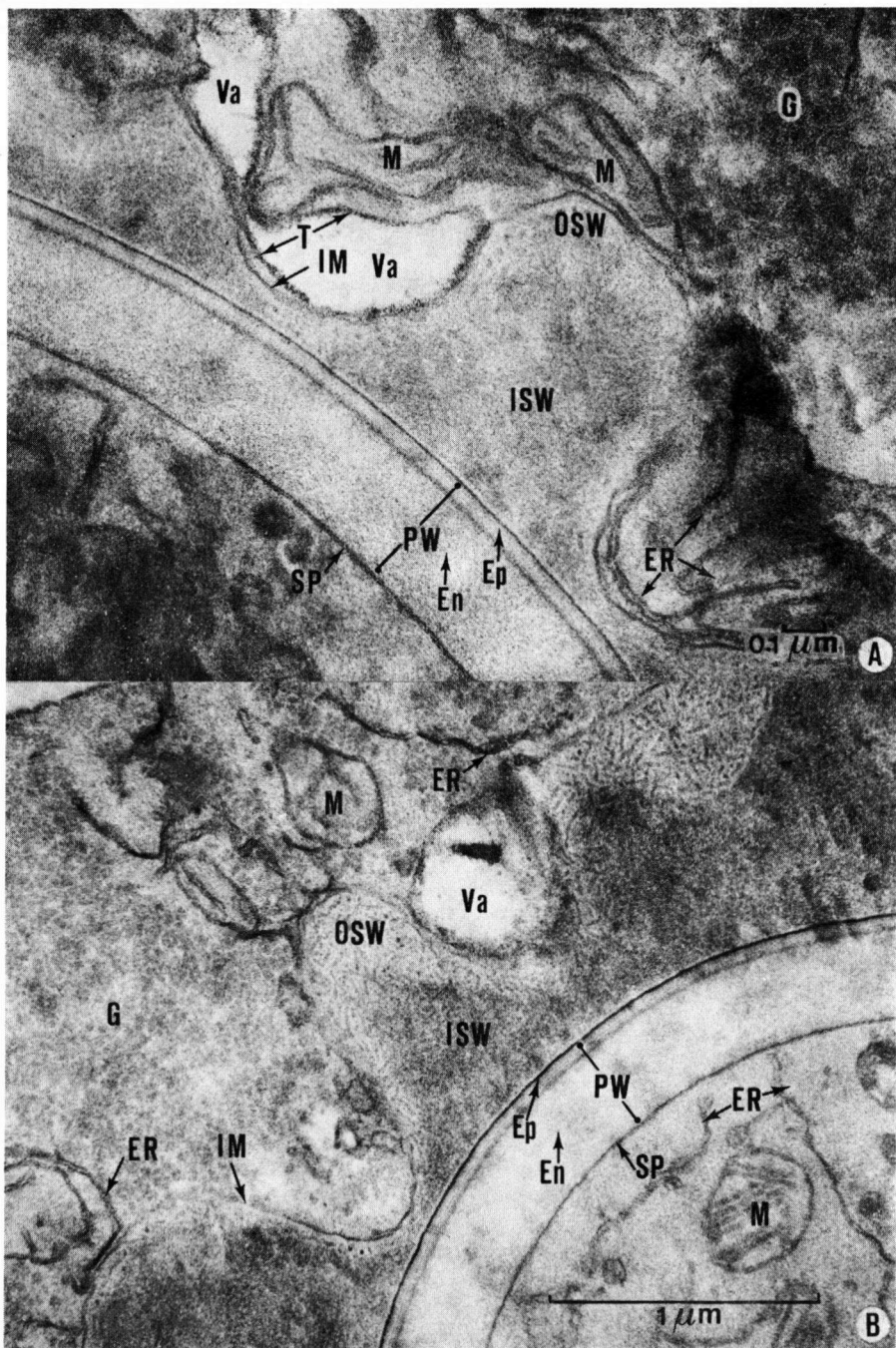


PLATE 38

FIGS. A-C. *Ascodesmis nigricans*, fixed in 1% KMnO_4 and stained with uranyl acetate: Fig. A. differentiation of the primary wall, and development of the secondary wall showing the internal structure of the inner and outer part, $\times 29,700$; Fig. B. id. $\times 57,700$; Fig. C. part of the secondary wall, $\times 57,700$.

En, endospore; Ep, episporium; ER, endoplasmic reticulum; G, glycogen; IM, investing membrane; ISW, inner secondary wall; M, mitochondrion; OSW, outer secondary wall; PW, primary wall; SP, sporoplasmalemma; T, tonoplast; Va, vacuole.

