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ULTRASTRUCTURE OF THE ASCUS AND THE ASCOSPORE WALL IN ELEUTHERASCUS AND ASCODESMIS (ASCOMYCOTINA)

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The ultrastructure of the ascus and the ascospore wall of *Eleutherascus lectardii*, *E. tuberculatus*, *E. peruvianus*, *Ascodesmis nigricans*, and *A. microscopica* is studied and compared. Especially the development of the primary and the secondary spore walls is exactly the same in *Eleutherascus* and *Ascodesmis*. *Eleutherascus* is placed as an extremely simple fungus in the Ascodesmidaceae (Pezizales).

Since the first species of *Eleutherascus* was described under the name *Arachniotus lectardii* by Nicot & Durand (1969), the taxonomic position of this fungus has been under consideration. It was placed in the family Gymnoascaceae (Eurotiales) by Nicot & Durand (l.c.) and von Arx (1971), but its resemblance with species of *Ascodesmis* Tiegh. (Pezizales) was also mentioned.

In her study of the life cycle of *Eleutherascus lectardii* Durand (1969) supposed a relationship of some representatives of the Gymnoascaceae with certain operculate discomycetes.

The doubtful taxonomic position of *Eleutherascus* was also reported and discussed by von Arx (1981, 1987a, 1987b), Huang (1975), Benny & Kimbrough (1980), von Arx & van der Walt (1986), and von Arx & Samson (1986).

There is a great resemblance in the early development of *Eleutherascus* (Durand, 1969) and *Ascodesmis* (van Tieghem, 1876; Delattre-Durand & Janex-Favre, 1979), especially in the process of gametangiogamy, where short antheridial and ascogonial branches coil around each other to produce ascogenous hyphae; but on the other hand important differences are found in the further development of the primordium, in the number of covering hyphae, and in the opening mechanism of the ascus.

In *Eleutherascus* four species have been described that can be distinguished by the ornamentation of the ascospores: *E. lectardii* (Nicot apud Nicot & Durand) Arx with spinulose spores (Nicot & Durand, 1969; von Arx, 1971), *E. tuberculatus* Samson & Luiten with tuberculate spores (Samson & Luiten, 1975), *E. peruvianus* Huang with spinulose spores having a large longitudinal crest (Huang, 1975), and *E. cristatus* Emden with spinulose spores having a subequatorial crest (van Emden, 1975). Similar patterns of ornamentation can be found in species of *Ascodesmis* (van Brummelen, 1981).

The aim of this study is the description of the ultrastructure of the ascus and the ascospore wall of species of *Eleutherascus* and a comparison with that in *Ascodesmis*.

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MATERIALS AND METHODS

The material of the species in the present study was obtained from cultures, for the main part received from the 'Centraalbureau voor Schimmelcultures' (CBS) at Baarn.

In the following list details are given about the origin of the cultures: *Eleutherascus lectardii* (Nicot apud Nicot & Durand) Arx isolated by P.Lectard from salty soil of Lorraine, comm. Lay near Chateau-Salin, dép. Moselle, France, Febr. 1968 (type culture CBS 626.71, Paris Cryptogamie 2022); *Eleutherascus peruvianus* Huang — isolated by C. Lang from soil, Urubamba, Peru, 1965 (type culture CBS 101.75, University of Wisconsin U 2709); *E. tuberculatus* Samson & Luiten — isolated by B. Luiten from sandy soil under *Calluna vulgaris*, Aalten, Netherlands, Jan. 1974 (type culture CBS 144.74); *Ascodesmis nigricans* Tiegh. — isolated by Tigelaar from sandy soil near Wageningen, Netherlands, May 1968 (culture CBS 389.68); *Ascodesmis microscopica* (Crouan) Seaver — isolated by M.J. Richardson, Edinburgh, Scotland.

Eleutherascus cristatus Emden was not included in this study because of its great similarity with *E. peruvianus* and the poor growth of the single culture available.

The cultures of *Eleutherascus* were grown on malt agar and oatmeal agar for 3 to 7 days at 30°C in the dark, those of *Ascodesmis* on yeast-extract agar for 7 to 10 days at 20°C under a diurnal photo regime of 12 hours of light and 12 hours of darkness.

As soon as asci were developing small blocks of agar with superficial growth and aerial mycelium were cut out from the plates and fixed. Various methods of fixation were applied.

ABBREVIATIONS USED.—A, ascus; AS, ascostome; AW, ascus wall; E, epiplasm; EN, endospore; EP, epispore; ER, endoplasmatic reticulum; FS, material fixed by rapid freezing, followed by freeze substitution; IL, inner layer of ascus wall; IM, investing membrane; ISW, inner zone of secondary spore wall; M, mitochondrion; N, nucleus; OL, outer layer of ascus wall; OSW, outer zone of secondary spore wall; P, periascus; PL, plugged septal pore; PM, plasmalemma; PW, primary spore wall; S, ascospore; SP, sporoplasm; SW, secondary spore wall; T, tonoplast; VA, vacuole.

If not otherwise stated, the illustrated material was fixed in 1% $KMnO_4$ and 1% OsO_4 and stained with uranyl acetate and lead citrate.

Figs. 1A, B. *Eleutherascus peruvianus*. — A. Bases of two asci, × 4800. — B. Detail of plugged pore at ascal base, × 29,000.

Fig. 1C. *Eleutherascus lectardii*, ascus wall, × 29,000.

Fig. 1D. *Eleutherascus tuberculatus*, ascus wall, × 29,000.

Fig. 1E. *Ascodesmis nigricans*, ascus wall near operculum, × 8100.

Fig. 2A *Eleutherascus lectardii*, advanced state in ascus development, × 5800.

Figs. 2B, C. *Eleutherascus peruvianus*. — B. Young ascus, uninucleate state, × 4800. — C. Advanced state in ascospore development, × 5800.

Fig. 2D. *Eleutherascus tuberculatus*, advanced state in ascospore development (FS), × 5800.

Fig. 3. *Eleutherascus lectardii*, ascospore development. — A. Showing two ripe ascospores, × 4800. — B, C. Development of endospore and epispore and differentiation of secondary spore wall, × 29,000. — D. Detail of spore wall, × 60,900.

Fig. 4. *Eleutherascus tuberculatus*, ascospore development. — A. Development of primary wall, × 4800. — B. Beginning of primary wall formation, × 29,000. — C. Advanced state in ascospore development, × 29,000. — D. Id., showing secondary wall.

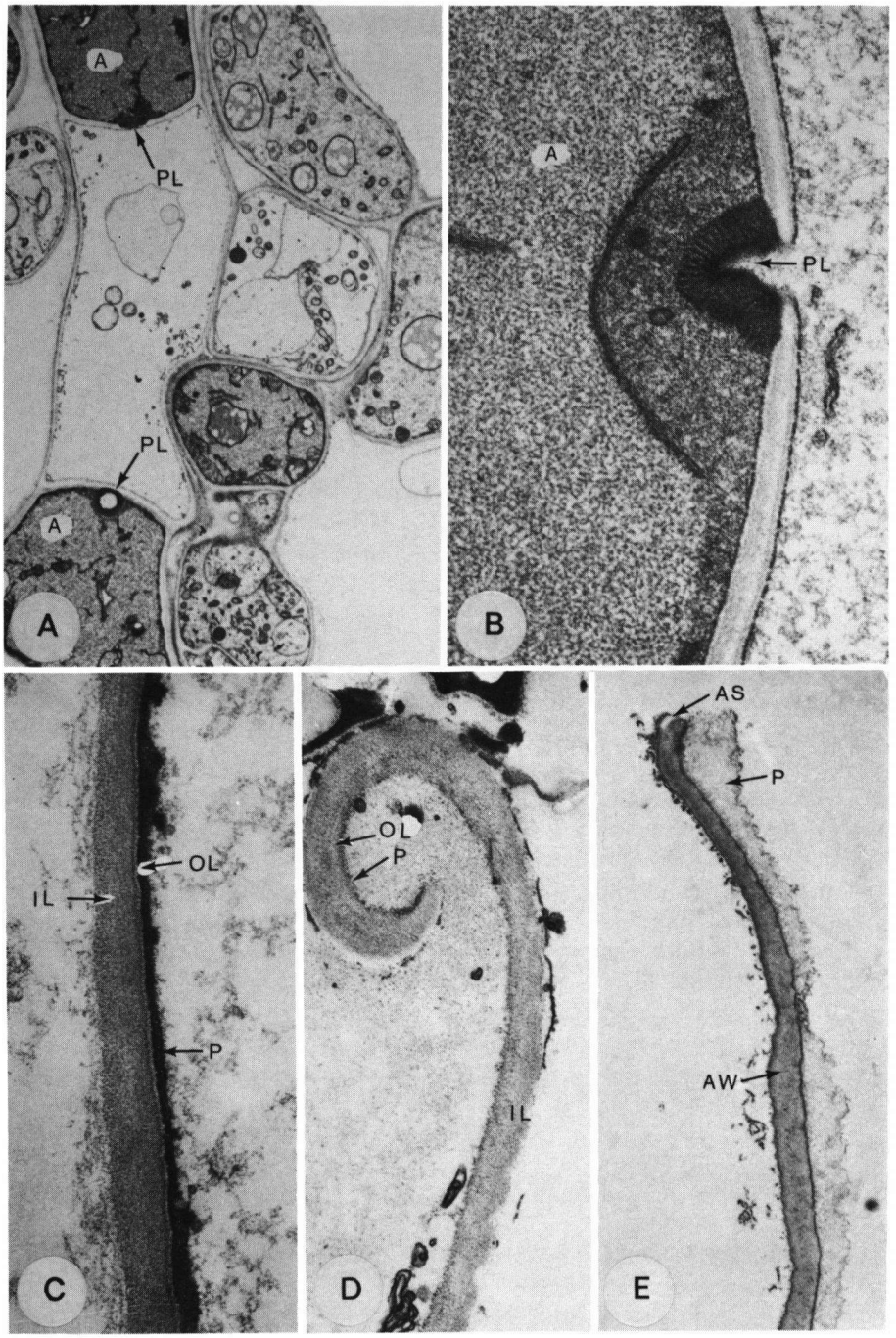

Fig. 1

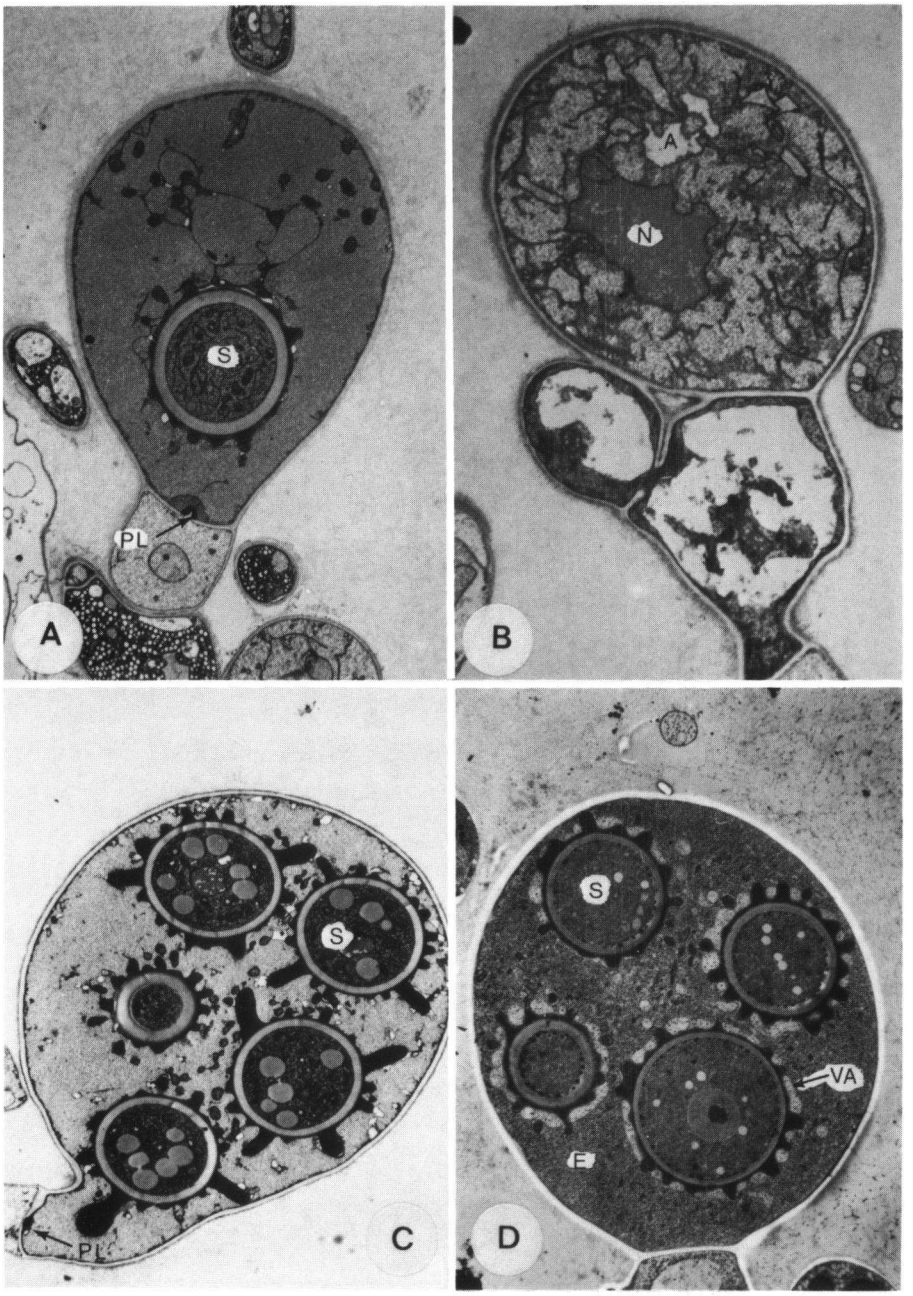

Fig. 2

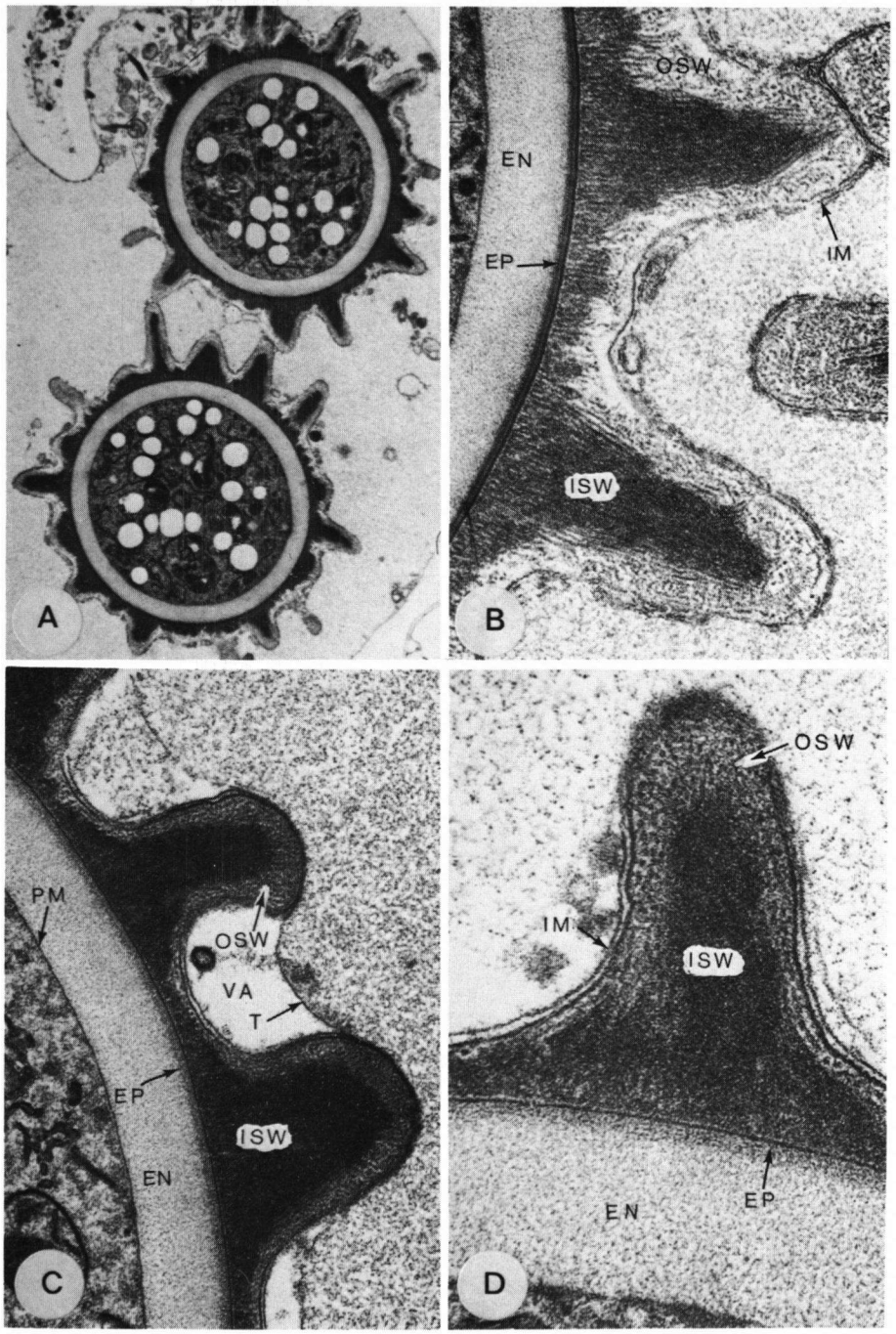

Fig. 3

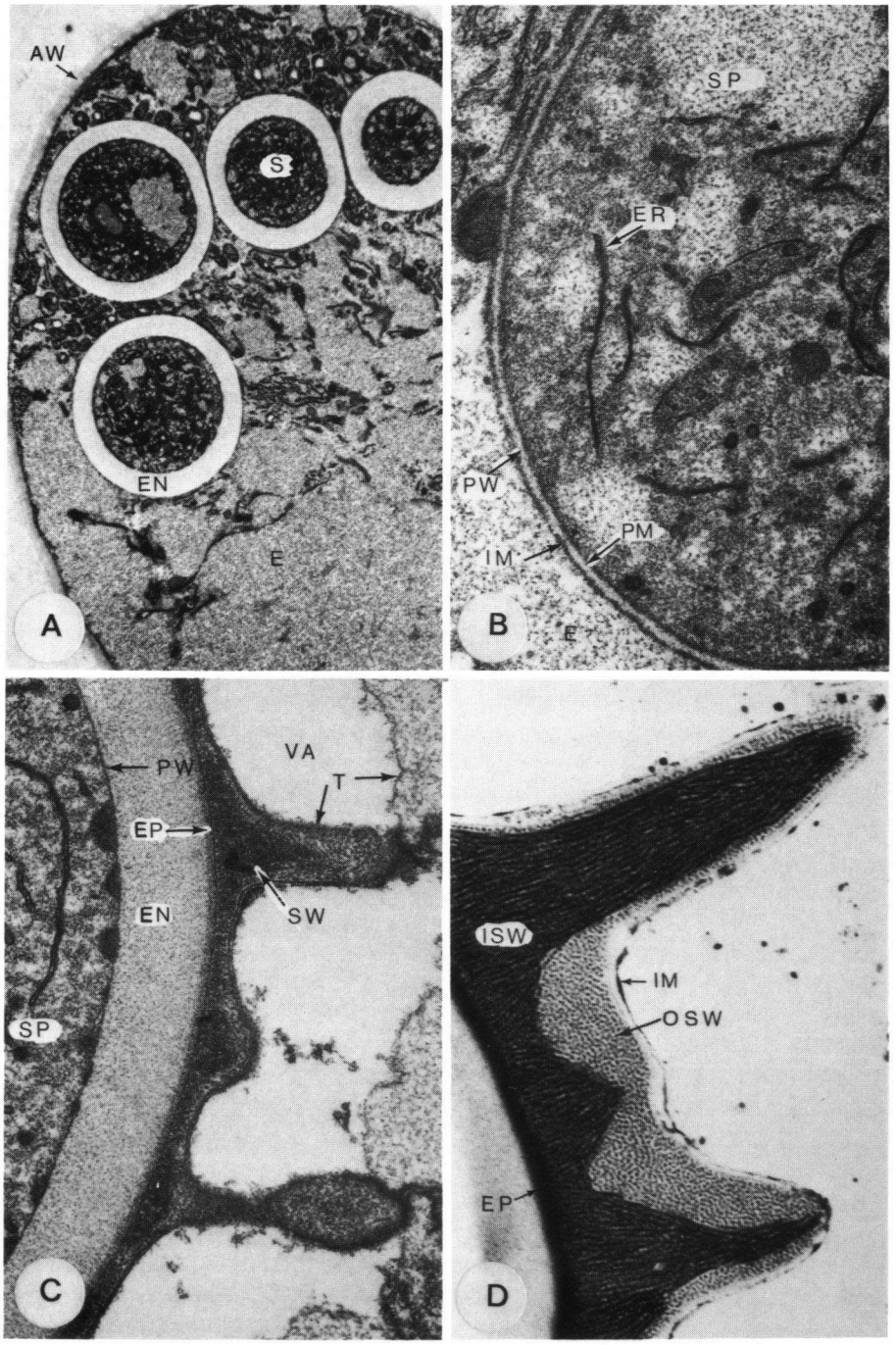

Fig. 4

For electron microscopy material of all species was fixed using chemical fixation procedures (van Brummelen, 1986). In addition, part of the material of *Eleutherascus lectardii* and *E. tuberculatus* was fixed by ultra-rapid freeze fixation (for a survey see Menco, 1986; Howard & O'Donnell, 1987).

For the rapid freeze fixation very small selected parts of aerial mycelium and superficial growth on agar were brought in a small container (2 x 1 x 1 mm) of wide-meshed fine copper wire and rapidly plunged in liquid propane at –180°C. After the specimens were frozen the containers were quickly placed in liquid nitrogen for transfer to precooled 4 ml polypropylene vials ('Eppendorf') containing 2 ml of a solution of 1% OsO_4 in anhydrous acetone.

Freeze substitution (FS) was carried out at –80°C for approximately 72 hours, after which the vials were allowed to warm up slowly during one night to –30°C and afterwards to room temperature during two hours. After several rinses in acetone the specimens were en bloc stained in 1% uranyl acetate in acetone for one hour and infiltrated gradually with Epon. Sections were stained with uranyl acetate and lead citrate before examination on a Philips EM 300 electron microscope.

To keep the terminology for ascospore layers as simple as possible close adherence is observed to the system of terms used for example by Carroll (1966, 1969), Wells (1972), Merkus (1973, 1976), and Beckett (1981).

OBSERVATIONS

THE ASCUS OF ELEUTHERASCUS

The early development of gametangia and asci can easily be followed in the species of *Eleutherascus* under observation, as these structures are freely exposed on the aerial mycelium or on the surface of the agar medium. This development fully agrees with the process described by Durand (1969) for *E. lectardii* and by Huang (1975) for *E. peruvianus*.

The young asci are subglobular to obovoid. In *E. lectardii* and *E. tuberculatus* they are often solitary and rarely in pairs, while they are rather frequently found in pairs or even small groups in *E. peruvianus* (Fig. 2).

After karyogamy a large fusion nucleus is formed in the central part of the ascus. At about this stage the septa formed at the base of the ascus between the ascus and the stalk cell and between the ascus and the terminal cell of the former crozier increase in thickness and become plugged. Initially the plugs are associated with endoplasmatic reticulum (ER). Subsequently they become electron dense and are situated as conspicuous hemispherical structures, of 700 to 1400 nm in diameter, at the ascal side of the septal pore. These plugs separate the contents of the ascus, rich in ascoplasm, from the degenerating contents of the stalk cell and the crozier end cell (Figs. 1A, B).

A fine radial striation can be observed in the ascal plugs after permanganate-OsO_4 fixation (Fig. 1B). On later development of the ascus the hemispherical plugs were often found collapsed or deformed, apparently by pressure from the ascal side.

On further development of the young diploid ascus different degrees of polarity can be observed. Meiosis may take place in the central part of the ascus in *E. peruvianus* (Fig. 2B) and

E. lectardii, but also in the extreme distal part of the ascus, as usually observed in *E. tuberculatus* (Fig. 4A) and occasionally in *E. lectardii*. In the latter case endoplasmatic reticulum, mitochondria, and small vacuoles concentrate in the distal part and large vacuoles can be found in the proximal part of the ascus.

After ascosporogenesis and subsequent spore maturation, the ascospores increase strongly in size and become usually located in the more central part of the ascus.

In all species of *Eleutherascus* studied mature ascospores remain in the ascus for a very long period. The full-grown asci are subglobular or obovoid and reach 40–50 × 35–40 µm in *E. lectardii* and *E. tuberculatus*, and 27–42 × 26–40 µm in *E. peruvianus* (Figs. 2A, C, D). In all species the ascal wall reaches a thickness of about 250 nm and consists of an inner 200–240 nm thick, relatively electron dense layer and an outer, about 15 nm thick, more rigid, electron transparent layer (Fig. 1A). The wall of the mature ascus gradually disintegrates and ruptures completely irregularly. As a result of different physical properties of both wall layers the wall curls outward along the fracturing line (Fig. 1D)

The ascospore wall

In all species studied, after three nuclear divisions eight nuclei are formed, which all or in part may develop into uninucleate ascospores. Each nucleus becomes surrounded by a double unit membrane. The primary wall is formed between both unit membranes (Fig. 4B); the inner delimiting membrane becoming the sporoplasmalemma and the outer delimiting unit membrane becoming the ascospore investing membrane. This primary wall consists of electron transparent material and gradually increases in thickness, reaching in permanganate fixed material in *E. lectardii* 370–450 nm (Figs. 3B–D), in *E. tuberculatus* 400–430 nm (Figs. 5B, C), and in *E. peruvianus* 370–450 nm (Figs. 6B, C).

During the formation of the secondary wall the primary wall shows a gradual internal differentiation into an inner electron transparent zone, the endospore, and an outer electron dense zone, the epispore.

When the epispore is fully differentiated it is sharply delimited and generally consists of about three closely spaced electron dense lamina separated by electron transparent ones. In *E. lectardii* even up to five electron dense lamina can be observed. This very constant epispore reaches a thickness of 35–45 nm in *E. lectardii* and *E. peruvianus* and 50–60 nm in *E. tuberculatus*. At full maturity the epispore forms a resistance against the penetration of chemical compounds of fixation and embedding. This usually causes poor results with ultratomy of mature ascospores after chemical fixation. Ultra-rapid freezing followed by freeze substitution proved to be a superior alternative.

The development of the primary wall and its differentiation shows a great similarity between the three species of *Eleutherascus* under observation. In the end also the endospore becomes slightly more electron dense, and especially in *E. tuberculatus* often a vague sublayering can be made visible after contrasting the sections with bariumpermanganate.

The development of the secondary wall shows a greater variation, corresponding with the differences in spore ornamentation.

ELEUTHERASCUS LECTARDII. — Fig. 3

In specimens fixed in glutaraldehyde-OsO_4, in permanganate-OsO_4, and after freeze substitution (FS) with OsO_4, as soon as the outer delimiting spore membrane or investing membrane, separates from the primary wall, the space between them is filled with new wall material. This secondary wall material is moderately electron dense for only a very short period and then differentiates into an inner zone of electron dense material directly on the outer surface of the epispore and an outer zone of fairly electron transparent material more or less parallel to the investing membrane. At a certain point the investing membrane becomes elevated to form rather narrow spines 270–1900 nm high.

The electron density of the inner zone is usually so high that it is difficult to reveal its structure. But in thin sections of OsO_4-fixed material the inner zone shows electron dense tubular structures with a diameter of 10–17 nm and a mutual distance of about 25 nm, arranged perpendicular to the epispore. The outer zone consists of the same type of tubular structures with about the same diameter and distance, but irregularly arranged or more or less parallel to the investing membrane.

On further development of the secondary wall the epiplasm changes; at first its organelles concentrate around the investing membrane and at the inner side of the ascoplasmalemma. The amount of glycogen granules increases.

On the outside of the ascospores the spaces between the spines are rather regularly filled with large vacuoles. These vacuoles develop simultaneously with the ornamentation and it is not clear whether the spines are the result or the cause of the pattern of vacuoles. Later these vacuoles fuse to form larger vacuoles, each surrounding a single spore. Finally this often results in a single enormous vacuole, filling the major part of the ascus and surrounding all mature spores. In the end all epiplasm disappears and the investing membrane is often no longer recognizable.

The sporoplasm of the mature spores has increased in electron density and has developed some small vacuoles.

ELEUTHERASCUS TUBERCULATUS. — Figs. 4, 5

The development of the secondary wall starts before the primary wall is fully differentiated. The investing membrane is locally lifted up and secondary wall material is deposited. In ascospores characteristic of *E. tuberculatus* this material is fairly electron dense from the beginning and differentiates very soon into two zones, an inner electron dense zone covering the whole of the epispore and an outer more electron transparent zone along the investing membrane.

The secondary wall takes the shape of a layer, 279–990 nm thick, with isolated rounded, truncate conical warts. Almost from the beginning the inner zone consists of electron dense, very closely placed, parallel tubular structures, 10–17 nm wide, at a mutual distance of 25–35 nm. The outer zone consists of very similar tubular structures, 10–17 nm wide and 20–25 nm apart, more irregularly arranged and more or less parallel to the investing membrane.

The investing membrane is rather persistent and often still visible in the mature spores. On the outside of the secondary spore wall the spaces between the warts are fully or only partly

filled with vacuoles. These images sustain the view that the shape, size, and position of the vacuoles depend on the position of the warts, and that these vacuoles do not play a primary role in the formation of the ornamentation.

During maturation of the spores large vacuoles are formed in the epiplasm, organelles and glycogen disappear, and in the end all epiplasm has dissolved completely. The sporoplasm then contains several small vacuoles and shows an increased electron density.

Especially the part of the material fixed by ultra-rapid freezing, followed by freeze substitution, shows superior images with a much better preservation of the structure of organelles, membranes, vacuoles, and tubules.

ELEUTHERASCUS PERUVIANUS. — Fig. 6

The development of the secondary wall is studied in material fixed in $KMnO_4$-OsO_4 or glutaraldehyde-OsO_4. The differentiation in this species proceeds extremely fast. When the investing membrane is locally lifted up the material deposited on the primary wall becomes almost fully electron opaque after poststaining the sections with uranyl and lead salts. Soon two zones can be distinguished within this secondary wall material. The differences between both zones are evident, but less clear and contrasting than in *E. lectardii* and *E. tuberculatus*.

The secondary wall takes the shape of blunt spines or warts (270–2400 nm high), one or a few short ridges and a single longitudinal or subequatorial crest (4–10 μm wide). At maturity the arrangement of the spores is such that the large subequatorial or longitudinal crests are more or less parallel to the ascus wall, while shorter ridges may point toward the centre.

THE ASCOSPORE WALL IN ASCODESMIS

For comparison material of two species of *Ascodesmis* has been observed after treatment with exactly the same procedures as used in the study of *Eleutherascus*. The material was fixed in $KMnO_4$-OsO_4 and in glutaraldehyde-OsO_4.

Ascodesmis nigricans has broadly ellipsoid ascospores (9.5–12.5 × 7.5–10 μm) ornamented with isolated or rarely connected spines (1–1.5 μm high), while *Ascodesmis microscopica* has also broadly ellipsoid ascospores (11–15.5 × 8–13.5 μm) which, however,

Fig. 5. *Eleutherascus tuberculatus*, ascospore development. — A. Development of endospore and epispore and differentiation of secondary wall (FS), × 9900. — B, C. Id., × 29,000. — D. Detail of secondary spore wall, fixed in 1% $KMnO_4$ and 1% OsO_4, × 60,900.

Fig. 6. *Eleutherascus peruvianus*, ascospore development. — A. Almost mature ascospore, × 6500. — B, C. Development of endospore and epispore and differentiation of secondary wall, × 29,000. — D. Id., also showing part of a ridge, × 29,000.

Fig. 7. *Ascodesmis nigricans*, ascospore development. — A. Part of an ascus in advanced state of spore development, × 4800. — B. Development of endospore and epispore and differentiation of secondary spore wall, × 29,000. — C, D. Details of differentiation of secondary spore wall, × 29,000.

Fig. 8. *Ascodesmis microscopica*, ascospore development, all × 29,000. — A, B. Development of endospore and epispore and differentiation of secondary spore wall. — C, D. Details of differentiation of secondary spore wall.

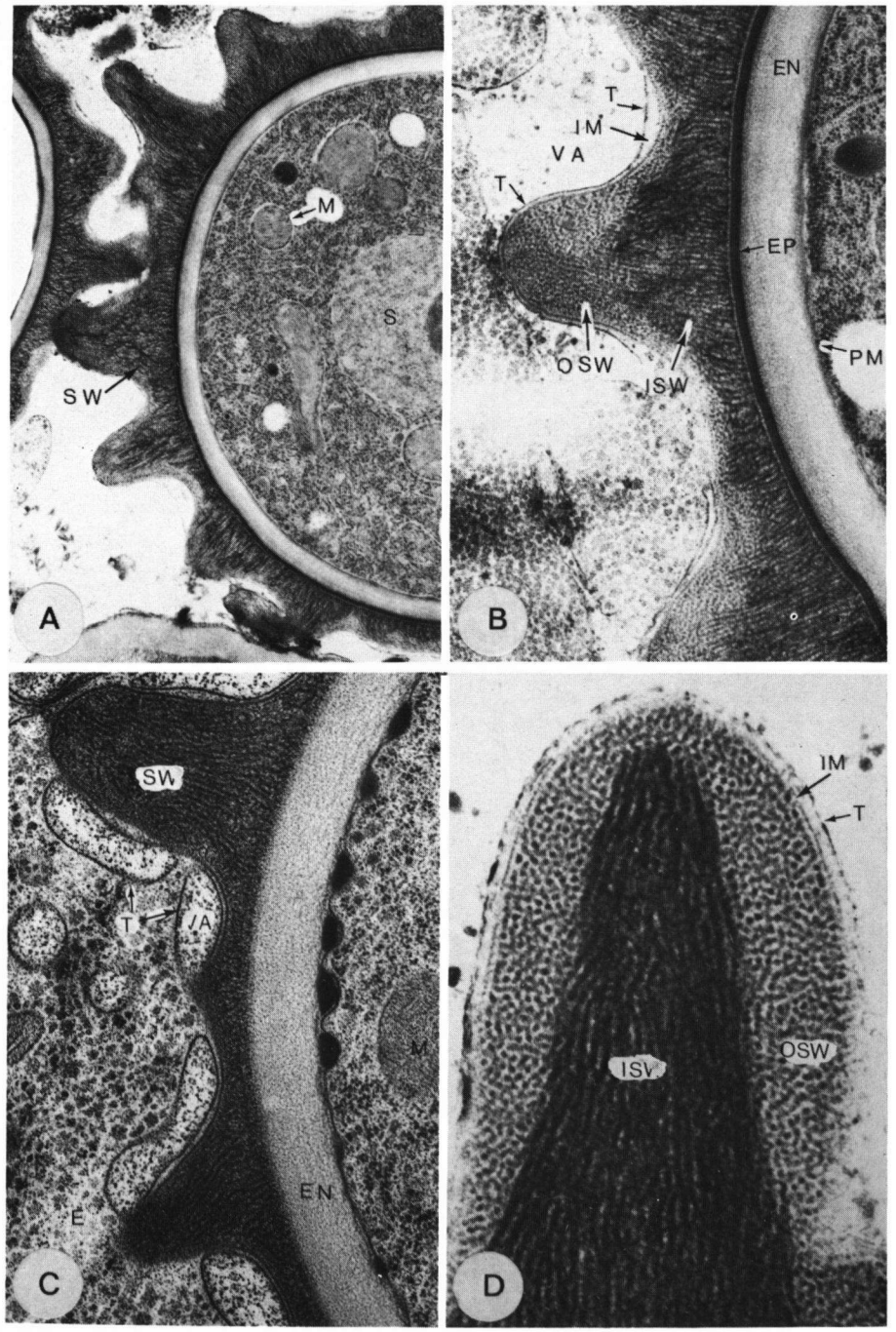

Fig. 5

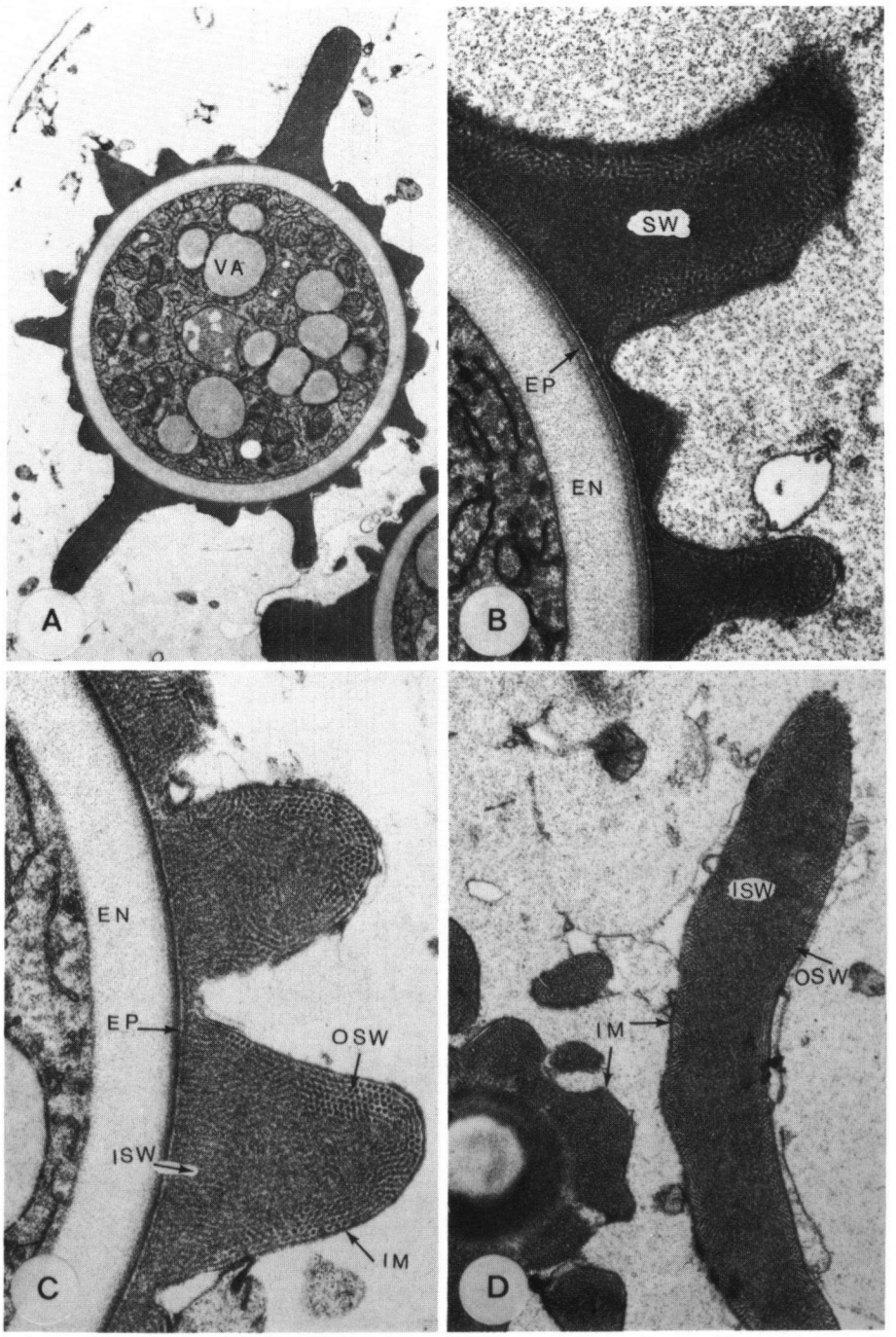

Fig. 6

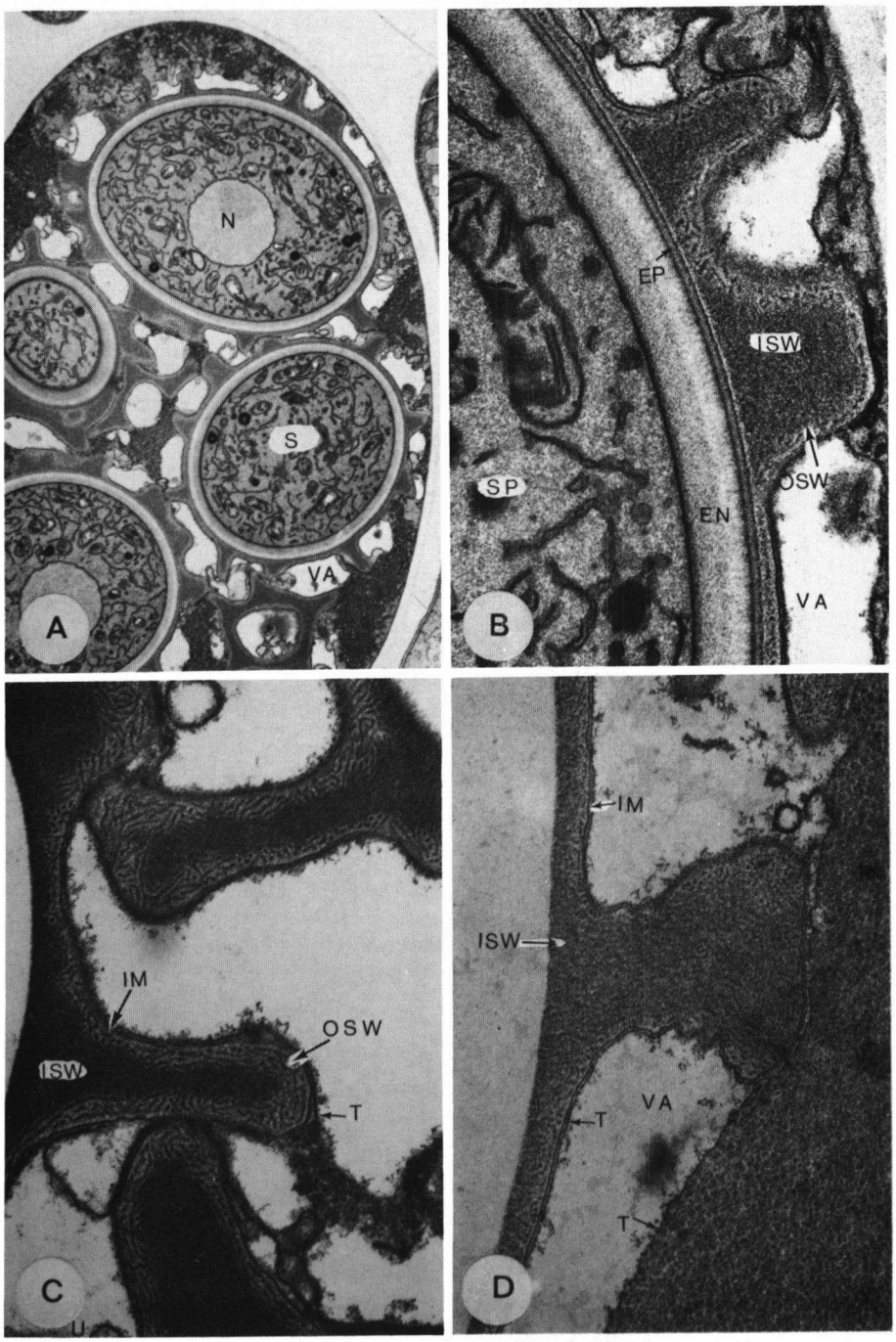

Fig. 7

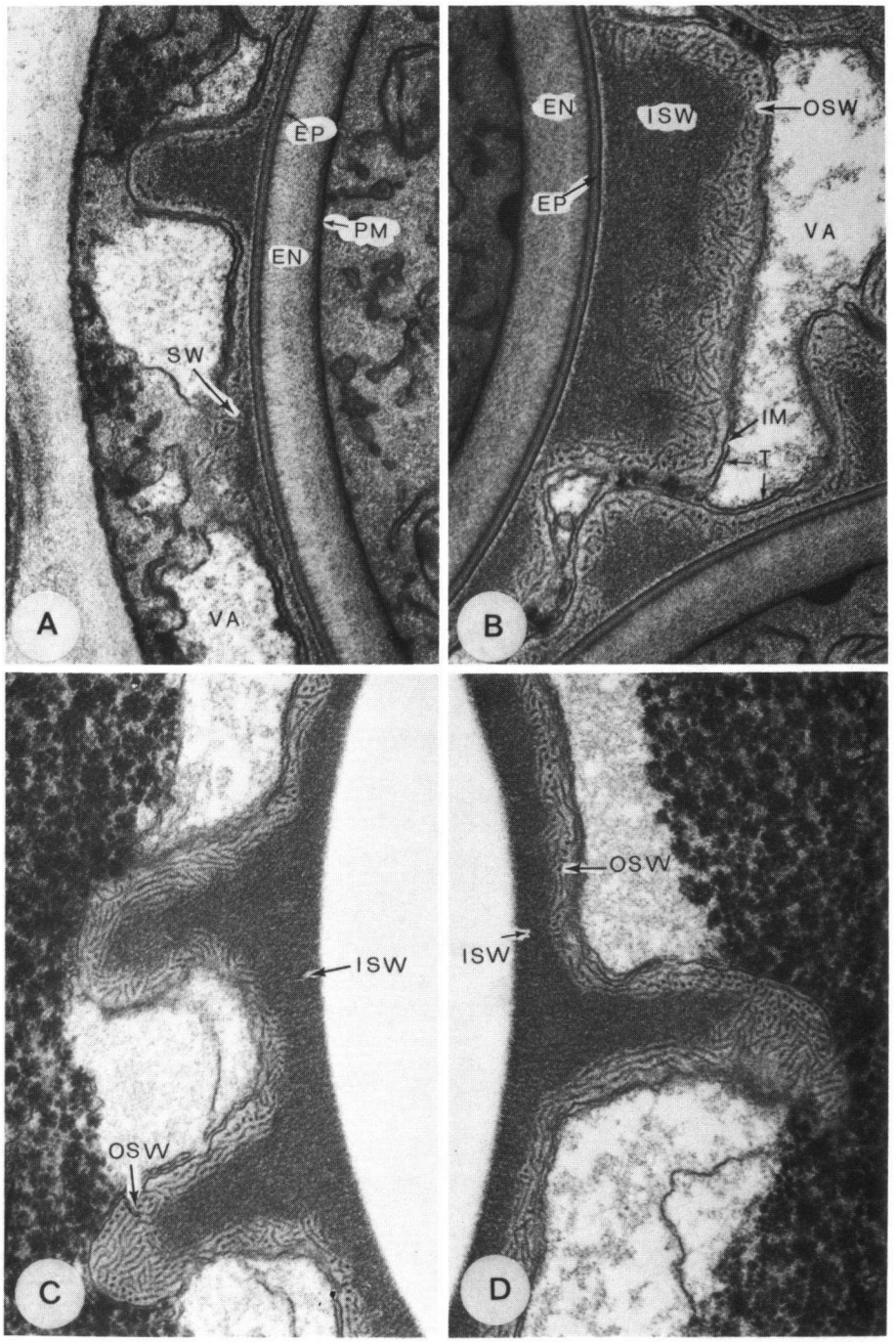

Fig. 8

are ornamented with a coarse network of ridges (up to 2 μm high), isolated short spines, and warts (cf. van Brummelen, 1981).

In both *A. nigricans* and *A. microscopica*, usually eight uninucleate ascospores are formed, according to the process described in detail by Merkus (1973).

The primary wall is formed between two unit membranes and consists of relatively electron transparent material, which becomes denser on ripening. It reaches a thickness of 300–350 nm in *A. nigricans* and 270–360 nm in *A. microscopica*. By internal redistribution of substances the primary wall becomes gradually differentiated into a more electron transparent endospore and an electron dense epispore. The fully differentiated epispore is sharply delimited and shows three to four electron dense lamina separated by electron transparent ones. It reaches a thickness of 40–60 nm in both species and becomes impermeable to chemicals used for fixation and embedding.

The mature endospore shows a vague sublayering after contrasting of the sections with uranylacetate, leadcitrate, and bariumpermanganate.

The development of the secondary wall shows some minor differences in both species of *Ascodesmis* studied.

ASCODESMIS NIGRICANS. — Fig. 7

The investing membrane is lifted up at certain points and soon afterwards also at places in between. The space formed is filled with moderately electron dense secondary wall material. This differentiates very soon into two zones, an inner more electron dense one and an outer more electron transparent one. The inner zone develops all around the primary spore wall. It is especially present in the spines but also as a thin basal zone in the lower regions between them. Only in critical observations its structure can be revealed. Then it shows electron dense tubular structures with a diameter of about 17 nm and a mutual distance of 25–35 nm, arranged perpendicular to the primary wall.

The outer zone shows tubular structures of the same diameter and distance, but more irregularly arranged or subparallel to the investing membrane. Especially in the spines the outer zone of the secondary wall becomes more important. At least one to three layers of tubules can be distinguished. The spaces between the spines are filled with large vacuoles which fuse during further ripening of the ascus. The epiplasm loses its organelles and disappears. The sporoplasm increases in electron density and shows no vacuoles or guttules.

ASCODESMIS MICROSCOPICA. — Fig. 8

Fairly electron dense material is deposited in spaces formed at the places where the investing membrane becomes lifted up from the underlying primary wall. This material differentiates almost directly into an inner electron dense zone and an outer more electron transparent one.

The inner zone is well developed in the ridges of the network and in the spines between them, while it is also present as a thin base of the secondary wall over the whole surface of the epispore. It consists of electron dense tubular structures about 17 nm wide at a mutual distance of 25–35 nm directed perpendicular to the primary wall.

The outer zone shows more clearly the same type of tubular structures of about the same

size and spacing, but irregularly arranged. In the thinnest parts of the secondary wall only a single layer of tubules is present in the outer zone, but in the spines and ridges up to nine layers of them may be counted.

The space between the ridges and the spines, just at the outside of the investing membrane, are filled up with rather large vacuoles. On further ripening these vacuoles continue to form larger ones until the major part of the ascus is filled by a single huge vacuole surrounding all eight ripe ascospores.

At this stage the epiplasm disappears almost completely. The sporoplasm becomes more electron dense and shows no vacuoles.

DISCUSSION

From the achieved results of this study and a comparison with those of previous studies (Merkus, 1973; van Brummelen, 1978) it may be concluded that, with the exception of the opening mechanism of the ascus, the ultrastructure and the development of asci and ascospores show considerable agreement between species of *Eleutherascus* and *Ascodesmis*.

Especially the development and the differentiation of the primary and secondary ascospore wall is the same in the finest details. The high degree of similarity of *Eleutherascus* with *Ascodesmis* is of special importance, since studies of Merkus (1973, 1976) have shown that the ontogeny of the secondary ascospore wall of *Ascodesmis* represents a fully unique type among the Pezizales.

In contrast to the other representatives of the Pezizales studied thus far, the secondary wall in *Ascodesmis* is internally differentiated and, moreover, the secondary wall material immediately constitutes the ornamentation without any form of condensation of secondary wall material. A permanent and rigid ornamentation is formed directly on the epispore.

Species of *Ascodesmis* represent extreme forms of fungi among the Pezizales with very simple eugymnohymenial ascomata (van Brummelen, 1967, 1972, 1981). The gametophytic system consists of a few basal cells and a bundle of paraphyses; an excipulum is completely absent. The ascogonia are formed at the end of branches that dichotomize usually twice. The sporophyte consists of a rather simple bundle of a few obovoid or broadly clavate asci.

Eleutherascus, as compared with *Ascodesmis* seems to represent a much simpler form. In *Eleutherascus* the gametophytic system consists of a very few short covering hyphae at the base; paraphyses are not formed. The ascogonia are mostly single. The sporophyte is represented by one or two naked asci only.

Despite the absence of an opening mechanism for the ascus, *Eleutherascus* is considered closely related to *Ascodesmis* and should be placed as a taxon with the simplest ascomata in the family Ascodesmidaceae of the Pezizales.

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