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Formation and Ultrastructure of *Mucor rouxii* Arthrospores

CECILIO R. BARRERA

Department of Biology, New Mexico State University, Las Cruces, New Mexico 88003

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The formation of arthrospores in *Mucor rouxii* was studied by transmission and scanning electron microscopy and light microscopy. The arthrospores formed in a random manner in terminal and internal regions of the hyphae. The earliest appearance of the arthrospores was seen by scanning electron microscopy as compartments delineated by double ridges. These ridges probably corresponded to the site of septal wall formation. The elongated compartments varied considerably in size. As the arthrospores matured, they tended to separate as a result of a gradual change in the shape of the arthrospores to a nearly spherical form and also as the result of simultaneous degradation of the outermost cell wall layer. The mature arthrospores were surrounded by a complex cell wall consisting of at least three distinct layers in addition to the original hyphal cell wall. Crystal-like structures were seen in the cytoplasm of some of the arthrospores in addition to the usual organelles such as mitochondria, nuclei, and ribosomes. Septum formation by centripetal cell wall growth from the lateral hyphal wall was documented by transmission electron microscopy. However, evidence was also found which suggested that not all internal cell wall development in the fungal hyphae during arthrosporogenesis necessarily led to the formation of mature arthrospores.

Arthrospores are asexual propagules which have been identified in a wide variety of fungi including *Mucor* spp. (3, 4), *Coccidioides* spp., *Trichosporon* spp. (1), *Trichophyton* spp. (6, 10, 11, 13), *Oidiodendron* spp., *Geotrichum* spp., *Sporendonema* spp. (8), and *Cephalosporium* spp. (17). In medically important fungi such as *Coccidioides* spp. and *Trichophyton* spp., these spores play crucial roles in the transmission of infection. However, the significance of arthrospores goes beyond the medical field. Arthrospores are important components of the life cycles of all of the fungi which produce them, they are important in the classification of these fungi, and they are also a potentially useful model for cellular differentiation. In comparison to what is known about other asexual propagules of fungi and bacteria, relatively little is known about the physiology and ultrastructural development of arthrospores, particularly in Zygomycetes. This paucity of information has been lamented as recently as 1978 (11).

Early physiological studies by Bartnicki-Garcia and Nickerson (3, 4) showed that arthrosporulation in *Mucor* spp. is stimulated by low pH, heavy inoculum, and high glucose concentrations. The findings of Jacobson (R. O. Jacobson, M.A. thesis, New Mexico State University, Las Cruces, N.M., 1977) supported some of these observations which indicated that arthrospore formation is associated with the growth of *Mucor* spp. under fermentable conditions. More recently, however, results from our laboratory have shown that the most extensive formation of arthrospores by *Mucor rouxii* occurs during growth in the absence of a fermentable carbon source in a medium consisting of yeast extract and peptone supplemented with potassium acetate (J. Serna, P. Olona, and C. R. Barrera, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, I132, p. 106). This stimulation of arthrosporulation by acetate is presently being investigated in our laboratory.

With respect to ultrastructural studies, Cole and Kendrick (7, 8) published a number of studies on the arthrospores of the Hyphomycetes *Oidiodendron* spp., *Geotrichum* spp., and *Sporendonema* spp. In *Geotrichum candidum*, Hashimoto et al. demonstrated the conversion of complex hyphal septa into arthrospore septa (12). A significant amount of work has also been done on the arthrospores of *Trichophyton* spp. including ultrastructural studies (6, 10, 11, 13). The only ultrastructural study of arthrospore development in a Zygomycete, however, is a freeze-fracture analysis of *Mucor javanicus* by Takeo and Nishiura (22). The present paper provides a detailed ultrastructural study of arthrospore formation in *M. rouxii*, with an emphasis on septum formation, by using transmission and scanning electron microscopy and light microscopy.

MATERIALS AND METHODS

Organism and growth conditions. *M. rouxii* (Calmette) Wehmer NRRL 1894 was grown in a medium containing 10 g of peptone, 3 g of yeast extract, and 25 g of potassium acetate per liter of medium. The pH was adjusted to 6.5 with sulfuric acid. This medium (YEP-acetate) was inoculated with 10^5 sporangiospores per ml, and growth occurred at 28°C for 24 h.

Light microscopy. Growth of *M. rouxii* was observed by light microscopy with a Leitz Ortholux microscope fitted with a Leitz interference contrast device T and 35-mm Leica camera. The organism was allowed to grow on a culture slide with a small amount of YEP-acetate medium.

Scanning electron microscopy. Approximately 10 ml of culture was passed through a Swinnex-25 syringe filter with a Gelman Metricel 0.45-μm filter. The cells were washed with three equal volumes of double distilled water. The mycelial mat was removed from the filter and placed in a solution of 0.1 M sodium cacodylate buffer (pH 7.4) with 5% (vol/vol) glutaraldehyde at 4°C for 2 h. The mycelial mat was then washed three times with 0.1 M sodium cacodylate buffer and treated with osmium tetroxide and thiocarbohydrazide by the procedure of Kelley et al. (14). The specimens were washed once with 0.1 M sodium cacodylate and twice with double distilled water. Dehydration of the material was accomplished by serial exposure to 20, 40, 70, 80, 95, and 100% ethanol. The specimens were then dried in a Ladd critical point dryer, mounted on aluminum stubs, and placed in a Philips SEM 501 for observation.

Transmission electron microscopy. Cultures were harvested, washed, and fixed with glutaraldehyde as described above. After fixation, the cells were washed and embedded in a small volume of Difco noble agar. Specimen-containing agar blocks approximately 2 mm by 2 mm by 2 mm were cut with a razor blade and placed in 1% osmium tetroxide in 0.1 M sodium phosphate buffer (pH 7.2) at 4°C for 2 h. The specimens were then washed three times with 0.1 M sodium phosphate buffer and three times with double distilled water. The material was then dehydrated by serial passage through 50, 70, 80, 95, and 100% ethanol and two changes of 100% propylene oxide. It was then placed in a 1:1 mixture of propylene oxide and Spurr embedding medium (21) overnight followed by 60 min in a 1:3 mixture of propylene oxide and embedding medium. The specimen material was finally placed in Spurr embedding medium which was allowed to cure at 70°C for 18 h. The embedded material was sectioned with an LKB ultramicrotome with a diamond knife. Silver sections were placed on copper grids and stained with uranyl acetate and lead citrate. The ultrathin sections were observed in a Siemens Elmiskop 1A electron microscope.

RESULTS

Figure 1 shows the appearance of arthrospore chains as seen by light, scanning, and transmission electron microscopy. The order of arthrospore formation, as determined by time-lapse light microscopy, was random rather than acropetal or basipetal. Furthermore, arthrospores formed terminally and subterminally as seen in Fig. 1A. Figure 1B clearly shows the great variation in arthrospore size during early stages of development. However, as will be emphasized later, it is unlikely that all compartments have the potential to develop into mature, viable arthrospores. As spore maturation progressed, the spores generally became more spherical. Figure 1C shows arthrospore chains and mycelia as seen by scanning electron microscopy, and Fig. 1D shows an ultrathin longitudinal section of an arthrospore chain as seen by transmission electron microscopy. The elongated appearance of the arthrospores in Fig. 1D indicated that they were at an early developmental stage before they became spherical.

Figure 2A is a scanning electron micrograph of an arthrospore chain at a very early stage of development. The immature spores were oblong, and ringlike structures, which probably corresponded to the site of the septal cell walls for each compartment as seen in Fig. 1D, were evident. It is likely that the septal walls appeared as ridges due to slight shrinkage of the hyphae during specimen preparation since no ridgelike structures were seen in the corresponding transmission electron micrographs.

Synthesis of the arthrospore cell wall apparently formed separate and distinct layers under the existing hyphal cell wall. This is clearly shown in Fig. 2B as seen by scanning electron microscopy and in Fig. 2C as seen by transmission electron microscopy. Note in Fig. 2C that the existing hyphal cell wall formed a continuous outer layer over the arthrospore compartments and that the arthrospore cell wall developed under the existing hyphal cell wall.

The complexity of arthrospore cell wall development is more clearly seen in Fig. 2D. This is a higher magnification transmission electron micrograph of two large arthrospore compartments separated by a very small compartment which in fact extended completely across the hypha. Note again that the outermost cell wall layer (Fig. 2D, a) was continuous over all three compartments. This layer appeared to be of a fibrillar nature. Under this layer were the cell wall layers of the arthrospore itself, and there appeared to be at least three distinct regions (Fig. 2D, b, c, and d). The outermost layer was eventually degraded during arthrospore maturation, allowing for separation of the arthrospores. The removal of this layer by what seemed to be enzymatic digestion is shown in Fig. 2E.

Formation of the arthrospore septa appeared to involve centripetal growth of the cytoplasmic membrane followed closely by deposition of cell wall material. The electron micrographs shown in Fig. 3 are of serial sections of a hyphal segment showing an incomplete septum. The sections progressed from a medial longitudinal

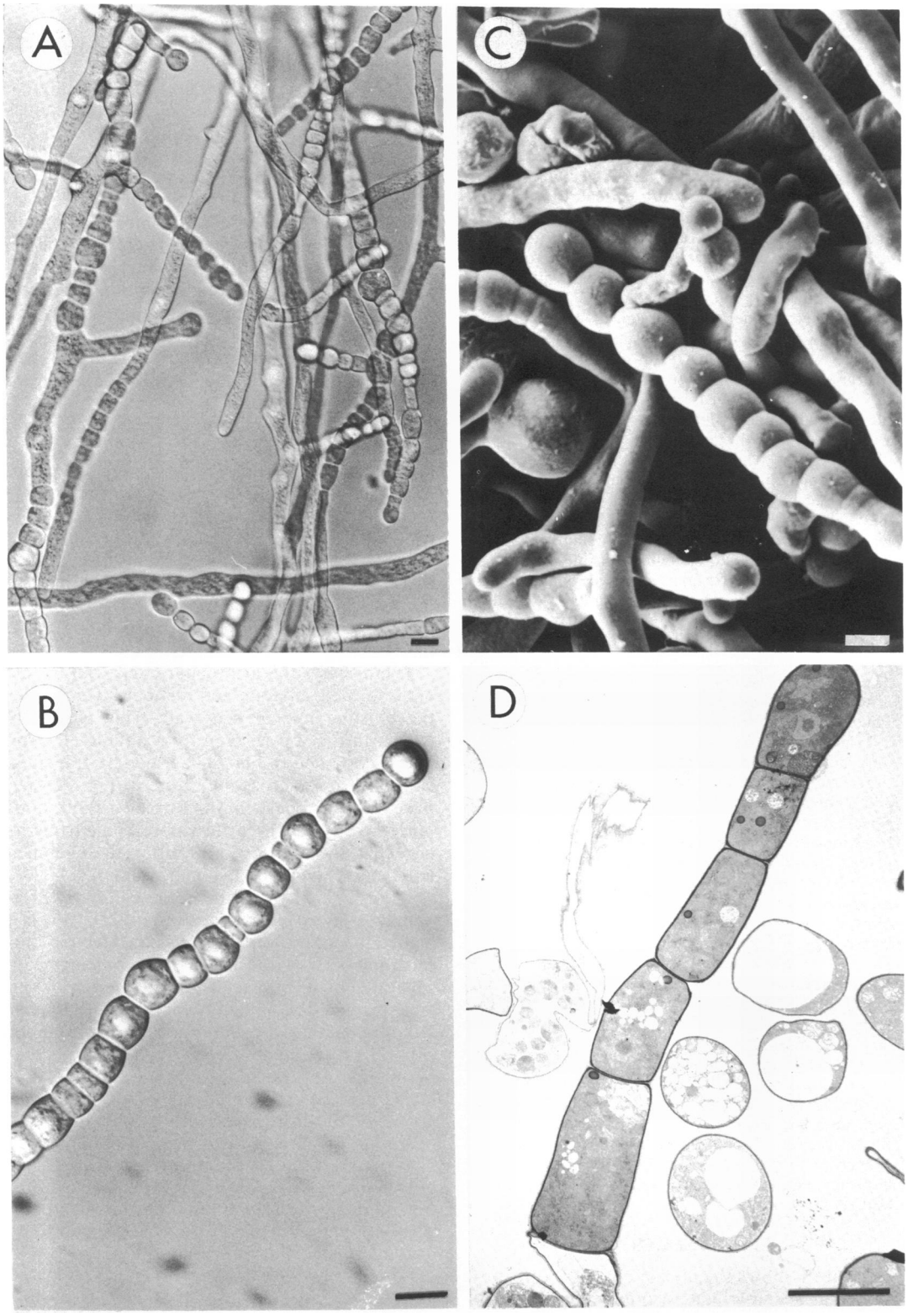

FIG. 1. Gross appearance of *M. rouxii* arthrospores. (A) Light microscopy revealed extensive arthrospore formation in yeast extract-peptone medium after aerobic growth for 24 h. Bar, 10 μm. (B) High-magnification light microscopy showed significant variation in arthrospore size and shape. Bar, 10 μm. (C) Scanning electron micrograph of arthrospores at various stages of maturity. Bar, 5 μm. (D) Transmission electron micrograph of arthrospore chain. Bar, 5 μm.

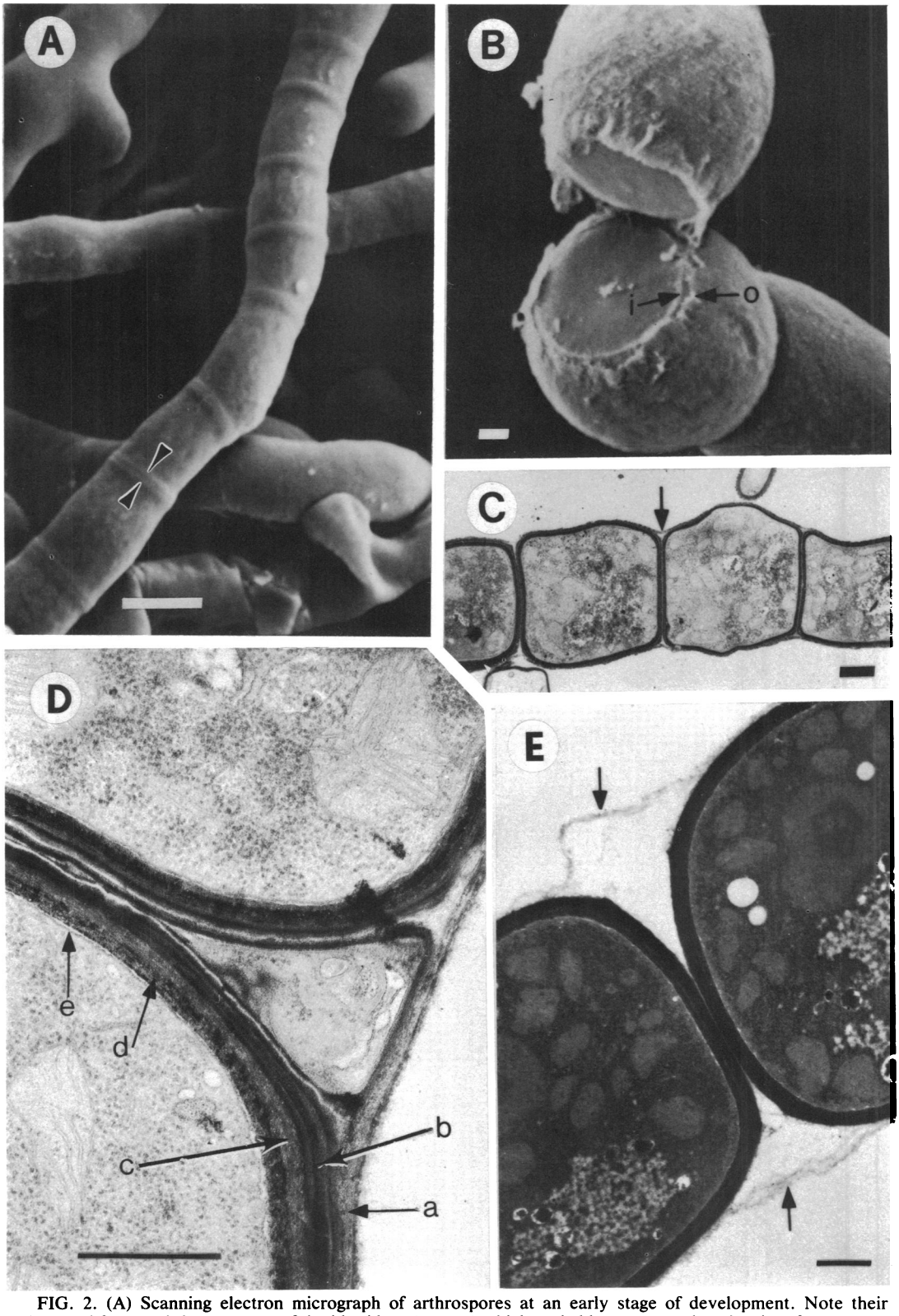

FIG. 2. (A) Scanning electron micrograph of arthrospores at an early stage of development. Note their elongated form and the presence of double ridges (arrows) which probably correspond to the site of transverse septa. Bar, 5 μm. (B) Scanning electron micrograph showing an outer cell wall layer (o) which probably corresponds to the original hyphal cell wall and an inner layer (i) which corresponds to the arthrospore cell wall proper. Bar, 1 μm. (C) Transmission electron micrograph showing a continuous outer cell wall layer (arrow) and a separate, electron-dense, inner layer which completely surrounds each arthrospore. Bar, 1 μm. (D) Ultrastructure of the arthrospore cell wall. Each arthrospore was enclosed by an outermost layer (a) followed by at least three internal layers (b, c and d). The cytoplasmic membrane is also indicated (e). Bar, 0.5 μm. (E) Degradation of outermost cell wall layer (arrows) as seen by transmission electron microscopy. The micrograph was overexposed to enhance the contrast of the cell wall remnants. Bar, 0.5 μm.

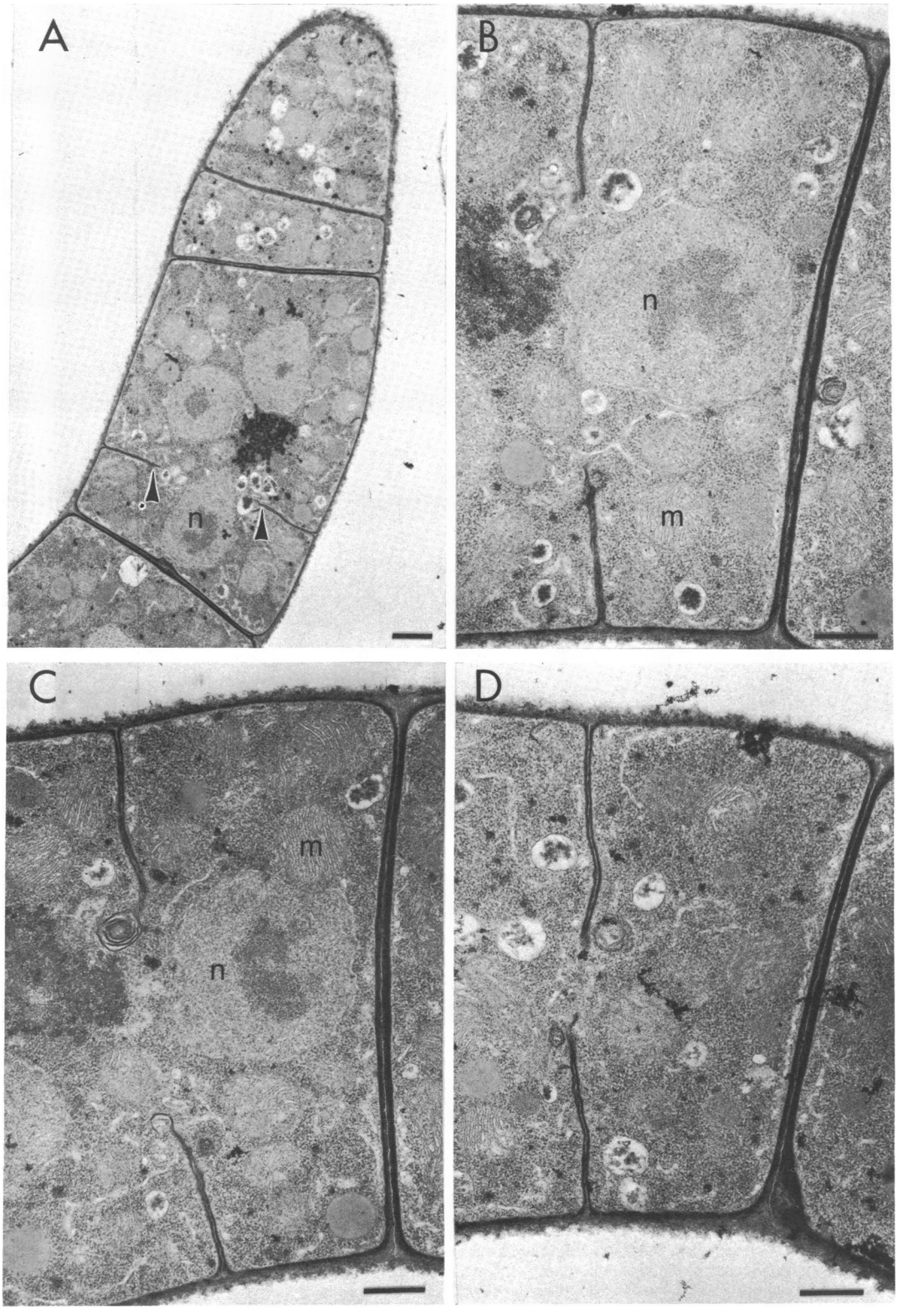

FIG. 3. Formation of an arthrospore septum by centripetal cell wall growth. (A) Overall appearance of hyphal region with three complete septa and one incomplete septum (arrowheads). Note the difference in size of each compartment. Bar, 1 μm. (B, C and D) Electron micrographs of nonconsecutive serial sections of the incomplete septum shown in (A). Note that the nucleus (n) seen in A, B, and C is not visible within the plane of the section shown in (D). Several mitochondria (m) are also visible. Bars, 0.5 μm.

plane in Fig. 3A to a lateral longitudinal plane in Fig. 3C and D. It is likely that a newly completed septum would be similar to that shown in Fig. 4A. The cytoplasmic membranes of the adjacent arthrospores were separated by a relatively small amount of cell wall material. Note that crystal-like structures are also seen in this figure. A variety of crystal-like inclusions have been observed in other fungi (2, 16, 18, 19), and the nature and possible significance of these structures in *M. rouxii* is presently under investigation in our laboratory.

There was also ample evidence that not all cell wall synthesis was directed toward the formation of readily recognizable arthrospore compartments. For example, Fig. 4B shows a peripheral area of a transverse section of a hypha, and Fig. 4C shows a longitudinal section.

Further evidence of the complexity of compartment formation in *M. rouxii* can be seen in Fig. 5. Figure 5A shows a region of arthrospore formation where a closed compartment was seen on one side of the hypha and an incomplete compartment appeared on the opposite side. Note the irregular appearance of the outermost cell wall layer which suggested partial degradation of this layer similar to the layer shown in Fig. 2E. Figure 5B is a higher magnification electron micrograph of the incomplete compartment shown in Fig. 5A. Note that the growth of the cell wall from the lateral hyphal wall ends in a complex membranous structure which may be the site of membrane and cell wall growth. A nonconsecutive serial section of the same area seen in Fig. 5B is shown in Fig. 5C. At this plane of the cell, the incomplete compartment was smaller, and the cell wall extension from the lateral cell wall almost joined an extension from the existing septal wall. Note that this extension from the existing septal wall was not present in the section shown in Fig. 5B. An electron micrograph of another nonconsecutive serial section of the area seen in Fig. 5B and C is shown in Fig. 5D. At this plane, the cell wall was continuous from the existing septal wall to the lateral wall of the hypha. Figure 5E is a lower magnification electron micrograph of the area seen in Fig. 5D and shows that at this plane there were closed compartments on both sides of the hyphal section.

DISCUSSION

The light microscopy studies presented here show that arthrospore production in *M. rouxii* did not follow either a basipetal or acropetal pattern. In this respect, *M. rouxii*, a Zygomycete, is similar to *Oidiodendron* spp., *Geotrichum* spp., and *Sporendonema* spp. (8), all of which are Hyphomycetes. Cole (7) also characterized the formation of arthrospores in these genera as being holothallic or enterothallic. The distinction is based on whether the arthrospore cell wall forms within the existing mycelium without disruption of the existing mycelial wall during septum formation. The transmission electron micrographs shown here indicate that the arthrospores of *M. rouxii* are endoarthrospores produced by enterothallic conidiogenesis.

The disarticulation of the arthrospores (Fig. 2B and E) was more similar to the process described by Cole for *Sporendonema purpurascens* than for *Briosa cubispora*, both of which produce endoarthrospores (7). Removal of the outermost layer of cell wall, along with a change in the shape of the arthrospore from an elongated form to a nearly spherical shape, seemed to contribute to the physical separation of the spores. It is also of some interest that unlike *Geotrichum* spp., which produce holothallic spores (7), and *Coccidioides* spp. (20), the hyphae of *M. rouxii* do not have fertile regions regularly interspersed with nonfertile regions. However, it was noted that not all compartments formed during the early stages of arthrosporogenesis in *M. rouxii* resulted in mature arthrospores. Thus, there were some nonfertile regions, but they did not occur frequently or regularly. In this respect *M. rouxii* was more similar to *B. cubispora*.

The transmission electron microscopic data indicate that the development of the arthrospore cell wall is a complex process, not only in regard to overall patterns of development, but also in regard to the composition and architecture of the cell wall. Figure 2D demonstrates that the arthrosporal cell wall in *M. rouxii* consisted of at least three regions. However, close examination shows that there may be more than these three major regions. The layer which was adjacent to the cell membrane had a mottled appearance with darkly stained granules embedded in it. The next layer was of a more homogenous nature, appeared to be fibrillar, and closely resembled the structure of the existing hyphal cell wall. Beyond the fibrillar layer were several layers of alternating electron-transparent and electron-translucent regions. Figure 2E provides evidence that these cell wall layers are not only structurally different from the hyphal cell wall, but that they are also chemically distinct. The electron micrograph shows that although the hyphal cell wall layer was completely removed eventually to allow disarticulation of the arthrospores, the arthrospore cell wall appeared to remain intact. Presumably, chemical differences between the hyphal cell wall and the arthrospore cell wall allowed differential cell wall digestion by specific hydrolytic enzymes.

Patterns of septum formation which were a departure from the classical centripetal mode of

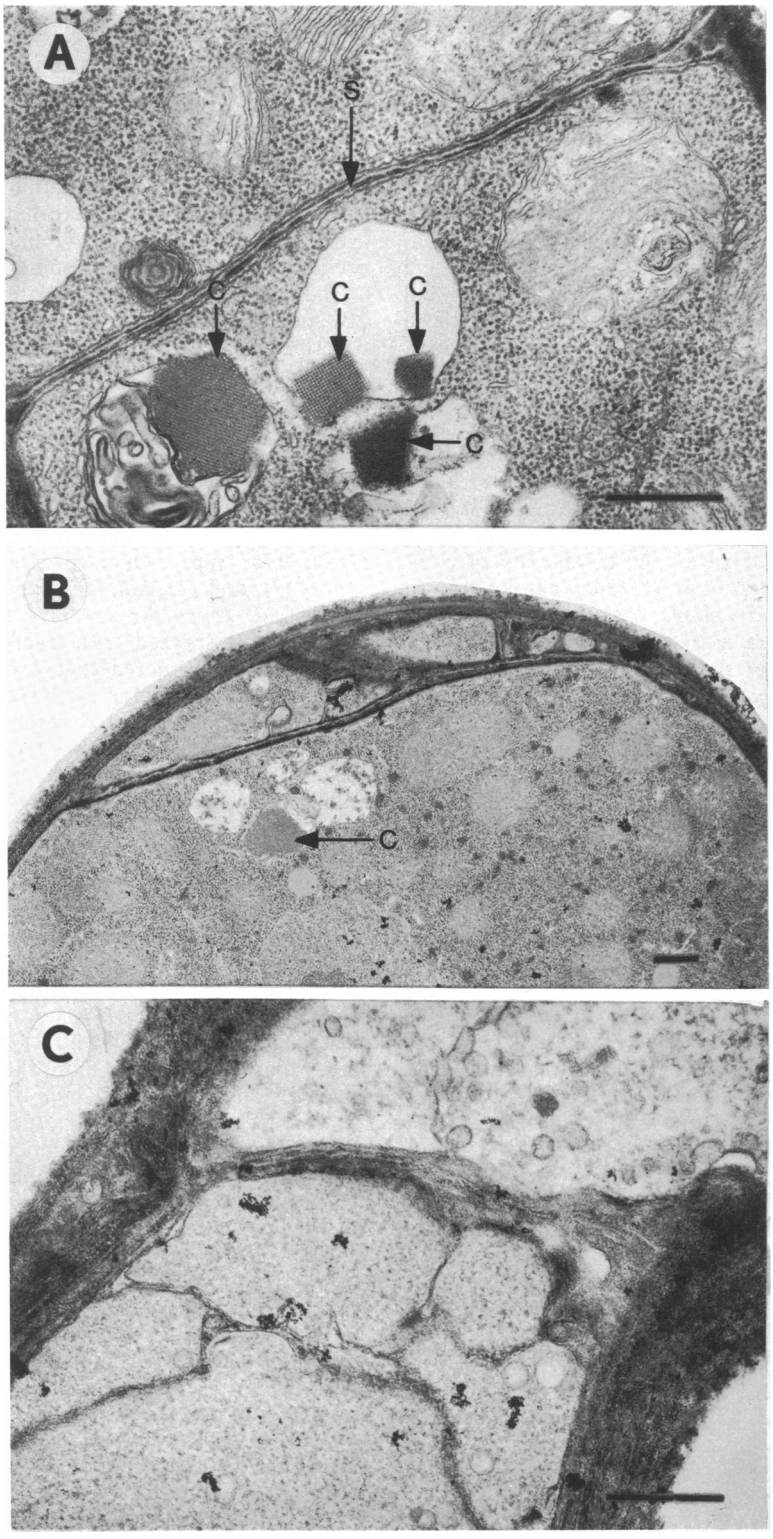

FIG. 4. (A) Transmission electron micrograph showing what could be a newly completed septum (s) with only a small amount of cell wall material deposited between the cytoplasmic membranes of adjacent arthrospores. Several crystal-like structures (c) are also visible. Transmission electron micrographs of transverse (B) and longitudinal (C) sections showing a variety of cell membrane and wall formations. A crystal-like structure (c) is also indicated in (B). Bars, 0.5 μm.

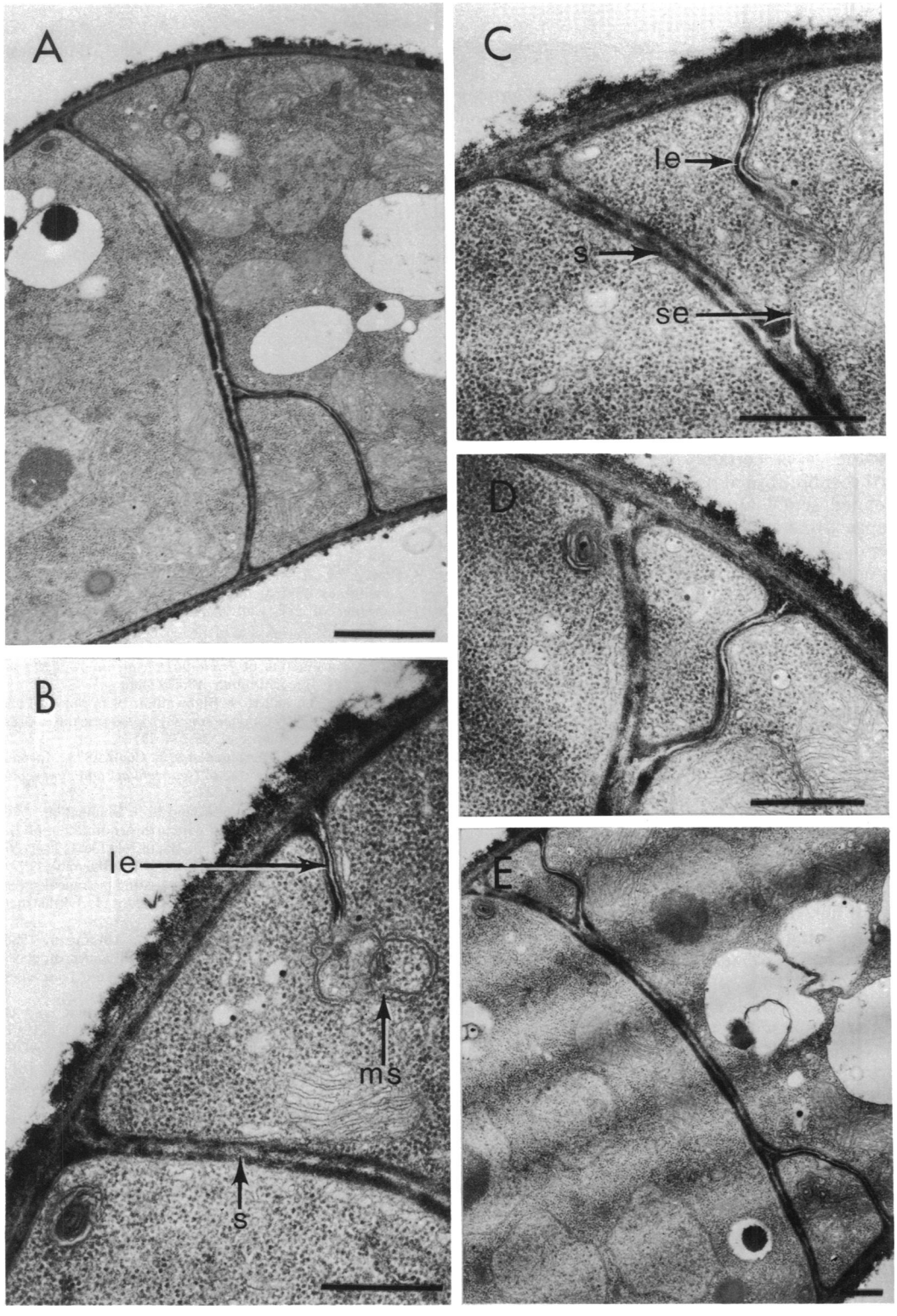

FIG. 5. Formation of a peripheral compartment around the circumference of a hypha. (A) Overall view of hyphal region with a completed peripheral compartment and an incomplete peripheral compartment. (B) Higher magnification view of the region of the incomplete compartment seen in (A). (C and D) Electron micrographs of nonconsecutive serial sections of the region seen in (B). (E) Lower magnification view of the section shown in (D). The nonconsecutive sections (B, C, and D) show a cell wall extension (le) originating from the lateral cell wall in (B) followed by formation of a septum extension (se) from an existing septum (s) in (C) and the eventual fusion of the lateral and septum extensions at the section plane in (D) to form a closed compartment. Note the membranous structures (ms) at the end of the lateral wall extension in (B). Bars, 0.5 μm.

septum formation were common, and examples are shown in Fig. 4 and 5. The series of electron micrographs in Fig. 5 suggests that it may be possible to have a peripheral compartment around the circumference of the hyphal cylinder adjacent to an existing septum. Numerous sections similar to that seen in Fig. 5E were observed during this study. It is possible that this cell wall configuration was an early stage of compartmentalization which was followed by centripetal growth of the ringlike compartment to form the typical compartment seen in Fig. 1D; however, this seems unlikely. In some cases it is clear that the compartments do not contain some of the essential organelles such as mitochondria and nuclei. The type of septum formation shown in Fig. 4 is certainly less likely to lead to arthrospore formation. The appearance of well-defined septal junctures seen in Fig. 2A does suggest that at least the peripheral region of the septa were completed well before the arthrospores began to change to a spherical shape. This is in agreement with the centripetal mode of septum formation illustrated in Fig. 3. The presence of an incomplete septum between two apparently completed septa in Fig. 3A also indicates the random mode of septum formation along a hypha.

Some of the studies now in progress in this laboratory deal with the physiological processes associated with arthrospore development and techniques for arthrospore separation. Isolated arthrospores will be used to study arthrospore resistance to chemical and physical agents as well as physiological and structural characteristics of arthrospore germination. Since some work has been done on the cell wall chemical composition of the yeast-like and filamentous forms of *M. rouxii* (5, 9) and on the cell wall composition of *Geotrichum* spp. (15) and *Coccidiodes* spp. (23), it would be desirable to investigate the chemical composition of *M. rouxii* arthrospore cell walls also. However, the data presented here indicate that this would be complicated by the presence of a hyphal cell wall layer on the outermost region of the arthrospore. It would be relatively difficult to completely remove this layer and to assure that arthrospore cell wall preparations would not be contaminated by hyphal cell wall components.

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