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Scanning Electron Microscopy of Surface and Internal Features of Developing Perithecia of *Neurospora crassa*

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Stages in the development of perithecia of *Neurospora crassa*, designated by the time elapsed after crossing, were investigated with the scanning electron microscope, from protoperithecia through perithecia. The usual examination of external features of whole specimens with this instrument was augmented by a freeze-fracture technique which allowed the viewing of development internally as well. Rapid increases in perithecial size soon after crossing were followed by the appearance, in section, of a centrum, at first undifferentiated but subsequently developing ascogenous hyphae. The perithecial beak appeared as a compact mass easily distinguishable in whole specimens from the surrounding hyphae by means of texture as well as shape. Two ascospores were photographed during emergence from an ostiole, but ostioles were found more frequently closed than open.

The development of the perithecium, which is a central event in the sexual cycle of *Neurospora crassa*, has been investigated in a variety of morphogenetic studies (7, 11, 15, 17). The scanning electron microscope (SEM), which allows three-dimensional visualization of specimens with high resolution, provides an auxilliary means of understanding morphogenetic changes.

Seale (13) recently presented an overview of the life cycle of *N. crassa* as studied with the SEM, but his treatment of perithecial development was necessarily limited by the breadth of his subject. Other SEM studies of *N. crassa* have concerned either conidia (16) or ascospores (1, 14).

The present investigation is restricted to stages in the development of the perithecium of *N. crassa*, as viewed by the SEM. These stages were observed using whole mounts and a modification of the freeze-fracture technique (8, 19). Thus we have been able to visualize perithecia both intact and in section.

MATERIALS AND METHODS

Organism and cultural conditions. *N. crassa* wild-type strain 74-OR8-1a was grown in an unlighted incubator at 25 C on Westergaard and Mitchell (18) medium in petri dishes and crossed on day 5 by adding a suspension of conidia of wild-type strain 74-OR23-1A. Prior to inoculating petri dishes with strain 74a, part of the agar surface was covered by sterile dialysis membrane (Union Carbide Corp., Chicago, Ill.). Both wild-type strains were obtained from the Fungal Genetics Stock Center, Humboldt State University, Aroata, Calif.

Microscopy. At successive intervals after crossing, small blocks of agar or squares of dialysis membrane supporting the specimens to be examined were removed with a sterile razor blade to a petri dish. No precautions were taken to shield specimens from light during sampling. A drop of aqueous 4% (wt/vol) osmium tetroxide fixative was placed in the dish without contacting the specimens, and the dish was sealed with tape and refrigerated for 12 to 96 h. After fixation, the specimens were immersed in demineralized water to which a drop of Tergitol (Union Carbide Corp., New York, N. Y.) had been added, rinsed in several changes of demineralized water, and dehydrated in a graded ethanol series.

At this point, agar blocks with specimens which were subsequently to be examined internally were subjected to a modified freeze-fracture technique (8, 19). The blocks were immersed in absolute ethanol in aluminum weighing pans, and the pans were floated on liquid nitrogen until the ethanol was frozen. The frozen blocks were fractured by random chops with a precooled razor blade. After thawing, which was hastened by addition of absolute ethanol at room temperature, the fragments were examined with a stereomicroscope, and perithecia which appeared to have been fractured were transferred to iso-amyl acetate-ethanol (50:50, vol/vol). Meanwhile, squares of dialysis membrane with specimens which were subsequently to be examined whole were transferred to the same kind of solvent mixture from the graded ethanol series. After a brief period of equilibration, the solvent mixture was replaced by iso-amyl acetate.

All specimens previously described were transferred to a critical point apparatus and subjected to critical point drying with CO_2. The dried specimens

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were attached by means of double-face tape to SEM stubs and uniformly coated with gold:palladium alloy (60:40 wt/wt) (Ladd Research Industries, Burlington, Vt.) by vacuum evaporation (Varian Vacuum Division, Portland, Ore.). Some mature perithecia, which were subsequently to be examined whole, were not fixed but were removed from the substrate and prepared by rinsing in demineralized water, air drying on SEM stubs, and metal coating as described above. All specimens were examined with a Cambridge Mark 2A Stereoscan Scanning Electron Microscope (Cambridge Scientific Instruments, Cambridge, England). The tilt angle of the specimens was 45° unless otherwise noted in figure legends.

RESULTS

The time designated for each developmental stage shown (except Fig. 1A) indicates the interval between crossing and beginning preparation of the specimen. It should be emphasized, however, that because perithecial development was not entirely synchronous, the time assigned to any given developmental stage shown may differ somewhat from the average time of that stage in the perithecial population.

Protoperithecia, which develop into perithecia after crossing, were visible on the 5-day-old mycelium of strain 74a prior to crossing. One such structure is believed to be represented in Fig. 1A, although identification is uncertain because recognition of the trichogyne is questionable. Six hours after crossing, enlarged structures believed to be incipient perithecia were evident, one of which is shown in Fig. 1B At 24 h (Fig. 1C), the perithecia had enlarged further and were surrounded by loosely woven hyphal strands. Our earliest freeze-fracture, which was performed at 24 h, revealed little internal development; an apparent incipient centrum was similar in texture to the surrounding perithecial wall (Fig. 1D). The fracture, in passing through the substrate, revealed the presence of hyphae penetrating the underlying agar. At a lower magnification at 36 h (Fig. 1E), hyphal strands are seen to span, as well as cover, the four ascocarps shown. A freeze-fractured perithecium at this same stage revealed that differentiation in the centrum had commenced (Fig. 1F).

At 48 h (Fig. 2A-C) ascogenous hyphae filled the centrum, with the rosette pattern characteristic of the asci of *N. crassa* already evident. At 60 h (Fig. 2D-F) the main developmental advancement seen was a further elongation and filling out of the asci.

In Fig. 3A (3 days) is shown the initial appearance of the perithecial beak, which became evident through the loose hyphal strands that continued to encompass the ascocarp. Up to then, the perithecia had maintained a generally spherical shape, but by 5 days (Fig. 3B) the pyriform shape characteristic of *N. crassa* was obvious, owing to the continued elongation of the beak. At a higher magnification of the beak at 5 days (Fig. 3C), the bleb-like texture of the surface and the almost complete absence of loose hyphal strands are shown. Freeze-fracture of the perithecium at 5 days (Fig. 3D) revealed the parallel arrangement of the asci containing immature ascospores, and the region of ascus attachment. Distally, the rind-like perithecial wall retained a more compacted appearance. A view of another perithecium at 5 days (Fig. 3E) shows that the walls of the ascospores were obliquely freeze-fractured, exposing the shrunken cytoplasm. (See interpretation of Fig. 4B and C, below.). The lower portion of a perithecium, sectioned at 7 days by a freeze-fracture rotated approximately 90° from that in Fig. 3D, is shown in Fig. 3F.

In Fig. 4A is seen a mature perithecium (10 days), which clearly shows the differentiation between body and beak. When this sample was taken, perithecia had already begun discharging ascospores. Freeze-fractures at 10 days near the base of the beak and at a somewhat lower plane are presented in Fig. 4B and C, respectively. In both figures, but especially at the higher magnification of Fig. 4C, the thickness of the wall of the ascospores is apparent in cross section. Our further interpretation of both these figures is that the ascus is not regularly visible in cross section and that the pithy appearance inside the wall of the ascospores was caused by shrinkage of the cytoplasm during preparation of the specimens. Ascospores are frequently discharged from perithecia with enough force to collect on the petri dish cover, and the direction of discharge is influenced by the phototropism of the beaks (2). The perithecium shown at 12 days in Fig. 4D, with the beak oriented horizontally, discharged its ascospores less forcefully in a pile a short distance away. Additional ascospores from this or other perithecia were scattered on the mycelial mat. An unfixed perithecium (the only one shown in this study) with open ostiole is seen at 12 days in Fig. 4E. The same ostiole is shown at higher magnification in Fig. 4F.

The emergence of two ascospores from an ostiole is seen at 12 days in Fig. 5A. Higher magnification of the same specimen in Fig. 5B indicates the close tolerance of the ostiole, which was only large enough to allow passage of the ascospores, one at a time, to the outside. The ascospores are ensheathed (the proximal ascospore, only partly so), preventing clear

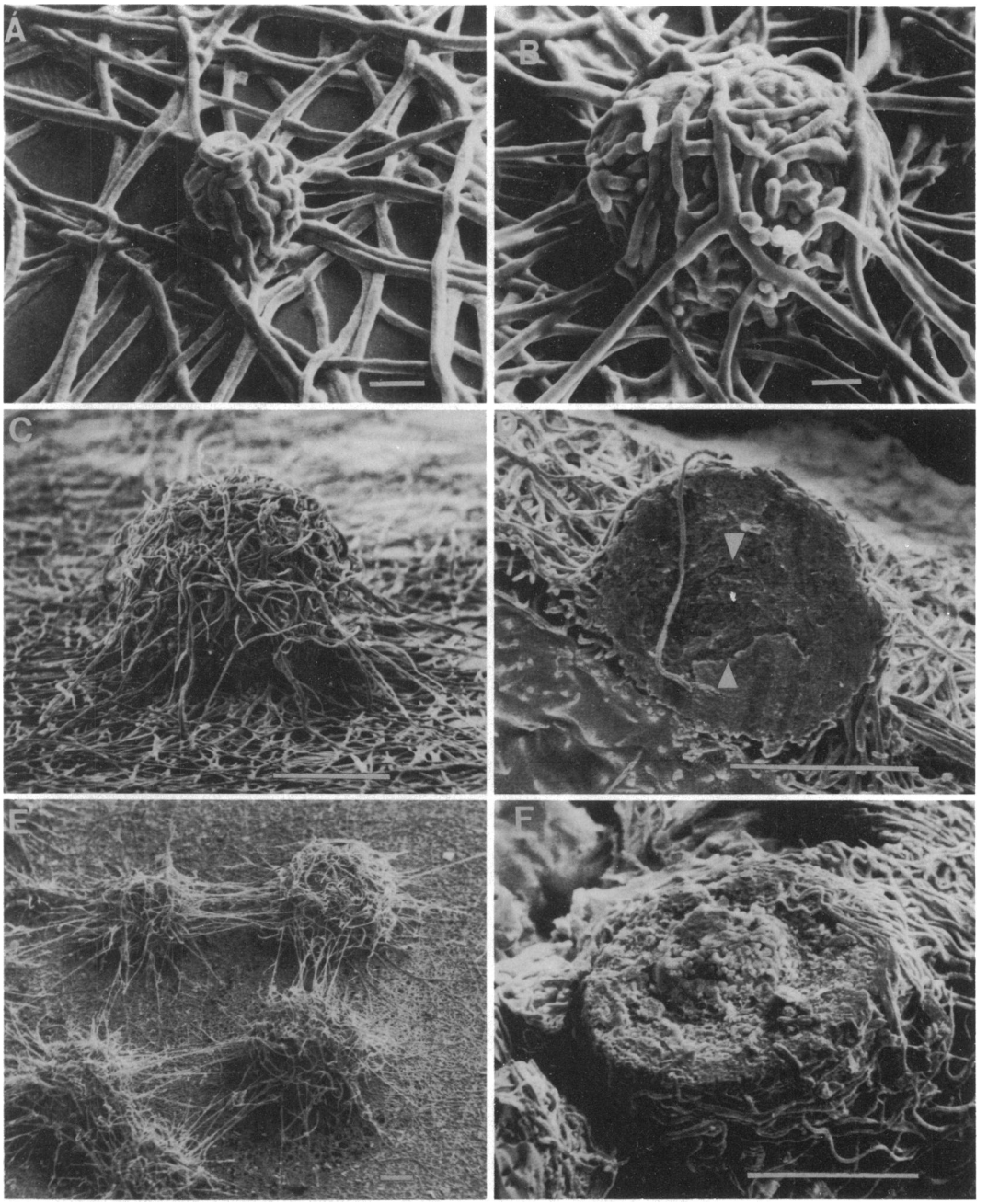

FIG. 1. *Perithecial development to 36 h. (A) Possible protoperithecium on 5-day-old mycelium of strain 74a, prior to crossing. (B) Incipient perithecium, 6 h. (C) External view of perithecium, 24 h. Tilt angle, 70°. (D) Internal view of perithecium, 24 h. Arrows indicate incipient centrum. (E-F) External and internal views, respectively, of perithecia, 36 h. Note degree of differentiation of the centrum in (F) compared to that in (D). Bars indicate 100 μm, except 10 μm in (A) and (B).*

distinction of the two adjacent ends, but nevertheless exposing the longitudinal ribs. The nature of the sheath has not been established but is probably either the ascus or a mucilaginous layer. Median sections of entire perithecia were infrequent, owing to the random freeze-fracture technique used. Two different views of a near-median section at 12 days are presented in Fig.

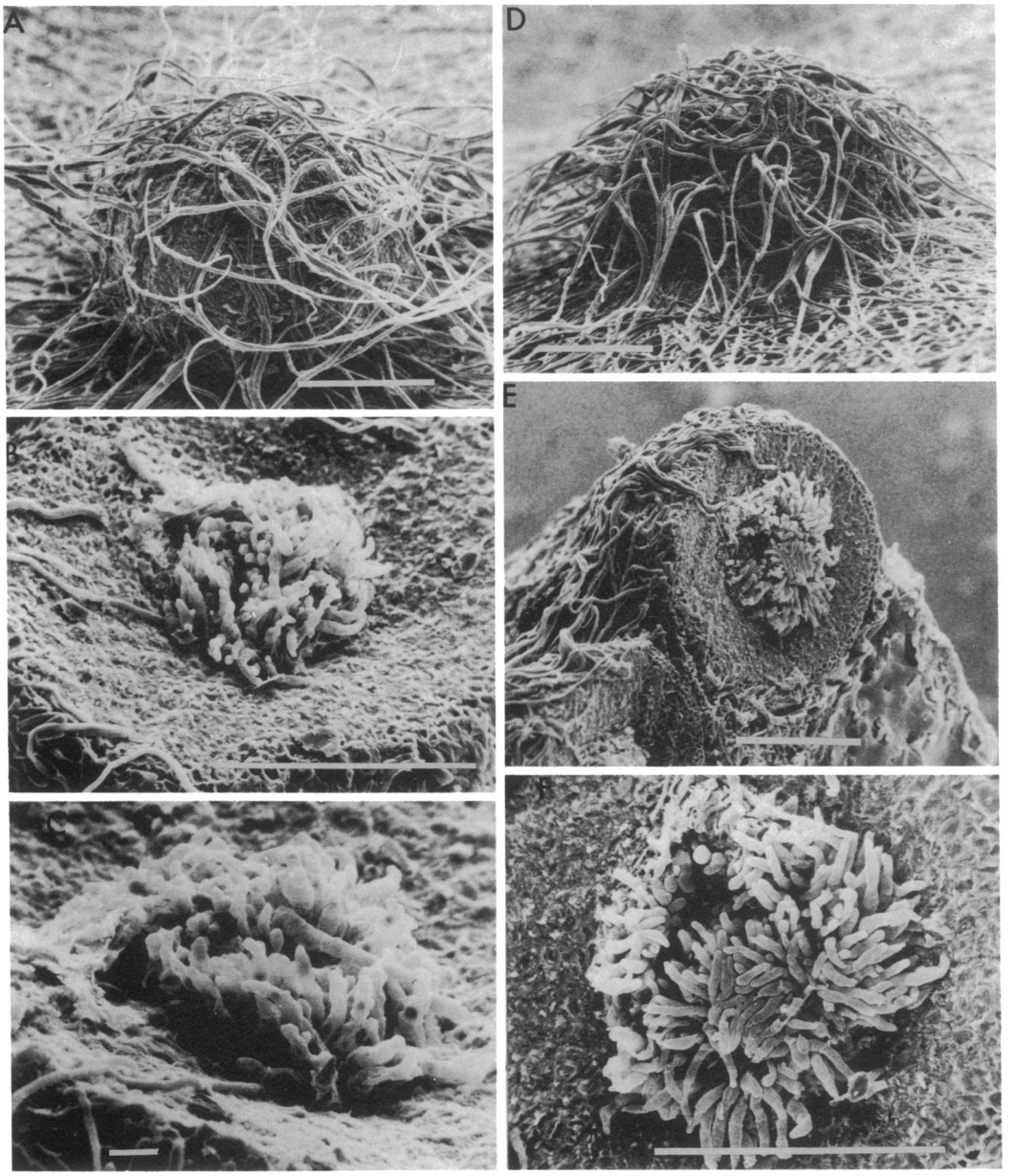

FIG. 2. *Perithecial development, 48-60 h. (A) External view of perithecium, 48 h. Tilt angle, 63°. (B) Internal view of perithecium showing ascogenous hyphae, 48 h. (C) Magnified view of ascogenous hyphae in previous figure. Tilt angle, 63°. (D) External view of perithecium, 60 h. Tilt angle, 70°. (E) Section of perithecium showing immature asci, 60 h. (F) Magnified view of asci in previous figure. Tilt angle, 54°. Bars indicate 100 μm, except 10 μm in (C).*

5C and D. Continued attachment of the base of the asci to the region of attachment inside the perithecium may be seen (Fig. 5C). Two unidentified pieces of material partly occluded the lumen of the beak. The upper arrow in both Fig. 5C and D indicates one of these two pieces, and the lower arrow in both figures indicates the other piece. The view in Fig. 5D reveals that the piece indicated by the lower arrow resembled an ascospore, but comparison with the ascospore located above the scale marker shows that this piece was longer and wider than an ascospore. Comparison of the width of the lumen in Fig. 5D with the relatively narrow ostiole in Fig. 5A and B suggests considerable tapering of the lumen toward the ostiolar end.

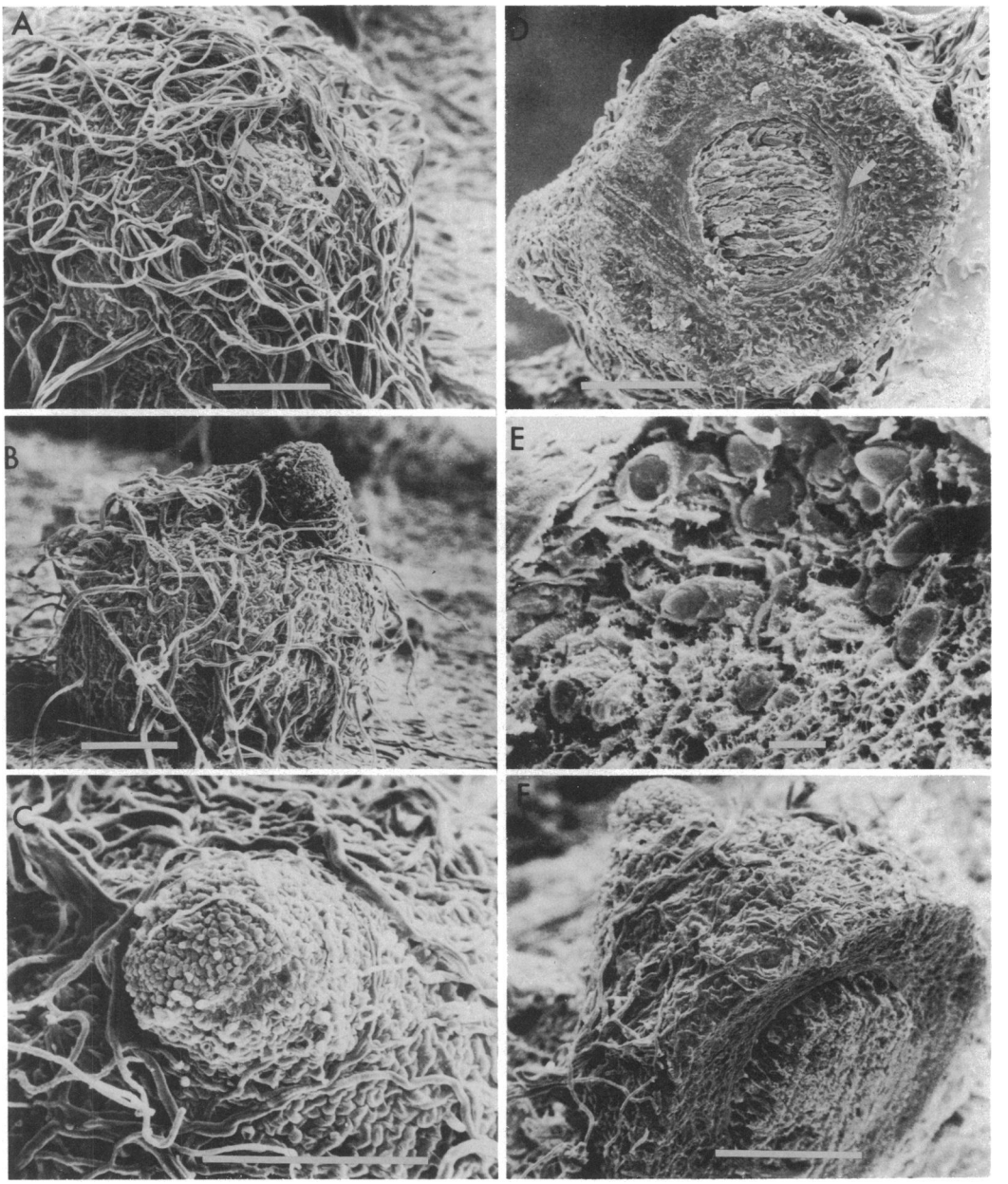

FIG. 3. *Perithecial development, 3-7 days. (A) Initial appearance of perithecial beak (between arrows), 3 days. (B) Elongation of beak, 5 days. Tilt angle, 80°. (C) Enlargement showing detail of bleb-like surface of beak and almost complete absence of loose hyphae, 5 days. (D) Section of perithecium showing region of ascus attachment (arrow), parallel arrangement of asci, and immature ascospores, 5 days, (E) Ascospores with walls obliquely freeze-fractured, 5 days. (F) Sectional view of lower portion of perithecial body revealing parallel asci, 7 days. Bars indicate 100 μm, excepting 10 μm in (E).*

DISCUSSION

Descriptive studies of the development of perithecia have been made by means of light microscopy in *N. tetrasperma* (5), in *N. sitophila* (3), and in *N. dodgei* and *N. terricola* (12). Seale (13) showed some stages in perithecial development in *N. crassa*, but his overview of the life cycle with the SEM placed major emphasis on ascospores, conidia, and their germinative events. We have presented a sequence of scanning electron micrographs, of both whole

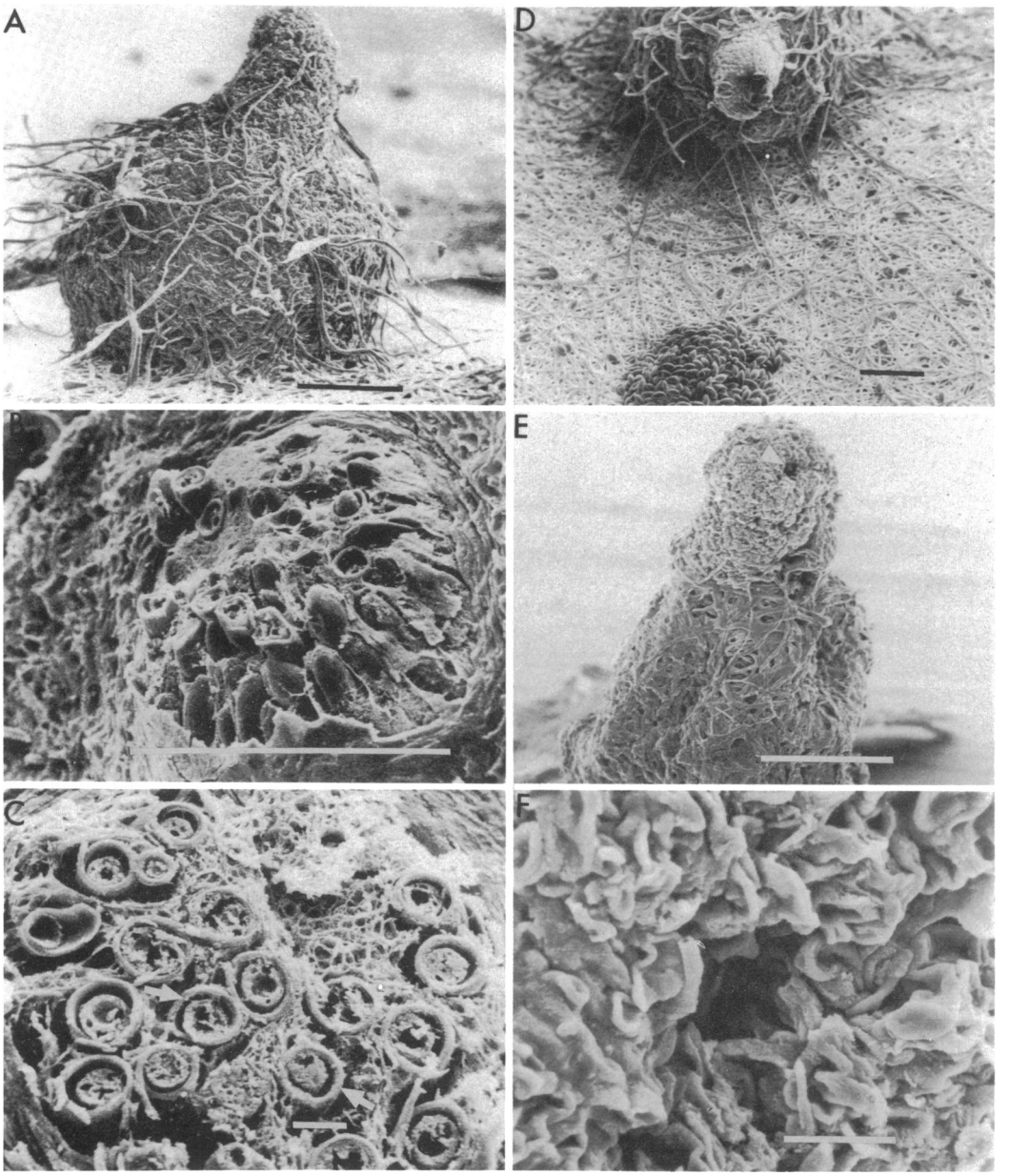

FIG. 4. *Perithecial development, 10–12 days. (A) Whole view of mature perithecium, 10 days. Tilt angle, 80°. (B) Oblique freeze-fracture of ascospores, 10 days. (C) Cross section of ascospores, 10 days. The two arrows indicate walls of two ascospores, with shrunken cytoplasm inside. Tilt angle, 16°. (D) A pile of ascospores discharged from the perithecium seen nearby, 12 days. (E) Perithecial beak with open ostiole (arrow), 12 days. Tilt angle, 75°. (F) Magnified view of open ostiole in previous figure. Tilt angle, 21°. Bars indicate 100 μm, except 10 μm in (C) and (F).*

mounts and sections, restricted to changes occurring during the development of the perithecium of *N. crassa*.

Limitation of the SEM (when operated in the secondary electron detection mode) to demonstrating surface features necessitates some method of exposing internal parts prior to examination, if more than external morphology is to be viewed with this instrument. Harris (6) showed some internal detail in broken perithe-

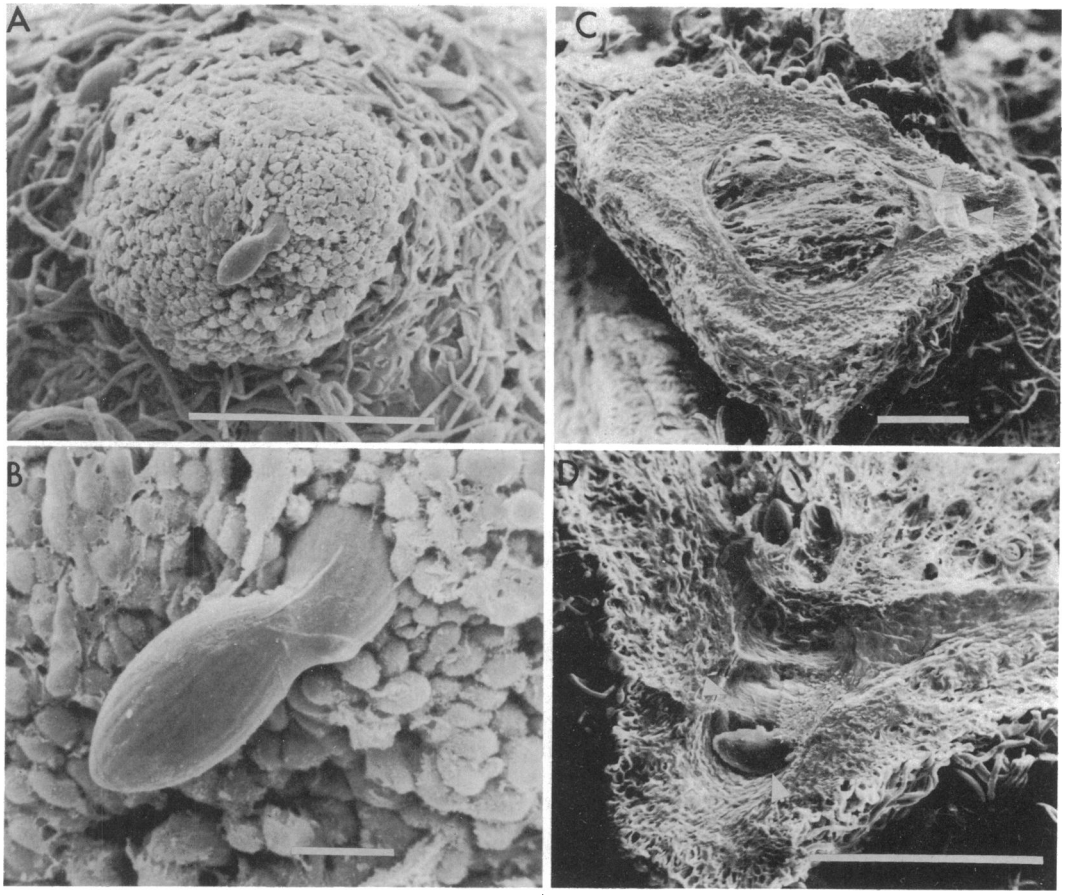

FIG. 5. *Perithecial development, 12 days. (A-B) Low and high magnifications, respectively, of two ascospores emerging from an ostiole. Tilt angle, 69°. (C-D) Two views of the same median section. Tilt angles, 12° and 70°, respectively. Arrows indicate two unidentified pieces of material which partly occluded the lumen of the beak. Bars indicate 100 μm, except 10 μm in (B).*

cial beaks of *Ceratocystis ulmi*. Corlett (4) sectioned embedded fungal spores of *Stemphylium* and examined them with the SEM after removal of the embedment, and Zeyen and Shearer (20) examined sectioned pycnidium-bearing fungi with the SEM. The freeze-fracture method (8, 19), applied in the present study, provides a simple means of obtaining information about internal structure.

An interesting feature of the freeze-fracture technique is shown in Fig. 1F, 2B-C, E-F, and 3F, in which the ascogenous cells protrude out of the plane of fracture. That the blade passed through at least part of the perithecium is evident in Fig. 2E, because the mark of the blade, to the left of the ascogenous hyphae, is continuous above and below them; also, the perithecial wall appears to be cut in the same plane on all sides of the hyphae. It seems likely that the plane of fracture might have preceded the blade and moved around most of the compacted and frozen hyphae, which were subsequently freed to expand into such configurations as those shown in Fig. 2E and F, upon thawing in ethanol. Moreover, some of the frozen hyphae might have been cut by the blade but subsequently lost their fluid and particulate contents upon thawing in ethanol and collapsed so that cut hyphal ends are not apparent.

The beak became morphogenically active after the rapid increase in perithecial size which characterized the first 2 days after crossing. At 3 days (Fig. 3A) the beak appeared as a compact mass bearing little resemblance to the surrounding filamentous cells. This difference suggests different physiological activities in the beak, and at least one has been reported, that of phototropism (2).

The mature ascospores were found to have a thick wall (Fig. 4C). Lowry and Sussman (10)

found the wall of the ascospore impenetrable to standard fixatives used for electron microscopy and succeeded in fixation only after mechanically breaking the outer wall. Osmium tetroxide, used as a fixative in the present study, may have penetrated the ascospores only slightly. Hence the cytoplasm in the ascospores appears to have been hardened insufficiently to prevent shrinkage and distortion during dehydration (Fig. 4C).

Ostiolar closure was investigated by examining at 12 days, after discharge of ascospores had commenced, 20 perithecia which were fixed and subjected to critical point drying, and eight perithecia which were unfixed and air dried. One open ostiole (Fig. 4E-F) was found among the eight unfixed perithecia and one open ostiole, from which ascospores were emerging (Fig. 5A-B), was found among the 20 fixed perithecia. Thus, closed ostioles were found much more frequently than open ostioles at 12 days, at least under present cultural conditions, whether fixation and critical point drying were used or not. The only open ostiole shown by Seale (13) was that of a specimen which had not been fixed.

The material which partly occluded the lumen of the beak (Fig. 5C-D) was not identified. However, this material could have been mucilaginous material of the type which Ingold (9) stated occurs in considerable amounts in the perithecia of some Pyrenomycetes.

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