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Life Cycle of *Neurospora crassa* Viewed by Scanning Electron Microscopy

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Scanning electron microscopy was used to examine the major stages of the life cycle of two wild-type strains of *Neurospora crassa* Shear and Dodge (St. Lawrence 3.1a and 74A): mycelia, protoperithecium formation, perithecia, ascospores, ascospore germination and outgrowth, macro and microconidia, and germination and outgrowth of macroconidia. Structures seen at the limit of resolution of bright-field and phase-contrast microscopes, e.g., the ribbed surface of ascospores, are well resolved. New details of conidial development and surface structure are revealed. There appears to be only one distinguishable morphological difference between the two strains. The pattern of germination and outgrowth which seems relatively constant for strain 74A or strain 3.1a, appears to be different for each. Conidia from strain 3.1a almost always germinate from a site between interconidial attachment points; whereas the germ tubes of strain 74A usually emerge from or very near the interconidial attachment site. These germination patterns usually do not segregate 2:2 in asci dissected in order. This observation suggests that conidial germination pattern is not under the control of a single gene.

The three-dimensional images produced by the scanning electron microscope can reveal intimate details of the anatomy of an organism and are useful in the resolution of changes in surface architecture during morphogenesis. These features establish scanning electron microscopy (SEM) as a useful adjunct for the description of developmental processes and for the analysis of morphological mutants. In microbial systems SEM has been utilized to investigate bacterial colony topography (28, 36), to aid in the classification of fungi (15), to detect differences in apparent membrane mutants (32), to visualize spore germination in bacteria and yeast (26, 25), and to follow developmental events in algae (4) and in fungi (13).

In this investigation I have examined with the scanning electron microscope most of the major stages of the life cycle of two wild-type strains of *Neurospora crassa* Shear and Dodge. The organism affords an opportunity to investigate the differentiation and morphogenesis of at least six distinct fungal structures: vegetative hyphae, conidia, ascogenous hyphae, protoperithecia, perithecia, and ascospores (Fig. 1). As it now appears probable that a close interrelationship exists between cell wall biosynthesis and fungal morphogenesis (2; S. Brody, *In* Cell differentiation, *in press*), a more detailed examination of surface structures may be profitable. The current study was undertaken to provide a baseline both for the further characterization of fundamental developmental stages and for the more detailed ultrastructural analysis of morphological mutants in *Neurospora*.

MATERIALS AND METHODS

Organism and cultural conditions. Two wild-type strains, 74A and 3.1a, of *N. crassa* Shear and Dodge obtained from the Fungal Genetics Stock Center, Humbolt State College, Arcata, California were used in this study. Vegetative cultures for the production of conidia were grown for 7 days at 25 C on slants of Vogel's minimal medium (34) solidified with 2% agar. Cultures for the production of sexual structures were grown on slants of Westergaard's crossing medium (35) solidified with 2% agar. Ascospores were heatshocked to induce germination by incubating them in a small volume of Vogel's minimal medium for 30 min at 60 C. Both conidia and ascospores were germinated in 50 ml of Vogel's liquid medium in a 250-ml flask and incubated on a gyrorotary shaker at 275 rpm at 37 C. In some cases, mycelial growth was examined by growing the mold on membrane filter disks (Millipore Corp.) placed on the surface of solid medium.

Electron microscopy. Adequate preservation of all structures was obtained with a freshly prepared

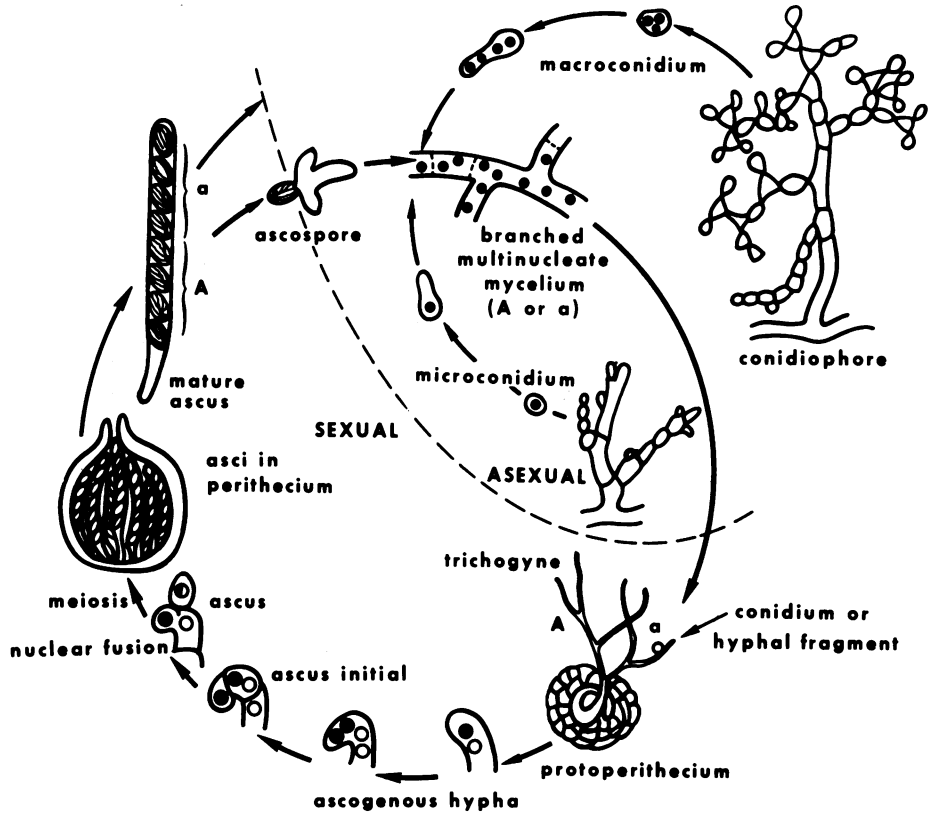

FIG. 1. *Life cycle of Neurospora crassa. Adapted in part from drawings of N. sitophila by Backus (1) and by Dodge (7).*

fixative composed of 2% glutaraldehyde (Polysciences Inc.) in 0.05 M sodium phosphate buffer at *p*H 7.0. Fixation was performed at room temperature (23 C ± 2 C) for times up to 3 hr, but 30 min of treatment gave adequate preservation and was normally employed. The quality of preservation was not enhanced by post fixation with osmium solutions or vapors, nor by quenching followed by freeze-drying. Spores were collected after fixation on membrane filters with a pore size of 0.45 μm. All fixed material was washed three times with 0.05 M sodium phosphate buffer (*p*H 7.0) followed by three washes in glass-distilled water. The membrane filters were air-dried at 23 C, and portions of the dried filters were mounted on aluminum specimen stubs with double-stick cellophane tape. The specimens were then vapor-coated with gold (20–40 nm thickness) while rotating at an angle of 45° to the vapor source. The specimens were immediately observed and photographed with a Cambridge Mark IIA Steroscan scanning electron microscope. Unless otherwise noted in the figures, the accelerating voltage was 20 kV, and the beam specimen angle was 45°.

RESULTS

Both unfixed and fixed material from each stage of the life cycle of *N. crassa* have been examined. In general, there is a dramatic improvement in the quality of preservation when fixed material is compared to unfixed. A 30 min fixation with 2% glutaraldehyde in 0.05 M phosphate buffer (*p*H 7.0) gives satisfactory preservation. In some systems, cryogenic techniques (e.g. quenching in liquid nitrogen followed by freeze-drying) applied after fixation result in improved specimen preservation (3, 20). No such improvement in the quality of ascospore, conidia or hyphae preservation was observed here. Therefore, only a one-step fixation procedure was employed throughout this study.

Figure 2 presents scanning electron micrographs of stages in the sexual differentiation of *N. crassa*. In Fig. 2A is seen the characteristically undifferentiated mass of anastomosing asexual hyphae of strain 3.1a grown on Westergaard's crossing medium at 25 C for 30 hr in the dark. No differentiation of protoperithecial initials is detectable, nor is there any demonstrable development of conidiophores or conidia at this time. Arrows point to the externally viewed, collar-like septa seen here in these slightly contracted hyphae. No differences are found between strains 3.1a and 74A grown

under identical conditions. By the fourth day, protoperithecia are readily detected and have the characteristic appearance shown in Fig. 2B. The tangled coil of hyphae initiating formation of the protoperithecium seems to be differentiated from one or a very few vegetative hyphae. In this picture, no obvious trichogyne is present, and the protoperithecium is immature. Figure 2C shows a mature protoperithecium photographed on the 6th day after inoculation. It is difficult to distinguish the trichogynes from the sterile hyphae, which grow out from the surface of the protoperithecial body. Figure 2D shows another protoperithecium at higher magnification with a possible trichogyne in evidence.

After fertilization, the protoperithecium rapidly enlarges and becomes melanized to form the perithecium. Figure 3A shows a portion of an unfixed, ripe obpyriform perithecium from a cross of 3.1a × 74A. At the apex, the ostium is clearly visible, and near it is a number of ejected ascospores, some of which have collapsed during coating with gold under vacuum. Numerous asexual hyphae are seen on the surface of the perithecium.

Ascospores display characteristic irregularly-spaced longitudinal grooves of varying depth

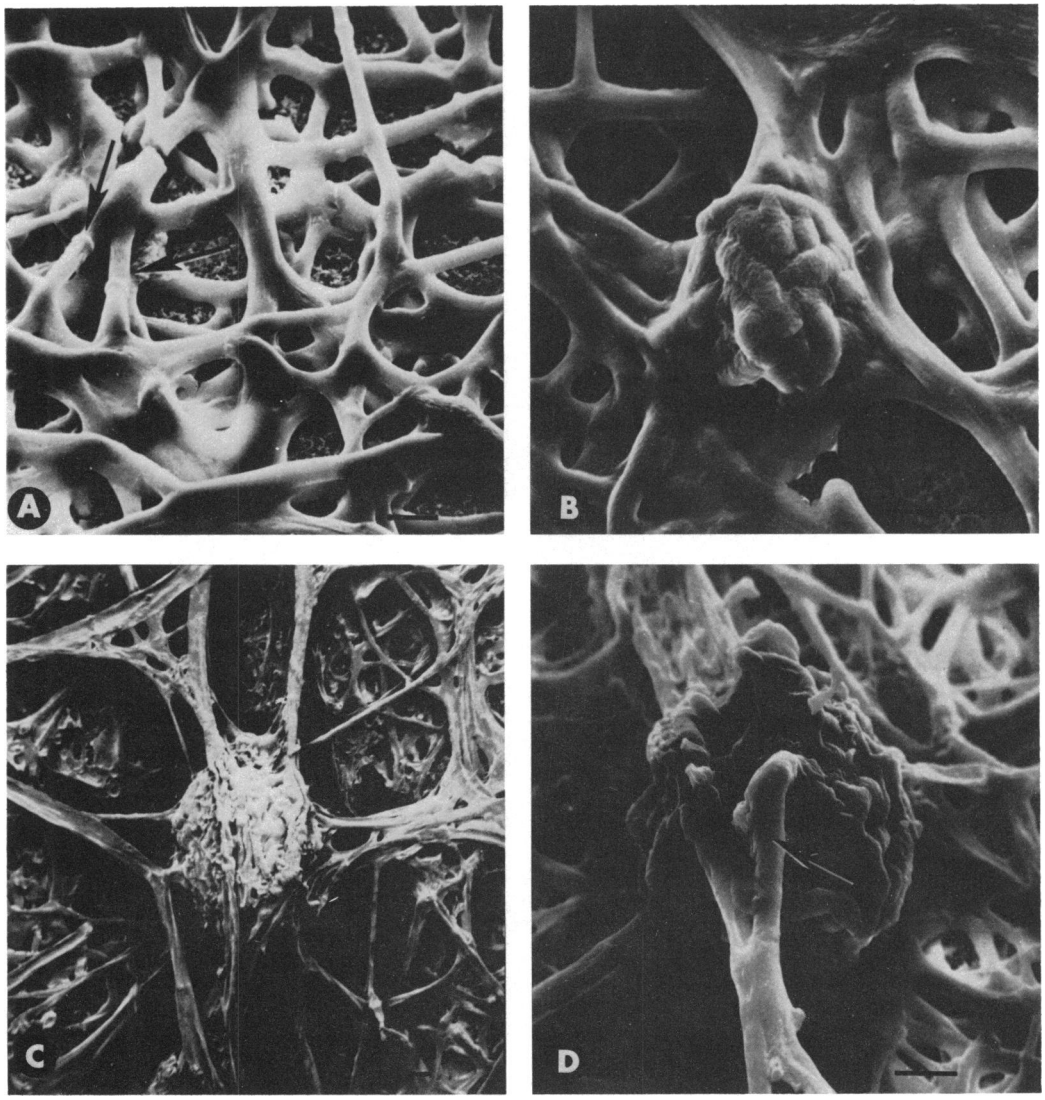

FIG. 2. *Early stages in the differentiation of sexual structures in N. crassa. A. Vegetative hyphae of strain 3.1a; arrows indicate septa. B. Immature protoperithecium. C. Mature protoperithecium; 0° tilt. D. Mature protoperithecium; Arrow indicates possible trichogyne. Scale markers are 11 μm.*

(Fig. 3B). The spore has a well-defined germination pore at each tip (Fig. 3B and C). Newly ejected ascospores are covered with a mucilaginous layer, which aids in their attachment to substrates after ejection from the perithecium (Fig. 3D). Older spores may show only remnants or none of this layer. Such spores, when collected from the surface of a culture tube, may appear to have a patch superficially resembling a bud scar where this layer was in contact with the glass.

Ascospores may germinate from one or both ends through the germ pore. The emerging germ tube is severely constricted at the pore but then balloons markedly and finally narrows to an average hyphal diameter (Fig. 4A and B). Essentially no surface structure is seen on the surface of the hyphal walls even at $\times$ 22,000. An early hyphal branch is seen on the left germ tube in Figure 4A. Beneath it is a collapsed conidium, which was killed by the heat shock used to activate the ascospores. Conidia, hyphae and hyphal fragments are often fused to the regions at, or near, the germ tube. This may indicate that regions actively synthesizing cell wall are especially adhesive, and that only one

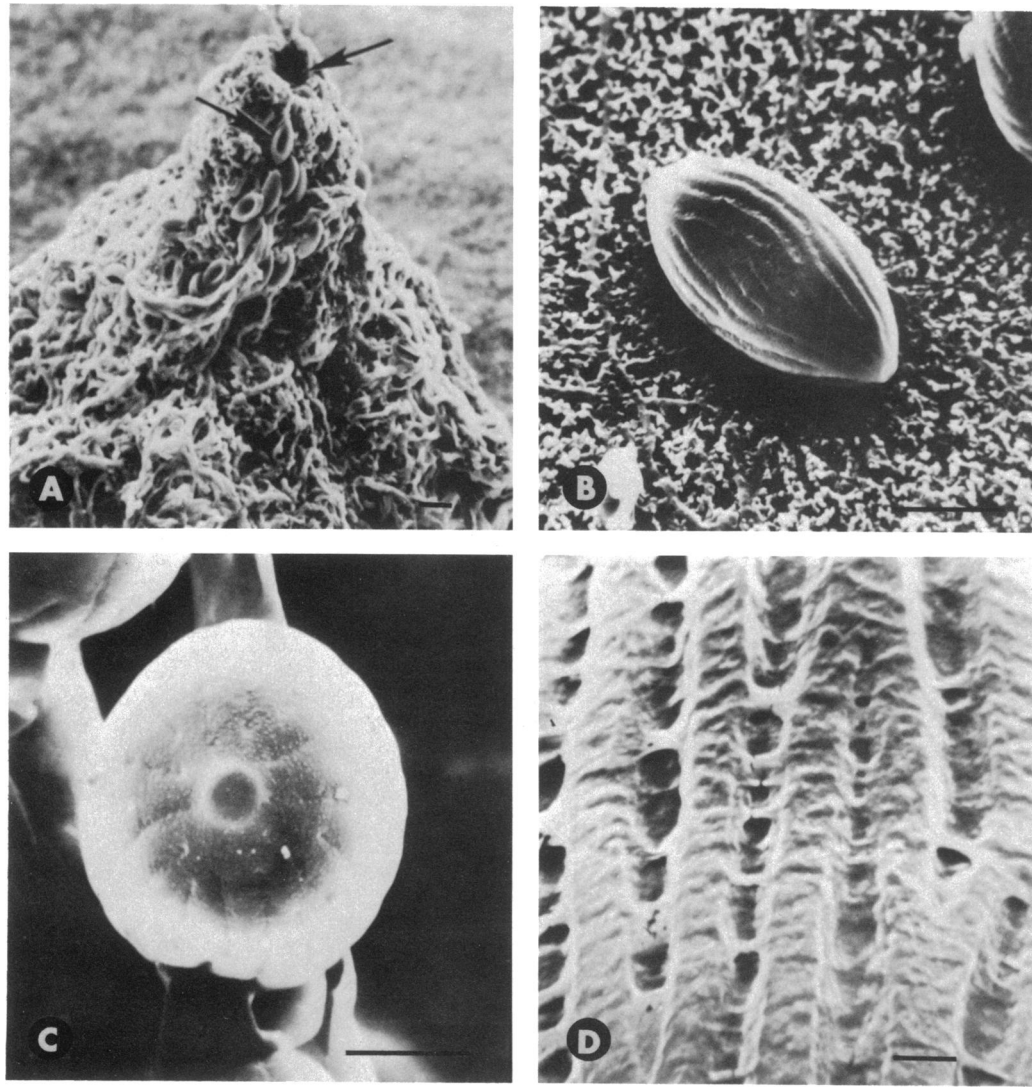

FIG. 3. *The perithecium and the ascospore of N. crassa. A. Ripe perithecium; arrows indicate ostium and ejected ascospores. Scale marker is 20 μm. B. Ascospore from a cross of 3.1a $\times$ 74A; 20 kv. Scale marker is 10 μm. C. End view of an ascospore showing germination pore; scale marker is 5 μm. D. The surface of a freshly shot ascospore showing surface mucilaginous layer; scale marker is 1 μm.*

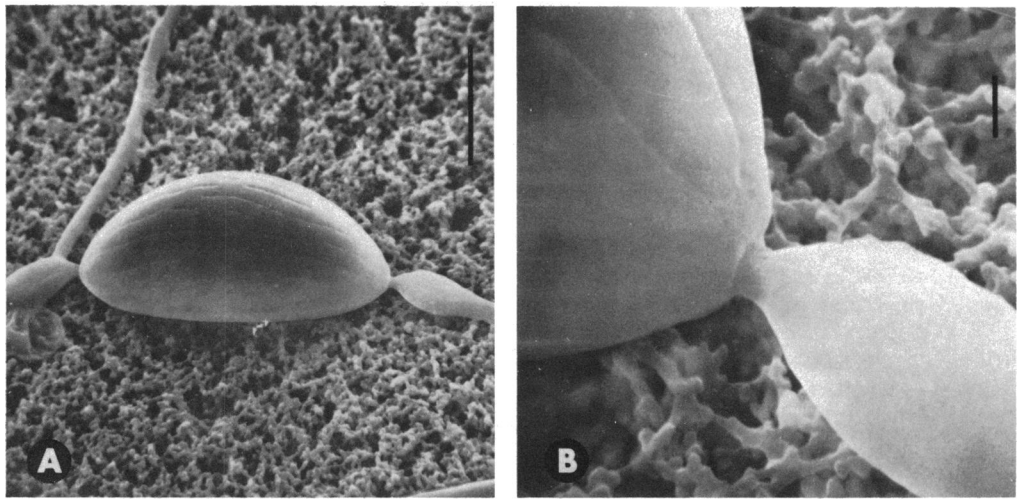

FIG. 4. *Germinating ascospore from N. crassa. A. Scale marker is 10 μm. B. Scale marker is 1 μm.*

element of a pair need be active in synthesis for wall fusion to occur.

The asexual portion of Neurospora's life cycle, which began with the germination of the ascospore, is examined in the remaining figures. Various views of the hyphal structure of strain 3.1a grown on Vogel's minimal medium for 18 hr at 30 C are shown in Fig. 5. No differences were discovered when identically grown hyphae from strain 74A were compared to those of strain 3.1a. A portion of the branching mycelium (Fig. 5A) shows little difference from that grown on nitrogen-deficient crossing medium (Fig. 2A). At higher magnification, a branch point shows no indication of external-wall differentiation at the site of branching (Fig. 5B). The surface of the hyphal cell wall shows almost no surface architecture, with the exception of very fine longitudinal striations. Hyphal fusions occur regularly as hyphae meet or overgrow one another or both (Fig. 5C). There appears to be an almost uninterrupted fusion of cell walls in such cases.

Vegetative hyphae can differentiate conidiophores and asexual reproductive spores called conidia. A portion of a conidiophore and a section of a rather thickened hypha are seen in Fig. 5D. This material was dry and unfixed, and was mounted directly upon a stub for viewing. The site of septum formation is evident in this collapsed and somewhat flattened hypha. It clearly provides structural support to wall areas associated with it. The macroconidia in this figure appear collapsed and very irregularly shaped. Similar observations are made when conidia are fixed immediately in suspension, dry-fixed with osmium vapors, or freeze-dried. Dry preparations of fresh conidia have a similar appearance when viewed with the phase contrast microscope. Attachment points and the specialized collar structure between conidia can also be seen (Fig. 5D).

The conidiophores and conidia of *N. crassa* are examined in Fig. 6. This material was suspended in liquid minimal-medium and incubated at 37 C with shaking for 30 min. The conidia swelled considerably during this period, presumably, because of the imbibition of water. There is considerable variation in macroconidial size. Conidia incubated in germination medium exhibit some surface irregularity (Fig. 6D) when compared to the smooth appearance of the outer surface of intact unincubated conidia (Fig. 5D). A probable microconidium is seen in the upper portion of Fig. 6A and in 6D. The latter microconidium possesses a very short stalk that is differentiated directly from a vegetative hypha. Macroconidia from a single conidiophore exhibit a complex branching pattern. The multiple interconidial attachment sites seen in these figures (3 per conidium in Fig. 6A and C, and 5 per conidium in Fig. 6B) suggest that a conidium may differentiate several other conidia from itself.

The biochemical events which lead to a commitment to germination culminate in the emergence of the germ tube from the conidium (Fig. 7). A conidium from strain 3.1a incubated for 2 hr in minimal medium shows continued swelling, and possibly an indication of the site from which the germ tube will emerge (Fig. 7A, arrow). Macroconidia from strain 3.1a nearly always put out a single germ tube, which emerges from between two interconidial at-

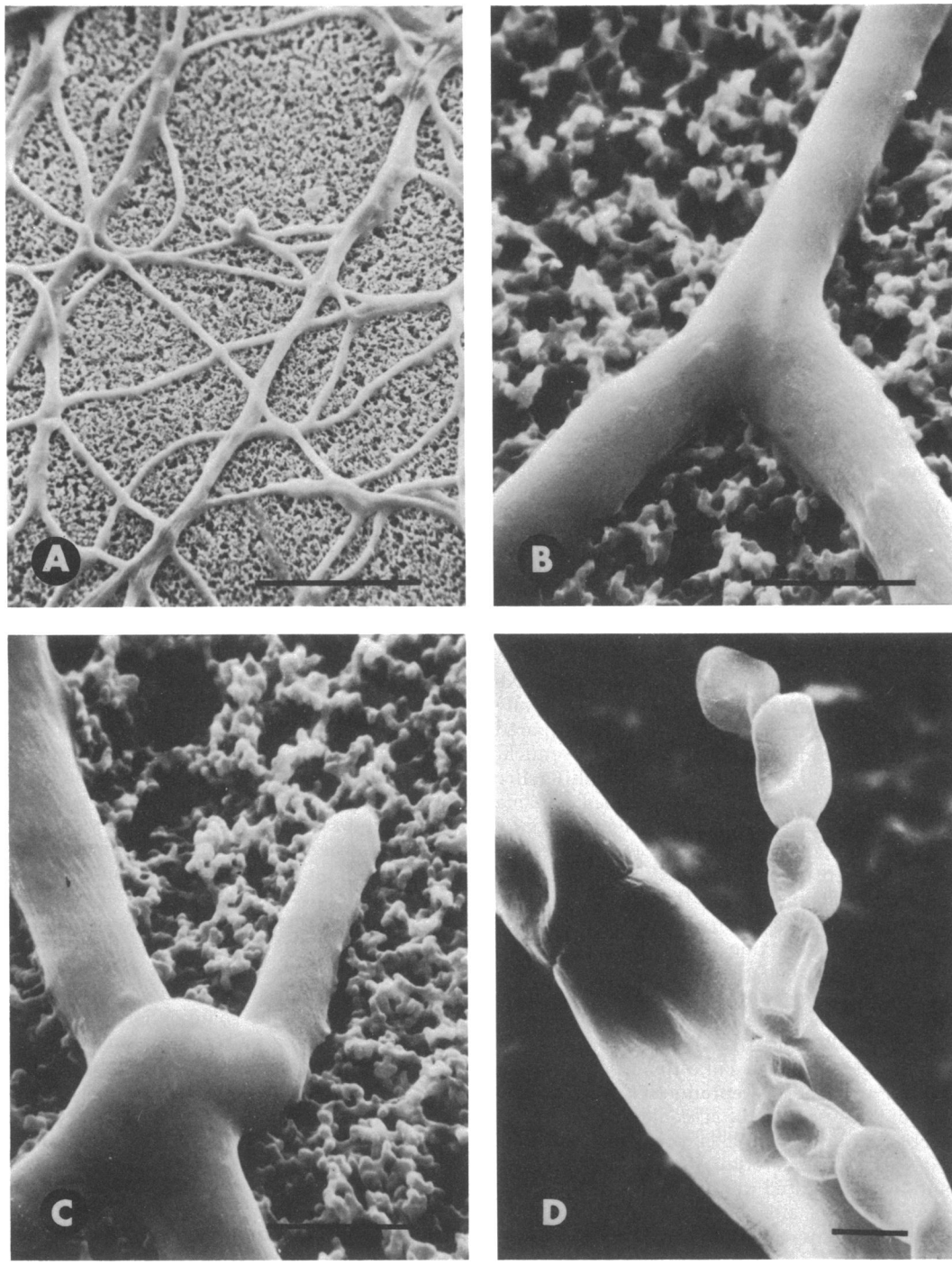

FIG. 5. *Vegetative hyphae in N. crassa. A. Mycelium of strain 3.1a; scale marker is 50 μm. B. Hyphal branching pattern; scale marker is 5 μm. C. Hyphal anastomosis; scale marker is 5 μm. D. Unfixed dry hypha with macroconidia; scale marker is 5 μm.*

tachment sites under the conditions employed here (Fig. 7B). Infrequently, (1% in our limited sample) two germ tubes may emerge from two sites (Fig. 7D). In contrast, macroconidia from strain 74A generally germinate from a site of interconidial attachment (Fig. 7C). This ob-

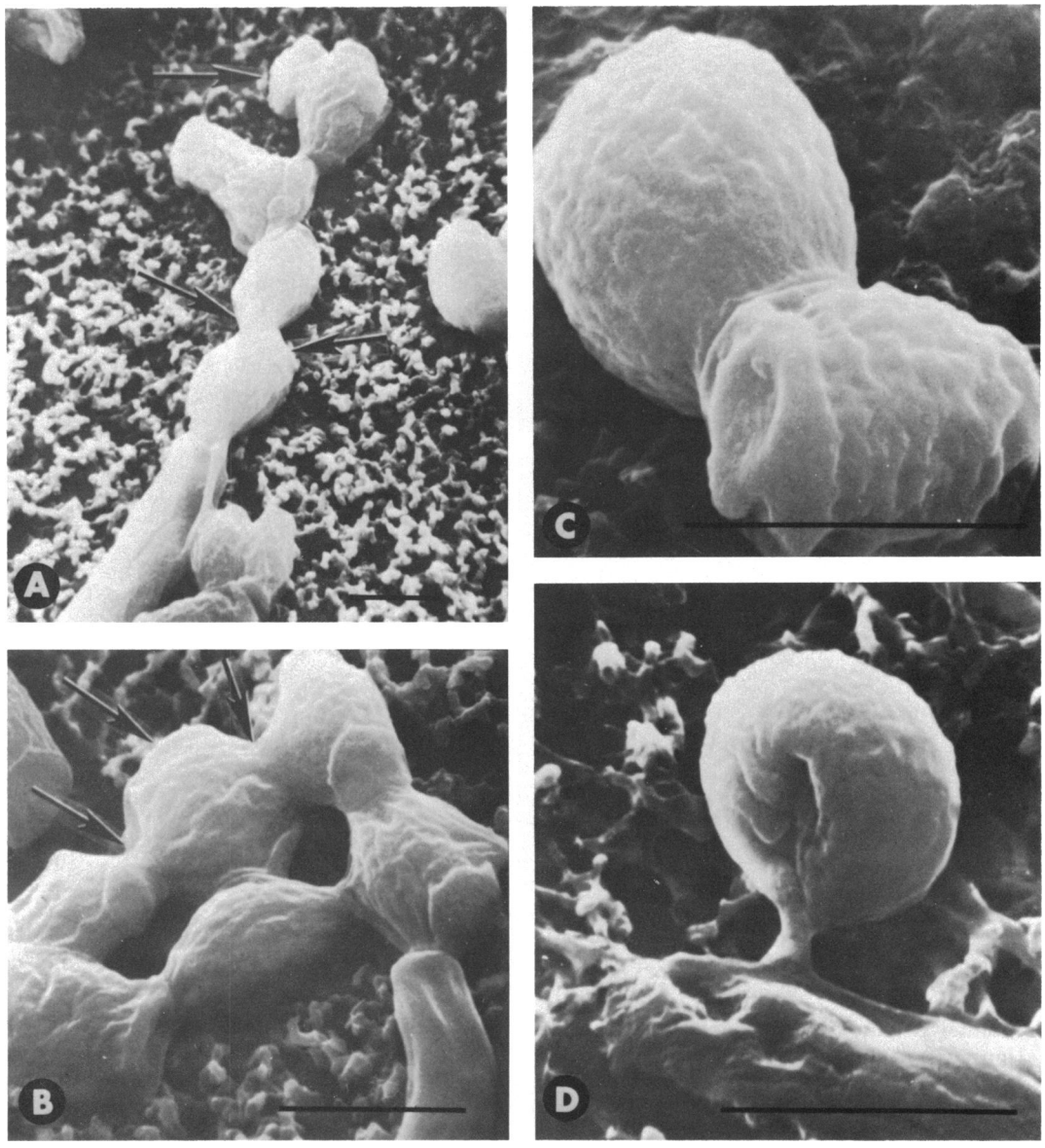

FIG. 6. *Conidiophores and conidia of N. crassa in germination medium. A. Portion of conidiophore with macroconidia; arrow at the top indicates microconidium; other arrows indicate conidial attachment points. B. Part of the macroconidial mass arising from a single conidiophore; arrows indicate sites of interconidial attachment. C. Two attached macroconidia. D. Probable microconidium attached to a short stalk that arises from a vegetative hypha; scale markers are 5 μm.*

servation has been confirmed for each strain with several different conidial preparations. Since strains 3.1a and 74A share a common St. Lawrence wild-type genetic background and are normally considered to be genetically very similar, if not identical except for the mating type locus (A or a), these results are unexpected. Segregation of the macroconidial germination pattern has been examined in cultures derived from 15 asci dissected in order. Conidial germination pattern does not regularly segregate 2:2. A culture derived from a single ascospore may as often have conidia which germinate in both manners, as it has conidia which germinate in only one.

Little differentiation of structure is seen in the growing hypha. The tip usually has a small pyramidal projection, which probably constitutes the expansion of the growing point (Fig. 8B). Finally, as hyphal growth proceeds after

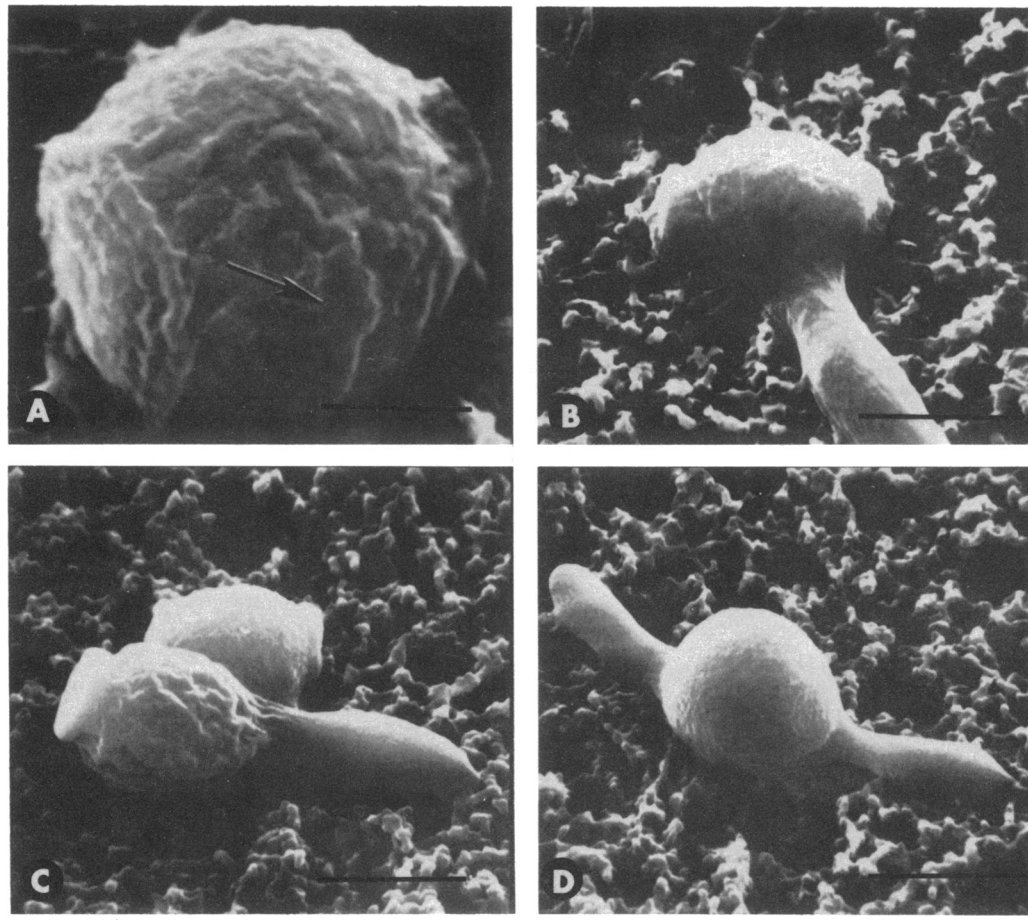

FIG. 7. *The germination of macroconidia in N. crassa. A. Swollen conidium of strain 3.1a with possible site of germ tube emergency (arrow); 2 hr in germination medium; scale marker is 2 μm. B. Germ tube emerging from conidium of strain 3.1a; scale marker is 5 μm. C. Germ tube emerging from conidium of strain 74A; scale marker is 5 μm. D. Germ tubes emerging from conidium of strain 3.1a; scale marker is 5 μm.*

conidial germination, fusions between hyphae (most often near the growing tip) become common (Fig. 8A). Growing regions seem to be especially "sticky" and account, at least in part, for the clumping which generally occurs in conidial cultures that are germinated in liquid medium.

DISCUSSION

This study constitutes an overview of the life cycle of *N. crassa* as seen with the resolution of the scanning electron microscope. It suggests that further, more-detailed SEM studies of the differentiation and germination of conidia, and the morphogenesis of protoperithecia would be a useful complement to biochemical and transmission electron microscope analyses of these developmental stages.

Essentially no surface architecture was revealed on vegetative hyphae, except in the apical region. At the hyphal tip, a pyramidal, cap-like apical body that probably corresponds to the growing point or spitzenkoper was often seen (Fig. 8A). The complex region of the spitzenkorper has been described by Girbardt (14) in a number of fungi, including *Neurospora*, with both the brightfield and electron microscope. No indication of hyphal surface alteration or differentiation at the site of nascent branch formation in hyphae was observed in our study. Asexual reproductive structures, conidiophores, and conidia, were seen with much better resolution and depth of field than is possible with the light microscope (7, 16). Both macroconidia and microconidia have been described at the light microscope and transmission electron microscope levels (16, 8, 7, 33, 21). Macroconidia arise directly from

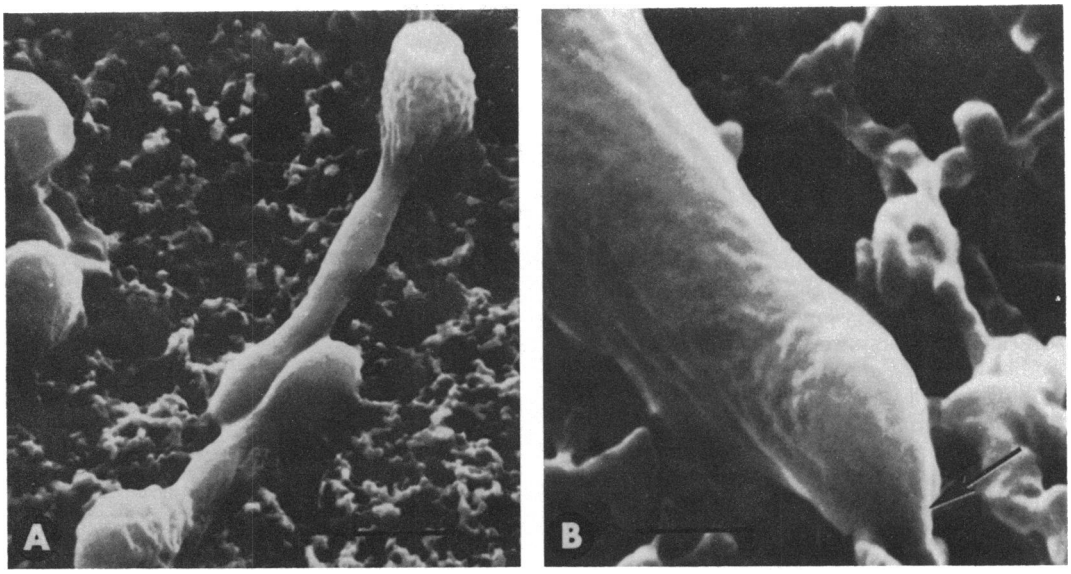

FIG. 8. *Germ tubes from conidia of N. crassa. A. Scale marker is 5 μm. B. Arrow indicates growing tip. Scale marker is 1 μm.*

a vegetative hyphal conidiophore and seem to be "acropetally formed by repeated constriction of the hyphae" (16). Both Lowry, Durkee, and Sussman (16) and Turian, and Matikian (33) show illustrations of the lateral formation of macroconidia from a pre-existing macroconidium as well as the usual terminal constriction of the conidiophore. This process, called "budding" by Turian and Matikian (33), was very common in our material (Fig. 6B). The few probable microconidia detected usually arose singly from a short specialized hypha, the microconidiophore (Fig. 6D; ref. 16). A clearly defined abscission layer, as well as a tubular structure which apparently interconnects adjacent conidia could be seen between conidia when they became separated (Fig. 5D and 6). The preservation of conidia in our experiments appeared to be equal to that of the more complex procedure explored by Travis et al. (32).

Neurospora conidia swelled markedly during the germination process, as do the spores of many other fungi (11, 23, 19). This is a characteristic of the spore germination process. The swelling of the spores is a consequence of the absorption of water. Spores are often in hygroscopic equilibrium with the environment and must absorb water to initiate the complex biochemical events leading to germination (5, 30, 37). The latter phenomenon probably accounts for the irregular, partially collapsed appearance of dry conidia. An interesting feature of the emergence of the germ tube from *Neurospora* upon germination was its position relative to interconidial attachment sites. Strain 3.1a conidia germ tubes usually arise from between two attachment sites, whereas those from strain 74A occurred from or very close to, an attachment site. First indications were that conidial germination pattern segregated 2:2 in asci dissected in order. However, re-examination of these four asci and scanning of eleven others clearly established that this germination phenotype normally does not segregate. Although apparent 2:2 patterns may occur occasionally, progeny ascospores more generally give rise to conidia of both types. If germination pattern is genetically controlled, it is not by a single gene in this case.

The development of the fruiting structure in *Neurospora* has been the subject of biochemical and genetic investigation (1, 8, 10, 29, 31). A surprising amount of the detail of protoperithecial and perithecial development in *N. tetrasperma* was elucidated by the work of Dodge (8) and Backus (1) with the light microscope. However, the potential for genetic analysis is much better in *N. crassa*. Further correlative work is necessary at both the transmission and scanning electron microscope level in *N. crassa*, although SEM will, probably, be of limited value because of the internal complexity of these structures. The protoperithecia seen here with the higher resolution of SEM are comparable to figures presented by others (1, 8).

Finally, we come to a consideration of the

sexual reproductive spore, the ascospore. The wall sculpturing of longitudinal striations, which particularly characterizes the genus *Neurospora* (27) was well resolved (Fig. 3). These striations or ribs are incompletely resolved by light microscopy but nevertheless, have been used as taxonomic markers (9, 12, 22, 24). SEM, clearly, can be of aid in the systematics of fungi (15). Excellent light micrographs showing that the outermost of three ascopore wall layers is the ribbed one have been published by Lowry and Sussman (17). Two additional layers were identified in later studies (18). The germ pore, seen clearly in this SEM work, was indicated in their micrographs. A more complete visualization of the ungerminated and germinated ascospore can be had by comparing the scanning electron photomicrographs with the transmission electron photomicrographs of Lowry and Sussman (18).

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