Ultrastructure of Microsclerotia of Verticillium albo-atrum

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Scientific Journal Paper No. 5772. Contribution of the Missouri Agriculture Experiment Station. Portions of this research made possible through CSRS Grant No. 816-15-18.

The authors gratefully acknowledge the technical assistance of Ray Faup and the assistance of John Fairing of Monsanto Chemical Company, St. Louis, for use of the scanning microscope.

Accepted for publication 22 October 1969.

ABSTRACT

The ontogeny of microsclerotia of *Verticillium albo-atrum* was examined by stereoscan and transmission electron microscopy. Microsclerotia originate from swollen hyphal cells of single or intermingled hyphae and increase in size by prolific budding from globose cells. The entire mass becomes enveloped in a pigmented mucilaginous matrix. Many peripheral cells degenerate early in the development of the microsclerotium, leaving nonfunctional hyaline cells embedded in the matrix among heavily pigmented functional cells. The pigmented cells are connected by means of septal pores and retain organized cytoplasmic contents, including a single nucleus, numerous mitochondria, lipid droplets, ribosomes, woronin bodies, and other inclusions. A possible mechanism of microsclerotial germination is proposed. Phytopathology 60:538-542.

Previous studies on the structure of microsclerotia (MS) have been confined to light microscopic techniques (3, 4, 8, 9), except for Nadakavukaren's (6). Controversy has arisen over the roles of the heavily pigmented vs. hyaline cells associated with the MS masses (8). It is generally considered that germination of pigmented cells [thick-walled cells of some authors (8)] does not occur, and that germination of the MS bodies originates only from hyaline cells.

This report offers evidence that illustrates the fine structure and functional nature of pigmented cells, and offers evidence that hyaline cells observed in peripheral areas of MS are incapable of germination. A possible explanation of the method of germination is offered.

MATERIALS AND METHODS.—Cultures of *V. albo-atrum* isolated from cotton (*Gossypium hirsutum* L.) were grown on cellophane discs over potato-dextrose agar (PDA) at room temperature (22-24 C) for periods up to 14 days. Microsclerotia were harvested and fixed with 6% glutaraldehyde in a 0.02 M phosphate buffer at pH 7.5 at 4 C for 4 hr, and rinsed in 0.25 M sucrose in a 0.02 M cacodylate buffer overnight at 4 C. Samples of MS were subsequently postfixed with 1% OsO_4 in the same buffer at 4 C for 2-4 hr, or in unbuffered 1% aqueous $KMnO_4$ for 1 hr at room temperature. A dehydration series of ethanol and propylene oxide was followed by infiltration under vacuum (15.24 cm of Hg) with an Epon-Araldite mixture 3:4 catalyzed with DMP-30 and polymerized at 65 C for 4 days. Ultrathin sections were cut on a Reichert ultramicrotome with a diamond knife. Sections of OsO_4-fixed MS were poststained with uranyl acetate and Reynold's lead citrate (7). Transmission micrographs were taken on an RCA EMU-3G electron microscope. Microsclerotia examined in the scanning microscope were fixed in buffered glutaraldehyde as indicated previously, except that they were stored at 4 C for 3 days and subsequently treated with cacodylate buffered OsO_4 for 4 days at 4 C. The MS were then rinsed in distilled water and air-dried at room temperature. The samples were coated with a Pt:Pd (80:20) alloy on a rotating stage in a Varian model VE-20 high vacuum evaporator. Scanning micrographs were taken on a Cambridge scanning microscope.

RESULTS.—Stereoscan and transmission electron micrographs show that MS develop initially by enlargement of individual hyphal cells of a single branching filament or adjacent parallel filaments (Fig. 1). From this cluster or chain of nearly globose cells, the MS increases in size by repeated budding from the globose cells as well as from the larger budded cells (Fig. 2, 3). Budding continues throughout the development of the MS, resulting in numerous buds on the exterior of the mature structure (Fig. 4, 5). Thus the MS consists of tightly compressed branching chains of nearly globose hyphal cells 8-15 μ in diam which are separated by simple single perforate septa (Fig. 7) characteristic of ascomycetous fungi (5). Transmission micrographs demonstrate (Fig. 8, 9) that all cells possess initially a single nucleus, numerous mitochondria, abundant lipid droplets, woronin bodies, ribosomes, and other typical organelles. The characteristically laminated septum connecting adjacent globose cells or terminal buds remains the width of the original hypha.

All the cells are enclosed in a mucilaginous pigmented

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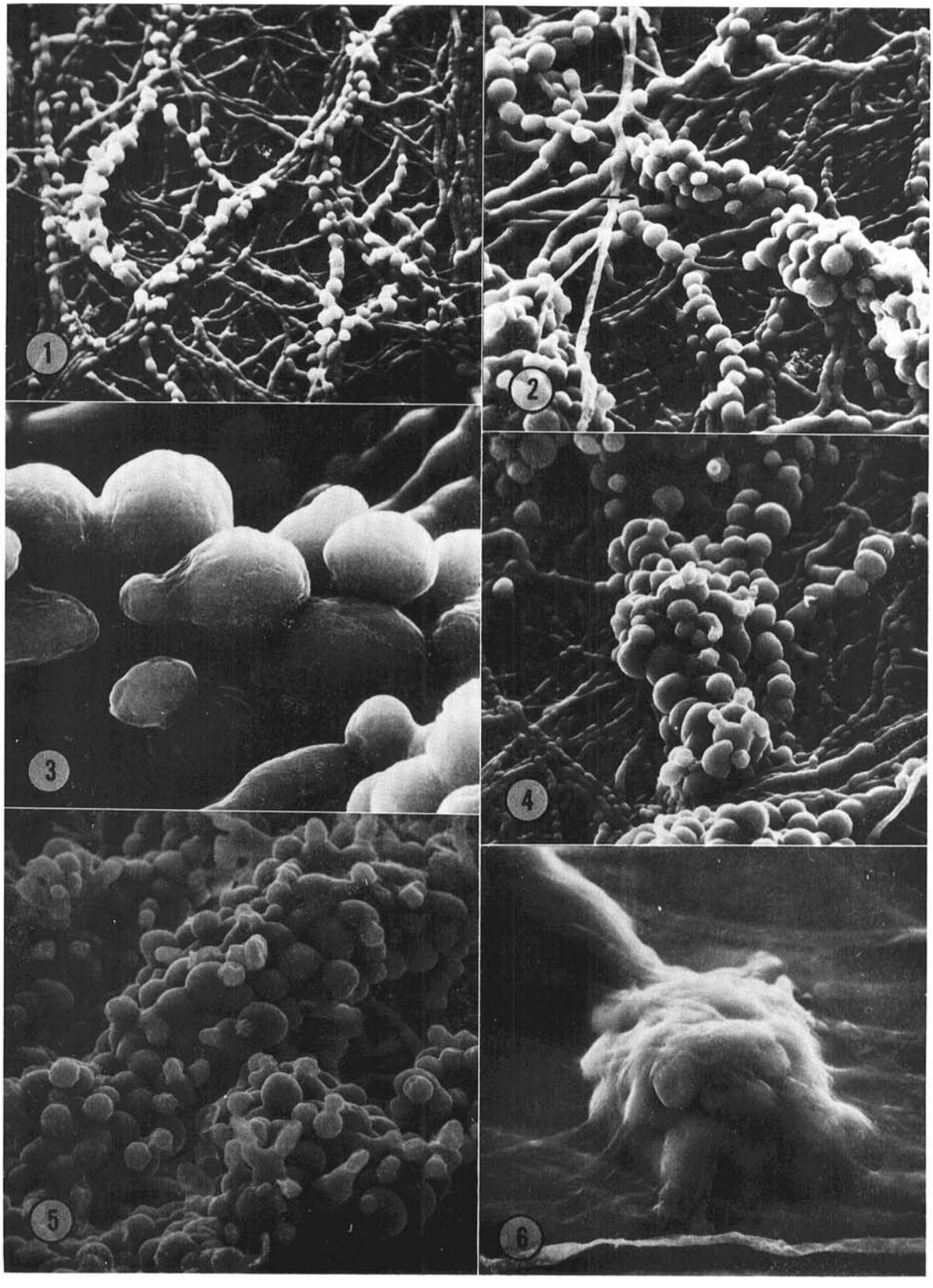
Fig. 1-6. Stereoscan electron micrograph of 4-day-old microsclerotial initials of *Verticillium albo-atrum*. (×660) Note the association of parallel hyphae and enlargement of adjacent hyphal cells to form chains of globose cells. **2)** A 6-day-old microsclerotium (×900), Note the proliferation of budded cells from globose primary cells and anastomosis of hyphal and globose cells (arrow). **3)** Higher magnification of a portion of Fig. 2, showing a chain of budded cells and accumulation of mucilage on the surface. (×4,140) **4)** An 8-day-old microsclerotium of *V. albo-atrum*. (×900) **5)** A 12-day-old microsclerotium. (×900) Note the sequential proliferation of budded cells forming a multicelled cluster in various stages of maturation and accumulation of encrusting mucilage between globose cells. **6)** Mature microsclerotium showing heavy deposits of mucilaginous material covering the exterior of the microsclerotium. (×1,240)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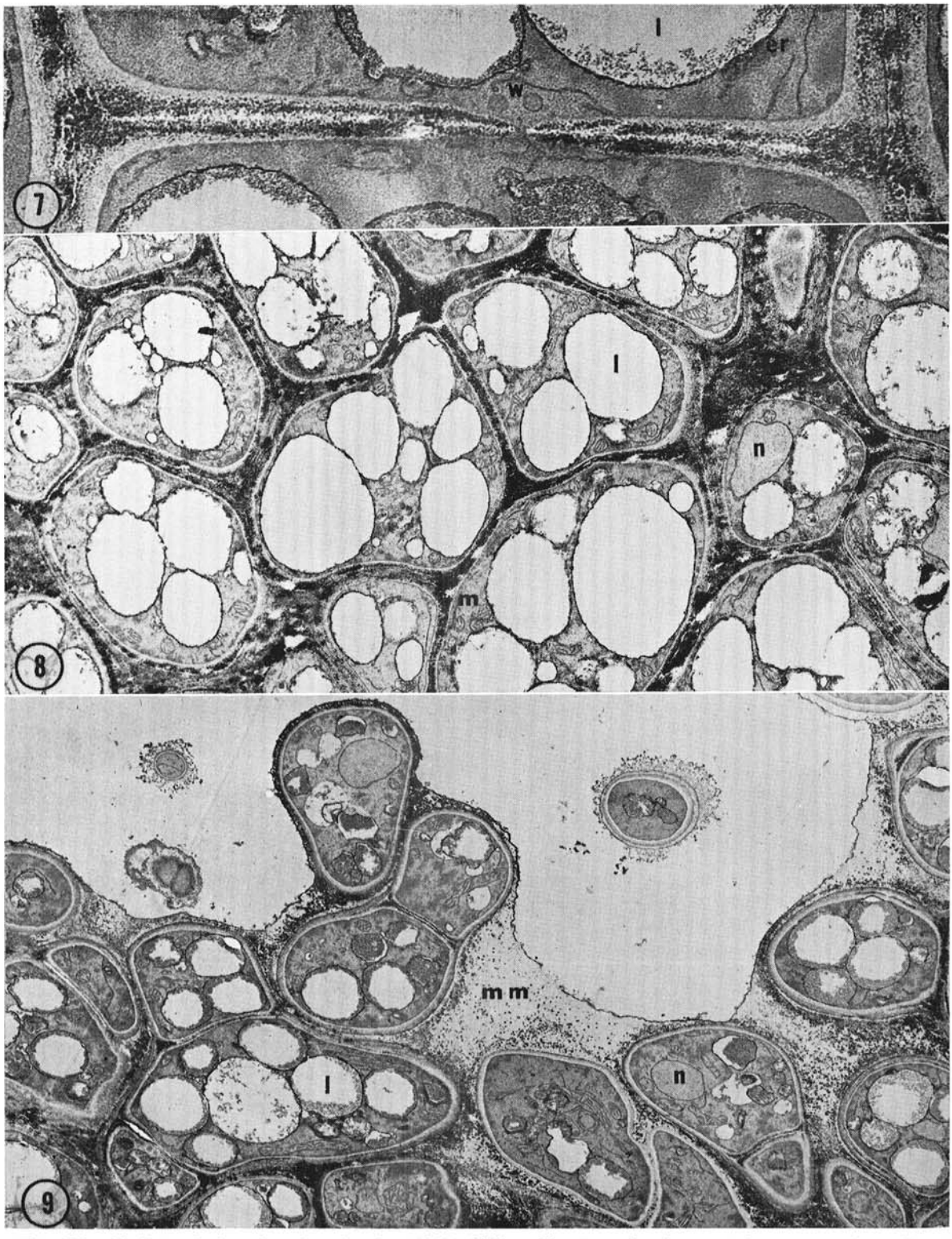

Fig. 7-9. 7) Internal view of a microsclerotium of *Verticillium albo-atrum* showing a septal pore connecting adjacent cells and associated woronin bodies (w); er = endoplasmic reticulum; l = lipid droplets. (×17,400) **8)** Typical cross section of a microsclerotium showing a single nucleus (n), numerous mitochondria (m), lipid droplets (l), and pigmentation between the cells and within cell walls. (×5,400) **9)** Peripheral region of a developing microsclerotium showing mucilaginous matrix (mm) and increasing degrees of pigmentation toward the interior of the structure. n = Nucleus; l = lipid droplet. (×3,600)

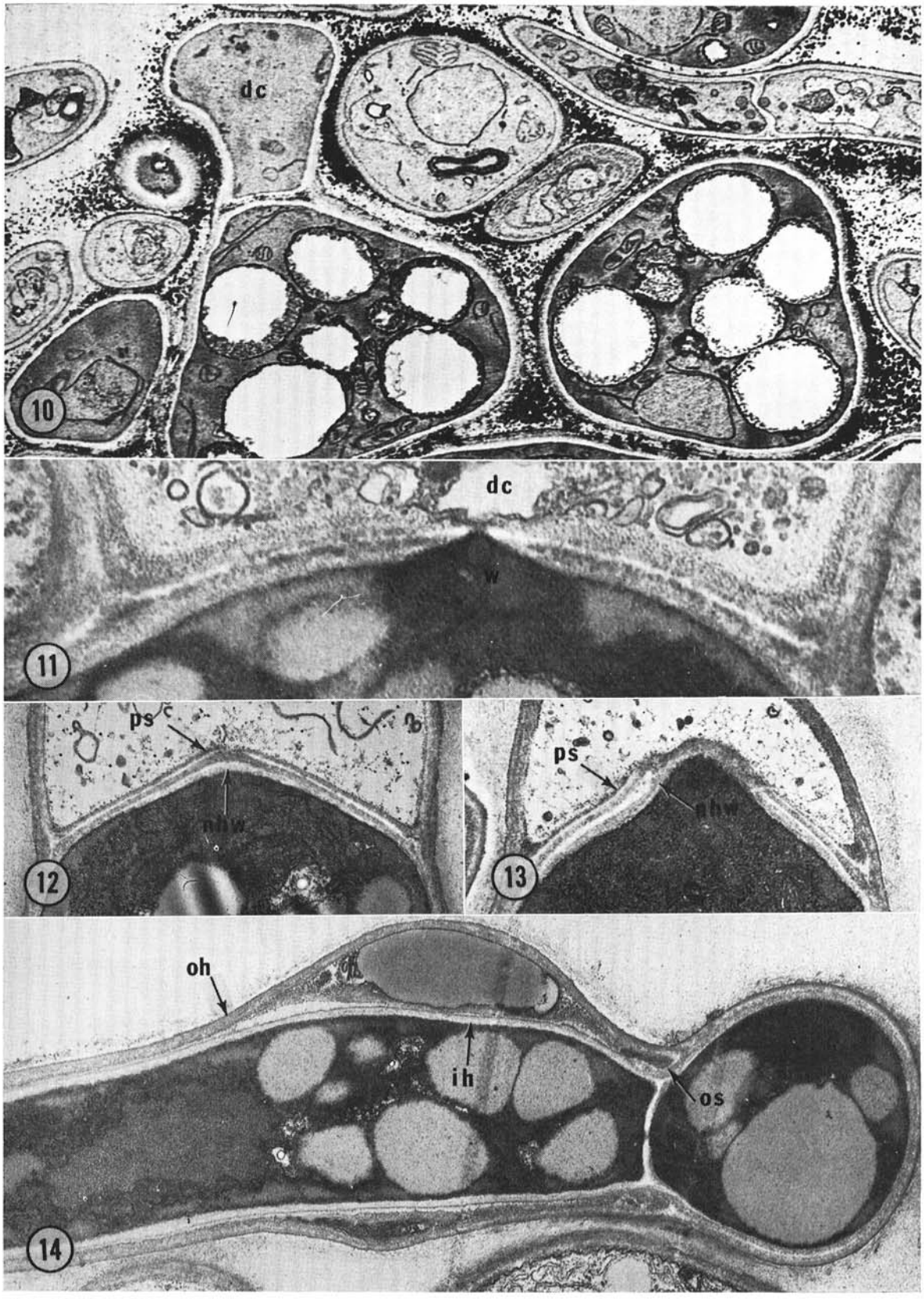

Fig. 10-14. **10)** Peripheral region of a microsclerotium of *Verticillium albo-atrum* showing the degeneration of peripheral cells. dc = Degenerating cells. (×4,900) **11)** Portion of a microsclerotium showing blockage of septal pore by a woronin body (w) between a degenerate and functional cell. dc = Degenerate cell. (×20,000) **12)** Transmission micrograph showing the initial expansion of an intact cell into a degenerate cell at the periphery of a microsclerotium of *Verticillium albo-atrum*. ps = Displaced parent septum; nhw = new hyphal wall. (×13,700) **13)** Expansion of an intact cell into a degenerate hyaline cell at the periphery of a microsclerotium. nhw = new hyphal wall; ps = parent septum. (×13,700) **14)** Intra-hyphal hyphae of *Verticillium albo-atrum* showing the remnants of the original septum (os); oh = old hyphal wall; ih = intra-hyphal hypha wall. (×17,400)

matrix which is thought to cement the cellular mass together (Fig. 9). As the MS matures, additional electron-dense material occurs between electron-transparent layers in the septum and wall proper as well as exterior to the individual cells (Fig. 8, 9). We consider the granular electron-dense material to be the result of melanin accumulation. Consequently, melanin accumulation is greatest around the interior cells of the MS (Fig. 8). Heavy deposits of mucilaginous material are shown covering the exterior of a mature microsclerotium (Fig. 6).

We observed that the cytoplasmic contents of terminal or penultimate cells of a hyphal strand composed of several swollen globose cells and associated "buds" frequently degenerate, leaving structurally intact walls but lacking organized membrane systems, nuclei, mitochondria, etc. These "hyaline" cells which must be nonfunctional are seen adjacent to or embedded between pigmented cells (Fig. 10). In our studies, only the cytoplasmic organization of pigmented cells appeared intact, and therefore apparently possessed the capability of germination in mature MS. The cytoplasm of adjacent heavily pigmented cells was continuous through a single septal pore 50-75 mμ in diam. Septal pores connecting degenerated nonpigmented cells and functional cells are blocked by membrane-bounded dense bodies (Fig. 11). During the process of maturation, germination of intact cells within the modified hyphal strands proceeds through the septal pore into degraded hyaline cells, and may extend through several cells in the form of intra-hyphal hyphae (Fig. 12, 13, 14).

DISCUSSION.—Nearly all of the information on the structure of MS or of most sclerotial structures of fungi have been limited to observations with the light microscope. Studies of sclerotial form and structure are limited (3, 8, 9). In the sole electron microscopic study of sclerotia, Nadakavukaren (6) confirmed the observations of Gordee & Porter (3) that MS of *V. albo-atrum* are composed of two types of cells. He reported that thick-walled cells contained food vacuoles and mitochondria but lacked nuclei, while thin-walled cells contained nuclei but no other organelles. These observations have led to the interpretation by Isaac (4) and Schnathorst (8) that the thick-walled cells may serve as reservoirs for food material, but that they are nonviable and incapable of germination. The function of germination, according to these workers, is performed by the thin-walled, hyaline cells. Nadakavukaren indicated, however, that many of the thin-walled cells were empty, and examination of his illustrations reveals that the cytoplasmic contents of those cells possessing nuclei are, in fact, in a state of degeneration. Our data indicate that although it is true that the thin-walled (hyaline) cells are degenerate, the thick-walled (heavily pigmented) cells possess the necessary organelles for germination, including nuclei, and they not only appear to be capable of germination, but, when they do so, may germinate through the septal pores into adjacent hyaline cells (Fig. 12, 13, 14).

Our data indicate that the microsclerotium of *V. albo-atrum* consists of an aggregation of spherical cells interconnected by septal pores; individual cells become heavily pigmented and possess all the organelles considered necessary for germination, growth, and survival. Only the heavily pigmented cells retain these organelles in mature MS, and consequently they are presumably capable of germination. Although hyaline cells were observed, usually at the periphery of dark pigmented cells or apparently embedded in the outer fringes of the MS, their cytoplasm was always degraded, and therefore these cells are incapable of further function. The functional cytoplasm of the pigmented cells was sealed off by plugging of the septal pore between normal and disorganized cells by electron-dense bodies. It is probable that these plugs are woronin bodies normally associated with the pore. Similar plugging in *Ascodesmis sphaerospora* has been observed (1).

Observing MS germination through the light microscope leads one to conclude that germination occurred from hyaline cells. However, our electron micrographs revealed that during maturation of the MS, extension of normal (pigmented) cells into adjacent degenerating (hyaline) cells through the intervening septum to form intra-hyphal hyphae occurs in a manner similar to that reported in *Sclerotinia fructigena* (2). It is possible, therefore, that what appeared to be germination of hyaline cells (8) was germination and subsequent development of heavily pigmented cells via the septal pores into and through adjacent degraded hyaline cells. Therefore, we suggest that the functional cells in all cases are the heavily pigmented ones, and that hyaline cells perform no function related to germination or survival.

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