Electron Microscopy of *Botrytis cinerea* Conidia

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ABSTRACT

BUCKLEY, PATRICIA M. (University of California, Davis), VIRGINIA E. SJAHOLM, AND N. F. SOMMER. Electron microscopy of *Botrytis cinerea* conidia. J. Bacteriol. 91:2037-2044. 1966.—Sections of germinating and nongerminating *Botrytis cinerea* conidia were examined with an electron microscope. Uranyl acetate or lead citrate provided contrast between membranes and cytoplasm. Membrane-bounded, dense inclusions previously unreported in dormant spores were termed "storage bodies." Whorled structures, spherules, granules, and membrane loops were seen within these inclusions. The various forms assumed by the enclosed materials closely resemble phospholipid inclusions described for other cells. It is suggested that the inclusions provide material for the assembly of membranous organelles during germination. Utilization of the stored material apparently results in extensive vacuolization in advanced germinants.

Few studies of the fine structure of fungal spores have been made, despite many improvements in techniques which permit better visualization of such material. A review by Carbonell and Pollak (1) covers work with spores of some fungi, with special reference to human pathogens. Williams and Ledingham (19) described the cytological components of wheat stem rust uredospores. Hawker and Abbott (6) and Necăs, Havelková, and Soudek (11) examined *Rhizopus* sporangiospores by electron microscopy. Hawker and Hendy (7) reported briefly on the subcellular structure of mature and germinating conidia of *Botrytis cinerea* Pers. ex Fr. The observations of *B. cinerea* presently reported agree generally with their findings. In this study, however, large inclusions not mentioned by Hawker and Hendy (7) were a striking feature of dormant conidia. These closely resembled other cell inclusions known to contain phospholipid or phospholipid and associated protein. This report describes the morphology and probable function of the inclusion bodies. The apparent relation of the inclusion bodies to vacuolization during germination is discussed. Also considered are subcellular structural differences between germinating and nongerminating conidia, some of which evidently relate to the utilization of inclusion material.

MATERIALS AND METHODS

Dormant conidia of *B. cinerea* were obtained from 2-week-old cultures grown on V-8 juice-agar (15).

Cultures were harvested by washing them with water containing 1 drop of Tween 80 per 100 ml. The suspension was strained through several layers of gauze to remove mycelial fragments, and centrifuged briefly to separate the conidia. These were immediately fixed for 1 to 3 hr in 4% formaldehyde or 6% glutaraldehyde, prepared in 0.1 M phosphate buffer solution at pH 7.2. Conidia were rinsed with fresh buffer solution and resuspended for 1 or 2 hr in 1 or 2% buffered KMnO₄ solution at room temperature. Osmic acid fixation, alone or after aldehyde stabilization, was tried without success. Williams and Ledingham (19) have also reported obtaining poor results with OsO₄ fixation of fungal spores. After dehydration in a graded isopropyl alcohol series (25 to 100%), the spores were transferred to propylene oxide and infiltrated with epon resin mixture during a 3- to 5-hr period preceding embedding. The infiltrating and embedding mixture was 85 parts dodecyl succinic anhydride (DDSA), 50 parts Araldite 6005, 19 parts Epon 812, and 2 parts dibutyl phthalate (DBP), by measured volumes. Polymerization was for 24 hr at about 70 C in vacuo. Thin sections were cut with a diamond knife and mounted on uncoated 200- or 300-mesh copper screens. They were stained with uranyl acetate in methanol, with diluted lead citrate stain (13), or with these in sequence, and were examined with an RCA EMU-3 microscope. When germinating conidia were required, spores harvested as described were soaked in potato-dextrose broth (PDB) and resuspended in water or diluted PDB and placed on a shaker to promote germination. When 60% or more of the conidia had germ tubes up to one spore diameter in length, they were prepared for sectioning in the same way as were the dormant cells.

RESULTS

Surveys of near median longitudinal sections of dormant conidia revealed features similar to those described by Hawker and Hendy (7). A representative conidium was typically oval in shape and had a darkly outlined outer wall and thick, light inner wall (Fig. 1). Bodies whose high electron density denoted avid acceptance of the lead stain were observed (Fig. 1, 2a–2d). In other kinds of cells, bodies which are strikingly similar in staining properties and appearance have been shown to contain lipid or phospholipid (3, 10). Spore germination was accompanied by progressive vacuolization of the bodies, suggesting a storage function. Consequently, they were termed “storage bodies” (SB). In nongerminating spores, the SB appeared to consist of coarsely granular material surrounded by a single membrane (Fig. 1, 2a). Sometimes myelinic membranes were included (Fig. 2b). In some spores, the bodies were lined with packed dense material or were completely solid and dark as in Fig. 2c. Still another manifestation of the inclusion material was a wormlike or whorled structure (Fig. 2a). In germinating cells, vacuoles which apparently develop from the evacuation and enlargement of storage bodies contained densely stained loops and circular forms (Fig. 2e, 2f). The dark loops often resembled the myelin figures (16) of animal cells. Ultimately the vacuoles enlarged so that they almost occupied the spore. Besides the SB, stellate vacuoles (7) were noted, lying usually near the cell margin (Fig. 1, 2b). Other single membrane-bounded inclusions were seen whose electron density was less than that of the SB (Fig. 1, 2d, 3, 4b). Their appearance did not change during germination.

At direct magnifications of 6,000 to 10,000 $\times$, organelles and membranes were readily visualized, especially after lead staining. The plasmalemma was usually well defined in the nongerminating conidium but not in advanced germinants (Fig. 1, 4b). Much of the endoplasmic reticulum (ER) of the dormant conidium occurred as short double strands lying near the cytoplasmic periphery, oriented so that it appeared to originate from the plasmalemma (Fig. 1, 2d). Germinants exhibited longer strands of ER more centrally arranged; as many as three double strands frequently lay near nuclei (Fig. 3). Mitochondria were numerous and pleomorphic; lobed, rod-shaped, oval, and “doughnut” outlines were seen (Fig. 1, 3, 4b, 4c). Early germinants had an abundance of lobed or doughnut forms which appear to result from the sectioning of cup-shaped mitochondria (4). The nuclear envelope appeared to consist of two membranes, and nuclear pores were numerous

(Fig. 1). The exact number of nuclei per spore was not ascertained; three to six were frequently found in dormant cell sections. Figure 4b shows eight nuclear outlines in a germinating spore, and more have been seen.

DISCUSSION

Dark-appearing storage bodies were a prominent and constant feature of the dormant conidia of *B. cinerea* in the present study. However, such inclusions were not discussed by Hawker and Hendy (7) and do not appear in their illustration of a mature conidium. That these inclusions contain phospholipid, possibly in combination with protein, is supported by (i) their resemblance to phospholipid-containing bodies in other cells (10), (ii) the resemblance of the enclosed material to the appearance of known materials treated similarly (12, 17), and (iii) their marked affinity for heavy metal staining which resembles that of cell membranes whose phospholipoprotein composition is generally accepted (13).

Chou (2) and Chou and Meek (3) identified several kinds of lipid globules in snail cells, and associated those containing phospholipid with electron-dense laminated and crescentic accumulations having an affinity for heavy metal deposition during fixation and staining. Mercer (10) showed that a variety of forms may be assumed by phospholipid inclusions. The characteristic whorled, wormlike, and laminated structures in the *B. cinerea* cells, their occurrence within vacuoles, and the rounded faces of some vacuolar dense inclusions are very like some of the forms he described. In Larson's excellent electron micrographs of pollen cells (9) stained with lead citrate, numerous similar dense inclusions are labeled lipid, but are only briefly discussed. Whaley, Mollenhauer, and Leech (18) showed dense lipid inclusions in meristematic cells of corn, some of which are associated with obvious Golgi structures and some of which have concentrically lamellar or multivesicular structures with differing degrees of density. Figure 2f shows what appears to be a multivesicular body within a partially vacuolized area of a germinating conidium.

Recently Hohl (8) described lipoprotein lamellar inclusion material in the food vacuoles of certain slime molds and suggested a possible mode of formation of membranes from amorphous precursor material. Stoeckinius (17) elegantly demonstrated the spontaneous association of phospholipid with globin from bovine erythrocytes to yield structures which under electron microscopy are identical in appearance to the natural membranes of cells. Mercer (10) has

FIG. 1. Section through dormant conidium shows extensive storage bodies (SB), numerous adjacent dense vesicles, stellate vacuole (SV), and less dense inclusion (S). Several mitochondria (m) lie near nuclei (N). Unlabeled arrows point to nuclear pores. Endoplasmic reticulum (er) strands marginally located. Uranyl acetate- and lead citrate-stained. $\times 21,200$.

FIG. 2a. Wormlike whorled structure within a storage body. Lead citrate-stained. $\times 39,600$.

FIG. 2b. Storage body with amorphous material and myelinic membranes. Clearly defined stellate vacuole (SV) may contain neutral fat. Uranyl acetate- and lead citrate-stained. $\times 24,000$.

FIG. 2c. Storage body group within dormant cell. Hollow vacuoles lined with dense material resemble inclusions containing hydrated phospholipid (10). Uranyl acetate- and lead citrate-stained. $\times 23,000$.

FIG. 2d. Storage bodies partially filled with dense spherules and inclusion (S) of different density. Uranyl acetate- and lead citrate-stained. $\times 23,000$.

FIG. 2e. Dense loops resembling myelin figures, in germinant. Lead citrate-stained. $\times 19,400$.

FIG. 2f. Multivesicular body (mvb) in extensively vacuolized region in a germinant. Lead citrate-stained. $\times 39,800$.

FIG. 3. Early germinant with slightly protruding germ tube (GT) and abscission scar (ab). Complex structure of SB shown by inclusion membranes and associated vesicles on right. Small vacuoles (V) and light inclusion (S) are present. Multiple double strands of endoplasmic reticulum (er) lie near a nucleus. Arrow indicates apparent continuity of endoplasmic reticulum with mitochondrion. Lead citrate-stained. $\times 21,600$.

FIG. 4a. Vacuoles in germinant show lacy reticulum and connecting membranes. $\times 18,200$.

FIG. 4b. Conidium with germ tube shows vacuolized areas (V) and numerous nuclei (N). Mitochondria (m) are lobed, oval, and doughnut-shaped. Uranyl acetate- and lead citrate-stained. $\times 19,400$.

FIG. 4c. Interior of germinant with a number of ring- or doughnut-shaped mitochondrial outlines. Lead citrate-stained. $\times 22,000$.

suggested that membranous organelles of cells in general could be formed by spontaneous dissolution of phospholipid accumulated in vacuoles early in a cell's history. He noted that such inclusions are common in embryonic cells and that their evolution into structured membrane systems has been traced in a few cases. In the light of this and the present concept that membranes are constructed of lipid bound hydrophobically and in micellar form to structural protein (14), the advantage to the fungal spore of stored phospholipid or phospholipoprotein would be rapid assembly of membranous components during germination.

Myelin figures, the densely staining loops and circular laminated structures representing hydrated phospholipid, have sometimes been taken to mean degeneration when found in the cytoplasm (5). However, the appearance of membranes within storage bodies would seem rather to indicate developmental potential when hydration and stimuli for germination are supplied. Loops and circular structures in the vacuoles of germinants (Fig. 2e, 2f, 3, 4a, 4b) probably represent stages in the mobilization of cellular reserves. The interior of a far advanced germinant becomes nearly filled with the vacuole(s) which formerly held the dense storage substance. Such a vacuole retains a bounding membrane; often a cobwebby reticulum and a few collapsed-looking dark membranes remain within. In Fig. 4a, loops overlap the boundaries of adjoining vacuolar spaces, as if the vacuoles are part of an interconnecting complex. Positioning of storage bodies in some dormant conidia contributes to the picture of an interconnecting system (Fig. 1, 2c, 2d). The idea that the vacuoles result from utilization of storage material differs from Hawker and Hendy's (7) thought that vacuolization resulted from the flow of spore contents into the germ tube.

The peripherally situated stellate vacuoles are thought likely to be storage regions for triglycerides, in accord with interpretations by Mercer (10) and Chou and Meek (3) for like structures with crenated edges and dark interiors. No role has been yet postulated for the less dense inclusions (S). Intact spores examined by light microscopy show numerous globules which are stained intensely by Sudan black, indicating the presence of lipid. However, cytochemical studies of the several kinds of inclusions are needed.

That the endoplasmic reticulum is more extensive and arranged differently in germinants than in nongerminants agrees with the observations of Williams and Ledingham (19) on uredospores of *Puccinia graminis* f. sp. *tritici*. The

proposed role for SB in *B. cinerea* conidia fits in well with their interpretation that connections of ER with lipid bodies represent structurally the access of lipid to catabolic sites.

The results of this study suggest that fungal conidia should be a rewarding source of more detailed information about developmental subcellular structure and function.

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LITERATURE CITED

1. CARBONELL, L. M., AND L. POLLAK. 1963. Ultraestructura de los hongos especialmente los patógenos. *Acta Cient. Venezolana Supl.* 1: 174-183.
2. CHOU, J. T. Y. 1957. The chemical composition of lipid globules in the neurones of *Helix aspersa*. *Quart. J. Microscop. Sci.* 98:59-64.
3. CHOU, J. T. Y., AND G. A. MEEK. 1958. The ultra-fine structure of lipid globules in the neurones of *Helix aspersa*. *Quart. J. Microscop. Sci.* 99:279-284.
4. CHRISTENSEN, A. K., AND G. B. CHAPMAN. 1959. Cup-shaped mitochondria in interstitial cells of the albino rat testis. *Exptl. Cell Res.* 18:576-579.
5. FREEMAN, J. A. 1964. Cellular fine structure, p. 75-76. McGraw-Hill Book Co., Inc., New York.
6. HAWKER, L. E., AND P. McV. ABBOTT. 1963. An electron microscope study of maturation and germination of sporangiospores of two species of *Rhizopus*. *J. Gen. Microbiol.* 32:295-298.
7. HAWKER, L. E., AND R. J. HENDY. 1963. An electron-microscope study of germination of conidia of *Botrytis cinerea*. *J. Gen. Microbiol.* 33:43-46.
8. HOHL, H. R. 1965. Nature and development of membrane systems in food vacuoles of cellular slime molds predatory upon bacteria. *J. Bacteriol.* 90:755-765.
9. LARSON, D. A. 1965. Fine structural changes in the cytoplasm of germinating pollen. *Am. J. Bot.* 52:139-154.
10. MERCER, E. H. 1962. The evolution of intracellular phospholipid membrane systems, p. 369-384. In R. J. C. Harris [ed.], *The interpretation of ultrastructure*. Academic Press, Inc., New York.
11. NECÁŠ, O., M. HAVELKOVÁ, AND D. SOUDEK. 1963. Submicroscopic morphology of *Rhizopus nigricans*. *Folia Microbiol. (Prague)* 8:290-292.
12. REVEL, J. P., S. ITO, AND D. W. FAWCETT. 1958. Electron micrographs of myelin figures of phospholipid-simulating intracellular membranes. *J. Biophys. Biochem. Cytol.* 4:495-496.
13. REYNOLDS, E. S. 1963. The use of lead citrate at

- high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* **17**:208-212.
14. RICHARDSON, S. H., H. O. HULTIN, AND D. E. GREEN. 1963. Structural proteins of membrane systems. *Proc. Natl. Acad. Sci. U.S.* **50**:821-827.
15. SOMMER, N. F., M. CREASY, R. J. ROMANI, AND E. C. MAXIE. 1964. An oxygen-dependent post-irradiation restoration of *Rhizopus stolonifer* sporangiospores. *Radiation Res.* **22**:21-28.
16. STOECKINIUS, W. 1959. An electron microscope study of myelin figures. *J. Biophys. Biochem. Cytol.* **5**:491-500.
17. STOECKINIUS, W. 1962. The molecular structure of lipid-water systems and cell membrane models studied with the electron microscope, p. 349-367. *In* R. J. C. Harris [ed.], *The interpretation of ultrastructure*. Academic Press, Inc., New York.
18. WHALEY, W. G., H. H. MOLLENHAUER, AND J. H. LEECH. 1960. The ultrastructure of the meristematic cell. *Am. J. Bot.* **47**:401-449.
19. WILLIAMS, P. G., AND G. A. LEDINGHAM. 1964. Fine structure of wheat stem rust uredospores. *Can. J. Bot.* **42**:1503-1508.