Electron Microscopic Observations on the Spore Wall and "Operculum" Formation in Chlamydospores of Thielaviopsis basicola

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ABSTRACT

Chlamydospores of *Thielaviopsis basicola* in a chain were enveloped by a wall consisting of two layers. Within the chain envelope, each spore had a thick wall also consisting of an electron-dense outer layer and an electron-transparent inner layer. In the areas where lateral and end walls joined, the inner layer extended, by wedging obliquely, into the thick, outer layer of the spore wall, thus creating a circular weak junction at the rims. The spore wall may dehisce at the rim during spore germination, resulting in a split between the lateral wall and the end wall and thereby forming the "operculum". Phytopathology 60:613-616.

The soil-borne fungal pathogen, *Thielaviopsis basicola* (Berk. & Br.) Ferr., produces dark, thick-walled chlamydospores in chains, usually of 3-5 spores. These spores are more resistant to adverse conditions than the hyaline, thin-walled "endoconidia" (phialospores), and are the main surviving propagules in soil (11). The chlamydospore chain breaks up into individual single-celled spores following aging (3, 10, 13), chitinase treatment (2, 6), or exposure to natural soil (9), presumably as the result of enzymic attack by soil microorganisms (2). Upon separation from the chain, individual spores are short cylindrical or drum-shaped with two flat end walls, except for the terminal spore which is dome-shaped with a round distal end. Each spore germinates by producing a germ tube which emerges at the junction of the cylindrical lateral wall and the flat end wall at either end of the drum-shaped spore or at the flat end of the terminal spore. This unique germination phenomenon, although implicated in crude illustrations of some earlier papers (3, 5, 10), was not described in detail until recently by Patrick et al. (9). They called this hinged, lidlike end wall the "operculum", and concluded that there must be "a weak point all around the rim at each end of the chlamydospore cell" (9).

There had been no studies on the ultrastructure of *T. basicola,* and the mechanism of operculum formation in the chlamydospores was not known. This paper reports some of the morphological features in the wall structures of *T. basicola* chlamydospores as observed under the electron microscope.

MATERIALS AND METHODS.—Chlamydospores of *T. basicola,* isolate T35, were obtained from 2-, 3-, and 5-week-old cultures grown at 25 C on cornmeal agar (Difco, 17 g/liter) in petri dishes. Chlamydospores were harvested by gently scraping, with a scalpel, the chlamydospore chains along with endoconidia and a portion of the mycelial mat off the agar surface. The harvested spore mass was gently compacted to spheres of about 3-4 mm in diam and individually wrapped and tied in a small piece of lens paper before fixation at room temperature. Three fixatives with a total of seven treatments were tested: (i) 3% $KMnO_4$ in phosphate buffer for 1, 3, or 6 hr; (ii) 3% $KMnO_4$-0.001 M $CaCl_2$ in phosphate buffer for 1, 3, or 6 hr; and (iii) 3% $KMnO_4$ in phosphate buffer for 1 hr followed by 1.5% solution of OsO_4 for 30 min. The phosphate buffer used in all three fixatives was at 0.067 M and at pH 7. An aspirator was used in the first 30 min of fixation to remove air and facilitate proper fixation. Fixed materials were dehydrated in a graded ethanol series and embedded in epoxy resin, Epon 812. Sections were cut with glass knives using a Jeolco JUM-5A or a Porter-Blum MT-2 ultramicrotome and examined under a Jeolco JEM-7 or an RCA EMU-3B electron microscope at 80 kv. It is well known that thick-walled fungal structures are difficult to fix; most of the above procedures resulted in poor fixation. Fixation in 3% $KMnO_4$ in phosphate buffer followed by 1.5% OsO_4 gave the best results in our study.

RESULTS AND DISCUSSION.—Electron microscopic observations of longitudinal sections of chlamydospore chains revealed that the spores in a chain were enveloped by a distinct outer wall (Fig. 1-A, C, D), verifying what was shown graphically in earlier papers by Zopf (13) and by Rosenbaum (10). Like the walls of hyphae and endoconidia, this outer wall of the spore chain, or "chain envelope", consisted of two layers: an electron-dense outer layer and an electron-transparent inner layer (Fig. 1-A, C, D). The chain envelope was an extension of the wall of the lighter colored basal cells of the chain and of the hypha from which the chlamydospore chain was formed.

Within this chain envelope, each chlamydospore had its own thick wall similarly consisting of an electron-dense outer layer and an electron-transparent inner layer, the latter adjoining the plasma membrane of a protoplasm rich in lipid bodies. The spore wall was usually 2-3 times as thick as the chain envelope, and was thickest at the corners where the lateral walls joined the end walls (Fig. 1-C, D). The distribution of the coarsely granular electron-dense wall material at these corner areas, however, was strikingly different from that at other wall areas, providing evidence for

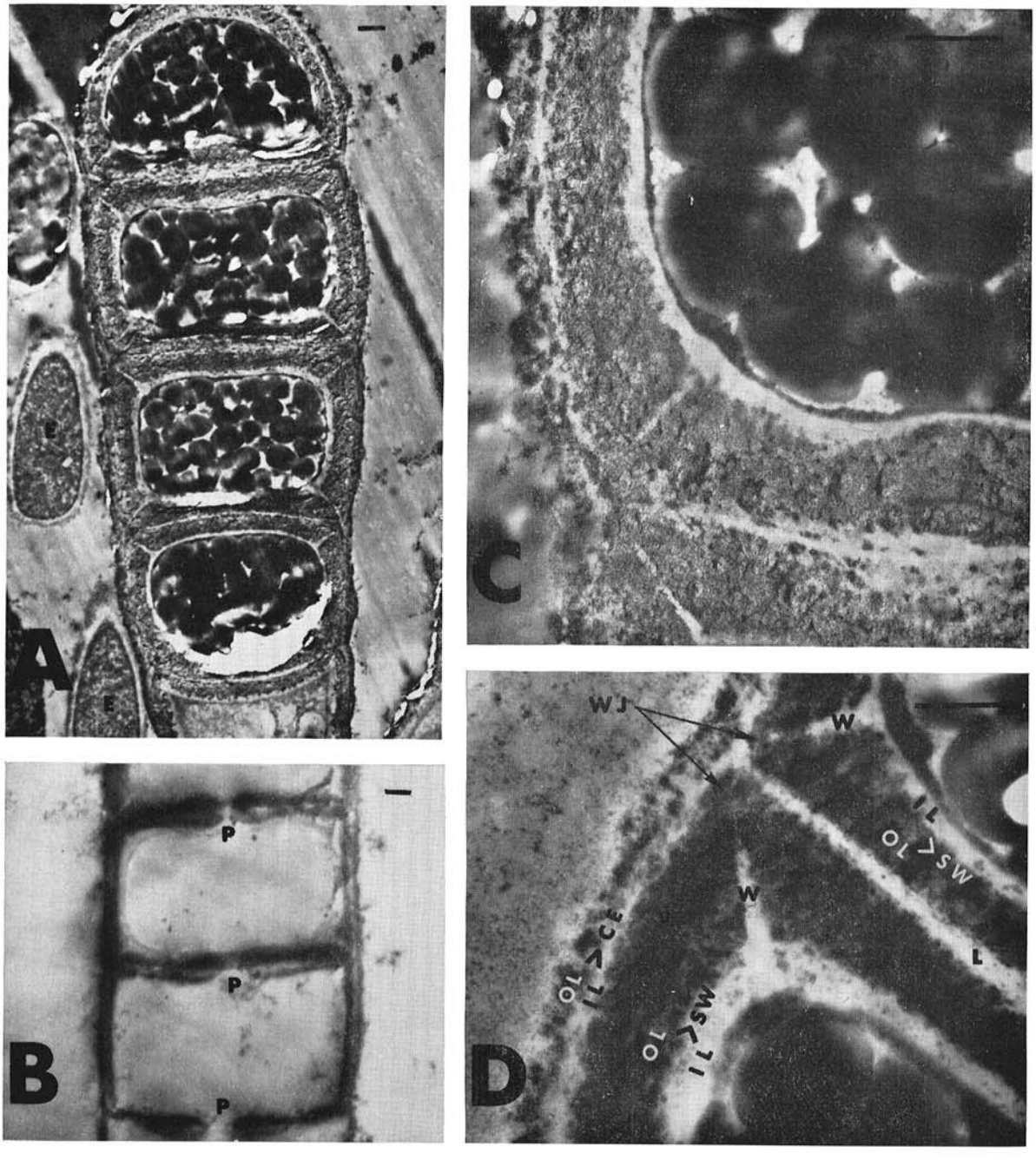

Fig. 1. Electron micrographs of chlamydospores of *Thielaviopsis basicola* in longitudinal sections. **A)** A chain of four mature chlamydospores within a chain envelope. At left are oblique sections of "endoconidia" (E). (×3,900) **B)** Median section of portion of a chain of immature chlamydospores showing incomplete wall formation and "septal pore" (P) in the end walls. The cell content was lost due to inadequate fixation. (×3,400) **C)** Chain envelope and spore wall at a higher magnification showing the granulation and electron densities of the various wall layers. (×14,500) **D)** Higher magnification of the corners of two adjoining chlamydospores showing the outer layer (OL) and inner layer (IL) of the chain envelope (CE) and of the spore wall (SW), the "wedge" (W) at each corner of the spore wall creating a weak junction (WJ), and the lamella (L) between the end walls of two adjoining spores. (×16,500) Figures A, C, and D involved the fixation procedure of 3% $KMnO_4$ in phosphate buffer for 1 hr, followed by 1.5% OsO_4 solution for 30 min. Figure B involved the fixation procedure of 3% $KMnO_4$ in phosphate buffer for 1 hr without postfixation with OsO_4, hence the loss in cell content. Chlamydospores in all four figures were from 3-week-old cultures of isolate T35. The scale lines represent 1 μ.

the operculum formation as described by Patrick et al. (9). At these four corners, the generally electron-transparent inner layer of the spore wall extended, by wedging obliquely at about a 45-degree angle, into the thick, electron-dense outer layer of the spore wall (Fig. 1-C, D). Dark granules were seen sparsely scattered in the transparent inner layer and in the wedge-shaped areas. Each "wedge" tapered gradually to a fine point just short of reaching the outer edge of the dark spore wall, thus creating a "weak junction" barely connecting the electron-dense portions of the lateral and end walls of each spore. This is most clearly illustrated in Fig. 1-D. The wedges at the four corners of each spore, as seen in the 2-dimensional longitudinal sections, were in fact two continuous, transversely located circular bands. Each band occurred around the rim at either end of the drum-shaped spore or, in the case of the dome-shaped terminal spore, around the rim of the proximal flat end wall (Fig. 1-A).

The oblique wedging by the inner layer into the four corners of the outer layer of the spore wall was observed also under the light microscope at ×1,000. Two other isolates, T491 from cotton and T492 from tobacco, together with T35 from citrus, were included in the study. Chlamydospore chains of the three isolates were fixed in Randolph's modified Navashin's solution, embedded in gelatin, and sectioned on a cryostat microtome at 6 μ. Transparent wedges identical in appearance to those in isolate T35 were observed in the four corners of the spore wall in both isolates T491 and T492, and were similar to those observed in T35 under the electron microscope.

The chemical nature of the chlamydospore wall of *T. basicola* has not been determined, but it is likely that chitin, along with glucans and other polysaccharides, is the main constituent of the spore wall as found in other higher fungi (1). Based on the findings of Durrell (4) on the composition and structure of dark spore walls of other fungi, the thick, electron-dense outer layer of *T. basicola* chlamydospore wall may contain melanin or melaninlike substances, and the thin, generally electron-transparent inner layer of the wall is probably the main chitinous layer containing little or no melanin. Pigmentless albino chlamydospores of *T. basicola* do not contain melanins. Linderman & Toussoun (8) reported that single, separated, albino chlamydospores "round up instead of retaining a rigid cylindrical shape, and that germination occurs through the wall rather than by means of an operculum". The wedge areas and the inner layer in the wall of a normal chlamydospore, therefore, are reasonably believed to be chemically less resistant and structurally weaker than the melanin-containing outer layer. During chlamydospore germination, the circular band may undergo dissolution as a result of endogenous enzymic action, and the internal pressure developed within the spore would then be sufficient to partially disjoin the weak junction at the rim. The dehiscence at the rim enables an end wall, which becomes the "operculum", to split in circumscissile fashion from the lateral wall at the weakest area, thereby allowing the emergence of the germ tube, as illustrated by Patrick et al. (9).

Patrick et al. (9) also reported the existence, in germinated chlamydospores, of an inner spore wall from which the hyphal wall of the germ tube is extended. This thin, hyaline inner wall with its germ tube can be separated freely from the dark spore "shell", and becomes detached. Based on existing knowledge of genesis of the germ tube wall in germinating spores of other fungi (1), this inner spore wall is either the electron-transparent, inner layer of the thick spore wall or could be formed de novo under the existing wall. We believe that the latter mode of genesis is involved in the case of *T. basicola* chlamydospores, but the exact nature awaits further study of germinating spores.

Between the end walls of two adjacent spores in the chlamydospore chain, there was a generally electron-transparent layer that resembled the inner layer of the spore wall as well as the inner layer of the chain envelope in both the degree of electron density and degree of granulation (Fig. 1). The middle-lamellalike layer was connected at the edge, in a right angle, to the inner layer of the chain envelope, and is perhaps the initial cross plate formed during cytokinesis of the spore chain. The formation of the lamella layer is probably the result of inward extension of the wall material of the chain envelope following membrane invagination when septations are formed, in acropetal successions, during the initial development of the chlamydospore chain (12). The lamella is believed to be the cementing "intercellular material" susceptible to chitinase digestion, as reported by Christias & Baker (2).

The "septal pore" at the center of the end wall or "operculum", as described by Patrick et al. (9), was often seen in median longitudinal sections of some spores under the electron microscope (Fig. 1-B). The electron microscopy of these septal pores, other cytological and morphological features of chlamydospore walls, and the ontogeny of chlamydospore chains will be reported elsewhere.

Germination of chlamydospores of *T. basicola* is enhanced when the spores are separated from the spore chain following chitinase treatment or enzymic attack by soil microorganisms (2, 6, 7, 9). The age of spores also affects their ability to germinate in the absence of nutrients (6, 7). The relationships of the morphological features in chlamydospore wall structure to separation of the spore chain and to the physiology of spore germination are discussed in a separate paper (7).

ADDENDUM

Since the submission of our manuscript in August 1969, two papers on electron microscopy of *T. basicola* have appeared. The abstract by C. Christias & K. F. Baker, 1969 (Phytopathology 59:1021), described the ultrastructure of chlamydospores. Although the wedges and weak junctions in the spore wall, as reported in our paper, were not mentioned in the brief abstract, they were present in the electron micrographs used in

their talk presented at the APS Meeting at Spokane, Washington. The paper by V. G. Del Vecchio et al., 1969 (J. Gen. Microbiol. 58:23-27), described the wall structure of immature chlamydospores from 4-day-old cultures. The prominent wedges in the walls of mature chlamydospores were, therefore, not present in their electron micrographs.

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