Bark beetle – fungal symbiosis. III. Ultrastructure of conidiogenesis in a *Sporothrix* ectosymbiont of the southern pine beetle

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SJB 133 is an isolate of a variety of *Ceratocystis minor* that is found in a *Sporothrix* imperfect state as an ectosymbiont of *Dendroctonus frontalis* Zimm. Within the mycangium (fungal transport pocket of the beetle), SJB 133 grows in yeast-like fashion. Cells contain prominent vacuoles which appear to bridge between dividing cells. Hypha-like transition cells are also present. In continuous culture on potato carrot agar, SJB 133 produces sympodial conidiophores. The resulting conidia are quite similar to the yeast-like cells in the mycangium and the conidiophores resemble some of the transition cells.

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SJB 133 est une souche d'une variété de *Ceratocystis minor* que l'on rencontre dans un stade imparfait *Sporothrix* comme ectosymbionte de *Dendroctonus frontalis* Zimm. A l'intérieur du mycangium (poche de l'insecte lui servant à transporter le champignon), SJB 133 croît comme une levure. Les cellules contiennent des vacuoles proéminentes qui semblent continues entre cellules en division. Des cellules de transition semblables à un hyphe sont également présentes. En culture continue sur un milieu gélosé de pomme de terre et de carotte, SJB 133 produit des conidiophores sympodiaux. Les conidies qui en résultent sont tout à fait semblables aux cellules de type levure dans le mycangium et les conidiophores ressemblent à certaines cellules de transition.

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Introduction

Insect–fungal associations are responsible for the destruction of large numbers of trees of both lumbering and horticultural significance. Among the most damaging associations are those which involve scolytid beetles, such as certain members of the genus *Dendroctonus*, which attack conifers, or as *Scolytus multistriatus*, which attacks elms. The complex associations among beetle, fungi, and host tree represent symbiosis in its broadest sense. A number of aspects of these associations remain unidentified, but the adult beetle is the fungal vector and the fungi contribute to, or may be required for, proper insect development (Norris 1972; French and Roper 1972; Barras 1973). The fungi may be transported within specialized integumental structures of the beetle; these fungal transport pockets have been termed mycangia (Batra 1963) or mycetangia (Francke-Grosmann 1967). Most mycangial fungi are pleiomorphic: within the mycangium, fungal growth is yeast-like, but in culture or in the tree, growth is usually mycelial. The mycangial microenvironment is selective so that specific species or forms of fungi flourish and 'weed' species are largely absent (Francke-Grosmann 1967). By definition mycangia are glandular, and apparently the secretions control the growth form and species composition of the mycangial microflora (Barras and Perry 1971; Happ *et al.* 1971). The secretions are allomones (Brown 1968), interspecific chemical signals which act, in this case, to promote a symbiotic association (see Happ (1973) for other examples).

The southern pine beetle, *Dendroctonus frontalis* Zimm., maintains a symbiotic association with two fungi (Barras and Perry 1972). One of these (SJB 122) is a basidiomycete derivative, with a multinucleate yeast stage and a hyphal

stage that shows clamp connections and dolipore septa (Barras and Perry 1972; Happ *et al.* 1975[1]). The second fungal ectosymbiont (SJB 133, CBS 737.70) is an isolate of a varietal *Ceratocystis minor* with a *Sporothrix* imperfect state (Barras and Taylor 1973). The hyphae and conidia of primary subcultures of SJB 133 are polymorphic; however, the conidia become monomorphic after several transfers on potato carrot agar. The perfect state has not been observed in nature but was produced in vitro after prolonged growth on potato glucose agar. In the present paper, we describe the fine structure of the conidia of SJB 133 within the mycangium and in continuous culture.

Materials and Methods

Adult beetles were collected and sexed as they emerged from bolts of naturally infested loblolly pines (*Pinus taeda* L.) sampled at various localities within Louisiana. The females, which possess a large glandular prothoracic mycangium (Barras and Perry 1971), were shipped to New York University or Colorado State University, where the anterior portions of the prothoraces were removed and fixed for transmission electron microscopy.

Tissues were fixed in phosphate-buffered glutaraldehyde or in cacodylate-buffered glutaraldehyde. For the phosphate technique, fixation in 5% glutaraldehyde (0.1 *M* sodium phosphate buffer, pH 7.4) at 0–4 °C for 2 to 6 h was followed by a 1-h wash in the same buffer with 10% sucrose added (Locke 1966) and postfixation in 1% osmium tetroxide buffered with phosphate (0.1 *M*, pH 7.4) and containing 4% sucrose. For the cacodylate technique, tissues were fixed in 2.5% glutaraldehyde (0.05 *M* cacodylate buffer pH 7.2, containing 0.15 *M* sucrose) at 0–4 °C for 2–12 h, washed in several changes of fresh buffer containing 0.5 *M* sucrose, and postfixed for 1 h in cold veronal-acetate-buffered 1% osmium tetroxide containing 0.4 *M* sucrose (Gupta and Smith 1969).

Tissues were dehydrated via an ethanol series and embedded in Epon 812 or Spurr's. Thin sections were stained routinely for 20 min with saturated uranyl acetate in ethanol–methanol (equal parts 70% ethanol and absolute methanol) followed by 5 min in lead citrate (Reynolds 1963). Sections were examined and photographed at 50 kV in an RCA EMU 2E or an RCA EMU 3D.

Cultures of the *Ceratocystis* isolate SJB 133 were obtained from earlier isolates (Barras and Perry 1971, 1972). Plates containing potato carrot agar were inoculated and after allowing 2 months for growth, the plates were flooded with cold glutaraldehyde in phosphate or cacodylate and allowed to fix for 2 h; small pieces were excised for postfixation in osmium. Processing, dehydration, etc., were as described above.

[1]HAPP, G. M., C. M. HAPP, and S. J. BARRAS. Bark beetle–symbiosis II. Fine structure of a basidiomycete ectosymbiont of the southern pine beetle. Submitted to Can. J. Bot.

Observations

Conidia within the Mycangium

The propagules within the mycangium are quite small (3–4 × 2 microns (μ)) and generally are ovate (Fig. 1). Several thousand fungal cells fill the lumen of the mycangium as a compact mass separate from the basidiomycetous symbiont.

As is characteristic of *Sporothrix* states (Thibaut 1970*a*; Lane *et al.* 1969), the yeast phase has a generally muddy appearance in transmission electron micrographs. The cell wall has a low affinity for the heavy metals used as stains in this study; however, a thin superficial layer, about 100 Å in thickness, is sometimes observed (Figs. 1, 5). The surrounding matrix consists of fine flocculent fibers (Figs. 1–5, 7) which are rather similar to those produced by the type 2 secretory cells which empty their products into the mycangium (Happ *et al.* 1971).

The plasma membrane is repeatedly invaginated to yield narrow channels which run 200–300 mμ toward the center of the cell (Figs. 1, 3–7). Inflated ends of these invaginations form paramural pockets (Figs. 1, 3–7), which may impinge upon the nucleus (Fig. 1) or upon the vacuole (Figs. 3–7). The channels might open into the vacuole to permit flux of material between the vacuole and the paramural spaces, but we have failed to detect such openings.

The vacuole is prominent in most cells. Its contents are usually flocculent (Figs. 1–7), of varying densities, and often include tubular membranous elements (Figs. 1–5, 7). Masses of dense material are frequently seen within the vacuole (Figs. 4, 5). In some cells, the vacuole is conspicuously constricted, which suggests that the vacuole may bridge between two cells in division (Fig. 5).

The individual yeast cells appear to be uninucleate. The nuclei are 0.5–1 μ in diameter and bounded by a distinct pair of membranes, which form the nuclear envelope (Figs. 1, 2, 4, 7), and the envelope is traversed by nuclear pores (Fig. 4). Several mitochondrial profiles are seen in any section through a cell (Figs. 1–5, 7); we do not know whether each profile represents an individual mitochondrion or whether the collective profiles are many slices through a single tortuous mitochondrion.

Although most of the cells are ovate, occasional cells appear pyriform (Fig. 3) or elongate

(Figs. 2, 4, 7). The elongate cells are perhaps the more interesting for they are of lower cytoplasmic density than other cell shapes and appear to be quite similar to the transition cells, intermediate between yeast and mycelial cells, described from *Sporotrichum schenckii* (=*Sporothrix schenckii*)[2] by Lane and Garrison (1970). Woronin bodies are adjacent to the septa which lie between these cells (Fig. 4). Paramural pockets containing inclusions, similar to lomasomes (Marchant and Moore 1973), are associated with the vacuoles of these transition cells (Figs. 2, 4, 7).

[2]We recognize there is some dispute over placement of this fungus. In this paper we follow Carmichael (1962), who compared type species of both *Sporothrix* and *Sporotrichum* and found sympodulate conidia only in *Sporothrix schenckii* Hetkoen and Perkins, type species of *Sporothrix*.

Conidia from Continuous Culture

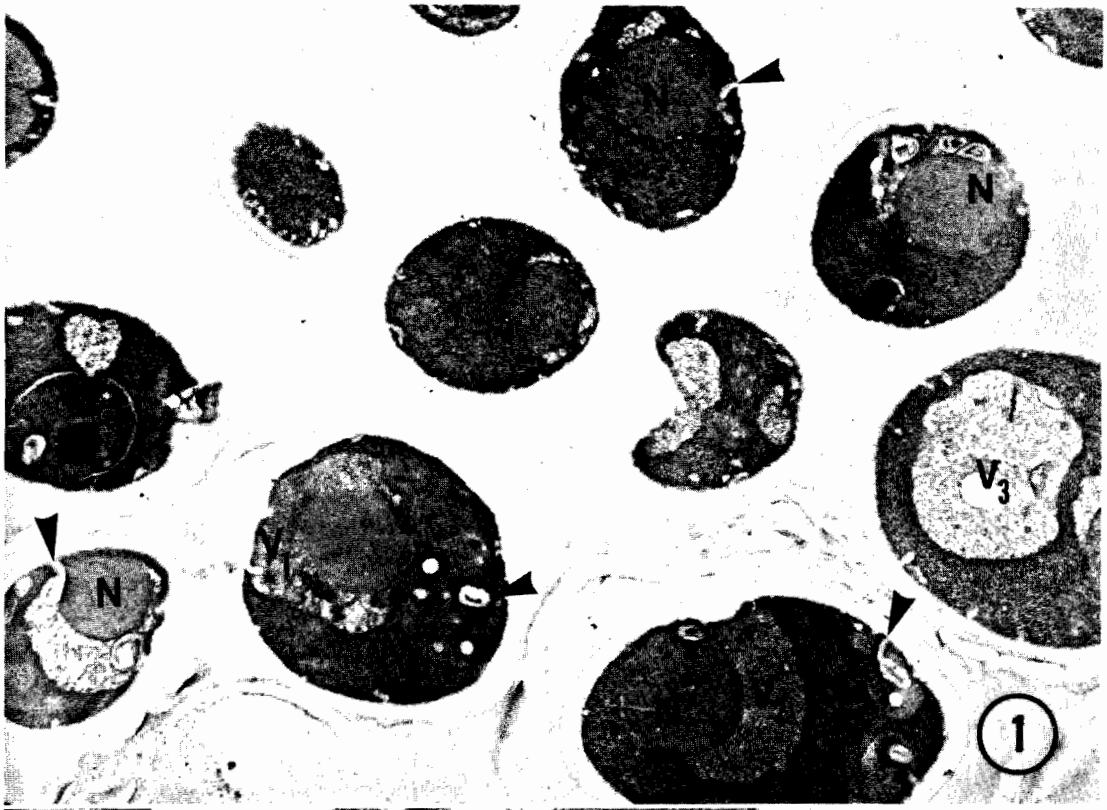
Conidia are sympodially produced from rachiform conidiophores (Kendrick and Carmichael 1973); each conidiophore is 1–2 μ in width and 10–15 μ in height (Fig. 8). The conidiogenous cells are sometimes adjacent to hyphal cells that are dead, but most frequently, the hyphal cell is viable and the intervening septum contains an electron-dense bilenticular plug (Figs. 9, 12, 13). Similar septal plugs have been

ABBREVIATIONS USED: C, conidium; *cb*, cylindrical body; CG, conidiogenous cell; *dm*, dense mass; *h*, hypha; *m*, mitochondrion; *mat*, matrix; N, nucleus; TC, transition cell; V, vacuole; *wb*, Woronin body; YC, yeast-like cell.

FIGS. 1–7. Fungi within the mycangium of the female southern pine beetle. Fig. 1. Uninucleate yeast-like cells are of various shapes. Prominent vacuoles of differing densities (compare V_1, V_2, V_3) are common. Arrowheads indicate paramural pockets. × 14 700. Fig. 2. Transition cells are elongate and form short hyphal chains The cytoplasmic density of the transition cell is lower than that of the yeast-like cell. An arrowhead indicates a paramural pocket. The fibrous matrix may represent secretions of the beetle. × 23 600.

Fig. 3. A lacrymoid yeast-like cell. Two paramural invaginations (solid arrows) project inward toward the vacuole and one of them abuts directly upon the membrane bounding the vacuole (hollow arrowhead). Membranous elements are present within the vacuole (small arrowhead). × 24 200. Fig. 4. A single nucleus (N) in the upper yeast-like cell communicates with the cytoplasm via nuclear pores (small arrowheads). The vacuole of the central yeast-like cell contains a prominent dense mass. Transition cells are at the lower corners of the photograph. The paramural pocket in the left cell contains lomasome-like elements (solid arrow). Next to the septum between two paramural cells on the right is a Woronin body. × 16 800. Fig. 5. A yeast-like cell with a central vacuole containing a dense mass and membranous elements (small arrowheads). The vacuole appears to be pinched at the position of the dense mass and to bridge between two halves of the cell, which suggests that cytokinesis is in progress. Paramural invaginations are seen at the solid arrow. An outer, electron-dense layer of the cell wall is bracketed by the hollow arrows. × 24 200. Fig. 6. Two paramural pockets (asterisks) formed by invaginations of the plasma membranes which project toward the vacuole. This figure is an enlargement of the region around the lower solid arrow in Fig. 3. × 120 000. Fig. 7. A vacuole within a transition cell with several distinct paramural pockets (arrowheads). The other cells in this photograph are yeast-like. × 16 800.

FIGS. 8–15. Conidia formation on potato carrot agar. Fig. 8. A hypha running across the photograph gives rise to a number of sympodial conidiogenous cells, each of which carries a cluster of conidia. × 1130. Fig. 9. A conidiogenous cell flanked by four conidia. Note that the lower conidium in each pair at the upper portion of the micrograph has an extensively infolded plasma membrane. The conidia at the lower right were produced by an adjacent conidiogenous cell. The septum between the conidiogenous cell and the hyphal cell is occluded by a double electron-dense plug (between large arrows). × 10 400. Fig. 10. A conidiogenous cell surrounded by four conidia, overexposed to reveal the structure of the cell wall. Cell walls laid down in the zones of abscission have an innermost layer of higher electron density (between arrows). × 13 700. Fig. 11. A vacuole of a conidiogenous cell containing a cylindrical body. The outermost layer (*a*) appears to be continous with the membrane bounding the vacuole. The intermediate layer (*b*) is of higher density and apparently runs farther into the vacuole than does the outermost layer. The innermost layer (*c*) is of lowest electron density. A paramural pocket is indicated by the asterisk. × 61 400. Fig. 12. Septum between a conidiogenous cell and a hyphal cell. The lower portion of the septal plug (arrow) is already formed. The circular profile indicated by the small arrowheads in the upper (conidiogenous) cell may be a precursor of the upper portion of the septal plug. × 36 000. Fig. 13. A conidium, apparently in the process of abscission from the conidiogenous cell. The septal plug (between hollow arrows) has formed and the abscission of the cross wall is largely complete. The two cells remain attached only via the outermost layers of the cell wall (thin arrows), which appear to be continuous between the conidium and the conidiogenous cell. The septal plug between the conidiogenous cell and the hyphal cell is below the white arrow. Note the paramural invaginations above the septal plug. Micrograph overexposed to show structure of cell wall. × 14 100.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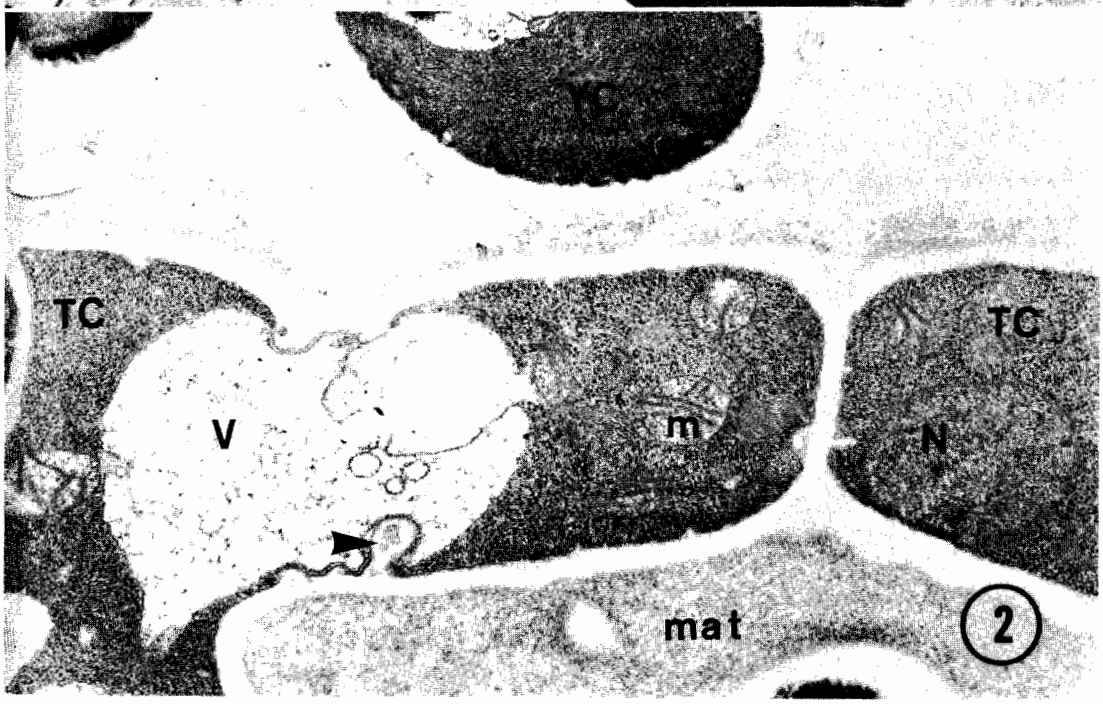
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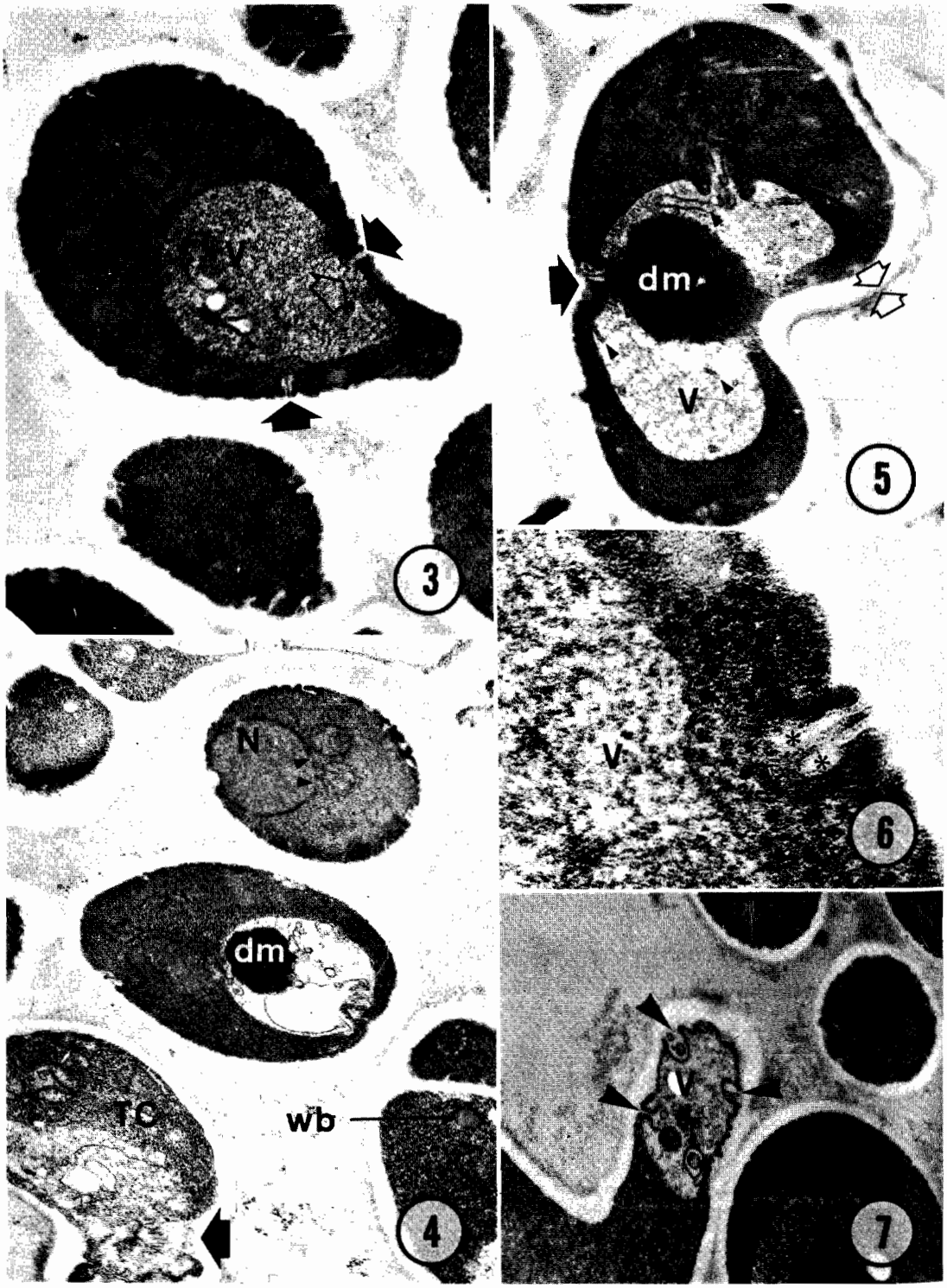
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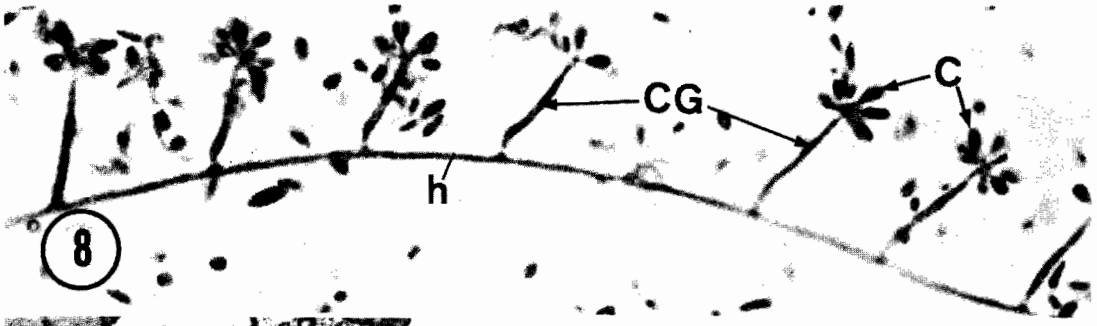
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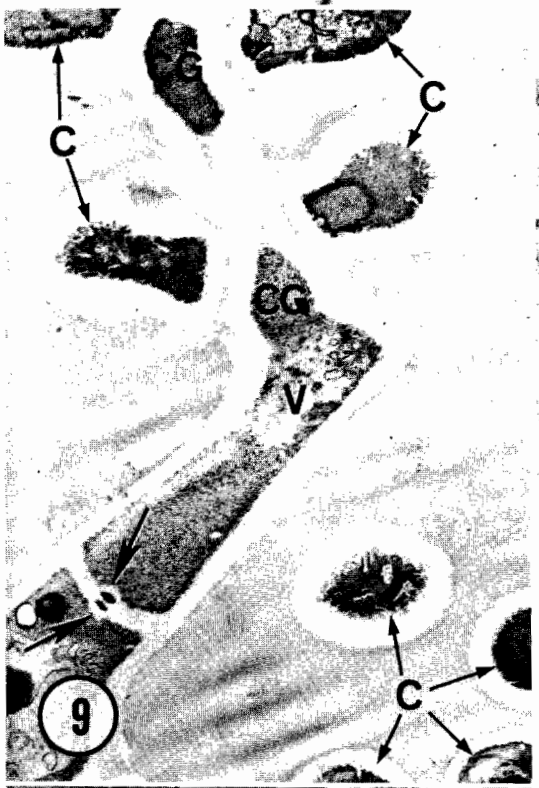
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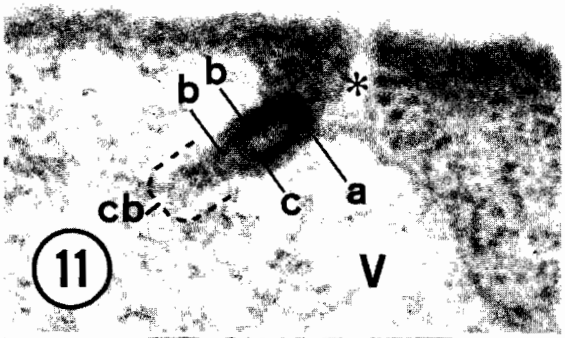
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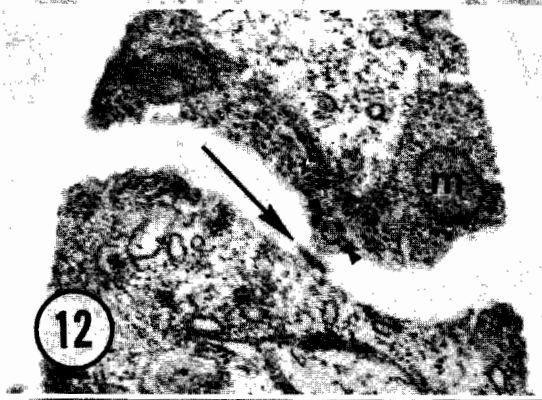
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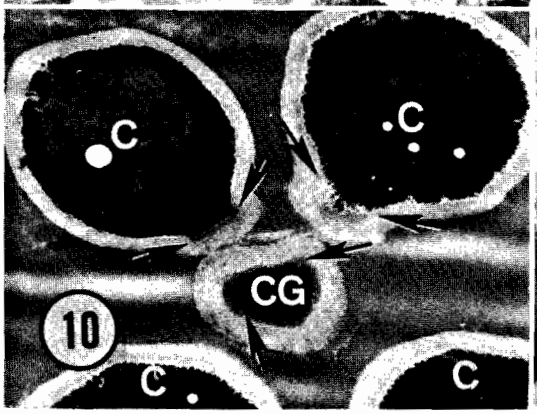
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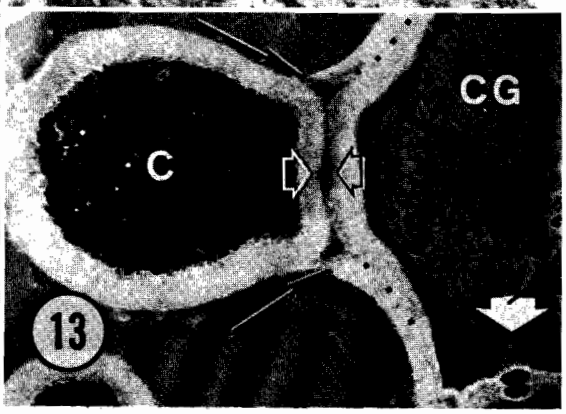
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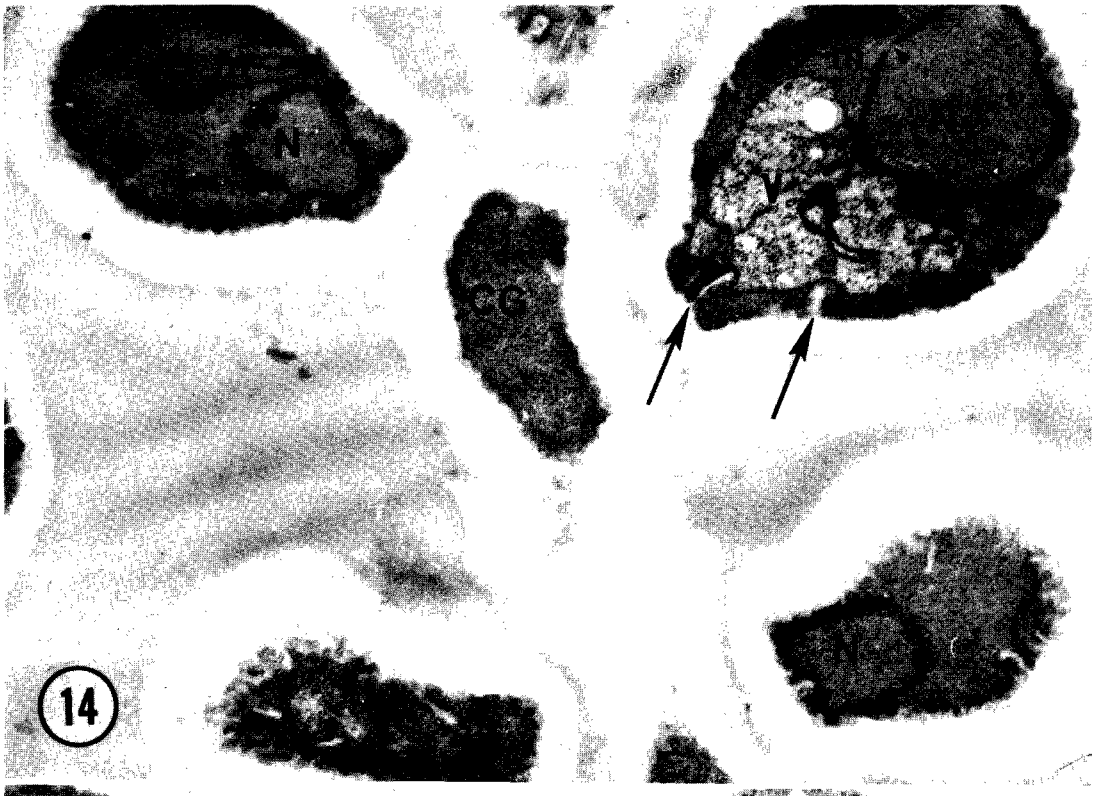
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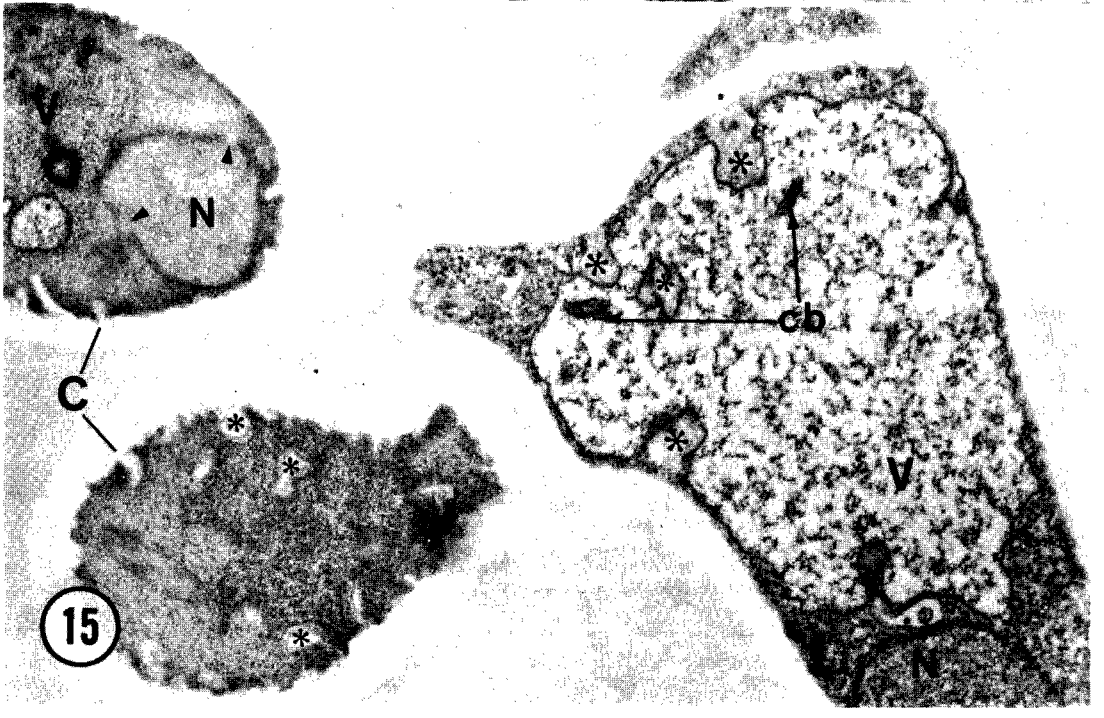
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described by Thibaut (1969). Each half of the electron-dense plugs may be derived from a circular organelle (Fig. 12), which may be a descendent of a Woronin body. The nuclei of the conidiogenous cells (Fig. 15) are often central and flanked by vacuoles.

The prominent vacuoles of the conidiogenous cells contain flocculent materials (Figs. 9, 15). The plasma membrane invaginations (paramural pockets) often impinge upon the vacuoles (Figs. 11, 15). The contents of the pockets may be homogeneous (Fig. 15), quite like the 'grey substance' of Carroll (1972), or the contents may include tubular or vesicular profiles of the lomosome variety (Marchant and Moore 1973). The paramural pockets of *Sporothrix schenckii* contain a diverse population of inclusions, which are quite similar to those in SJB 133. Often paramural pockets impinge upon the vacuole adjacent to presumed sites of conidial detachment (Fig. 15). Within the vacuole, more or less aligned with the growth axis of the conidium initial, is a cylindrical body (Fig. 15), which appears to consist of invaginated vacuole membranes, a sleeve of fine microfilaments, and an electron-transparent center. The role of this cylindrical body remains moot; however, parallels with the sheaves of microfibrils described by Cole (1972) are appealing.

The mature conidia are bluntly obpyriform in shape. Usually, the nucleus is basal (Figs. 9, 10, 13–15), but in a few sections (Figs. 10, 14) it is apical. Nuclear pores are quite common (Figs. 14, 15). The mitochondria tend to lie lateral to the nucleus, in a cytoplasm of moderate electron density (Fig. 14). The vacuole may be apical (Fig. 15) or basal (Fig. 14) and often contains membranous inclusions (Fig. 14).

The conidia are blastic, growing by deposition of new cell walls. The outermost layers of the conidial cell walls appear to be continuous with the outermost layers of the conidiogenic cell (Fig. 13); the innermost layers do not (Fig. 13). At the site of abscission, the innermost layer of the cell wall is of higher electron density than the rest. Smaller (and presumably younger) conidia tend to be more elongate than the ovate mature conidia. It appears that longitudinal growth may occur first and then the conidium initial may swell to become ovate.

Discussion

The structure of the yeast stage of SJB 133 is similar to that reported in *Sporotrichum schenckii* (=*Sporothrix schenckii*) (Lane *et al.* 1969; Thibaut 1970*a*). The appearance of the cytoplasmic organelles, the nucleus, and the overall density of the cytoplasm are similar in our *Sporothrix* state and in *S. schenckii*. SJB 133 differs from the previously described yeast phase in several respects: (1) the cell wall of the yeast-stage SJB 133 had low affinity for electron stains, unlike *S. schenckii*; (2) the cells of SJB 133 tended to be pyriform rather than fusiform; and (3) SJB 133 showed very distinct paramural pockets which were not reported in the yeast phase of *S. schenckii* (Thibaut 1970*a*; Lane *et al.* 1969), although similar structures have been seen in the mycelial phase (Thibaut 1970*b*). In the yeast–mycelial transition of *S. schenckii*, the fusiform cells become 'transition cells,' intermediate between yeast and mycelial cells in their characteristics (Lane and Garrison 1970). We find certain cells within the mycangial lumen that seem to have the characteristics of the transition cell class. These elongate transition cells are of lower cytoplasmic density than the pyriform or ovate propagules and contain Woronin bodies (Fig. 4).

To our knowledge, the sympodial production of conidia has not been previously described at the ultrastructural level for *Sporothrix*. However, the process has been examined in other species with sympodial conidiophores (Cole 1973), and our data are quite consistent with his. Unfortunately, our lack of precise information on the

Fig. 14. Micrograph at higher magnification of the upper portion of the sympodial conidiogenous cell shown in Fig. 9. Within the conidia, nuclei may be apical (as in upper right), but more commonly, the nucleus is basal (upper left and lower right). Paramural invaginations which impinge upon the vacuole are indicated by the arrows. The nuclear membrane contains pores (arrowhead). × 28 300. Fig. 15. A conidiogenous cell (right) and two conidia (left). Asterisks mark the extensive paramural invaginations in the lower (presumably younger) conidium and in the conidiogenous cell. In the latter, the paramural pockets appear to contain material of intermediate electron density. Two cylindrical bodies lie in the vacuole of the conidiogenous cell. Small arrowheads indicate pores in the nuclear membrane of the conidium (upper left). × 25 100.

layers of the cell wall and their deposition makes a detailed comparison difficult.

The propagules of SJB 133 which lie within the mycangium of the beetle and the conidia which are sympodially produced in stable cultures differ very little from one another in ultrastructure. The mycangial propagules and the conidia are of similar size and have similar wall structure. Both contain a single nucleus and a vacuole. In the cultural conidia, the vacuole tends to remain packed with dense material, whereas the mycangial propagules have a looser organization of their vacuolar contents. In appearance, the vacuoles of the mycangial propagules are quite like the vacuoles of the conidiogenous cells. In the mycangial propagules, the vacuoles often lie at the narrow end of pyriform cells and in the central zone of 'dumbbell-shaped' cells. In these mycangial propagules, our micrographs suggest that the growth of the cell is correlated with an increase in size of the vacuole; perhaps the osmotic properties of the vacuole allow it to 'inflate,' and thus to expand the cell wall. In sympodial conidiogenesis, passage of the organelles and cytoplasmic ground substance from the conidiophore into the conidium initial was also accompanied by an expansion of the vacuole. Thus, the conidial morphology and presumably the mechanisms of conidiogenesis are quite similar in the two growth stages. In *Sporothrix schenckii*, the production of 'secondary' conidia has been reported and their production has been shown to be acropetal or sympodial (Taylor 1970). Certainly, our ultrastructural data on SJB 133, also a *Sporothrix*, are consistent with Taylor's report for *S. schenckii*. Furthermore, the vacuole of some transition cells (Fig. 7) is rather like that of sympodial conidiophores (Fig. 15).

The mycangial lumen can be viewed as a specialized microenvironment which regulates both species composition and growth stage of the ectosymbionts. In their gross anatomy, the fungal ectosymbionts of the southern pine beetle are dimorphic: in the mycangium their growth is as a budding yeast while in culture or in the host tree the fungi form a mycelial mat. Clearly, this dimorphism is adaptive: maximizing the number of propagules carried by the vector while emphasizing coherent growth of the plant body in culture. At the ultrastructural level, the two ectosymbionts of the southern pine beetle, namely SJB 122 and SJB 133, effect this yeast–mycelial 'dimorphism' in strikingly different ways. In SJB 133, the subject of the present study, the 'yeast' phase is simply production of secondary conidia. Growth in culture is pleiomorphic, and in a sense, the 'yeast stage' represents only a particular sample from the continuum encountered in culture. The second major fungal ectosymbiont of the southern pine beetle is SJB 122, a basidiomycetous yeast (Barras and Perry 1972; Happ *et al.* 1975). In malt-extract culture, SJB 122 develops in a mycelial mat with dolipore septa and clamp connections and is therefore presumed to be dikaryotic. In the mycangium, SJB 122 is a multinucleate yeast which divides mostly by fission. Similar cells were induced in culture using techniques described in Leonard and Dick (1973). Cytoplasmic organization, septal adaptations, and wall organization differ sharply as one compares yeast stage and mycelial stage. In SJB 122, morphological and cytological dimorphism appears to be related to changes in ploidy during a complex life cycle perhaps related to lower Tremellales or Ustilaginales (Kreger-van Rij 1973). In SJB 133, dimorphism merely reflects selection and maintenance of asexual reproduction in the mycangium. The efficiency of this selection is shown when transition cells do not go on to produce mycelia in the mycangium.

Acknowledgments

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