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Pycnidial development of *Phyllosticta harai* and *Sphaeropsis* sp.

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The development of conidiomatal structures is divided into three stages: primordia, cavity formation, and conidiogenesis. These ontogenetic features of conidiomata indicate diversity. This study clearly shows the difference in pycnidial development between *Phyllosticta harai* and a species of *Sphaeropsis*. In *P. harai*, a cavity is formed at the center of the pycnidium following the meristogenous or symphogenous primordium formation. This process is characterized by autolysis of cells at the pycnidium center. The pycnidial primordium of *Sphaeropsis* sp. is meristogenous. It can be assumed that the cavity is formed by dispersion and spacing of original hypha, with subsequent hypha filling the spaces between hyphal cells. The cavity enlarges gradually due to the mechanical force caused by successive conidium production and increasing conidial size.

Key Words——cavity formation; developmental morphology; *Phyllosticta harai*; pycnidial primordium; *Sphaeropsis* sp.

Sutton (1973) suggested that the mode of fructification in coelomycetes fungi is diverse and may reflect their evolution. DiCosmo and Cole (1980) also noted that pycnidial development should provide valuable taxonomic criteria. In particular, in the pycnidial lichenicolous fungi, both the shape of pycnidium, and the mode of pycnidial development support classification into five types (Vobis and Hawksworth, 1981). The modes of pycnidial development in coelomycetous fungi also are varied (Mercer, 1913; Dodge, 1923; Archer, 1926; Chippindale, 1929; Harris, 1935; Punithalingam, 1966; Mass et al., 1979; DiCosmo and Cole, 1980). Most studies of pycnidial development have used light microscopy and, as a result, photography and schema of many features are obscure. We have been unable to obtain satisfactory information from the literature about the relationship between the pycnidial development of pycnidial fungi and their taxonomic value.

This study clarifies the mode of conidiomatal development in two pycnidial fungi as part of a research series with similar objectives. Pycnidial development differs between *Phyllosticta harai* Togashi, the pathogen of brown spot of Japanese laurel (*Aucuba japonica* Thunb.), and a species of *Sphaeropsis*, the pathogen of the leaf and fruit disease of pomegranate (*Punica granatum* L.).

Materials and Methods

Strains and culture conditions Two fungal isolates were used: *Phyllosticta harai* (MAFF236703) isolated from Japanese laurel (*Aucuba japonica*), and *Sphaeropsis* sp. (MAFF237167) isolated from pomegranate (*Punica grantum*). Both fungi were cultured on potato dextrose agar (PDA) under 60-W white fluorescent lamps at 23–25°C.

Light microscopy (LM) (a) Pycnidia were fixed in 8% formaldehyde at 4°C. Specimens were frozen on stage at −25°C and sectioned at 10 μm with a sliding microtome (MCR802A). (b) Pycnidia were fixed in buffered 4% glutaraldehyde for 3 h at 4°C, washed five times in buffer solution, then postfixed in buffered 1% osmium tetroxide for 3 h at 4°C. The materials were dehydrated through an ethanol series for 15 min, infiltrated with acetone, then embedded in Spurr low-viscosity plastic (Spurr, 1969). Thin sections (0.3–0.5 μm) were cut with an ultra-microtome (JEOL JUM7) using a glass knife. These sections were stained with 1% toluidine blue O (Sigma).

Transmission electron microscopy (TEM) The LM (b) specimens were also used for TEM. They were sectioned with an ultra-microtome (JEOL JUM7) using a diamond knife. The sections were picked up on sheet meshes, then poststained for 40 min on droplets of 0.5% uranyl acetate followed by 7 min on lead acetate. They were observed with a TEM (JEOL 100s) at 80 kv.

Scanning electron microscopy (SEM) The pycnidia were fixed in phosphate buffered 1% osmium tetroxide for 2 h at 4°C, and dehydrated through an ethanol series. The dehydrated specimens were placed in liquid nitrogen for instantaneous freezing, and cut with a chisel. They were hydrated through an ethanol series, then fixed in phosphate buffered 0.1% osmium tetroxide for 24 h at room temperature and in phosphate buffered 1% osmium tetroxide for 1 h. These specimens were dehydrated

again through an ethanol series. They were then critical point dried (Eiko DX-1), coated with gold (JEOL JFC 1100), and examined with a SEM (JEOL 5200) at 10–25 kV.

Results

Phyllosticta harai Pycnidia were formed as soon as 3 d after inoculation on PDA. Young pycnidia were white to gray; older pycnidia were greenish to blackish brown.

Pycnidial primordia originated from single or plural hyphae and can be divided into three types: meristogenous, arising from swelling of single hyphal cells (Fig. 1A); symphogenous, arising from swelling of two or more hyphal cells (Fig. 1B); and the hyphal-coiling type (Fig. 1C). In the latter type, hyphae branched and hyphal cells swelled at the inside of the hyphal coil. All primordia grew by hyphal accumulation at their surface (Fig. 2) and developed into the typical pycnidia of the genus *Phyllosticta*.

In the next stage, hyphal cells composing the inner pycnidium swelled and changed into a simple cell mass (Fig. 3). At this time, the outer cell layers turned black and constructed a pycnidial wall. In the pycnidium, each of the swollen central cells divided into two or more cells, and the central portion of pycnidia consisted of small cells (Fig. 4). These cells included large amounts of lipids, large vacuoles containing myelin figures, and low-dense cytoplasm with a few ribosomes (Fig. 5). As shown in Fig. 6, a cavity containing numerous lipids appeared at the central part of the pycnidium following cell disorganization. The cavity filled with mucilage and numerous large lipid globules (Fig. 7). The cavity then enlarged peripherally as a result of cell collapse. Cells in the inside layer of the pycnidium proliferated towards the center and formed new cells (Fig. 8). The new cells had high-dense cytoplasm and several large lipids, and appeared very active (Fig. 9). These new cells converted into conidiogenous cells and produced conidia (Fig. 10).

SEM observation of mature pycnidia showed conidiogenous cells lining the inner layer of the pycnidial wall. Many conidia were observed in the cavity (Fig. 10). There was a round pore (ostiole) at the apex of the pycnidium (Fig. 11).

A conidium originated in a swelling at the apex of a conidiogenous cell (Figs. 12, 13). The swelling was delimited by a transverse septum. Figure 14 shows that a conidiogenous cell has three annellations formed by disintegration of the cell wall between the conidium and the conidiogenous cell. The third conidium is forming at the top of the conidiogenous cell. Later, the conidium became covered with mucilage and bore a mucilaginous appendage showing the typical morphological characteristics of the genus *Phyllosticta* (Fig. 15).

***Sphaeropsis* sp.** Pycnidia were formed 3 d after inoculation on PDA. They were pale blackish brown at all stages.

The primordium in the early stage was meristogenous with a simple texture. Later another hypha fused with it (Fig. 16). In ultra-thin sections of the initial pycnidium, the hypha composing the pycnidium swelled and its cells were characterized by a large vesicle, high-dense cytoplasm and nuclei (Fig. 17). In the next stage, although not distinctly shown here, each of the cells may be separate. Conidia were formed from the original hyphal cells forming the pycnidia (Figs. 18,19). The young pycnidium contains young conidia of distortion in its cavity (Fig. 20). The pycnidium increased in size as swollen hyphae adhere to its outer surface (Fig. 21). At further stage of pycnidial development, the cavity enlarged by successive disintegration of conidiogenous cells after conidium formation. Mature pycnidia had very thin walls. Cells at the inner layers of the wall converted into conidiogenous cells and formed conidia. The cavities filled with many conidia formed successively (Fig. 22).

The conidium formation was holoblastic. The original hyphal cells of the inner layer of the pycnidium converted gradually into conidiogenous cells and proliferated toward the central portion of the cavity. The tip of the proliferated portion swelled (Fig. 24) and was delimited by a septum. In the SEM, the mature conidium was globose but had a small projection from a ruptured scar (Fig. 23).

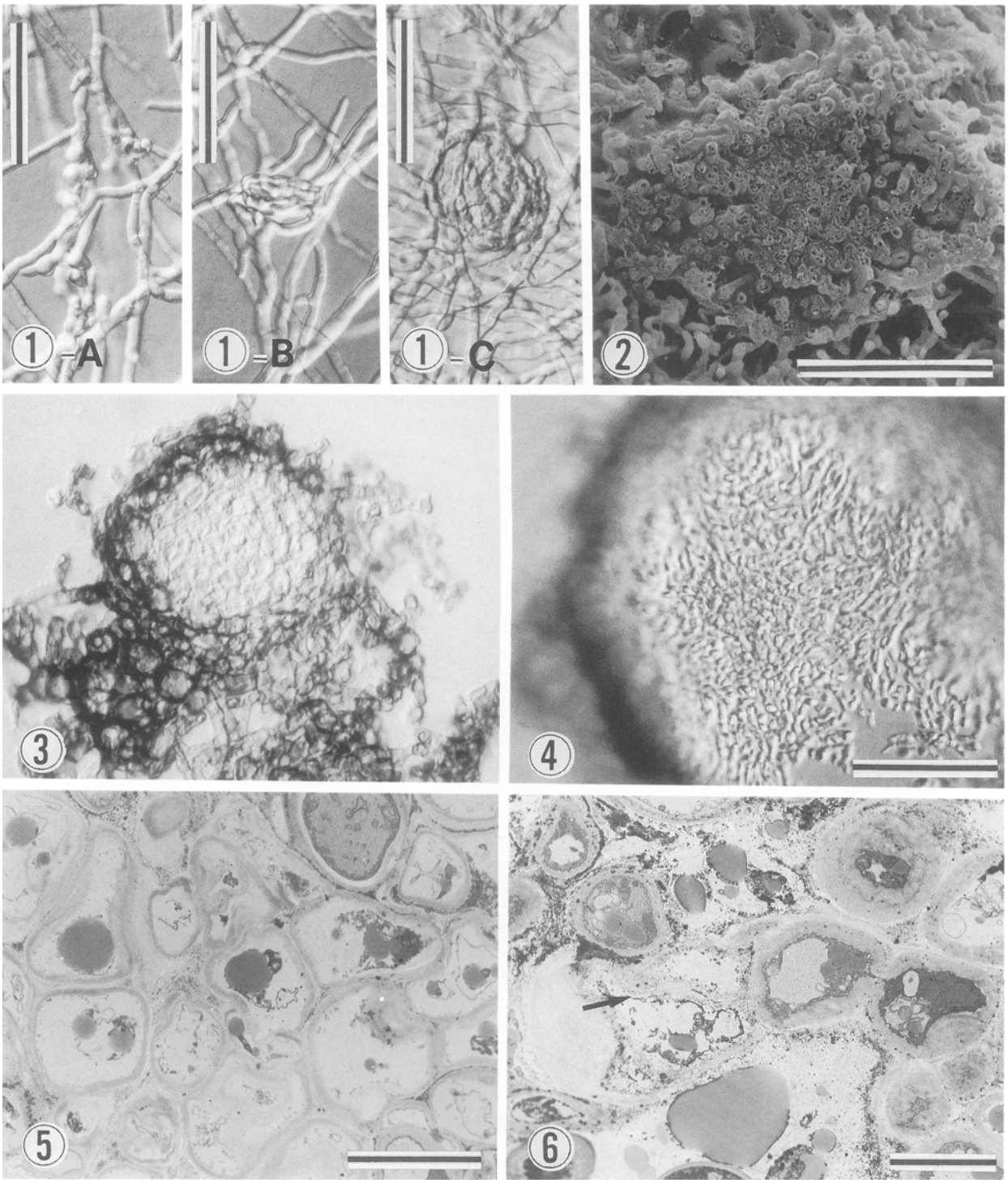
Discussion

Pycnidial primordium Types of pycnidial primordia were systematized by Kempton (1919), as those of simple meristogenous, compound meristogenous and symphogenous. In *Phyllosticta harai*, three types of primordia were observed: hyphal coiling not mentioned by Kempton (1919), typical simple meristogenous type, and typical simple symphogenous type. The hyphal-coiling type besides simple meristogenous and simple symphogenous types were recorded in *Phyllosticta antirrhini* P. Syd. by Maiello and Peterson (1976). Our results for *P. harai* agree well with their results and reconfirm the existence of hyphal-coiling type of primordia.

In *Sphaeropsis* sp., the primordium was simple symphogenous, although pycnidia developed by adhesion of some of hyphae and hyphal swellings during the pycnidial formation. Kempton (1919) showed that primordia of *Sphaeropsis malorum* Pk. were compound meristogenous and symphogenous, and primordia of *Sphaeropsis citricola* McAlpine were simple meristogenous and compound meristogenous. The genus *Sphaeropsis* has irregular types of primordium which depend on the species. The genus *Sphaeropsis* may be divided into several groups by the mode of pycnidial development.

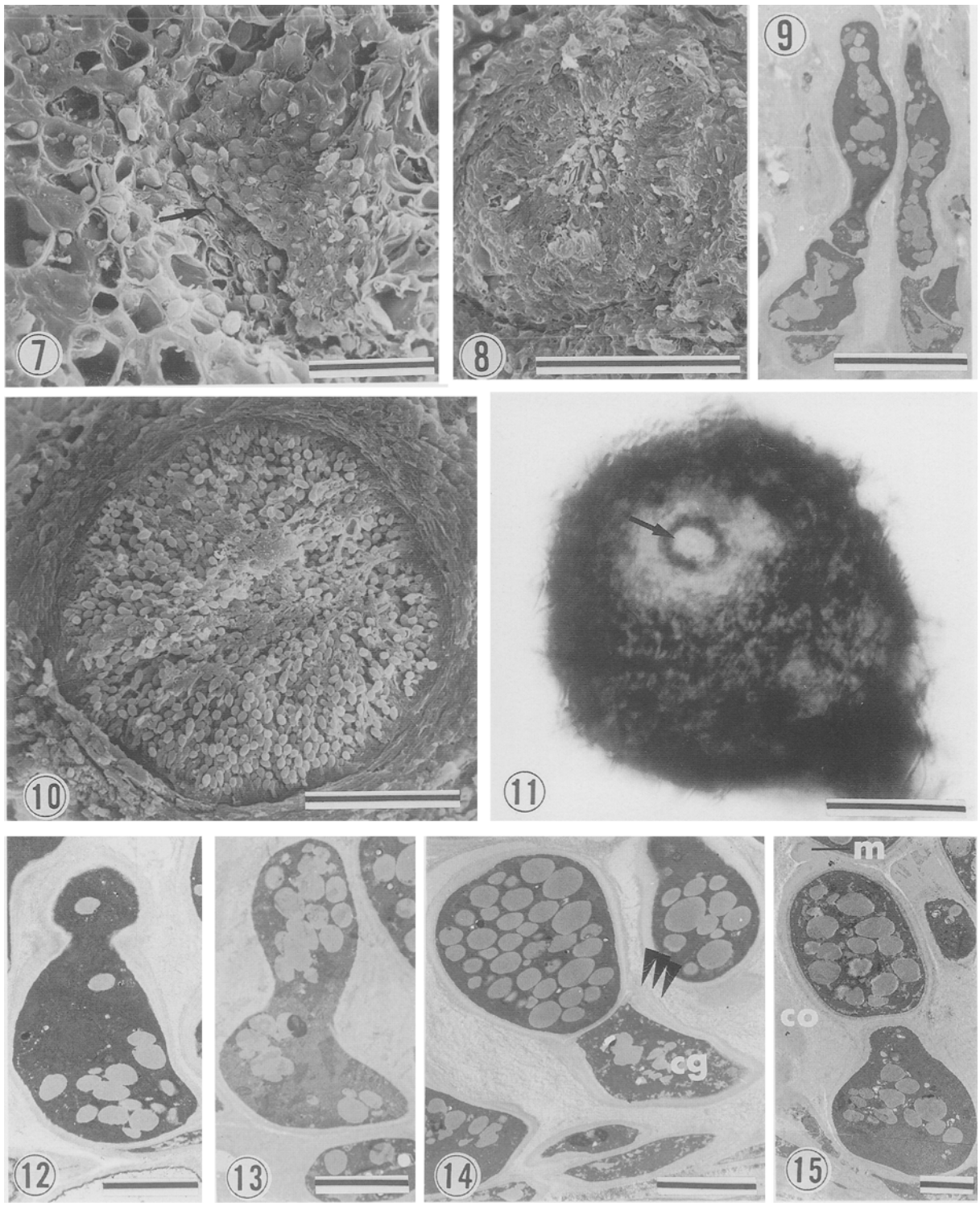
Cavity formation Cavity formation in the pycnidium and stromatic conidioma is schizogenous, lysigenous, or a combination of both (Nag Raj, 1981). Each fungus has a particular type of cavity formation (Harris, 1935). In cavity development of *P. harai* and *Sphaeropsis* sp., the most distinct processes are the time for cavity formation and increase in the pycnidial size.

In *P. harai*, cavity formation begins after the pycnidia are grown almost to the mature size. This cavity formation is lysigenous. Similar cavity formation has been

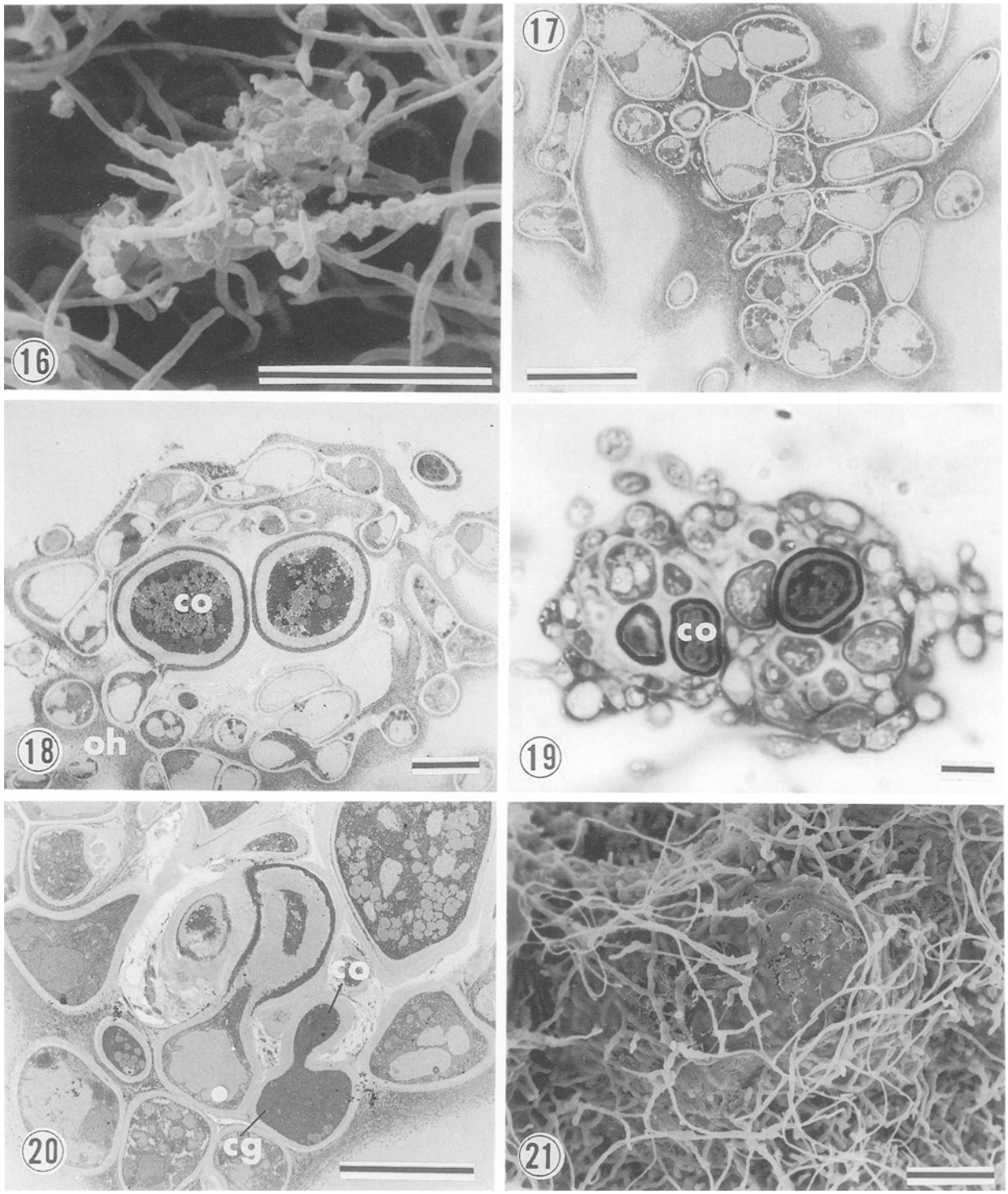


Figs. 1–6. Pycnidial development of *Phyllosticta harai*.
1A–C. Pycnidial primordia on PDA. 1A. Meristogenous type. 1B. Schizogenous type. 1C. Hyphal-coiling type. 2. Swelling of hyphal cell mass. 3. A pycnidium of early stage. 4. Central small cells of pycnidium after swelling cells divided. 5. Cells of pycnidium center with low-density cytoplasm (TEM). 6. Cell wall break down at center of pycnidium (TEM). Arrow indicates wall breakdown. Scale bars: 1–4=50 μm; 5, 6=5 μm.

reported in *Phyllosticta carpogena* (Shear) van der Aa (Dodge, 1923). In further observations on *P. harai*, we show here that cytoplasm of cells in the central portion of pycnidia broke down, and the photography by TEM agrees with photography of the autolysis phase of cells in *Penicillium chrysogenum* Thom (Trinci and Righelato,



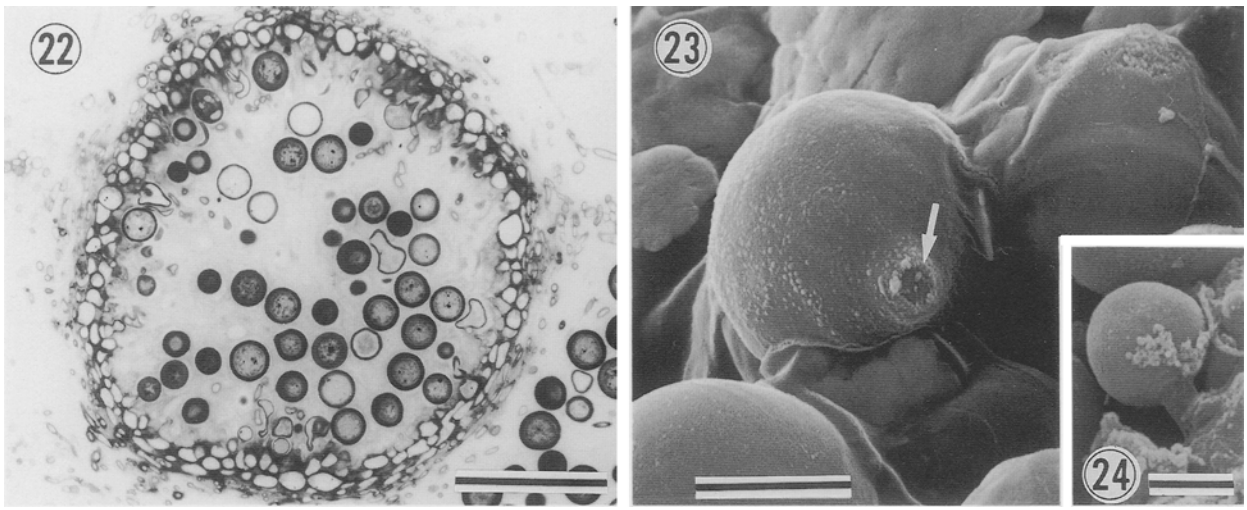
Figs. 7–15. Pycnidial development of *Phyllosticta harai*.
7. Pycnidium cavity at center. This cavity is full of mucilage and lipids (arrow) (SEM). 8, 9. Proliferated new cells formed from inside layer of pycnidium. 8. SEM. 9.TEM. 10. Section of a mature pycnidial section (SEM). The proliferated cells convert to conidiogenous cells forming conidia. 11. Mature pycnidium with an ostiole (arrow). 12, 13. Apical swelling of a conidiogenous cell (TEM). 14. Apical portion of a conidiogenous cell (cg) delimited by transverse septum. The conidium has numerous glycogens and lipids (TEM). Arrowheads indicate annellations. 15. Conidium (co) covered by mucilage with mucilaginous appendage (m) (TEM). Scale bars: 7=5 μm; 8, 10, 11=50 μm; 9=3 μm; 12–15=2 μm.



Figs. 16–21. Pycnidial development of *Sphaeropsis* sp.
16. Pycnidial primordium (SEM). 17. Pycnidial primordium (TEM). 18, 19. Conidia (co) formed from original hyphal cells (oh). 18. Residual matter formed by cell breakdown. 19. Cavity filled by conidium. 20. Conidiogenous cells (cg) forming conidia (co) in narrow space (TEM). 21. Hyphae accumulating at pycnidium surface (SEM). Scale bars: 16, 21 = 50 μm; 17–19 = 10 μm; 20 = 3 μm.

1970). In the pycnidia of the genus *Phyllosticta*, cell destruction in this autolysis phase seems to be prerequisite for their cavity formation.

In *Sphaeropsis* sp., cavity formation occurs as the pycnidium size increases. This mode is both lysigenous and schizogenous. At first, hyphal cells forming the



Figs. 22–24. Mature pycnidium of *Sphaeropsis* sp.
22. Pycnidial wall of one or two layers. 23. Mature conidium with remaining ruptured wall (arrow) (SEM). 24. Swelling of a conidiogenous cell (SEM). Scale bar: 22=50 μm; 23, 24=5 μm.

pycnidium disperse, then the cavity enlarges by the breaking down of conidiogenous cells at the inner surface of the pycnidium. *Sphaeropsis* sp. had variously shaped conidia in the early cavity. The conidia could not become globose until the cavity enlarged sufficiently. In addition, organelles of any disintegrated cells were not observed in the early cavity. The mechanical force produced when the conidia became globose may have helped enlarge the cavity.

Generally speaking, most pycnidial development in coelomycetes can be classified into three stages: primordium formation, increase in pycnidial size and cavity formation, and conidium formation. In our results, *P. harai* and *Sphaeropsis* sp. differ greatly in all these stages.

Dodge (1930) and DiCosmo and Cole (1980) presented similar photographs of cavity formation in different species of *Chaetomella* , although their interpretations were different. Dodge (1930) observed cavity formation of *Chaetomella raphigera* Swift by light microscopy and noted disorganized central filaments. DiCosmo and Cole (1980) using SEM photographs noted that the cavity of *Chaetomella acutiseta* Sutton et Sarbhoy was formed by the internal hyphae of pycnidium becoming loose and pulling away. This results are caused by the difference of their interpretations. It is clear that *C. raphigera* and *C. acutiseta* have same development process in their photographs.

Harris (1935) and Punithalingam (1966) showed that the pycnidial development was meristogenous and schizogenous in seven species of the genus *Septoria*. Conidiogenesis in these species is diverse, being holoblastic sympodial or enteroblastic phialidic (Sutton, 1980). This result indicates that the evolution of pycnidial development is not related to the direction of conidium formation.

Our experiments and other reports clarify that pycnidial development is diverse. Notably, the direction and timing of cavity formation are complex. However, our results and other reports do not indicate a clear correlation between type of pycnidial development and genus on taxonomy. This is because it is unknown if asexual morphology evolved with teleomorph lineage, or whether it evolved in a short term, or a long term after the loss of sexuality (Berbee and Taylor, 1993). More data on clearly distinct taxa are required to understand the relationship between pycnidial development and the systematics of coelomycetes.

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