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Studies on Conidiomata developmental morphology of *Pestalotiopsis disseminata*

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This study shows the conidiomatal development of *Pestalotiopsis disseminata* (Thuen.) Stey., using light and transmission electron microscope. Light microscopic study showed the presence of non-ostiolate pycnidial conidiomata in culture, whereas it is known to produce only acervular conidiomata on leaves. Interestingly, the fungus showed the ultrastructure of the conidial wall in the coloured cells as well as the basal and apical hyaline cells with the appendages. *P. disseminata* was studied for the first time on the development of the conidiomata and conidia by light and transmission electron microscopes.

Key words: Coelomycetes, Conidiomata, conidium ontogeny, appendages.

INTRODUCTION

Traditional classification of coelomycetes was based on morphology and was thus subjective, often resulting in artificial generic and species boundaries (Wijayawardene et al., 2012). Coelomycetes have conidia formed within a cavity lined by fungal or fungal host tissue. The conidia-bearing structure (conidioma) is classified into five types according to exterior morphology: pycnidial, pycnothyrial, acervular, cuplate and eustromatic (Hawksworth et al., 1995). The genus *Pestalotiopsis* steyaert is a heterogeneous group of coelomycetous consisting of 205 described species that are differentiated primarily on conidial characteristics such as size, septation, pigmentation and presence or absence of appendages (Sutton, 1980). These facts suggest that the morphogenesis of conidioma has taxonomic value. In view of the variability and diversity, it is not easy to be classified based on conidiomata, satisfactorily. Pycnidial-type conidiomatal development has been described (Maas et al., 1979; Punithalingam, 1966). Development is divided into three stages: primordia, cavity formation and conidiogenesis with each pycnidial fungus having a determinate mode in each stage.

In describing acervular development, Archer (1926) noted that the pseudo- acervulus in the genus *Pestalotia* (written as *Pestalozzia)* was formed by the breaking open of the pycnidial wall to form a structure similar in appearance to an acervulus. Also, another manner of acervulus development in which the upper cells of a cell aggregation proliferate and produce conidia was noted. Generally, acervuli are formed by the breaking open

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of the wall of a pycnidium-like structure after it has developed as a pycnidium.

Cryptosporiopsis radicicola produces only excipular covering conidioma-like tissue with adhesive amorphous material and setae. Synnematous conidiomata with abundant macroconidia dominate the colony of *Cryptosporiopsis ericae*. *Cryptosporiopsis rhizophila* is different in its globose to subglobose conidiomata, consisting of loosely aggregated vegetative hyphae developing macroconidial conidiophores. *Cryptosporiopsis grisea*, being the only teleomorph-connected species, differs from the others in its distinct columnar surface (Wang, 2011).

Therefore, as more and more data on this effect becomes available, the distinction between Hyphomycetes and Coelomycetes may be abandoned because of the presence of intermediary stages between hyphomycetes, acervular, stromatic and cupulate conidiomata. The present investigation revealed that *P. disseminata* produced pycnidial conidiomata in culture and acervular conidiomata on natural hosts.

The various stages of development of the conidiomata were investi-gated in culture and they also resembled that of a typical pycnidial conidioma which were already studied. The developmental morphology of the conidiomata in *P. disseminata* in culture was described in this study.

MATERIALS AND METHODS

Culture character and identification

The *Pestalotiopsis disseminata* (Thuen.) Stey. was isolated from leaf of *Syzygium amotica* collected in Kodaikanal, India. *P. disseminata* isolates derived from single spores were grown on PDA. Cultures were incubated at 24°C in continuous light, and cultural morphology was examined after seven days. Spore size was determined by measuring the length and width of 30 to 40 arbitrarily selected conidia from a conidial suspension. The isolates were identified initially by comparing morphological and cultural characteristics (size of conidia, color and length of median cells, thickness and length of apical appendages, and length of basal appendage).

Germination study

The initial stages of the development of conidiomatal primordia were studied by slide cultures (Riddell, 1950). For germination studies, conidia were collected aseptically from the teased out conidiomata in 1% glucose solution and allowed to germinate in cavity slides kept at room temperature (28°C) and were examined every 5 h for 36 h to study germination.

Light and transmission electron microscopy (TEM)

Selected conidiomata with agar were trimmed into 2 mm square blocks and fixed in 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 2 h at room temperature (27°C) and 1 h at 4°C and post fixed for 12 h in 1% osmium tetroxide. Specimen were dehydrated through an ascending series of acetone (30-100%) at room temperature, each changes at 30 min intervals, followed by 2-3 changes in fresh spurr in the ratio of 3:1 (acetone : spurr) for 6 h, followed by two changes with absolute spurr mixture for 24 h each lasting for 8 h and polymerised in fresh spurr at 70°C for 8 h. The samples infiltrated with the resin were transferred to a vacuum chamber for 1 h for complete removal of air bubbles. Thin sections (0.5 µm) were cut from these blocks and stained with 1% aqueous toluidine blue to study the development of conidiomata and conidiogenesis under the light microscope. The same specimens as LM were used for TEM. Ultrathin sections were collected on copper girds (400 mesh) and excess water in the grids was removed by filter paper. The sections were picked up on mesh sheets, and then post stained for 40 min on droplets of 0.5% uranyl acetate followed by lead acetate. Transmission elelctron micrographs were taken by using Philips CM 10 at 40 and 60 KV.

RESULTS

Description of the fungus in culture

Conidiomatal acervuli stomatic, is black, 75-250 µm in diameter, conidia 5-celled, elliptic to clavate, fusiform, tapering to the base to slightly curved, 23 x 8 µm, median coloured cells unequally coloured, sometimes two upper cells slightly darker. The basal cells hyaline have a single unbranched appendage, 5 µm. Apical cell hyaline has 3 apical appendages, 22 µm. The apical cell hyaline has 3 apical appendages, 22 µm. The apical and basal cells are conic to cylindric, 5 µm (Figure 1A).

Light microscopic study

Development of conidiomatal initial

In the germination study, conidia became swollen and resulted in the breakage of outer wall and the germ tube emerged. The germ tubes mainly arise from the lower most median cells. One to two germ tubes arises from each cell (Figure 1B). The conidiomatal initials were first evident as small knots of fungal hyphae. Some of the cells became swollen and thick-walled and multiply by repeated divisions to form the knot-like primordial. This type of primordium formation is referred to as "meristogenous type" (Figure 1C). The primordium is also initiated by "symphogenous type" where the cells of adjacent hyphae by continued cross and longitudinal divisions, form the primordium. Also, intertwining of several hyphae resulted in primordia formation. Initially, the cells constituting the primordium are spherical to subspherical and are hyaline which stain deeply when compared with the cells of the surrounding hyphae (Figure 1D).

During further development, the primordium continuously increased in size by continued transverse and transverse and longitudinal divisions of the cells. As the primordial initials increase in size, several layers of the promordium became differentiated into morphologically distinct layers. The outermost one or two layers were

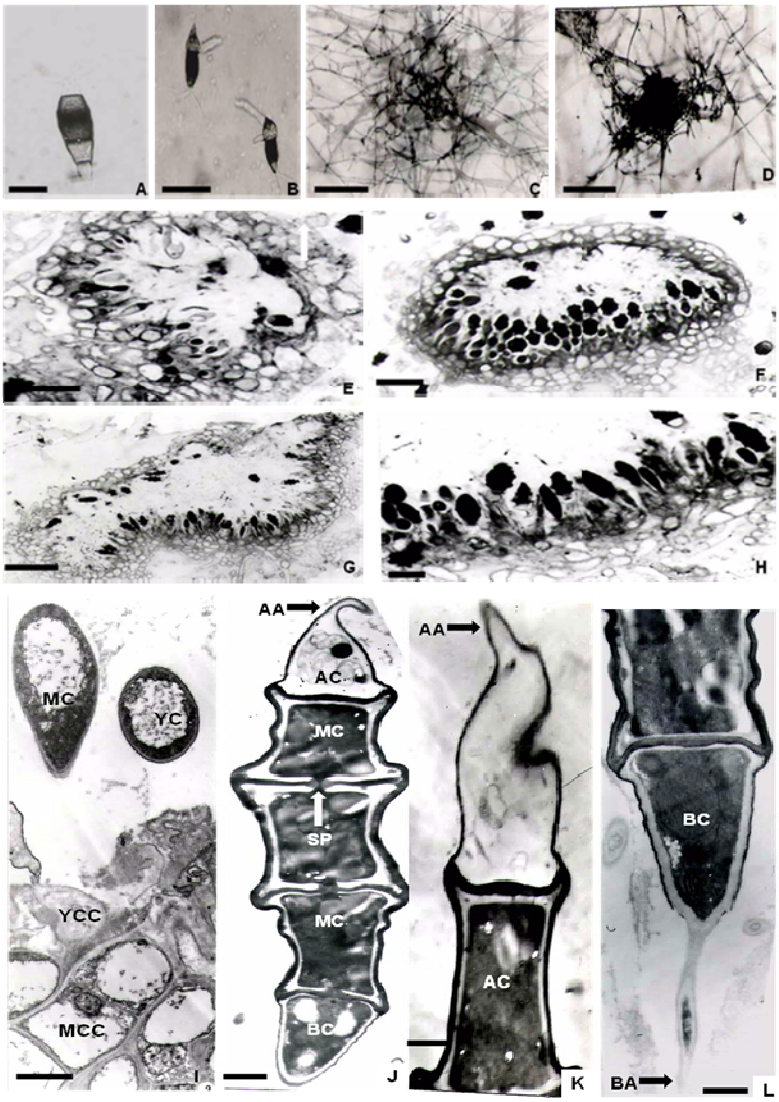

Figure 1. A. Mature conidia, bar = 12.5 µm; B. Germinating conidia producing germ tube from the lowermost cell and basal hyaline cells, bar = 100 µm; C. Early stages of simple symphogenous method of conidiomatal formation, bar = 25 µm; D. Aggregation of hyphal to form the conidiomatal primordium, bar = 50 µm; E. Section through a young primordium showing cavity formation, bar = 100 µm; F. Section of the conidioma showing temporary conidiogenous cells and the conidia, bar = 50 µm; G. Section of conidioma showing permanent conidiogenous cells and conidia, bar = 50 µm; H. Section of the pycnidia showing different stages of development of conidia, bar = 50 µm; I. TEM picture showing young conidiogenous cells with conidia, bar = 1 µm; J. Section through the apical part of the conidia showing the apical appendage. Note the continuation of outer electron dense layer of median cells to the end cells as well as appendage in the form of a thin layer, bar = 1 µm; K. Longitudinal section of a conidium showing the apical cell with the appendage, bar = 1 µm; L. Longitudinal section of a conidium showing the basal cell with the appendage and the upper most coloured cell, bar = 1 µm. CC- Conidiogenous cell, MCC- matured conidiogenous cell, YCC- young conidiogenous cell, YC- young conidium, MC- median cell, SP- septal pore, AC- apical cell, BC- basal cell, AA- apical appendage, BA- basal appendage.

pseudoparenchymatous with thick, lightly pigmented walls, which form the outer wall layer of the conidiomata (Figure 1E). Inside this outer wall, there are four compact layer of cells.

Formation of cavity and sporogenous tissue

The central cells in the primordium showed sign of schizogenous and lysigenous activity to form the central cavity. Further developmental stages showed the formation of the conidium simultaneously with the cavity formation. As the conidia nature, they were released from the conidiogenous cells to fill up the cavity (Figure 1F). The later formed conidiogenous cells were typically cylindrical in shape with one to three annellation (Figure 1G). The conidioma become flattened in shape during the later stages of development. The mature conidioma produces conidiogenous cells which line only the flattened basal region but not the sides and upper region of the condioma (Figure 1H). There was no regularly formed ostiole found in this species. After maturity the upper layers of the conidioma open quite irregularly to release the conidia.

Electron microscopic studies

Conidiogenous cell

The initial of the conidium arises as a small protrusion of the apex of the conidiogenous cell (Figure 1I) and develops holoblastically. Cell organelles migrate into the developing conidium until a delimiting septum was formed more or less near the base of the conidium initial. As the conidium enlarges, the conidium wall forms an electron-opaque outer layer, which starts from the base of the conidium.

The inner transparent layer of the conidium was continuous with the wall of the conidiogenous cell. The conidiogenous cell itself does not develop an electron-opaque outer wall layer. Successive conidia develop just at or below the level at which the preceding conidium was delimited. The conidia were produced from the annellides and more than 3 annellations were observed in some conidiogenous cells.

Mature conidia

As sections through young conidiomata showed, conidia arise from spherical to subspherical conidiogenous cells lining their cavity (Figure 1J). The conidia consist of three thick-walled median cells with thin apical and basal hyaline cells. The wall layers of the three median cells appear granular and pale brown. Prior to septation, the conidium initial has a thin electron transparent wall. During septation, the wall increases in thickness. The first septum was laid normally near the base of the conidium (Figure 1K).

Gradually, the peripheral region of the conidial wall becomes electron dense by the deposition probably of melanin in the wall matrix.

Median coloured cells

During the growth of a membrane across the conidium, wall material is continuously produced through the cell wall developed by each membrane. The septal pores are present between the cells of the conidium (Figure 1J and L). The septal pore is formed as a result of cessation of wall deposition at the junction where the plasma membranes from either side remain fused to form the trans-septal membrane. Simultaneous to the septal formation, wall deposition occurs over the entire inner surface of the conidium. Peripheral walls and the septa of the conidia becomes distinctly electron dense. The cell wall is thicker in the upper two median cells than in the lower median cell. The lower median cell is structurally different from the other two median cells of the conidium in that it showed pronounced wrinkling of the wall (Figure 1J). Probably because of the difference in the nature of the wall, it appears pale brown in colour under the light microscope.

Apical and basal cells with appendages

The apical and basal cells are morphologically indistinguishable from the median cells during the early stages of the development of conidium. At maturity, the end cells showed cytolysis and the cytoplasmic content completely disappears from these cells. The thickness of the electron-dense layer of the apical and basal cells gradually decreases (Figure 1J). The apical appendage originated from the apex of the conidium as a simple elongation of a small bud produced at an early stage of the development of the conidium (Figure 1K). Occasionally, the basal appendage of the developing conidium was observed within the annellation, which proves that the basal appendage is endogenously produced (Figure 1L).

DISCUSSION

The fungus is known to produce only acervular conidiomata on its host whereas in artificial culture media, pycnidial conidiomata were formed. The morphology of conidia, appressoria and cultural characters of the ex-neotype culture was provided (Liua, 2011). The development of the pycnidial primordium (the earliest stage of pycnidial development) was systematized as simple meristogenous, compound meristogenous, symphogenous (Kempton, 1919) and hyphal coiling (Maiello and Peterson, 1976). Primordia grow by aggregation of cells due to hyphae accumulating at the primordial surface. We were unable to observe pycnidial primordia. However, the aggregation of swollen cells in our specimens was observed under the leaf tissue or on the leaf surface, indicating that the cell aggregation

originated from hyphae accumulating in the same way as in pycnidial primordia. As the primordium increases in size, the central cavity was formed because the primordium compactly packed pseudoparenchymatous tissue. The central cells in the primordium showed signs of schizogenous and lysigenous activity to form the central cavity. Sutton (1961) also reported the developmental studies on *Pestalotiopsis* with various stages in spore development in some species.

The remaining portions of the cells often gelatinize and fill up the cavity with the mucilagenous matrix. Probably, the matrix provides nutrition to the developing conidiogenous cells and young conidia. In *Pestalotiopsis guepinii* and *Pestalotiopsis neglecta,* cavity formation was mainly lysigenous. The origin of the cavity was due to cytolysis of the central cells of the cell aggregation. Nag (1981) suggested that the slime which originates through lysis occurring during cavity formation play an important role in conidium dispersal. The slimy matrix also plays an indirect role in the dispersal of the conidia by insects. During the later stages of development of the conidiomata, secondary conidia are formed enteroblastically showing annellidic conidiogenesis. The conidiogenous cells or annellides became cylindrical with 4-5 annellations. This showed that the first formed conidia may be morphologically different from the secondary conidia formed successively from the conidiogenous cell. The present study showed that the disorganized central cells gelatinize to form the slimy matrix in *P. disseminate*. However, initially formed conidiogenous cells after releasing the conidia showed signs of degeneration inside the cavity. The present observation clearly revealed that *Pestalotiopsis* species have mucilagenous matrix arising either through lysis occurring during cavity formation or formed by the degenerating temporary conidiogenous cells which play an important role in the survival of the fungus. The presence of mucilagenous matrix during the formation of the pycnidial conidiomata was also reported in other pycnidial coelomycetes (Murugan and Muthumary, 2003).

The production of temporary and permanent conidiogenous cells were observed in species of *Pestalotiopsis.* The dual conidiation process was reported in various fungi, *Phyllosticta caryota* and *Ascochyta* species in grown culture (Punithalingam, 1979).

The fine structure of the conidiogenous cells and conidia were demonstrated in six coelomycetes species (Griffiths and Swart, 1974a, 1974b). Longitudinal sections through the conidia of *P. disseminata* showed massive and highly pigmented conidial walls. The transverse septa vary in the degree of pigmentation, perhaps, due to the varying sequences of development within the maturing conidium. The conidial wall was characterized by the deposition of electron dense material in the outer layers of the septa. The basal and apical cells have partly pigmented and partly unpigmented walls, which were clearly distinct from the central cells. Externally, the conidia are sheathed in an electron-dense outer wall and an electron-transparent inner wall. The conidial cells showed perforation between individual cells by a simple septal pore (Figure 1K). The conidium in *Pestalotiopsis* has an outer electron-dense layer and an inner electron transparent layer in the wall. The thicknesses of the two wall layers differ in that the median cells have a thick inner wall layer and a thin outer wall layer when compared with the end cells of the conidium. Therefore, the outer zone of the wall is melanized while the inner zone remains unpigmented. The transverse septa arise as outgrowths of the conidial wall and new wall material is deposited outside the invagination of the plasma membrane. In mature conidia, the transconidial septum consists of a thin, central, electron dense layer flanked on either side by a hyaline layer. A distinct pore formed centrally, following cessation of wall material deposition perforates the septum. When all the four septa are formed, the conidium has pigmented peripheral walls and the transconidial septa. The well-pronounced wrinkling observed in the outer-pigmented wall of the lowermost median cell implies that it is structurally different from the rest of the cells.

Conclusion

This study clearly shows the presence of non-ostiolate pycnidial conidiomata in culture whereas the fungus is known to produce only acervular conidiomata on leaves. Ultrastructure of the conidial wall in the coloured cells as well as the basal and apical hyaline cells with the appendages are shown.

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