Light and transmission electron microscopic studies on the conidiomatal development and conidia of *Pestalotiopsis cruenta*

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Abstract. This study has clarified the conidiomatal development of *Pestalotiopsis cruenta* by using light and transmission electron microscopes. Based on light microscopic studies of the isolated fungus, it was shown that this species forms non-ostiolate pycnidial conidiomata in culture, whereas it is known to produce only acervular conidiomata on leaves. One of the interesting observations is the ultrastructure of the conidial wall in the pigmented cells as well as the basal and apical hyaline cells with the appendages. To the authors' knowledge, this is the first ultrastrucural examination of conidiomata and conidia of *Pestalotiopsis cruenta*.

Key words: acervular and pycnidial conidiomata, appendicular coelomycetes, conidium ontogeny, ultrastructure

Introduction

In view of the variability and diversity in the form of the coelomycete conidiomata, it is no easy task to accommodate them in a system of classification solely based on conidiomata satisfactorily. Coelomycetous fungi have conidia formed within a cavity lined by fungal or fungal/host tissue (Hawksworth *et al.* 1995). The conidia bearing structure (conidioma) is classified into five types according to exterior morphology: pycnidial, pycnothyrial, acervular, cuplate, and eu-stromatic (Hawksworth *et al.* 1995). The morphological structure is one of the most important keys in classification of the *Coelomycetes* (Sutton 1980). Conidiogenous cells are formed directly from the mycelium in culture though such fungi are known to produce pycnidial conidiomata in nature (Subramanian & Reddy 1974; Baxter 1981; Baxter *et al.* 1985). Therefore, as more and more data to 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 genus *Pestalotiopsis* Steyaert is a heterogeneous group of coelomycetous fungi 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; Nag Rag 1993; CABI Bioscience database 2001). *Pestalotiopsis* is characterized by conidia having mostly four-euseptate and pigmented median cells with two to four apical appendages arising as tubular extensions from the apical cell and a centric basal appendage (Jeewon *et al.* 2002). However, *Pestalotiopsis* is a complex genus, which is difficult to be classified to the species level because characters such as growth rate, conidial morphology, and fruiting structure characteristics tend to vary within species (Karakaya 2001).

In this study, the developmental ontogeny and morphology of *Pestalotiopsis cruenta* (Syd.) Steyaert, which has pycnidial conidiomata in culture and acervular conidiomata on natural hosts, is described based on examinations *in vitro.*

Materials and Methods

Pestalotiopsis cruenta was isolated from leaf of *Michelia champaca* from Kodaikanal, Tamil Nadu, in August 1999 (MUBL No. 442). Cultures of *P. cruenta* were grown on oat meal agar (OMA) and potato dextrose agar (PDA) in Petri dishes at room temperature (28 °C). The initial stages of the

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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 to 36 hours.

To study the development of conidiomata, selected conidiomata with agar were trimmed into 2 mm square blocks and fixed in 2 % glutaraldehyde (Sigma) in 0.1 M phosphate buffer (pH 7.2) for 2 h at room temperature (27 °C) and 1 hour at 4 °C and post fixed for 12 hours in 1 % osmium tetroxide (Sigma). Specimens were dehydrated through an ascending series of acetone (30-100 %) at room temperature, each change at 30 minutes intervals, followed by 2-3 changes in fresh Spurr in the ratio of 3:1 (Acetone : Spurr) for 6 hours, followed by two changes with absolute Spurr mixture for 24 hours each lasting for 8 hours and polymerised in fresh Spurr at 70 °C for 8 hours. Semithin sections (0.5 µm) were cut through ultramicrotome (Reichert-Jung) from these blocks and stained with 1 % aqueous toluidine blue (Sigma) to study the development of conidiomata and conidiogenesis under the light microscope (Nikon "Labophot" model HFX11). Ultrathin sections were collected on 400 mesh copper girds (Sigma) and excess water in the grids was removed by filter paper. Photomicrographs (Kodak film, Sigma) were taken using transmission electron micrographs (Philips CM 10) at 40 and 60 KV.

Results

Description of the fungus in culture

Pustules punctiform, black, 120-180 µm diam., gregarious. Conidia fusiform, 5-celled, 20-24 (–23) × 6-7 (–6.5) µm, hardly constricted at septa; pigmented cells 12-15 µm, central or upper two umber, lowest olivaceous, guttulate; exterior cells hyaline, conical; apical appendages 1-4, usually 2-3, 8-8.5 (–8.4) µm long, divergent, basal appendage up to 8 µm long, usually 5-5.3 µm (Fig. 1).

Development of conidiomatal initial

The germination study showed that 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 primordium. This type of primordium formation is referred to as "meristogenous type" (Fig. 2). 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 primordial formation. Initially the cells constituting the primordium are spherical to subspherical and are hyaline which stain deeply when compared to the cells of the surrounding hyphae (Fig. 3). During the further development, the primordium continuously increased in size by continued transverse and longitudinal divisions of the cells. As the primordial initials increase in size, the several layers of the promordium became differentiated into morphologically distinct layers. The outermost one or two layers were pseudoparenchymatous with thick, lightly pigmented walls, which form the outer wall layer of the conidiomata (Fig. 4). Inside this outer wall there were three to four compact layers of cells.

Temporary conidioma formation

Either during or at the completion of the growth of the pseudoparenchymatous conidiomatal initial, certain cells in the middle region produce conidia (Fig. 5). Very often the initially produced conidia are supported by the same hyphae that give rise to the conidiomatal initials. The conidia produced from the temporary conidiomata resemble those formed later inside mature conidiomata in all aspects. The conidiomata thus formed are spherical to subspherical and consist of a wall, which is only 1-2 layered (Fig. 5). Very few conidia are produced from such conidiomata because of their temporary nature.

Formation of cavity and sporogenous tissue

The central cells in the primordium showed signs of a schizogenous and lysigenous activity to form the central cavity. Further developmental stages showed the formation of the conidia simultaneously with the cavity formation (Fig. 6). As the conidia nature they are released from the conidiogenous cells filling up the cavity (Figs 7-8). In *P. cruenta* the cells lining the pycnidial cavity commence conidial formation. Although initially indistinguishable from the cells lining the cavity, the conidiogenous cells differed in remaining thin-walled and hyaline, whereas the wall cells became pigmented and thick-walled (Fig. 9). The processes of conidiation and dislodgement of conidiogenous cells continued till the cavity was filled up. The temporary conidiogenous cells resemble more or less the pycnidial wall cells in size and shape (Figs 10-11). Subsequently, these temporary conidiogenous cells were replaced by the formation of permanent conidiogenous cells which were easily distinguished by their size and shape from the temporary conidiogenous cells. The permanent conidiogenous cells are cylindrical and annellidic with one or two annellations. The conidiogenous cells formed later are typically cylindrical in shape showing 1-3 annellations. The conidioma becomes 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 conidioma. There is no regularly formed ostiole found in this species. After maturity the upper layers of the conidioma open quite irregularly to release the conidia.

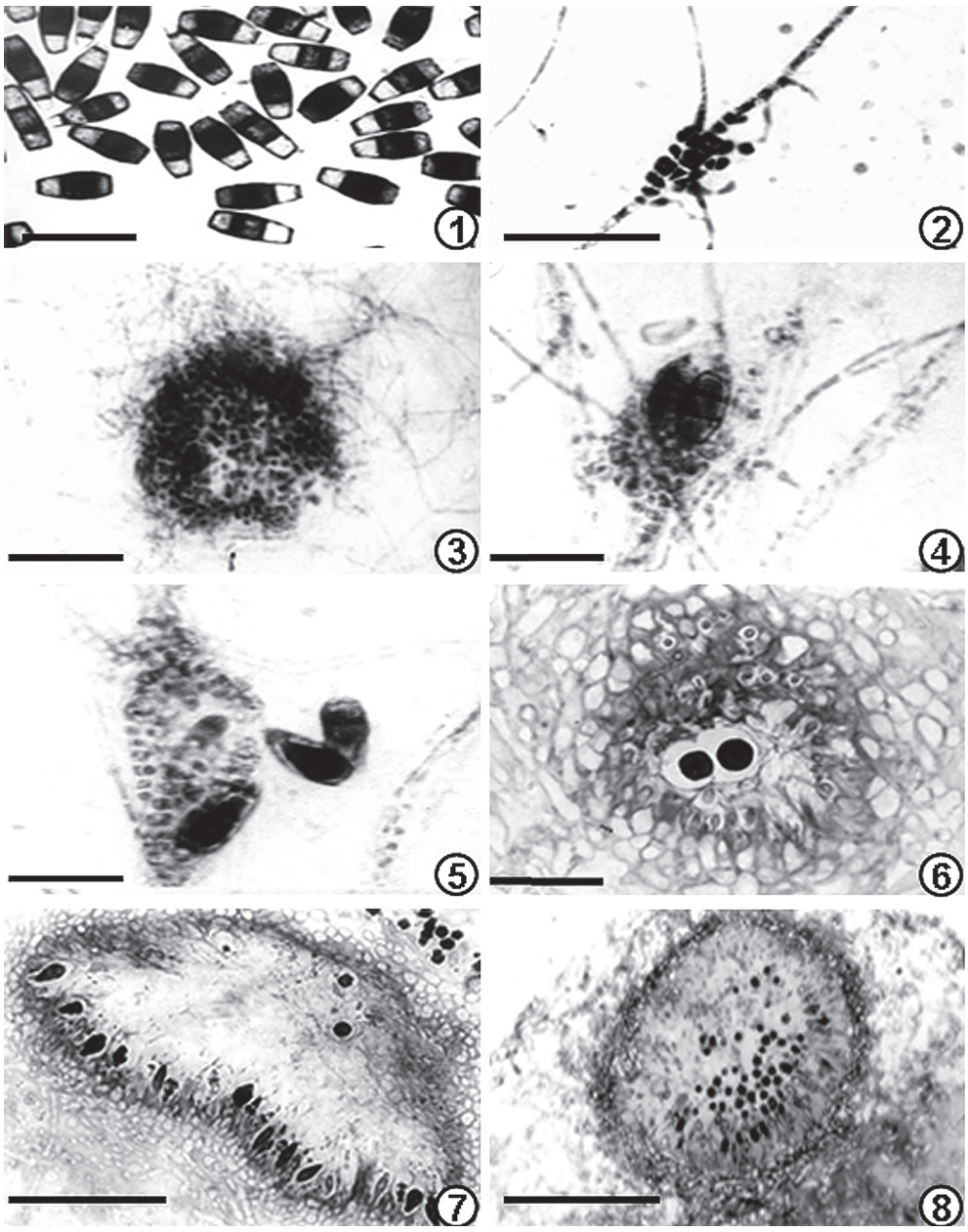


Fig. 1. Mature conidia. Bar = 12.5 µm. **Fig. 2.** Meristogenous methods of conidiomatal formation. Bar = 25 µm. **Figs 3-4.** Early stages of simple symphogenous method of conidiomatal formation. Bar = 25 µm. **Fig. 5.** Formation of temporary conidiomata. Bar = 25 µm. **Fig. 6.** Vertical section of young primordial showing the dissolution of central cells to form the cavity. Bar = 50 µm. **Fig. 7.** Vertical section showing temporary conidiogenous cells. Bar = 50 µm. **Fig. 8.** Vertical section of mature pycnidium. Bar = 50 µm

Electron microscopical studies

Conidiogenous cell

The initial of the conidium arises as a small protrusion of the apex of the conidiogeous cell (Fig. 12) and develops holoblastically. Cell organelles migrate into the developing conidium until a delimiting septum is 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 is continuous with the wall of the conidiogenous cell. The conidiogenous cell itself does not develop an electron-opaque outer wall layer. Successive conidium develops from a point 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 (Figs 13-14).

Mature conidia

Sections through the young conidiomata showed that the conidia arise from spherical to subspherical conidiogenous cells lining their cavities (Fig. 12). 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 is bounded by a thin electron transparent wall. During septation the wall increases in thickness. The first septum is laid

normally near the base of the conidium (Fig. 15). Gradually the peripheral region of the conidial wall becomes electron dense by the deposition of melanin in the wall matrix.

Median coloured cells

Transconidial septa arise as outgrowths of the conidial wall and new wall material is deposited external to the invaginations. 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 (Fig. 15). The septal pore is formed as a result of cessation of wall deposition at the junction where the plasma membranes from either side fuse 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 become distinctly electron dense. The thickness of the cell wall is more in the upper two-median cells when compared to lower median cell. The lower median cell is structurally different from the other two cells of the conidium in that it showed pronounced wrinkling of the outer-pigmented wall (Fig. 15). 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

The apical and basal cells are morphologically indistinguishable from the median cells during the early stages of the conidial development. 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 (Figs 15-17).

Apical and basal appendages

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 (Fig. 16). The conidia were produced from the annellides and more than 3 annellations were observed in some conidiogenous cells. Occasionally the basal appendage of the developing conidium was observed within the annellation, which proves that the basal appendage is endogenously produced (Fig. 17).

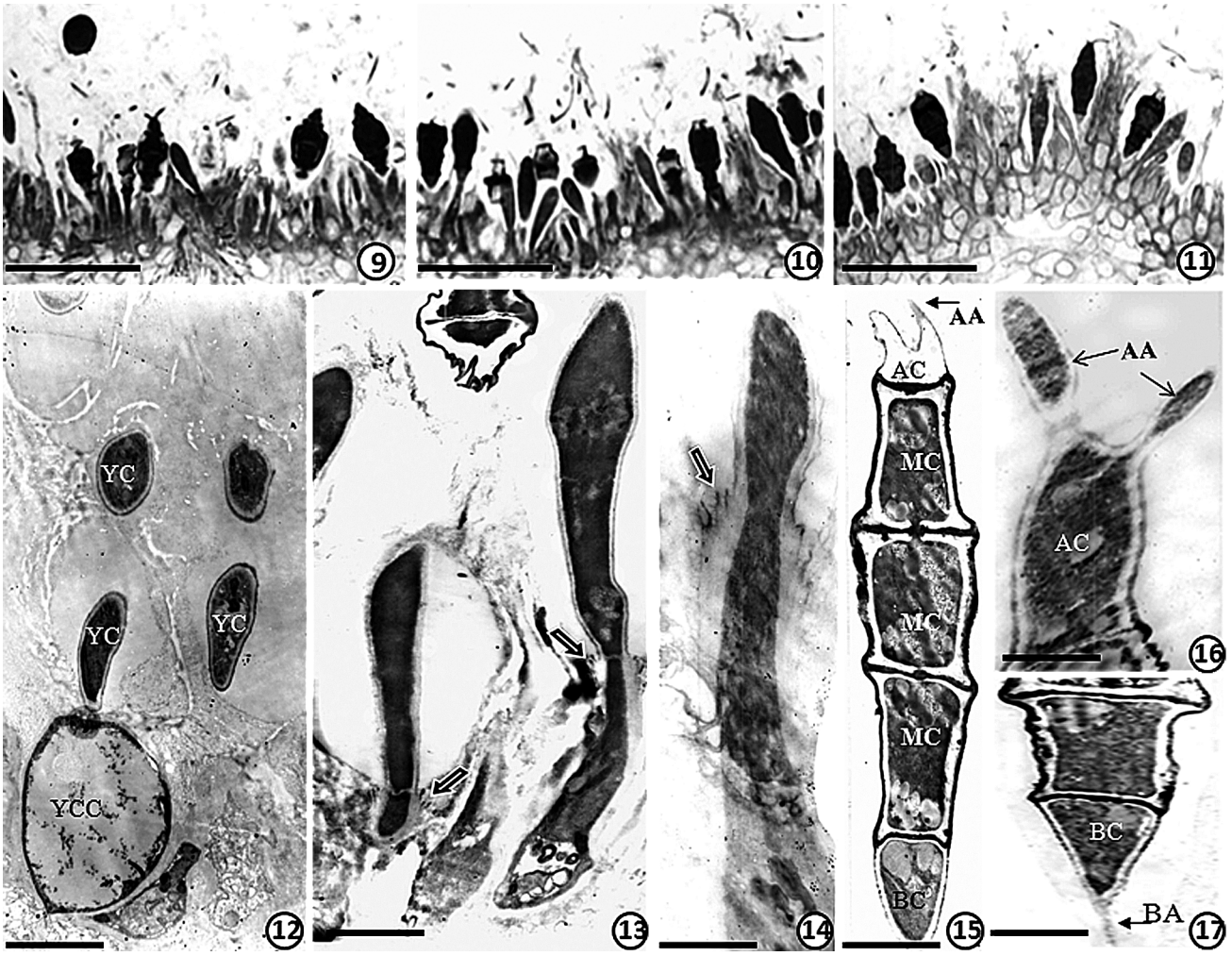
Discussion

Species of *Pestalotiopsis* have not been studied with reference to the development of conidiomata. Sutton (1961) in his developmental studies on *Pestalotiopsis* reported various stages in spore development in some species. The present study reports the various stages of development of conidiomata in *Pestalotiopsis cruenta* with interesting observations. The fungus is known to produce only acervular conidiomata on its host whereas in artificial culture media, pycnidial conidiomata are formed. As the primordium increases in size, the central cavity is to be formed because the primordium is a compactly packed pseudoparenchymatous tissue. The central cells in the primordium showed signs of schizogenous and lysigenous activity to form the central cavity. The remaining portions of the cells often gelatinize and fill up the cavity with a mucilagenous matrix. Probably, the matrix provides nutrition to the developing conidiogenous cells and young conidia. Nag Raj (1981) suggested that the slime, which originates through lysis occurring during cavity formation, plays an important role in conidium dispersal. The slimy matrix also plays an indirect role in the dispersal of the conidia by insects.

In the present investigation it was observed that initially conidiogenous cells are not annellides because the first conidia formed (primary conidia) are holoblastic and only during the later stages of development of the conidiomata secondary conidia are formed enteroblastically showing annellidic conidiogenesis. Later on conidiogenous cells are formed which are cylindrical with 4-5 annellations. This shows that the primary conidia may be morphologically different from the secondary conidia formed successively from the conidiogenous cell. The dual conidiation process was also observed in other species of *Pestalotiopsis* examined in our lab. Furthermore, it was observed that in *P. cruneta* the disorganized central cells gelatinize to form the slimy matrix. But in *P. disseminata* (unpublished data) the temporary conidiogenous cells after releasing the conidia showed signs of degeneration inside the cavity. The formation of a slimy matrix by the dissolution of central cells of the primordia was not very clear in *P. maculans* (Murugan & Muthumary 2001) and *P. cruenta, P. uvicola* (Murugan & Muthumary 2003) resembles *P. rhododendri* (unpublished data) in that the temporary conidiogenous cells showed degenerative activity after releasing the conidia into the cavity.

From the above observation, it is clear that in *Pestalotiopsis* species studied in the present investigation, the presence of mucilagenous matrix, arising either through lysis occurring during cavity formation or formed by the degenerating temporary conidiogenous cells plays an important role in the survival of the fungus. The presence of mucilagenous matrix during the formation of the pycnidial conidiomata was reported in certain other pycnidial coelomycetes also (Masilamani & Muthumary 1994, 1995, 1996; Murugan & Muthumary 2001, 2003, 2007). The formation of conidiogenous cells inside the cavity of the conidiomata is another interesting feature studied in the species of *Pestalotiopsis.* It was the pioneering work of Punithalingam (1979) who studied in detail the process of conidiation in *Ascochyta* species in culture.

The fine structure of the conidiogenous cells and conidia were demonstrated in four coelomycete species (Campbell 1968; Sutton & Sandhu 1969; Griffiths & Swart 1974a, b).



Figs 9-11. Section of conidioma showing permanent conidiogenous cells and conidia. Bar = 50 µm. **Fig. 12.** Section showing the young conidia and condiogenous cells (in TEM). Bar = 1 µm. **Figs 13-14.** Section through young conidium showing the annellides (in TEM). Notes annellation (arrow indication). Bar = 1 µm. **Fig. 15.** Longitudinal section of a 5 celled mature conidium (in TEM). Bar = 1.5 µm. **Fig. 16.** Section of the apical cell with two appendages (in TEM). Bar = 2 µm. **Fig. 17.** Section of the basal cell with appendage (in TEM). Bar = 1 µm. **Abbreviations:** 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

Generally, these studies were intended to extend light microscopic observations. Some of the features, which could not be resolved adequately by the light microscope, could be demonstrated with the help of electron microscope. Obviously, in the present investigation, the fine structural details observed with the electron microscope in *Pestalotiopsis cruenta* have demonstrated some interesting findings. Longitudinal sections through the conidia of *Pestalotiopsis cruenta* 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 is 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 are 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 (Fig. 9). The conidial wall consists of an outer layer bounding an inner layer, which consists of a finely granular matrix. According to Griffiths & Swart (1974a), in *Pestalotiopsis* the conidial wall is single-layered though differentiated into an outer melanized zone and a much thicker, inner hyaline zone. Sutton (1969) on the basis of light microscopy suggested that they are two distinct wall layers – the outer pigmented and the inner, non-pigmented. It is true that it is not possible to observe melanization of a portion of a thin cell wall by light microscopy. But mycologists hold different views regarding the accurate description of the wall layers in *Pestalotiopsis.* Therefore, Griffiths & Swart (1974a) described the pigmented and non-pigmented areas of the wall as "zones" rather than "layers".

In the present investigation, following Sutton (1969), the outer melanized wall and the hyaline inner wall layer are treated

separately. 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, having a thick inner wall layer and a thin outer wall layer when compared to the end cells of the conidium. According to Griffiths & Swart (1974a), during septation of the conidium the peripheral zone of the conidial wall becomes electron dense by the deposition of melanin in the wall matrix, while the remaining portion of the wall remains hyaline. Therefore, they concluded that the outer zone of the wall is melanized while the inner zone remains unpigmented. The transconidial septa arise as outgrowths of the conidial wall and new wall material is deposited externally to the invaginations 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 consists of the 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. Griffiths & Swart (1974a) noticed that this lower most median cell is frequently binucleate and is characterized by the presence of large round, electron-dense particles within the hyaline wall layer.

The apical and basal cells, which are hyaline, are morphologically indistinguishable from the median cells at the ultrastructural level. During the later stages of development, the end cells undergo cytolysis and in mature conidia they are devoid of cytoplasmic contents. Cytolysis occurs very soon in the apical cell since it bears the appendages and only during the later stages in the basal hyaline cell because of its attachment to the conidiogenous cell. The conidia are produced from annellides and up to five anellations were observed. The basal appendage of the developing conidium was sometimes observed within the annellide. The basal cell along with the appendage remained viable long after the cytolysis of the apical cell. To our knowledge, this is the first ultrastructural study of conidiomata and conidia of *Pestalotiopsis cruenta.*

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References

CABI Bioscience database 2001. Published online.

Baxter, A.P. 1981. A study of the morphology and taxonomy of certain South African species of *Colletotrichum corda.* M.Sc. thesis. University of Pretoria, Pretoria, South Africa.

Baxter, A.P., van Der Westhuizen, G.C.A. & Eicker, A. 1985. A review of literature on the taxonomy, morphology and biology of the fungal genus *Colletotrichum.* – Phytophylactica **17**: 15-18.

Campbell, R. 1968. An electron microscope study of spore structure and development in *Alternaria brassicicola.* – Journal of General Microbiology **54**: 381-392.

Griffiths, D.A. & Swart, H.J. 1974a. Conidial structure in two species of *Pestalotiopsis.* – Transactions of the British Mycological Society **62**: 295-304.

Griffiths, D.A. & Swart, H.J. 1974b. Conidial structure in *Pestalotia pezizoides.* – Transactions of the British Mycological Society **63**: 169-173.

Hawksworth, D.L., Kirk, P.M., Sutton, B.C. & Pegler, D.N. 1995. Ainsworth and Bisby's Dictionary of the fungi, 8th edn, pp. 104-105. CAB International, Wallingford, U.K.

Jeewon, R., Liew, E.C.Y. & Hyde, K.D. 2002. Phylogenetic relationships of *Pestalotiopsis* and allied genera inferred from ribosomal DNA sequences and morphological characters. – Molecular Phylogenetics and Evolution **25**: 378-392.

Karakaya, A. 2001. First report of infection of kiwifruit by *Pestalotiopsis* sp. in Turkey. – Plant Disease **85**: 1028.

Masilamani, S. & Muthumary, J. 1994. Development of conidiomata in *Ciliochorella mangiferae* (*Coelomycetes*). – Mycological Research **98**: 857-861.

Masilamani, S. & Muthumary, J. 1995. Pycnidium ontogeny in *Coleophoma cylindrospora.* – Mycological Research **99**: 693-969.

Masilamani, S. & Muthumary, J. 1996. Development of conidiomata in *Botryodiplodia theobromae.* – Mycological Research **100**: 1383-1387.

Murugan, M. & Muthumary, J. 2001. Developmental morphology and ultrastructure of *Pestalotiopsis maculans.* – Mycotaxon **79**: 55-465.

Murugan, M. & Muthumary, J. 2003. Studies on developmental morphology of the conidiomata in *Pestalotiopsis uvicola* with a note on the ultrastructure of the conidia and conidiogenous cell. – Journal of Mycology and Plant Pathology **33**(2): 204-211.

Murugan, M., Gangadevi, V. & Muthumary, J. 2007. Light and transmission electron microscopic studies on conidiomata developmental morphology in *Pestaloiopsis rhododendri.* – Indian Journal of Science and Technology **1**(2): 1-7.

Nag Raj, T.R. 1981. Coelomycete systematics. – In: G.T. Cole & B. Kendrick [eds]. Biology of conidial fungi. Vol. **1**. Pp. 43-84. Academic Press. New York.

Nag Raj, T.R. 1993. Coelomycetous anamorphs with appendage-bearing conidia. Mycologue Publications **331**. Pp. 43-79. Daleview PI. Wateloo, Ontario, Canada.

Punithalingam, E. 1979. Graminicolous *Ascochyta* species. Commonwealth Mycological Institute.

Riddell, R.B. 1950. Permanent stained mycological preparations obtained by slide cultures. – Mycologia **42**: 265-270.

Subramanian, C.V. & Reddy, K.R.C. 1974. The genus *Discosia* I. Taxonomy. – Kavaka **2**: 57-89.

Sutton, B.C. 1961. *Coelomycetes.* I. – Mycological Papers **80**: 1-16.

Sutton, B.C. 1969. Forest microfungi III. The heterogenecity of *Pestalotia* de Not. Section *Sexloculatae* Klebahn sensu Guba. – Canadian Journal of Botany **48**: 2083-2094.

Sutton, B.C. 1980. The *Coelomycetes.* Fungi imperfecti with pycnidia, acervuli and stromata. Commonwealth Mycological Institute, Kew, Surrey, England.

Sutton, B.C. & Sandhu, D.K. 1969. Electron microscopy of conidium development and secession in *Cryptosporiopsis* sp., *Phoma fumosa, Melanconium bicolor* and *M. apiocarpum.* – Canadian Journal of Botany **47**: 745-749.