Ultrastructural Studies of Conidiogenesis of *Ascochyta rabiei*, the Causal Organism of Chickpea Blight

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Studies employing transmission (TEM) and scanning (SEM) electron microscopy revealed that conidiogenous cells of the chickpea blight fungus, *Ascochyta rabiei* (Pass.) Labr., resembled phialides in most of their features. The phialides were generally of a simple type, but occasionally they proliferated in a percurrent fashion. There is enough evidence to suggest that the fungus is better placed in genus *Phoma*. SEM has been employed for the first time to study internal details of fructifications of a closed type in coelomycete fungi.
KEY WORDS: *Ascochyta rabiei*; chickpea blight; ultrastructure; taxonomy; chickpea; scanning electron microscopy; transmission electron microscopy.

INTRODUCTION

Chickpea is attacked by a large number of diseases (28). Among them, blight caused by *Ascochyta rabiei* (Pass.) Labr. (teleomorph: *Didymella rabiei* (Kov.) V. Arx) is the most devastating and is widely prevalent in most of the chickpea-growing areas of the world. The taxonomy of this fungus is controversial (33). The asexual stage of the fungus is commonly referred to in the literature by the names *Ascochyta rabiei* (Pass.) Labr., *Phoma rabiei* (Pass.) Khune & Kapoor, and *Phyllosticta rabiei* (Pass.) Trot. The name *A. rabiei* has been adopted by most workers. In the light of newer developments, however, there are many gaps in our knowledge on the taxonomy of this important pathogen. In deuteromycete taxonomy, recently, emphasis is laid particularly on the mode of conidiogenesis. Development is reported to be phialidic in *Phoma* spp. but annellidic in *Ascochyta* spp. (4). Therefore, keeping this developmental aspect in mind, studies were performed under transmission (TEM) and scanning (SEM) electron microscopes to reveal the mode of conidiogenesis in the fungus *A. rabiei*.

MATERIALS AND METHODS

Medium

The culture of *A. rabiei* was raised on potato dextrose–chickpea seed–agar medium, PDCA (peeled potatoes, 200 g; dextrose, 20 g; agar-agar, 20 g; water to make 1 *l*; split chickpea seeds with testa intact, two per test tube), at 20±1°C.

Part of the Ph.D. thesis submitted by the senior author to the Indian Agricultural Research Institute, New Delhi 110 001, India. Received Oct. 25, 1996; received in final form May 21, 1997.
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Preparation of specimens for TEM

Small blocks of agar (1.5-mm cubes) from PDCA supporting 12-day-old pycnidial growth of the fungus were fixed for 3 h in 6% glutaraldehyde in sodium cacodylate – HCl buffer (pH 7.2). The samples were washed several times in the buffer and post-fixed in 1% osmium tetroxide for 3 h at 4–8°C in the dark. They were then prestained for 20 min in a 10% EtOH solution of 1% uranyl acetate. After prestaining, specimens were dehydrated in graded-EtOH series of 50%, 70%, 95% and 100% for 10 min each, followed by a final change in absolute EtOH for 5 min. Samples were then infiltrated in a mixture of Spurr low viscosity resin (14) and acetone in proportions of 1:3, 1:1 and 3:1 for 30 min each. Lastly, the specimens were placed in pure Spurr resin in Beem capsules, oriented properly, labelled, and cured at 60°C for 2 h in a C. Reichert KT 100 polymerization oven. Ultrathin sections were cut with a glass knife on an LKB Ultrotome, decompressed with xylene fumes in water boats, mounted on nitrocellulose coated copper grids and poststained with lead citrate in oxygen-free atmosphere for 20 min, followed by uranyl acetate (1% soln) for 10 min. The specimen-mounted grids were dried and viewed in a Philips EM 300 electron microscope at 80 kV.

Preparation of specimens for SEM

Pycnidia produced on PDCA were picked up individually, cut with a sharp-edged blade under a stereo-microscope, and fixed for 2 h at 4°C in the dark in 6% glutaraldehyde plus 1% osmium tetroxide (1:1) in sodium cacodylate – HCl buffer (pH 7.2). They were buffer-washed once and refixed in 1% osmium tetroxide for 2 h at 4°C, washed twice in cacodylate buffer, for 15 min each time, and dehydrated in an ethanol series of 30%, 50%, 70%, 90% and 100% for 15 min each, followed by a final change in absolute ethanol for 10 min. Following this, the samples were critical-point-dried in an E-3000 Polaran Critical Point Drier, mounted on specimen stubs, coated with silver in an Edwards Coater 306 and examined in a Cambridge Stereoscan S4-10 SEM operating at 20 kV.

RESULTS

TEM studies

The development of the first conidium started by a papillate thickening of the wall at the apex of the conidiogenous cell (Fig. 1). Further growth and differentiation allowed the protoplast to push through the apical thickening (Fig. 2). Within this apical thickening the inner wall layer was distinguishable from the outer covering layer, and was continuous with the inner wall of the mother cell. The exact timing of breaking of the outer wall, whether it occurred during an early stage of development of the conidium initial or at the time of secession of the primary conidium, could not be pinpointed. As a consequence of formation of the first conidium, a collarette was established at the neck region of the conidiogenous cell (Fig. 3). The inner wall of the conidiogenous cell extended through the collarette thus formed and developed into a conidium (Fig. 4). In the majority of the conidiogenous cells examined, only one collarette was observed; rarely two (Fig. 5) or three (Fig. 6) collarettes could also be found. Each successive collarette formed at a higher level than the previous, as a result of percurrent proliferation of the conidiogenous locus. The collarettes were usually some distance apart, not closely spaced like scars in typical annellides. It appeared that they formed in a way very similar to the first one.

Figures 1–19: Transmission electron micrographs of *Ascochyta rabiei*.
Fig. 1. Apical thickening (Ap) of a conidiogenous cell (× 8340). **Fig. 2.** Different wall layers of a conidiogenous cell: outer (O), middle (M) and inner (I) in the apical thickening (Ap) (× 10,175). **Fig. 3.** Formation of collarette (C) at neck region of a conidiogenous cell (× 27,470). **Fig. 4.** Almost fully mature conidium still attached to the conidiogenous cell (× 8340). **Fig. 5.** Two collarettes (C_1, C_2) at apical end of a conidiogenous cell, C_2 having been formed at a higher level by percurrent proliferation of the conidiogenous locus (× 10,175). **Fig. 6.** Three collarettes (C_1, C_2, C_3) at apical end of a conidiogenous cell (× 10,175). **Fig. 7.** Delimitation of a conidium by formation of a septum (S) (× 27,470). **Fig. 8.** Nearly complete separation of a conidium from a conidiogenous mother cell (× 16,280). **Fig. 9.** A highly constricted channel at the neck region of a conidiogenous cell (× 6920). **Fig. 10.** Abundant granular material (GM) in cavity of a conidioma (× 12,730). **Fig. 11.** Degeneration (arrows) of the old conidiogenous cells due to lysigenous activity (× 6920). **Fig. 12.** A fertile conidiogenous cell below degenerated conidiogenous cells (asterisks) in a conidioma (× 8340). **Fig. 13.** An interconnection (Ic) in pseudoparenchymatous cells of a pycnidial conidioma (× 10,175). **Fig. 14.** Pycnidial hymenium showing presence of a Woronin body (Wb) near an interconnection (× 12,720). **Fig. 15.** Formation of a tong-shaped structure above collarette of a conidiogenous cell (× 8340). **Fig. 16.** Globoid structure (arrow) in a conidiogenous cell (× 12,720). **Fig. 17.** A conidiogenous cell showing delimitation of a conidium primordium by a septum at a very early stage of development (× 10,175). **Fig. 18.** Absence of collarettes at neck region of a conidiogenous cell. Note sterile protoplasmic masses formed by this development process (× 16,280). **Fig. 19.** A very early stage of formation of septum (S) in a conidium. Note that septa are already thick (× 12,720).

Figures 20–26: Scanning electron micrographs of *Ascochyta rabiei*.
Fig. 20. Cut-away view of a pycnidium (× 417). **Fig. 21.** Bottle-shaped conidiogenous cells (× 1696). **Fig. 22.** Pitcher-shaped conidiogenous cells. Note a single unruffled collarette (C) around the neck of the conidiogenous cell (× 1578). **Fig. 23.** Conidiogenous cells with one (C) and two (C_1 and C_2) collarettes (× 1696). **Fig. 24.** Portion of a conidium (Ps) inside a conidiogenous cell, indicating that the conidiogenous locus is some distance below the neck region (× 1487). **Fig. 25.** Membranous protoplasmic layers at apical ends of conidiogenous cells (single arrows). Note several conidia (Ps) inside one of the conidiogenous cells and a ruffled collarette (double arrows) in another conidiogenous cell (× 1585). **Fig. 26.** Degenerated conidiogenous cells. A pore in the old, lysed conidiogenous cell (single arrow) provides evidence that there is a juvenile conidiogenous cell underneath (× 2318).

Additional Abbreviations Used in Electron Micrographs
CW = cell wall; CWI = inner cell wall; CW2 = outer cell wall; ER = endoplasmic reticulum; L = lipid droplet; M = mitochondria; Mb = microbody; PM = plasmamembrane; R = ribosomal matrix; SP = septal pore; V = vacuole; Vs = vesicles.

The enlargement given in parentheses for each figure, indicates the original enlargement of each electron micrograph. The printed size is approximately 30% smaller.

Fig. 1. NONE

Fig. 2. NONE

Fig. 3. NONE

Each conidium was delimited at its base from the conidiogenous cell by a septum and seceded as a result of a circumscissile tear at the median electron-transparent area (Figs. 7,8). The upper part of the septum became the base of the conidium, while the lower part formed the growing apex of the conidiogenous cell. Many conidia did not have a well-marked basal septum, probably due to a highly constricted channel (Fig. 9). Basal frills were not observed on seceded conidia.

The conidial wall and the inner conidiogenous cell wall above the conidiogenous locus were not firmly bound together (Figs. 4,8,9). Woronin bodies were not found associated with conidium-delimiting septa; only in one or two of the cells was their presence suspected (Fig. 7). Lipid droplets were observed frequently in the conidiogenous cells and conidia, both young and old (Figs. 1,4,9). Plasmalemma in the developing conidia and apical parts of the mother cells was found to be highly corrugated (Figs. 3,6). In a good number of conidiogenous cells examined, only one nucleus per cell was observed that appeared to migrate towards the apex some time before formation of the delimiting septum (Figs. 6,10). Granular electron-opaque material was frequently observed in cavities of the pycnidial conidiomata (Figs. 6,9,10).

Eventually conidiogenous cells of the innermost layer of the pycnidial hymenium exhausted their capacity to produce conidia (Fig. 11), degenerated, and the underlying fertile layers of the cells started producing conidia (Fig. 12).

Pseudoparenchymatous cells of the conidiomata possessed interconnections for cytoplasmic exchange (Fig. 13). Few such connecting streams had Woronin bodies associated with them (Fig. 14).

Apart from the normal mode of conidiogenesis, some rare structures/exceptions were also observed, for instance (i) formation of a tong-shaped structure above a collarette (Fig. 15), (ii) presence of a globoid structure inside a conidiogenous cell (Fig. 16), (iii) delimitation of a conidium primordium by a septum at a very initial stage (Fig. 17), and (iv) absence of a collarette around the neck region of a conidiogenous cell and demarcation of sterile protoplasmic masses of an irregular shape by a septum (Fig. 18).

Conidial septa were rarely encountered in ultrastructure studies. Apparently from the very start they attained the thickness of a final septum (Fig. 19). They developed by an invagination of the primary wall of conidia in a centripetal fashion.

SEM studies

These studies supplemented to a large extent the information obtained by TEM. A view of the cavities as seen by SEM is presented (Fig. 20). The conidiogenous cells were bottle- (Fig. 21) to pitcher-shaped (Fig. 22), *i.e.*, more or less flask-shaped, and were arranged in the form of a hymenium inside the cavity. A large majority of them had only one collarette (Figs. 21,22); two collarettes were observed rarely (Fig. 23). Some conidiogenous cells had small pores at their apical region and collarettes in an unfinished state (Fig. 25), whereas others possessed bigger pores with collarettes in a more uniform, rounded-off condition (Fig. 22). Not even a single conidiogenous cell was observed in a stage which could reveal that the first conidium formed holoblastically.

It is apparent from the electron micrographs that the conidiogenous locus was located some distance below the collarette in the neck region of the conidiogenous cell (Fig. 24). A membranous protoplasmic layer enclosing the apical end of the conidiogenous cell was noticed in some of the cells (Fig. 25). Broken conidiogenous cells were observed in many

cavities of pycnidial conidiomata (Figs. 24,26). Some conidia happened to fall into these broken cells. In a limited number of cases two or three conidia could also be observed inside conidiogenous cells (Fig. 25).

DISCUSSION

Conidiogenesis is reported to be phialidic in *Phoma* spp. but annellidic in *Ascochyta* spp. (4). Several distinctions between phialides and annellides have been proposed from time to time by various workers (5,7,8,9,10,17,23,25,30,35). The characters on which these distinctions are based are discussed here in light of the present investigations made on *A. rabiei*.

Apical thickening of the conidiogenous cell

Marked apical thickening of the conidiogenous cells was recorded prior to conidium formation in *A. rabiei*. Such pronounced apical thickening has been recorded in *Phoma* spp. previously and it is believed to be a typical feature of the genus (4).

Mode of development of the first conidium

Boerema and Bollen (4) stated that according to Kendrick (17) the first conidium in a phialide is formed enteroblastically. Probably Kendrick was not of this view since holoblastic development of the first conidium has been reported (25) in phialides of the fungus *Cryptocline effusa*. In *A. rabiei* it could not be established whether the first conidium formed enteroblastically or holoblastically. However, it is now believed that the mode of formation of the first conidium in phialides and annellides is essentially the same (7,9).

Mode of development of successive conidia

The mode of development of successive conidia of the fungus was clearly enteroblastic. It has been stated (17) that annellidic conidia are holoblastic and not enteroblastic, which indirectly supports our view. The mode of development of the successive conidia is, however, also considered controversial at present (4,10,30).

Position of the circumscissile tear in the periclinal wall

The relative positions of the circumscissile tear in the periclinal wall and the basal septum of the first conidium, have important implications in the taxonomy of this group of fungi (8,9,10). Cole and Samson (9) distinguish between annellidic *vs* phialidic thus: “the outer annellide wall usually ruptures at the base of the first-formed conidium, adjacent to the double layered septum leaving an annellation, while the outer phialide wall may rupture at any point above the basal septum, leaving a collarette.” In the present fungus the exact position of the tear could not be ascertained, but it is quite evident from the site at which the subsequent conidia begin to form (*i.e.*, deep inside the collarette) that the tear occurs at a higher level than the septum. Therefore, the fungus under study can be said to possess a phialidic mode of conidial development.

Involvement of the half septum in formation of the wall of secondary conidia

Morgan-Jones *et al.* (25) reported that in annellidic development the percurrent proliferation of the conidiogenous cell must involve the half septum left behind after

secession of the previous conidium, whereas in phialidic development the wall of the conidium arises *de novo*. In *A. rabiei*, the lower half of the septum was found involved in formation of the wall of the subsequent conidia. Such an involvement of the lower half septum is evident in *Phoma* species recorded by Boerema and Bollen (4, Pl. 21). Therefore, it is obviously not a very reliable criterion for differentiating between the two genera.

The apical channel in some of the conidiogenous cells examined was so narrow that conidium-delimiting septa were not visible. Formation of phialoconidia by protoplasmic attenuation and constriction has been reported earlier (25).

Presence of basal frills on seceded conidia

Basal frills could not be observed on seceded conidia of *A. rabiei.* They have not been recorded on phialoconidia of *Phoma* spp. (4,37), but have been observed on annelloconidia of *Ascochyta* spp. (4). The present fungus, therefore, resembles *Phoma* in this character.

Cloudy substance formation

Boerema and Bollen (4) reported the presence of an abundant mucilaginous mass (cloudy substance) surrounding the conidia of *Phoma* spp. However, from their electron micrographs it is apparent that the cloudy substance was not equally abundantly formed in all the *Phoma* spp. (*cf. P. lycopersici* and *P. exigua*, in which, respectively, it is almost absent and super-abundantly formed). In *A. rabiei*, mucilaginous mass of the sort shown in *P. exigua* was not observed. However, granular electron-dense matrix of the type shown in several other fungi in which the phialidic mode of conidiogenesis operates (16), was frequently recorded. Therefore, separation of *Phoma* and *Ascochyta* on the basis of this character appears not to be justified.

Degree of structural association between the conidial wall and the conidiogenous cell distal to the conidiogenous locus

Cole and Samson (9) reported that in annellides the outer conidial wall and the inner conidiogenous cell wall distal to the conidiogenous locus are bound firmly together, whereas in phialides they are not. In *A. rabiei* these wall layers were not bound firmly together, and therefore their conidiogenous cells are, in this regard, phialides.

Association of Woronin bodies with basal septa

The conidium-delimiting septa in *A. rabiei* most probably did not have Woronin bodies associated with their pores. The absence of Woronin bodies with the septa is considered to be typical of phialides (6,9,12), although exceptions to this feature are also known (13,18).

Conidial septation

A limited number of conidial septa that were observed in electron microscope studies originated as a result of ingrowth of the lateral wall and from the very start attained the thickness of a final septum. Boerema and Bollen (4) classified such septa as eusepta and believed them to be typical of *Phoma* spp. *A. rabiei* therefore deserves its placement in *Phoma*. Roberts and Swart (31) compared distoseptate conidia of *Helminthosporium maydis* (40) and euseptate conidia of *Seridium* (38) and found a structural similarity between the two types. Therefore, they stressed that these two types of conidia require

further elucidation before they can be safely applied to indicate distinctions of taxonomic importance.

Most conidia of *A. rabiei*, *in vitro* and *in vivo*, were one-celled. Similar observations have been recorded by several workers (20,21,22,32,34). Boerema and Bollen (4) reported that "the mature conidia of true *Ascochyta* species are always *in vivo* as well as *in vitro* two- or more-celled; whereas *Phoma* species in culture produce mainly one-celled conidia." Sutton (36) mentions that in genus *Ascochyta* the conidia are "medianly 1 septate" (p. 409), whereas in genus *Phoma* they are "aseptate or occasionally septate" (p. 378). According to the differences proposed by these researchers, *A. rabiei* deserves its placement in the genus *Phoma*.

Number of conidia produced at each conidiogenous locus

Some workers believed that two or three ridges or layers that sometimes occurred in periclinal thickening of *Phoma* spp. corresponded to the number of conidia released (3,37). Subsequently, it was reported (4) that these ridges represent different layers of the original papilla (outer, middle and inner). However, Reisinger *et al.* (30), who used Thiery's TCH technique, believed that striations in the collarettes resulted from deposits left in the course of successive conidium production. Hence, they thought that a number of conidia were produced from a single conidiogenous locus.

An extremely large number of annellations has been demonstrated in some fungi such as *Trichurus spiralis* (11) and *Scopulariopsis brevicaulis* (15). In *A. rabiei* most of the conidiogenous cells possessed one collar; two or three collars formed very rarely. We do not have direct evidence that several conidia are produced from a single conidiogenous locus, but speculate (from the ruffled nature of collarettes and abundant production of pycnidial ooze by the fungus in culture medium) that this is the case.

The number of conidia produced through each successively formed collarette is considered a decisive factor in differentiating percurrently proliferating phialides from annellides (7). Determining the number of conidia produced at each level of proliferation is considered to be the crux of the problem (26). Sutton (36) believes that this problem is particularly associated with coelomycetes, since their conidiomata are closed structures and conidiogenous cells are much smaller and difficult to interpret. In the present study a novel technique was developed which comprised dissecting the conidiomata and viewing them under a SEM. This technique has been employed for the first time to study internal details of fructifications of a closed type in the coelomycete fungi. The technique supplemented the information obtained by TEM but direct evidence of production of multiple conidia from a conidiogenous cell is still not available, since living plant material cannot be viewed by SEM and moreover the so-called basipetal arrangement of conidia is likely to be disrupted by the sample preparation technique.

During the process of conidiogenesis some of the conidiogenous cells were observed in a degenerated or broken state. It is believed that when these cells age, they exhaust their capacity to produce more conidia, disintegrate, and their contents leak out. As a consequence of this lysigenous activity more space is created inside the cavity of the conidioma and pseudoparenchymatous cells lying below the degenerated cells get an opportunity to produce conidia with fresh vigor; this process continues until barely one or two outer peripheral layers remain. As a result of dissolution of the exhausted conidiogenous cells some mucilage may be produced, which may play a

role in expulsion of the spores. Madelin (24) believes that in enteroblastic development conidia are not produced very swiftly, and there is a pause sufficiently long for the wall at the conidiogenous locus to lose its capacity for direct involvement in further growth and differentiation, unlike that in holoblastic development. He postulated that in the enteroblastic process of conidiogenesis, mature parts of the wall are lysed, to allow the egress of a juvenile layer from below. In *A. rabiei*, this process of lysis of the old cells has been amply demonstrated and Madelin's distinction lends further support to the belief that the enteroblastic mode of development operates in this fungus. One or several conidia that were at times noticed inside the conidiogenous cells in *A. rabiei* are believed to have been produced by some underlying juvenile conidiogenous cell and had happened to lie inside the old conidiogenous cell whose cellular contents had run out but whose walls were yet to dissolve.

In rare instances conidium-delimiting septa were observed in a very early stage of conidium formation. If it is presumed that such a conidial primordium develops into full size, the mode of development of conidia will then be interpreted differently. In a very few electron micrographs, tong-shaped structures were also observed above the collar region. Similarly, in very rare cases sterile protoplasmic masses of an irregular shape were abstricted by a septum. All these three occurrences are believed to be aberrations and probably result from exhaustion of nutrients near the apical region in the aged conidiogenous cells. In nature, some abnormalities due to genetic disturbances may also be expected. Therefore, we believe that these abnormalities were exceptions, since they were of rare occurrence.

Boerema and Bollen (4) held the view that true Ascochytas are mostly true parasites and restricted in their host range, whereas *Phoma* spp. are plurivorus in nature. According to this view, *A. rabiei* belongs to the genus *Ascochyta*, but it is doubted if the parasitic/saprophytic ability of a fungus has to be regarded as a character of taxonomic importance at generic level.

From the foregoing discussion it is evident that in most of its traits *A. rabiei* resembles *Phoma*, not *Ascochyta*. The transfer of the fungus *Ascochyta rabiei* (Pass.) Labr. to *Phoma rabiei* (Pass.) Khune & Kapoor has been proposed before (19). We now provide additional ultrastructural evidence in favor of the latter name.

Some workers have preferred the name *Phyllosticta rabiei* (Pass.) Trot. for chickpea blight fungus (1,2,21,39). SEM and TEM studies have shown that there is no apical appendage and a surrounding gelatinous sheath on the conidia of this fungus, which are considered to be typical features of *Phyllosticta* spp. (29). Hence, it is clear that this fungus does not belong to the genus *Phyllosticta*. It is, therefore, proposed that *Phyllosticta rabiei* (Pass.) Trot. be considered a misnomer. The earlier concept that *Phoma* is parasitic on stem and roots while *Phyllosticta* spp. are leaf parasites, is now considered obsolete (27).

ACKNOWLEDGMENTS

The authors express their thanks to the Head, Division of Plant Pathology, Indian Agricultural Research Institute (I.A.R.I.), New Delhi; Project Director, Nuclear Research Laboratories, I.A.R.I., New Delhi; and Dean, Postgraduate Studies, Indian Institute of Hauz Khas, New Delhi, for providing the necessary facilities. The senior author is especially grateful to I.A.R.I. for financial assistance toward his doctoral studies.

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