Cytology of Chlamydospore Germination in *Protomyces macrosporus* Unger

M. S. Pavgi and A. N. Mukhopadhyay

Faculty of Agriculture, Banaras Hindu University, India

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Introduction

Species of the genus *Protomyces* Unger have evoked interest amongst the mycologists since its establishment for their fairly widespread occurrence and parasitism on the Compositae and Umbelliferae in the world regions. Preliminary observations on cytology of the species of this genus were reported by earlier workers at the turn of the century (Dangeard 1906, Popta 1899, Sappin-Trouffy 1897). Büren (1915, 1922) carried out extensive investigations into the pattern of life cycle in the genus *Protomyces* represented by several species including *P. macrosporus*, but was unable to precisely determine the site of meiosis. He described the gross cytological features with meagre data on various stages in the sequence. He observed the vesicle of a germinating chlamydospore in *P. macrosporus* containing a number of centrally oriented vacuoles, which coalesced and pushed the cytoplasm and nuclei to a peripheral layer. This was believed the place where reduction division of the diploid nuclei possibly occurred cleaving the protoplasm into tetrads, each cell finally forming into a uninucleate spore. Tubaki (1957) working on 3 other species of *Protomyces* stated that the nuclei in *Protomyces* were by no means well-defined and neither the nucleolus nor the chromatin could be differentiated in preparations stained with Heidenhain's hematoxylin. Valadon *et al.* (1961, 1962, 1962a) attempted to obtain evidence for the site of meiosis in *P. inundatus* Dangeard but without success. Increase in the number of nuclei during the later part of chlamydospore germination led them to assume it a result of meiotic nuclear division. Cytological changes in sequence during the chlamydospore germination in *P. macrosporus* were studied, observations on which are presented here.

Materials and methods

Diseased stems of coriander (*Coriandrum sativum* L.) bearing mature galls by *Protomyces macrosporus* were collected and stored at 10°C. Chlamydospores scraped out of these galls were fixed onto slides by alternate wetting and drying technique (Thirumalachar *et al.* 1950, 1953), treated in weakly alkalined water (Mukhopadhyay and Pavgi 1964) and incubated at 22-24°C. Germinating chlamydospores were fixed at various stage of develop-

ment in weak chrom-acetic acid (10% aqueous chromic acid 2.5 ml, 10% aqueous acetic acid 5 ml, and dist. water 100 ml; 16 hrs), washed thoroughly overnight (15 hrs. or more) in running water, rinsed in dist. water and mordanted in 2% iron alum 'AnalaR' (30 min.), rinsed in running water (30 min.) and stained with Heidenhain's hematoxylin (30 min.) (Johansen 1940). They were washed in running water (10-15 min.) and rinsed in dist. water; destaining was done in saturated picric acid soln. (10 min. or more). The slides were thoroughly washed in running water and passed through an ascending alcohol series and xylol: alcohol grades (3-5 min. each) and mounted in euparal or in Canada balsam. The preparations were supplemented with another set of slides fixed in Carnoy's fluid (3 parts 97% ethyl alcohol, 2 parts chloroform and 1 part glacial acetic acid) and stained with Belling's aceto-carmin (0.5 g carmin in 100 ml or 45% acetic acid) after mordanting with 2% iron alum (10-15 min.) and thorough washing for complementary observations.

Observations

A mature chlamydospore was spherical to subspherical in shape having a thick wall showing 3 distinct layers (exospore, mesospore and endospore) and containing 50 or more resting diploid nuclei (Fig. 1). The cytoplasm was uniformly granular perforated with a number of small evenly distributed vacuoles. The nuclei were spherical in shape containing a single dark staining nucleolus and a distinct nuclear membrane. The endospore along with the protoplasm contracted from all sides, separating from the mesospore with the initiation of the germinating process (Fig. 2). The contracted endospore moved onto a pole touching the mesospore wall inside (Figs. 3, 4). The nuclei remained evenly distributed apparently without any morphological change. The vacuoles moved and collected towards the centre and later fused into larger ones (Fig. 4). There was no change in the number of nuclei during these stages. The mesospore and exospore ruptured at the point of contact apparently due to an enzymatic action and a portion of the endospore pushed out of the orifice (Fig. 5). Nearly one third portion of the endospore pushed out distending the ruptured walls. The vacuoles coalesced further and were mostly oriented in the central cytoplasm well separated from each other (Fig. 6). The endospore pushed out further and the vacuoles closed in pushing the cytoplasm along with most of the nuclei to the periphery (Fig. 7).

*endospore as a vesicle and peripheration of nuclei. 11-12, formation and enlargement of a large central vacuole and final peripheration of cytoplasm and nuclei. 13, uneven distribution of nuclei along the periphery. 14, enlargement of vacuole and uniform distribution of nuclei along the periphery. 15, nuclei arranged in a single layer in the parietal cytoplasmic layer. Segmentation of cytoplasm enclosing a diploid nucleus. 16, formation of uninucleate cells along the periphery; many of the nuclei in interphase. 17, a complete vesicle showing various stages (diakinesis, metaphase I, anaphase I) of meiotic nuclear division in different segments.

Figs. 1-17. Meiotic nuclear division in the germinating chlamydospores of *Protomyces macrosporus*. 1, chlamydospore containing numerous diploid nuclei. 2-3, contraction and movement of protoplasm in the chlamydospore. 4, condensed protoplasm touching the inner surface of mesospore. 5, rupture of outer 2 walls (exospore and mesospore). 6-8, emergence of endospore in stages and centring of vacuoles pushing the uniformly distributed nuclei to the periphery. 9-10, coalescence of vacuoles at the centre; final emergence of*

Only few vacuoles persisted and the dense cytoplasm was being gradually replaced by a thin cell sap (Figs. 8, 9). Gradually the whole endospore pushed out in the form of a spherical to oval or cylindrical vesicle with the nuclei distributed mainly along the periphery (Fig. 10). The vacuoles further coalesced into a large central one making the cytoplasm denser in consistency in the peripheral region (Fig. 10). The vacuolar space extended pushing the protoplasm further into a thin parietal layer. Depth of the layer varied with the amount of protoplasm, size and shape of the vesicle (endospore) and size of the large vacuole (Figs. 11, 12). Centring of the vacuole was not a general character and variation in its orientation often led to uneven distribution of nuclei in the peripheral layer (Fig. 13). The vesicle enlarged and distended fully, the vacuole also enlarged proportionally and the nuclei organized into a single layer along the periphery (Fig. 14). Radial septation took place enclosing a nucleus in each cell (Fig. 15). The process of septation was discontinuous and was completed within a short period and the contents were circumscribed into a single layer of uninucleate cells along the periphery of the vesicle, often freed and raised above the base of the vesicle (Fig. 16).

Meiotic nuclear division was initiated during the subsequent developmental stages. The nucleus expanded during interphase and meiosis I proceeded rapidly. Interphase nuclei were morphologically similar to those in higher plants. The nucleus at this stage was composed of a light staining, round to oval mass of chromatin material surrounding the deeply staining round nucleolus both bounded by a thin membranous wall (Figs. 16, 18). All the nuclei were not observed in the interphase stage at a time and several were delayed, showing that meiosis was not initiated in all the nuclei simultaneously in the germinating chlamyospore. It was not possible to determine all the stages in the sequence of prophase I of the division due to extreme minuteness of the chromosomes and unsatisfactory staining reaction of the chromatin. Diakinesis was characterized by the condensed bivalents lying well-spaced out in the nuclear vacuole (Figs. 17, 19). The chromosomes occupied the central portion of the nuclear vacuole and the nucleolus was no more evident or was perhaps very faintly stained. Two dark stained short thick, condensed bivalents were distinguished (Figs. 17, 19). They had the form of X and O apparently indicating the number and location of chiasmata. A bivalent with 1 chiasma had the form of an X, whereas the one with 2 or more near its terminals appeared a loop. Two homologues of each bivalent were obscurely distinct. Distinctive morphological characters of the chromosomes could not be recognized due to their extreme contraction at this stage.

The chromosomes rapidly passed through a prometaphase into the metaphase. The nuclear membrane disappeared with the initiation of prometaphase leaving the chromatin material in a nuclear vacuole surrounded by dense cytoplasm. The indistinct spindle with ill-defined fibrils became oriented radially to the vesicular wall. Orientation of 2 bivalents was regular and they

were distinct in all the preparations (Figs. 17, 20). Anaphase was initiated in continuum of the divisional process. The chromatids of the bivalents

Figs. 18-33. Semi-diagrammatic representation of meiotic nuclear division in the germinating chlamydospores of *Protomyces macrosporus* (contd.). 18, diploid nucleus in interphase. 19, diakinesis. 20, metaphase I. 21-22, anaphase I. 23, telophase I. 24, cytokinesis and organization of the 2 nuclei. 25, dyad formation. 26, interphase II in the dyad. 27, metaphase II. 28, metaphase II and anaphase II in the dyad. 29, anaphase II. 30, anaphase II and telophase II. 31, telophase II. 32, reorganization of nuclei and cytokinesis. 33, quartet formation.

separated and the 2 sets moved towards the 2 poles. Anaphasic movement of the chromosomes was erratic and observed infrequently. Late anaphase was observed with the 2 chromosomes at the 2 poles (Figs. 17, 21, 22). Chromosome counts at late anaphase revealed 2 chromosomes and their morphology was hardly displayed at this stage. The chromosomes became

Figs. 34-42. Meiotic nuclear division in the germinating chlamydospore of *Protomyces macrosporus* (contd.). 34, vesicle showing various stages of nuclear division (anaphase I, telophase I, cytokinesis and dyad formation) in the cytoplasmic segments along the periphery. 35, vesicle with peripheral dyads. 36, vesicle showing various stages of nuclear division (metaphase II, anaphase II, telophase II) in dyad cells. 37, vesicle showing various stages of nuclear division (anaphase II, telophase II, cytokinesis and reorganization of nuclei) in dyad cells. 38, formation of quartets along the periphery. 39, separation of cells in the quartets and formation of uninucleate spores. 40, accumulation of spores towards centre of the vesicle. 41, accumulation of spores in a mass and its movement to tip of the vesicle. 42, rupture of the vesicle; expulsion of spore mass and collapse of the empty vesicle.

aggregated into dense chromatin masses at telophase and spindle fibres coalesced into a single long strand (Figs. 23, 24). The strands disappeared and the telophase of a very short duration merged quickly into interphase with faintly stainable nucleoli in each chromatin reticulum with a thin nuclear membrane (Figs. 25, 34). Division of the cytoplasmic portion of the protoplast (cytokinesis) varied. In most of the preparations it followed after telophase (Fig. 24), whereas in few of the preparations, it began immediately with the initiation of telophase I. In this case telophase and cytokinesis appeared as 2 events of the same process. Cytokinesis was accomplished by constriction or furrowing. The result was a 2-celled and 2-nucleate dyad (of 2 uninucleate cytoplasmic units) (Figs. 25, 35).

Prophase II being of very short duration was not observed in any of the preparations. Metaphase II became initiated with the organization of spindles and orientation of chromosomes on the plate of each spindle. The 2 nuclei in each dyad underwent the second division simultaneously (Figs. 27, 36), or not infrequently one of them started dividing earlier than the other (Figs. 26, 36). Orientation of the spindles at the metaphase II was invariably parallel to the vesicular wall as well as to the dense membrane partitioning the 2 units of the dyad. The 2 chromosomes representing the haploid complement of *P. macrosporus* were evident again at this stage. They appeared short, stubby and rod-shaped.

The 2 sets of sister chromatids moved to the 2 poles with elongation of the spindle (Figs. 28, 29, 37). This constituted anaphase II, which being of very short duration quickly merged into telophase II with the aggregation of chromosomes at the 2 poles (Figs. 30, 37). The aggregation at spindle poles in the dyad was asynchronous occasionally (Figs. 30, 31, 37). The few spindle fibrils again coalesced into a single long strand (Figs. 30, 31), chromosomes reorganized in the 2 cells of the dyad and 2 nuclei with a nuclear membrane and a delicate chromatin were formed (Figs. 32, 37). The 4 groups of chromosomes now reorganized as a quartet of nuclei, each of which had the haploid chromosome number ($n=2$). Cytokinesis followed and the 2 daughter nuclei in each cell of the dyad became separated from each other by a new partition, thus forming a quartet of uninucleate units (Figs. 33, 38). Each unit later separated and matured into a uninucleate spore.

The uninucleate spores remained along the periphery of the vesicle for some time (Fig. 39), gradually moved and accumulated into a mass at the centre enveloped within a thin layer of unused cytoplasm (Fig. 40). The spore mass partially matured inside and moved upward touching the vesicular wall (Fig. 41). The vesicle became perforated at the point of contact apparently through enzymatic action and ruptured; the spores were ejected out to some distance, while a few spores lagged behind adhering to the inner surface of the vesicle (Fig. 42). The spores matured finally and became separated from one another. The vesicle shrank and gradually collapsed. The spores

were typically oval to ellipsoid, variable in shape, hyaline, thinwalled, single-celled and uninucleate measuring $2.5-5 \times 1.25-2.5 \mu$.

Discussion

Certain aspects of the cytology of the genus *Protomyces* Unger have for some time remained controversial (Büren 1915, 1922, Dangeard 1906, Popta 1899, Sappin-Trouffy 1897, Valadon *et al.* 1961, 1962, 1962a). This has mainly centred around the site of meiosis in the life cycle of the pathogen. Cytological observations revealed that each chlamydospore of *P. macrosporus* contained 50 or more diploid nuclei and the diploid condition in the life cycle of the fungus was not terminated, until meiosis took place in the vesicle of the germinating chlamydospores. Meiotic nuclear division of those nuclei resulting in quartets of uninucleate units led to the formation of spores, confirming the assumption of earlier workers (Büren 1915, 1922, Valadon *et al.* 1961, 1962, 1962a) that meiosis took place in the vesicle of the germinated chlamydospores. Spore formation took place through separation of each unit of the quartet resulting after the nuclear divisions. The spores were always uninucleate.

Complete emergence of the vesicle, movement of all the nuclei and cytoplasm to the periphery of the vesicle, coalescence of small vacuoles into a large central vacuole and radial septation of the protoplasmic layer appeared prerequisite for initiation of the meiosis normally. Cytological evidence indicated that the sequence in the nuclear divisional stages was regular. The haploid number of the chromosome complement appeared to be 2 ($n=2$). Morphologically, the 2 chromosomes appeared relatively similar.

Placement of Protomycetaceae in the Ascomycetes remained uncertain in the absence of knowledge about initiation of the diploid stage in the life cycle and exact position of meiosis (Bessey 1950, Fitzpatrick 1930). Valadon *et al.* (1961, 1962, 1962a) observed copulation between spores (blastospores), thereby bringing about the diploid stage in the life cycle of *P. inundatus* Dangeard, but were unable to determine the site of meiosis. They placed Protomycetaceae in the Ascomycetes on the assumption that meiosis might have taken place in the chlamydospore during germination. Affinities of the family Protomycetaceae now appear a little clearer with the present findings. Observations on the cytology of the culture spores of *P. macrosporus* revealed copulation between compatible spores and fusion of nuclei resulting in diploid condition. The meiotic nuclear division and the quartet formation of haploid uninucleate units similar to the naked asci in the vesicles of germinated chlamydospores may validate the morphology of the whole vesicle as a synascus. Tendency to develop the endospores in quartets appears reminiscent of a similar development in species of the genus *Basidiobolus* Eidam. (Entomophthorales) supporting the view of monophyletic trend of evolution of Ascomycetes from the Phycomycetes through the Entomophthorales.

Summary

Observations are presented on the cytology of chlamydospore germination and sporogenesis in *Protomyces macrosporus* Unger. A resting chlamydospore contains 50 or more diploid nuclei. The chlamydospore on germination produces a thinwalled vesicle and the nuclei become arranged along the periphery of the vesicle. Meiotic division of these nuclei results in the formation of quartets. Radial septation takes place enclosing a nucleus in each cell, which develops into a uninucleate haploid spore. Two chromosomes appear to be the haploid number ($n=2$) in the complement of the species. Taxonomic aspects of the genus *Protomyces* in the fam. Protomycetaceae are discussed in light of these observations.

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