CYTOLOGICAL STUDIES ON THE SPORANGE DEVELOPMENT AND GAMETOGENESIS IN BREVILEGния DICLINA HARVEY

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INTRODUCTION

Primary interest has been directed to the sexuality of the Saprolegniaceae and for many years there has been considerable discussion as to whether or not fertilization does actually occur.

Pringsheim (1855, 1858, 1860), working with various members of the Saprolegniaceae, while he did not actually observe fertilization, believed that it took place. Zoog (1882, 1883) declared that Pringsheim did not have fertilization, but that what he saw were “spermamoeben” zoospores of a parasitic fungus. Cornu (1872) believed fertilization was present in Achlya polyandra Hild. and Achlya racemosa Hild. He demonstrated that the structures which Pringsheim considered antherids were the sporanges of parasites, probably of a Chytrid.

De Bary (1884) did not share Pringsheim’s views and in his “Comparative Morphology and Biology” stated that while the antherid may approach the oogone and penetrate it and even continue on through and pass out the other side, it does not discharge its protoplastic content.

Humphrey (1892) was the first investigator to fix, imbed, cut, and stain his material. He described the behavior of the nuclei within the oogones of Achlya americana Humphrey and Achlya apiculata de Bary. He stated that the nuclei did not divide, but repeatedly fused in pairs until each egg contained only one nucleus. He agreed with de Bary that the fertilization tube may enter the oogone and approach the egg, but that it does not discharge the male gamete–nucleus.

Hartog (1892, 1895), staining in toto, agreed with Humphrey that the nuclei in the oogone fused until each egg
contained a single nucleus and that there were no mitotic divisions within the oogene. He, too, insisted that fertilization did not occur; antheridia approached the oogones, but there was no discharge of their protoplasmic contents.

Trow (1895) disagreed with Hartog as to the fusion of nuclei and as to whether there was an actual fertilization. He described nuclear divisions in the oogones and antherids of Saprolegnia dioica and observed where the fertilization tube had discharged the male gamete–nucleus into the egg. From 1896 to 1899 there was a bitter controversy between Hartog and Trow as to the behavior of the nuclei and as to the occurrence of fertilization, but they came to no agreement.

Davis (1904) worked with Saprolegnia mixta which he believed to be apogamous. He described one division within the oogonium. The chromosome number was four and there were no centrosomes present. He found in the early formation of the egg a structure which he called a coenocentrum, from which delicate fibrillae radiated. The coenocentra were formed de novo, one for each spore origin. Its function, he believed, was concerned with the “balling” or rounding up of the egg and that it exerted a chemotactic influence over nuclei that were near it. These nuclei were nourished by the coenocentrum as they increased in size. Generally one nucleus persisted in the egg, but occasionally two or three and in such instances the eggs were bi- or tri-nucleate.

Trow (1904) worked on Achiya polyandra Hildebrand and observed a fertilization tube in direct open communication with a young egg. In Achiya debaryana Humphrey the nuclei undergo two divisions within the oogene, the second of which Trow believed to be in the nature of a reduction division. Fertilization occurred in this species.

Claussen (1908) with Saprolegnia monoica (Prings.), Mücke (1900) with Achiya polyandra de Bary and Kasanowsky (1911), with Aphanomyces laevis de Bary have observed fertilization to be present.

Carlson (1925) with Achiya racemosa Hildebrand described the divisions of the nuclei within the oogene. She believed that the central vacuole in the young oogene was the active agent in the cutting out of the egg initials and
not the coenocentrum described by Davis (1905). She observed a body that might be termed a coenocentrum, but observed it only a few times. As complete evidence for fertilization she described the discharge of the male gamete-nucleus into the egg, the migration of the male gamete-nucleus to the female gamete nucleus, and finally the union of the two.

Couch (1925) with Leptolegnia caudata de Bary observed fertilization in this species. The penetration path left in the cytoplasm by the male gamete nucleus is of interest as it has not been previously recorded.

**MATERIALS AND METHODS**

The material was secured from Mr. J. V. Harvey who isolated it from soil gathered in the vicinity of Madison, Wisconsin. Cultures were grown on boiled hemp seed in distilled water. Single sport cultures were made and within three days to a week oogonia became abundant. The material was identified as Brevilegnia declina Harvey. It was fixed in formol-acetic-alcohol. The material was imbedded by the paraffin method and sectioned to a thickness of 5 microns. Haidenhain’s iron–alum haemotoxylin stain with a counter stain of light green gave the best results.

**DEVELOPMENT OF THE SPORANGES**

The mycelium of Brevilegnia declina Harvey is dense and rather opaque, the hyphae are straight and sparingly branched. The sporanges appear within a day in cultures growing on boiled hemp seed, at nearly all the hyphal tips, but also arise from the same point in sympodial groups.

The writer, while cooperating with Mr. Harvey in the examination of material, observed two characteristic types of primary sporanges, one long and slender, with the spores arranged in a single row except for a small cluster of spores at the tip of the sporange, the other ovate to long club-shaped as in Thraustotheca clavata (de Bary) Humphrey. Secondary sporanges appear in dense sympodial clusters terminally or frequently below the primary sporanges. As in Thraustotheca (Weston, 1918), typical sporanges are
broadly clavate and lack papillae of dehiscence. The sporanges of Brevilegnia develop on the dimunition of the food supply, agreeing in this respect with the general law established by Klebs (1899).

Sporange formation begins with the gradual streaming of the protoplasm to the hyphal tip which, as a result, begins to swell slowly. In time the accumulation of protoplasm fills the sporange initial and a cross wall is then formed across the base of the sporange. The contents now begin to differentiate into spores. In the sporange having the spores arranged in a single row in section view, there appears at the tip of the sporange a vacuole which by enlargement and furrowing cuts out the spore initials (fig. 2). The formation of the spore initials in the remaining part of the sporange is caused probably by a furrowing of vacuolar and plasma membranes.

In longitudinal sections of the larger club-shaped type of sporange, the vacuole first appears near the tip and continues on down through the center of the sporange (fig. 5). Here, as in the other type of sporange, the enlargement and furrowing of the vacuole cuts out the polygonal spore initials. In both types of sporanges the spore initials are at first distinct and then for a space become slightly indistinct, due probably to the readjusting of the spore initials and the movement of the liquid in the vacuole. The spore initials, now granular in appearance, form a wall about themselves and become more and more distinct. Weston, in Thraustotheca, states that the sporangiospores imbibes water and swell, but in Brevilegnia, on the contrary, the spores throw off water and the cytoplasm appears to contract. This is now evidently an encysted state. The completed spores occupy the same position as did the spore initials.

In this form, as in Thraustotheca, it is to be noted that there is no evidence of an intersporal substance, a condition in agreement with the observations of Rothert (1890) and Humphrey (1893) for other Saprolegniaceae.

It is interesting to note that in the formation of the spore initials the vacuole does not cut out uninucleate masses of protoplasm as has been observed in all other of the Saprolegniaceae (fig. 2). In section view the sporangiospores
(aplanospores) show a multinucleate condition. The nuclei are quite distinct, each with a definite nuclear membrane and a conspicuous irregularly shaped nucleole. A fine granular network appears to run from the nucleole to chromatin material at the periphery of the nucleus. The nuclei vary slightly in size; the smaller ones, however, show no indications of degeneration. Weston uses the term sporangiospore to indicate a non-motile spore, thereby distinguishing it from the term zoospore. Since the sporangiospores are monoplanetic the term sporangiospore is not quite applicable in this case. In Brevilegnum the writer suggests the term aplanospore as the spores are non-motile at all times.

The method of the liberation of the spores agrees partially with that of Thraustotheca which Weston believes differs from that of any other genus in the Saprolegniaceae. As has been mentioned, the papillae of dehiscence which are present in most members of the family are lacking in Brevilegnum, and the aplanospores escape by the disintegration of the sporangae walls. In a cross section of the larger club-shaped type of sporangae, the spores are arranged about the central vacuole. There is no rupture of the wall by the swollen sporangiospore as in Thraustotheca, but rather a disintegration of the wall here and there due in all probability to either chemical or enzyme action (fig. 6). The aplanospores are shed either singly or more commonly in small groups of two or three, often with portions of the sporangae wall adhering to them. Upon leaving the sporangae they round up, clinging together. This is in accordance with Weston, who observes a distinct adhesion and mutual attraction among the spores. Aplanospores remain sticking to any hyphae with which they may come in contact. They now have definite membranes or walls with the protoplasm slightly contracted (fig. 7). The cytoplasm is finely granular with small vacuoles, nuclei many, 7–15 having observed in some instances.

The aplanospores float about in the water, showing absolutely no signs of motility. Germinating spores or zoospores were not observed. Harvey believes that the aplanospores undergo a period of rest before germination, omitting the zoospore stage, and produce a germ tube di-
rectly. Couch, however, succeeded in producing zoospores from aplanospores by placing some of them in distilled water to a pH of about 4, or filtering them through animal charcoal. Gemmae were not observed by Harvey or the writer.

DEVELOPMENT OF THE OOGONES

The oogones arise singly and apically from hyphae on short lateral branches of the mycelium. In the formation of the oogone there is a streaming of the protoplasm into the hyphal tip, forming in it a marked swelling. The streaming continues until the swelling has caused the hyphal tip to become almost spherical. A cross wall is now laid down at the base of this swelling and the oogone is thus formed (figs. 8–10). The cytoplasm at this stage is multinucleate; conspicuous vacuoles are present surrounded by cytoplasm containing granules (mitochondria) of varying size and shape. A conspicuous, dark-staining body resembling a nucleolus is observed in each of the nuclei. This body seems to be identical with that described by Hartog as a chromatin body, by Trow as a combination of a chromosome and a nucleolus, by Davis, Clausen, Mücke, and Carlson as a nucleolus.

A vacuole remains in the center of the oogone after the formation of the cross wall at the base. Many of the nuclei migrate to the periphery of the oogone, where they very soon show indications of degeneration. The remaining nuclei appear to enlarge slightly and to stand out clearly.

These nuclei appear to undergo one mitotic division only. That there is only one division occurring within the oogone (figs. 8, 9) agrees with the results found by Davis, Clausen, Mücke, Kasanowsky, and Carlson in regard to closely related species. Trow (1904) describes the occurrence of two divisions in the oogone, the second of which he believes to be a reduction division. Contrary to other workers who state that the nuclei divide simultaneously, the writer observed nuclei in resting stage and in various stages of division within a single oogone (figs. 8, 9). The chromatin-linin network becomes very massed in the prophase and with the nucleole still quite prominent it is very difficult to make accurate determinations of chromosome behavior.
The metaphase has been observed frequently (figs. 8-10). The chromosomes are massed together at the equator of an intranuclear spindle. Few spindle fibers are present. Asters with only the suggestion of rays could be seen (fig. 9). In the anaphase the chromosome masses are to be noted. The chromosomes can be seen at this stage but they are so crowded it is impossible to make accurate counts. The writer believes the number to be comparatively few (4-8) and in no instance as many as 14-16, mentioned by Clausen. The telophase has been observed in a few instances and only a massing of cytoplasm at the poles could be made out.

After the nuclear divisions, the oogene evidently throws off water because there is a marked contraction of the cytoplasm from the wall. The vacuole in the center disappears and the cytoplasm becomes uniform throughout. This aggregated mass of cytoplasm becomes now the single egg which is constant in this species.

**FORMATION OF THE EGGS**

Davis, working with an oogene containing several eggs, found a body which he terms a coenocentrum and which he thinks is instrumental in the rounding up of the eggs. The cytoplasm in the oogene remains adherent to the wall and the central vacuole, by enlarging and furrowing, cuts out masses of cytoplasm, each with its respective nucleus. Beside each nucleus a body appears, the coenocentrum, which he thinks aids in the rounding up or “balling” of the cytoplasm. This body acts as a dynamic center which draws the cytoplasm to it and rounds it up into a spheroid mass. Miss Carlson and Clausen observed a body which they said might be termed a coenocentrum but observed it only a few times. In Brevilegnesia, on the contrary, with the contraction of the cytoplasm and the disappearance of the central vacuole, there is no indication of a coenocentrum (fig. 12). The loss of water from the oogene causes the rounding up of the cytoplasm.

The nuclei that have recently undergone division are fairly evenly distributed throughout the cytoplasm, but there is a tendency for them to arrange themselves at the
periphery of the egg (fig. 12). All the nuclei except one shortly begin to show signs of degeneration. The remaining nucleus enlarges slightly and is now the female gamete-nucleus. The nucleus becomes slightly flattened and the chromatin-linin network appears to radiate from the nucleol to the nuclear membrane where the chromatin appears to be more aggregated. The vacuoles in the egg are fairly uniform in shape, those near the center being somewhat larger than those at the periphery. The degenerated nuclei persist until after fertilization. The mature egg is found generally at one side of the oogone just prior to fertilization.

**Behavior of the Antherids**

During the formation of the egg in the oogone, antherids have been approaching the oogone from the same hyphae or from neighboring hyphae. Antherids have been observed to attach themselves to the oogones after the formation of the egg, but more frequently they are present at the stage of egg formation. The development of the male gametes within the antherid was not observed in Brevileg-nia, but on the basis of the observation of other workers it is thought comparable with the changes taking place within the oogone during the formation of the egg. The antherid upon coming in contact with the oogone may indent the oogone. The rupture of the wall and discharge of its contents into the oogone was not observed. The presence of a fertilization tube has not been observed in this form. The antherid which adheres to the wall of the oogone at the spot where the egg touches the wall appears to be the functional antherid. There is evidently a dissolution of the wall of the oogone at the point of contact of the antherid and the egg. Apparently one of the gamete nuclei migrates into the egg. Several nuclei are often present within the antherid and the terminal nucleus appears slightly larger than the others and is probably the nucleus that functions as the male gamete-nucleus (fig. 15). Upon the discharge of the male gamete-nucleus into the egg, the other nuclei in the antherid show signs of degeneration.

After the formation of the egg, the wall of the oogone is
quite irregular with several papillae. Shortly before fertilization the oogone wall stretches slightly and the papillae disappear. Previous workers have observed that the wall of the oogone in a number of species varies in thickness. The oogone wall, in section view, appears to be made up of many circular thickened plates at the intersection of which are thin-walled regions. In section view, this gave the appearance of thickened areas separated by thin membranes. It has been thought for a long time that the antherid paused at a thin area or that the fertilization tube penetrated the thinner areas only, due probably to some chemotactic stimulus. Couch, in his studies with the water molds, has observed that the fertilization tubes penetrated the thickened areas as well as the thinner ones, and so believes that there is no ground for the assumption that the fertilization tubes penetrate the thinner areas only.

Fertilization

After the male gamete nucleus has been discharged into the egg it migrates toward the female gamete nucleus. The male gamete nucleus can be readily recognized within the egg (fig. 17). It is almost spherical and slightly smaller than the female gamete nucleus. A nucleolus can be seen within each of the nuclei. It was not uncommon to find the female gamete nucleus at a distance from the center of the egg. In many preparations, while it may not be characteristic for the species, the writer has observed that the male gamete nucleus was invariably discharged into the egg (fig. 17) at the side farthest away from the female gamete nucleus.

Several stages have been observed of the male gamete nucleus on its way to the female gamete nucleus. The male nucleus leaves in its wake a path of dense cytoplasm, which Couch calls the "penetration path" (figs. 17, 18, 19). Couch describes such a path in _Leptolegnia caudata_ de Bary. The male gamete nucleus comes to lie next to the female gamete nucleus, one slightly overlapping the other. Upon the union of the gamete nuclei the egg becomes a zygote. A wall now appears about the zygote. The cytoplasm becomes quite vacuolate. Several large vacuoles are found
in the vicinity of the zygote nucleus, with many smaller ones at the periphery. Shortly before fertilization a small opaque body appears in the egg and after fertilization it enlarges rapidly (figs. 23, 24). Occasionally there may be more than one. These bodies are easily recognized as oil bodies because of their definite outline and because of their staining reaction. Faint fibrillar lines are present over their surfaces. An oil body may enlarge until in some instances it is about two-thirds the size of the zygote.

The zygote evidently undergoes a period of rest before germination. Germinating zygotes were not observed. In several zygotes a multinucleate condition has been observed with resting nuclei (fig. 25), but a most careful study of a very large number of preparations has given no evidence as to when such division begins. No one has as yet given a complete story of the processes which intervene between fertilization and the germination of the zygote in any of the Saprolegniaceae.

**Summary**

In Brevilegnia the development of the sporanges agrees with other members of the family.

The sporangiospores are multinucleate, a fact which has been observed in only one other genus of the Saprolegniaceae.

The method of spore liberation is by the dissolution of the sporangial wall and not by the rupture of the wall, by the contraction and rupture of the wall by the enlarging zoospores, nor by the assistance of an intersporal substance as has been noted by earlier observers.

The sporangiospores are non-ciliate at all times. They float about for a time, settle into substrate and germinate directly into hyphae.

Prior to the liberation of the sporangiospores from the sporangium the spores appear to encyst as there is a marked contraction of the cytoplasm from the wall of the spore.

A single division of the nuclei takes place in the young oogone which is in agreement with the results of recent workers in other members of the family.
The oogone contains a single egg only, which is found generally at one side of the oogone.

The antherids approach the oogone during the formation of the egg. After attaching themselves to the oogone, the antherid nearest the egg is the functional antherid. The antherid possesses no fertilization tube. The separating walls dissolve and the male gamete nucleus passes directly from the antherid into the egg and migrates to the female gamete nucleus leaving in its wake a penetration path.

The gamete nuclei fuse and the egg becomes the zygote.

No nuclear divisions were observed in the zygotes, but several zygotes were observed in a multinucleate stage.

The writer wishes to express his appreciation to Prof. E. M. Gilbert for his advice and encouragement during this investigation.

**EXPLANATION OF PLATES**

All drawings were made with the aid of a camera lucida at table level with a Leitz No. 4 ocular and a 1/16 oil immersion, making a magnification of 1750.

**PLATE 3**

**FIG. 1.** Tip of young multinucleate sporangium with the first appearance of the central vacuole.

**FIG. 2.** Sporangium with the spores in a row. Aplanospores being cut out by the central vacuole and by the furrowing from the side of the sporangium. Aplanospore initials present.

**FIGS. 3, 4.** Cross section of the club-shaped type of sporangium with the aplanospores arranged about the central vacuole. Cytoplasm has contracted slightly, evidently an encysted condition.

**FIG. 5.** Longitudinal section of the club-shaped type of sporangium showing the position of the central vacuole.

**FIG. 6.** Aplanospores of the sporangium bearing the spores in a single row, showing the irregular manner of the disintegration of the sporangium wall; the spores remaining clustered together.

**FIG. 7.** Club-shaped type of sporangium at maturity, anterior portion of sporangium disintegrated and the aplanospores about to be liberated.

**PLATE 4**

**FIGS. 8, 9.** Longitudinal sections of an oogone prior to its assuming typical spherical shape. Nuclei in various stages of division; some nuclei in the resting stage.
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FIGS. 10, 11. Longitudinal sections of young oogones which have rounded up with the nuclei in various stages of division.

FIG. 12. Multinucleate egg after the division of the nuclei. The cytoplasm contracted, the vacuole has disappeared.

FIGS. 13, 14. Uninucleate eggs with the supernumerary nuclei disintegrating.

FIG. 15. Antherids from adjacent hyphae attached to oogone.

FIG. 16. Antherid from same hypha in contact with oogone wall opposite egg, showing position assumed at time of fertilization.

PLATE 5

FIG. 17. Egg with the male gamete nucleus approaching the female gamete nucleus, leaving in its wake a penetration path.

FIG. 18. Egg with the gamete nuclei lying in contact. The penetration path is still in evidence. Antherid attached to oogone wall.

FIG. 19. Egg with gamete nuclei about to fuse. Penetration path still present.

FIGS. 20, 21, 22. Zygotes with the gamete nuclei about to fuse.

FIG. 23. An abnormal zygote in which the gamete nuclei have not fused. The presence of the oil bodies indicates that some time has elapsed since the male gamete nucleus entered the egg.

FIG. 24. Fully matured zygote, with fusion nucleus and well developed oil bodies.

FIG. 25. Zygote in a multinucleate stage prior to germination. Large oil body present.

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