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Cytology of Glomerella.

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CYTOLOGY OF GLOMERELLA

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Botany,
Bacteriology, and Plant Pathology

by

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B.S., Pennsylvania State College, 1940
M.S., Louisiana State University, 1942
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ABSTRACT

The Ascomycete Glomerella is a genus of fungi which includes parasitic forms involved in diseases of higher plants, including cotton, apple, pepper, Ipomoea and many others. This dissertation reports an investigation of the cytology of Glomerella cingulata (Stoneman) Sp. & v. Shr., the chromosomal condition of the fungus and the sequence of events in ascus and ascospore formation. Such information seemed vital in relation to the solution of problems of inheritance, since the fungus appears to include both homothallic and heterothallic strains.

Perithecia formed by mating various strains were studied as well as perithecia formed by various strains when grown alone. Both smears and microtome sections were made of the perithecia. These were stained with aceto-carmine, aceto-crocin, the Feulgen stain, iron alum haematoxylin and crystal violet. The crystal violet stain gave the best results and was used the most. The perithecia formed by mating various strains could not be distinguished from perithecia formed by various strains when grown alone.

Asci in perithecia of all strains, except one, were observed in all stages of development. No crozier formation was observed. Apparently the asci arise directly from the ascogenous hyphae in the base of the perithecium. Each young ascus contains two haploid nuclei. These unite to form a large fusion nucleus which contains a conspicuous fusion nucleolus. This nucleolus disappears before metaphase I. No nucleolus is again evident until after the third
ascus division occurs, at which time a small nucleolus appears in the nucleus of each ascospore. The three divisions in the ascus follow each other rapidly and the divisions apparently are anastral. No central body, centrosome or centriole was observed. It was not determined how the ascospores were delimited. The maturation divisions in the ascus appear to be intranuclear; division I occurs in the longitudinal axis of the ascus. Division II is either longitudinal or oblique, while the third division is either transverse or oblique.

Indications are that the haploid number of chromosomes in Glomerella is four. The chromosomes at division I are much longer than at the third division. The longest chromosome is the one to which the nucleolus is attached. At prophase I this chromosome is approximately 4 microns in length, at telophase of the third division it appears to be no more than 0.6 microns in length. So far as is known this is the first time such data have been presented on the cytology of Glomerella.

Mature ascospores and conidia when subjected to various stains were seen to be uninucleate. Perithecia of one strain were malformed and few were of mature size. The few asci in these perithecia were small and abnormal. The nuclear condition of these asci was not determined. These data are in agreement with the results of similar investigations carried out by others.
INTRODUCTION

Investigations in recent years have shown that certain fungi are ideally suited in many respects for studies of inheritance, chromosome behavior and the mechanics of gene action. The studies on Neurospora and Venturia inaequalis (Cooke) Winter are but two examples of fungi furnishing valuable basic contributions in this respect. These two fungi and many others spend much of their life cycle in the haplophase or monoaecyotic condition and therefore offer a splendid opportunity for studying the action of one set of genes in an organism without the masking effect of a complementary genome.

Glomerella cingulata (Stoneman) Sp. & v. Shr. is a fungus peculiar in that certain strains, arising from single uni-nucleate haploid ascospores, can complete the life cycle, including the perfect stage, without mating with another strain, i.e. certain strains of Glomerella are homothallic. Other strains arising from single uni-haploid spores cannot complete the life cycle including the perfect stage unless mated with another strain, i.e. certain strains of Glomerella are heterothallic. Some strains of Glomerella produce perithecia; in others these structures have never been observed. Some strains of Glomerella produce perithecia with numerous ascospores, while other strains produce perithecia with few or no ascospores. These facts indicate that in this fungus sex is not a simple, easily explained phenomenon, but may require a highly complex interaction or group of interactions for the perfect stage to be initiated and completed.
Data interpreted as evidence of fertilization between different strains of *Glomerella* have been obtained. It would thus appear that Mendelian principles apply to inheritance in this fungus just as in higher plants. However, it was thought that a knowledge of the chromosomal condition and the sequence of events in the formation of the perithecia, asci and ascospores might prove helpful in explaining the laws which govern inheritance in *Glomerella*. Realizing the potential importance of this information, this study on the cytology of *Glomerella* was undertaken with the hope that some facts of value might be unearthed.
HISTORICAL REVIEW

Anton de Bary (12), in 1863, was the first to suggest that perithecial formation in the Ascomycetes was associated with the sexual processes of these fungi. He also was the first to report the occurrence of nuclei in the asci of Pyronema confluens (Pers.) Tulasne. He described the condition as follows:

"the young asus is filled with finely granular protoplasm containing vacuoles; a nucleus may be seen in the center of the protoplasm in the form of a clear spherical body, the center of which is another smaller and strongly refringent body. It has yet to be learnt whether the whole body should be called the nucleus and the inner and smaller body the nucleolus, or whether the latter alone is the true cell-nucleus."

However, it was not until 1894 that Dangeard (11) first observed nuclear fusions in the ascus of Peziza vesiculosa Bull. ex Fr. The following year Harper (25) published on the fusion of nuclei in the oogonium of Sphaerotheca humuli (DC.) Burill as well as in the asus itself. Harper and Gwynne-Vaughn (22) representing the Anglo-Saxon school, postulated that in the asus a "double reduction" division took place and thus each ascospore received a haploid set of chromosomes, even though the fusion nucleus in the asus was tetraploid. Clausen (6) denied that a fusion of nuclei took place in the oogonium. He believed that fusion occurred only in the asus. Since then the question has been much discussed. Various investigators have contributed evidence to favor the one viewpoint or the other. However, the investigations of Dodge (14), Colson (9), Lindgren (26) and Wilcox (40)
have indicated that in Neurospora there is only one fusion and one reductional division in the life cycle.

In the investigations of the powdery mildews (23), Neurospora (9), and certain yeasts (30) conspicuous centrosomes and astral rays have been figured and described. Harper (23) called the centrosome a central body and described for Erysiphe "a peculiar and characteristic attachment of the chromatin of the nucleus to the central body, giving the nucleus a characteristic polar rather than radial structure." Lindegren (30) also described such a central body (centriole) in Saccharomyces cerevisiae Hansen. In the powdery mildews and Neurospora the astral rays surrounding the centriole aid in delimiting the spores. In his description of the division in the ascus of Phyllactinia, Harper showed that after the first division in the ascus the resulting nuclei each formed a nuclear membrane, the nucleolus in each reappeared, and the "chromosomes in passing over into the so-called resting stage in the reconstitution of the daughter nuclei do not lose their connection with the central body". He also stated that the daughter nuclei never reached the size of the primary (fusion) nucleus and as a rule they divided again immediately, though in some cases apparently a considerable period might intervene.

Colson (9) stated that in Neurospora tetrasperma Dodge the divisions in the ascus were intranuclear. In her illustrations she showed conspicuous nucleoli which did not appear to be attached to any chromosome. These nucleoli appeared to persist through the ascus divisions. How the nucleoli divided she did not state. Fraser and Welsford (20) showed similar figures for Otidea aurantia Pers. ex
S. F. Gray and Peziza vesiculosa Bull. ex Fr. and Bagooee (1) for Pustularia bolaricoides Ramsb. Brooks (4) stated that in Gnomonia erythrostoma (Pers.) Auerwald "in the reconstruction of the two daughter-nuclei a distinct nucleolus was formed. Sax (35) in her study of Philospora coerulescens Rehm stated that the nuclei apparently entered a resting stage at the close of each successive division. She also reported that the ascus divisions were intranuclear and that the nucleus contained a nucleolus and central body. Backus and Keitt (2) stated that the primary nucleus in the ascus of Venturia inaequalis (Cooke) Winter had a diameter equal to more than three-fourths that of the ascus and contained a large nucleolus. They reported that the three ascus divisions were intranuclear and that a dark staining granule appeared at each end of the spindle. These granules they thought were probably centrosomes. They reported in the telophase of division I the presence of a granule which was located adjacent to the spindle but in the cytoplasm. They thought this granule to be the remains of the nucleolus. Their figures also indicated that at the end of the first division the nuclei were reconstituted and formed definite nuclear membranes. In the second maturation division the remnants of the nucleoli again appeared adjacent to the spindle in the cytoplasm. In the third division of the ascus the same situation occurred. The eight free nuclei resulting from the third division each contained a chromatin network and nucleolus. They did not determine how the spores were delimited, although they reported that the presence of beaked nuclei and astral radiations was suggested in a few asci.
McClintock (33), in her study of the chromosomes of Neurospora, gave a somewhat divergent account of the behavior of the nucleolus. She stated that at metaphase I the nucleolus became smaller but did not disappear. At anaphase I the chromosomes migrated toward the poles but the nucleolus might be dragged toward one pole or stretched between the poles because the nucleolus organizers of one or both dyad chromatids had not been released from their attachment to the nucleolus. The nucleolus became detached before telophase I began and might be seen subsequently in the cytoplasm of the ascus. No true resting nucleus was formed before division II set in. However, a new nucleolus was produced by and remained attached to the nucleolus organizer of chromosome 2. At telophase II in each nucleus a new nucleolus was again formed on chromosome 2.

In the Ascomycetes the position of the daughter nuclei and orientation of the spindles in the three divisions furnishes, when taken in connection with the results of culture studies, a seemingly reliable basis for determining the time at which segregation of sex factors takes place. Wilcox (40) gave a summary of spindle orientation in the asci of 30 different Ascomycetes. She found that in those fungi which had long asci the spindles of the first division were most frequently longitudinal. The spindles of the second division were also longitudinal but less constantly so; while the spindles of the third division were commonly transversely placed. Several exceptions occurred. The spindles of division I of Neurospora sitophila (Mont.) Shear & Dodge were longitudinal, those of division II were longitudinal while those of the third division were transverse. In Venturia inaequalis (Cooke) Winter
the first division spindle was longitudinal while the second and third division spindles might be longitudinal, oblique or nearly transverse. Mature ascospores were uniseriate in the ascos.

Up to the present time little work has been done on the cytology of *Glomerella*. Several investigators including Clinton (7), Edgerton (17), Stoneman (39) and Shear and Wood (38), have described mature asci, ascospores and perithecia of *Glomerella*, but so far as is known no detailed study of ascus formation and development has been made.

Edgerton (17), in describing mature perithecia of *Glomerella* isolated from apple, stated as follows:

"The perithecial wall was composed of three to four layers of narrow, long, black thicewalled cells. Sometimes two perithecia fused, so that there was one internal cavity with two ostiules. At the base of the perithecia there were generally large dark-colored hyphae made up of nearly globose cells 5 - 8 μ in diameter... The perithecial wall gradually blends into the pseudoparenchymatous tissue."

Hüttig (24), working with *Glomerella lycopersici* Krüger, illustrated the initiation of perithecia by antheridia and oogonia. His figures were not convincing.

A review of the literature indicates that considerable work has been done on the cytology of the ascus in a number of the Ascomycetes. A conspicuous nucleolus has been reported as occurring in the large fusion nucleus of the ascus of many genera. However, the fate of this nucleolus in the resulting divisions has not been clearly determined. In the powdery mildews and *Neurospora* the ascus divisions are of the astral type and the astral rays assist in delimiting the spores. A conspicuous "central body" has been reported as occurring in the powdery mildews and *Saccharomyces cerevisiae* Hansen. The chromosomes
in these fungi appeared to be attached to this central body. The ascus
divisions of many Ascomycetes are intranuclear with spindles clearly
evident. The chromosomes of the Ascomycetes are much smaller than
those of higher plants. Experimental evidence indicates that in some
Ascomycetes at least, there is but one nuclear fusion and that this
occurs in the ascus.
MATERIALS AND METHODS

The cultures of Glomerella used in these studies were the progeny of an original isolate made from a dead stem of Ipomoea as described by Lucas (31). Four different strains were used. These were designated as 5-16-1 (plus B), 17-F-5 (minus B), 4-17-1-1 (minus A) and 36-D-3 (conidial B).

Culture 5-16-1 (plus B) when grown on oatmeal agar produces perithecia in scattered glomerulate masses. The perithecia form well developed asci and ascospores in abundance. However, the asci when analyzed by culturing individual ascospores, ordinarily produce cultures of the plus type (plus B) and apparently none of the minus type. This is in contrast to cultures of the plus A type whose asci when analyzed by culturing individual ascospores ordinarily produce four cultures of the plus type (plus A) and four of the minus type (minus A), or else eight of the minus (minus A). The plus B strain (5-16-1) is macroscopically indistinguishable from the plus A strain.

Culture 4-17-1-1 (minus A) produces numerous perithecia, singly or in groups of two or three scattered over the surface of the agar. The perithecia ordinarily are sterile, only an occasional one producing a few asci. The ascospores on germination produce only minus A colonies. A few conidia are produced by culture 4-17-1-1.

Culture 17-F-5 (minus B) is also called the fertile minus type. It produces numerous perithecia singly or in groups of two or three...
scattered over the surface of the agar. Most of the perithecia are filled with well-developed asci and ascospores. These ascospores when isolated produce colonies of the minus B strain.

Culture 36-D-3 (conidial B) produces no perithecia. It does produce conidia, but the conidial masses are not so numerous as the conidial masses formed by the conidial A strain. The conidial A strain was not used in these studies.

Studies were made first of perithecia formed by mating the conidial B strain, 36-D-3, with the plus B strain, 5-16-1, in a petri dish (Plate I, Fig. 1) on oatmeal agar and incubating at room temperature as described by Chilton et al. (6). This was done for two reasons. First, a heavy line of perithecia was formed at the contact line between the two strains and second, studies on cultures arising from ascospores isolated from 65 different asci from these perithecia supplied evidence that a cross had occurred. Seven to 14 days after inoculating the plates, perithecia were abundant at the fusion line between the two colonies. In an effort to obtain different stages of the maturation divisions in the asci the petri dishes in which the two strains were mated were subjected to temperature changes as follows. Twelve to 24 hours before the perithecia were to be killed and fixed the petri dishes were placed in a refrigerator kept at approximately 10° C. At the end of this time the petri dishes were removed from the refrigerator and the blocks of agar about one-fourth inch in length, containing the perithecia, were excised and dropped into the killing fluid at one minute intervals up to 10 minutes after removal. The killing fluid used was weak Flemming's solution as recommended by Chamberlain. After
killing and fixing, the perithecia were washed in water, run up through
an ethyl alcohol series, imbedded in paraffin and cut with a microtome
into sections six microns thick.

In another series of experiments the petri dishes were left in
the refrigerator overnight. Upon removal the perithecia were excised
and dropped into a warm aqueous solution (0.1%) of colchicine for
30 minutes. They were then placed in the killing fluid and imbedded
as the others.

In the studies of the minus A strain (4-17-1-1), minus B strain
(17-F-5) and plus B strain (5-18-1), each strain was inoculated alone
into a petri dish on oatmeal agar. At the end of five to ten days,
when perithecia were visible to the naked eye, agar blocks containing
perithecia were killed, imbedded and sectioned with the microtome as
described above.

Use of Aceto-Carmine

Aceto-carmine stain was made by dropping one gram of carmine
dye (alum lake) into 100 cc. of 45% acetic acid in a reflux condenser.
The suspension was allowed to boil gently for one hour. It was then
cooled and filtered through coarse filter paper. The volume was brought
back to 100 cc. by the addition of 50% acetic acid. Three to four
drops of ferric acetate were then added. This stock solution was
kept in a refrigerator (10° C.) and used as needed.

Smears were made of perithecia in all stages of development of
the plus B strain, the minus A strain, the minus B strain and perithecia
formed at the fusion line of the plus B strain and conidial B strain.
A small square of agar containing the perithecia was teased apart on a
glass slide. The perithecia were crushed to cause the asci to emerge. A drop of aceto-carmine was placed on the material and the slide was then warmed gently for 30 to 60 seconds. A cover slip was placed on the mount and the excess stain was replaced by water introduced under the cover slip. The preparation was then examined under the microscope. Smears were also made of conidia of the conidial B strain and stained.

Use of Aceto-Cresin

Aceto-cresin stain was prepared according to the procedure given by LaCour (25). Smears were made of perithecia and conidia of the various strains as described above.

Use of the Feulgen Stain

One-half gram of basic fuchsin was dissolved by pouring over it 100 cc. of boiling distilled water. It was shaken thoroughly, cooled to 50° C. and filtered. To the filtrate was added one gram of potassium metabisulphate and 10 cc. of normal HCl. This was allowed to bleach for 24 hours. One-half gram of Norit (a descolorizing carbon manufactured by the American Norit Co.) was added, the solution shaken thoroughly and filtered through coarse filter paper. This colorless stock solution was then stoppered in a brown bottle and stored in the dark to be used as needed.

Smear preparations using the Feulgen stain were treated as follows: The agar blocks containing the perithecia were killed in normal HCl for 30 minutes. They were then hydrolyzed at 60° C. in normal HCl for 2, 5, 8, 10, 12, 15, 20, 25 or 30 minutes. After hydrolysis they were cooled for two to three minutes in cold normal HCl
and washed in distilled water. They were placed in the fuchsin stain and allowed to remain from 30 minutes to six hours. The perithecia were then placed on a glass slide in 45% acetic acid and crushed to force out the asci. A cover slip was placed on the preparation and the slide examined under the microscope.

The microtome sections were treated according to the procedure given by De Tomasi (13) for the Feulgen stain.

Use of Crystal Violet

The crystal violet procedure used was the one recommended by Sass (34, p. 76). A one percent aqueous solution of crystal violet was used to stain the sections. They were destained with picric acid and clove oil. This stain gave the best results and was used the most. Perithecia of all the strains were stained.

Use of Miscellaneous Stains

Sections of perithecia formed by mating the plus B strain and conidial B strain were stained with iron-alum haematoxylin. Smears of perithecia of all strains and conidia of the conidial B strain were mounted in aqueous methylene blue and aniline blue in lactophenol and observed.
EXPERIMENTAL RESULTS

Results with Aceto-Carmine

Mature ascospores of all strains stained with aceto-carmine were seen to be uninucleate. The nucleus was located in the approximate center of the allantoid spore. The nuclear area stained pink with the aceto-carmine while the cytoplasm stained lightly or not at all. It was not determined whether the entire nucleus was stained or just the nucleolus. Occasionally very old ascospores were seen to be two celled with one nucleus per cell. Mature conidia were also uninucleate. The nucleus occupied the center of the spore. Here again it was not determined whether the nucleolus alone was stained or the entire nucleus. No chromosomes were seen in the aceto-carmine preparations.

Results with Aceto-orcein

Mature ascospores and conidia stained with aceto-orcein gave the same appearance as those stained with aceto-carmine. The spores were uninucleate, with the nuclear area visible as a pink sphere.

Young asci were observed in which the fusion nucleus was defined as an almost hyaline, oval area. The cytoplasm of the ascus stained pink. Within the hyaline area distinct threads, interpreted as chromosomes, were visible. Three asci were seen in this condition. Several asci (16 to 20) were seen with the hyaline area, but no threads were visible. One ascus was seen in which there were two hyaline oval areas but in which no chromosomes were visible. Mature asci with
eight well-defined spores were observed. Even before the spores were discharged from the ascus the pink nuclear area of each spore was visible.

Results with the Feulgen Stain

Ascospores from perithecia of the minus B strain and perithecia formed by mating the conidial B and plus B strain extruded from ripe asci and stained by the Feulgen method were seen to be uninnucleate. They appeared similar to spores stained with aceto-carmine or aceto-cresin. However, in general the results obtained with the use of this stain were disappointing. In neither the smears nor the sections were chromosomes seen in any ascus of any age. Even though the perithecia were hydrolyzed at 60° C. in normal HCl (a supposedly critical step in the procedure) from 2½ to 30 minutes no good results were obtained. The perithecia were also allowed to remain in the fuchsin stain from 30 minutes to 6 hours, but no satisfactory staining reaction was obtained.

Use of Crystal Violet

The microtome sections of perithecia formed by mating the conidial B strain with the plus B strain and stained with crystal violet gave excellent results. However, placing the petri plates in the refrigerator overnight and killing the perithecia at one minute intervals after removal gave no better figures than untreated perithecia. Perithecia dropped into colchicine also gave no better results than untreated perithecia.

Upon examination the perithecia formed by crossing the conidial B strain with the glomerate B strain, the perithecia formed by the
glomerate B strain grown alone and the minus B strain grown alone were seen to be strikingly similar. In the sections examined perithecia were seen in all stages of development and asci within the perithecia were seen in all stages of development. Some perithecia contained asci at all stages of development from ascogenous hyphae to asci with eight mature ascospores. Since the figures seen in all perithecia, whether arising from a cross of the conidial B x glomerate B, the glomerate B grown alone or the minus B grown alone, were so similar the sequence of events in ascus and ascospore formation reported in the following description will apply to perithecia formed in any of the three ways mentioned above. The perithecia formed by the minus A strain grown alone will be considered separately.

In a large peritheciuim, before the asci are formed, the interior of the peritheciuim is occupied by slender vegetative hyphae called pseudoparenchymatous hyphae by Edgerton (17) which appear to arise from the base of the inside perithecial wall (Plate I, Fig. 2). These hyphae have lenticular nuclei and seem to be multicellular. As the asci mature they push their way up through these pseudoparenchymatous hyphae and gradually replace them. In a peritheciuim containing many asci there are few or no pseudoparenchymatous hyphae. The function of these vegetative hyphae could not be determined, nor the manner (lysis, digestion, etc.) in which they were displaced by the asci.

The asci arise from a fertile region (Plate I, Fig. 3) at the base of the peritheciuim. The branching of the hyphae of the fertile region becomes so complicated that it is impossible to follow the course of any one hypha. No crozier formation was seen however, and it
cannot be stated definitely that all the hyphae in the fertile region are ascogenous hyphae. In any event from this mass of fertile hyphae young asci are produced, each containing two supposedly haploid nuclei (Plate II, Fig. 1). The most conspicuous feature of these nuclei is the large, round deeply staining nucleolus. The nucleolus is surrounded by a clear hyaline area. As the two nuclei prepare to fuse chromatin material becomes visible and the nucleolus of each increases in size. Soon the spiremes become evident; the chromosome to which the nucleolus is attached is quite conspicuous. At this prophase stage the two nuclei come to lie side by side with the nuclear membranes in contact (Plate II, Fig. 3) and with the chromosomes of each nucleus intermingled. Sometimes the two nucleoli can be seen within the same nuclear area before they fuse (Plate II, Fig. 2). Eventually the two nucleoli join to form a large "fusion nucleolus” which is approximately twice the diameter of a haploid nucleolus. Measurements of 50 of these "fusion nucleoli” showed them to range from 1.4 to 1.6 microns in diameter. The fusion nucleolus is remarkably consistent in size. Frequently the two chromosomes (one contributed by each nucleolus) can be seen attached to the "fusion nucleolus” (Plate II, Fig. 4). The fusion nucleus is quite large and conspicuous. Its diameter is just slightly less than that of the ascus. Many fusion nuclei with their large nucleoli have been observed. Frequently the chromatin material seems scattered around the periphery of the nuclear area but most often the spiremes are clearly evident twisting and coiling throughout the nuclear area. Often it cannot be determined where one chromosomal thread leaves off and another begins. Sometimes, too, the spiremes appear to be clustered or aggregated
at one side of the nucleus with the nucleolus at the other side and connected to the aggregate by a single spireme. At this stage, leptotene, the chromosomes reach their maximum length. However, no ascus was seen in which the fusion nucleus had a centrosome, or "central body" or centriole to which the chromosomes were attached giving them a polarized appearance. No nuclear membrane of any fusion nucleus observed appeared to be conspicuously thickened at any one point or region where a central body might have been located.

An analysis of the nuclear figures at this stage gave some indication of the different lengths of the chromosomes. The chromosome to which the nucleolus is attached appears to be the longest chromosome. This chromosome is approximately four microns long at prophase I and may be designated as chromosome 1. Chromosome 2 is slightly shorter than chromosome 1. Chromosome 3 is approximately one-half the length of chromosome 1 while chromosome 4, the shortest of all, is perhaps no more than one micron long. At this stage also the ascus apparently is at its full length. Gradually the "fusion nucleolus" disappears until only the spiremes are evident (Plate II, Fig. 5, 6). These become associated in pairs at synaptotene and begin to shorten and thicken at the pachytene stage. Although it cannot be stated with certainty, at this stage, there appears to be four pairs of chromosomes (Plate III, Fig. 1). Some figures show only seven chromosomes with the possibility of one coiled thread composed of two chromosomes.

First Maturation Division

The first maturation division occurs along the longitudinal axis of the ascus (Plate II, Fig. 7). No figures of division I were observed
in which the spindle fibers were clearly evident although figures were seen similar to those of Colson (9) for Neurospora. Frequently the chromosomes at late anaphase or early telophase appear to be located in a clear vacuolar area. No nucleoli are visible after metaphase I and it is very probable that the chromosomes pass from telophase of division I to prophase of division II without the nuclei being completely reformed. At least no such figures have been noticed in the many asci observed. This indicates that division II closely follows division I. At early telophase of division I figures were observed which indicated the haploid number of chromosomes to be four (Plate II, Fig. 7).

Second Maturation Division

The second maturation division may occur either longitudinally or diagonally. Figures of division II occur much less frequently than the fusion nucleus stage. At telophase I the chromosomes may be shortened and thickened, but at prophase II they have elongated and may be about the same length as in prophase I (Plate II, Fig. 8). They appear thinner, however, and more bead-like than in prophase I. At anaphase II the four groups of chromosomes may be located at some distance from each other (Plate II, Fig. 9) or again they may occupy the center of the ascus and be grouped close together (Plate IV, Fig. 8). Also in some asci they appear to be located in a clear area while again they may not. No prominent spindles have been seen but figures suggesting spindles have been observed. In all cases these structures were located in a vacuolar area. No nucleoli were seen in figures of division II and no figures were seen in which the daughter nuclei were reformed with chromatin strands and nucleoli as has been reported in
several other fungi (2, 9, 23). It is very probable that division II is followed very quickly by the third division and possibly the chromosomes pass directly from telophase II to prophase of the third division. Because the chromosomes are so small and thin it is difficult to detect any apparent doubleness of the chromosomes. No figures of division II were observed in which the chromosome number could be stated with certainty.

Third Ascus Division

As stated above, the third division quickly follows division II. Due to the absence of conspicuous spindles it is difficult to state with certainty if the divisions are transverse or diagonal. However, by comparing the appearance of the chromosome groups, it is sometimes possible to determine sister groups of chromosomes. This was possible because in some asci the chromosomes at late anaphase or early telophase were grouped in such a way that chromosome 4 was visible as a small round dot (Plate IV, Fig. 3). By noting the position of chromosome 4 in adjacent chromosome groups it was possible to determine which groups had been derived from the same nucleus of division II. The chromosome groups at telophase of the third division may form any of several patterns. Most frequently they appear as either two evenly biseriate rows with the pairs of nuclei directly opposite each other, four in each row, or diagonal to each other in two rows of four each (Plate IV, Fig. 4). However, asci were seen in which the eight nuclei resulting from the third division were clustered at the distal end of the ascus. Also, asci were seen with one nucleus in each end of the ascus and six
nuclei in two rows of three each occupying the central portion of the ascus. No doubt shifting of the nuclei occurs in the asci of *Glomerella.*

In the third ascus division the chromosomes frequently appeared to be located in a hyaline or vacuolar area. In several instances four distinct chromosomes (Plate IV, Fig. 2) were clearly visible. At this stage the chromosomes are very small and the largest no more than six-tenths of a micron long. The chromosomes are clustered so close together at late telophase that if the preparation is somewhat overstained, the chromosome group or genome appears as a round black mass, eight of which occupied an ascus (Plate IV, Fig. 4). In such figures each group of overstained chromosomes had the appearance of and was about the same diameter as a fusion nucleolus.

**Ascospore Formation**

At late telophase of the third division the nucleolus of each ascospore nucleus began to appear. As the chromosomes began to lose their conspicuous staining the nucleolus becomes more prominent. Generally coincident with the appearance of the nucleolus is the delimitation of the ascospore. Occasional asci have been seen in which the nucleolus of each ascospore and two small chromosomes were visible. As stated above, at this stage the chromosomes are very small, in fact they approach in size the limits of visibility. In some cases the chromosome on which the nucleolus is reformed is smaller than the nucleolus itself. Eventually all the chromosomes disappear until in a mature ascospore (Plate IV, Fig. 4) only the nucleolus itself stains. At this stage the nucleolus is approximately one-half a micron or less in diameter.
It was not possible to determine how the ascospore walls were delimited. Frequently ascospores were seen outside the ascus in which the chromosomes were still visible and the nucleolus not yet delimited. At maturity the ascospores are slightly curved, rounded at the ends and are biseriate in the ascus. Frequently the pairs of ascospores overlap each other. After the ascospores have matured the ascus wall deliquesces or ruptures and the spores are extruded. Often the spores can be seen clustered in the neck and at the ostiole of the peritheciun. After the asci have all matured and ruptured and the ascospores expelled, the peritheciun is completely empty and appears as an empty shell.

Peritheciun of the Minus A Strain

Sections of peritheciun formed by the minus A strain (4-17-1-1) grown alone when examined did not yield much information. Many peritheciun were seen but they were malformed and few were of mature size. It was impossible to determine the nuclear condition of the asci or to find sufficient figures where the chromosomes could be definitely seen. The asci themselves (of the few seen) seem somewhat small and misformed and the organization of the hyphae within the peritheciun was abnormal and irregular.
DISCUSSION

Use of the Feulgen Reaction

It is strange that the Feulgen stain gave such generally poor results. It is true that the nuclear area of mature ascospores stained a light pink color but in no ascus were any chromosomes seen stained the bright pink color which is usually obtained with this reaction when using the technique on chromosomes of higher plants. At first it was believed that the stain was not good, hence a new solution was prepared. This was used to stain root tips of alligator weed, Alternanthera philexeroides Griseb. and good results were obtained, revealing many mitotic figures. Again, the perithecia were hydrolyzed at 60⁰ C. for different lengths of time and allowed to remain in the stain from 30 minutes to six hours with poor results. In many cases the cells composing the inner two or three layers of the peritheium took on a pink tinge, but no chromosomes were visible in asci of the same peritheium. The Feulgen technique was used on both smears and microtome sections, with negative results. In one case, sections originally subjected to the Feulgen technique were restained with crystal violet and good mitotic figures were obtained. Plate IV, Fig. 2 is a photomicrograph of an ascus at telephase of the postmeiotic division. The chromosome groups were not visible with the Feulgen stain but did become visible when crystal violet was used.

It is admitted that the technique may have been faulty and certainly it should be tried again, especially since the crystal violet
stain has shown chromosomes to be present. However, Lindgren (30), working with yeasts, also obtained poor results with the Feulgen stain. He made the statement "that the general rules concerning the specificity of the Feulgen stain for chromosomes do not hold in yeasts." Such a possibility should not be overlooked as existing in Glomerella.

Anastral Meiosis and Mitosis

In the powdery mildews and Neurospora many investigators have commented upon the conspicuous centrioles and astral rays which are present in the maturation divisions in the ascii of these fungi. Astral mitosis and meiosis is uncommon in plants and the powdery mildews and Neurospora are unusual in having such types of division. In the present investigations no evidence of astral rays could be found, even though the same fixing agent (weak Flemming solution) and similar stains were used that the other investigators employed. Little information was found concerning how the ascospores of Glomerella were delimited.

Considerable mention, too, has been made of the "polarized" appearance of the chromosomes of the powdery mildews, Neurospora and certain yeasts, and of their attachment to a "central body" or spot located on the periphery of the nuclear membrane. In this respect, also the above mentioned fungi are unusual, for such a condition is seldom, if ever, found in other plants. No such condition was found in Glomerella. It is true that figures were seen in which the spiremes were aggregated or clustered together, but this could be attributed to a number of factors such as the killing, fixation, dehydration, etc. However, in no case were the chromosomes seen attached to a central body.
or "polarized" with one end of each chromosome attached to a spot on
the nuclear membrane.

Only a few figures were seen in the asci in which spindle
formation was suggested. Since the divisions are probably intra-
nuclear and the chromosomes small it is reasonable to assume that
the spindle fibers themselves would be delicate and hard to fix and
stain properly.

Chromosome Number

It is significant that the same number of chromosomes were seen
in asci of all perithecia whether they came from a cross of the
conidial B x glomerate B, the glomerate B alone, or the minus B alone.
Indeed the figures were so similar that one would not be able to
distinguish perithecia formed by a cross of the conidial B x glomerate B,
the glomerate B alone or the minus B alone. Although a detailed and
minute scrutiny of individual chromosomes was not possible, it seems
evident that the difference between the plus B strain and minus B
strain is a genic difference and not due to a difference in chromosome
number or aberration. This suggested, too, that the ability of one
strain to cross with another was genically controlled and not due to
difference in chromosome number, chromosome aberration or mechanical
or physical inability of chromosomes to pair.

The Role of the Nucleolus

A nucleolus has been described as occurring in the asci of many
fungi. Its function has been discussed and debated for many years.
In some organisms (9, 23) it has been shown to be of a different
chemical structure than the chromosomes themselves. In many Ascomycetes investigators (1, 2, 9, 10, 23) have represented the nucleolus as persisting through at least the first division in the ascus and sometimes the second. In some cases, as in Neurospora, the nucleolus becomes detached and can be seen in the cytoplasm. In other cases the nucleolus may be reconstituted in the daughter nuclei of telophase I and again at the telophase of division II. Glomerella does not fit any of these situations. It seems quite definite that the nucleolus of Glomerella disappears at late prophase of division I and does not reappear until it is reformed at the telophase of the third division when the ascospores are being delimited. The nucleolus of an ascospore is probably no more than one-third the diameter of a fusion nucleolus.

In a young ascus when the haploid nuclei are preparing to fuse the nucleoli seem to expand or grow larger than when the haploid nuclei are first associated in the ascus. This increase in size would be in accord with the theory that the nucleolus is a reservoir of surplus nucleic acids which are readily available for transformation to chromosomal material. Thus at the time of division in the ascus the large fusion nucleolus would contain a large supply of readily available material for building chromosomes.

Expansion and Contraction of the Chromosomes

Not only does the nucleolus grow larger at the beginning of the divisions in the ascus but the chromosomes themselves exhibit a remarkable increase and subsequent decrease in length during the divisions. At prophase I (Plate III, Fig. 2) the chromosomes are quite long (the longest being at least four microns) conspicuous and
heavily stained. By metaphase I they have shortened and thickened considerably. At prophase II they are again elongated but perhaps not quite so long as in prophase I. However, at anaphase II the chromosomes are markedly decreased in both length and width and by telophase of the third mitosis (Plate III, Fig. 2) they have shrunken to almost the limits of visibility, the longest chromosome being no more than 0.6 microns in length.

In view of the fact that the maturation divisions follow each other so rapidly one is forcibly impressed by the manner and speed with which the chromosomes contract to less than one-fourth their length in the short interval (a matter of minutes perhaps) from prophase I to telophase of the third division and have disappeared altogether by the time the ascospore is formed.
SUMMARY

1. Smears and sections of perithecia of *Glomerella* were treated with a number of stains. In general crystal violet stain gave the best results.

2. The perithecia formed by mating various strains were studied as well as perithecia formed by several strains when grown alone.

3. Ascii in perithecia of all strains (except one) were seen in all stages of development from ascogenous hyphae to mature ascii containing eight ascospores. No difference in appearance of the perithecia could be observed no matter whether the perithecia arose as a result of a cross, or from individual strains growing alone.

4. No crozier formation was observed. Apparently the ascii arise directly from ascogenous hyphae at the base of the perithecium.

5. Each young ascus contains two haploid nuclei. These fuse to form a large, complex fusion nucleus which contains a conspicuous fusion nucleolus. This fusion nucleolus disappears before metaphase I. No nucleolus is again evident until after the third ascus division occurs at which time a small nucleolus appears in the nucleus of each mature ascospore.

6. The three divisions in the ascus follow each other rapidly. At prophase I the chromosomes reach their maximum length. At this stage the chromosome to which the nucleolus is attached is about four microns long. At metaphase I they have shortened and thickened. At prophase II the chromosomes are again elongated. After metaphase II,
however, the chromosomes are considerably smaller than at any stage in the first division and by the time telophase of the third division is reached the chromosomes are much reduced in size, the longest chromosome being no more than 0.6 microns in length.

7. Indications are that the haploid number of chromosomes is four.

8. The divisions in the ascus of Glomerella are anastral. No central body to which the chromosomes might be attached was observed. No centriole or astral rays were seen. It was not determined how the ascospores were delimited.

9. Spindle formation was difficult to observe. The divisions appear to be intranuclear. Division I occurs in the longitudinal axis of the ascus. Division II may be either longitudinal or oblique while the third ascus division is either transverse or oblique.

10. Mature ascospores and conidia of Glomerella are uninucleate.

11. Perithecia of one strain were found to be malformed and few reached maturity. The relatively few asci seen in these perithecia were small and abnormal. The nuclear structure of such asci was not determined.


40. Wilcox, H. S. The sexuality and arrangement of spores in the ascus of Neurospora sitophila. Myc. 20: 3-17. 1928.
George Blanchard Lucas was born on March 8, 1915 at Philipsburg, Pennsylvania. He was graduated from the Philipsburg High School in May, 1931. He entered the National Farm School at Doylestown, Pennsylvania, in April, 1932 and was graduated from that institution in April, 1935. In September, 1936, he entered the Pennsylvania State College and received a Bachelor of Science degree in the Botany curriculum in June, 1940. He accepted a graduate assistantship in the Department of Botany, Bacteriology and Plant Pathology at Louisiana State University in September, 1940, and received the Master of Science degree in June, 1942. In November, 1942, he entered the U. S. Navy and served as a deck officer until released from the Service in September, 1945. He reentered the Louisiana State University in October, 1945, and is a candidate for the Ph. D. degree in August, 1946.
LEGEND FOR PLATES

PLATE I  PERITHECIA OF GLOMERELLA

Fig. 1. Petri dish containing culture 5-16-1 (plus B) mated with culture 38-D-3 (conidial B). Note heavy ridge of perithecia.

Fig. 2. Young perithecium filled with pseudoparenchymatous hyphae. Asci not yet formed. x 625.

Fig. 3. Mature perithecium showing how asci arise from fertile hyphae at base of perithecium. x 490.

PLATE II  DIVISIONS IN THE ASCUS OF GLOMERELLA

Fig. 1. Young ascus showing nuclei before fusion. Note conspicuous nucleolus. x 2000.

Fig. 2. Nuclei just before fusing. Note the two nucleoli, not yet fused, with attached chromosomes. x 2000.

Fig. 3. Nuclei in contact but chromosomes not yet intermingled. x 2000.

Fig. 4. Fusion nucleolus with two attached chromosomes. x 2000.

Fig. 5. Prophase I, leptotene. x 2000.

Fig. 6. Prophase I, leptotene. Nucleolus has disappeared. x 2000.

Fig. 7. Telophase I. x 2000.

Fig. 8. Prophase II. x 2000.

Fig. 9. Telophase II. Nuclear division along longitudinal axis of ascus. x 2000.

PLATE III  DIVISIONS IN THE ASCUS OF GLOMERELLA

Fig. 1. Camera lucida drawing of asci shown in figure 2.
A. Metaphase I.  B. Prophase I, synaptotene.
C. Prophase I, synaptotene. Nucleolus still evident.  D. Ascus to left in third division showing eight groups of chromosomes. Ascus to right showing fusion nucleus. Note difference in chromosome lengths in Prophase I and in the third division.
Fig. 2. Same as shown in figure 1. A. Metaphase I. B. Prophase I, synaptotene. C. Prophase I, synaptotene. Nucleolus still evident. D. Ascus to left in third division, ascus to right showing fusion nucleus. Note difference in chromosome lengths in the two asci. x 2000.

PLATE IV  DIVISIONS IN THE ASCUS OF GLOMERELLA

Fig. 1. Telophase II. Division along transverse axis of ascus. x 2000.

Fig. 2. Telophase of third division with four chromosomes evident. x 2000.

Fig. 3. Telophase of third division. Note small dot chromosomes. x 2000.

Fig. 4. Ascus to left containing mature ascospores. Note the small nucleolus of center ascospore. Ascus to right at telophase of third division. x 2000.
PLATE I

Fig. 1

Fig. 2

Fig. 3
EXAMINATION AND THESIS REPORT.

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Major Field: Botany and Cytology

Title of Thesis: Cytology of Glomerella

Approved:

[Signatures and titles of the examiner and committee members]

EXAMINING COMMITTEE:

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Date of Examination:

June 28, 1946