

A STUDY OF SOME ASPECTS OF MORPHOLOGY, GENETICS, AND CULTURAL
BEHAVIOR OF THE HETEROTHALLIC PYRENO MYCETE GELASINOSPORA
CALOSPORA (MOUTON) MOREAU ET MOREAU, VAR. AUTOSTEIRA
(ALEXOPOULOS ET SUN) ALEXOPOULOS ET SUN

By

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AN ABSTRACT

Submitted to the School for Advanced Graduate Studies of
Michigan State University of Agriculture and Applied
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DOCTOR OF PHILOSOPHY

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The morphology of protoperithecia with "trichogynes" and spermatophores and spermatia is described for Gelasinospora calospora var. autosteira. The organism is hermaphroditic and strictly heterothallic.

Environmental factors such as light, temperature, and culture medium were found to influence fruiting. Cultures incubated in total darkness produce few if any perithecia whereas when light is supplied abundant perithecia may be produced. Light was also found to influence the inception of protoperithecia, in that few if any of these structures were formed in the dark. The pigmentation of the mycelium and the production of aerial mycelium were more pronounced in cultures incubated in the dark.

The organism grew well at all temperatures tested which ranged from 12 to 37° C. Neither protoperithecia nor perithecia were produced at 37° C. Other conditions being favorable, best ascospore production occurred at 20° C.

Difco corn meal agar was found to be most satisfactory for cultivation of this fungus. The quantities of culture medium employed in Petri dishes was found to effect the number and maturation of perithecia produced.

The spores from 142 asci were analyzed for mating type, pigmentation, and protoperithecia. The G and Mt loci were found to be linked and located in the same arm of the

chromosome. The protoperithecial factor segregated in a 1:1 ratio in 86 of the 142 asci dissected, and was found to be too complex for analysis at this time. Segregation for protoperithecia contributed to the sterility noted between compatible strains.

Two general patterns of the distribution of perithecia in Petri dishes were established. Depending upon the protoperithecial potential of the strains involved, perithecia may be produced on the one or both sides of the culture dish.

The advantages which make Gelasinospora a particularly valuable tool for the study of heredity in the fungi are discussed.

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CHAPTER I

INTRODUCTION

Gelasinospora calospora var. autosteira was found to be heterothallic when it was first described by Alexopoulos and Sun (1) in 1950. The writers removed the spores from two asci and found the compatibility factor segregated at first division. They also noticed a variation in the intensity of the pigment produced by the isolates, and suggested that this character may be linked to the compatibility factor. In addition, during the course of their culture studies, they observed considerable variation in:

- 1) the length of time required for perithecial development,
- 2) the number of perithecia produced, and
- 3) the distribution of perithecia in Petri dish matings.

It was suggested that these factors may be correlated with certain parental crosses or environmental conditions.

No publications dealing with either cultural and environmental or genetic studies of Gelasinospora calospora var. autosteira have appeared since the original work of Alexopoulos and Sun (1). Furthermore, as far as the writer is aware, there has been no genetic study attempted in any of the species of Gelasinospora.

Hence, in view of the above account this study was undertaken to determine:

- 1) the linkage relationships of such characters as pigmentation of the mycelium, formation of protoperithecia, and compatability factor,
- 2) the factor or factors responsible for certain patterns of perithecial distribution in the Petri dish matings, and
- 3) the cultural behavior of the fungus under certain environmental conditions.

Also included, is a report of some additional morphological findings in Gelasinospora calospora var. autosteira.

Literature Review

Gelasinospora calospora var. autosteira was first described by Alexopoulos and Sun (1) as Gelasinospora autosteira. The organism developed in moist chamber on Spanish moss, Tillandsia usneoides, collected in Natchez, Mississippi in 1949. At the same time this species was described, the writers presented evidence demonstrating that this fungus consists of two types of strains designated A and B which are self-sterile but interfertile. In the course of their investigation Alexopoulos and Sun (1) observed certain variations in pigmentation of mycelium, formation of protoperithecia, maturation of perithecia, and distribution of perithecia in Petri dish matings; neither conidia nor spermatia were reported.

Taxonomy of the genus Gelasinospora has been reviewed in two publications: one by Cain (8) and another by Moreau and Moreau (32). Nevertheless, some confusion still exists with respect to three species: Gelasinospora calospora (Mouton) Moreau and Moreau, Gelasinospora adjuncta Cain, and Gelasinospora autosteira Alexopoulos and Sun, all of which were described by three groups of independent investigators at about the same time. The first mentioned species is homothallic whereas the latter two are heterothallic. On the basis of the morphological similarity of these three species, Moreau and Moreau (23) reduced both, G. adjuncta and G. autosteira, to synonyms of G. calospora. Sun, Alexopoulos, and Wilson (44) questioned this point of view. In a comparison between G. calospora and G. autosteira they found no significant morphological or cytological differences which may set these organisms apart as separate species, but, nevertheless, proposed that the name Gelasinospora calospora var. autosteira be used to separate the heterothallic form (G. autosteira) from the homothallic (G. calospora). No publication dealing with G. adjuncta has appeared since the original paper by Cain (8) in 1950. According to Sun (43) this species differs from G. calospora var. autosteira only in that it produces spermatia.

Conidia have not been observed in any of the species of Gelasinospora described to date. Spermatia, however,

have been reported in one species. Cain (8) found that G. adjuncta produced spermatophores and spermatia on certain media under the conditions of his experiments. According to him, the spermatia are budded off one at a time from minute projections on the short cells of branched spermatophores. It was suggested the minute projections from which the spermatia are budded may actually be reduced phialides. Cain (8) also noted that spermatophores were produced on certain media, particularly those low in sugar.

Although considerable genetic data are available for such pyrenomycetes as Neurospora, Pleurogea, and Bombardia, a survey of the literature indicates that no genetic studies of Gelasinospora have been made. However, cytological data are available for three species and one variety. Dodge (18) studied Gelasinospora tetrasperma and found spindle orientation and spore delimitation to be very much like that described for Neurospora tetrasperma. Sun, Alexopoulos and Wilson (44) studied the cytology of ascus development of three forms of Gelasinospora: G. cerealis, G. calospora, and G. calospora var. autosteira. They found that during first division meiosis in the young ascus the spindle apparatus is always oriented parallel to the long axis of the ascus. In second division meiosis, although spindles had not been stained, they found that the arrangement of daughter nuclei indicated the orientation of the spindle may be either

parallel or oblique to the long axis of the ascus. Haploid counts of 6 chromosomes were obtained for G. calospora and G. calospora var. autosteira. In general, they found that the ascal cytology of the three forms they studied closely resembles that of Neurospora and other pyrenomyetes.

CHAPTER II

MATERIALS AND METHODS

A. Cultural Methods

1. Source of Material for Study

The stock of Gelasinospora calospora var. autosteira employed in this study is that described by Alexopoulos and Sun (1) as having been developed in moist chamber on Spanish moss which had been collected in Natchez, Mississippi by Alexopoulos in March 1949. All cultures were derived from single ascospores obtained by mating descendants of some of the original isolates made by Alexopoulos.

As a matter of introduction to the organism, the original description of Alexopoulos and Sun (1) is herein included as follows:

Mycelium profusely branched, hyphae 3-14.6 μ diam.; perithecia superficial, pyriform, membranous, 0.42-0.61 mm. diam., 0.65-0.71 mm. tall, black, glabrous; beak prominent, cylindric, characteristically bent or hooked at the tip; asci normally octosporous, cylindric, persistent, 14.1-18.3 μ x 218.6-244 μ ; ascus walls thickened at the apex around a central canal which terminates in a round pore; paraphyses lacking; ascospores oval

hyaline and transparent when young, changing to yellow and finally to very dark brown or black, opaque, 10.6-14.1 μ x 16.7-26.5 μ , binucleate at maturity; characteristic pits in ascospore walls best visible just before maturity of ascospores has been attained; conidia and spermatia unknown.

Thalli self-incompatible, species consisting of two inter-fertile strains designated A and B.

2. Maintaining Stock Cultures

All stock cultures were maintained on Difco corn meal agar in test tubes stored in a refrigerator at 6° C. Subcultures were established by transferring blocks of agar containing mycelium to fresh agar slants, this being the only method available since conidia are not produced. Certain stock cultures of G. calospora var. autosteira, particularly isolates which normally produce protoperithecia, upon subculturing, lost their ability to produce protoperithecia, and did not form perithecia when mated with certain other isolates. This presented a serious problem, particularly in the genetic analysis, so that special care had to be exercised in maintaining such strains. To circumvent this difficulty subculturing was held to a minimum. It was found that even after two years without subculturing, many stocks kept at 6° C. in the refrigerator remained viable and fertile.

Whenever subculturing became necessary, the resultant mycelial growth was checked particularly in those isolates which produced protoperithecia. If protoperithecia were formed in abundance the cultures were utilized, but if few or no protoperithecia were produced the subculture was discarded. As a further precaution viable ascospores were always kept on hand to serve as a source of material should the single ascosporic isolates become infertile.

3. Temperature Control

In those experiments in which temperature was uncontrolled, the cultures were incubated on a laboratory shelf where the temperature varied from 20 to 30° C throughout the year. For more critical temperature control, laboratory incubators (plus or minus 1/2° C) were employed.

4. Media Used

Difco corn meal agar, prepared according to the manufacturer's specifications, proved to be the most satisfactory medium and was used extensively throughout this study.

Another medium, Spanish moss agar, employed particularly for the morphological study had advantages similar to those observed for corn meal agar. It is prepared by steaming 15 gms. of dry Spanish moss in 500 ml. of water for one-half

hour. The resultant decoction is filtered and made up to 500 ml. to which 15 gms. agar is added and the solution is then sterilized at 15 lbs. for 20 min.

5. Light Control

For experiments in which artificial light was used a Sylvania 14-watt cool light standard flourescent tube was employed. The tube was placed at an average distance of 28 cm. from the culture level. When the light was employed within an incubator, the ballast unit was removed and maintained outside the incubator in order to minimize local temperature fluctuations.

6. Standard Culture Conditions

Certain conditions found to be most favorable for cultivation of G. calospora var. autosteira, hereafter designated Standard Culture Conditions, are as follows:

- (1) Medium: Difco corn meal agar, 17 gms. in a liter of distilled water; sterilized at 15 lbs. for 15 min. When culturing was done in Petri dishes, 20 ml. of Difco corn meal agar per plate was employed.
- (2) Temperature: 20° C, obtained with the use of a B.O.D. temperature control cabinet.

- (3) Light: artificial, supplied as described in section on light control.

B. Techniques Employed in Genetic Analysis

1. Preparation and Handling of Material for Dissection

The most satisfactory perithecia for the dissection of asci were obtained from cultures, of cross (A-3 x a-12), incubated at standard culture conditions for 25 days. After that period, the ripe ascospores are ejected daily in great numbers and the material becomes progressively less suitable for study. When it is necessary to keep cultures for some time after they have fully matured their ascospores, the following technique may be employed: after 25 days the cultures are removed from the 20° C incubators and placed in a refrigerator, where they may be stored for one to two weeks without appreciable ascospore discharge. When material for dissection is desired the appropriate culture is removed, the perithecia are picked off, and the plate is returned to the refrigerator as soon as possible. If the plate is not returned to the refrigerator the majority of the ripe ascospores will discharge in from 15 to 45 minutes at room temperature.

2. Isolation of Individual Asci

With the aid of sterile forceps one or more perithecia are transferred from the mature culture to the surface of a sterile flat rubber disc cut to fit into the base of a standard 10 cm Petri dish. The opaque rubber mat provides a surface which is firm enough so that perithecia may be crushed against its surface and yet it is elastic enough so that there is little chance of injuring the ascospores. The most advantageous feature of such a mat, however, is the working surface obtainable. In the preparation of the rubber mat, glycerol is rubbed on the upper surface in such a way as to obtain a gradient between a pool of glycerol on the one side and a dry surface on the other. The rubber mats so prepared are sterilized for 30 minutes under an ultraviolet light.

The fluid area of the mat surface is particularly favorable for crushing the perithecia and separating the asci. Under magnifications of 15x and 45x obtained with a binocular dissecting microscope and with the aid of fine glass needles fashioned on a de Fonbrune Microforge, the asci are separated free hand. After separation from each other and the clusters, the asci are transferred to the viscid central area of the rubber mat, where they tend to remain in a fixed position even when the ascospores are being pushed out from the end of the ascus.

3. Dissection of the Asci

The individual asci were dissected free hand, employing fine glass needles and magnifications of 45x and 90x obtained with a binocular dissecting microscope. Each ascospore was pushed out one at a time by inserting the terminal portion of the needle between the ascospore nearest the end of the ascus and the ascospores immediately below it. A single needle was employed in the operation for the ascus remained stationary on the viscid surface. The ascospores were kept separated outside the ascus, care being taken to retain the exact order of removal. After all eight spores were removed from an ascus they were transferred to the centers of circles which had been previously marked on the bottom surface of the Petri dishes containing Difco corn meal agar with 1:100,000 distilled Furfural. The circles were numbered to retain the exact order of the spores as they were removed from the ascus and placed on the plate.

4. Stimulation of Ascospore Germination

The Petri dishes containing the ascospores in separate known areas were then placed in an oven at 60° C for one-half hour to stimulate germination. By employing the furfural stimulation technique of Emmerson (23) and the heat treatment method of Dodge (10) excellent germination was obtained.

After heat treatment the plates were incubated for 6-16 hours on a laboratory shelf at room temperature and checked for germination of ascospores. Agar blocks containing the sporelings were then cut out and transferred to corn meal agar slants in test tubes for incubation.

5. Designation System for Isolates

The numbering system employed in the tetrad analysis was as follows: each segregant was given a number in three sections, 000.00.0. The first section (000.) of the number was the number of the perithecium from which the ascus was taken; the second section (.00.) was the number of the ascus, and the third section (.0) was the sequential number given to the spore as it was removed in the serial dissection of the ascus. For example, one of the segregants was designated 238.5.3. Hence this number indicates that the isolate was derived from the third ascospore (.3) dissected from the fifth ascus (.5.) taken from perithecium 238.

No set pattern was employed in numbering isolates obtained from spores selected at random.

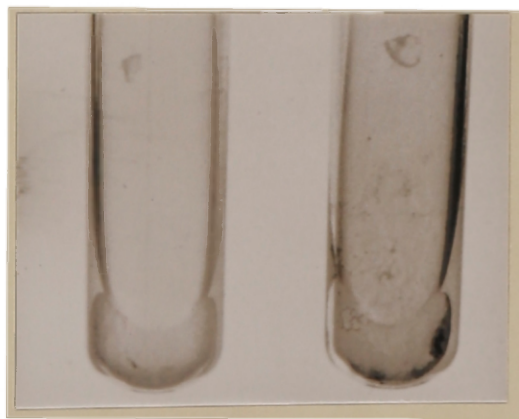
6. Genetic Symbols Employed

The genetic symbolism used by Catcheside (9) has been employed. The mating type alleles are assigned the symbols

Mt⁻ and Mt⁺. For the Green locus studied, the symbol G⁺ denotes the allele responsible for the production of a green pigment in the mycelium, and G⁻ the allele without such activity.

7. Description of Isolates Employed

Two single ascosporic isolates, a-12 and A-3, were used throughout this study. Another isolate, 207.1.5 was employed in the genetic analysis as a tester strain for mating type determination. These isolates are described as follows: a-12 was selected for use because it was highly fertile, particularly in combination with isolate A-3. It normally produces protoperithecia (small dark area in bottom of tube, see test figure 1), spermatophores, and produces a green pigment (G) in the mycelium. It is of the Mt⁻ mating type. On occasion this strain lost its ability to produce protoperithecia when subcultured.



Text Figure 1. Characteristics of two compatible isolates used throughout the study. To the left, isolate A-3, non-pigmented; to the right, a-12, pigmented with protoperithecia at base of agar slant.

A-3 This isolate was found to be highly fertile when mated with a-12. Its compatibility relationship is opposite that of a-12, being designated Mt^+ . It produces a mycelium lacking the green pigment and it was never observed to produce protoperithecia but spermatophores are normally produced (text figure 1). When this strain is mated with a-12 the perithecia are always formed on the a-12 side of the culture.

207.1.5 This isolate was obtained as an F 1 segment from cross a-12 x A-3. It is pigmented, produces both protoperithecia and spermatophores and is of the Mt^+ compatibility group. In matings with a-12 perithecia are produced on both thalli.

CHAPTER III

RESULTS AND OBSERVATION

A. Morphology

1. General Morphological Features

The initial observations of Alexopoulos and Sun (1) on the morphology of Gelasinospora calospora var. autosteira (G. autosteira) have been verified throughout this study. However, further investigation has yielded additional information, particularly with regard to the formation of spermatia and receptive hyphae. From the morphological study presented here, it seems likely that a trichogyne-spermatium relationship exists in Gelasinospora calospora var. autosteira. However, the facts concerning cell fusions and nuclear migrations have not been investigated, but these aspects are now under study by other investigators in this laboratory.

2. Spermatia and Spermatiphores

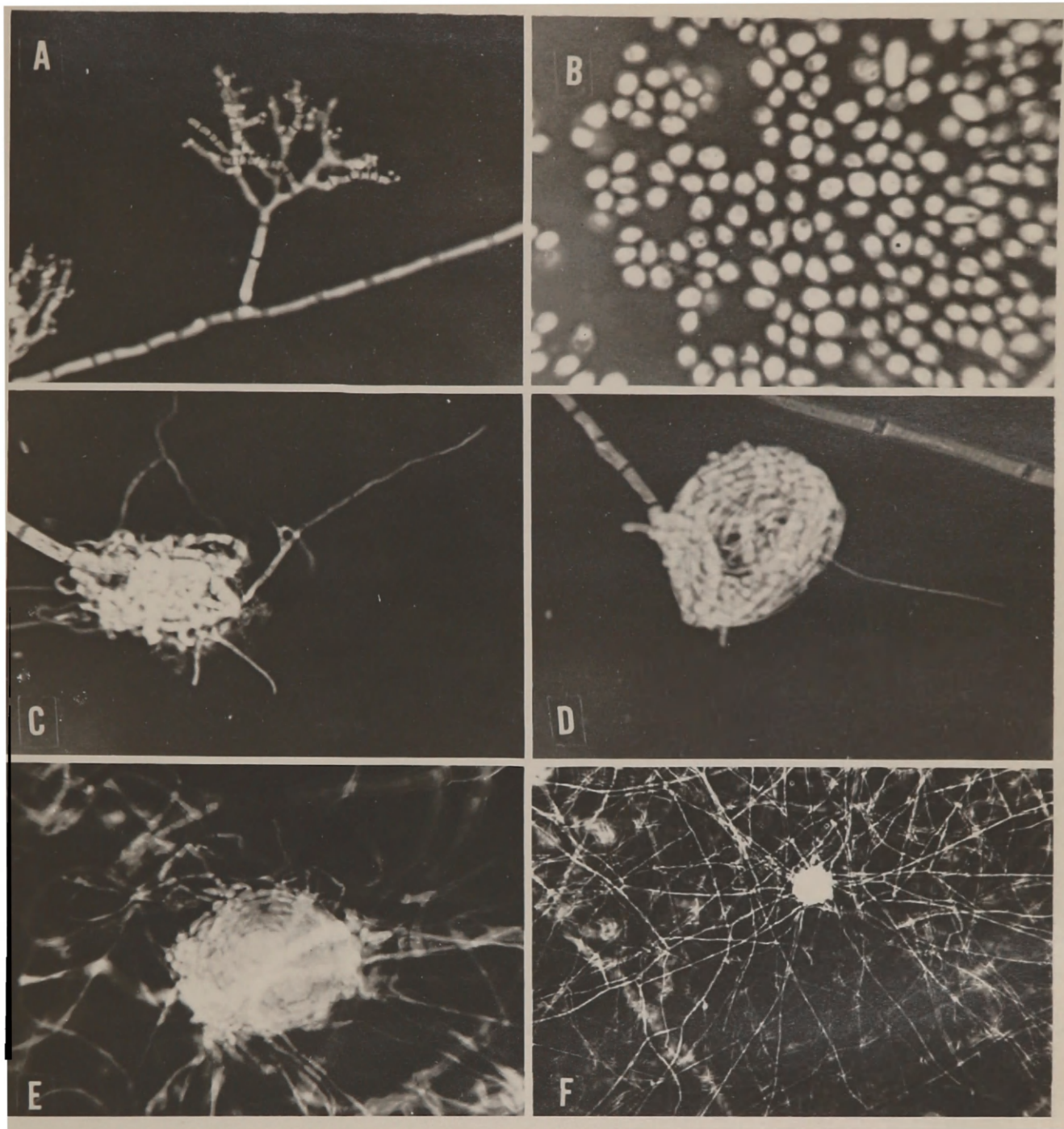
When single ascospores are isolated in separate test tubes or Petri dishes and the mycelium is allowed to mature, some of the cultures produce minute conidia-like structures,

which in their morphology are similar to the spermatia and spermatophores of Cain's Gelasinospora adjuncta. Furthermore, since their morphology and manner of formation is similar to those of other pyrenomycetes which are known to produce spermatia (Dodge, 17), it is suggested that these structures be designated spermatia and spermatophores. Further evidence, although admittedly circumstantial, in support of the view that these structures are spermatia, is afforded by the fact that it has been difficult to germinate the small cells to produce mycelia on various media and in hanging drop cultures. In one case, long and slender germ tubes were developed from the spermatia in a hanging drop suspension. Most of the germ tubes reached a length of approximately five times the diameter of the spermatium, but some were longer, before development stopped. In no case have they been found to be able to reproduce the organism as is the case with the microconidia of Neurospora (Dodge, 14).

The spermatophores and spermatia are morphologically well-defined and easily differentiated from other morphological characters. The spermatia are produced on aerial spermatophores 90-115 u high, which by their method of branching assume a dendroid appearance as shown in Plate I, Figure A. The branches of the spermatophores consist of short cells, 6 x 12 u, each of which bears a single projection

PLATE I

- Figures A-F. Various stages of spermatial and protoperithecial formation in Gelasinospora calospora var. autosteira.
- Figure A. A typical spermatophore, showing the method of branching. The spermatia are budded-off from the short cells of the branches. Two day old slide culture, of isolate a-12. Ca. x400.
- Figure B. Spermatia as observed under oil immersion objective. The cells are subglobose and minute, averaging $3.4\ \mu$ in diameter. Ca. x1000.
- Figure C. A young protoperithecium with what may be a "trichogyne" extending outward from the protoperithecium toward the lower right hand corner of the photograph. Ca. x440.
- Figure D. A slender, septate, unbranched "trichogyne" arising from the internal portion of the protoperithecium. The point of attachment of the "trichogyne" to the ascogone is difficult to distinguish for the ascogone is not differentiated at this stage. Sterile hyphae have not developed yet. Ca. x440.
- Figure E. A mature protoperithecium, of which the internal area is occupied by the enlarged coiled ascogonium surrounded by a pseudo-parenchymatous wall with numerous sterile hyphae. A "trichogyne" is distinguished with difficulty at this stage because of the numerous other hyphae present. Ca. x440.
- Figure F. The branched "trichogyne" may be seen extending from the protoperithecium toward the lower left hand corner and the letter (F). It is of considerable length and branched dichotomously. Ca. x100.



from which the spermatia are budded off. These cells, all of which are potentially spermogonia, are more densely protoplasmic and stain darker than the basal, much larger segments of the spermatophore.

The spermatia do not appear to be organized endogenously, but are budded off one at a time from the projections on the short cells of the spermatophores. In the first stages of development the spermatia may be observed as subglobose enlargements at the ends of the projections. The buds enlarge and become constricted at the base as development proceeds until, finally, they enlarge into globose cells which are released and pushed to the side as other buds develop from below. After their formation the spermatia may form small clusters about their point of origin. When placed in water for observation such clusters readily break apart. The globose to subglobose spermatia, Plate I Figure B, average $3.5\ \mu$ in diameter. Variation in size and shape may be encountered on some occasions.

On Difco corn meal agar or Spanish moss agar, the individual spermatophores may arise directly from ordinary hyphae on the surface of the agar or they may be produced in clusters near the terminal portions of specialized stout hyphae which grow up and intermingle with sterile aerial mycelium. These hyphae may often reach a length of approximately one millimeter or more. In some isolates a cushion-

shaped spermodochium may be formed particularly near the edge of the colony or on the glass walls of the culture vessel. In test tube cultures the spermatophores may be scattered over the entire slant or, as more often happens, they are confined to the upper edge of the agar slant where the agar tends to dry out rapidly.

A study of approximately 1300 single ascosporic isolates grown on agar slants for genetic analysis or for other reasons revealed the variation which may be encountered. In some isolates spermatia were abundantly produced, in others they were sparingly produced and in still others spermatia could not be observed at all. This latter group, though small, was probably a heterogeneous one since it was found occasionally that such "non-spermatial" isolates produced spermatia under certain conditions such as when the mycelium is injured during the cutting out of an agar block for subculture. In such instances the spermatia are observed in that area where the agar block had been removed, and not in any of the other areas of the slant. In addition, it was often noticed that isolates which produced spermatophores under one set of culture conditions often did not produce such structures under another set of culture conditions. Some information regarding the influence of environment on spermatophore formation is given in the chapter devoted to environmental studies.

3. Protoperithecia and "Trichogynes"

A general account of the formation of protoperithecia was reported by Sun (43) and published by Alexopoulos and Sun (1). However, the possibility that receptive hyphae may be produced by strains of G. calospora var. autosteira has not been given much attention. In view of the fact that spermatia were found to be produced by this organism, it was thought desirable to study the development of protoperithecia rather critically to determine if receptive structures are produced. Such a study indicates that there are produced certain structures which on a morphological basis may be interpreted as trichogynes. However, actual cell fusions involving trichogynes have not been observed.

The "trichogyne" is best distinguished before the protoperithecium has matured. In early stages of protoperithecial development the ascogonium develops as a terminal or lateral coil which very soon in its development is enveloped by adjoining hyphae to form a young protoperithecium. It is at this young stage, before the pseudo-parenchymatous wall is formed with its numerous sterile hyphae arising from the outer edge, that certain long flexuous hyphae may first be seen arising from within this mass of knotted hyphae. Such "trichogynes", shown in Plate I Figure C and Figure D, are slender and septate. They arise internally, presumably from the ascogonium, and extend outward where they may be observed

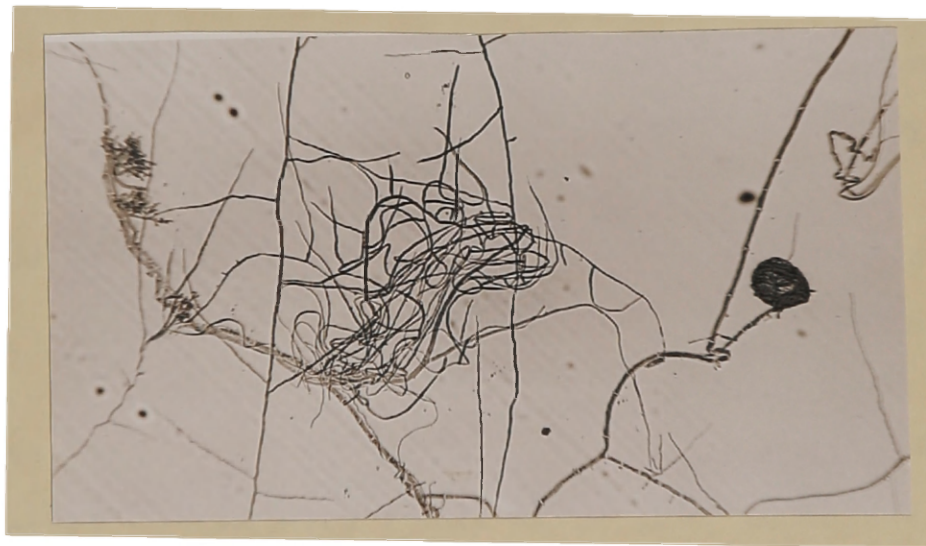
to branch dichotomously particularly as they elongate, Plate I Figure F. It was not possible to determine with certainty the attachment of the trichogyne to the ascogone for, at early stages of development, the ascogone is not differentiated from the other hyphae within the protoperithecium, see Plate I Figures C and D. When the protoperithecium matures the ascogone is plainly visible and assumes the characteristic enlarged coiled shape shown in Plate I Figure E. However, at this stage study of the "trichogynes" is made most difficult by the many sterile hyphae arising from the outer layer of the pseudoparenchymatous wall.

On the basis of the following morphological distinctions it is suggested that such long flexuous hyphae are analogous to the trichogynes of other ascomycetes and may function in the development of a protoperithecium into a perithecium.

- (1) The "trichogynes" are more densely stained with cotton blue especially when the stain is first applied.
- (2) They originate from within the protoperithecium and not from the outer layer as is the case with the other hyphae.
- (3) Their method of branching is characteristically dichotomous, thus differing from the random method of branching observed in other hyphae.

4. Hermaphroditism

Gelasinospora calospora var. autosteira is basically an hermaphroditic organism since it can be demonstrated that single ascosporic isolates of both compatibility groups are equally endowed with the capacity to produce both spermatophores and protoperithecia, text figure 2. However, it shall be shown later that certain isolates producing only protoperithecia under the condition of study may be encountered.



Text Figure 2. Single ascosporic thallus a-12 bearing spermatophores (left), and a protoperithecium (right). Ca. x100.

B. Environmental Studies

1. Nature of Study

Environmental studies preceeded the genetic analysis because very little was known of the

influence of the environment on growth, sporulation and cultural characteristics of G. calospora var. autosteira. Furthermore, as Lindegren (29) has pointed out, in any genetic study employing microorganisms of this nature the environmental conditions should be critically controlled if reliable results are to be obtained. Accordingly, experiments of this nature were conducted to determine:

- (1) the optimum culture conditions for growth and reproduction,
- (2) to what extent such variable characters as pigmentation, protoperithecial formation and spermatophore production were influenced by the culture conditions, and
- (3) the relationship between the environment and certain perithecial distribution patterns obtained in Petri dish cultures.

Of those environmental factors studied, temperature, light and culture medium were found to be most important in these respects.

2. Temperature Effects

To demonstrate that temperature may influence reproduction and general cultural characteristics of G. calospora var. autosteira the following experiment is described:

The two isolates a-12 and A-3, which normally produce perithecia when mated, were inoculated opposite each other and singly in Petri dishes containing 20 ml. Difco corn meal agar per plate. Six Petri dishes of each: (1) a-12 and A-3 in mated fashion, (2) a-12 grown singly, and (3) A-3 grown singly, were cultured at 12, 16, 20, 24, 28, 30, and 37° C in incubators wherein light was not controlled. Because it was physically impossible to control the light in the incubators it was thought that additional information could be obtained by incubating some cultures under controlled lighting conditions. Accordingly, a set of cultures was incubated at the same time at room temperature (24-26° C.) on a laboratory shelf provided with light as described under light control, in materials and methods.

Various stages of perithecial development, encountered at the different temperatures, complicated the counting of the perithecia produced in the Petri dish matings. It was arbitrarily decided that an ascocarp in question was not a perithecium unless there was evidence of beak formation. A binocular dissecting microscope employing 45x and 95x magnifications was used for the determination. Such a criterion seemed justified for such ascocarps with young beaks generally developed into mature perithecia with ascospores upon continued incubation, other conditions being favorable.

For the determination of the relative abundance of ascospores produced in perithecia at the different temperatures

the following method was employed: several perithecia, (at least ten) from each plate were removed from all the plates incubated at each temperature and crushed to expose the spores for observation under the microscope. No attempt at obtaining accurate numerical counts was made. If the perithecia in the culture contained few mature ascospores and rarely an ascus with eight mature ascospores the culture was classed as poor in ascospore production. Likewise if the perithecia contained many ascospores and asci with eight mature ascospores were not uncommon the culture was designated as fair. Cultures which produced perithecia with both abundant mature ascospores and many asci with all eight mature spores were designated as good. The presence and relative abundance of spermatophores and protoperithecia was determined subjectively after a study of the cultures with the unaided eye and with a binocular dissecting microscope.

a. General cultural characteristics at the temperatures employed. Aside from noticeable differences in growth rate the organism grew well at all temperatures tested. Growth was most rapid at 30° C at which temperature after 36 hours, the mycelium had completely covered the surface of the agar plates. Cultures incubated at lower temperatures 20, 16, and 12° C produced consistently more aerial mycelium than those grown at the higher temperatures. In this experiment pigmentation of the mycelium was not critically studied.

b. Effect of temperature on the length of time required for perithecia to appear in mated cultures. Reference to Table I shows that perithecia can be observed in those cultures in which A-3 and a-12 were mated, in as early as 3 days at 24-26° C when supplementary light was provided. When such light was not provided, perithecia did not appear until after 6 days in cultures incubated at 24, 28, and 30° C, after 9 days in cultures incubated at 20 and 16° C, and after 21 days in those incubated at 12° C. At 37° C growth consisted of vegetative mycelium only, there being no evidence of proto-perithecia or perithecia in any of the cultures.

c. Effect of temperature on the production of perithecia and ascospores. Information regarding the relationship between temperature and fertility was obtained by counting the number of perithecia which were produced. Table II lists the totals obtained for six Petri dishes at each of the eight temperatures employed. The perithecial counts were made after a two-week culture period. This was done because cultures incubated at higher temperatures grew and matured more rapidly and had a tendency to dry out sooner than the cultures incubated at the lower temperatures where growth and maturation was retarded. The greatest number of perithecia were counted in cultures incubated at room temperature 24-26° C with light provided. In contrast, at 37° C the mycelium was sterile, with neither protoperithecia nor perithecia being produced.

TABLE I
EFFECT OF INCUBATION TEMPERATURE ON THE TIME OF THE
APPEARANCE OF PERITHECIA IN PETRI DISH MATINGS

Incubation Temperature	Period of Incubation					
	3 days	6 days	9 days	14 days	18 days	21 days
12° C.	-	-	-	-	-	+
16° C.	-	-	+	+	+	+
20° C.	-	-	+	+	+	+
24° C.	-	+	+	+	+	+
25-26° C.*	+	+	+	+	+	+
28° C.	-	+	+	+	+	+
30° C.	-	+	+	+	+	+
37° C.	-	-	-	-	-	-

* In these cultures the light was controlled as explained in text.

+ = perithecia present as judged by the formation of beaks.

- = no perithecia present.

Medium: Difco corn meal agar

TABLE II
EFFECT OF TEMPERATURE ON THE NUMBER OF PERITHECIA AND
ABUNDANCE OF ASCOSPORES PRODUCED

Temperature of Incubation	Total Number of Perithecia Counted in Six Petri Dishes at Each Temperature	Ascospore Production
12° C.	0	None
16° C.	966	Poor
20° C.	858	Good
24° C.	811	Poor
28° C.	128	Poor
30° C.	376	None
37° C.	0	None
24-26° C.* Light controlled	1326	Fair

* Light was controlled in these cultures as explained in the text.

Incubation period: two weeks for perithecial determination; four weeks for ascospore determination.

Isolates: A-3 and a-12 mated in standard Petri dishes

Medium: Difco corn meal agar, 20 ml per plate

Light: not controlled, except as noted.

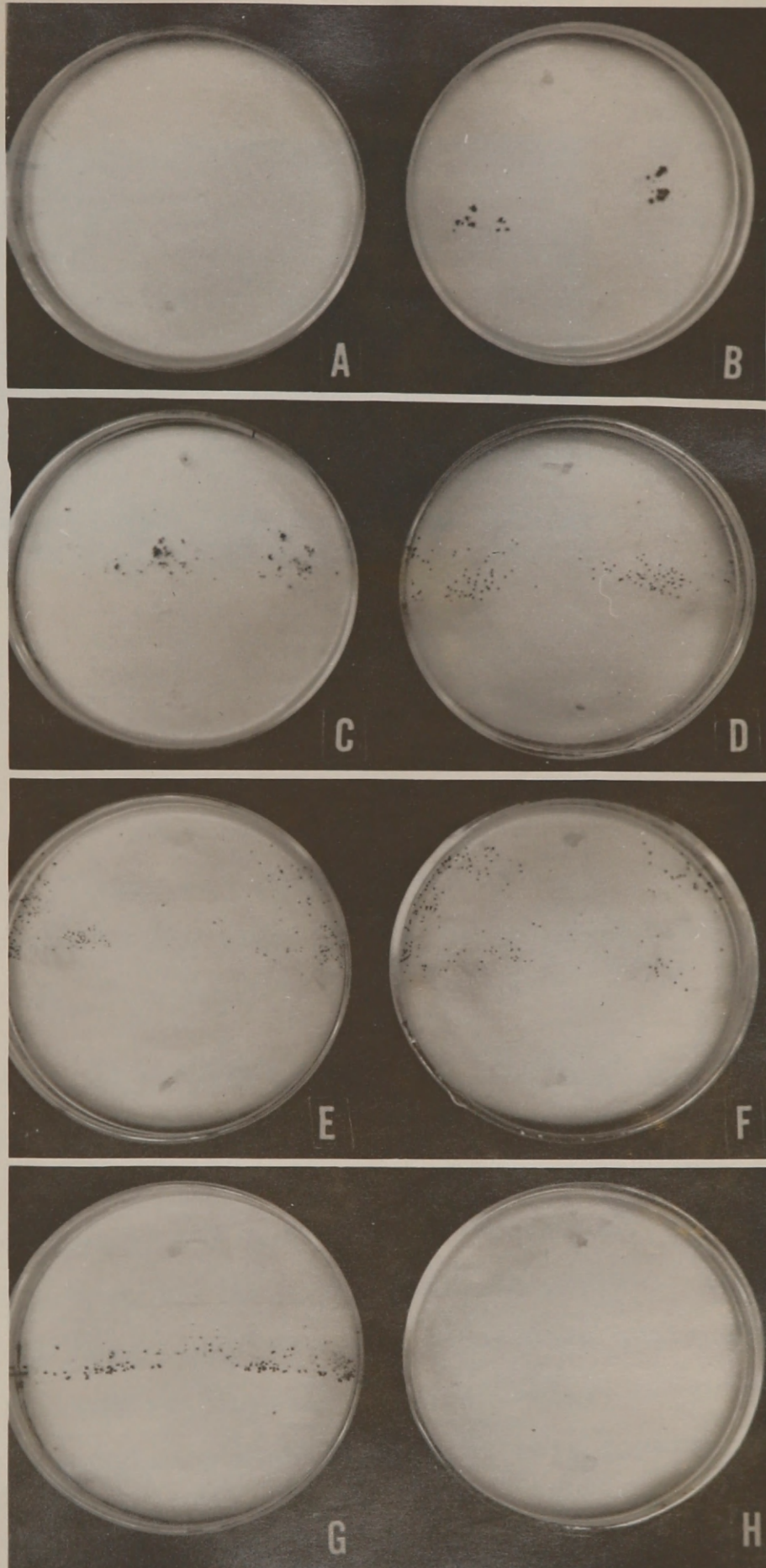
Of those matings incubated with occasional light, most perithecia were produced at room temperature of 24°C and below; of this group, cultures incubated at 16°C produced a slightly higher number of perithecia. After incubation for 28 days the perithecia which had been previously counted were then examined for ascospores. Reference to Table II, column three, shows that best ascospore production occurred at 20°C . Mature ascospores were not evident in perithecia obtained at incubation temperatures of 12, 30, and 37°C . It is of interest to note that although most perithecia were produced in cultures incubated at $24\text{-}26^{\circ}\text{C}$ with supplementary light, ascospores were most abundantly produced at 20°C with occasional light.

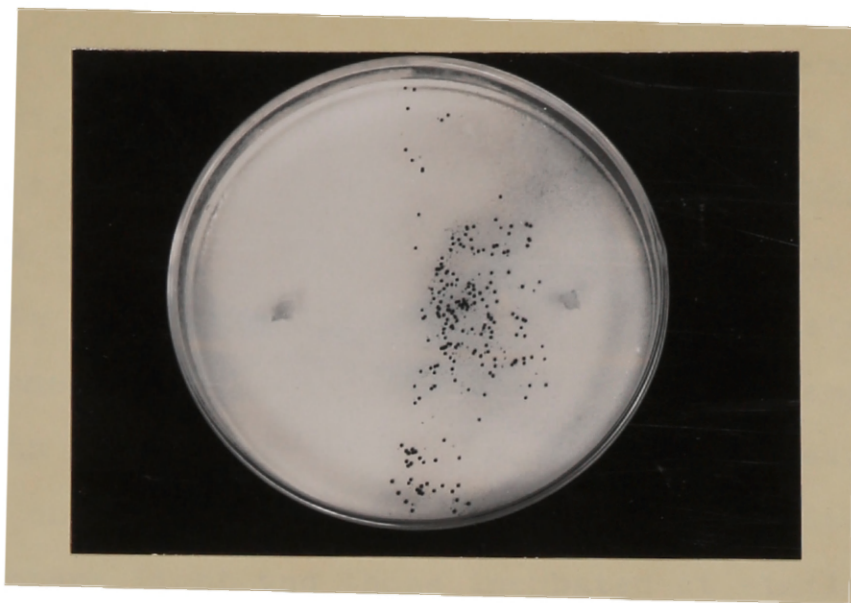
d. Effect of temperature on distribution of perithecia in Petri dish matings. The cultures were also examined for changes in perithecial distribution patterns. Isolates a-12 and A-3 when mated opposite each other in a Petri dish, normally produce perithecia distributed in a scattered fashion on the a-12 side of the culture at standard conditions. This pattern is shown in text figure 3.

The changes and modification of this pattern caused by the temperature are shown in Plate II. It can be noticed that at 28 and 30°C the perithecia showed a marked tendency to form clumps. At $24\text{-}26^{\circ}\text{C}$ with supplementary light, the

PLATE II

- Figures A-H. Modification in fruiting obtained at different incubation temperatures. In each figure, a-12 was inoculated in the top position, and A-3 in the lower position. Note that in cases where perithecia were produced, they were formed consistently on the a-12 side. Growth was obtained in all plates at all temperatures. Incubation: two weeks, on Difco corn meal agar, 20 ml/plate; light uncontrolled except as noted.
- Figure A. 37° C. Cultures were completely sterile, protoperithecia spermatophores, and perithecia were not observed. The organism grew well, however, and filled the plate with mycelium.
- Figure B. 30° C. A marked tendency for the perithecia to clump was noted. No mature ascospores were observed after four weeks.
- Figure C. 28° C. Some perithecial clumping observed, but the condition is not as evident as at 30° C. Occasional mature ascospores were produced.
- Figure D. 24° C. The perithecia are somewhat clustered, but not clumped. Ascospore production poor.
- Figure E. 20° C. The perithecia somewhat more scattered on the a-12 side than had been observed at other temperatures. Ascospore production good.
- Figure F. 16° C. Approximately the same degree of scattering of perithecia on the a-12 side as noted at 20° C.
- Figure G. 24-26° C. These cultures differed from the others in that a supplementary light source was supplied. Cultures produced a broad band of perithecia along the line of meeting. Perithecia appeared in as early as 3 days. Ascospore production was fair.
- Figure H. 12° C. No perithecia were produced. However, upon prolonged incubation, protoperithecia and perithecia may be produced.





Text Figure 3. Perithecial distribution pattern obtained by mating A-3 (left) x a-12 (right). The perithecia are scattered and restricted on the a-12 side of the culture.

perithecia were produced in the region where the two isolates met. In all cultures the perithecia were consistently formed on the a-12 side of the culture, no change from this pattern being observed.

e. Effect of temperature on the formation of proto-perithecia and spermatophores. Those cultures in which A-3 and a-12 were grown separately were used specifically for the determination of the influence of temperature on the formation of protoperithecia and spermatophores. Isolate A-3, which had never been observed to produce protoperithecia throughout the entire investigation, likewise, produced no protoperithecia under the conditions of this experiment.

Attention was directed to strain a-12 which normally produced protoperithecia and spermatiphores. The results obtained for this strain are tabulated in Table III. After two weeks incubation neither spermatiphores nor protoperithecia could be observed in cultures incubated at the two extremes 12° C and 37° C. In the median temperature range both structures were produced. No noticeable difference was observed between those cultures incubated with supplementary light and those incubated at similar temperatures where the light was not provided.

3. Light Effects

Some preliminary studies with light, which were conducted during temperature studies, yielded some information regarding the action of light on G. calospora var. autosteira. However, to demonstrate further the importance of light in the fruiting process of this fungus the following experiment is pertinent:

Random monospore isolates, 2-5 and 3-R-25, which normally produced perithecia on the 2-5 side of the culture when mated, were inoculated opposite each other on Difco corn meal agar in Petri dishes and incubated at room temperature (24-26° C). Five dishes so inoculated were cultured in a cabinet which had been sealed to exclude light. To serve as controls five other plates were placed in a similar locker, and

TABLE III
EFFECT OF TEMPERATURE ON THE FORMATION OF
OF PROTOPERITHECIA AND SPERMATIA

Temperature of Incubation	Protoperithecia	Spermatia
12° C.	- ¹	-
16° C.	++	-
20° C.	++	+
24-26° C.*	++	++
24° C.	++	++
28° C.	+	++
30° C.	+	+
37° C.	-	-

* In this set of cultures light was controlled as explained in text.

Incubation period: two weeks

Medium: Difco corn meal agar

Light: not controlled or as specified

Strain: a-12

¹Protoperithecia were eventually produced upon prolonged incubation of one month or more.

exposed to diffuse daylight for at least 15 minutes each day throughout the culture period.

After 14 days one of the dishes was removed from that group grown in the dark and compared to the control cultures incubated with some light. This comparison revealed that the culture from the dark had neither protoperithecia nor perithecia and, in addition, the pigmentation was more pronounced than in the cultures incubated in the light. The control cultures produced perithecia abundantly and consistently on the 2-5 side. A second dish was removed from the dark after 19 days of incubation and the same results were obtained as noted in the first dish. After 28 days the remaining three dishes were removed from the dark. In two of these last three dishes the reaction was similar to that in cultures removed after 14 and 19 days. However, in one plate several protoperithecia and two perithecia were observed. The perithecia appeared to be normal and contained some mature ascospores.

Pigmentation and mycelial growth differed in the cultures incubated under the two conditions. Although, in the dark, both strains produced a dark green pigment, strain 2-5 was most heavily pigmented. In the cultures incubated with occasional light, pigmentation was not as pronounced; a green pigment was noted in isolate 2-5 but not easily detected in strain 3-R-25. The mycelium of cultures incubated in the dark was rather uniformly distributed in the plates

so that no line indicating the place where the two isolates had met was discernible, and there was a general overall tendency for more aerial mycelium to be produced. In cultures grown with some available light, there was little or no aerial mycelium and the area at which isolates 2-5 and 3-R-25 met was easily distinguishable.

Of interest here are the results obtained when those cultures incubated in the dark were removed and placed in the light for an additional incubation period. After four or five days protoperithecia and spermatophores became evident, and upon continued incubation, perithecia were produced in abundance completely scattered over the plates and not restricted to the 2-5 side of the cultures as was observed in the controls. Eventually mature ascospores were obtained.

In another experiment isolates a-12 and A-3 were inoculated on Difco corn meal agar slants in 75 ml culture tubes. Six of the tubes were coated with black paint to exclude light, and six other tubes were left uncoated as control. The cultures were incubated at standard culture conditions for three weeks. The same results were obtained as in the previous experiment. Few or no perithecia were formed when culturing was done in the dark whereas abundant perithecia were formed when light was available during the culture period.

4. Media Effects

Gelasinospora calospora var. autosteira was grown on such different media as potato dextrose agar, malt agar, Czapek's solution agar, dung agar, Westergaard and Mitchell medium (45), Spanish moss agar, and Difco corn meal agar. The organism grew well and fruited on most media. However, Difco corn meal agar proved to be the most satisfactory for culture of this fungus and was used extensively because of the following reasons:

- (1) substantial growth and sporulation was obtained on this medium;
- (2) little or no aerial mycelium was produced, and
- (3) pigmentation of the mycelium which obscured the study of morphological structures was reduced to a minimum.

Since extensive experimentation was conducted in standard Petri dishes, it was thought desirable to determine the optimum quantity of agar to be employed in such cultures. Accordingly the following experiments were conducted:

Petri dishes containing 10, 15, 20, 25, 30, 35, 40, 45, and 50 ml of medium per plate were inoculated with isolates a-12 and A-3 singly, and in mated fashion. Incubation was conducted at 24° C with occasional light entering when the incubator door was opened. The methods of counting perithecia and estimating the relative abundance of protoperithecia and

spermatophores were the same as described in temperature studies.

Four plates were used for each of the different quantities of medium employed. All of the plates containing 10 ml of medium were sealed with tape to prevent drying. For comparative purposes, one plate from each of the remaining groups of four plates was sealed in the same way.

The results are summarized in Table IV. Excluding for the moment those cultures which were sealed, it is observed that the number of perithecia produced, increased with the increased amounts of medium employed, to a maximum at 50 ml. It was also observed that when small quantities of medium were used fewer larger perithecia were formed and the rate of maturation of perithecia was more rapid than when greater amounts were used. Ascospore production increased somewhat with increased amounts of medium up to a certain point. At the level of 50 ml of medium, per plate, ascospores were not observed in 28 days because the perithecia had not yet matured.

The results obtained from the sealed plates differed markedly from the others. The numbers of perithecia produced in all these dishes were consistently lower than in the unsealed dishes. Furthermore, mature ascospores were either rare or absent in most plates.

The data obtained when both isolates, a-12 and A-3, are grown separately in Petri dishes containing different

TABLE IV

EFFECT OF DEPTH OF DIFCO CORN MEAL AGAR ON
PERITHECIAL FORMATION IN PETRI DISH MATINGS

Amount of Medium per Plate	Plate No.	Number of Perithecia	Total (3)	Ascospores
10 cc	1	75		+
	2	161	347	+
	3	70		+
	4	41		+
15	1	156	415	+
	2	128		+
	3	131		+
	4	70 sealed		-
20	1	122	429	+
	2	154		+
	3	153		+
	4	84 sealed		-
25	1	267	708	+
	2	226		+
	3	215		+
	4	116 sealed		-
30	1	215	699	++
	2	245		++
	3	239		++
	4	111 sealed		--
35	1	336	857	++
	2	262		++
	3	259		++
	4	8 sealed		-
40	1	289	837	++
	2	243		++
	3	305		++
	4	237 sealed		+
45	1	417	1352	++
	2	512		++
	3	423		++
	4	32 sealed		-
50	1	473	1584	-
	2	540		-
	3	571		-
	4	143 sealed		-

Cultures were sealed to prevent drying by taping the edge of the dish with masking tape.

Four plates of Difco corn meal agar at the various depths were inoculated with strains a-12 and A-3 in mated fashion, incubated 28 days, 24° C, light not controlled.

amounts of medium are shown in Table V. At all amounts tested, isolate A-3 produced spermatophores but no protoperithecia. Isolate a-12 however, produced spermatophores and protoperithecia in all plates except those with 10 ml of medium. A comparison of Tables IV and V reveals a strong correlation between the quantity of protoperithecia produced by isolate a-12 and the quantity of perithecia produced on the a-12 side of matings (a-12 x A-3).

C. Genetic Studies

A preliminary study of approximately 200 random single ascosporic isolates and monospore cultures derived from the serial dissection of 18 asci, revealed that the following characters appeared to show some segregational pattern: (1) pigmentation of the mycelium, (2) production of protoperithecia, (3) formation of spermatia, and (4) the production of aerial mycelium. Not all the characteristics just mentioned proved to be suitable for genetic analysis within the limits of available time and facilities.

The genetic information presented in this report was accumulated from the single cross (a-12 x A-3). It may be recalled, that these parent isolates, a-12 and A-3, selected for tetrad analysis, are the same which had been employed extensively in the environmental studies. All culturing for the genetic analysis was conducted under standard culture

TABLE V
EFFECT OF DEPTH OF MEDIUM ON THE FORMATION OF SPERMATIA
AND PROTOPERITHECIA BY ISOLATES a-12 AND A-3

Amount of Medium		Protoperithecia	Spermata
ml.			
a-12	10	+ *	- *
	15	++	++
	20	++	+
	25	++	++
	30	++	++
	35	++	++
	40	++	++
	45	+++	+++
	50	+++	+++
- - - - -		- - - - -	- - - - -
A-3	10	-	+
	15	-	+
	20	-	+
	25	-	+
	30	-	++
	35	-	++
	40	-	+
	45	-	+
	50	-	+

* Cultures dried out in 6 days.

Medium: Difco corn meal agar

Incubation: 2 weeks, at 24° C, light not controlled

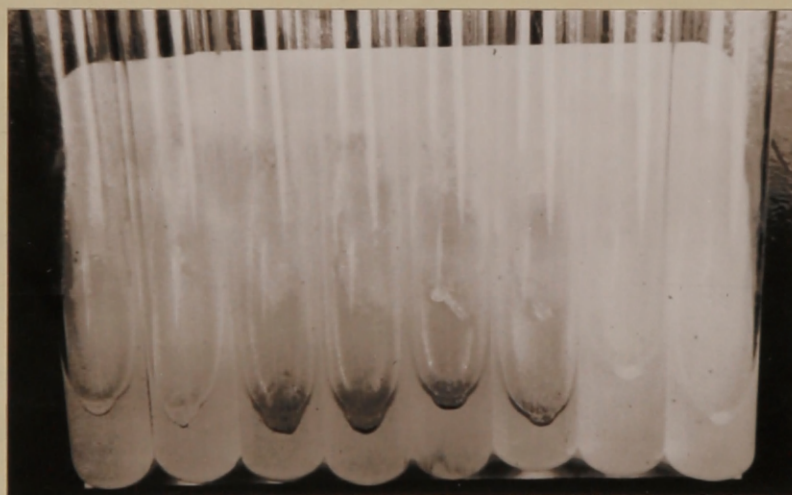
conditions, which are reported in materials and methods. Likewise, the reader is referred to materials and methods for details regarding the techniques employed in genetic analysis. Mating type was determined by backcrossing the F 1 segregants to the parents A-3 and a-12. Isolate 207.1.5, selected from the F 1, was also employed, particularly when a segregant reacted negatively with both a-12 and A-3.

1. Segregation of the Pigmentation Factor

One hundred and forty-two asci were dissected and analyzed. From the segregation patterns obtained it became apparent that the pigmentation of the mycelium was affected by more than one gene. All of the factors involved were not analyzed. One of the genes studied was Green (G), a factor which imparts a green color to the mycelium in cultures incubated at standard culture conditions for 6-8 days. The length of time the cultures are incubated is important, for in some cultures, beginning after 6-8 days, segregation for G is obscured by secondary pigmentation reactions, which, as yet, have not been analyzed. Of the total of 142 asci studied, 112 showed first division, and 30 second division segregation for G, see text figures 4 and 5.



Text Figure 4. Ascal segregation pattern showing first division segregation for G.



Text Figure 5. Ascal segregation pattern showing second division segregation for G.

2. Linkage Relation to the Mg and G Loci

As in most other Ascomycetes the compatibility reaction has been found to be controlled by a single factor pair.

Mutations at the Mt locus were not encountered in this study. When both Mt and G are considered simultaneously, nine segregation patterns were obtained. These are listed in text figure 6. The reader is referred to materials and methods for the explanation of genetic symbols employed. If the genotype of the parents employed is designated as follows:

$$a-12 = Mt^-G^+$$

$$A-3 = Mt^+G^-$$

then the genotypes and arrangement of ascospores in asci derived from this cross may be tabulated as in text figure 6. Since no significant differences were noted between adjacent spore pairs in an ascus the genotypes of the asci shown in text figures 6 and 7 are simply represented by tabulation of spore pairs rather than individual spores.

Linkage is indicated by the large number (112) of asci obtained in which the ascospores are genotypically like the parents, see text figure 6, column 1, row 1. However, linkage may be more readily demonstrated if the patterns of ascus segregation are grouped into ascus segregation classes (Catcheside, 9). Text figure 7 shows the seven segregation classes obtainable for two factors segregating independently. Such a grouping disregards: 1) orientation with respect to base and apex of the ascus, and 2) the way segregations are directed in the second division spindles with respect to one another.

GENOTYPE AND ARRANGEMENT OF ASCSPORES
IN ASCI DERIVED FROM CROSS: $MT^+ G \times MT^- G^+$

ARRANGEMENT	$MT^+ G$ $MT^+ G$ $MT^- G^+$ $MT^- G^+$	$MT^+ G$ $MT^+ G$ $MT^- G^+$ $MT^- G$	$MT^+ G$ $MT^+ G$ $MT^- G^+$ $MT^- G$	$MT^+ G$ $MT^+ G$ $MT^- G^+$ $MT^- G$	$MT^+ G$ $MT^+ G$ $MT^- G^+$ $MT^- G$	$MT^+ G$ $MT^+ G$ $MT^- G^+$ $MT^- G$
NO. OF ASCI OBSERVED	112 (2)	7 (3)	6 (3)	5 (3)	5 (3)	5 (3)
ARRANGEMENT	$MT^+ G$ $MT^- G$ $MT^+ G$ $MT^- G$	$MT^+ G$ $MT^- G$ $MT^+ G$ $MT^- G$	$MT^+ G$ $MT^- G$ $MT^+ G$ $MT^- G$	$MT^+ G$ $MT^- G$ $MT^+ G$ $MT^- G$	$MT^+ G$ $MT^- G$ $MT^+ G$ $MT^- G$	$MT^+ G$ $MT^- G$ $MT^+ G$ $MT^- G$
NO. OF ASCI OBSERVED	1 (4)	4 (6)	1 (6)	1 (7)	1 (7)	1 (7)

Text Figure 6

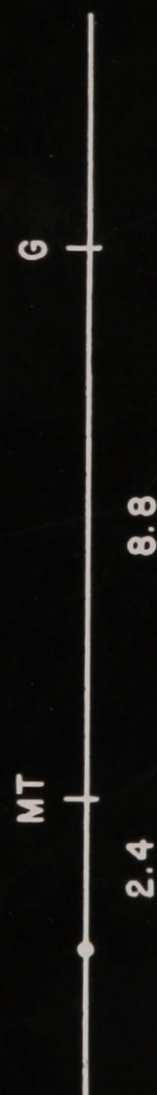
SEGREGATION CLASSES	1	2	3	4	5	6	7	TOTAL
	MT ⁺ G ⁺ MT ⁺ G ⁺ MT ⁻ G ⁻ MT ⁻ G ⁻	MT ⁺ G ⁺ MT ⁺ G ⁺ MT ⁻ G ⁺ MT ⁻ G ⁺	MT ⁺ G ⁺ MT ⁺ G ⁺ MT ⁻ G ⁺ MT ⁻ G ⁺	MT ⁺ G ⁺ MT ⁻ G ⁺ MT ⁺ G ⁺ MT ⁻ G ⁺	MT ⁺ G ⁺ MT ⁻ G ⁺ MT ⁺ G ⁺ MT ⁻ G ⁺	MT ⁺ G ⁺ MT ⁻ G ⁺ MT ⁺ G ⁺ MT ⁻ G ⁺	MT ⁺ G ⁺ MT ⁻ G ⁺ MT ⁺ G ⁺ MT ⁻ G ⁺	
ASCI	0	112	23	1	0	5	1	142
2ND DIV. SEG. FOR MT	0	0	0	1	0	5	1	7
2ND DIV. SEG. FOR G	0	0	23	1	0	5	1	30
RECOMBINANT SPORE PAIRS	0	0	46	2	0	0	2	50

Text Figure 7. Segregation classes and frequency of asci obtained in each class.

The observed segregation patterns shown in text figure 6 were grouped into the appropriate segregation classes shown in text figure 7. The small figures appearing in brackets in text figure 6 indicate the segregation class into which the ascal types may be grouped. That the G and Mt loci are linked is indicated by the large disparity between segregation classes 1 and 2 shown in text figure 7. If the loci were on separate chromosomes we would expect these two classes to appear with equal frequency. Additional evidence for linkage is found in the high frequency of asci in class 6 where a crossover had occurred but the spore pairs still have a composition like the parents.

From the data in text figure 7 it can be seen that in 30 asci, second division segregation for the G locus occurred, and for the Mt locus, 7 asci showed second division segregation. On this basis the calculated recombination between the loci and centromere (one-half second division segregation percentage) is 2.4 for Mt, and 10.5 for the G. The recombination between Mt and G loci as calculated by determining the percent of recombinant spore pairs is 8.8. Since Mt and G are linked, two relationships are equally possible as regards the relative position of the loci on the chromosome. They may be: a) in the same arm of the chromosome, or b) in the opposite arms of the chromosome. Referring to text figure 8 this relationship is determined in the following

A	B	TOTAL SPORE PAIRS	RECOMBINANT SPORE PAIRS MT - G		A + B	A - B
ONE-HALF 2ND DIV. SEG. FOR MT %	ONE - HALF 2ND DIV. SEG. FOR G %		TOTAL	%		
2.4	10.5	568	50	8.8	12.9	8.1



Text Figure 8. Recombination data and chromosome map.

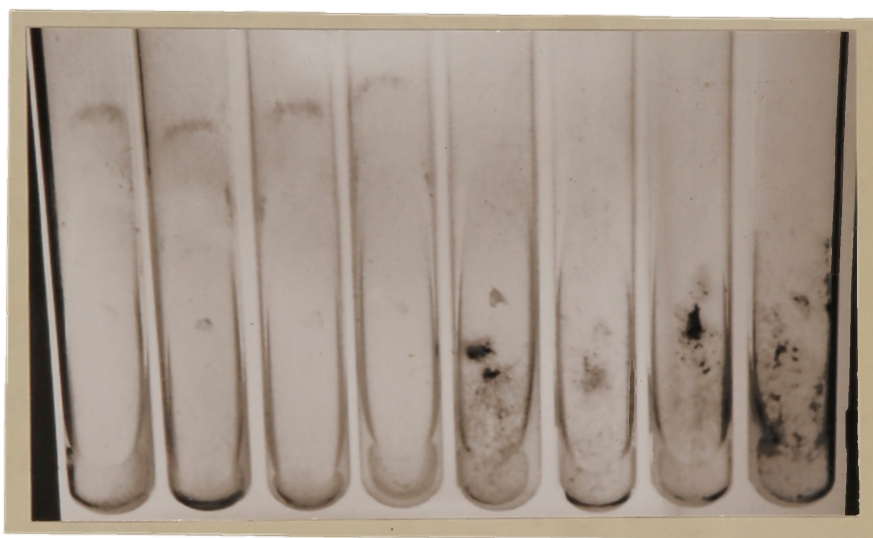
way. If the genes are in the opposite arm of the chromosome then the recombination between them (8.8) should be more nearly the sum (12.9) of the recombination between the respective loci and the centromere; text figure 8, column A (2.4) plus column B (10.5). If the loci are in the same arm of the chromosome the recombination between them (8.8) will be more nearly equal to (8.1) the difference of the recombination values between the loci and the centromere; text figure 8, column B (10.5) minus column A (2.4). This latter case applies here and hence the loci are established as being in the same arm of the chromosome.

Using the recombination values, a chromosome map, uncorrected for double crossovers, may be constructed as shown in text figure 8. The mating type locus is established as 2.4 units from the centromere and the G locus as 8.8 units from the mating type locus, as judged by the recombination between the two loci.

3. Segregation of Protoperithecia

Segregation for protoperithecia was observed, but it was not as well defined as the 1:1 segregation obtained for both the Mt and G loci. When the total 142 asci analyzed were studied for the production of protoperithecia, the ascus segregation patterns as judged with the unaided eye fell into five groups:

- (1) Segregation patterns which showed a 1:1 segregation ratio for protoperithecia. Eighty-six asci were of this type, 78 of which showed first division segregation (see text figure 9) and 8 of which showed second division segregation.



Text Figure 9. Segregation pattern showing first division segregation for protoperithecia.

- (2) Segregation patterns in which it was observed that none of the isolates produced protoperithecia. Thirty asci were of this type.
- (3) Segregation patterns in which all eight ascospore isolates produced protoperithecia in numbers which might in any way approach those produced in group (1). Four asci were of this type.
- (4) Segregation patterns in which two of the eight spores from an ascus yielded protoperithecial

cultures and the other six yielded non-protoperithecial cultures. Sixteen asci of this kind were observed.

- (5) Segregation patterns in which six of the spores from an ascus yielded protoperithecial cultures and the other two gave non-protoperithecial cultures. Six asci of this type were detected.

If a binocular dissecting microscope were employed to ascertain the presence or the absence of protoperithecia in the isolates, the classification would not correspond to the system just outlined wherein scoring was done with the unaided eye.

Because of the peculiar segregation ratios obtained from the total 142 asci, a conclusive analysis of the protoperithecial factor cannot be made. However certain information was obtained, which may be of value in further studies.

Some of the segregants obtained were new types which had not been recognized before. Assigning the phenotypic symbol P to cultures producing protoperithecia and using the genetic symbols for mating type and pigment, the isolates can be tabulated as in Table VI.

4. Probable Protoperithecial Linkage Relationship

More than one-half of the 142 asci analyzed showed 1:1 segregation for protoperithecia. By a closer examination of this group it was hoped to ascertain the relationship of

TABLE VI
A LISTING OF RECOMBINANT ISOLATES OBTAINED
FROM CROSS A-3 x a-12

Mating Type	Pigmentation	Protoperithecia	Isolate Number
Mt ⁺	G ⁻	p	A-3 (parent)
Mt ⁺	G ⁻	P	214.15.4
Mt ⁺	G ⁺	p	248.5.6
Mt ⁺	G ⁺	P	207.1.5
Mt ⁻	G ⁺	P	a-12 (parent)
Mt ⁻	G ⁺	p	214.4.1
Mt ⁻	G ⁻	P	208.5.2
Mt ⁻	G ⁻	p	207.1.3

P = phenotypic symbol; protoperithecia present

p = protoperithecia absent

Mt and G = genotypic symbols

this factor complex with the Mt and G loci. Table VII lists the segregant patterns obtained in the group of 86 asci which showed 1:1 segregation for protoperithecia. The symbol P was used to designate protoperithecial strains. It seems probable that the P factor complex is on the same chromosome as Mt and G, as judged from the large number of ascal types (64) in which the spores are like the parents.

5. Spermatophore Segregation

Spermatophores and spermatia were not taken into account when this cross was made. Both parents, a-12 and A-3, produced spermatophores. An examination of the segregants for this character indicated a quantitative segregation for spermatophores was being obtained. This was particularly noticed in those segregation patterns (86 asci) in which the P complex segregated in a 1:1 fashion. Those isolates which formed no protoperithecia generally produced noticeably more spermatophore and spermatia than the P isolates.

D. Cultural Behavior

1. Sterility

Considerable sterility was encountered, particularly at the outset of the investigation, so that substantial difficulty was encountered in attempts to obtain mature

TABLE VII

ASCAL SEGREGATION PATTERNS OBTAINED IN 68 ASCI SHOWING
1:1 SEGREGATION FOR PROTOPERITHECIA (P)*

Pattern	Number of Asci Observed	Pattern	Number of Asci Observed	Pattern	Number of Asci Observed
Mt ⁺ G ⁻ Mt ⁺ G ⁻ Mt ⁻ G ⁺ P Mt ⁻ G ⁺ P	61	Mt ⁺ G ⁻ P Mt ⁺ G ⁻ P Mt ⁻ G ⁺ Mt ⁻ G ⁺	6	Mt ⁺ G ⁻ Mt ⁺ G ⁻ P Mt ⁻ G ⁺ P Mt ⁻ G ⁺	2
Mt ⁺ G ⁺ Mt ⁺ G ⁻ Mt ⁻ G ⁺ P Mt ⁻ G ⁻ P	1	Mt ⁺ G ⁻ P Mt ⁺ G ⁺ P Mt ⁻ G ⁻ Mt ⁻ G ⁺	1	Mt ⁺ G ⁻ P Mt ⁺ G ⁻ Mt ⁻ G ⁺ Mt ⁻ G ⁺ P	2
Mt ⁺ G ⁺ Mt ⁺ G ⁻ Mt ⁻ G ⁻ P Mt ⁻ G ⁺ P	1	Mt ⁺ G ⁺ P Mt ⁺ G ⁻ P Mt ⁻ G ⁻ Mt ⁻ G ⁺	1	Mt ⁺ G ⁻ Mt ⁻ G ⁺ P Mt ⁺ G ⁻ Mt ⁻ G ⁺ P	3
Mt ⁺ G ⁻ Mt ⁺ G ⁺ Mt ⁻ G ⁺ P Mt ⁻ G ⁻ P	4	Mt ⁺ G ⁻ P Mt ⁺ G ⁻ Mt ⁻ G ⁺ P Mt ⁻ G ⁺	1	Mt ⁺ G ⁻ Mt ⁻ G ⁺ Mt ⁻ G ⁺ P Mt ⁺ G ⁻ P	1

* P = protoperithecial complex; protoperithecia present

Total Asci = 86

1st Div. P = 78

2nd Div. P = 8

fruiting bodies and ascospores. Some of the factors which may produce this sterility are as follows:

- (1) Compatibility factor: Other conditions being favorable, only strains of opposite compatibility groups are fertile.
- (2) Environmental conditions: It has been demonstrated in the environmental studies that the culture conditions may influence the fertility of a cross.
- (3) Capacity to produce protoperithecia: During the course of the investigation it became apparent that this factor was of great importance in determining the sterility or fertility of certain crosses. Furthermore it became more significant when it was observed that, aside from known genetic segregation for protoperithecia, the capacity to produce protoperithecia could be modified by sub-culturing. When such modification was encountered it was consistently in the direction Protoperithecia ---->non-protoperithecia. Concomitant with this change a loss in pigmentation occurs. Such non-protoperithecial strains were then only fertile in combination with protoperithecial strains, of the opposite compatibility.

Genetic segregation for protoperithecia also determined the sterility or fertility of certain

crosses. Data accumulated when the F 1 segregants were backcrossed to parents A-3 and a-12 indicate that, excluding other factors, only those matings are fertile in which at least one of the strains has the capacity to produce protoperithecia, see Plate III. The non-protoperithecial segregants were fertile with a-12 but infertile with A-3, in crosses where the appropriate compatibility factors were present. When crossed with 207.1.5, a protoperithecial isolate, the non-protoperithecial segregants which were infertile with A-3 were then fertile.

- (4) Ascospore abortion: In certain matings aborted spores may be observed. During the isolation of asci for tetrad analysis, occasional perithecia obtained from cross a-12 x A-3 contained asci in which only four of the spores matured and the other four aborted.

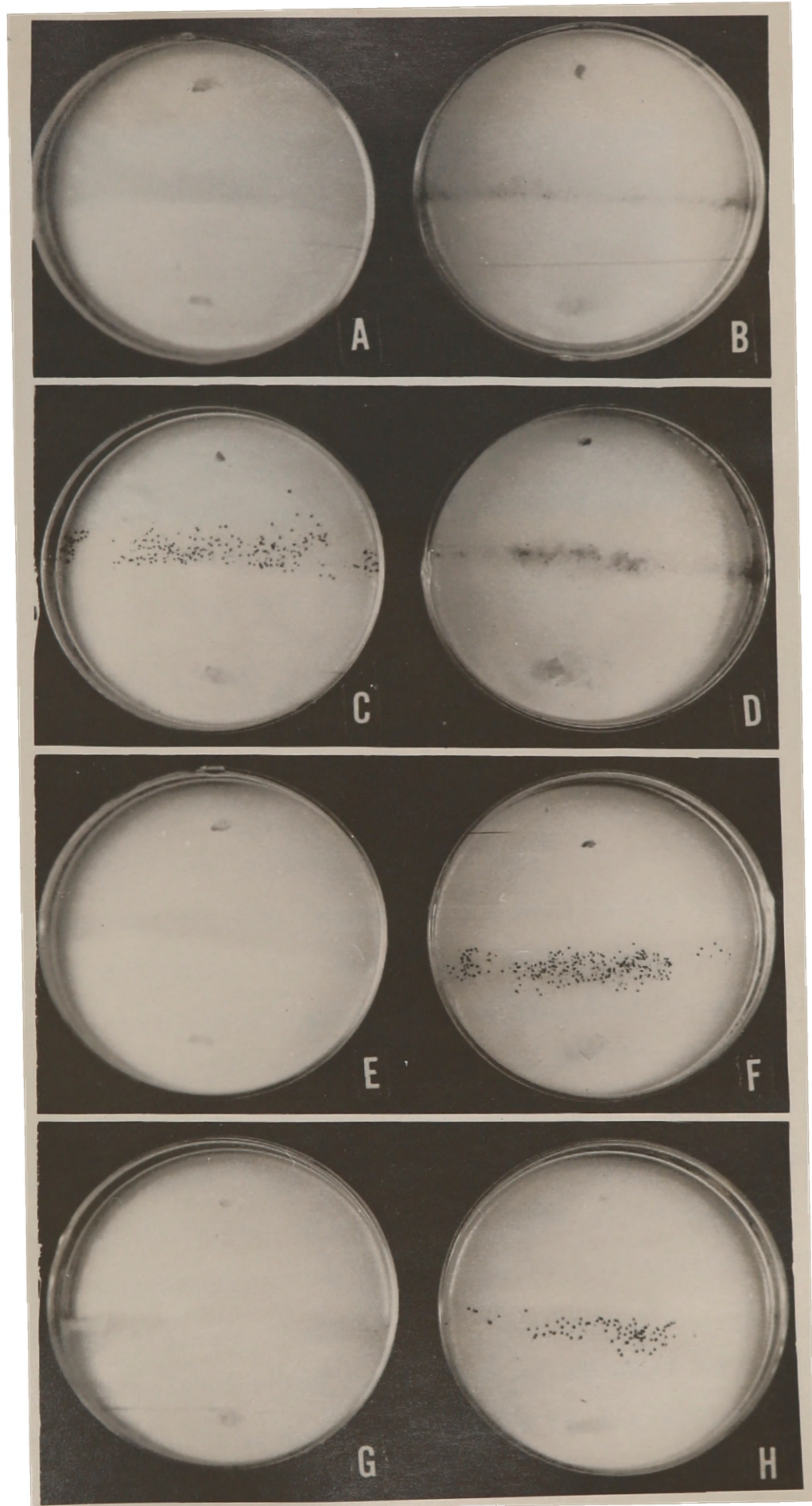
2. The Distribution of Perithecia in Petri Dish Matings

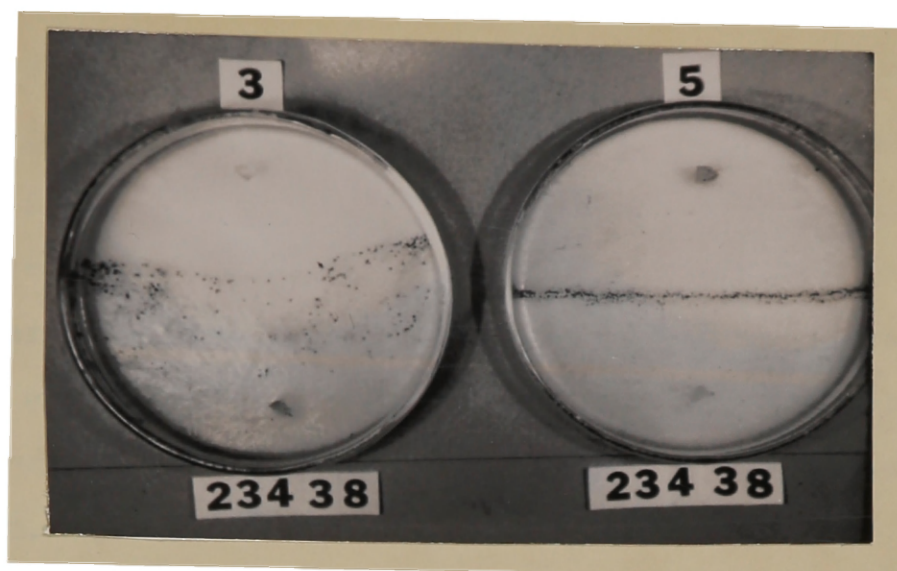
The distribution pattern of perithecia produced in Petri dish matings differed with the isolates employed. However for any two strains the pattern was constant at standard culture conditions. Two general types are recognized:

PLATE III

- Figures A-H. Results obtained when a representative four of the eight isolates obtained from a single ascus are backcrossed to parents A-3 and a-12. In Petri dishes, A, C, E, G, isolate A-3 was inoculated near the bottom side, and in B, D, F, H, a-12 was inoculated near the bottom side. Pairs of cultures as shown were inoculated: in each series of two, from top to bottom, A, B with isolates 117.1.1; C, D with 117.1.3; E, F with 117.1.5; and G, H with 117.1.7; all near the upper side of the culture dish.
- Figures A-B. Isolate 117.1.1 is sterile with both A-3 and a-12. However, the sterility observed in Figure A is not due to compatibility differences, but to the protoperithecial potential of the two strains involved. Neither of the isolates in Figure A produce protoperithecia and consequently perithecia are not produced.
- Figure C. Isolate 117.1.3, to top, produced protoperithecia upon isolation and gives the typical pattern when a protoperithecial and non-protoperithecial isolate are mated. The perithecia appear restricted on the 117.1.3 side of the dish.
- Figure D. This cross 117.1.3 x a-12 is sterile because the isolates are of the same mating type. The dark spots in the region of the confrontation are protoperithecia.
- Figure E. Cross 117.1.5 x A-3 sterile because isolates are of the same mating type.
- Figure F. Cross 117.1.5 x a-12 fertile, with perithecia on a-12 side, compare with Figure C.
- Figure G. Sterile because the isolates are of the same mating type as noted in Figure E.
- Figure H. Cross 117.1.7 x a-12, fertile, reaction normal and of the same kind as noted in Figure C and F.

PLATE III





Text Figure 10. Two basic perithecial distribution patterns:

Left: Cross A-3 x 234.3.8
yields perithecia
scattered to 234.3.8
side only.

Right: Cross 207.1.5
x 234.3.8 gives
perithecia to
both sides of a
narrow line.

Type I, represented by cross 3 x 234.3.8 in text figure 10, is characterized by the formation of perithecia to the one side of the culture. (In this case strain 234.3.8 is the protoperithecial strain and 3 is the non-protoperithecial strain); and Type II as shown by cross 5 x 234.3.8 is distinguished by the formation of perithecia on both sides of the culture generally as a narrow line where the two strains meet. As cultures become older the line of perithecia may broaden by the progressive formation of perithecia on both sides of the culture dish.

The protoperithecial potential of strains employed in such mating was found to be important in the determination

of the pattern. When only one of the isolates involved in a cross produces protoperithecia, a Type I pattern results. Type II pattern is obtained when two protoperithecial isolates are mated. Two non-protoperithecial isolates, when mated, are sterile and yield no perithecia to form a pattern.

It may be recalled that environmental factors such as light and temperature were also found to modify the patterns produced.

CHAPTER IV

DISCUSSION

Taxonomy and Morphology

The discovery of spermatia of Gelasinospora calospora var. autosteira, as reported in this investigation, removes any distinction between G. calospora var. autosteira and G. adjuncta. In all probability Cain's G. adjuncta represents a separate isolate of G. calospora var. autosteira. On the basis of morphological descriptions alone, Moreau and Moreau (32) have reduced G. autosteira (later changed to G. calospora var. autosteira by Sun, Alexopoulos, and Wilson (44)) and G. adjuncta to synonyms of G. calospora. However, until the two heterothallic species G. adjuncta and G. calospora var. autosteira are critically compared the taxonomy will remain somewhat uncertain. If they are the same species, as appears to be likely, it is of interest to note the distribution of the isolates. G. calospora var. autosteira was isolated from Spanish moss collected in Natchez, Mississippi (1), and G. adjuncta was isolated from dog dung collected in Germany (8). It is possible that other isolates of this strictly heterothallic Gelasinospora will be made in the intermediate area since it seems unlikely

that the fungus should be restricted to such widely separated localities.

The sexual structures produced when the isolates of G. calospora var. autosteira develop to their fullest potential are: 1) protoperithecia with trichogyne-like outgrowths, and 2) spermatia on spermatiphores. The former corresponds to the female and the latter to the male components of the mycelium. Hermaphroditic isolates are of either mating type, which indicates that the factors for sex and compatibility are separate entities. Basically G. calospora var. autosteira is hermaphroditic. Therefore, such unisexual isolates as A-3 are best considered as mutants which arose from a normally hermaphroditic isolate. This point of view is in agreement with that of Whitehouse (46) who also believes that such unisexual strains are mutants of normally monocious fungi. This interpretation seems most likely when one considers that the change from hermaphrodite to unisexual occurred with high frequency in certain isolates employed in this investigation.

Because the spermatia and trichogyne-like structures resemble those of other ascomycetes, it is very likely that future experiments will reveal that the sexual behavior of this species is similar to that of Bombardia lunata (48, 49, 50), Pleuraea anserina (2, 3, 4, 21, 35, 36), and Sclerotinia gladioli (20) all of which exhibit some form of heterothallism and produce both functional trichogynes

and spermatia. However, it was also observed, that when certain isolates were mated, few if any spermatia were produced and yet abundant mature perithecia developed. In this case it is evident that the compatible nuclei were brought into association by some other mechanism than spermatization. It is likely that the trichogyne finding no spermatium, fused with a hypha of the opposite strain. Sun (43) believed the ascogonial coil copulated with a hypha of the opposite strain, although she did not observe trichogynes or spermatia. It is unlikely that such plasticity as observed in *Neurospora* (5, 6, 11, 12, 13, 14, 15, 16, 19, 28, 30, 33, 34, 39, 40, 45) wherein the receptive hyphae may copulate with any portion of the thallus of the compatible strain will be demonstrated for Gelasinospora.

Influence of Environmental Factors

It has been repeatedly shown by numerous investigators that the environment may influence the expression of sexuality in fungi, plants, and animals. In the fungi, interest has centered about such pyrenomycetous fungi as Neurospora and Gelasinospora which are suitable for environmental as well as genetic studies. In G. calospora var. autosteira the expression of femaleness (protoperithecia) is influenced by the environmental as well as genetic factors. The formation of protoperithecia and perithecia was inhibited at 37° C

by some unknown mechanism. Hirsch (27) observed a similar temperature effect in Neurospora crassa and in addition found that a rather strong correlation existed between melanin production and protoperithecial formation. At higher temperatures where melanin production was reduced protoperithecial development was inhibited. It is possible such a relationship as just noted may also occur in Gelasinospora. However, the pigmentation of the isolates grown at different temperatures was not critically studied. It was noted, nevertheless, that aside from the light effects, all isolates producing abundant functional protoperithecia were always pigmented.

Genetic Segregation of Morphological Sex Factors

In certain ascomycetes the factors responsible for the differentiation of morphologically distinct sex organs or gametes and those determining compatibility may both be segregated during meiosis. So far as has been investigated it has always been found that these factors are at separate loci and may or may not be linked (35). Heterothallic forms sensu Dodge (16) of this nature may segregate to produce unisexual strains as exemplified by: Bombardia lunata (46, 47, 48), Hypomyces solani f. cucurbitae (22, 25, 26), Neurospora sitophila (5, 19), and Chalara quercina (7).

In most of these forms there is still a question as to whether or not the production of protoperithecia or asco-carp initials is controlled by a single factor pair.

The results with Gelasinospora calospora var. autosteira, obtained in this investigation indicate that a more complex segregation is occurring than can be explained by single factor segregation. This situation finds a parallel in some other heterothallic fungi. For example Aronescu (5) was unable to reach any definite conclusions about protoperithecial segregations in Neurospora sitophila because of the unusual ascal segregation patterns obtained through the study of serially isolated ascospores. But, using the same strain material Dodge (19) reports a simple 1:1 segregation for protoperithecia. However, Dodge (19) used random single ascospores whereas Aronescu used serially isolated ascospores which may account for the difference in results. Dodge (19) studied other wild type strains which he had, and could not find the same phenomenon occurring: he states: "the writer, after some study of the wild-type races mentioned above is not in a position to warrant any conclusion whatever on this matter of production of protoperithecia. Receptive bodies may often consist merely of differentiated special hyphal branches which could be called ascogonia". In Bombardia lunata, Zickler (49) found that for the most part a 1:1 segregation for ascocarp initials occurs, but he indicated that sometimes ascocarp initials are produced by the lan (male) strains. His bulb strains produce both ascogonia and spermatia. The incomplete segregation noticed by these workers as well as that obtained in this investigation would

seem to indicate that the factors controlling sex are extremely complex and one may doubt as to whether complete segregation will ever be obtained. On the other hand, however, there are other investigators (7, 22, 25, 26) who have reported a complete segregation for sex.

Perhaps the clearest case of segregation for ascocarp initials (female) and non-ascocarp initials (male) was obtained by El-Ani (22) in his revaluation of the data of Hanson and Snyder (25) and Hirsch (26) on Hypomyces solani f. cucurbitae. El-Ani (22) isolated the eight ascospores from each of 42 asci and determined the sex by employing a function criterion, e.g., mating to suitable tester strains. Excluding the male factor, which is of no value in this discussion, he found a definite 1:1 segregation (138 isolates female; 138 isolates male) for perithecial initials. It is believed that if a functional rather than a morphological criterion is used for determining the presence of protoperithecia in G. calospora var. autosteira it is not improbable that a definite segregation ratio may be obtained as is the case in Hypomyces.

During this investigation the writer has been convinced that the genus Gelasinospora or species thereof can be developed into exceedingly important tools for the study of heredity in the fungi; and that particularly G. calospora and its variant autosteira has such great possibilities so

as to even exceed those which have been exploited in Neurospora. The characteristics which make this genus of particular interest in genetic studies are:

- (1) Species of Gelasinospora are remarkably similar and closely related to Neurospora, about which considerable information is already known. Furthermore, all the advantages for heredity studies which are exhibited by Neurospora are also exhibited by Gelasinospora.
- (2) The unique situation in which both strictly homothallic and strictly heterothallic strains are both present in the same species, G. calospora, affords a unique opportunity to investigate the fundamental nature of hetero- and homothallism, and its relationship to morphologically distinct sex structures.
- (3) The conidia of Neurospora are often objectionable because they are easily carried by air currents and thereby may serve as a source of contamination. These objections are avoided since Gelasinospora does not produce conidia.

CHAPTER IV

SUMMARY AND CONCLUSIONS

1. A study of the heterothallic Ascomycete, Gelasinospora calospora var. autosteira, revealed the presence of both spermatia and protoperithecia with trichogyne-like structures on mycelium derived from single ascospores of both mating types. Accordingly, G. calospora var. autosteira consists of strains which, in theory at least, are hermaphroditic and self-sterile; perithecia are formed only when strains of opposite mating type are mated. The morphology of spermatia and spermatophores and protoperithecia with "trichogynes" is described.

2. A study of environmental factors which may influence fruiting in Gelasinospora calospora var. autosteira indicates that temperature, light, and media are important. Growth was obtained at all temperatures ranging from 12 to 37° C. At 37° C neither protoperithecia nor perithecia were produced by isolates A-3 and a-12. Best ascospore production occurred at 20° C, other conditions being favorable.

Light was found to be important in that few or no perithecia or protoperithecia were produced in cultures incubated in the dark. Pigmentation and aerial mycelium were more pronounced in cultures grown in the dark.

The number of perithecia and protoperithecia increased when the quantities of medium employed in Petri dishes was increased up to 50 ml, the limit employed in the study.

The distribution of perithecia in Petri dish cultures was strongly modified by culturing in the dark, and only partly modified by temperatures of 30 and 28° C.

In culturing G. calospora var. autosteira, such environmental factors must be taken into account. The most suitable conditions of cultures which were successfully employed are as follows: Difco corn meal agar, 25 ml per plate, 20° C, with natural or artificial light supplied throughout the culture period.

3. One hundred and forty-two asci from cross A-3 and a-12 were dissected and their spores were analyzed for mating type, pigmentation, and protoperithecia. The Mt and G loci were found to be linked and located in the same arm of the chromosome. The Mt locus is proximal to the centromere and 2.4 units away, whereas the G locus is 8.8 units from the Mt locus and distal to the centromere. Segregation for protoperithecia was incomplete.

The factor or factors responsible for the production of protoperithecia segregated in a 1:1 ratio in 86 of the 142 asci analyzed. The P factor complex appeared to be linked to Mt and G. Although the segregation of protoperithecia is incomplete and perhaps too complicated for analysis at this

time, account should be taken of the fact that the segregation which does occur contributes to sterility. If isolates are unable to produce protoperithecia they will be sterile in certain combinations regardless of the mating type factor.

4. Cultural behavior studies made with G. calospora var. autosteira indicate that certain definite distribution patterns of perithecia may be observed in Petri dish matings. These patterns may be classed as follows: 1) perithecia distributed in such a fashion so that they are all restricted to one thallus of the two strains employed in the cross, and 2) perithecia distributed to both sides, appearing on both thalli of the strains involved in the cross. These distribution patterns are correlated with the capacity of the individual isolates to produce protoperithecia when grown alone.

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