THE FUNCTIONS OF THE MICROSPORES OF THE HETEROTHALLIC PYRENOMYCETE GELASINOSPORA CALOSPORA (MOUTON) MOREAU ET MOREAU, VAR. AUTOSTEIRA (ALEXOPOULOS ET SUN) SUN, ALEXOPOULOS ET WILSON

by

Bernard Joseph Sloan

AN ABSTRACT

Submitted to the School for Advanced Graduate Studies of Michigan State University of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Department of Botany and Plant Pathology

Year 1957

Approved ________________________________

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The functions of the microspores of *Gelasinospora calospora* var. *autosteira* are discussed. Evidence is presented which demonstrates that the microspores function primarily as male sexual cells (spermatia) in fertilization. Circumstantial evidence is also presented which strongly indicates that the microspores may function as asexual propagative cells (microconidia) under certain environmental conditions.

Results obtained from spermatization, cross spermatization, and localized perithecial development, suggest that a trichogyne-microspore relationship is functional in this fungus. Additional results obtained from experiments involving a study of the u-tube and hyphal tip subcultures indicate that compatible nuclei of this heterothallic fungus are not brought into close associations with one another by somatic anastomoses and nuclear migrations.

It is suggested that compatible nuclei occur in close associations only in the ascogonia by way of the trichogyne

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SECTION I

INTRODUCTION

Gelasinospora calospora var. *autosteira* as described by Sun, Alexopoulos and Wilson (33) in 1954, was originally found to be heterothallic by Alexopoulos and Sun (1). In 1955, Tylutki (35) demonstrated further that this fungus was fundamentally hermaphroditic since single ascospores of both compatibility groups normally are equally able to produce both spermatia and protoperithecia in culture. The latter author also described at this time the formation of spermatia by most isolates of this fungus, particularly under certain environmental conditions, and indicated the possible existence of a trichogyne-spermatium relationship. Cain (9) in 1950 reported spermatial production in *Gelasinospora adjuncta* but no work has been done to show how these structures function. Since there had been no study made in relation to the function of the spermatium-like bodies produced by *Gelasinospora calospora* var. *autosteira*, it was suggested that an investigation be conducted to determine the possible function of these structures and the mechanism whereby plasmogamy was initiated. With these aims in mind, research was directed toward determining whether:

(1) the microspores functioned as sexual male cells (spermatia),

(2) the microspores functioned as asexual cells capable of reproducing the organism (micronconidia),

(3) the microspores functioned as both asexual and sexual structures.

Additional research was directed toward determing whether spermatization is the only method of insuring cross fertilization in this heterothallic fungus. Dodge (13) has expressed the opinion that those who have discovered spermatia in the past tend to magnify their importance and ignore the numerous other ways in which heterothallic fungi are able to become cross-fertilized. Since macroconidial production has never been reported for any of the several described species of Gelasinospora, unlike the situation in the related genus Neurospora, the only possible methods available other than spermatization, whereby cross-fertilization could be accomplished, would seem to be through somatogamy, or copulations between trichogynes and vegetative hyphae of compatible strains.

Because of these possibilities, additional lines of investigation were initiated to determine whether:

(1) a trichogyne-microspore relationship was existent in this fungus (see Plate I), or

(2) nuclei of opposite mating types were transferred through somatic hyphae.

PLATE I

Figures A. C. Sexual structures of <u>Gelasinospora</u> <u>calospora</u> var. <u>autosteria</u>.

Figure A. Young protoperitheical fundament with its single unbranced trichogyne

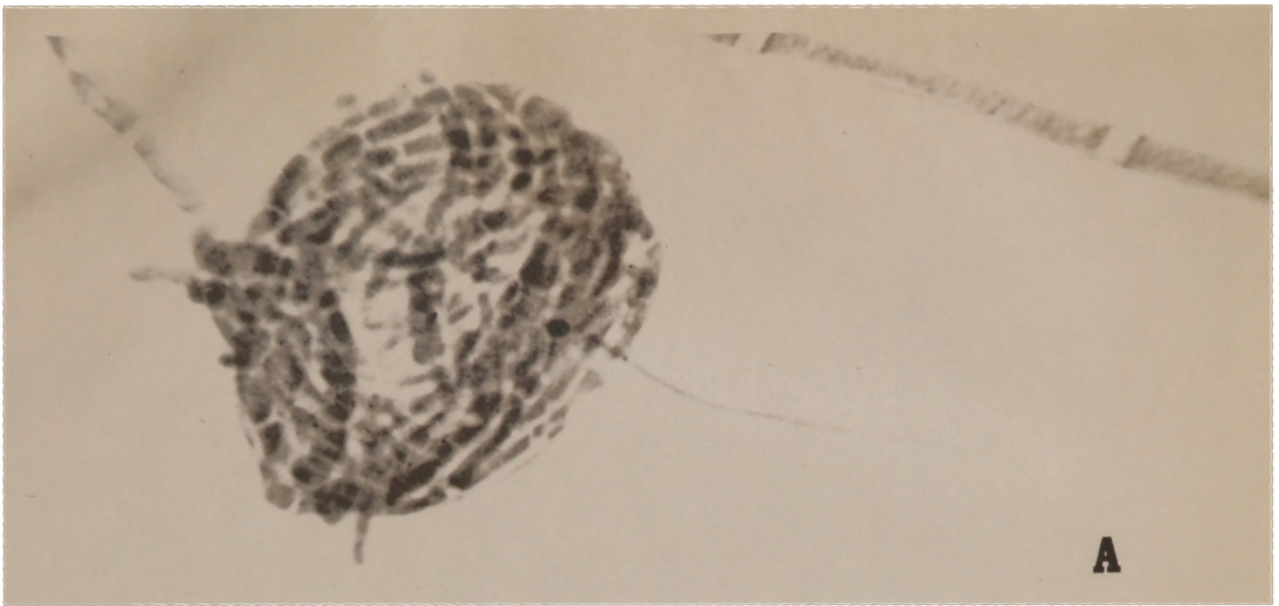
ca. x 440

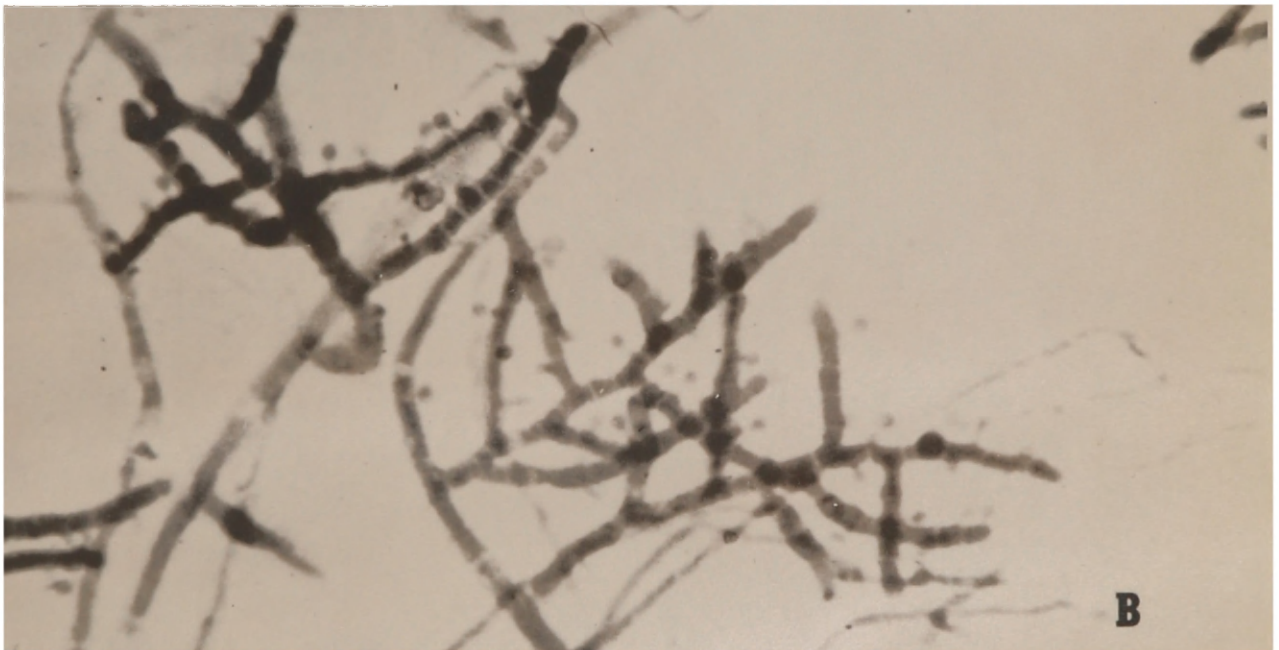
Figure B. Several dichotomously branched microsporophores. The microspores are produced from the individual cells of the microsporophores

ca. x 1000

Figure C. A single germinating microspore. The microspore wall still remains attached to the long germ tube.

ca. x 1000


A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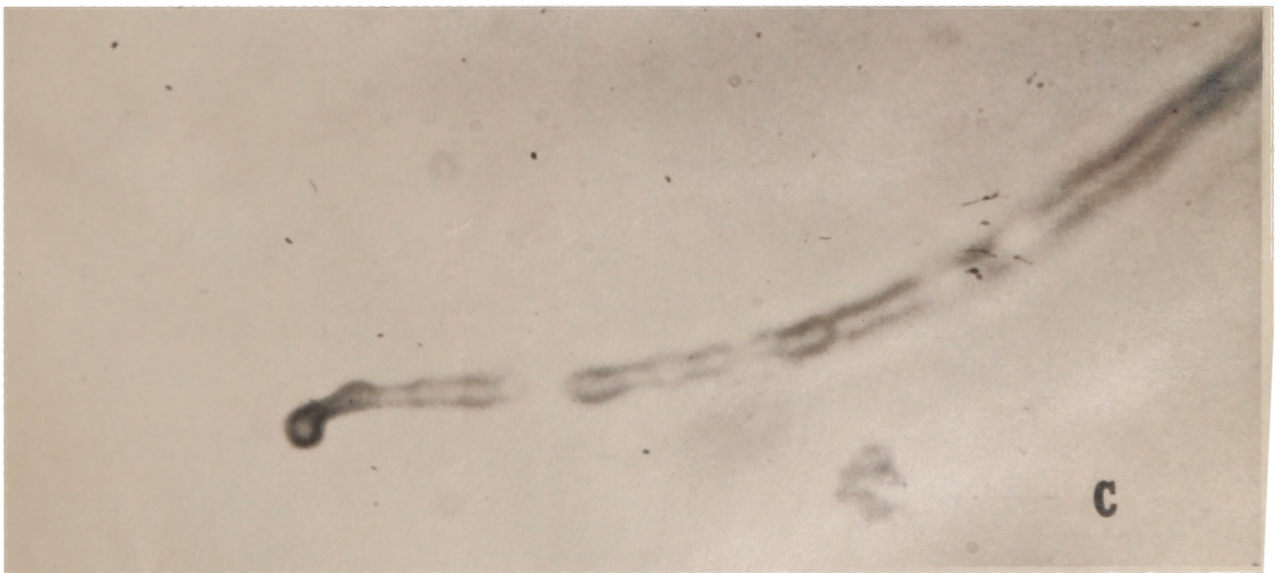
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Literature Review

Since the literature concerning methods of fertilization in the fungi in general have been discussed recently by Olive (30), only those cases pertinent to the problem will be discussed here.

Microspores (spermatia, pycniospores, or microconidia) are known to be found characteristically in certain groups of the algae (Florideae), fungi (rusts, ascomycetes), and in lichens.

Definite proof that microspores in the ascomycetes may function as fertilizing agents did not appear in the literature until the early thirties. According to Backus (7), Itzigsohn in 1850, discovered small bodies produced by some ascolichens, and indicated that these structures may prove to be male elements. Tulasne, a year later, according to Drayton (19), in describing these small structures, coined the word spermatium, and prophesied in 1861 that some day these small structures would be shown to function like pollen.

Stahl, according to Backus (6), even went further than Tulasne. He actually described the process whereby spermatia of the lichen fungi effect fertilization by attaching and fusing with tips of trichogynes. Humphrey (23) in 1893 described how the microconidia of Sclerotinia fructigena germinated in his cultures and produced mycelia. However, many have questioned his work.

In the Laboulbeniales, Thaxter (34) has suggested that in most species described, development of the ascocarps depends upon the direct attachment of spermatia to trichogynes and subsequent passage of nuclei down into the ascogonia. However, cytological data to confirm these findings are lacking.

Higgins (21, 22) working with two species of *Mycosphar-ella* described the formation of trichogynes and spermogonia and even illustrated spermatia attached to trichogynes, but the author was unable to find any direct evidence that the spermatia play any part in perithecial development.

Backus (6) in 1934, working with the fungus *Coccomyces hiemalis*, described how microconidia attach themselves to tri-chogynes but no actual fusions between the spermatial bodies and the trichogynes were ever seen.

Thus as indicated from the previous remarks, no reports actually appeared in the literature up to 1930 which demon-strate the ability of microspores to effect fertilization. However, numerous descriptions of various types of spermatial or microconidial bodies reportedly produced in the ascomycetes and imperfects, had appeared in the literature prior to this date.

Drayton (19), Dodge (11) and Ames (3) in the year 1932, demonstrated conclusively that in *Sclerotinia gladioli*, *Neuro-spora sitophila*, *Neurospora tetrasperma*, and *Pleurage anserina*, the microspores act in a fertilizing capacity.

In Neurospora sps., Dodge demonstrated that the microspores will not only effect fertilization but will also germinate and give rise to mycelia which in turn produce protoperithecia and microconidia. These can be mated together with a compatible strain to produce ascocarps.

However, this is to be contrasted to the situation observed in Sclerotinia gladioli and Pleurage anserina in which it has been demonstrated that the microspores do not germinate but function only as male gametes which when placed on receptive bodies bring about the formation of ascocarps.

Zickler (38) isolated a coprophilous ascomycete from horse dung in 1934 and described it as a new species, Bombardia lunata. In the year 1937, Zickler (39) described how colourless uninucleate spermatia were produced in flask shaped spermogonia which were developed laterally on the hyphae produced by Bombardia lunata. These spermatia, like those produced by Pleurage anserina and Sclerotinia gladioli, were similarly shown to be incapable of germination to form a colony but functioned solely as fertilizing agents.

In 1950, Alexopoulos and Sun (1) described a new species of Gelasinospora, G. autosteira, which was found to be heterothallic, self-sterile, but cross-fertile. In the authors' original description, spermatia were not reported. Following a cytological investigation of the genus Gelasinospora, a few years later, Sun, Alexopoulos and Wilson (33) proposed that the name Gelasinospora calospora var. autosteira

be used rather than the original described name, to separate the heterothallic form (*G. autosteira*) from the homothallic form (*G. calospora*).

Later, while conducting a morphological and genetical investigation of *Gelasinospora calospora* var. *autosteira*, Tylutki (35) reported that certain strains of this fungus produce spermatia regularly in culture. Although an excellent morphological description of the spermatia was presented by this author, the function of the spermatium-like bodies was not investigated, though it was suggested that a trichogyne-spermatium relation does exist.

Of the remaining six species of *Gelasinospora*, spermatial production has been reported only in *Gelasinospora adjuncta* as described by Cain (9). Although Cain was able to produce spermatia in this fungus under certain cultural conditions, no work was done to show how these structures function.

SECTION II

MATERIALS AND METHODS

A. Cultural Conditions

A description of the original isolates from which the strains employed in this study were obtained, as well as the method used in maintaining stock cultures has been previously reported (34). Furthermore the standard cultural conditions as outlined by Tylutki (35) were employed here in growing cultures of the protoperithecial strain. These standard cultural conditions are as follows:

(1) Medium:

Difco corn meal agar, 17 gms. in a liter of agar.

(2) Temperature:

Twenty degree C, obtained by using a B.O.D. temperature control cabinet.

(3) Light:

A Sylvania 14-watt cool light standard florescent tube, with the ballast unit removed and placed outside the incubator. The tube was placed approximately 28 cm. from the culture level.

The microspore-producing strains were grown in large test tubes containing 20 ml. of Difco corn meal agar. These test tube corn meal agar slant cultures were placed inside a 28°C incubator and incubated for approximately ten days at which time sufficient quantity of microspores were produced.

B. Technique Employed in Obtaining Microspore Suspension

1. Apparatus

The most satisfactory method of obtaining an aqueous suspension of the microspores, free of viable mycelial fragments, was to filter the microspore suspension through several layers of filter paper. A description of the filter apparatus used is as follows:

> The glass tube of a glass funnel was fitted with a No. 9 one-hole rubber stopper, and this was inserted into the neck of a 500 ml. Erlenmeyer flask containing a large test tube which had been previously placed inside the Erlenmeyer flask. Four or five layers of 12 cm. No. I filter paper were placed inside the funnel. Then the base of the funnel and neck region of the flask were wrapped with cotton and a 400-ml. beaker was placed over the entire apparatus.

In preparing a microspore suspension, the filtering apparatus was first sterilized in an autoclave for thirty minutes at fifteen pounds pressure per square inch. The filtering apparatus is illustrated in text figure 1.

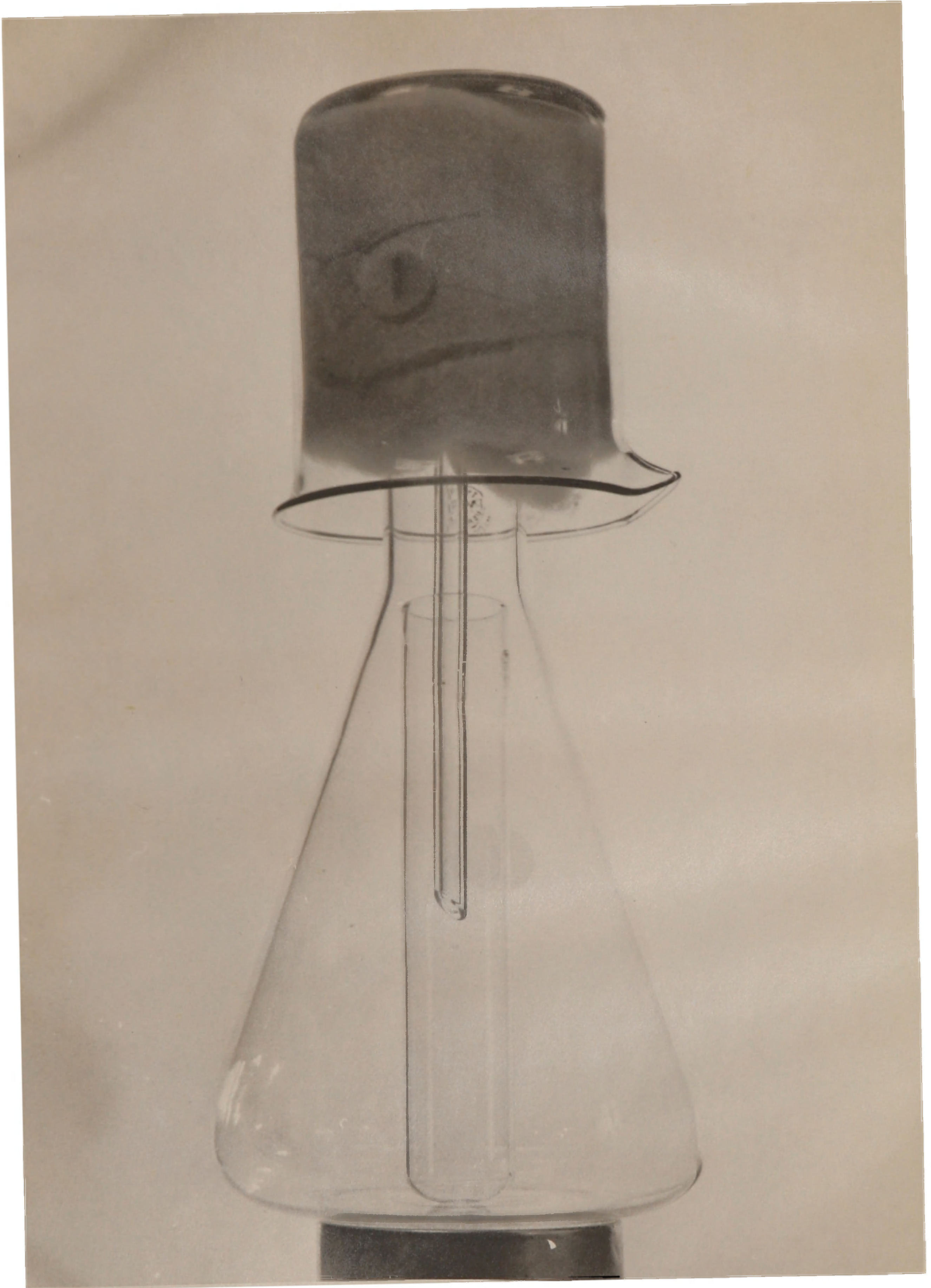


Figure 1. Filtering Apparatus Used to Obtain Mycelial-free Suspension of Microspores.

2. Procedure

All strains employed for microspore production were grown in test tubes on slants of Difco corn meal agar, incubated at 28°C (±°C) for approximately ten days. When microspore production became discernible with a binocular dissecting microscope, sterile distilled water was pipetted into the test tube cultures until the entire agar slants became completely inundated.

The test tube cultures were then placed between the palms of both hands and rolled back and forth approximately fifty times. This mild manipulation was found to be quite effective in dislodging the microspores from the microsporophores without disrupting in any serious degree the somatic mycelial growth covering the agar slants. Therefore, this procedure minimized the actual amount of viable mycelial fragments which may be obtained with the microspore suspension prior to the filtration procedure.

The aqueous microspore suspension within each test tube was then poured through several layers of filter paper and collected inside sterile test tubes located within the Erlenmeyer flask of each filtering apparatus. Sterile plugs were then inserted by aid of sterile forceps into the mouth of the test tubes. Microscopic check of the microspores in suspension on slides were made to see if hyphae were present before spermatization. No hyphal fragments were detected in any of the microspore suspension samples tested. The

microspore suspensions were then labeled and ready for use.

3. Dilution Technique

In order to spermatize with a definite number of spores, microspore suspensions were prepared and microspore counts were made by use of a Howard Corpuscle counting chamber. This chamber is divided into a total of twenty-five squares, each of which is subdivided into sixteen divisions. In determining a microspore count, the outermost square of each corner of the chamber and the square located in the exact center of the chamber were selected for use. This involved a total of five squares each of which was further subdivided into sixteen divisions making a grand total of eighty squares. Repeated trial spore counts were made until an average was maintained. Then the actual spore count was derived for each microspore suspension. Following this procedure, a series of microspore dilutions was prepared.

C. U-Tube Experiments

U-tubes were used in this study in an attempt to determine whether nuclear migration and mycelia dikaryotic for opposite mating types, could occur in *Gelasinospora calospora* var. *autosteira*.

The u-tubes were heated in an oven for approximately three hours at 160°C and then corn meal agar which had been previously prepared and held in test tubes for a few days,

was remelted and poured into the u-tubes and allowed to solidify. Following inoculation of the arms of the u-tubes with blocks of agar containing mycelia, or with a suspension of the microspores, the u-tubes were placed inside a large dessicator containing sterile distilled water in the bottom. This served as a moist chamber. The moist chamber was then placed inside a 20°C ($\pm$1°C) incubator. This procedure was quite effective in preventing drying out of the agar medium in the u-tubes. Even after months of incubation, the u-tubes were still found to be free of air spaces.

SECTION III

RESULTS AND OBSERVATIONS

A. Microspores

I. Size and Morphology

The size, morphology and formation of spermatium-like bodies on unbranched and dichotomously branched spermatiophores as well as a description of the formation of the protoperithecial fundaments by many strains of *Gelasinospora calospora* var. *autosteira* (*G. autosteira*) have been recently described by Tylutki (35). It was also indicated in that report that a possible trichogyne-spermatium relationship exists in this fungus. In this investigation, the results obtained by the previously mentioned authors in relation to the size and morphology of the spermatium-like bodies, have been verified. In addition, however, facts concerning the function of the spermatium-like bodies and the possibility of nuclear migrations occurring through somatic hyphae have been investigated and are presented.

II. Function

An attempt was made to determine whether the spermatium-like bodies, which henceforth shall be designated as microspores, of *Gelasinospora calospora* var. *autosteira* (*G.*

autosteira) function as male sex cells (spermatia), as asexual cells (microconidia) endowed with the capacity to reproduce the organism, or as both. Since some isolates have been found to produce only protoperithecia or microspores in culture while others have been found to be completely cross sterile or weakly fertile in compatible matings, only two strains of both mating types designated as A and B were selected for study:

(1) a protoperithecial, microspore producing "A" strain, "214.1.3,"

(2) a protoperithecial, microspore producing "B" strain, "227.3.7,"

(3) and two non-protoperithecial, microspore producing "B" strains, "214.1.1," and "106.1.4."

The description of the original strains from which these isolates were obtained as well as the designation system employed for each of the four isolates, has been previously reported by Tylutki (35). It is 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.

a. Role of Microspores in Fertilization

In an attempt to determine the possible funtion of the microspores of Gelasinospora calospora var. autosteira (G.

autosteira) in fertilization, the following series of experiments was employed.

The two perithecial, microspore-producing strains were grown separately on 20 ml. of Difco corn meal agar in Petri dish cultures. These cultures were kept at 20°C($\pm$1°C) inside a laboratory incubator under constant illumination for approximately eight days. The two microspore-producing strains were grown in test tubes on corn meal agar slants. These test tube cultures were incubated at 28°C since this higher temperature was found to favor maximum microspore formation. The formation of microspores usually occurred within twenty-four hours following incubation at this particular temperature. However, in order to obtain a sufficient quantity of the microspores, the test tube cultures were incubated for at least ten days. Observation of the cultures at this time revealed the formation of microsporophores quite abundantly at the upper end of the agar slants. Some, however, were also found scattered over the entire slant. An aqueous suspension of the microspores was prepared as described above. A microscopic check for hyphal fragments was made prior to using the suspension for spermatization. No such fragments were ever detected.

When protoperithecia were produced on the plate cultures of corn meal agar, and were easily discernible with the unaided eye, four sterile glass rings were placed on the agar over the areas where protoperithecia had been produced, and 0.2 ml. of an aqueous suspension of microspores of the

opposite mating type were placed in two of the glass rings. The remaining glass rings received 0.2 ml. of sterile distilled water and served as a control in the experiment. In order to maintain a control against possible carryover of mycelial fragments in the microspore suspension, one or two ml. of the microspore suspension was poured over several sterile corn meal agar plates. All cultures in Petri plates were then returned to the 20°C incubator and kept under constant illumination. Within twenty-four to thirty-six hours following this treatment, young perithecia were produced over the small areas inside the rings where drops of water carrying the microspores had been placed, Plate II. When mature, these perithecia contained asci with mature dark, pitted ascospores. No perithecia were ever produced in those rings which had received drops of sterile distilled water. This experiment has been set up in replicates of twenty or more Petri plates and repeated numerous times. In every instance, identical results were obtained.

The several corn meal agar plates which had received one or two ml. of the microspore suspension, remained sterile indicating:

(1) lack of any viable mycelial fragments in the microspore suspension,

(2) inability of microspores to germinate and produce vegetative mycelium on Difco corn meal agar at least under the environmental conditions employed.

PLATE II

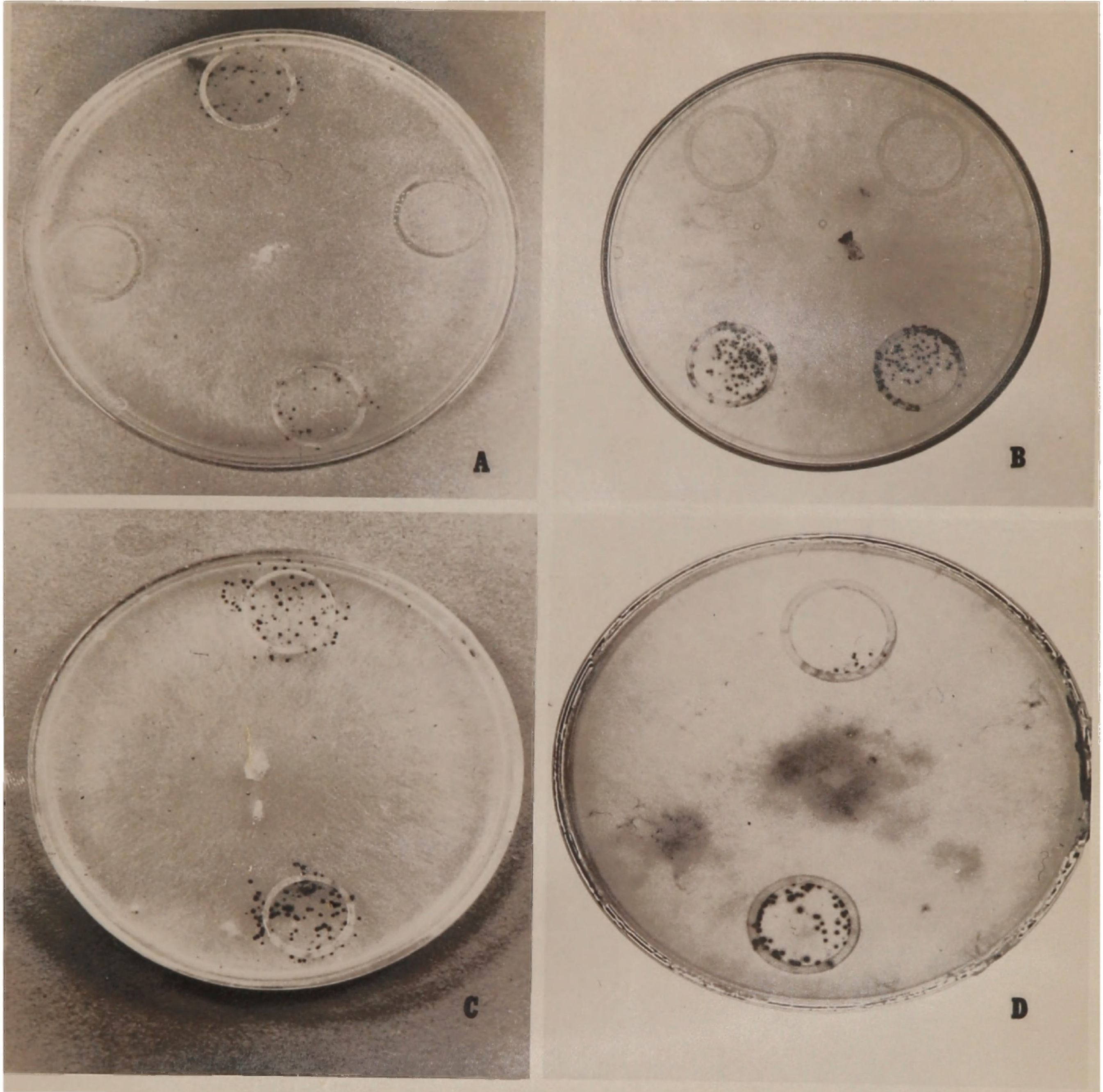
Figures A-D. Perithecial formation resulting from the placing of microspores of one mating type over protoperithecia of the opposite mating type.

Figure A. Perithecia produced in rings resulting from the spermatization of protoperithecia of strain 214.1.3 with a suspension of microspores of strain 214.1.1. Perithecia failed to develop in the control rings.

Figure B. Same as in Figure A.

Figure C. Reciprocal cross between two hermaphroditic strains in which the microspores of strain 214.1.3 were placed on the protoperithecia of strain 227.3.7.

Figure D. Reciprocal cross between two hermaphroditic strains in which the microspores of strain 227.3.7 were placed on the protoperithecia of strain 214.1.3.


A
B
C
D

1. Reciprocal cross

Since it was desirable to make a reciprocal spermatization cross between two self-sterile, but cross-fertile hermaphroditic strains, an experiment was conducted in which the microspores of strain 214.1.3 were employed to artificially fertilize the protoperithecia of strain 227.3.7 and the microspores of the latter strain to fertilize the protoperithecia of the former. Within thirty-six hours perithecia were produced in the immediate areas delineated by the rings where the drops of water containing the microspores had been placed, Plate II. In relation to the previously described experiments on spermatization as well as the reciprocal spermatization just described, it was noted that the microspores never produced mycelia when an aqueous suspension of the microspores was placed on Difco corn meal agar. The microspores swelled to several times their normal volume on agar and some were even detected producing extremely long slender germ tubes. However, at no time were these germ tubes found capable of reproducing the organism even after several weeks under cultural conditions which were apparently favorable for mycelial growth of this organism.

In view of the results obtained at this time, it appeared certain that the microspores of *Gelasinospora calospora* var. *autosteira* (*G. autosteira*) functioned only in the capacity of sexual male propagative cells and therefore, the microspores were designated as spermatia. Furthermore, the localized

perithecial development within the rings when microspores of the appropriate mating type were placed on the protoperithecial areas of the mycelium tends to suggest a possible trichogyne-spermatium relationship existent in this fungus.

2. Localized development of perithecia.

The localized development of perithecia has been obtained in various other ways, in addition to the method previously described. This is evident from the results of two experiments now to be described.

Droplets (0.2 ml.) of the microspore suspension of strain 214.1.1 was placed inside glass rings on young cultures of strain 214.1.3 where the mycelium had grown out but where no protoperithecia had yet developed. As soon as protoperithecia were produced in these areas, the placing of microspores of the opposite mating type on these structures resulted in the localized development of perithecia inside the glass rings. In another similar experiment, when microspores of strain 214.1.1 were placed on one side of sterile corn meal agar plates, and the opposite sides of the sterile plates were inoculated with strain 214.1.3 one week later, the same localized development of perithecia occurred on the sides of the plates containing the microspores, but only after the mycelium of strain 214.1.3 had grown over the microspores and produced protoperithecia in the areas. This suggests that the microspores remain viable and that they

function as sexual fertilizing agents as soon as the female sexual structures develop.

3. Spermatization experiments involving definite number of spores.

In an attempt to obtain additional data which might offer further evidence of a possible trichogyne-spermatium relationship, a series of experiments were conducted involving over sixty-five plate cultures of either strain 214.1.3 or strain 113.1.1. In this series of experiments, the approximate number of microspores of strains 214.1.1 or 106.1.4 to be placed inside glass rings on cultures of the opposite mating type, was determined. The plate cultures of strain 214.1.3 were incubated for approximately eight days at 20°C(±°C) under constant illumination. The microspore-producing strains, 214.1.1, and 106.1.4, were grown in test tubes on corn meal agar slants at approximately 28°C for ten days. Microspore suspensions were then prepared and microspore counts made by use of a Howard Corpuscle counting chamber as described in Section II. The serial, two-fold tube dilution technique, was used to prepare the series of microspore dilutions in both experiments, Tables I and II.

Two sterile glass rings were placed on the agar over groups of protoperithecia and 0.4 ml. of the microspore suspension of the opposite mating type was placed in each of the rings. With one exception, replicas of four petri dishes were set up for each of the microspore dilutions employed. In the

TABLE I

EFFECT OF MICROSPORE CONCENTRATION OF STRAIN 106.1.4
ON PERITHECIAL PRODUCTION OF STRAIN 113.1.1

	Total Number of Microspores used to Spermatize+					
	0	8	80	800	8,000	80,000
Plate No.						
1	-	-	-	10	2	28
2	-	-	-	2	-	23
3	-	-	-	-	-	*
4	-	-	-	-	-	*

- = no perithecia present

* = no culture spermatized

+ = amount of liquid used to spermatize (0.4cc.)

Medium = Difco corn meal agar

TABLE II

EFFECT OF MICROSPORE CONCENTRATION OF STRAIN 214.1.1 ON PERITHECIAL DEVELOPMENT OF STRAIN 214.1.3

	Total Number of Microspores used to Spermatize+						
	0	2	12	120	1,200	12,000	120,000
Plate No.							
1	-	-	2	-	1	1	66
2	-	-	-	-	-	9	145
3	-	-	-	-	-	16	103
4	-	-	-	-	-	2	1
5	-	-	-	-	-	1	3
6	-	-	-	-	-	4	16

- = no perithecia present

+ = amount of liquid used to spermatize (0.2cc.)

Medium = Difco corn meal agar

one exception, only two plates were spermatized at this particular microspore dilution rather than four, Table I.

In a later series of experiments, 0.2 ml. of microspore suspension of the opposite mating type was placed in each of the rings. Each plate was set up in replicas of six for each microspore dilution used, Table II.

In both series of experiments conducted, the customary controls were maintained. All plates were allowed to incubate at 20°C($\pm$°C) under constant illumination for thirty hours after which time the plates were examined. Reference to Tables I and II discloses that Petri plates containing glass rings which received approximately eighty microspores or fewer failed to produce perithecia. Likewise perithecial production was extremely poor even though microspore concentration added to the rings was as high as eight thousand. Good production of perithecia was evident, however, when the quantity of microspores numbered approximately eighty thousand and above, Tables I and II.

On the basis of these results, it would appear that the quantity of microspores placed inside the glass rings exerts a direct influence on the number of perithecia eventually produced. Although the quantity of perithecia formed in each ring depends to a large measure on the number of protoperithecia located in that area, it does not necessarily hold true that every protoperithecial fundament within the ring will develop into a mature perithecium. Rather, it is

believed that only those protoperithecial bodies, whose trichogynous extensions have come in direct contact with one or more microspores of the opposite mating type, are capable of being fertilized and developing into mature perithecia. The dilution experiment data appear to indicate that a great quantity of male sexual cells must be added to the rings in order to insure the possibility of some coming into contact with the trichogynes. Apparently the majority of the microspores fail to make contact and lie dormant on the mycelium or at the base of many protoperithecia. Backus (7) and other investigators have shown that microspores of *Neurospora sitophila* which normally are capable of germinating and producing mycelial growth on sterile agar, are incapable of germination when placed in an area already occupied by mycelium.

b. Role of Microspores in Asexual Reproduction

Cultural studies have been conducted to determine whether microspores were capable of germinating and producing mycelial growth on corn meal agar. One ml. of an aqueous suspension of microspores of strain 214.1.1 was placed inside sterile glass rings on sterile corn meal agar plates. Following this treatment the sterile Petri plates were placed in triplicate inside incubators and cultured at 12, 16, 20, 24, 28 and 30°C. With the exception of Petri dishes cultured at 20°C, light was not constant in this experiment. The customary control was maintained in this experiment by spermatizing

protoperithecia contained inside glass rings on eight-day-old cultures of strain 214.1.3.

The Petri dish cultures were incubated for two weeks and then examined. Microscopic examination revealed the complete lack of mycelial growth on any of the Petri dish cultures at the various incubation temperatures. Microscopic examination under high power revealed that the majority of microspores failed to germinate. However, some had produced short germ tubes.

Mature perithecia containing typical ascospores were produced on the control plates.

A further attempt was made to determine whether the microspores of Gelasinospora calospora var. autosteira (G. autosteira) functioned only as male cells in fertilization, or whether they could be induced to function as microconidia under certain environmental conditions.

Accordingly a series of experiments was set up involving over seventy-five Erlenmeyer flasks containing from 5 ml. to as much as 40 ml. of corn meal broth per flask. Each flask received 5 ml. of water suspension containing microspores of one of the three strains 214.1.1, 214.1.3, and 227.3.7. The flasks containing corn meal broth were placed either on a horizontal shaker or on a shelf located in a constant temperature room. To maintain a control in this series of experiments, in order to illustrate that the microspores used were viable in functioning as spermatizing

cells and incapable of producing mycelial growth on corn meal agar, 2 ml. of microspore suspension was poured onto several corn meal agar plates. Also protoperithecia of eight day old cultures of strain 214.1.3 contained withing glass rings were spermatized with 0.2 ml. of either strain 214.1.1 or 227.3.7.

All plates were incubated at 20°C(±1°C) for at least eight days or longer.

Within thirty-six hours after the shake flasks containing corn meal broth were inoculated, hyphal strands appeared in the flasks and were easily discernible by the unaided eye.

When two mycelia of opposite mating type obtained from shake culture flasks originally sowed with microspores of strains 214.1.1 and 214.1.3, were planted together in Petri dishes containing corn meal agar and incubated at 20°C(±1°C) perithecia containing typical ascospores were eventually produced. An additional mating of mycelia, obtained from flasks originally sowed with microspores of strains 214.1.1 and 227.3.7, was made. No perithecia were produced in this mating. However, this was to be expected since these two strains were of the same mating type, namely "A." On the basis of the results obtained it appears certain that the hyphal strands and mycelia produced in the shake culture flasks are those belonging to *Gelasinospora calospora* var. *autosteira* (*G. autosteira*). The stationary corn meal broth flasks, which were likewise inoculated with microspores from one of the three strains used, also produced hyphal strands and mycelial

growth inside the flasks but only after an incubation period of approximately forty-eight hours or longer. Therefore, it would appear that some other factor or a combination of several environmental factors other than aeration or oxygen may play an important role in influencing the germination of the microspores. Those control plates which received 2 ml. of the microspore suspension remained sterile. The control plates of strain 214.1.3 which were spermatized produced typical perithecia within the rings.

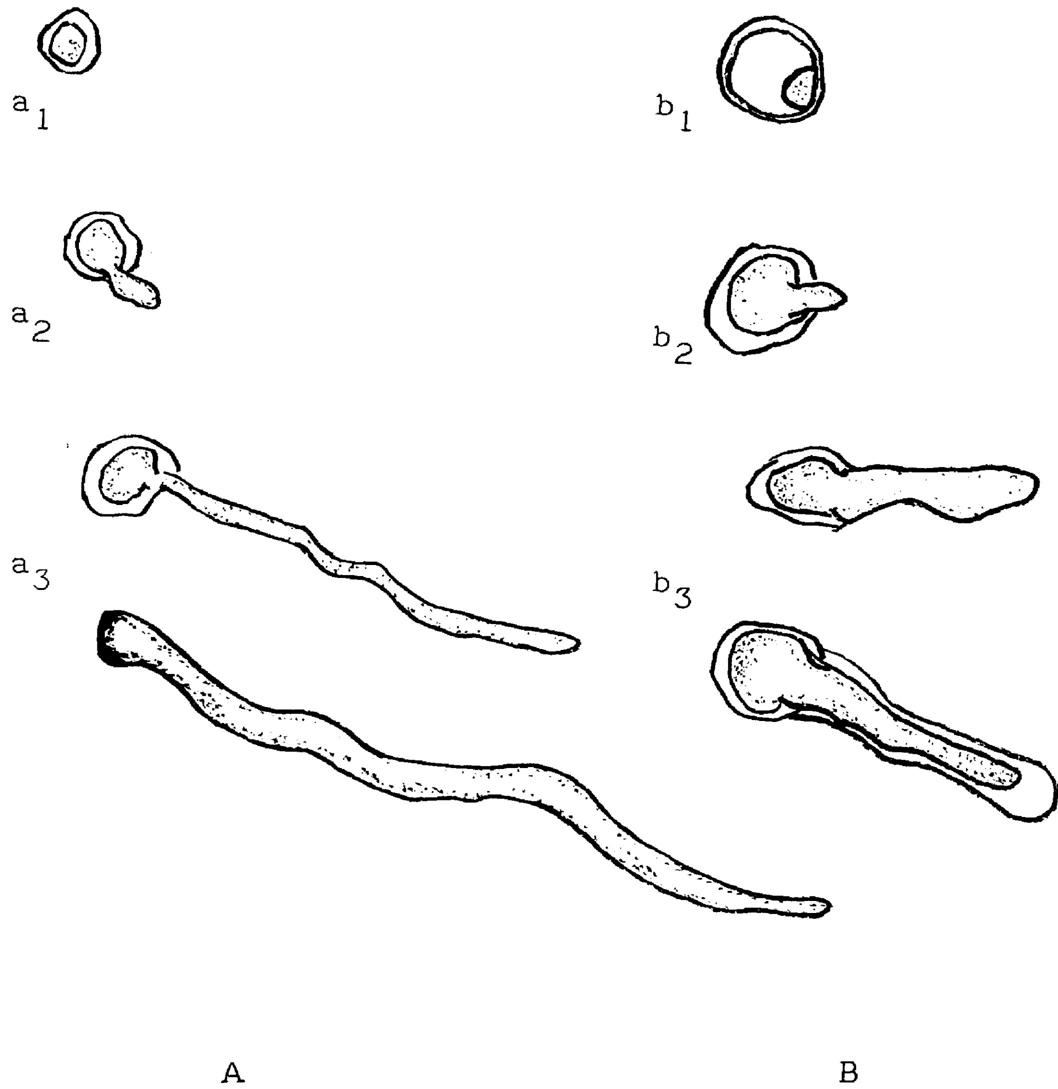
The fact that microspores when planted in corn meal broth resulted in the production of hyphal strands and mycelia belonging to *Gelasinospora calospora* var. *autosteira* (*G. autosteira*), presents strong evidence that the microspores of this organism do function as microconidia and are capable of reproducing the organism as to those produced by *Neurospora* sp. and other ascomycetes, providing cultural conditions are suitable.

Since the evidence submitted is admittedly circumstantial, attempts were made to further substantiate this conclusion. Germinating microspores with the spore wall still attached were retrieved from broth shaker flasks and placed on sterile corn meal agar. The micorspores continued to develop long germ tubes for a short time and then ceased development. However, none of the germ tubes were observed producing side branches. In over two thousand slide preparations of microspores seeded in liquid culture, a germinating

microspore possessing a single side branch has never been observed. Microspores planted on moistened agar failed to produce mycelial growth. Furthermore the number of microspores actually found germinating and producing germ tubes was relatively small in comparison to the hundreds of thousands which remained dormant. Text figure 2 illustrates a series of stages of germinating microspores. Some microspores which commenced to produce germ tubes on solid agar were placed into liquid shake culture flasks. These flasks failed to show any evidence of mycelial growth.

Hanging drop preparations of the microspores were also set up and kept inside Petri dishes containing moistened filter paper. Some of the microspores produced germ tubes but failed to develop further. It is of extreme importance to reiterate that an extremely small proportion of the total quantity of microspores actually produce germ tubes either on solid or in liquid medium. In view of this fact, the potential possibility of discovering a germinating microspore with a single side branch would be extremely small. Perhaps one would even have to prepare a hundred thousand slides rather than two thousand to catch this particular stage of microspore germination.

Dodge (11) has pointed out that any spore, if capable of producing a germ tube should also be potentially capable of producing vegetative mycelium under certain cultural conditions.



Text Figure 2. Various Stages of Successive Microspore Germination on Solid Agar as drawn by Camera Lucida. ca. x 1000.

It appears strange that the microspores of *Gelasinospora calospora* var. *autosteira* (*G. autosteira*), some of which are capable of producing long unbranched germ tubes, are unable to produce mycelial growth on agar. Perhaps some critical environmental factor is not supplied to the microspores on agar.

When the microspores are placed in corn meal broth in shake and stationary culture, a few germinate and produce mycelia representative of the appropriate mating type in each flask. Therefore, it appears certain, although admittedly circumstantial, that the microspores of this fungus are capable, at least under certain cultural conditions, of functioning as microconidia. Perhaps an inhibitor may be present in the agar and not in the corn meal broth. This interesting problem should be investigated further.

B. Migrating Nuclei

Sexual reproduction in *Gelasinospora calospora* var. *autosteira* (*G. autosteira*) involves at least two types of reactions, namely the production of protoperithecia and fusion of nuclei of opposite mating-type in the developing asci.

Furthermore, the preliminary step leading to sexual reproduction depends upon an interaction between mycelia of opposite mating type resulting in plasmogamy. A trichogyne microconidial relationship has been suggested as the mechanism whereby plasmogamy is initiated in this fungus.

With this particular hypothesis in mind, an attempt was made to determine whether the production of somatic heterokaryons involving nuclei of opposite mating-types could be produced in this fungus. If sexual reproduction is to occur in this fungus only through the agency of a trichogyne-microconidial relationship, and not by somatogamy, then it should not be possible to isolate dicaryotic mycelia comprising nuclei of both mating-types.

The experiments to be described were made to determine whether compatible nuclei could migrate from hyphae of one mating type to those of another giving rise to dicaryotic mycelia.

A number of u-tube experiments was carried out in which two percent corn meal agar was carefully poured to fill the medium cross arm and one-quarter of both upright arms of each u-tube.

All u-tubes were treated in such a manner that both arms of each u-tube were inoculated with mycelium of either the protoperithecial strain 214.1.3 or the microspore producing strain 214.1.1. Thirty-five such u-tube cultures were prepared and placed inside a moist chamber which was placed inside a 20°C($\pm$1°C) incubator. Some of these u-tubes were kept under observation for as long as three months.

A few days after the inoculations were made in the arms of the u-tubes, the hyphae from the opposite arms were observed to grow down the arms and appeared to meet somewhere

in the middle band of each u-tube. Observation of the u-tubes by aid of a dissecting binocular microscope usually revealed the occurrence of some intermingling of hyphae presumably of opposite mating types, Plate III, figure A_1.

With but a single exception, no perithecia were produced in any of the u-tubes where the hyphae coming from opposite arms of the u-tubes had appeared to make contact. It is difficult at this time to offer an explanation as to the reason why perithecia did develop in one solitary u-tube. Perhaps a small air space did develop in this u-tube allowing for microspore development and subsequent spermatization. However, no such air space was conspicuously discernible. Perhaps also, a few trichogyne-vegetative hyphal copulations took place. To offer an explanation in terms of somatogany would raise the question as to why did the protoperithecia in the one arm of the u-tubes fail to develop further?

Two months later some of the u-tubes were broken in the medium arm region of each u-tube with aid of a small file and a small chunk of agar was taken from this region and planted on the center of sterile corn meal agar in Petri plates. These plates were incubated at 20°C($\pm$1°C). Within ten days, mature perithecia containing typical ascospores were produced in over one-half of these plates indicating that intermingling of hyphae of the two compatible strains had occurred in the medium arm of some of the u-tubes.

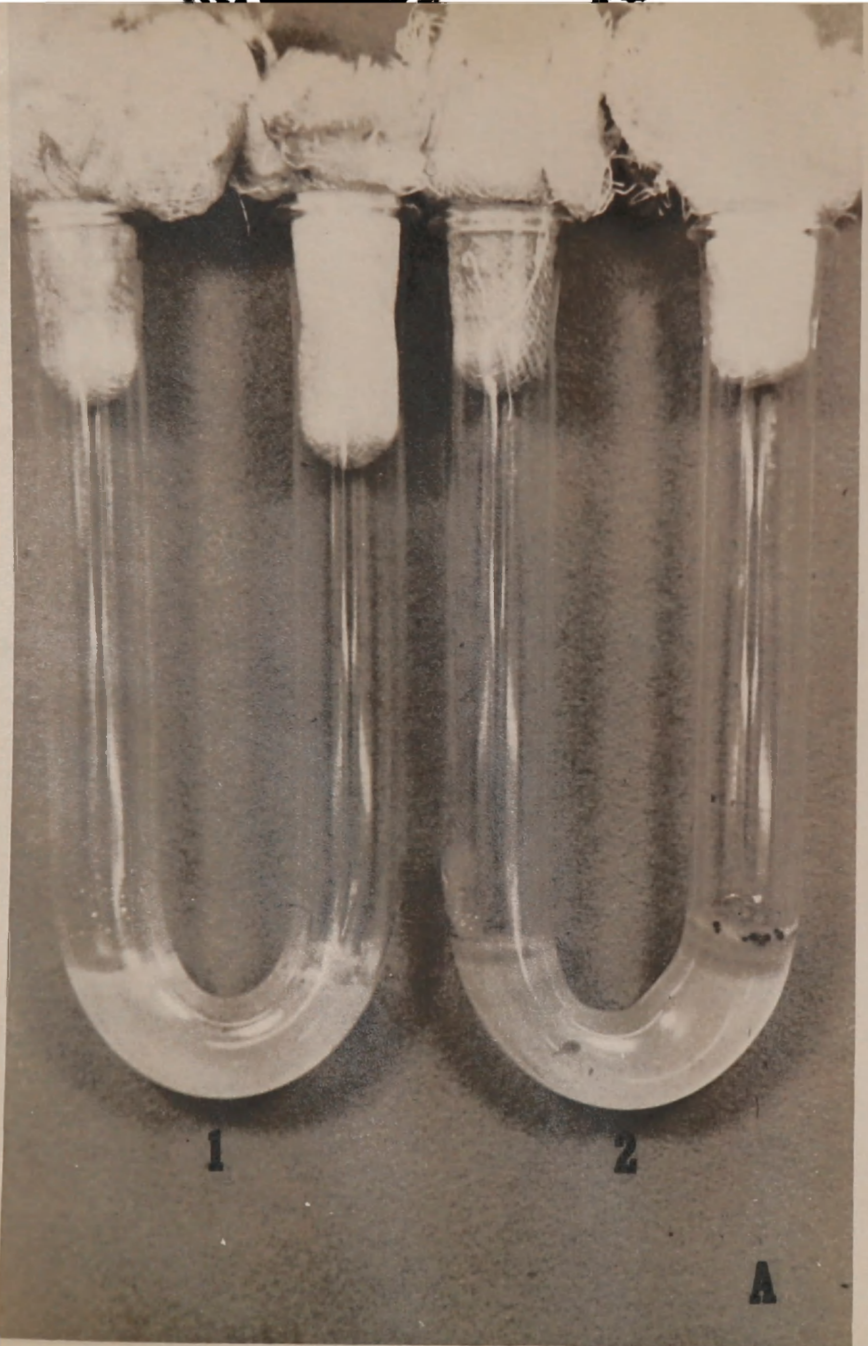
PLATE III

Figures A-B. u-tube and hyphal tip experiments.

Figure A_1. This represents one of the thirty-five u-tubes inoculated in one of the arms with strain 214.1.3 and in the opposite arm with strain 214.1.1. With one exception, no perithecia were produced in these u-tubes.

Figure A_2. One of several u-tubes, one arm of which was inoculated with strain 214.1.3 and the other arm with a microspore suspension of strain 214.1.1. Perithecia developed only in those arms containing the microspore suspension.

Figure B. The outermost plates represent four of the hyphal tip subcultures, derived from the inner center plate, which failed to develop perithecia. Numerous perithecia developed in the latter plate but only after protoperithecia and microspores made their appearance.


1
2
A

B

Since Dodge (10) has suggested that perithecia form in the bend of the u-tubes only when an air pocket forms in the tubes allowing sufficient oxygen to be available for perithecial formation, some of the u-tubes were allowed to air dry at room temperature. Even though the air pockets were formed in the tubes, the agar eventually drying up, no perithecia were ever produced on the agar in the u-tubes. Perhaps, however, the mycelium was too old to be capable of forming perithecia at this time.

It should be noted at this time, however, that no perithecia were produced in those arms of the u-tubes which had originally been inoculated with the protoperithecial strain 214.1.3. Although protoperithecia were produced on the agar surface in these arms, the structures failed to develop further.

In another series of u-tube experiments, one arm of each u-tube was inoculated with mycelium of a protoperithecial strain 214.1.3, and the other arm with a suspension of microconidia of strain 214.1.1. The u-tubes were kept inside a moist chamber placed inside a 20°C($\pm$°C) incubator under constant illumination.

Within two days, hyphae originating from the arm of the u-tubes inoculated with the protoperithecial strain had grown down and around the middle arm of the u-tubes and had made its appearance at the surface of the opposite arms forming a mycelial mat of hyphae within the microspore suspension.

Shortly afterwards protoperithecia made their appearance on those hyphal mats. Eventually many of the protoperithecia developed into mature perithecia containing typical ascospores. The protoperithecia produced in the opposite arms of the u-tubes originally inoculated with hyphae of the protoperithecial strain, failed to develop further, Plate III, figure A_2.

It is of special interest to note that no mycelial growth issuing from the microconidial suspension could be detected.

On the basis of the above results it appears certain that fusions or anastomoses occurring between mycelia of opposite mating types do not represent the fundamental mechanism whereby young protoperithecia receive nuclei of opposite mating types in this fungus.

Dodge (12) states that in heterothallic species of *Neurospora* nuclei of opposite mating types are normally kept apart until reproduction.

Beadle and Coonradt (8) suggest that the failure of formation of somatic heterokaryons between two strains of the opposite mating-types probably is due to the "tendency of the hyphae containing nuclei of the two sexes to form fruiting bodies (perithecia) rather than grow in the manner of strictly vegetative hyphae." This may be a possible explanation for the occurence of the one exception in the u-tube experiments.

However, an experiment now to be described tends to make this unlikely.

In a further attempt to determine whether compatible nuclei could be transferred from somatic hyphae of one mating type to those of another, as well as to investigate whether compatible nuclei become localized in the region of protoperithecial formation, over two hundred hyphal tips were isolated from cultures containing both mating types together.

In the first experiment of this type the two mating types, namely, strains 214.1.3 and 214.1.1, were inoculated adjacent to one another so that intermingling and anastomosing of compatible hyphae would be hastened. When intermingling of hyphae became evident, with the aid of a dissecting binocular microscope, small discs of agar containing intermingled hyphae of both mating types were cut out and transferred to the center of Petri dishes containing sterile corn meal agar. These plates were incubated at 20°C($\pm$1°C) for twenty-four hours after which time single hyphal tips were isolated and transferred to sterile corn meal plates. Of the ninety-nine cultures developed from these hyphal tips, none produced perithecia. The original mixed culture containing hyphae of both mating types, however, produced numerous mature perithecia at the periphery of the Petri plate, Plate III, figure B.

In a second experiment of this type, a small agar block containing young perithecia produced from mixed mycelia, which had been inoculated on the corn meal agar ten days previously,

was placed in the center of a Petri dish containing corn meal agar. The Petri dish was then incubated at 20°C(±1°C) under constant illumination for twenty-four hours.

Following this procedure, one hundred and six hyphal tips were obtained from hyphae growing out from the base of the perithecium and each was placed in test tubes on sterile corn meal agar slants. As in the previous experiment, none of these cultures produced perithecia even after incubation at 20°C(±1°C) under constant illumination for several weeks.

When these cultures were spermatized with 0.5 ml. aqueous suspension of microspores belonging to strain 214.1.1, mature perithecia containing typical ascospores were produced in forty-seven of these test tubes.

These results indicate that hyphal tips of both mating types were retrieved illustrating that mycelia of both mating types were present at the base of the perithecium and consequently ample opportunity was available for hyphal anastomoses between opposite mating types to occur.

Failure, however, to discover a dikaryon produced from any of the hyphal tips isolated tends to suggest that compatible nuclei do not necessarily occur localized around areas of perithecial development. More likely, compatible nuclei occur in associations only in the ascogonia by way of the trichogynes. The results tend to further substantiate the aforementioned concept that plasmogamy is initiated in *Gelasinospora calospora* var. *autosteira* (*G. autosteira*) only through the agency of the functional sexual structures.

SECTION IV

DISCUSSION AND CONCLUSION

Function of the Microspores

Numerous fungi have been reported in the literature by Olive (30) and others, to be capable of producing some form of microspores under certain cultural conditions. However, in most of the cases reported, adequate cytological tests as well as tests to determine the specific function of these microspores have been neglected.

In this study, an attempt was made to determine the function of the spermatium-like bodies produced by *Gelasinospora calospora* var. *autosteira*, as reported by Tylutki (35).

I. Sexual Reproduction

1. Microspores (free of hyphal fragments) have been shown to function as male sexual cells (spermatia) when placed in the immediate vicinity of protoperithecia.

2. The possibility of a trichogyne-microspore relationship functioning in this organism still remains questionable. However, in favor of this assumption are the following facts:

   a. There is no evidence of somatogamy indicated by the numerous u-tube experiments reported.

b. There is no evidence of dikaryotization existing in this organism as demonstrated by hyphal tip subcultures. Lack of dicaryons in hyphal tips taken at the base of young developing perithecia further substantiate this point.

c. The distribution pattern of developing perithecia is directly correlated with areas of the cultures spermatized with the microspores as well as with the distribution of protoperithecia.

d. Quantity of microspores used to spermatize appears to have a direct effect upon the number of perithecia produced. This could indicate that a large number of microspores is needed when spermatizing to insure that some come in contact with the receptive trichogynes.

e. In a negative approach to the problem, nuclei of different types when present in the same mycelium, are capable of migrating throughout the mycelium. Therefore, if somatogamy were functional we should expect to find evidence of nuclear migrations. However, we did not.

Wilson and Alexopoulos (36) have recently illustrated this particular point. Mutant nuclei affecting spore maturation in the homothallic fungus, _Gelasinospora calospora_, were detected in the heterokaryotic mycelium, hyphal tip transfers

yielding colonies which produced non-segregating and segregating perithecia randomly distributed.

Thus, apparently only two valid theories of mechanisms of fertilization in Gelasinospora calospora var. autosteira remain. Either there is a microspore-trichogyne relationship and this represents the only method by which fertilization can be accomplished or frequent copulation between trichogynes and vegetative hyphae of compatible strains takes place.

From the results reported in this paper, it appears quite certain that the association of compatible nuclei in this heterothallic fungus occurs only in the ascogonia and ascogenous hyphae by way of the trichogynes.

Because of the difficulties encountered in this study in attempting to determine possible relations of microspore attachments to trichogynes, and because of the vital importance of this particular point in the life cycle of the organism, future research should be directed toward a refinement of techniques possibly at the micromanipulator level, in order to investigate this particular aspect more thoroughly. According to some authors, the formation of fruiting bodies is the result of hormone action rather than sexual fusion of compatible nuclei. Moreau and Moruzi (26) state that perithecial formation in Neurospora sitophila is cause by hormonal stimulation. Dodge (10) and Aronescu (5) repeated these experiments and found no evidence of hormonal stimulation. In fact Aronescu (5) dissected and grew ascospores from single

asci and in every case found a segregation of the two mating types in a 1:1 mendelian ratio.

In _Gelasinospora_ _calospora_ var. _autosteira_, Tylutki (34) in a genetical study of this organism has likewise demonstrated that fertilization is a direct result of nuclear fusion of compatible nuclei. Ascospores taken randomly as well as ascospores taken from one hundred and forty-two asci were analyzed. Both, mating type and pigmentation loci were found to segregate in a 1:1 mendelian fashion. If sexual reproduction in this organism were stimulated by hormone action, then all ascospores should theoretically be of one mating type. This was not so.

In order to further demonstrate that perithecial formation following spermatization was the result of fusion of two nuclei of opposite mating types and not due to hormonal action, a series of experiments was set up, in which portions of the colony of a protoperithecial strain were treated with a Seitz filtered suspension of microspores of the opposite mating type. No perithecia were produced by these cultures. Control plates treated with the microspore suspension prior to Seitz filtration developed perithecia.

In another experiment, when 0.2 ml. suspensions of microspores, previously exposed to u.v. light for three consecutive days for ten minute periods, was added to protoperithecia contained within glass rings, perithecia failed to develop. Control plates developed mature perithecia.

Four cultures of one of the protoperithecial strains were allowed to incubate for seven days. Then sterile circular disks of wettable cellophane were placed over these colonies and an aqueous suspension of microspores was placed on the cellophane. These Petri plates were then returned to the incubator. Forty-five days later, examination of the plates revealed no perithecia present.

The u-tube experiments mentioned earlier also tend to support the above results. For, if a diffusible hormone were produced by the mycelium of one mating type and stimulated the mycelium of the other mating type, it would be expected that young developing protoperithecia produced in one arm of the u-tubes would be stimulated into perithecial development.

In view of these results, it appears unlikely that protoperithecia are stimulated into becoming mature perithecia by any hormonal action.

II. Asexual Reproduction

Again it has been definitely shown that microspores free from mycelial contaminations when placed in liquid culture, result in the production of mycelial growth within the liquid culture flasks within forty-eight hours. This mycelial growth is furthermore demonstrable of the mating type strain corresponding to the inoculum.

All attempts to demonstrate conclusively that any given microspore is capable of going on and producing mycelium, have

failed. However, since it appears that only a very small proportion of the microspores actually do germinate, the chance of picking a microspore which will go on and produce mycelium would be very small.

On the basis of work reported here, it is concluded that the microspores of *Gelasinospora calospora* var. *autosteira* function primarily as male sexual cells, but under certain as yet unspecified environmental conditions they are capable of functioning as microconidia.

SECTION V

SUMMARY

1. A study of the function of microspores produced by the heterothallic Ascomycete, *Gelasinospora calospora* var. *autosteira*, revealed that these particular structures are capable of functioning as spermatizing sexual propagative cells (*spermatia*).

Evidence is also presented which strongly indicates that the microspores may function as asexual propagative cells (microconidia) endowed with the capacity to reproduce the organism.

2. In connection with the experiments on spermatization, cross spermatization, and localized perithecial development, when microspores were placed on compatible protoperithecia, the development of these structures into perithecia occurred within forty-eight to seventy hours. This would appear to indicate that protoperithecia develop very rapidly into perithecia only after being fertilized by compatible microspores.

A trichogyne-microspore relationship does appear to be operative in *Gelasinospora calospora* var. *autosteira*. However, the cytological details of cell and nuclear fusions occurring between trichogynes and compatible microspores still need to be worked out.

3. In a study involving over two hundred hyphal tips and thirty-four u-tube cultures, results were obtained that tend to indicate that compatible nuclei in the heterothallic ascomycete *Gelasinospora calospora* var. *autosteira* are not brought into close associations with one another by vegetative anastomoses and nuclear migration.

Compatible nuclei occur in close associations in this fungus probably only in the ascogonia by way of the trichogynes. Plasmogamy and karyogamy is accomplished probably only through the sexual structures. Therefore, a trichogyne-microspore relationship must be operative in this fungus.

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