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Introduction.--No biological problem is of more importance than that dealing with the developmental process occurring during the growth of the organism from its inception to maturity. The end product of morphogenesis is fairly well known from the efforts of students of morphology and anatomy. The impetus given studies of heredity by Mendel and later investigators in the field of cytogenetics has largely clarified the mode of transmission of factors determining the final form of the adult organism. Between the inception of the individual and its adult form lies a sequence of developmental events toward the understanding of which biological research is now largely directed. This relatively new research approach is toward an understanding of how the inherited units interplay and influence development to produce the pattern of the adult. The term morphogenesis is applied to the whole of the developmental processes that bring about the unfolding of the finished organism. It differs from morphology in that morphology's main concern is with the finished product of maturation.

The object of an morphogentic approach is to discover how the units acquired at fertilization express themselves during the growth of the egg thus determining the form of the adult. Genetics is of course concerned with the transmission of the internal factors determining form.

Many component and interacting factors contribute to the end product of development in any organism. Genetic, physical, and ecological factors all enter into the developmental recipe of any species. Each factor is in itself all important, yet a harmonious interaction between each factor of development is essential for the normal growth and maturation of the organism. In a morphogenetic study it is important to note the role of genetic, ecological, and environmental factors. All of which contribute to every stage of development. In such an analysis it is also helpful, if not essential to the experimenter to be able to control one or more of these factors.

The student of morphogenesis is at once confronted with the problem of a suitable choice of experimental material. The ideal experimental object must be such that it is, as far as possible, controlled by its genetic potentialities and begins its development with a minimum of accessory substances such as food acquired from the parent. It should be able to develop on synthetic media which can be varied by the experimenter. Ideally the observer should be able to record development at intervals of time during growth and be able to trace the development of individual cells and other structures.

These requirements nearly rule out animal material since the individual cells are not clearly delimited due to the thin membranes forming the cell outlines. Moreover, the animal egg begins its development with a large amount of stored food and unknown substances carried over from the female parent.

The fern spore is an excellent subject for experimental work because it carries little of its previous environment with it ~~since~~ since its size limits the contents of the spore to a minimum. Therefore, any experimental environment designed by the investigator will not be contaminated by large amounts of unknown substances which would be present in a seed or in an animal egg. Each gene in a haploid organism plays its role in development since the effect of recessive genes cannot be masked by the presence of dominants as is the case in heterozygous diploid organisms.

Another aspect in favor of the fern spore as an experimental object in a physiological study is that the experimenter can begin observations with the single-celled spore instead of the complexity of cells found in the seed. From this single celled spore the growth and development of the multicellular prothallium can be observed.

A fern prothalium further renders itself favorable for research in that, contrary to most botanical organisms it is only one cell thick except for the

archegonial cushion near the notch of the prothalium, which is approximately four cells thick. This facilicates microscopic or camera study, since essentially all of its cells can be observed in the living condition and the formation of new cells and organs can be observed directly.

The small size of the prothallia is an additional advantage, in that many hundreds of them can be grown in a limited space.

The use of the fern prothallium as an experimental object is not new. The older literature concerning its development is summed up by Bower (1923). More recently, Albaum (1938) carried out a series of surgical experiments to determine the mode of growth control in the prothallium and reviewed the literature to that time. Albaum found that removal of the apical notch resulted in the production of adventitious outgrowths on the basal portion of the prothallium. These outgrowths were inhibited by the application of auxin solutions or auxin in lanolin. Thus it was concluded that auxin produced by the apical notch inhibits the formation of such outgrowths on the intact prothallium. Ward and Wetmore (1954) further employed surgical treatment of prothallial tissue to partially separate the zygote from enveloping gametophytic tissue. The embryo subsequently outgrew the calyptra much earlier than normal. This release resulted in a slower growing of the embryonic mass, thus leading to an assumption that the auxin centers are in the archegonial region. Perhaps the most recent physiological studies of fern prothallia were published by Steeves, Sussex, and Partanen (1955). Steeves described the two types of abnormal proliferations of fern prothallia grown in vitro and Partanen described these abnormalities cytologically. An apparent correlation was found between the frequency of archegonial abnormalities and the ability to produce normal organized growth under the prevailing conditions.

This report has several purposes. It will primarily describe some preliminary experiments undertaken to perfect techniques for growing and observing developing

fern prothallia. Methods of controlling the environment such as lighting, nutrition, etc., will be presented as well as photographic techniques.

THE DEVELOPMENT OF THE GAMETOPHYTE

Spores of Pteridium aquilinum obtained from the Carolina Biological Supply Company were grown on a medium containing NH_4NO_3, 0.5 gms.; $KHPO_4$, 0.2 gms.; $MgSO_4$, 0.2 gms.; $CaCl_2$, 0.1 gms. and five drops of a one per-cent solution of $FeCl_3$ per liter.

In earlier experiments 1.5 per-cent agar was added and the medium was plated. Spores were sifted through cheese cloth onto the surface of the agar. It was found however, that the agar dried out after several weeks and the developing prothallia died. For this reason the agar medium was abandoned and spores were floated on the surface of the liquid medium in stentor dishes.

The dishes were placed on a table near a west window. The heat from the sun damaged the prothallial tissue, and growth was variable, possibly because the illumination was not constant. To insure a constant photoperiod and to eliminate intense heat, found on days when the sun was very bright, the dishes were illuminated by florescent lights, two 45 watt daylight tubes at room temperature 25°-27° centegrade. The dishes were placed 43 centimeters from the light source. A time switch controlled the lights so they were on from 6 A.M. to 6 P.M. daily. The shades were drawn in the room to keep out additional light. The arrangement of the culture dishes and lamps is shown in figure 1.

Crowding, which occurred when too many spores were sifted into a dish (fig. 2), resulted in reduced development and produced filamentous, pin cushion and coralloid proliferations as described by Steeves et al (B.C.). These abnormal colonies produced seemingly functional sex organs but prothallial development was slower.

Prothallia from each culture were observed, microscopically, once every week and records were kept of germination of the spore to the formation of the

sporling. Drawings of the prothallia were made at intervals with the aid of a camera lucida.

The following method was employed for photographic recording of growth. A water slide of the material to be photographed was placed on the stage of a microscope on a horizontal position (stage vertical) and the image was projected onto sensitized paper. The microscope and its light source was set up in a dark room, thus the arrangement is essentially a photoenlarger. A 10X objective and a 5X ocular were used. The distance from ocular to paper holder were adjusted to give a magnification of 100X.

Germination of the spores occurred in this investigation from 5 - 8 days after the sifting of spores onto the surface of the nutrient solution. Rhizoids were first formed from the spores, followed by single prothallial cells. These cells divided to become mature heart-shaped prothallia bearing antheridia in about 35 days after germination.

The antheridia matured from 5 - 7 days after their formation, and released swimming antherozoids. Each antheridium released an average of 50 antherozoids.

Archegonia developed near the archegonial notch of the prothallium, after all the antheridia had finished their release of antherozoids. This occurred about 45 days after germination. The ripening of the sex organs at different times prohibited self fertilization. Several antherozoids from other prothallia in the culture swarmed around each archegonium. Fertilization was accomplished, when an antherozoid entered the canal and united with the egg. Two-leafed sporlings were produced about 70 days after germination. Figures 3, 4, 5, 6, 7, 8 and 9 present a series of developmental stages from the spore to the mature gametophyte.

In attempting to follow one spore through its development to a mature gametophyte, germinated spores were placed in thin moist chambers. These chambers were constructed by cutting an opening in a thin piece of lucite and

by affixing cover slips to the opposite sides of the opening. Strips of filter paper acted as a wick to keep the inner surface of the cover slips moist. The detailed construction of the chamber is shown in figure 10.

Agar held the germinated spore to the cover slip. Photographs were taken at three day intervals. However, this procedure was unsuccessful because the agar covering the slide did not permit clear photographs. Mold contamination produced further difficulty.

In a further attempt to follow the developmental processes of a single spore, spores were placed in depression slides containing nutrient solutions. Drying and mold rendered this unsuccessful.

Thus it appears that to successfully follow the development of a single spore, some method must be found to sterilize the spores prior to planting.

Steeves, Sussex and Partanen, in a paper just published (l.c.) described a method of spore sterilization. In this process, spores were agitated for 10 minutes in a 3.5 per-cent calcium hypochlorite solution and subsequently washed several times in distilled water.

The possibilities of camera studies are illustrated in figure 11, from which it can be seen that the cell walls are clearly defined and the occurrence of new cell walls can be readily observed.

EXPERIMENTS WITH GROWTH SUBSTANCES

This study is devoted to the observations of the effects of indole - 3 - acetic acid (auxin) and 2, - 4, - 6 - trichlorophenoxy - acetic acid (2 - 4 - 6 T) on the development of the gametophyte. Auxin is the best known growth regulator in plants. So far as is known it is produced in all flowering plants and ferns. Growth and development depend upon the amount and availability of the substance (Thimann 1954).

In most cases, if the natural source of auxin is cut off from the plant and auxin is exogenously supplied, the amount of growth is related to the concentration of auxin applied. High concentrations of applied auxin may inhibit growth or even be toxic to the normal metabolic and maturation processes. In low concentrations, however, auxin may be a limiting factor. For most plants there is an optimum concentration which induces maximum growth.

2, 4, 6 T apparently has the properties of an "anti-auxin" (McRae and Bonner 1952, Hoffman 1953). It is antagonistic to many auxins, thus inhibiting normal auxin effects whether applied alone or in mixtures with auxin.

It is supposed that auxin formation in the fern prothallium occurs in the apical notch (Albaum l. c.), the archegonium and derived calyptra (Ward and Whetmore l.c.). This investigation was made to determine whether 2, 4, 6 T would have an inhibitory effect on the naturally occuring auxin, and to determine whether or not the exogenous addition of auxin would influence the normal course of development.

2, 4, 6 T was added to the nutrient solutions to make the following concentrations; 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 0.5, 1.0, 1.5, 2, 3, 4, 5, 6, 8, 10, 100, and 250 milligrams per liter.

It was found that solutions above four milligrams per liter were toxic to the spores, preventing germination. Solutions above 0.5 milligrams per liter supported the growth of mold on the spores. The mold did not prevent germination or growth, but it appeared to cause some inhibition of normal growth and development.

Twenty-five separate experiments were run with cultures in solutions from 10^{-8} to 10^{-2} milligrams of 2 - 4 - 6 T per liter. Often the results were inconsistant due to factors which will be discussed later, but the overall growth and development appeared to be equal to that of prothallia grown in control solutions.

There was a negligible indication that antheridia formation was retarded in the higher concentrations of solutions in the series. The results of these experiments are summarized in figure 12.

Experimental results using solutions from 10^{-1} to 4 milligrams per liter indicate an above normal progressive stimulation of development.

An above normal progressive stimulation of growth was noted in cultures grown in 10^{-1} to 4 milligrams per liter solutions. Development in the 10^{-1} mg liter cultures was slightly above normal. Growth stimulation was progressively greater as the concentration strength increased. Prothallia grown in the 4 mg. liter cultures developed antheridia two weeks earlier than normal. Thus a positive stimulation of growth could be indicated in this series of experiments, however, there are factors which prohibit a conclusive statement to that effect. The solutions were used in only five experiments; although all experiments indecated positive results, mold contamination was such that death occurred before sporelings could be produced, thus making the results incomplete.

Six experiments with auxin were run using solutions of the same concentrations as the 2 , 4 , 6 T solutions. Auxin in series from 10^{-8} to 10^{-2} milligrams per liter appeared to retard development. Antheridia developed one week later in auxin cultures in this series than in control solutions. Prothallial cells in auxin cultures appeared to be slightly larger than prothallial cells found in control and 2 , 4 , 6 T cultures. Thiaman (1949) theorized that auxin entering the cell with great rapidity, accelerated protoplasmic streaming, which in turn brought about an increased intake of solutes, and a greater cell wall plasticity. Thus the increased solute (water) uptake coupled with changes in cell wall properties increased cell size.

Auxin in solutions above 10 mg. per liter prevented the spore's germination. Growth and development of prothallia in solutions from 10^{-1} to 10 mg. per liter were retarded slightly more than that in solutions from 10^{-8} to 10^{-2}. Only four

experiments were carried out using these auxin concentrations. The prevalence of mold destroyed the cultures before their maturity and discouraged further experiments until some agent is found to eliminate mold.

Results from both auxin and 2 , 4 , 6 T experiments were rather nebulous and inconsistent. The rate of development was not consistent in the series when they were repeated, as can be illustrated in figure 12. The large variation in developmental stages found in prothallia in the same culture or in a duplicate culture rendered definite conclusions difficult.

Certain physical factors may account for inconsistent results. One such factor being the presence and abundance or absence of sporangia sifted into the stentor dishes with the spores. It was noted in several instances that sporangia were present in large numbers in cultures where development was normal. It was noted also that duplicate cultures with few sporangia or lacking sporangia were slowing in their development. After this observation an experiment was carried out wherein all sporangia were removed from a control nutrient culture and a control culture was set up containing sporangia. This test resulted in normal or above normal growth in the dish containing sporangia and retarded growth in the dish without sporangia. (figure 13)

Algae, which grew in the cultures, appeared to have an inhibitory effect in cultures where it was prevalent. In cultures where algal growth was present but not so prolific no apparent effect was noted.

EXPERIMENTS WITH ULTRA-VIOLET LIGHT

Experiments with ultra-violet light were suggested by the sponsor and considered worthwhile by the investigator. Ultra-violet radiations are known to cause an abnormal increase in cell size in Timothy roots (Brumfield 1954) and to induce cytological changes in certain test objects. Ultra-violet light penetrates to such a limited extent that its cytological effects are restricted to very thin ob-

jects such as grasshopper neuroblasts, pollen tubes, bacteria and fungi. It was assumed in this investigation that prothallia might be suitable test objects for radiations of limited penetration.

In experiments with ultra-violet, the prothallia were radiated by a 15 watt General Electric germicidal lamp which delivers about 80 per cent of its energy in the 2537 Å region. The prothallia were exposed for 1, 2, 3, 5, 8 and 10 minutes at an intensity of 8 ergs /mm² / sec.

Prothallia (45, 37, and 15 days after their germination) were exposed to radiation. Exposure from 5 to 10 minutes appeared after one week to increase cell size and number, and it seemed to hasten sporophyte development about one week before normal in the 45-day old prothallia. Cell size and number increase was rated in the 37 day old prothallia.

Chromosomes were studied in the 15 day prothallia one day after exposure.

For cytological study, prothallia were cleared ~~by placement~~ in 95% alcohol for 20 minutes. The cleared prothallia were then placed in a paradichlorobenzene solution (5ml 1 bi water) for twenty minutes to spread the chromosomes. The material was stained with aceto-carmine and temporary slides were prepared. A Wratten green filter was used to facilitate microscope observation.

Chromosomal change was not evidenced in this investigation; however, only one experiment was carried out. Further research might produce positive results.

According to Britton (1953) Pteridium Aquilinum has 52 chromosomes. See figure 14 for camera lucida drawing showing chromosomal arrangement in a metaphase plate.

SUMMARY

This investigator believes that fern prothallia are good subjects for a physiological study because they are haploid, are one cell thick, can be studied in a living condition, ~~possess an~~ their environment ~~that~~ can be easily controlled, and are small enough to grow large numbers in limited space.

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DESCRIPTION OF FIGURES

Fig. 1. This light set up was used for maintaining constant photoperiod in growing prothallia.

Fig. 2. Culture (left) shows effect of crowding. Note filamentous projections on outside of culture. Duplicate culture (right) shows normal uncrowded development.

Fig. 3-9. Camera lucida drawings (Ca 600X) show development of a prothalliun. Fig. 3-8 show growth of a single germinated spore over a 3 week period. Fig. 9 drawing illustrates a typical prothallium bearing antheridia.

Fig. 10. Figure shows construction of a lucite chamber, used in attempting to follow a single germinated spore through its developmental activities.

Fig. 11. Prothallium, showing clearly defined cell walls, illustrates possibilities of camera study.

Fig. 12. Bar graph indicates development time of prothallia in four (a,b,c,d) typical experiments using concentrations from 10^{-8} to 10^{-2} mg/L: 2-4-6T and run with control culture.

Fig. 13. Control Culture (left) which contained sporangia. Sporangia are absent in culture (right).

Fig. 14. Camera lucida drawing (Ca 2,000) shows chromosomal arrangement in metaphase plate.

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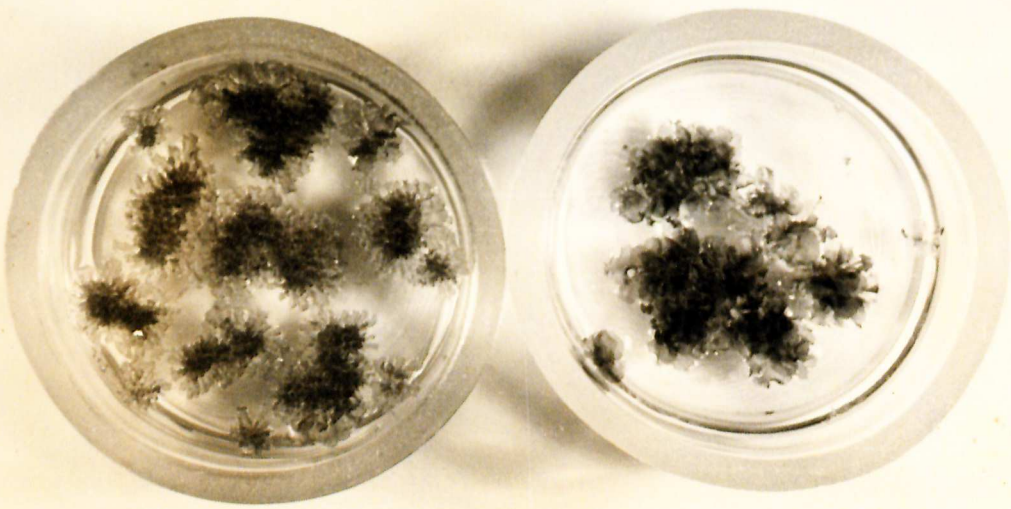

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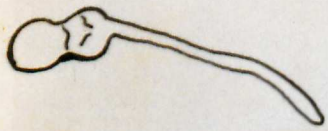

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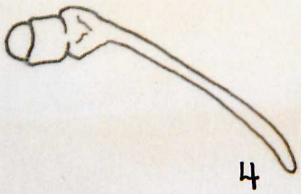

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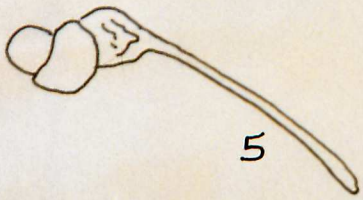

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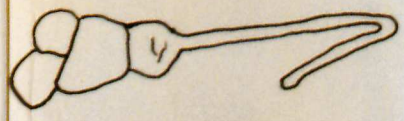

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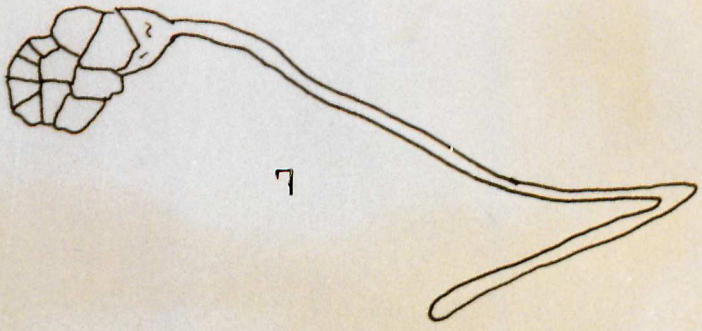

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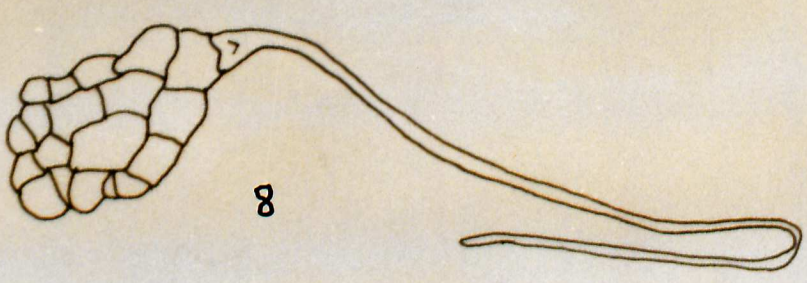

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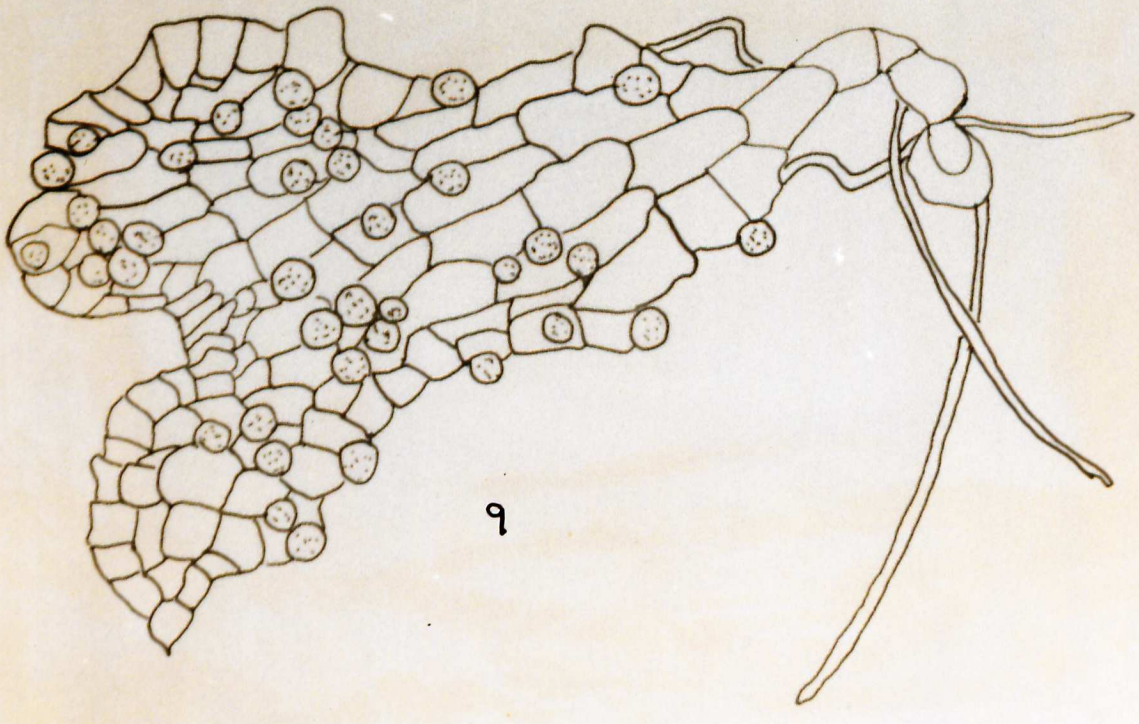

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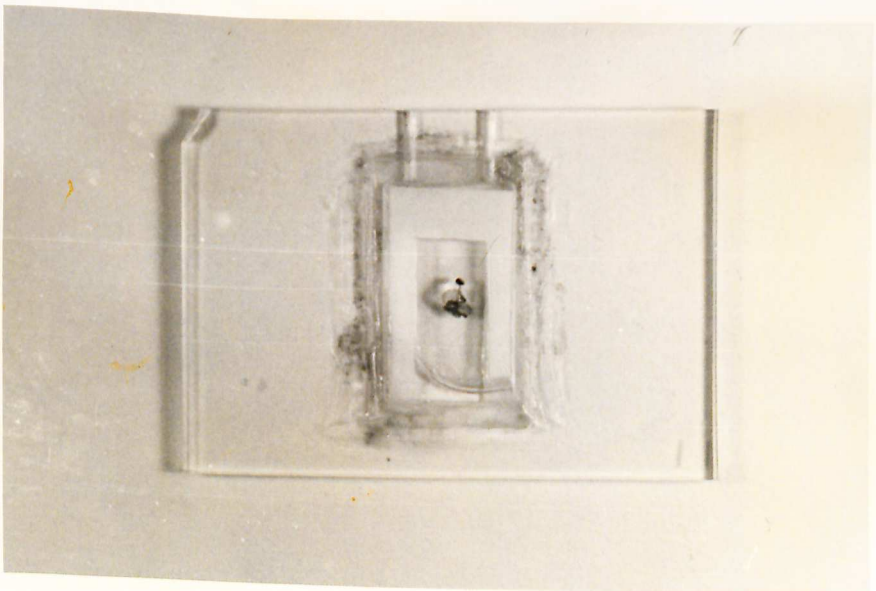

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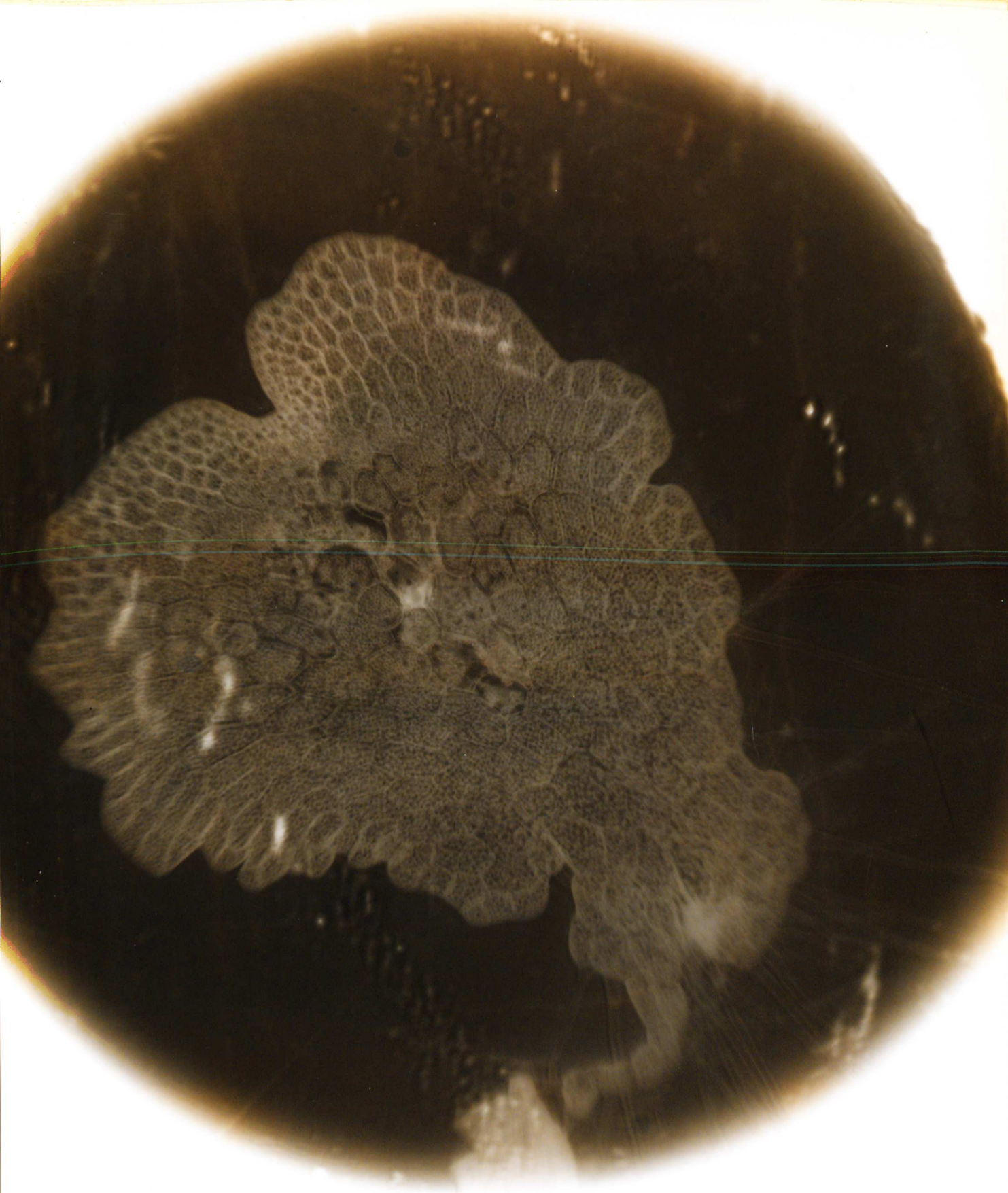
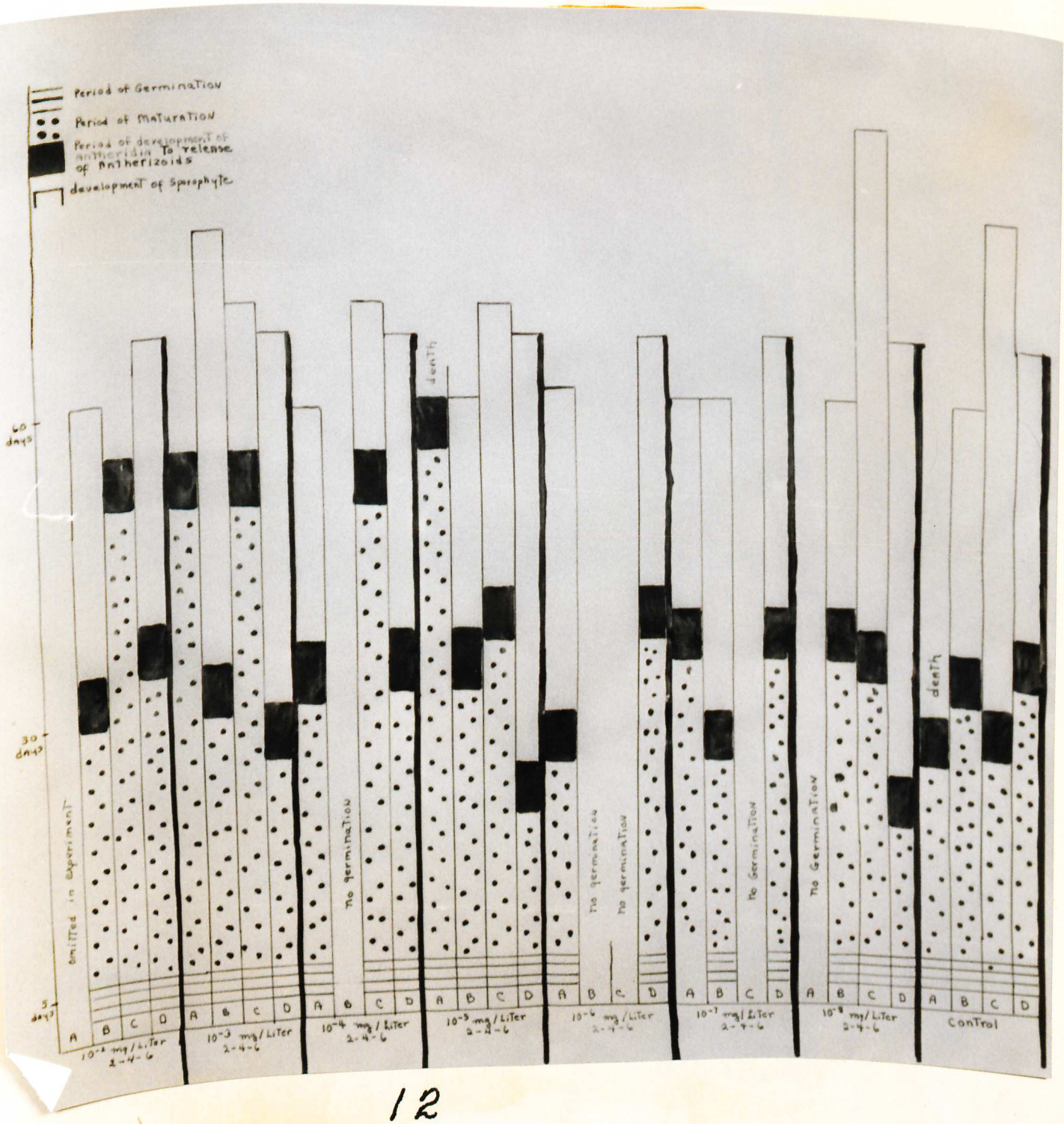
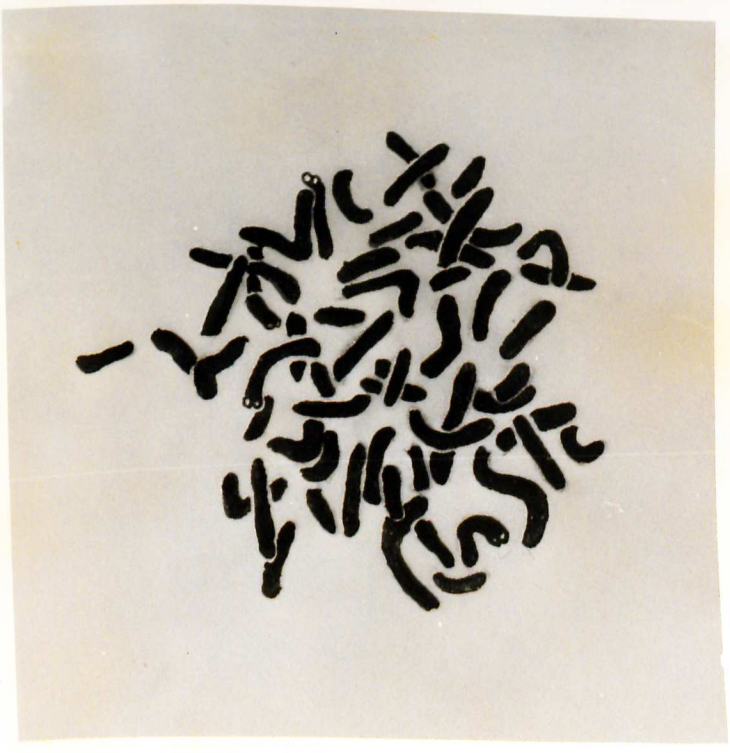



Period of Germination
Period of maturation
Period of development of antheridia To release of Antherizoids
development of Sporophyte
60 days
30 days
5 days
omitted in Experiment
A
B
C
D
10⁻² mg/Liter 2-4-6
10⁻³ mg/Liter 2-4-6
10⁻⁴ mg/Liter 2-4-6
10⁻⁵ mg/Liter 2-4-6
10⁻⁶ mg/Liter 2-4-6
10⁻⁷ mg/Liter 2-4-6
10⁻⁸ mg/Liter 2-4-6
Control
No germination
death

13



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