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# STUDIES *IN VITRO* ON THE POLLEN OF *GINKGO BILOBA*

by

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# ABSTRACT

## Studies *In Vitro* on the Pollen of *Ginkgo biloba*

by Walter R. Tulecke

The purpose of the present work was to study the development of the male gametophyte of *Ginkgo biloba* in culture and to investigate the occurrence and the cytology of a tissue derived from the pollen.

Two sources of pollen were used for culturing: 1) fresh pollen was cultured by surface-sterilizing intact sporangia and placing them on the agar surface, and 2) stored pollen was obtained in powder form by causing the sterilized sporangia to dehisce in vials over calcium chloride at 5° C. The stored pollen was inoculated on media by dusting or it was grown in hanging drop cultures. A modified White's (1943) medium with 0.25% yeast extract and 1 milligram indoleacetic acid per liter was the most successful medium used, since it enabled the gametophytes to grow to the immature sperm cell stage and also served as a substrate for the growth of the pollen tissue.

In addition to development to the immature sperm cell stage, there was one instance in which the blepharoplasts of a pair of sperm cells differentiated to form spiral bands of two revolutions, but they lacked cilia. The microgametophytes in culture followed closely the

cytological details of development as it occurs *in vivo*, but there were differences in growth rate and size. The body cells and immature sperm cells formed *in vitro* were smaller than those observed *in vivo* and they were formed in a shorter time.

Several types of abnormalities were observed in the cultures of the male gametophyte. These included 1) septate tube cells, 2) multinucleate coenocytic gametophytes, 3) intercalary divisions of the stalk cell, 4) cytoplasmic units cut off from the body cell, and 5) the outgrowth of a tissue from the masses of germinated microgametophytes. The most active cell in forming the abnormalities was the tube cell, and it was largely responsible for the septate tube cells and the coenocytic gametophytes.

The most striking abnormality to arise from the cultures was the tissue derived from the pollen. This tissue appeared repeatedly and, when isolated and subcultured, it proliferated very readily by means of meristematic groups of cells. The chromosome complement of the tissue was basically haploid in the meristematic centers, but the cultures also exhibited polyploid and multinucleate cells. Tissue initials were found frequently in cultures of the stored pollen and on certain media but cultures of the fresh pollen also produced the tissue.

The studies on the origin of the tissue have not been conclusive, but they indicate that the prothallial cells are not active in tissue initiation. The abnormalities suggest that both the spermatogenous and tube cells of the gametophyte are possible sources of the tissue, with the weight of evidence indicating an origin from the tube cell.

The two principal results reported here are the development *in vitro* of the male gametophyte up to the immature sperm cell stage and the isolation and sustained culture of a tissue derived from the pollen of *Ginkgo biloba*.

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# INTRODUCTION

Since Amici's discovery of the pollen tube in 1824, studies *in vitro* on angiosperm pollen have been carried on by numerous investigators. Comparatively little work, however, has been done on the culture of gymnosperm pollen, probably because of the long period usually required for the growth and development of the microgametophyte. Moreover, early attempts to grow gymnosperm pollen were not very successful. Juranyi (1872), Strasburger (1892), Lopriore (1905) and Chamberlain (1910) cultured various cycad pollens with only limited results. More recently, Branscheidt (1939) and LaRue (in press) have applied modern tissue culture techniques to the problem of gymnosperm pollen development *in vitro*, and they have obtained rather striking results. Branscheidt found that *Taxus* pollen could be grown to maturity of the microgametophyte, and LaRue discovered that *Zamia* gametophytes would develop to the immature sperm cell stage, and, in addition, would undergo extra divisions in the gametophyte cells to give rise to intragametophytic cell masses.

It was, in fact, LaRue's success with *Zamia* that led to the present investigations on *Ginkgo* pollen development *in vitro*. The author had previously been studying microsporogenesis in *Ginkgo*, when it occurred to him that

it might be possible to study the development of the microgametophyte by employing suitable culture techniques. Preliminary studies on pollen growth on a nutrient medium were successful, and they suggested a cytological study of pollen germination and microgametophyte development *in vitro*. Such a study seemed particularly desirable in view of Newcomer's (1939) report that he had been unable to obtain the germination of preserved *Ginkgo* pollen on any of a variety of culture media.

Additional impetus was given the investigation when a tissue was discovered arising from a mass of the germinating pollen grains. Immediately, there followed the question of the tissue's origin, its ability to continue growth in culture, and its cytology. These and other questions gave the problem its final form: the study of the development of *Ginkgo* pollen *in vitro* with special emphasis on tissue formation.

# A REVIEW OF THE LITERATURE

One of the first cultures of gymnosperm pollen was reported by Juranyi (1872). He dusted the pollen of *Ceratozamia longifolia* upon pieces of ripe juicy pears and twenty four hours later observed their germination. A large and rapid accumulation of starch was noted by him, but he described no development of the gametophyte beyond germination. Somewhat later, Belajeff (1891) germinated the pollens of *Taxus*, *Zamia*, and *Juniperus* on sugar solutions. He reported no maturation of the gametophytes beyond the formation of the generative and tube nuclei in *Taxus* and concluded that maturation of the microgametophyte *in vitro* was impossible.

Strasburger (1892) obtained results with *Ceratozamia* similar to those of Juranyi. However, he improved on the latter's technique by sterilizing the pieces of pear before sowing the pollen. Tube elongation was as much as twice that reported by Juranyi, and Strasburger mentioned one case of nuclear division. Unfortunately, his observations were terminated when the pollen tubes exhausted their stored food reserves and subsequently underwent plasmolysis. In this same paper, Strasburger gave the first account of pollen development in *Ginkgo*. His description was based upon observations which he had made on the development of the gametophyte in the nucellus

of the ovule. Some of his observations, however, were not substantiated by later workers. One of the major discrepancies was his report that the derivatives of the body cell (presumably these would be sperm cells, although he called them generative cells) occasionally entered the tube cell. Shortly thereafter, it was shown that the sperms of Ginkgo were motile, hence, they would enter the tube cell only when fully mature and ciliated, and Strasburger's account did not include the mention of motile sperm. In fact, it was four years later that Hirase (1896) first described motile sperm in Ginkgo biloba. Later the same year Ikeno(1896) discovered spermatozoids in Cycas revoluta, and this was followed by Webber's (1897) report of motile sperm in Zamia integrifolia.

Lopriore (1905) was able to obtain germination of Araucaria bidwelli pollen on both pear juice and a twelve percent sucrose solution. A temperature of 25°-30° C. was maintained, and it was observed that the pollen grew best when the grains were sown in thin layers on the medium. Lopriore also made the striking observation that the gametophytes contained up to forty nuclei, which he interpreted as sperm nuclei. Subsequent investigation by Jeffrey and Chrysler (1907) indicated that these nuclei were liberated from many prothallial cells shortly before the pollen was shed.

The investigation of Tischler (1910) dealt almost

exclusively with the accumulation of starch by pollens during their initial stages of germination. He studied angiosperm pollen for the most part, but he included two species each of Podocarpus and Pinus. The gymnosperm pollens also germinated readily in sugar solutions, but Tischler did not attempt to study their development in detail.

Pollen studies in the United States were first seriously undertaken by Brink (1924, 1925). In a series of papers, he presented his results on the development and physiology of angiosperm pollen grown in culture. His work included a study of the nutrient requirements for pollen growth, chemotropic pollen responses, the role of salts in pollen tube growth, and pH studies. Brink noted a very considerable improvement in pollen tube growth with the addition of small amounts of sterilized yeast. Pollen grains in groups germinated and developed better than those planted singly on the agar surface, a result which he ascribed to the retardation of leaching of some diffusible growth-promoting substance. Further, he underlined the variability of pollen growth in culture by noting, for example, that pollen from different anthers of the same flower showed extreme differences in germination and growth. Brink also compared pollen tube growth in vivo with that obtained in culture and remarked (1924 III, p. 354), "The observations

as a whole indicate very close agreement between the behavior of the nuclei of pollen tubes growing *in vitro* and those growing *in vivo*." He likewise called attention to the fact that (1924 III, p. 353), "Few observations have been made on the behavior of nuclei in pollen tubes growing on artificial media."

Other physiological studies were carried out on angiosperm pollen by Branscheidt (1930). He utilized a hanging drop technique, rather than the agar method of Brink. Branscheidt observed the effects of stigma and pollen extracts on pollen tube growth as well as the effects of one pollen on the germination of another. Later, one of his students, Kuhlwein (1937), extended this pollen work to the gymnosperms, including such genera as *Taxus*, *Chamaecyparis*, *Thuja*, *Juniperus*, *Pinus*, *Picea*, *Larix*, and *Ginkgo*. Kuhlwein was interested primarily in securing data on germination and pollen tube length on various sugar concentrations and in relation to time. *Ginkgo* pollen was found to store starch for about three days and then send out a germination tube. This was true only for hanging drop cultures, since Kuhlwein reported that he was unable to germinate the pollen on agar media. However, *Ginkgo* pollen was shown to germinate and grow in sucrose concentrations of 5 to 40 percent. In the case of the various genera studies, Kuhlwein called attention to the fact that germination was obtained on concentrations of sucrose ranging from 2 to 60 percent

(and as high as 80 percent in Chamaecyparis). Two to twenty percent was normally used, and the lower concentrations gave the best results. Kuhlwein's data on pH for gymnosperm pollen revealed an optimum of pH 5.0-6.0 for germination, and this was only slightly more acid than Brink's (1925) observation that angiosperm pollen germinated best at pH 6.0-7.0 and grew best at about 6.0.

Following Kuhlwein's initial success with gymnosperm pollen, Branscheidt (1939) made one of the most detailed and successful studies of the development of gymnosperm pollen in vitro. For his material, he chose the pollen of Taxus baccata and employed the hanging drop technique and a supplemented sucrose medium. The pollen was placed in culture at maturity and in the uninucleate condition; within two or three days the first division took place to form the generative and tube cells (no prothallial cells are formed). Later, the generative cell divided to form the body and stalk cells. Two sperm nuclei were then formed from the body cell as early as 20 days after the pollen was placed in culture. This was the first report of a microgametophyte of a gymnosperm grown to maturity in vitro.

More recent studies by LaRue (in press) on Zamia floridana pollen secured some very interesting results. He obtained development of the microgametophyte in culture up to the immature sperm cell stage and also reported

small embryonic masses of cells within the tube cell of the gametophyte. In addition, LaRue described abnormal divisions in the prothallial, stalk, tube, and body cells of the microgametophyte. LaRue's technique differed from the hanging drop used by Branscheidt and Kuhlwein in that he grew the pollen on the surface of agar and used a more complete nutrient medium.

In the contemporary criticisms of *in vitro* studies, it is usually stated that abnormalities are to be expected because of the treatment given the material. This objection is best answered by admitting its validity and also pointing out that the culture technique simply offers another approach to the problems of normal and abnormal development. That normal variation exists in most biological material is readily admitted, and it is useful, in this connection, to indicate some of the abnormalities that are known to occur among pollen cells.

It was early found by Chamberlain (1897) that the pollen of *Lilium philadelphicum* occasionally produced three sperm nuclei, two tube nuclei, or rarely, a prothallial cell. Smith (1898), working on the pollen of *Eichhornia crassipes*, demonstrated two tube nuclei in 50 percent of the pollen grains. Strasburger (1892) noted that *Ginkgo* pollen, instead of having the normally single persistent prothallial cell, showed three such

cells, the basal one of the set sometimes undergoing an anticlinal division. Rarely, did he observe the stalk cell divide into two cells.

The variations in the pollen of Picea excelsa were the subject of an extensive paper by Pollock (1906). Among the prothallial cells(normally two disorganizing cells) Pollock found 66.5 percent of the grains with only one prothallial cell and 15.7 percent with two cells and a small number with three such cells. He also observed several grains in which there were two stalk cells.

Embryo-sac-like giant pollen grains were first described in Hyacinthus by Nemec (1898), but were later studied in detail by Stow (1930, 1933). Stow was able to induce the abnormality by subjecting bulbs of the plant to a temperature of 25° C. during meiosis. Examination of the anthers and germination of the pollen revealed a mortality of 90 percent. The remaining 10 percent contained, in part, the embryo-sac-like giant pollen grains. The latter were figured by Stow as having a bulging elliptical intine and containing eight nuclei. Three cells were located at the exine end (corresponding to the micropylar end), three cells at the distal end (antipodals), and two polar nuclei in the central region. The formation of these presumptive embryo sacs was believed, by Stow, to be the result of three successive divisions

of the original microspore cell. Stow also described a normal microgametophyte, which had coiled around one of the embryo-sac-like gametophytes, discharging its sperm into one of abnormal pollen grains. On the basis of such evidence, Stow suggested not only a morphological but a physiological female tendency in the embryo-sac-like grains. Other abnormal pollen grains were found containing four to as many as sixteen nuclei, the latter without a definite arrangement. Geitler (1941) reported a similar occurrence of the embryo-sac-like giant pollen grains in Ornithogalum nutans, and he also observed four, eight, and sixteen nucleate microgametophytes.

Polynucleate pollen mother cells were reported in Trillium erectum by Stern (1946). He placed the material on a sucrose medium and observed that the peripheral gel layer of the cells underwent solution, which, in turn, permitted fusions of the cells. As a result, as many as 32 nuclei were found in a giant pollen mother cell formed in this manner. The cells were in early leptotene stage of development when the fusions took place, and Stern suggested that this may be one mechanism which gives rise to polyploid pollen, although he did not report further development of the pollen.

Other experiments with excised anthers grown in vitro were performed by Shimakura (1934), Taylor (1950), and Lima de Faria (1950). Shimakura called attention to

continuing divisions in the pollen mother cells of Tradescantia virginiana in sucrose solutions. Taylor obtained transitions from meiosis to mitosis and the reverse in cultured anthers of Tradescantia paludosa. And Lima de Faria, using anthers of Secale cereale, described conditions for controlled meiosis and pollen mitosis in vitro.

Since Hirase's (1896) discovery of the spermatozoids in Ginkgo biloba, a considerable number of papers have been published on various aspects of the tree, some of which are pertinent to this work.

Shortly after Sprecher's (1907) comprehensive study on Ginkgo biloba, Ishikawa (1910) cleared up the discrepancies in chromosome number previously reported for the species. The haploid complement was determined to be twelve, and this was confirmed later by Sax and Sax (1933), who worked with divisions in the female gametophyte.

A re-investigation of microgametogenesis in Ginkgo was reported by Herzfeld (1927), and her results corroborated many of Hirase's (1898) observations. Herzfeld used von Wettstein's terminology for the cells of the gametophyte and challenged the correctness of Strasburger's terminology, which had been adopted almost universally by workers publishing in the English language. Wettstein, for example, called the sister cell of the

spermatogenous cell the wall cell, whereas, Strasburger labeled it the stalk cell. Wettstein believed that the cell was analogous with the wall cell of an antheridium. Strasburger, on the other hand, held that, since the cell was intercalary between the prothallial cells and the spermatogenous cells, it was analogous with the stalk cells of an antheridium. In this view Chamberlain (1910, p. 142) concurred, stating, "The stalk cell.... may be regarded as representing the actual stalk cell of an antheridium." However, Downie (1928), one of Chamberlain's collaborators, observed that in Microcycas calocoma neither Wettstein's nor Strasburger's view was applicable to the stalk cell. Downie found that the generative cell in Microcycas divided in the normal manner to produce the stalk and body cells, but that the true nature of the stalk cell was revealed in its subsequent activity. It divided repeatedly to give eight body cells, each in turn giving rise to two sperm cells for a total of sixteen motile sperm. Downie believed that the generative cell was, in reality, the primary spermatogenous cell and that the stalk and body cells were both secondarily spermatogenous.

Support for Downie's interpretation in Microcycas is indicated in the reports of multiple sperm cells in members of the Coniferales. Reference is made to the work of Juel (1904) and Doak (1932), both of whom studied species of Cupressus. Juel found 4 to 20 (usually 8 to 10)

cells derived from the body cell of Cupressus goveniana. He termed these cells sperm cells and considered their development normal for the species. Phylogenetically, he believed that ancestral polyspermy had been retained in the plant. Juel's findings were long considered abnormal and exceptional, until Doak confirmed his observations by reporting polyspermy in Cupressus arizonica. Doak also found 4 to 14 cells produced by the body cell, although occasionally he found the two male cells which are typical for the family. Doak interpreted the condition of many sperm cells as a reversion of the reduced two male cell type to the state of polyspermy. Unfortunately, Doak did not comment on Downie's observations in Microcycas, although Downie commented briefly on Juel's work with Cupressus. Downie attributed the activity of the body cell in Cupressus to the sterile and degenerate condition of the stalk cell. She cited this condition of the stalk cell as part of the reduction seen in the male gametophyte of the Cupressineae as a whole, where prothallial cells are lacking and normally only two sperm cells are formed.

To summarize, Downie considered the generative cell as the primary spermatogenous cell. Its two derivatives, the stalk and body cells, were thought of as actual spermatogenous cells, although they might be modified in different ways. For example, the stalk cell could produce many body cells and thus lead to polyspermy as in

*Microcycas*, or, if the stalk cell were inactive, the sperm cells derived from the body cell might proliferate to give the polyspermous condition, as in *Cupressus*.

Several studies *in vitro* on *Ginkgo* have been reported by Li (1934), Li and Shen (1934), and Radforth (1936). Li studied the growth of the *Ginkgo* embryo on Knudsen's medium and showed that there was no dormant or overwintering phase in embryo development either *in vitro* or in the intact seed. He also described excrescences from the lenticels of the embryonic cotyledons and the hypocotyl. Li and Shen were interested in the effect of wheat bran extract (pantothenic acid) on the growth of yeast and the growth of the embryonic root of Ginkgo. The extract was inhibitory to the radicle and stimulatory to the yeast, while an extract of the female gametophyte was beneficial to the growth of the radicle.

Finally, Radforth was interested in the interrelation of the suspensor and the proembryo. He adopted Crone's solution for his use and added an extract of Brewers' yeast to complete the culture medium. He was able to show that suspensor formation may be delayed *in vitro* while the proembryo increases in size.

# MATERIALS AND METHODS

From the outset, it was apparent that any work on the growth of tree pollen *in vitro* would be hindered by the seasonal aspects of pollen production. Certainly this was true of *Ginkgo*, since its pollen is usually shed within a four-day period once a year, anthesis occurring during the first week of May in Ann Arbor. Thus, while an abundance of pollen could be cultured at this time, there would be neither a period for preliminary experimentation nor any recourse for repeating experiments until anthesis of the following year. Fortunately, there were two solutions to this problem: either the twigs could be forced into early strobilus production or the mature pollen could be held in a viable condition by some method. Both of these alternatives were utilized and both of them were important to the problem.

## A. Forcing Twigs Into Early Strobilus Production

The expedient of forcing twigs into early strobilus production required no special treatment. Branches of male *Ginkgo* trees were collected at about the middle of the dormant season (January) and placed with their basal ends in tap water in a warm greenhouse. A little over a month was necessary to produce pollen from these cuttings, and the time became progressively shorter as the

growing season approached. Meiosis appeared normal in the sporangia and the pollen produced in this manner germinated readily in culture.

This technique of breaking dormancy is apparently associated with the rise in temperature and the increased availability of water. The importance of water in the maturation of the male strobilus was indicated by the work of Sosa-Bourdoil (1949). He took the spur branches of male and female Ginkgo trees and compared the water uptake in these branches to their initial weight. He found that the male spur branches had a significantly greater affinity for water than either the vegetative or the female branches. Seasonally, the affinity was lowest in December and then gradually increased to a maximum at pollen maturation.

The pollen obtained by forcing twigs was used entirely for preliminary studies. Various media, sugar concentrations, and growth substances were tried with this material and the promising experiments were repeated with either fresh or preserved pollen. None of the data included in pollen development in vitro, however, were taken from these preliminary experiments. Perhaps the most important role of this forced pollen was its use in modifying and adapting the pollen storage technique of Newcomer (1939) so as to permit pollen development in culture.

## B. Pollen Preservation

Holman and Brubaker (1926) showed that pollen could be stored over extended periods by several methods. They were able to keep the pollen of Typha latifolia for 336 days over calcium chloride at 18°-21° C. and Pinus sylvestris pollen for 279 days by air-drying. In Becquerel's (1932) work, gradual drying was important for pollen grains of Antirrhinum majus, spores of Aspidium filix-mas, and thalli of lichens. Becquerel slowly air-dried the material, placed it in a dessicator, and then exposed it to liquid nitrogen temperatures of -268° to -271° C. for one to seven hours. After the return to room temperature, Becquerel found that germination was better than in material held at room temperature. Pollen longevity tests by Nebel (1939) indicated that it was possible to keep pollen of sour cherry for five and one-half years at 2°-8° C. and 50% humidity. From such studies as these, one may conclude that pollen storage depends to a great extent on proper drying and low temperatures.

Newcomer (1939) experimented with the storage of Ginkgo pollen and found that he could, apparently, maintain viability from one season to the next. The conditions Newcomer used differed little from those given above. The pollen was collected after dehiscence of the sporangia, placed in a vial, and suspended over calcium chloride at

7° C. Samples of the pollen were withdrawn from time to time to test for viability, but Newcomer reported that he was unable to get the pollen to germinate, despite the use of a variety of media and growth substances. The following spring the pollen was placed on the ovules at the time of exudation of the pollen droplet and subsequent ovule development appeared normal. Newcomer took ovule development as evidence of pollen viability, but he did not check the ovules for pollen germination. It is possible that ovule development may have taken place by means of a stimulating agent in dead pollen, rather than through pollen germination.

The pollen preservation technique used in the present study was a modification of Newcomer's method. Preliminary experiments on pollen storage were made by using the pollen collected from twigs forced into early strobilus production. Some of the pollen was cultured as controls and some was preserved and then cultured. Eventually, a suitable method was devised and it was applied to the pollen which had matured on the tree. The procedure was as follows. On May 5, 1952, mature strobili were hand-picked from a male Ginkgo tree and brought into the laboratory. The strobili were selected for yellowness and unopened sporangia and then placed in half-pint packer bottles. The strobili were surface-sterilized by immersion in 70-95% ethyl alcohol and briefly agitated; the alcohol was then poured off (duration in alcohol of 15

seconds, maximum) and replaced by a 20% solution of commercial Chlorox. The bottles were closed and shaken at intervals, followed by the removal of the Chlorox after 20 minutes. The sterile strobili were removed to sterile paper toweling in order to soak up the excess Chlorox solution and to reduce the amount of water being introduced into the dessicator units. The bottles receiving the strobili were miniature dessicators consisting of one-half ounce Armstrong Capsule Bottles with a one-half inch layer of calcium chloride on the bottom. On top of the dessicant a pollen carrier made of aluminum foil was placed; the bottle was closed with a screw-cap top (Fig. 1). Previously, the dessicators were sterilized in an oven at 200° C. for 15 minutes, which also effectively drove off any water taken up by the calcium chloride during preparation. One or two strobili were placed in the pollen carrier of each miniature dessicator, and the latter were capped and packed into a larger dessicator and immediately placed in the cold room at 5° C. Dehiscence of the sporangia and release of the pollen was complete within 12 to 30 hours.

Two details may be singled out from this method of pollen storage which distinguish it from Newcomer's original procedure. The first, of course, is the sterile technique, which not only permits sterile culture of the pollen, but also protects it from the attacks of psychrophilic fungi and bacteria. Second, is the matter of

obtaining anthesis in the cold. Dehiscence at low temperature reduces the metabolic activities of the pollen grains while they are still contained in the sporangium and the method maintains the low metabolic level after the grains are released. Thus, there is little chance for the damaging effects of simultaneous high temperature and dessication to operate to the detriment of pollen viability.

Up to the present time, the modified Newcomer method of pollen preservation has been satisfactory, with some loss in germination percentage, for a total of twelve months.

## C. Pollen Culture Technique

It is the purpose of this section to describe the techniques applied to the growth of *Ginkgo* pollen *in vitro*. It should be emphasized that the objective in these procedures was to obtain suitable conditions for the development of the male gametophyte. For this purpose, those conditions shown by previous workers to have an important role in pollen growth (such as sugar concentration and growth substances) were experimentally evaluated in the establishment of a basal medium for pollen growth. Obviously, not all the factors important from a physiological standpoint were the subject of experimentation, since such studies were beyond the immediate scope of the problem.

## 1. Culture Methods

The fresh pollen of Ginkgo was prepared for culture by surface-sterilizing entire strobili and placing them on the nutrient agar. Pollen germination occurred within the sporangia and anthesis took place when the development of the gametophytes broke open the sporangium wall. Occasionally, the sporangia were stripped from the strobili and planted directly on the culture medium, or the pollen grains were squeezed out of the sterile strobili upon the agar surface. In all instances, the pollen germinated readily and there was little contamination.

The stored pollen was in powder form and inoculation of the culture media was accomplished by dusting the pollen on the agar surface. Sometimes sterile cellophane, which was permeable, was placed on the agar surface to receive the pollen. The cellophane method facilitated observations and made recovery and fixation of the pollen easier than when the pollen was placed directly on the agar surface.

The hanging drop technique was used quite successfully as a means of following the early stages of gametophyte development. Stored pollen was employed as the inoculum in these cultures.

The present study was aided by two conspicuous

advantages: large populations of gametophytes were available and the observations could be made with comparative ease. Neither of these advantages are attainable from microgametophytes *in vivo*. Usually fewer than eight microgametophytes will develop in an ovule and to observe these, in all except the advanced stages, it is necessary to fix, imbed, section, and stain the material, or to make successful squash preparations.

## 2. Nutrient Media

Since the time of Haberlandt's (1902) unsuccessful attempts at plant tissue culture and the later successes of Harrison (1907), Burrows (1910), and Carrel (1911) with the growth of animal tissue *in vitro*, much effort has been directed toward the cultivation of plant tissues on nutrient media. The basic research on plant tissues in culture was done in the period from 1920-1945 and includes the work of Robbins (1922), Kotte (1922), White (1932), Gautheret (1932), LaRue (1936), Nobecourt (1937) and many others. White (1939) developed the first completely synthetic medium for sustaining the growth of plant tissues in culture; later, other investigators developed special media which permitted the growth of particular tissue explants such as cambium, carrot callus, and crown gall tissues of various plants. However, as helpful as these media have been to later workers, it is admitted that no medium answers the growth requirements of all plant tissues. There remains

a certain amount of empirical procedure, trial and error, which is needed to determine the specific nutrient demands of a new tissue culture.

The first step in compounding a medium for *in vitro* studies on a new tissue or plant organ usually involves the modification of a known basal medium containing inorganic salts, trace elements, and perhaps vitamins. To this solution an extract of yeast or malt, tomato juice, or coconut milk is added in various concentrations. In this way a medium favorable to the tissue under investigation may be devised, although a large number of unknown factors, such as vitamins, amino acids, purines, pyrimidines, and even enzymes, may be introduced by the extract. Such a medium is unsuitable for critical nutritional studies, but it is adequate for certain experimental and developmental work.

LaRue (1949), for example, was able to obtain continuous growth of corn endosperm by culturing tissue of the proper age on a modified White's (1943) medium. One third of the water in White's medium was replaced by canned tomato juice and the acidity was adjusted from pH 3-4 to pH 6.2-6.5. This medium was the one used for the 1951 cultures of *Ginkgo* pollen, and it was found to support germination and development of the microgametophyte very well. White's Standard plus tomato juice

(WS-T) was subsequently modified. In order to show the modifications made, the composition of White's (1943) Standard medium is given below.

White's Standard (WS)

| Salts | Mg/Liter |
|---|---|
| $MgSO_4$ | 360.00 |
| $Ca(NO_3)_2$ | 200.00 |
| $Na_2SO_4$ | 200.00 |
| $KNO_3$ | 80.00 |
| KCl | 65.00 |
| $NaH_2PO_4 \cdot H_2O$ | 16.50 |
| $Fe_2(SO_4)_3$ | 2.50 |
| $MnSO_4$ | 4.50 |
| $ZnSO_4$ | 1.50 |
| $H_3BO_3$ | 1.50 |
| KI | .75 |
| Sucrose | 20,000.00 |
| Glycine | 3.00 |
| Nicotinic acid | 0.50 |
| Pyridoxine | 0.10 |
| Thiamine | 0.10 |

In LaRue's laboratory, Straus modified White's medium in several ways(using information from correspondence with Wetmore). The manganese sulfate, zinc sulfate, boric acid, and potassium iodide of White's medium were omitted and Nitsch's (1951) minor elements were substituted as follows:

Nitsch's Minor Elements

| Salts | Quantity |
|---|---|
| $H_2SO_4$ | 0.5 ml |
| $MnSO_4 \cdot 4H_2O$ | 3000.0 mg |
| $ZnSO_4 \cdot 7H_2O$ | 500.0 mg |
| $H_3BO_3$ | 500.0 mg |
| $CuSO_4 \cdot 5H_2O$ | 25.0 mg |
| $NaMoO_4 \cdot 2H_2O$ | 25.0 mg |
| $CoCl_2$ | 25.0 mg |
| $H_2O$ | 1000.0 ml |

(Use one ml of this stock per liter of medium)

This substitution introduces cobalt, molybdenum, and copper into the medium as trace elements. A further change resulted when the ferric sulfate of White's medium was replaced by ferric citrate. A stock solution of the ferric citrate was made up at a concentration of 2.5 gms per liter of distilled water, and 4 ml of this stock solution was used per liter of medium.

The original WS-T medium of LaRue was found to be improved by increasing the vitamins and glycine content above that used by White. An assay was run using the corn endosperm tissue at levels of 1.0, 2.5, 5.0, 7.5, and 10.0 times the vitamins and glycine listed in White's Standard. Growth of the tissue was best at the 2.5 level, and this was henceforth incorporated into the WS-T medium and subsequent media as a modification. The vitamins and glycine were also prepared in a stock solution which was sterilized by Seitz filtration.

Vitamins and Glycine

| Constituent | Mg/Liter | Stock Solution |
|---|---|---|
| Glycine | 7.50 | 750.0 mg/L |
| Nicotinic acid | 1.25 | 125.0 mg/L |
| Pyridoxine | .25 | 25.0 mg/L |
| Thiamin | .25 | 25.0 mg/L |

(Use 10 ml of stock solution per liter of medium)

The stock solution was poured into sterile one-half ounce bottles in 10 ml aliquots after Seitz filtration and stored at 5° C. until used.

Yeast extract was successfully employed by Brink (1924 IV) for the growth of the pollen of Vinca minor. White (1943), LaRue (1949), and others also added an extract of yeast to their culture media as a non-specific growth factor rich in vitamins and amino acids. Similarly, in the case of Ginkgo pollen, it was found that yeast extract was beneficial to gametophyte growth and maturation. The yeast extract was a better supplement than tomato juice, and it was therefore substituted for tomato juice in some of the media that were made up. A concentration of 0.5% yeast extract was used initially, but 0.25% was later found adequate.

Indoleacetic acid was also added to some of the media as a further modification of White's Standard medium. Addicott (1943) has shown that pollen growth in Milla biflora and Tropaeolum majus was promoted by the addition of various plant hormones, pyrimidines, purines, and vitamins, among which was indoleacetic acid (IAA). The optimal concentration of IAA for Ginkgo pollen was 1 mg per liter.

The yeast extract, indoleacetic acid, and vitamin components of the nutrient media were considered as possibly heat-labile. They were therefore sterilized by Seitz-filtration and then combined with the remainder of the medium, which had previously been sterilized by

autoclaving.

The basal medium used in the present study has been derived from some of the modifications of White's Standard medium listed above. This basal medium has been labeled White's Modified (WM) to distinguish it from the original White's Standard. Its composition is given below:

White's Modified (WM)

| Constituents | | Amount Per Liter |
|---|---|---|
| $MgSO_4$ | | 360.0 mg |
| $Ca(NO_3)_2$ | | 200.0 mg |
| $Na_2SO_4$ | | 200.0 mg |
| $KNO_3$ | | 80.0 mg |
| KCl | | 65.0 mg |
| $NaH_2PO_4 \cdot H_2O$ | | 16.5 mg |
| Ferric citrate | (stock) | 4.0 ml |
| Nitsch's Minor Elements | (stock) | 1.0 ml |
| Glycine and Vitamins | (stock) | 10.0 ml |
| Sucrose | | 20.0 gm |
| Agar | | 8.0 gm |
| Water (double distilled) | | 985.0 ml |

Combinations of WM with other substances provided a number of media for pollen growth. By far the most successful and widely used of these was one known as WM-0.25%YE-IAA, which contained White's Modified, 0.25% yeast extract, and 1 mg of indoleacetic acid per liter. On this medium the pollen not only germinated readily, but it developed up to the immature sperm cell stage. Most of the observations of microgametophyte development were made from material grown on this medium. In this connection, it should be noted that this medium is not

the best that could be devised, since it has not been established that each constituent of the medium is either essential or at the optimal concentration and some factors may be lacking entirely. However, the WM-0.25%YE-IAA medium is significant in view of Newcomer's (1939, p. 121) statement that, ".....all attempts to germinate the (Ginkgo) pollen failed, although efforts were made at regular intervals for a period of more than four months with a variety of media and stimulatory substances." Newcomer was working with pollen which he had preserved; it is not known whether or not he attempted the culture of fresh pollen. The WM-0.25%YE-IAA medium supported germination and development of both the fresh and the preserved pollen.

Another culture medium was used in addition to the WM-0.25%YE-IAA medium and the WS-T medium. This additional medium was known as Moore's Modified (MM) medium because Moore's inorganic salts were used, as indicated below.

Moore's Modified (MM)

| Constituents | | Amount Per Liter |
|---|---|---|
| Moore's inorganic salts | | |
| $NH_4NO_3$ | | 500.0 mg |
| $KH_2PO_4$ | | 200.0 mg |
| $MgSO_4$ | | 200.0 mg |
| $CaCl_2$ | | 75.0 mg |
| Ferric citrate | (stock) | 4.0 ml |
| Vitamins and glycine | (stock) | 10.0 ml |
| Nitsch's minor elements | (stock) | 1.0 ml |
| Sucrose | | 20.0 gm |
| Agar | | 8.0 gm |
| Water | | 985.0 ml |

Germination and growth of the microgametophytes took place in the MM medium with only two conspicuous differences from that observed in other media. The masses of microgametophytes showed primarily a spherical habit of growth and they were loose and easily separable when mounted on a slide for observation. The fact that the gametophytes normally tended to stick together in a medium such as WM-0.25%YE-IAA and separated easily from one another in the MM medium suggests that the difference in calcium in the two media may be significant. Moore's Modified medium contains considerably less calcium than the WM-0.25%YE-IAA medium, even if the calcium in the yeast extract in the latter medium is disregarded. If the amount of calcium present in the organic salts of each medium is calculated, one finds that the MM medium contains 27.0 mg and the WM-0.25%YE-IAA medium 48.8 mg of the metal per liter. The role of calcium in pectate formation and the importance of pectates as cementing substances suggests that the loose fashion of growth of the microgametophytes in Moore's Modified medium may be the result of the low level of calcium.

Naphthaleneacetic acid (NAA) and calcium pantothenate (CP) were used by Morel (1948) in his modification of Berthelot's (1934) solution for the growth of grape stem callus. Morel's concentration of .1 mg

naphthaleneacetic acid and 1 mg calcium pantothenate per liter of medium were used in the preparation of some of the media for the growth of the pollen of Ginkgo.

A summary of the composition of the media used in the present investigation is given below. The list is intended to serve as a reference, since the symbols shown below are used throughout the remainder of the thesis.

Constituents of the Media

| Symbol | Meaning |
|---|---|
| WS | White's Standard |
| WM | White's Modified |
| T | One-third tomato juice per liter |
| CP | 1 mg/L calcium pantothenate |
| NAA | 0.1 mg/L naphthaleneacetic acid |
| IAA | 1 mg/L indoleacetic acid |
| MM | Moore's Modified |
| 0.5% YE | 0.5% yeast extract per liter |
| 0.25% YE | 0.25% yeast extract per liter |

List of Media Used

WS-T
WM-CP
WM-CP-NAA
MM-IAA
WM-IAA
WM-0.5%YE
WM-0.25%YE
WM-0.25%YE-IAA

Only the WS-T medium was used in the 1951 cultures, and the inoculum was fresh pollen, since the technique for pollen storage was not devised until the following year. The successful germination and growth of the micro-

gametophytes in 1951 led to the use of the other media listed above in 1952, and both fresh and stored pollen were available at that time. As previously mentioned, pollen germination and microgametophyte growth were best on the WM-0.25%YE-IAA medium. Good growth of the tissue derived from the pollen was also obtained on this medium.

### 3. Hydrogen Ion Concentration (pH)

Brink (1925), working with Lathyrus odoratus pollen and using phosphate or amino acid buffer, found an optimum pH range for pollen germination of 6.0-7.0. LaRue's (unpublished) WS-T medium, first used in 1951 for the germination of the pollen of Ginkgo, was adjusted from the original pH of 3.0 to 6.2-6.5. Later, in 1952, a pH series was set up within the range of the natural buffering system of the tomato juice of the WS-T medium. The hydrogen ion concentration was adjusted by the addition of potassium hydroxide to the WS-T medium to give a range of pH 3.0 to 6.8. The experiment consisted of six culture bottles at each pH level: 3.0, 3.7, 4.2, 5.0, 5.6, 6.0, 6.5, 6.8. Intact strobili were surface-sterilized and placed on the medium on May 2, 1952 and a qualitative evaluation of the results was made by periodically observing the cultures. At pH 3.0 there was no germination and at pH 3.7 and 6.8 germination was poor.

The optimum was from pH 5.6 to 6.5. The latter range was adopted as suitable for pollen culture purposes, and the media subsequently used were brought within this range. The media showed some variations in pH after autoclaving, but, in general, they were quite stable. Readings on some of the important media after autoclaving are listed below.

| Medium | pH |
|---|---|
| WM-0.25%YE-IAA | 5.8-6.4 |
| WS-T | 6.2-6.5 |
| WM-CP-NAA | 5.2-6.8 |
| MM-IAA | 6.2 |

A macerate of dehisced pollen in water gave a pH of 6.0, corresponding rather closely with the pH of the most useful WM-0.25%YE-IAA medium which was 5.8-6.4.

### 4. The Optimum Sucrose Concentration

Sucrose has been widely used for studies on pollen *in vitro* because most pollens will germinate on media containing this sugar. Moreover, Paton (1921) has shown that in eighteen species tested for invertase in the pollen all gave a positive test, and it would seem that sucrose could be utilized by many, if not all, pollens.

The pollen of *Ginkgo* was cultured on a series of sucrose concentrations in three experiments. Pollen obtained by forcing twigs into early strobilus production was used on a sucrose series of Moore's Modified medium;

fresh pollen was cultured on WM-0.25%YE-IAA nutrient agar, and preserved pollen was inoculated into hanging drops of the liquid WM-0.25%YE-IAA medium. In every instance, the germination and growth of the microgametophytes was best at a concentration of one to four percent. The results for one such experiment are shown in Table 1.

TABLE 1

THE EFFECT OF SUCROSE CONCENTRATION ON THE GERMINATION OF GINKGO POLLEN

Fresh pollen was placed on the WM-0.25%YE-IAA medium on May 4, 1952. Germination results are indicated as 4-excellent, 3-good, 2-fair, 1-poor, and 0-none.

| Date | Percentage Sucrose Concentration | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0 | .25 | .5 | 1.0 | 2.0 | 4.0 | 8 | 16 | 32 |
| May 19, 1952 | 0 | 1 | 2 | 3 | 4 | 3 | 2 | 0 | 0 |
| June 4, 1952 | 1 | 2 | 2 | 4 | 4 | 4 | 3 | 2 | 1 |

The two percent level of sucrose was chosen from the optimum range of 1-4% because the sucrose of the 1% cultures was utilized rather quickly and the 4% concentration seemed a little high. Very few pollen grains germinated on the 0% or 32% sucrose media; in the former the starch reserves of the pollen grain were consumed within a short time, whereas, in the latter, starch was rapidly accumulated in great quantities.

## 5. Plant Growth Substances

Beneficial effects of yeast extract on pollen

germination were found by Brink (1924 II) and later confirmed by Beck and Joly (1941). Brink attributed the effect to a growth promoting substance. Smith (1939) found that indoleacetic acid at a concentration of 1 mg per liter increased the percentage of germination and stimulated pollen tube elongation. Smith also observed that Pinus austriaca pollen did not germinate on control cultures lacking the hormone, but germinated well on a medium containing IAA. Beck and Joly (1941) likewise reported that growth substances were required for the germination of pine pollen.

Preliminary experiments with the pollen of Ginkgo obtained by the forcing method indicated that a concentration of 0.01 to 1 mg of indoleacetic acid was stimulatory to pollen germination and microgametophyte development. A similar experiment was set up with fresh pollen inoculated upon the WM-0.25%YE medium containing several concentrations of the hormone: 0.001, 0.01, 0.1, or 1 mg per liter. Periodic examination of the pollen revealed no sharply defined or critical hormone level within the limits of the experiment. The highest level of IAA (1 mg) seemed to produce the greatest number of gametophytes showing advanced body cell development, and this concentration was therefore incorporated into some of the nutrient media. In this way the IAA of the WM-0.25%YE-IAA medium supplemented

the unknown growth factor in the yeast extract.

## 6. Temperature

Many studies have been made on the effect of temperature on pollen development, germination, and growth. The work of Sax (1935) is cited here to show that extremes in temperature may cause abnormalities at meiosis and during pollen germination.

Sax subjected Tradescantia plants to high (35° C.) and low (6° C.) temperatures and found that these temperatures caused a number of aberrancies. The tube nucleus of some pollen grains was induced to divide under these conditions. In other pollen grains, the axis of division of the microspore was often in the longitudinal rather than in the dorsoventral axis. This abnormal polarity disrupted the vacuolar and cytoplasmic systems of the grains. Sax also observed pollen grains germinating in the anther prior to nuclear maturation; these grains enlarged and suggested the giant embryo-sac-like pollen grains reported for Hyacinthus by Stow (1930). Further experiments included gymnosperm material in the form of branches of Pseudolarix amabilis; the branches were placed in water in a warm greenhouse and brought into early strobilus formation. Sax described unusual orientations of the early division figures. For instance, if the microspore nucleus divided in the plane of the

wings of the pollen grain rather than at right angles to it, then no prothallial cell was cut off. Instead, two nuclei of equal size were produced within the microspore and no cell plate was formed.

The fresh and stored pollen of Ginkgo was kept in a dark constant temperature room at 25°-27° C. during the culture period, except when the cultures were removed for examination. However, the stored pollen underwent a period at 5° C. before being cultured, and it received, in effect, a low temperature treatment. The treatment was applied to the pollen after it was mature, but the possibility remains that the low temperature may have had some effect on the cells of the mature pollen grains.

## D. Microtechnique

Harris' hematoxylin (Johansen, 1940) proved to be the best stain for permanent whole mounts of the microgametophytes. The developing gametophytes were taken from the cultures for routine examination and stained with iron acetocarmine or they were observed in vital neutral red. Those cultures selected for making permanent mounts were fixed in Randolph's modification of Navashin's fluid and later processed through the schedule for Harris' hematoxylin. Handling of the material was facilitated by centrifugation, so that the gametophytes were held in centrifuge tubes from the time of washing

out the fixative with 70% alcohol to mounting from xylol. The gametophytes were pipetted to clean slides and mounted in Permount.

Material for sectioning was also fixed in Navashin's solution and it was dehydrated by using Johansen's tertiary butyl alcohol technique. Haupt's adhesive was used for affixation, and various stains, principally Harris' hematoxylin, Heidenhain's hematoxylin, and safranin-fast green, were employed. For chromosome counts of the pollen tissue, the acetocarmine squash technique was used with satisfactory results.

## E. Photography

Records of temporary mounts of the microgametophytes were obtained photographically whenever possible, otherwise, camera lucida drawings were made. With permanent mounts, the above mentioned methods were used or a carbon arc microprojector was substituted to facilitate drawing. Photographic results were best when 35 mm Kodack Micro-File film was used. A reflex-focusing Kine Exakta II camera body was mounted on extension tubes and coupled to the microscope with a homemade attachment. Critical lighting was approximated with the use of a 50 watt concentrated filament projection bulb, a focusing lens at the source, an improvised light source diaphragm, and the proper microscope adjustments.

# RESULTS

## A. Normal Growth of the Male Gametophyte In Vitro

Normal growth *in vitro* is construed to include the growth process of those gametophytes which develop in a manner comparable to the classical account of gametogenesis as reported by Hirase (1898), Miyake (1902), and Herzfeld (1927). The details and sequences of maturation are those which are found in the microgametophytes *in vivo*. Abnormal development, on the contrary, includes all the departures from the accepted mode of development.

Before presenting the pattern of development *in vitro*, it seems desirable to estimate the number of pollen grains in a culture bottle, the germination percentage, and the proportion of normal and abnormal development. An approximation of the number of pollen grains within one of the ovoid sporangia is obtained by counting the number of pollen grain diameters in the least radius of the sporangium, and then calculating the number of grains in a sphere with a volume of $4/3\ R^3$ (Chamberlain, 1935). About 4000 pollen grains are estimated per sporangium by this method. In placing the pollen in culture, the contents of an entire strobilus is distributed to six culture bottles. Therefore, in order to find the pollen grains per bottle, it is necessary to compute the number per strobilus. An average of 60 pairs or couplets of sporangia are borne on a strobilus and this gives an

estimate of 480,000 (4000 X 2 X 60), or 80,000 pollen grains for each of the six culture bottles.

Pollen grains which showed a bulging of the intine were listed as germinated, and the germination percentage was found to vary from 35-45%. In numbers this means that about 32,000 out of 80,000 pollen grains in a culture bottle germinated. Only one-half or 16,000 of those that germinated underwent growth and some degree of maturation; the remainder ceased growth soon after germination began. The pollen which developed abnormally is estimated between 0.05% and 1% of the 16,000 which underwent some degree of maturation.

### 1. Germination and Pollen Tube Growth

At anthesis, the pollen of Ginkgo is shed as a four-celled male gametophyte. The pollen grain contains an evanescent prothallial cell, a persistent prothallial cell, the generative cell, and the tube cell (Fig. 3). All of the cells are contained within the outer wall of the pollen grain, the exine layer. The exine does not entirely close around the tube cell, however, since one finds that the exine is open at the ventral side (Fig.2). The opening consists of a longitudinal furrow through which the germination tube eventually grows.

Germination in a suitable nutrient medium, such as WM-0.25%YE-IAA, begins with an uptake of water, which

causes the grains to swell and become spherical. At the same time, the small and variable starch reserve originally present in the grains is augmented by the absorption of sucrose and its deposition as starch. An iodine-starch test readily confirms the nature of the storage substance. Vacuolation of the cytoplasm accompanies the water uptake and the deposition of starch so that within 48-72 hours the intine bulges through the slit in the exine. This marks the beginning of tube formation in the gametophyte.

Mature pollen grains average about 26 microns in diameter. The microgametophytes enlarge by one-half their diameter within 24 hours and double this dimension in three days (Fig. 4). Five days after inoculation the initial bulge of the intine is very conspicuous, and at eight days the tubular form may be seen. When growth of the microgametophyte continues apically, one obtains what is called a "tubular gametophyte". Other gametophytes develop projections from the main tube and may be considered "haustorial gametophytes" (Fig. 5). The latter type correspond most closely to the development occurring in the plant. In vitro, the haustorial type is encountered most commonly in low sucrose concentrations of 0.25%-0.5%. A final type of microgametophyte enlarges as a sphere and these are called "spherical gametophytes"; this type predominates in the higher

sucrose concentrations of 4%-32%. The development of the microgametophytes in a given culture is never restricted to one particular type of tube cell formation, and the tubular form is found more or less throughout the range of sucrose concentrations.

The growth rate of the microgametophytes varies considerably in the cultures. Thus, various stages in development may be seen when a mount is made from the growing material. A certain stage or size may predominate, but a spectrum of the extent of development is usually present. The greatest size attained rarely exceeds 1000 microns, and generally the size is within a range of 200-600 microns.

## 2. Gametophyte Development

One of the first noticeable changes after germination has begun is the movement of the tube nucleus out into the area of the bulging intine of the tube cell. The nucleus is usually centrally located in the tube in the early stages of growth and is suspended amidst the vacuolar system by a network of cytoplasmic strands. The tube nucleus enlarges slightly and measures from 15-17 microns in diameter. The starch that is deposited early during germination tends to aggregate around the tube nucleus and to make it obscure, at least for a time. The tube nucleus remains in the distal portion of the

tube until tube growth has ceased; then it becomes located either centrally or peripherally in the cytoplasm. Later, with the maturation of the cells of the microgametophyte, the tube nucleus often migrates to the area adjacent to the exine.

Conspicuous areas of granular cytoplasm are found at the apices of the pollen tubes and haustoria during early microgametophyte development. These areas stain readily with acetocarmine, but they are noticeably absent from the spherical gametophytes. The aggregation of cytoplasm appears to be associated with the axial growth of the tubular and haustorial gametophytes, since it is absent when growth is isodiametric, as in the spherical gametophytes.

Maturation of the cells at the exine end of the gametophyte occurs, for the most part, after the main growth of the tube cell has taken place. The evanescent prothallial cell normally does not enlarge or become conspicuous at any time during development in vitro. However, the persistent prothallial cell enlarges and becomes a dome-shaped cell which fills the area immediately adjacent to the exine. Ventral to the persistent prothallial cell is the generative cell which undergoes vacuolation and then protrudes ventrally into the tube cell; its nucleus may be as much as three times the diameter of the prothallial cell nucleus.

## PLATE 1

### EARLY STAGES OF NORMAL DEVELOPMENT OF THE MALE GAMETOPHYTE IN VITRO

Figure 1. The method used for storing the pollen of Ginkgo. The one-half ounce capsule bottle contains the dessicant, calcium chloride, and the aluminum foil container for the pollen. natural size

Figure 2. Ventral view of the monocolpate pollen grain showing the longitudinal slit in the exine wall (E) and the intine layer (I), which is visible through this slit. 640X

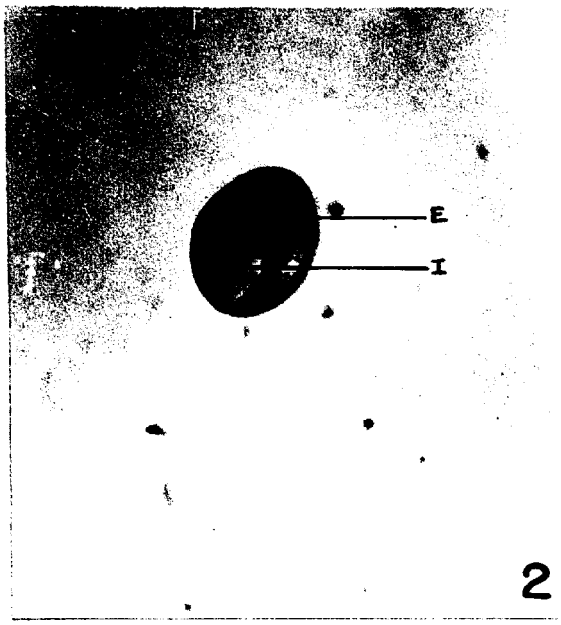
Figure 3. The pollen at shedding stage: shown are the evanescent prothallial cell nucleus $P_1$, the persistent prothallial cell nucleus $P_2$, the generative cell nucleus G, and the tube nucleus H. 1500X

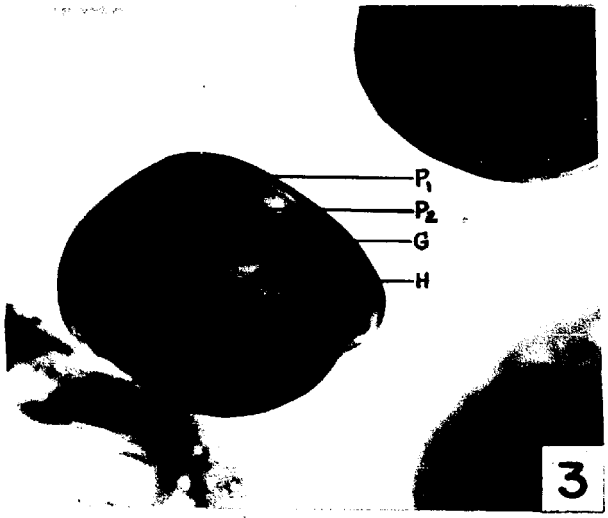
Figure 4. An initial stage in pollen germination in which the tube nucleus has moved out into the bulge in the intine. Note the accumulation of starch. 170X

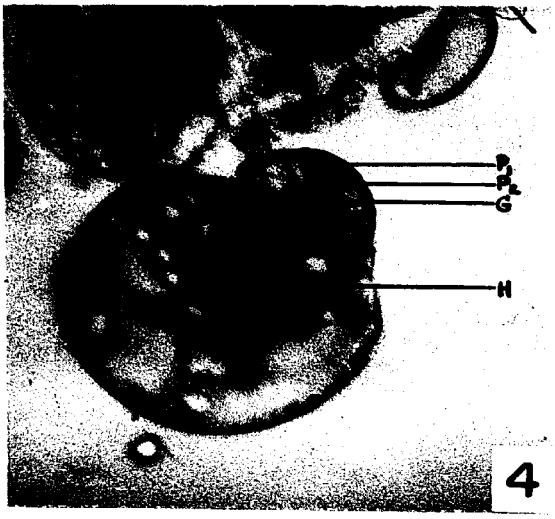
Figure 5. Extensive haustorial development from the tube cell of a microgametophyte. 140X

Figure 6. Early growth of the cells at the exine: the prothallial cell (P) is bulging into the recently formed stalk cell (S) and the body cell (B) is projecting into the large tube cell. 640X

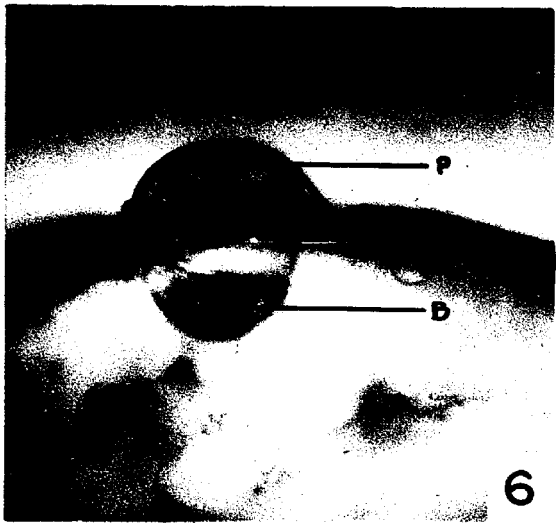
# PLATE I





Zimmermann (1896), Hirase (1898), and Herzfeld (1927) are agreed that the division of the generative cell nucleus in Ginkgo is not accompanied immediately by cell plate formation. They point out that the stalk nucleus is displaced toward the exine of the gametophyte and that the major portion of the generative cell cytoplasm is incorporated into the body cell. Indications of this peculiar behavior were found in some of the cultures (Fig. 24), but the evidence was by no means conclusive. Further observations on this phase of development are needed in order to clarify the manner in which the stalk and body cells are formed.

The nuclei of the stalk and body cell are initially equal in size, and, as both cells enlarge, the nucleus of the stalk cell remains essentially unchanged while that of the body cell enlarges to many times its original diameter. Accompanying these changes in the body cell nucleus are changes which occur in the cytoplasm. Leucoplasts were frequently found in the early stages of body cell development and they functioned in starch formation for a brief time. The presence of the starch was substantiated by an iodine-starch test (Fig. 7). The growing body cell soon consumed the starch and the leucoplasts were never observed to function again. In this connection, attention should be called to the lineage of the plastids in the microgametophyte. Mann

(1924) has demonstrated that plastids in the microspore mother cells of Ginkgo were distributed at random to the four microspores. In the cells of the mature pollen grain, however, one observes that only the tube cell contains numerous plastids. Therefore, it is of interest to note that plastids are present in the body cell of the microgametophyte because this implies that the sperm cells also receive them and that, ultimately, some male plastid material is carried to the egg cell by the sperm cell.

As the body cell develops, the persistent prothallial cell elongates and extends itself into the stalk cell. The growth of the prothallial cell often further invaginates the stalk cell and both the stalk and prothallial cells protrude into the body cell. This forms what appears to be a ring-like stalk cell surrounding a columnar prothallial cell, and both cells supporting the body cell (Fig. 8). It is improbable that the elongating prothallial cell actually penetrates the stalk cell, as Downie (1928) has suggested for Microcycas calocoma. Rather, it seems to be a case of extreme invagination of the stalk cell by the prothallial cell. The work of Davie (1951) on the antheridium in the Polypodiaceae clearly shows that the "ring cell" of the antheridium is formed by the enlargement of the central cell, instead of by a funnel-shaped division.

Much the same type of cellular enlargement is responsible for the relationship of the prothallial and stalk cells in Ginkgo.

Some of the most interesting changes in microgametophyte development are related to the advanced stages of body cell development. The body cell and its nucleus reach a very considerable size when fully developed (Figs. 8 and 9); the cells measure from 80-150 microns in diameter and their nuclei 30-80 microns. Two blepharoplasts or cilia-forming structures and two cytoplasmic bodies of an unknown nature appear in the body cell cytoplasm (Fig. 10). Both structures have been described by previous investigators. The blepharoplasts arise at the prothallial cell side of the body cell nucleus and then migrate laterally to take up their position on either side of the nucleus and at the poles of the slightly elongated cell. They are thus situated on an axis at right angles to the longitudinal axis of the microgametophyte and are located outside of the two cytoplasmic bodies. Little is known about the cytoplasmic bodies, although Herzfeld (1927) described their origin from the nucleolus and called them extra-nuclear nucleoli. These bodies stain slightly with Harris' hematoxylin but not at all with acetocarmine. They are spherical structures which are always present in the mature body cell. They are

distributed to each of the immature sperm cells during body cell division and they also persist in the mature sperm cells. However, nothing is known of their function either in the sperm cell or during fertilization.

The mature body cell with its blepharoplasts, cytoplasmic bodies and large nucleus (Fig. 10) undergoes nuclear division and cytokinesis to form two immature sperm cells. The body cell nucleus is at first granular in appearance, but it becomes somewhat hyaline as an early prophase nucleus is formed.(Fig. 11). The subsequent stages in body cell division *in vitro* were not well-defined in the material observed. In some cases, it was found that the nuclear membrane and two nucleoli were dissolved and that a fragmentary spindle was formed. The chromosomes, however, were obscure and the details could not be distinguished.

Unorganized sperm cell formation *in vitro* was not rare or exceptional. Observations on 56 pairs of these cells revealed that the orientation of the two daughter cells to the stalk cell, prothallial cell and exine was precisely what one finds *in vivo*. The nuclei of the immature sperm cells were mostly equal, and the cytoplasmic bodies and blepharoplasts were distributed one per cell (Figs. 12 and 13).

A comparison of the development of the body cells and immature sperm cells *in vitro* with what is found *in vivo* reveals some important differences. These are shown in Table 2.

TABLE 2

A COMPARISON OF THE SIZE OF BODY CELLS AND IMMATURE SPERM CELLS *IN VITRO* AND *IN VIVO*

All measurements are of the greatest diameter in microns. BC indicates the body cell and ISC the immature sperm cell.

| *In vitro* Cultured May 2, 1952 Observed July 5, 1952 | | *In vivo* Pollinated May 6, 1952 Observed Sept. 12, 1952 | |
|---|---|---|---|
| BC | ISC | BC | ISC |
| 77 | 62 | 144 | --- |
| 91 | 79 | 160 | --- |
| 67 | 62 | 160 | 96 |
| 67 | 50 | 164 | 120 |
| Average 75 | 63 | 157 | 108 |

The comparison in Table 2 shows that immature sperm cells appear *in vitro* in about two months, whereas the same stage of development *in vivo* requires four months. In addition to the time element, the microgametophytes under the two conditions show a marked difference in body cell size and in immature sperm cell size. Body cells averaged 75 microns *in vitro* and 157 microns *in vivo*; immature sperm cells averaged 63 microns *in vitro* and 108 microns *in vivo*. It is apparent that there was an acceleration of development *in vitro* as compared to what was observed *in vivo*, but at the expense of size.

# PLATE 2

## ADVANCED NORMAL DEVELOPMENT OF THE MALE GAMETOPHYTE IN VITRO

Figure 7. An iodine-starch test identifies starch and leucoplasts in the early body cell. E, exine, B, body cell, ST starch. 640X

Figure 8. Advanced body cell development is seen here at the exine end of a microgametophyte. E, exine, $P_2$, a columnar prothallial cell, S, the ring-like stalk cell, and B, the body cell. 640X

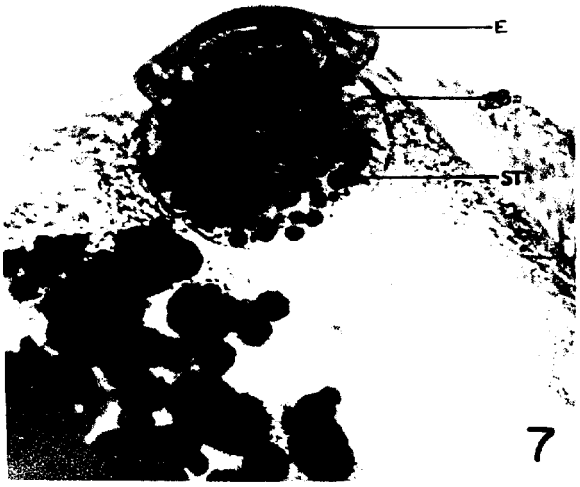
Figure 9. A large body cell and an unidentified cell next to the exine. 640X

Figure 10. Body cell in dorsal view immediately prior to division to form two immature sperm cells. BL, blepharoplasts, BC, body cell, NU, nucleolus, N, nucleus, and BCCB, cytoplasmic bodies of the body cell. 640X

Figure 11. A close-up of a body cell nucleus shows the nucleolus and chromonemata at early prophase. 730X

Figure 12. A side view of a pair of immature sperm cells; portions of the prothallial and stalk cells are seen above the cells. 640X

# PLATE 2

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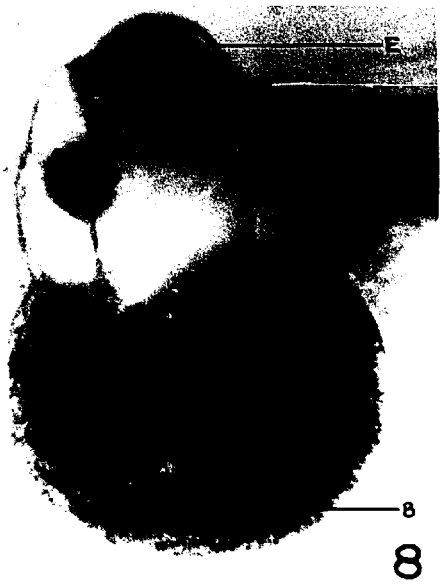


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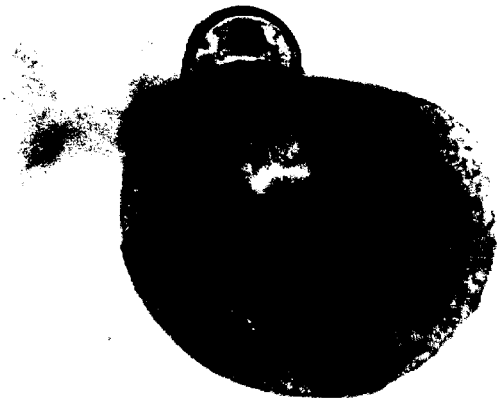

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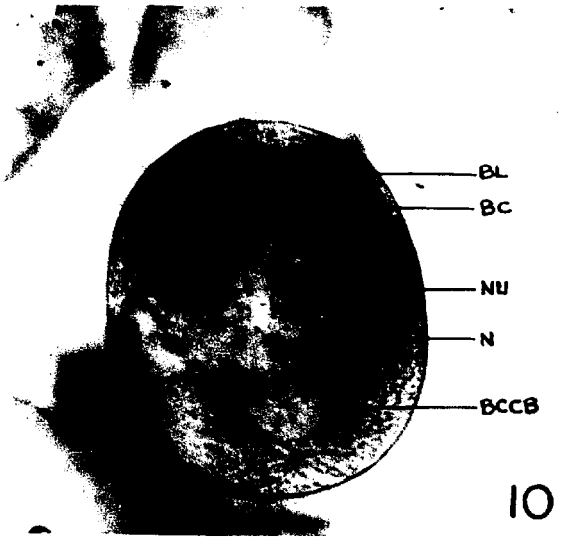


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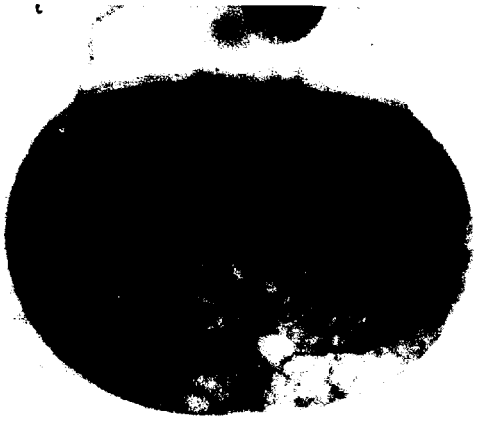

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In vivo the unorganized sperm cells undergo a metamorphosis and become mature sperm cells. The principal change involves the development of an apical band of cilia from the blepharoplast in each of the two daughter cells. After this development the sperm cells are liberated from the original body cell wall as naked motile cells, and they may then function in fertilizing an egg.

In vitro only one microgametophyte was found in which the pair of immature sperm cells were partly differentiated. The blepharoplast of each sperm cell was arranged as a spiral band at the apex of each cell and it consisted of about two revolutions. The spiral nature was unmistakeable, but no cilia could be discerned in the fixed and stained whole-mount preparation. The configuration of the blepharoplast resembled a conical cork-screw very similar to Shimamura's (1937) text-figure 4, 1a. This single occurence of sperm cells in culture was obtained on the WM-0.5%YE-IAA medium from inoculations with fresh pollen. The pollen was cultured on May 2, 1952 and the sperm cells were fixed on July 5, 1952. The body cell measured 72 microns in diameter and the sperm cells 55 microns. This one instance of sperm cell formation was the limit of normal differentiation thus far obtained in the culture of the microgametophyte of Ginkgo. It is noted that the

sperm cells were imperfect in lacking cilia and in their small size, but, nevertheless, their development represents a substantial step toward full maturation of the microgametophyte *in vitro*.

A summary of normal development *in vitro* has been compiled as Table 3 which is given below.

TABLE 3

THE CHRONOLOGICAL DEVELOPMENT OF THE MALE GAMETOPHYTE *IN VITRO*

| Stage of Development | Time Interval | Average Size in Microns |
|---|---|---|
| Mature pollen grain | May 5, 1952 | 26 |
| Germination | | |
| Starch accumulation | 1-72 hours | 26-30 |
| Swelling | 12-24 hours | 35-40 |
| Initial bulge | 18-72 hours | 40-60 |
| Growth of the tube cell | 1-30 days | 50-500 |
| Division of generative cell | 1-3 weeks | 20-25 |
| Small body cell | 2-4 weeks | 25-40 |
| Medium body cell | 3-6 weeks | 35-60 |
| Large body cell | 6-8 weeks | 60-150 |
| Immature sperm cells | 8-10 weeks | 30-80 |

## B. Abnormalities Occurring in Male Gametophytes Grown In Vitro

### 1. The Prothallial Cells

Few instances of possible prothallial cell division were found in all the observed material (Figs 27 and 35). Part of the difficulty in determining where the cells or nuclei arose lies in the fact that there is always the possibility that both of the original prothallial cells persisted during development. It could also be that the persistent prothallial cell divided. In any event, the comparative inactivity of these cells is an interesting contrast to what happens to the other cell components of the microgametophyte. Although little significance can be attached to these cells in either normal or abnormal development, their essentially non-active nature is of some importance from the standpoint of tissue ontogeny, as will be pointed out later.

### 2. Intercalary Divisions of the Stalk Cell

It has been assumed for purposes of discussion that the generative cell is the primary spermatogenous cell of the microgametophyte and that the stalk and body cells are potentially spermatogenous derivatives of this initial cell. The work of Downie (1928) on *Microcycas* and Doak (1932) on *Cupressus* has already been cited as evidence supporting this interpretation. The two derivatives of the body cell, the immature sperm cells, are

also considered spermatogenous in the sense that they must undergo a metamorphosis before they become mature sperm cells. Thus there is a rather large cellular complex composed of the generative cell, the stalk cell, the body cell, and the immature sperm cells which are considered potentially spermatogenous.

The generative cell has only been seen to divide to form the normal stalk and body cells. No unorganized divisions of this cell to form cell masses or abnormal configurations have been noted.

Both the stalk cell and the body cell have been observed to divide after their formation from the generative cell (Figs. 25 and 26). On a comparative basis the body cell has been found less abnormal than the stalk cell, although it sometimes forms groups of several cells or nuclei. The stalk cell or nucleus, as the case may be, frequently divides to form several intercalary cells or nuclei (Figs. 35, 36, 37, 38). These are the so-called intercalary divisions of the stalk cell, and they recall the situation in Microcycas calocoma, where the stalk cell divides repeatedly to form eight body cells, each of which forms two sperm cells at maturity. Unlike Microcycas, however, the stalk divisions in Ginkgo are sometimes confined to the nucleus and the number of nuclei produced is commonly less than eight.

### 3. The Septate Tube

Gametophytes which develop in the tubular manner sometimes have a septate or several-celled tube. The process involves nuclear division and cytokinesis within the tube cell to form additional cells (Figs. 14, 15, and 16). The tube may be several hundred microns in length and the number of vegetative cells may be 2 to 8 or more in number (Fig. 44). Some of the cells thus formed may divide again anticlinally or the nuclei of the cells may divide without cytokinesis to form cells with several nuclei.

Fresh cultured pollen showed fewer examples of the septate tube development than the stored pollen. For example, in one experiment the preserved pollen was cultured on December 17, 1952 and was observed five days later. A single acetocarmine mount revealed a total of 2094 germinated pollen grains and 22 occurrences of the septate tube condition. Thirteen of the gametophytes were 2-celled, six were 3-celled, two were 5-celled, and one was 6-celled.

### 4. Coenocytic Gametophytes

Coenocytic gametophytes appear frequently in the cultures of pollen. They are readily distinguished from the normal microgametophytes by the few to many nuclei which are scattered throughout the cytoplasm of what was

# PLATE 3

## THE SEPTATE TUBE AND CYTOPLASMIC UNIT ABNORMALITIES

Figure 13. A dorsal view of two immature sperm cells, their nuclei, and the cytoplasmic bodies. 640X

Figure 14. A male gametophyte with the tube cell divided into three cells. 140X

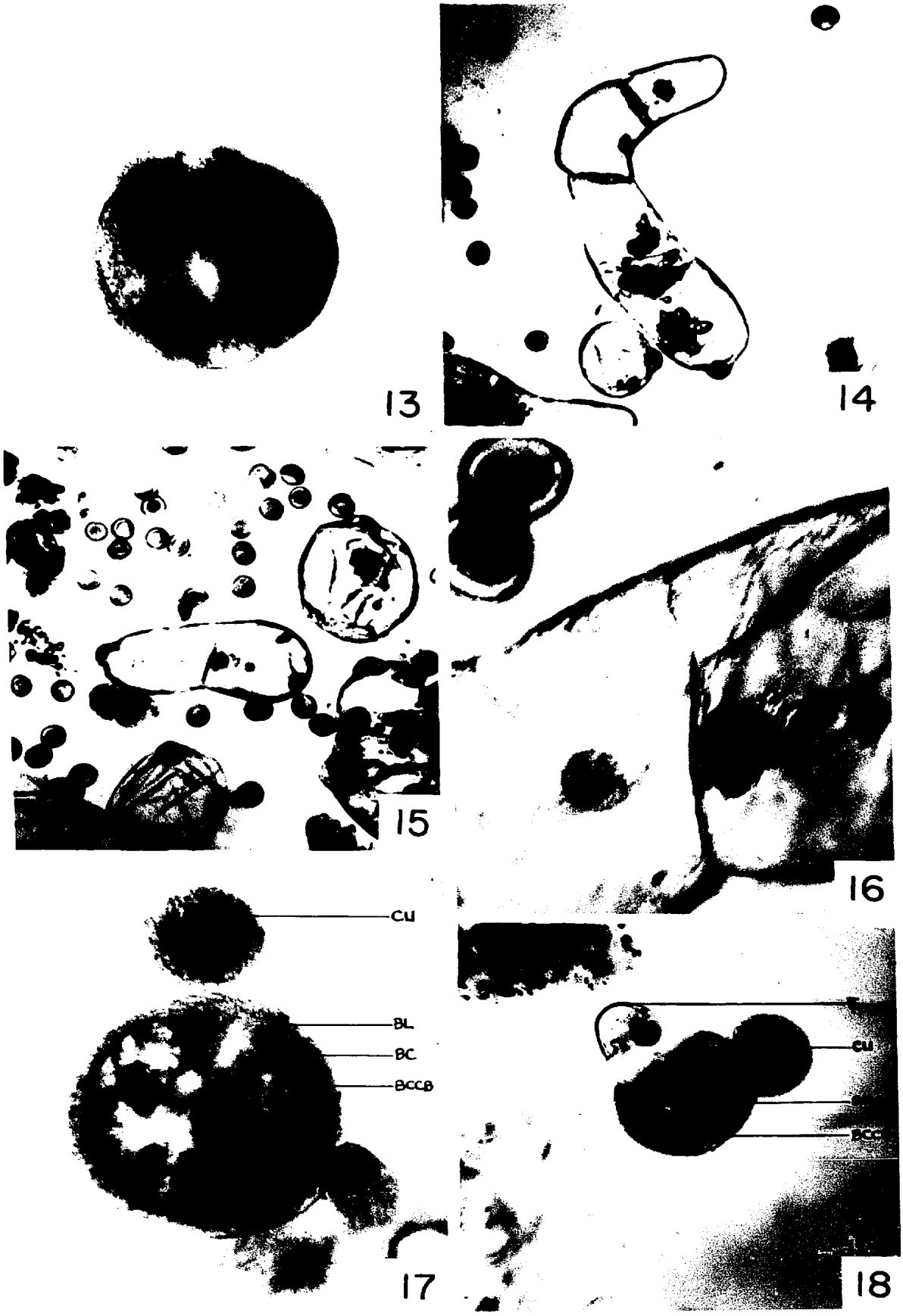
Figure 15. The gametophyte in the center is another example of a septate tube cell. 140X

Figure 16. An enlargement of the preceding figure illustrating a recent division of the tube nucleus. 640X

Figure 17. The cytoplasmic units cut off from the body cell present this aspect. BC, body cell, BL, blepharoplasts, BCCB, the body cell cytoplasmic body, and CU, the cytoplasmic unit. 640X

Figure 18. This illustrates more clearly the relationship of the cytoplasmic unit to the rest of the gametophyte. E, exine, CU, cytoplasmic unit, SMC, immature sperm cell, and BCCB, the body cell cytoplasmic bodies. 380X

PLATE 3



previously the tube cell of the gametophyte (Figs. 21, 22, and 23). Over 72 such gametophytes have been observed in permanent mounts and others in temporary mounts, and the number of their nuclei has varied from 2 to 40 or more. Invariably, these coenocytes develop from the spherical type of gametophytes rather than from the haustorial or tubular types. The reason for the development from thespherical gametophytes seems to be connected with the fact that, in all the multinucleate coenocytic gametophytes, there has been no evidence of complete cell plate formation. Only fragments of a cell plate have been observed, and it is apparent that the cell plate cannot span the expanses of the large spherical cells.

When first observed in 1951 the coenocytes were old and large, and the other cells of the gametophytes were unidentifiable. Little could be deduced about the origin of the coenocytes and they were quite variable in nuclear size and number (Figs. 30 and 32).

In 1952 early stages of coenocyte formation were found and it was then possible to identify the cells of the gametophyte. Figures 45 and 46 illustrate two coenocytes which developed in an experiment involving hanging drop cultures. The experiment was set up on February 4, 1953 by using stored pollen and a liquid WM-0.25%YE-IAA medium. The coenocytes were abundant within 24 days

after the cultures were started. A temporary aceto-carmine mount showed a large number of gametophytes with several tube nuclei, and clearly visible at the exine were the other cells of the gametophyte, the persistent prothallial cell, the generative cell, and in some instances, the evanescent prothallial cell. In this single mount there were 7 gametophytes with 2 tube nuclei, 4 with 4 tube nuclei, 3 with 6, 1 with 7, 1 with 14, and 2 with 15 tube nuclei.

As indicated above, these observations suggest an origin of the coenocytic gametophytes from the tube nucleus. However, one cannot entirely rule out the possibility that other cells of the gametophyte may also take part in coenocyte formation. At least the prothallial, stalk, and body cells at the exine are sometimes absent from the older coenocytic gametophytes (Figs. 30 and 32). It is presumed that the latter cells were absorbed by the tube cell or broken down and their nuclei released into the tube cell, or perhaps they never existed, in which case the divisions to form the coenocytes would have taken place beginning with the nucleus of the microspore. The latter possibility seems very unlikely in view of the evidence already presented on early coenocyte development, but it is mentioned here because Stow (1930) believed that his embryo-sac-like giant pollen grains in Hyacinthus were derived from the

nucleus of the microspore.

The multinucleate nature of these male gametophytes of Ginkgo is not unique. One finds a similar pattern in the early female gametophyte and proembryo of Ginkgo. In both of the latter structures wall formation is delayed until after the 256-nucleate stage. In the development of the Ginkgo proembryo, for example, Favre-Duchartre (1950) called attention to the transitory phragmoplasts which were formed at the interphases of the 2 to 64-nucleate stages of proembryo growth. Complete cell wall formation occurred after the 256-nucleate condition was reached. Stow (1930) found that there were multinucleate gametophytes among the embryo-sac-like pollen grains of Hyacinthus which contained as many as 16 nuclei and showed no apparent organization. Jeffrey and Chrysler (1907) reported a coenocytic condition in Araucaria microgametophytes which was arrived at through the liberation of several prothallial cell nuclei into the tube cell.

The multinucleate coenocytic gametophytes which develop in vitro have been correlated with the spherical type of pollen tube growth and with the adverse effect of the pollen storage technique on the prothallial and spermatogenous cells. The activity of the tube nucleus has been suggested as the origin of this abnormality, and the activity is apparently related to either a re-

# PLATE 4

## GAMETOPHYTE INCLUSIONS AND THE MULTINUCLEATE COENOCYTIC GAMETOPHYTES

Figure 19. A Sudan III test for lipids was made on male gametophytes which had been in culture for two months. The upper microgametophyte shows oil droplets in the peripheral cytoplasm; the lower one is in optical section and shows the aggregation of starch in the central part of the gametophyte. 170X

Figure 20. Unidentified crystals were sometimes found in gametophytes from older cultures. The gametophyte shown here was from a culture that was five months old. 170X

Figures 21-23. Three multinucleate coenocytic microgametophytes: the first contains approximately 10 nuclei, the second about 21 nuclei, and the last one over 40 nuclei. 140X

# PLATE 4

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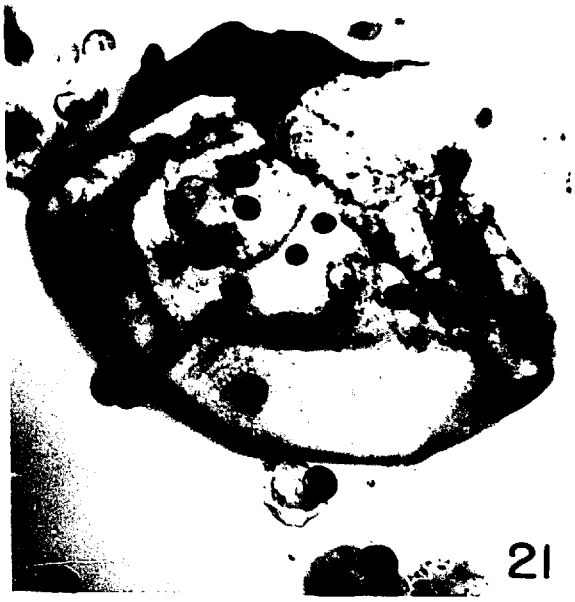

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lease from some inhibitory agent or to a stimulatory effect of the culture medium.

### 5. Cytoplasmic Units

Body cells in the later stages of maturation were found to eliminate a portion of their contents by furrowing. For the most part, such formations lacked a nucleus and they were therefore called,"cytoplasmic units" (Figs. 17 and 31). Gametophyte development progressed in the normal manner until the body cell was nearly ready for division; instead of dividing, however, a furrowing process intervened (Fig. 29) and a small portion of the body cell cytoplasm was separated from the cell. The blepharoplasts, the cytoplasmic bodies, and the body cell nucleus remained within the larger part of the body cell. In this way, one or two cytoplasmic units were subtracted from a body cell (Fig. 31); rarely, a portion of the body cell nucleus or a blepharoplast was included in the cytoplasmic unit.

The cytoplasmic units were not very abundant in the cultures, but their peculiar nature warranted their inclusion as one of the types of abnormalities. The relative abundance of the cytoplasmic units and other abnormality types are included in Table 4. The type of organization of the different abnormalities is also indicated.

TABLE 4

THE COMPARATIVE FREQUENCY OF ABNORMALITY TYPES IN CULTURES OF THE GINKGO MALE GAMETOPHYTE

| Abnormality Type | No. Counted | Organization |
|---|---|---|
| Extra prothallial cells (?) | 3 | 2 cells |
| Extra body cells | 7 | 2-5 cells |
| Extra stalk cells | 23 | 2-8 cells |
| Extra immature sperm cells | 2 | 3,4 cells |
| Extra tube cells | 100 plus | 2-15 cells |
| Coenocytic gametophytes | 100 plus | 2-40 nuclei |
| Cytoplasmic units | 24 | 1-3,usually 1 |

## 6. Tissue Formation

The most obvious abnormality that appeared in the pollen cultures was the outgrowth of a tissue from a mass of the developing microgametophytes. The tissue arose as a white cluster of cells projecting from a slightly discolored group of gametophytes, and its occurrence was initially limited to a single isolated piece in one culture bottle. Under the circumstances, many questions arose concerning the tissue.

Where did the tissue come from? Would it be induced to form in subsequent cultures? Could it be excised and subcultured? And what were its cytological attributes? These and other questions provided the orientation for the study of this particular abnormality. Some of the results of this investigation are reported in the section which follows.

# PLATE 5

## SOME ASPECTS OF NORMAL AND ABNORMAL DEVELOPMENT

All figures show cells at the exine end only.

Figure 24. Normal: at the exine (E) the persistent prothallial cell (P) has enlarged and the stalk (S) and body (B) nuclei have been formed. Note that the latter two nuclei have not been separated by a cross-wall. 570X

Figure 25. Abnormal: a prothallial cell nucleus is present (P) and the stalk (S) and body (B) cell nuclei have been separated by a cross-wall. Both of the nuclei have divided again. 570X

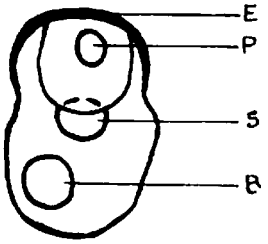
Figure 26. Abnormal: the prothallial cell is absent and the stalk cell (S) has divided to form two cells while the body cell has become binucleate. 570X

Figure 27. Abnormal: this exine (E) end of a gametophyte shows two prothallial cells (P), two stalk cells (S), and a well-developed body cell (B). 570X

Figure 28. Normal: the body cell (B) prior to the formation of the two immature sperm cells. E, exine, P, prothallial cell, S stalk cell, BCB, cytoplasmic body of the body cell, BL, blepharoplast. 570X

Figure 29. Abnormal: a cytoplasmic unit is being formed from a large body cell. E, exine, P, prothallial cell, BL, blepharoplast, BCB, cytoplasmic body of the body cell, and B, the body cell nucleus. 570X

# PLATE 5

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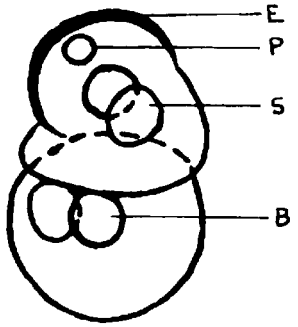


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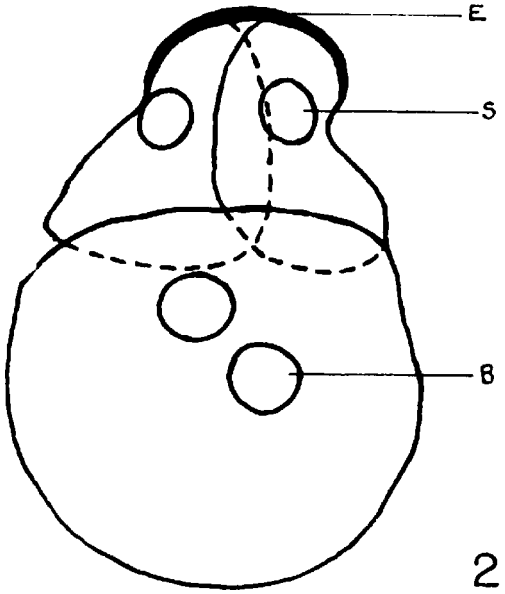


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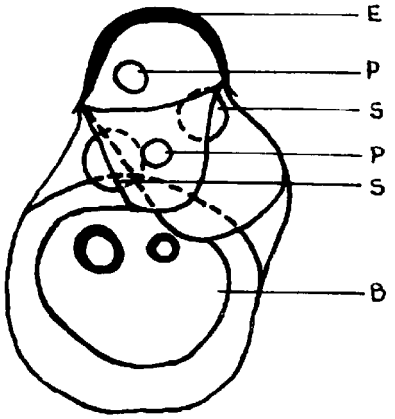


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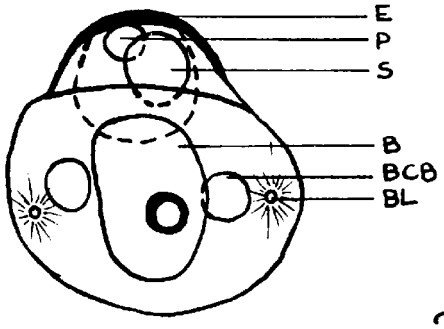


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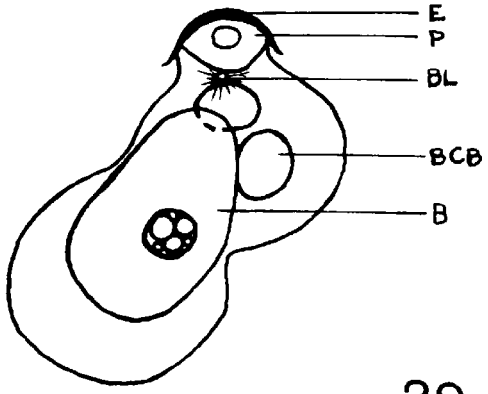


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# PLATE 6

## COENOCYTES, CYTOPLASMIC UNITS, AND THE TISSUE DERIVED FROM THE POLLEN

Figure 30. Five-nucleate coenocytic gametophyte with only the prothallial cell nucleus identified from its smaller size. 570X

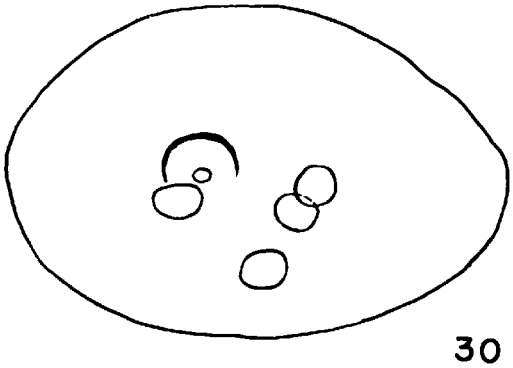
Figure 31. Intact gametophyte showing the relative size of the parts. Two cytoplasmic units (CU) have been cut off from the body cell (BC). E, exine, CB, cytoplasmic bodies, S, stalk nucleus, P, prothallial nucleus, T, tube nucleus, and BL, blepharoplast. 570X

Figure 32. A 16-nucleate coenocyte with nuclei of rather uniform size. 570X

Figure 33. This is interpreted as an early stage of tissue formation, although the derivation of the cells is unknown. Only the exine and the wall of the gametophyte may be identified. 570X

Figure 34. Meristematic cells of the pollen tissue. 570X

# PLATE 6



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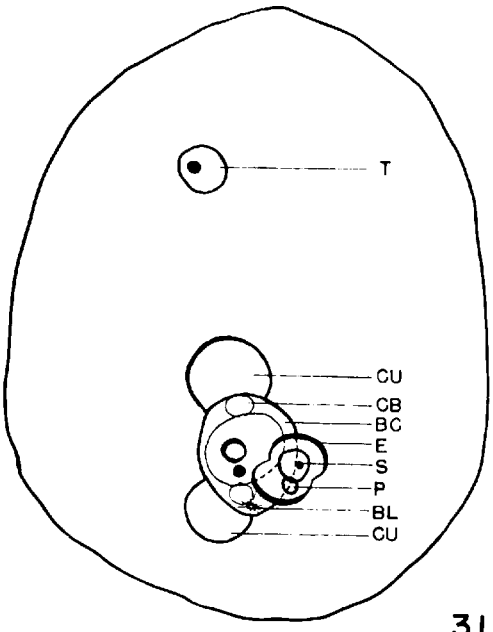


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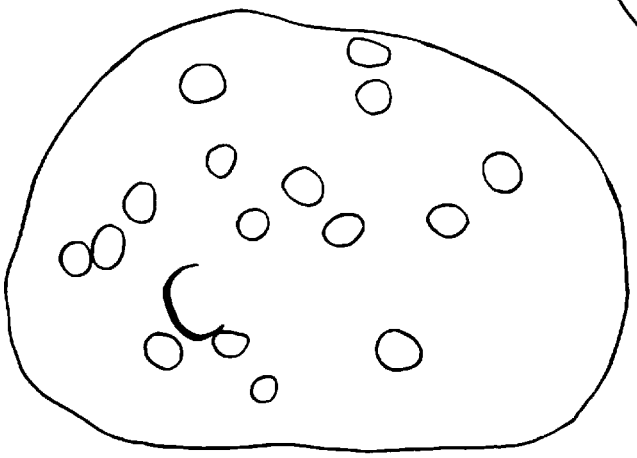

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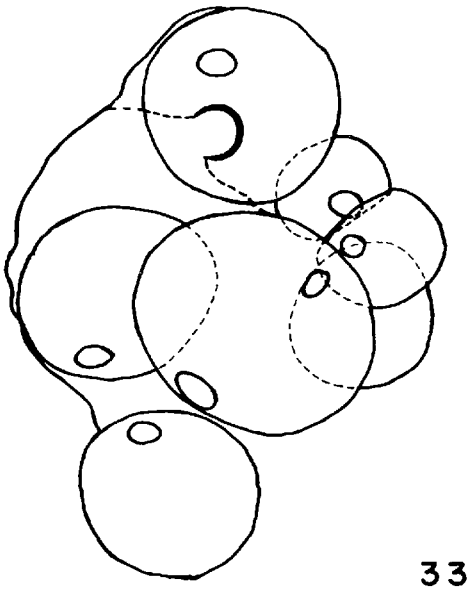

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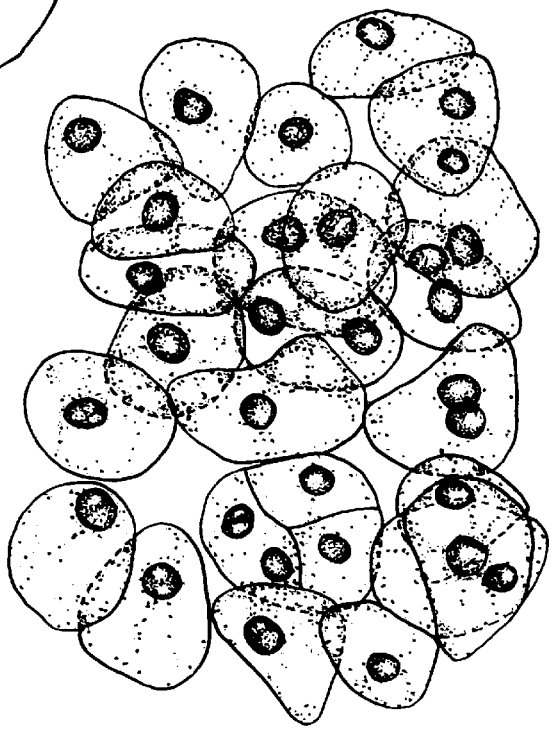

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# PLATE 7

## INTERCALARY DIVISIONS OF THE STALK CELL

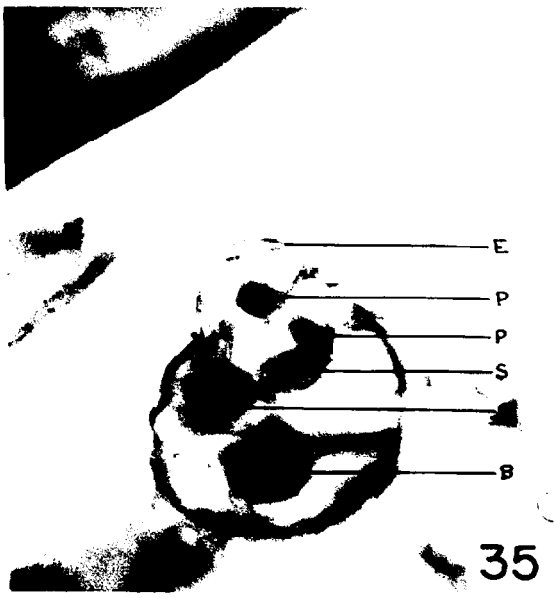
Figure 35. Both the stalk nucleus (S) and the prothallial nucleus (P) have divided. The body cell (B), meanwhile, is at a relatively early stage of development. 730X

Figure 36. Three large stalk nuclei (S), body cell (B) nearly mature, and the tube nucleus (H). E is the exine. 730X

Figure 37. This shows the gross aspect of a gametophyte with numerous cells at the exine end. 140X

Figure 38. This is an enlargement of part of the preceding figure. The exine (E), the prothallial cell (P), the body cell (B), and the stalk cell (S) are all normal in their relation to each other, except that there has been an intercalation of cells and nuclei between the body cell and the prothallial cell. These intercalated cells are believed to be derived from the stalk cell. 730X

# PLATE 7

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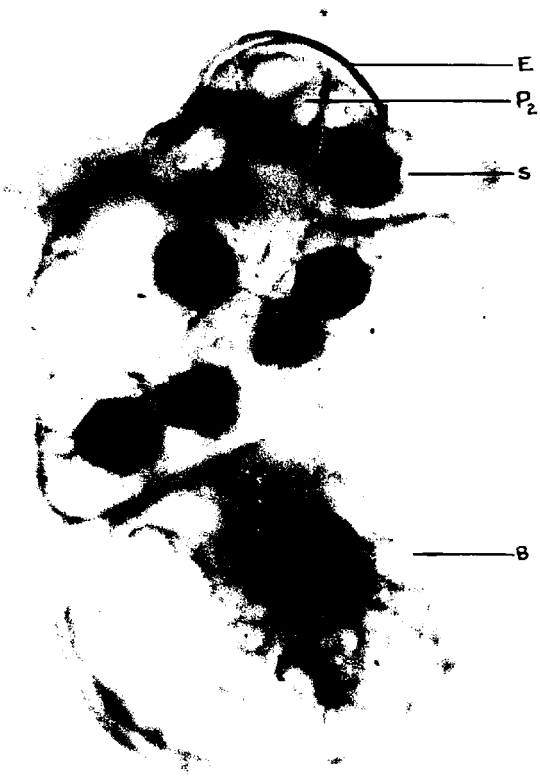


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## C. The Tissue Derived from the Pollen

### 1. Occurrence and Growth

The first pollen tissue appeared in culture as a white mass of cells growing out from a sporangium. The sporangium was placed on the agar surface as part of an intact strobilus just prior to anthesis on May 3, 1951 and the tissue was noticed on February 2, 1952. Nine months of culturing elapsed before the appearance of the tissue. No other cases of tissue formation were found during this first year of culturing the male gametophyte (May, 1951-May, 1952).

The medium on which the tissue was produced was White's Standard and one-third tomato juice by volume, WS-T. At the time the tissue appeared, the WS-T medium was considerably dehydrated and a transfer of the tissue was necessary. The piece of tissue (6-7 $mm^3$) was therefore divided into five fragments and placed on a new medium, WM-0.5%YE. It was reasoned that, since the microgametophytes developed better on this new medium, the growth of the pollen tissue might also be benefited by the new substrate. As it happened, only two of the five cultures actively proliferated and the growth in these was very slow. Two months elapsed before the two active cultures were large enough to furnish material for subculturing. Transfers were made to several media,

and the WM-0.25%YE-IAA medium was finally found to support growth very well. The latter medium was used to establish and maintain the first clone of the pollen tissue, Clone 1. Table 5 gives a history of this clone of the tissue.

TABLE 5

TRANSFERS OF GINKGO POLLEN TISSUE, CLONE 1

| Transfer Number | Date of Transfer | Culture Medium* | | Number of Bottles |
|---|---|---|---|---|
| 0 | 2-2-52 | WS-T | | 1 |
| 1 | 2-7-52 | WM-0.5%YE | | 5 |
| 2 | 4-17-52 | WM-0.25%YE | | 4 |
| 3 | 5-6-52 | WM-0.25%YE | | 7 |
| 4 | 6-18-52 | WM-0.25%YE-IAA | 3 | 13 |
| | | WM-IAA | 3 | |
| | | MM-IAA | 4 | |
| 5 | 7-3-52 | WM-CP-NAA | | 6 |
| 6 | 7-20-52 | WM-0.25%YE | | 34 |
| 7 | 8-25-52 | WM-0.25%YE | 24 | 53 |
| | | WM-0.25%YE-IAA | 29 | |
| 8 | 10-19-52 | WM-0.25%YE-IAA | | 20 |
| 9 | 10-30-52 | WM-0.25%YE-IAA | | 90 |
| 10 | 12-10-52 | WM-0.25%YE-IAA | | 70 |
| 11 | 12-27-52 | WM-0.25%YE-IAA | | 60 |
| 12 | 1-17-53 | WM-0.25%YE-IAA | | 60 |
| 13 | 2-17-53 | WM-0.25%YE-IAA | | 60 |
| 14 | 3-17-53 | WM-0.25%YE-IAA | | 60 |

*For the composition of culture media see page 30.

One other tissue initial occurred in the 1951 cultures in June, 1952, thirteen months after the pollen was placed on the nutrient agar. This tissue was subcultured on different media, but it did not grow well. It lacked sufficient vigor to establish a clone of tissue.

Considering the very limited occurrence of tissue initials in cultures from the 1951 season, an effort was made in 1952 to increase the number of these initials. The nutrient substrate was varied, an improved basal medium (WM) was used, and the stored pollen was available for culture purposes. More tissue initials were obtained under these conditions, as is shown in Table 6.

TABLE 6

THE FREQUENCY AND TIME INTERVAL OF TISSUE FORMATION

| Culture Medium** | Date Started | Incubation Time in Months | Bottles in Experiment | Bottles with Tissue |
|---|---|---|---|---|
| WS-T | 5-3-51 | 9 | 100* | 1* |
| WS-T | 5-3-51 | 13 | 100* | 1* |
| WS-T | 5-2-52 | 5 | 48 | 2 |
| WM-CP-NAA | 5-7-52 | 5 | 4 | 1 |
| WM-0.25%YE-IAA | 5-25-52 | 6 | 15 | 1 |
| WM-0.5%YE | 5-27-52 | 6 | 15 | 3 |
| WM-0.5%YE | 5-27-52 | 10 | 15 | 3 |
| WM-0.25%YE-IAA | 6-24-52 | 5 | 5 | 1 |
| WM-CP | 6-24-52 | 5 | 5 | 1 |
| WM-CP-NAA | 6-24-52 | 5 | 5 | 1 |
| MM-IAA | 6-24-52 | 5 | 5 | 1 |
| WM-IAA | 7-18-52 | 3½ | 2 | 1 |
| WM-CP-NAA | 7-18-52 | 3½ | 8 | 2 |
| MM-IAA | 7-24-52 | 4 | 12 | 2 |
| WM-0.25%YE | 7-24-52 | 4 | 3 | 1 |
| WM-0.25%YE | 7-24-52 | 8 | 3 | 1 |
| WM-IAA | 7-24-52 | 4 | 54 | 1 |
| MM-IAA | 7-24-52 | 6 | 6 | 1 |
| WM-0.25%YE-IAA | 7-24-52 | 4 | 6 | 1 |
| WM-IAA | 8-12-52 | 3 | 15 | 1 |
| WM-IAA | 8-12-52 | 8 | 15 | 3 |
| WM-0.25%YE-IAA | 9-15-52 | 2 | 15 | 1 |
| WM-0.25%YE-IAA | 9-15-52 | 3 | 15 | 1 |
| WM-0.25%YE-IAA | 10-31-52 | 2½ | 30 | 1 |
| WM-0.25%YE | 10-31-52 | 4½ | 30 | 1 |
| WM-CP-NAA | 11-8-52 | 1½ | 2 | 1 |
| WM-CP-NAA | 11-8-52 | 4 | 2 | 1 |
| WM-0.25%YE-IAA | 12-17-52 | 4 | 30 | 2 |
| | | Average 5 | Total 365 | Total 36 |

*1951 cultures omitted from total
**Composition of media on page 30

Table 6 shows 36 tissue initials formed in 365 cultures for a frequency of 9.8%. These figures include only those experiments which showed tissue formation at some time or other. The frequency of tissue initials in all experiments was 4% of the total number of bottles inoculated with the pollen. There appears to be a lack of medium specificity in tissue formation, although some media seem to be better than others. The data on the basis of media are given in Table 7.

TABLE 7

THE EFFECT OF NUTRIENT MEDIUM ON TISSUE FORMATION

| Culture Medium* | Number of Cultures | Interval in Months | Tissue Initials | % |
|---|---|---|---|---|
| WM-CP-NAA | 27 | 4.0 | 7 | 25.9 |
| WM-0.5%YE- | 30 | 8.0 | 6 | 20.0 |
| MM-IAA | 23 | 5.0 | 4 | 17.5 |
| WM-0.25%YE | 36 | 5.5 | 3 | 8.3 |
| WM-IAA | 86 | 4.8 | 6 | 7.0 |
| WM-0.25%YE-IAA | 116 | 3.8 | 8 | 6.9 |

*For the composition of media see page 30.

Table 7 shows the occurrence of tissue initials in the WM-CP-NAA medium (25.9%) to be considerably above the average of 9.8% shown in Table 6. On the other hand, the WM-0.25%YE-IAA medium, which initiated tissue formation in the shortest time, shows the lowest incidence of tissue initiation in the group (6.9%). These data indicate a substrate effect of some sort, and it is possible that tissue production is increased by media beneficial to

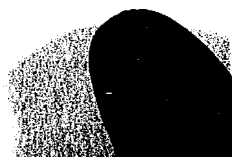
to pollen growth or by media supporting only poor growth and inducing abnormalities. In any event, the substrate effect is obscure, since the first three media in Table 7 have little in common as far as composition is concerned, and there appears to be no positive correlation between the components of the media and tissue initiation. All that may be concluded is that the WM-CP-NAA medium produced a large percentage of initials for the number of cultures made and in a relatively short time. It would seem to be the best medium for obtaining tissue initials.

Stored pollen gave a higher frequency of tissue formation than did fresh pollen. All of the 1951 cultures were set up with fresh pollen and only two tissue masses were obtained from several hundred culture bottles. Out of ten experimental set-ups with fresh pollen in 1952 only two gave tissue initials. The two experiments showed 3 bottles out of 52 with tissue initials (5.8%). In the stored pollen cultures, 19 out of 24 experiments produced tissue initials. The nineteen experiments showed 32 initials out of 313 bottles (10.2%). Consequently, there appears to be an advantage in using preserved pollen for obtaining tissue formation, and this infers that some of the stored pollen is altered by the preservation technique.

Three clones of pollen tissue have been established

from the initials mentioned in Table 6. Clone 1 was the original isolation and it was derived from fresh pollen cultures made in 1951. Clone 2 was isolated from fresh pollen cultured in 1952, and Clone 3 originated from pollen preserved May 5, 1952 and cultured May 27, 1952. The first subculture to form the clones took place at different times for each clone. Clone 1 was obtained on February 2, 1952; Clone 2 on October 26, 1952; Clone 3 on November 15, 1952. All of the clones were maintained as continuous cultures on the WM-0.25%-YE-IAA medium by subculturing every 3-6 weeks. Up to the present time, Clone 1 has passed through 14 subcultures and the remaining two clones have passed through 5 transfers each. Occasionally unproliferative pieces of tissue turned up in the cultures, but by and large, there has been no apparent diminution of growth activity in the clones.

Growth in the several clones appears to be identical. Subculturing consists of taking an inoculum of approximately 5 $mm^3$ of the tissue and placing it on a fresh nutrient agar (WM-0.25%YE-IAA). There is a lag of several days before the tissue recovers from the transfer process and begins to divide. Within 7-12 days, however, the tissue spreads away from the original inoculum and upward, and it fills the 1/2 ounce culture bottle to a height of 7-10 mm by the end of the third week. The



tissue usually maintains itself in this condition for some time if not subcultured. Later, at 6-8 weeks, the tissue gradually changes from a chalk white to a translucent mass and then becomes brown and collapses.

Differentiation of the tissue has been limited to the storage parenchyma cell type. No effort has yet been made to induce transformations of the tissue cells either by chemical means or by the implementation of grafting techniques.

A single experiment on the growth of the tissue in shake culture indicates that the tissue will proliferate rapidly under such conditions. The tissue (2.15 gm) was inoculated into 10 125 ml Erlenmeyer flasks containing a liquid WM-0.25%YE-IAA medium; it was then aerated by the movement of a reciprocal shaker for a period of 15 days. One contaminated culture was discarded and the results were calculated on the basis of the nine remaining bottles. The tissue was filtered out of the liquid medium and then washed and weighed. The harvest amounted to 12.95 gm wet weight after blotting. The initial inoculum for the nine flasks was 1.93 gm, a percentage increase of 670% over the initial wet weight. The dry weight of the harvested tissue was .425 gm or 3.5% of the wet weight so that a very great part of the tissue growth was concerned with water uptake. The significance of the experiment, however, is the fact that it demon-

strates the ability of the tissue meristems to continue functioning in an aerated liquid medium. In addition, the tissue showed the capacity to maintain its proliferative nature while in a dispersed, non-aggregated condition.

## 2. Cytology

In its gross aspect the pollen tissue may be characterized as a loose, white, parenchymatous cell mass (Fig. 39). Tissue growth is irregular in form, since granular outgrowths are produced over the entire exposed surface. In texture, the mature cells of the tissue seem not to adhere to one another and are quite friable. The small meristematic areas of the tissue are relatively compact and adherent (Fig. 34). The meristems are commonly composed of 25-100 cells which measure from 25-50 microns in diameter and have nuclei of 12-20 microns in diameter. Cells of the meristems are usually devoid of starch when the tissue is proliferating, but the cells derived from them accumulate the substance in great quantities as they enlarge through hydration and vacuolation. The mature cells may reach a size of 500 microns or more, but the usual range is from 75-200 microns; the nucleus enlarges only slightly to 12-25 microns. Invariably the cells are spherical and highly vacuolate, and their nuclei are located in the central or peripheral cytoplasm.

Multinucleate cells are commonly encountered in the tissue (Figs. 40 and 43). The nuclei may be two to twenty or more, and they are usually dispersed throughout the cell. Presumably nuclear division goes on very rapidly in some of the cells derived from the meristematic centers, but without cytokinesis. Whether these multinucleate cells are later divided into many smaller cells by wall formation has not been established; at least no wall formation has been observed.

Leucoplasts in the tissue cells are known to function in starch accumulation, but when the tissue is placed in light they do not become pigmented. Even on media with minimal or low carbohydrate content the plastids of the tissue cells remained unpigmented. In contrast to this, the female gametophyte of Ginkgo has large functional chloroplasts, and the gametophyte is known to intensify its greenness in light. Hence, there appears to be a distinct difference in potentiality between the plastids found in the pollen tissue and those found in the female gametophyte.

Heteroploid cells are reported for many plant tissues and for plant tissues grown in vitro. The alteration of the chromosome complement often occurs normally, or it may be induced by colchicine, which acts to inhibit chromosome separation at metaphase. That other chemical agents may act to produce heteroploid cells has also

been demonstrated. Greenleaf (1938) induced polyploid cells in Nicotiana callus through treatment with indoleacetic acid, and Dermen (1941) reported a similar response in Phaseolus tissues to naphthaleneacetic acid. In tissue cultures of corn endosperm, Lowry (unpublished) found chromosome numbers of 10, 20, 30, 60, and 100, and Straus (unpublished) found additional counts of 45 and 48 chromosomes and 105 double chromosomes.

The pollen tissue also possesses nuclei with increased chromosome complements. The haploid chromosome number of the tissue was established at twelve shortly after it was produced, and this figure agreed with that obtained by Ishikawa (1910) and Sax and Sax (1933). More recent squash preparations of the tissue shows a basic haploid complement (Fig. 48). However, diploid and higher complements have also been observed from time to time (Fig. 47). It is believed that the meristematic centers remain haploid and that the derivatives from these centers occasionally become heteroploid during later abnormal nuclear divisions.

### 3. Ontogeny of the Pollen Tissue

It has been well established that the tissue has its origin from the microgametophytes. It was true that there existed some doubt of this fact at the beginning, when the sporangia were cultured intact, for it was

# PLATE 8

## TISSUE DERIVED FROM THE POLLEN OF GINKGO

Figure 39. A 125 ml Erlenmeyer flask containing the pollen tissue. This tissue represents one month's growth from an initial inoculum approximately 6 $mm^3$. natural size

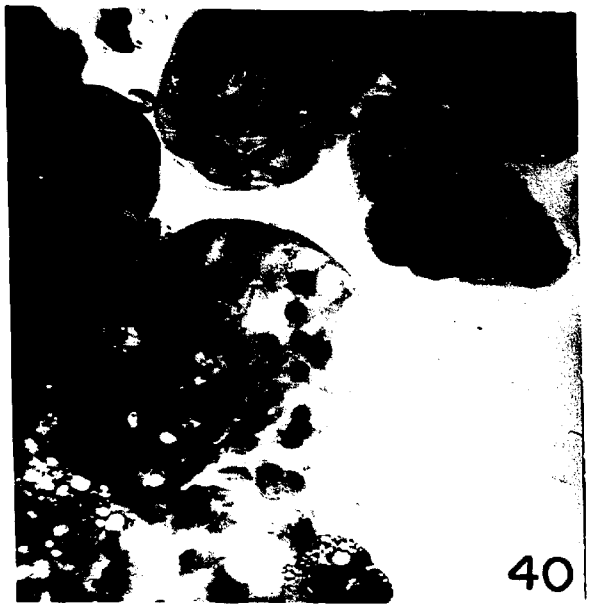
Figure 40. A coenocytic cell of the tissue with about 20 nuclei. 140X

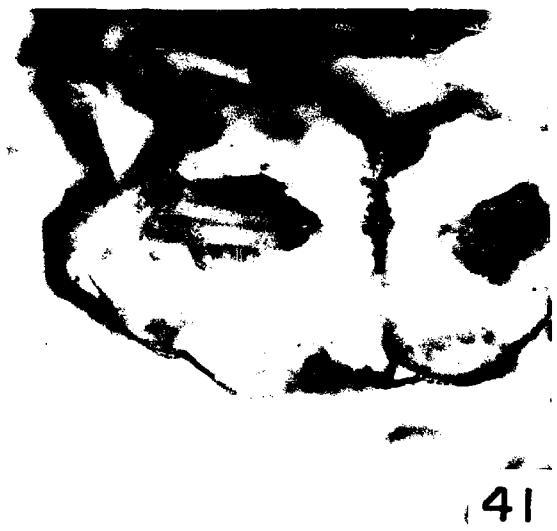
Figure 41. Telophase stage of a dividing tissue cell showing cell plate formation. 640X

Figure 42. Synchronized divisions in a meristematic area. 730X

Figure 43. Vacuolate and mature pollen tissue cells are variable in size and may contain one or several nuclei. The large cell, for example, contains one cluster of six nuclei and another of two nuclei. The diameter of the cell is approximately one-half millimeter. 90X

# PLATE 8



# PLATE 9

## ADDITIONAL ASPECTS OF GAMETOPHYTE ABNORMALITIES AND THE TISSUE

Figure 44. A tubular type of gametophyte which has become septate. The exine (E) is seen at the right, but the other cells of the gametophyte are not shown. 170X

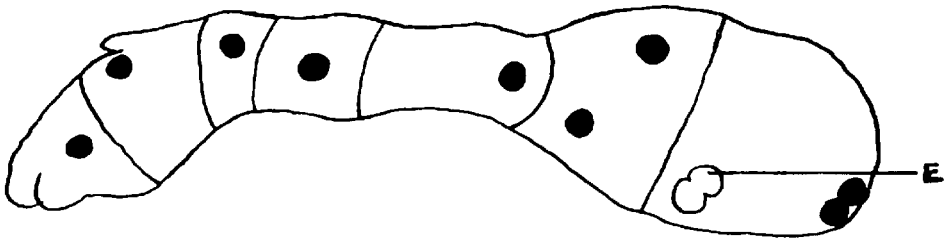
Figure 45. A spherical type of gametophyte with eight tube nuclei. Next to the exine (E) is the evanescent prothallial cell, the persistent prothallial cell, and the generative cell. 270X

Figure 46. Another coenocytic gametophyte showing some variability in nuclear size. Next to the exine (E) is the persistent prothallial cell and beyond the generative cell. 270X

Figure 47. A diploid nucleus of a pollen tissue cell with 48 chromosomes at metaphase. 1700X

Figure 48. A tissue cell with a complement of twelve chromosomes. 2700X

PLATE 9

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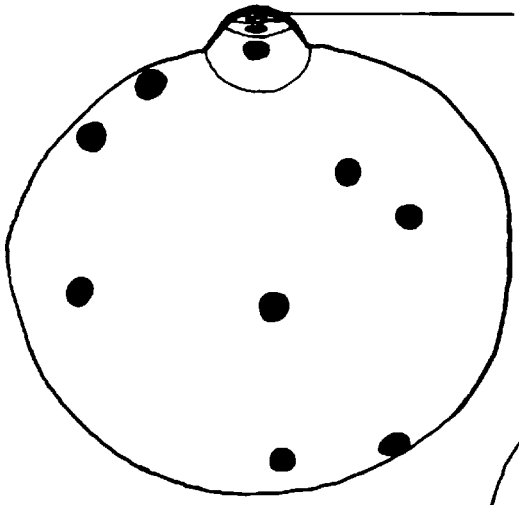


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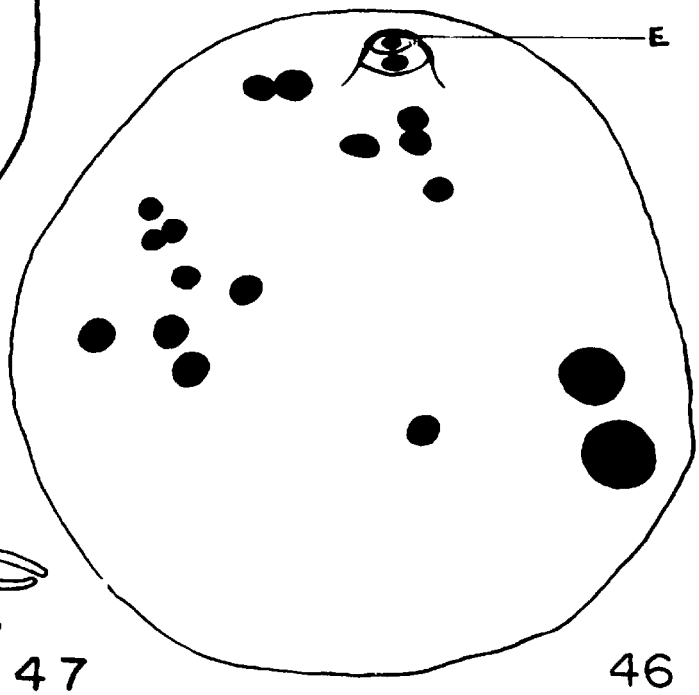


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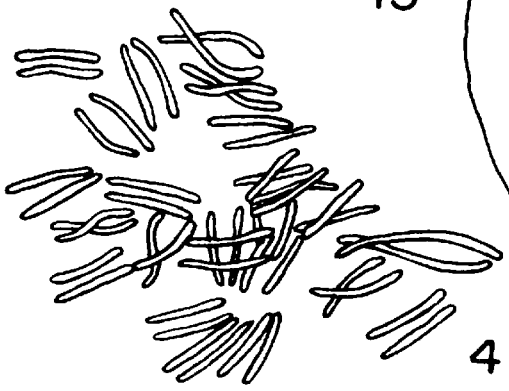

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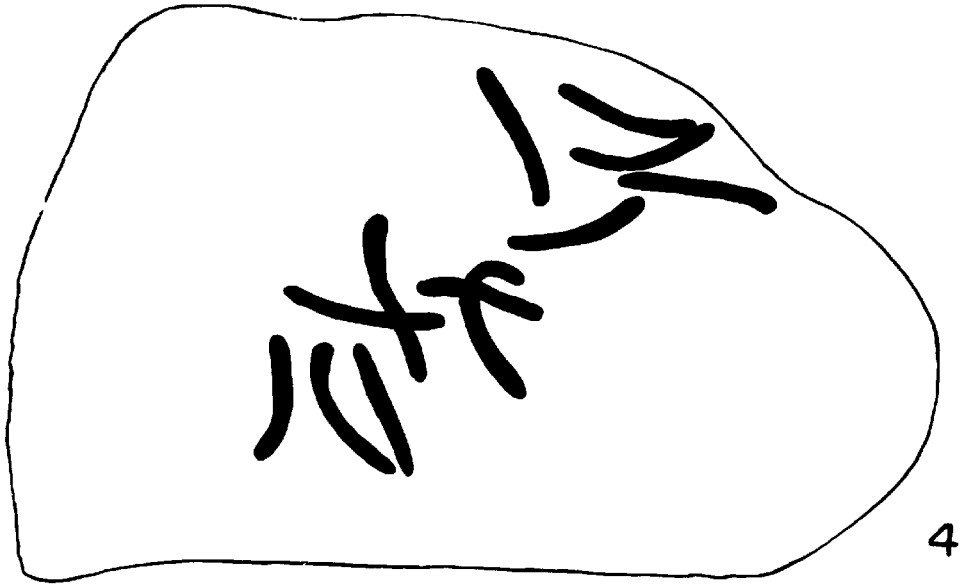

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considered possible that the cells of the sporangium might have formed a callus-like tissue. This suggestion was discarded when the chromosome complement of the tissue was determined to be haploid. In addition, tissue initials were also produced by dusting the stored pollen on the surface of the nutrient agar, and it was concluded that the tissue did in fact arise from the masses of germinated pollen.

Fixing the microgametophytes as soon as a tissue was visible macroscopically provided some information about tissue formation, but the original gametophyte or gametophytes involved in forming the tissue could not be identified. The closest approach to a young tissue initial was found in a permanent slide preparation (Fig. 33). The exine and expanded intine of the microgametophyte were present, but the other components were missing, and only seven spherical tissue cells were visible. Such an isolated, potentially proliferative, group of cells could be interpreted as a tissue initial, but the interpretation actually sheds little light on the origin.

It is necessary to refer to the abnormalities which occur in the cultures in order to determine which cell or cells are active in tissue production. The abnormalities are believed to be directly related to tissue formation because the unusual growth of some components of the gametophyte gives rise to the tissue, and the ab-

normalities are the best evidence of this unusual growth.

Indications of prothallial cell division were few. No more than two prothallial cells were seen in a gametophyte, and it was uncertain whether the two cells were the original prothallial cells or whether the persistent cell had divided (Figs. 27 and 35). The overall role of the prothallial cells in tissue formation seems to be an inactive one, and it is considered unlikely that these cells are responsible for tissue initiation.

The potentially spermatogenous cells included the generative cell, its two derivatives, the stalk cell and the body cell, and the two immature sperm cells. The only cells in the complex at all active in abnormality production were the stalk cell and the body cell, and the stalk cell was the more active of the two. Soon after their formation both cells showed a tendency to divide again (Figs. 25 and 26), but it was during their early growth and before the body cell enlarged to any extent that the stalk cell divided several times to give a group of four or as many as eight nuclei or cells (Figs. 35, 36, and 38). It is possible that such a group of cells derived from the stalk cell could provide the basis for tissue formation.

The activity of the tube cell in the production of abnormalities was the greatest of all the cells of the

microgametophyte. The formation of the coenocytic gametophyte abnormality and the septate tube abnormality have been attributed to the activity of the tube nucleus. Together, these two abnormalities comprise by far the majority of all abnormalities found, and this suggests that the tube nucleus has a role in tissue initiation. If the origin was from the coenocytic gametophytes, then they would have to become septate to form the tissue, but, if the origin was from the septate tube gametophytes, it is relatively easy to visualize their continued growth into a tissue.

In summary, it should be made clear that the origin of the tissue might be from different cells of the gametophyte in different tissue initials. However, the observations on the microgametophytes indicate that some cells are more active than others in the production of abnormalities, and from this evidence certain remarks can be made. The comparative inactivity of the prothallial cells suggests that they are probably not responsible for tissue initiation. The demonstrated activity of the stalk cell, on the other hand, makes it a possible source of the initials. However, the conspicuous activity of the tube cell in forming the coenocytic and septate tube gametophytes offers the best explanation of tissue initiation.

# DISCUSSION

The development of a pair of sperm cells with spiral blepharoplasts in the cultures of microgametophytes of Ginkgo represents a significant step in the growth of gymnosperm pollen in vitro. Heretofore, Branscheidt (1939) had grown Taxus pollen to maturity and LaRue (in press) had obtained development to the immature sperm cell stage in Zamia. These results from members of the Coniferales, Cycadales, and Ginkgoales are substantial evidence that the microgametophytes of gymnosperms possess an autonomous developmental pattern which is independent of the influences of the ovule. The results further indicate that a nutrient medium such as the WM-0.25%YE-IAA medium is sufficient for development of this kind. The inadequacies of the medium are revealed in the failure of the sperm cells to reach full maturity, but the results are encouraging enough to suggest that fully mature motile sperm may be produced when the proper nutrient substrate is furnished.

The abnormalities encountered in the microgametophytes in vitro may be interpreted as an effect of the culture medium on the cells of the gametophytes. The septate tube, the coenocytic gametophyte, the intercalary divisions of the stalk cell, and tissue formation would then be labeled as substrate effects. An alternative

interpretation is to regard the abnormalities as normal variation. Unfortunately, adequate information is lacking on the abnormalities which occur regularly *in vivo* so that no comparison can be made. However, it is quite certain that the type and number of abnormalities *in vitro* reaches unusual numbers, especially in the case of the septate tube, coenocytic gametophyte, and tissue formation, none of which have been reported by previous workers as occurring *in vivo*. If these abnormalities are labeled as substrate effects, then there is the difficulty of reconciling this effect with the fact that normal development usually prevails in the cultures. The substrate would have to be thought of as exerting a selective action in affecting some gametophytes and not others. But this is not unreasonable to imagine if it is recognized that the pollen grains are the products of genic segregation at meiosis. The great masses of pollen which were cultured would then represent a population of diverse genic composition, and some pollen grains would be better adapted to growth on the nutrient substrate than others. The abnormalities would be formed through the effect of the substrate in stimulating, inhibiting, or otherwise altering the normal maturation processes of the gametophytes. Such a mechanism for abnormality production would also explain the greater number of abnormalities in the preserved pollen. In this instance, the conditions of the storage technique would

exert a selective action apart from the substrate. Many pollen grains would be essentially unaffected by both the storage and substrate, but others would be altered because of their particular genic composition.

The microgametophyte of Ginkgo has demonstrated a capacity for unlimited vegetative growth in culture by producing a tissue. Phylogenetically and physiologically this capacity is of considerable interest, since the microgametophyte is of a reduced type. In the liverworts and mosses, we find that the gametophytes have a capacity to grow in an unlimited vegetative manner. In fact, this is the principal method of vegetative reproduction in the Bryophyta and the thallus and gemma are examples of this proliferative ability. Fern gametophytes may also live on for years, as has been reported by Walp and Proctor (1946), especially if no sporophytes are formed. The long-lived tuberous gametophytes of Lycopodium are another example of the vegetative growth of gametophytes. In recent studies in vitro Morel and Wetmore (1951) have reported that Osmunda gametophytes will produce a green callus on a supplemented Knudson's medium and will then continue to grow indefinitely as a tissue. Similar results were found for Selaginella megagametophytes by Wetmore and Morel (1951). In the case of seed plants, one observes that the gametophytes are greatly reduced in size, but an ability to proliferate has been demonstrated in the rather large megagametophytes of the gymnosperms. LaRue (1948, 1950) has shown

that Zamia and Cycas megagametophytes will continue to grow and will regenerate new plants under the proper cultural conditions. The growth of a tissue from the microgametophyte of Ginkgo is believed to be the first report of vegetative growth from a male gametophyte of a seed plant, and it extends the concept of gametophyte growth potential to the microgametophyte and to the Ginkgoales. It is not unlikely that this capacity for vegetative growth may also exist in the gametophytes of other conifers and angiosperms.

The question arises as to the nature of the pollen tissue. How should it be regarded? Is it simply an extension of the male gametophyte? The tissue is basically haploid, but contains polyploid cells. It is further characterized as unorganized and parenchymatous, and it grows by means of islet meristems scattered throughout the tissue mass. The tissue differs from the haploid callus of Osmunda obtained by Morel and Wetmore (1951) in lacking chlorophyll and in having no tracheary elements. It is similar to the corn endosperm tissue of LaRue (1949) in its general growth habit and appearance, and distinguished from the latter by the form of the starch grains, by the chromosome number, and in nuclear and cell size. The pollen tissue is distinct in its characteristics and it seems appropriate to consider it as an extension of the microgametophyte.

The spermatogenous and tube cells of the microgametophytes have been suggested as possible origins of the tissue, with the tube cells the more likely origin. To the hypothesis that the tube cell is responsible for tissue initiation may be added a certain amount of circumstantial evidence. Reference is made to the fact that both the pollen tissue cells and the tube cell of the gametophyte are well-adapted to convert a suitable nutrient substrate to their use. This is evident in the tissue cells by their ability to grow rapidly in vitro. It is also evident in the tube cells when they obtain from the substrate the metabolites, trace elements, and other factors necessary for microgametophyte growth. The tube cells surround the spermatogenous cells and maintain an environment for them (the tube cell cytoplasm) which is isolated and different from that of the substrate. Theoretically, at least, the spermatogenous cells would have to be modified or undergo some degree of dedifferentiation in order to function as a tube cell. The conclusion is reached that the cells of the tissue resemble the tube cell of the gametophyte more than the spermatogenous cells in their ability to obtain from the nutrient substrate the materials required for their continued growth.

## CONCLUSIONS AND SUMMARY

1. Microgametophyte development *in vitro* progressed up to the immature sperm cell stage. The cytological details of maturation were precisely those which obtain *in vivo*.

2. Development *in vitro* differed from that *in vivo* in the faster growth rate of the body cells and in their smaller size at division. Immature sperm cells were formed in culture in two months, whereas four months were required in the plant.

3. A medium suited to the growth requirements of the pollen was devised. The medium not only supported normal gametophyte growth, but it served as a substrate for subcultures of a tissue derived from the pollen. It was composed of a modified White's (1943) medium to which 0.25% yeast extract and 1 mg indoleacetic acid per liter were added.

4. Cultures of the microgametophytes from mass inoculations of pollen gave rise to tissue initials, which, when subcultured, continued to proliferate. Several clones of the tissue were established and some of these have passed through as many as 14 subcultures over a period of more than a year. The tissue was found to be basically haploid, although some degree of polyploidy

also existed.

5. Plastids in the early body cell were found to function as leucoplasts during a brief phase of body cell growth. With the work of Mann (1924), this indicates a continuity of plastid material from the microspore mother cells to the sperm cells.

6. The abnormalities most conspicuous among the cultured microgametophytes included intercalary divisions of the stalk cell, divisions of the tube nucleus to form coenocytic and septate gametophytes, and the separation of cytoplasmic units from the body cell. The several cells of the microgametophyte varied in their activity in the production of abnormalities; the tube nucleus was the most active and the stalk cell next, followed by the body cell and the relatively inactive prothallial cells.

7. The pollen preservation technique of Newcomer (1939) was adapted to permit the pollen to be cultured under sterile conditions. The stored pollen was maintained in a viable condition for over a year, but with some loss in the percentage of germination.

8. One pair of sperm cells was observed in which each cell had a spiral blepharoplast of two revolutions. The cells contained a large sperm nucleus and a cytoplasmic

body, but no cilia.

8. The origin of the pollen tissue has not been determined. The observations on microgametophyte abnormalities indicate that the prothallial cells are not responsible for tissue initiation, but the potentially spermatogenous cells suggest one possible origin. The weight of evidence, however, points toward the tube cell as the most probable source of pollen tissue initials.

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