THE DEVELOPMENT IN VITRO OF THE PROEMBRYO

OF PINUS

A STUDY OF THE MORPHOLOGICAL STATES IN THE

IN VITRO DEVELOPMENT OF THE PROEMBRYO OF

PINUS NIGRA ARN. VAR. AUSTRIACA A. & GR.

By

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Preface

The earliest embryonic stages, because of the simplicity of their organization and cellular structure, are logical starting points for developmental studies of plants as a whole. The zygote and early post-zygote phases of embryonic development are periods of minimum cell form differentiation. If the cells possess a potentiality to develop in a number of different ways at any stage in their development, it is in all likelihood at this early date. Becoming more and more specialized with continued growth, they finally form tissues and organs with the capacity for specific functions in the division of labour of the mature plant organization. It was felt that the more specialized the cells became, the less impressionable they would be to the influence of an _in-vitro_ environment. Accordingly, this treatise deals with the culture in artificial medium of the early post-fertilization periods in the embryonic growth of _Pinus nigra_, var. _austriaca_, in an effort to discover and assess those factors which influence and govern the embryonic developmental pattern.

The present study was begun in 1951 at the suggestion of Professor N. W. Radforth. I wish to express my appreciation to Professor Radforth for his guidance in

conducting my research, without which this investigation could not have been carried out.

This work was undertaken as part of a programme which has been assisted through a grant from the Ontario Cancer Treatment and Research Foundation, for which Dr. Radforth is the grantee.

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Introduction

The great necessity for experimental study in the field of embryonic development has long been appreciated. Numerous postulates have been made with regard to the biological factors fundamental to the patterns of differentiation observed in the embryonic tissues of plants in the living state. Some of these theories have gained widespread approval and general acceptance despite their uncertain origins. Other more or less contradictory speculations or suggestions, born in a similar fashion, while commanding less universal respect, have enjoyed a more limited, but nonetheless devoted following. Huge superstructures of subsequent investigation have arisen with their foundations resting on the assumed validity of one or more of these controversial hypotheses. As a natural, but unfortunate consequence, much confusion and argument has resulted. It is obvious from the foregoing, that no true evaluation of the situation can be derived without basis in experiment.

Bower (1) advanced the theory that filamentous organization is fundamental to embryonic development in multi-cellular organisms throughout the plant kingdom. He then proceeded to an analysis of the polarity of the early filament from a detailed study of numerous plants in

various taxonomic groups. Relationships between the axis of the filamentous structure and that of the archegonium were studied. It became increasingly apparent that the presence or absence of a suspensor was a factor in this relationship. The orientation of the mature adult plant also seemed to have a definite bearing on the axis of the embryonic filament. The factor, however, which has come to be accepted generally as the chief consideration under-lying this polarity is the biological advantage of a source of nutrition in the richest area of the gametophytic tissue. It was suggested that the suspensor, obviously under tension in the life situation, is designed to push the young embryo into this highly nutritious portion of the prothallium.

The need for new evidence which will emphasize the justification in accepting filamentous or radial develop-ment becomes more significant when it is recalled that much of the recent work of developmental morphology of conifers relates to polyembryony as a measure of primitiveness or specialization in fundamental growth trends prevalent in conifers.

Buchholz (2) represents the asexual pattern as the more primitive in pine and the forerunner of simple embryo-geny, and expresses the belief that the asexual pattern does not vary with environment. These postulates were not based on any experimental evidence.

R. B. Thomson (10) contends that the morphological expression of any hereditary feature cannot be used to determine the course of phylogeny, until the extent of its variability under different conditions has been determined and taken into account.

Acknowledging the importance of such investigation, part of this work has been undertaken in an effort to gain a better understanding of those factors which govern the developmental pattern of differentiation of the proembryo of Pinus. In sharp contrast to the more primitive gymnosperms such as Ginkgo and Cycas, Pinus has a true filamentous suspensor, thereby offering more opportunity for studying those factors underlying this algal type of organization.

This study entailed the possibility of in-vitro culture of all the early growth stages following fertilization, and once achieved, a comparison of morphological states in this material with those already established for in-vivo cases at comparable stages.

The cells of the gametophytic tissue are extremely delicate, the cell walls being very thin. The difficulty in excising early embryonic tissue in organic association with parent tissue in, say, mammals can be appreciated, but in plants there are cell walls in place of membranes, which can better protect the cell contents against mechanical injury. In Pinus, therefore, the physical situation is

such that it is just possible the excision might be accomplished. Once separated and placed in a satisfactory nutritive medium, the early embryo would be in an entirely new environment. The embryo would be surrounded equally on all sides by nutrient, in contrast to a specifically situated prothallial matrix.

Experiencing considerable difficulty, Radforth (8) was ultimately successful in conducting a similar investigation with Ginkgo. This, and numerous successful attempts at growing more differentiated excised tissues in culture encouraged the adoption of the foregoing design.

Materials and Procedure

Pinus nigra var. *austriaca* was chosen because of the relatively large size of its ovule and embryo sac, the former being about 10 mm. in length, and the latter 6 mm.

Two procedures of preparation were ultimately adopted. The first consisted in making a lateral severance across the distal end of the sporangium with a sharp knife, after having removed the prothallium encompassed by the nucellus from the integument. This cut was made a short distance from the archegonial cluster. The second involved removing the entire sporangium from the enveloping integument without any excision being made. In either case, great care was exercised in the removal of the gametophytic tissue from the integument, to avoid injury to the prothallial apex. In both techniques, the excised tissue was dropped into a seven percent solution of chloride of lime, which had been filtered after standing three hours. The tissue was then transferred to the culture medium. The thin-bladed scalpel used to sever the prothallium in the first method was flamed and cooled in the same calcium hypochlorite solution, and the whole operation was conducted as quickly as possible.

Of primary importance, too, was the selection of a culture medium which would support life, and possibly sustain

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growth. Two media, both highly recommended for embryonic tissue culture were used in the earlier experiments. The first was Knudson's formula, for which Morel and Wetmore (7) claimed excellent results in their growth studies of fern callus tissue. This medium was used simultaneously with a modified Crone's solution, employed successfully by Dr. Radforth in growing the proembryo of Ginkgo. Later, the former was discarded in favour of the latter.

The constituents of the modified Crone's solution were as follows: 20 g. potassium chloride; 5 g. calcium sulphate; 5 g. magnesium sulphate; 5 g. magnesium phosphate; 5 g. iron phosphate. Of this mixture, 1.5 g. were added to 1 litre of water to give a '100 percent' solution, and the hydrogen ion concentration was adjusted to a pH of 5.5. Dextrose was then added in the proportion of 2 g. dextrose to 100 c.c. of solution. Ordinary 20 c.c. test tubes were utilized as culture containers. Each was supplied with about 8 c.c. of the medium, and then sealed with a plug of cotton wool. The test tubes were then autoclaved for 20 minutes at 15 pounds pressure. After a period of trial, a dilution of the original medium to 25 percent with water was found to be the optimal strength for the maintenance of healthy cells.

Tissues were allowed to remain in the nutritive medium for periods ranging from one to twelve days.

The fixative used in the earlier work was formalin-

aceto-alcohol (F.A.A.) after Johansen (6), but this was later substituted by weak chrom-osmo-acetic (C.O.A.) (Johansen).

The butyl alcohol series was used throughout in the embedding process, and the sections were cut to a thickness of twelve microns.

Stains used were Erlich's haematoxylin, recommended by Dr. Radforth, and safranin and fast green, utilized by Schopf (9) in staining embryonic tissues of _Larix_. The latter was used exclusively in the later work.

Results

Effects of Dissection and Transfer to Medium:

Repeated attempts to excise the proembryo from the prothallium with a micromanipulator, prior to insertion in the culture medium, resulted in devastating shock, through which we failed to preserve the extremely delicate embryonic cells, as Radforth had succeeded in doing with Ginkgo.

This led ultimately to the first procedure of preparation outlined in the previous section. In cutting across the prothallium some distance from the archegonial end, rather than attempting a removal of the proembryo itself, a great number of cells were left between the proembryo and the nutritive medium, which it was hoped would buffer the shock of transfer. Due to the ready infiltration of medium through the severed and loosened cells, it was felt safe to assume that the young embryo would be supplied with an unlimited supply of nutrient on all sides. This technique also avoided any possibility of damaging the proembryos in the region of the archegonia.

Despite every precaution, contamination proved a serious impediment, and the second method, a modification of the first, was developed. It was observed under the

microscope that in the post-fertilization period, the nucellus is a very thin membrane, and in addition to being normally semi-permeable, or even permeable, it is discontinuous, with many tears and breaks. Consequently, it seemed reasonable to conclude that this surface would allow for ready infiltration of the culture medium on all sides. This eliminated the necessity of cutting, and speeded up the process considerably by reducing the handling. The first technique was not abandoned, however, since it was not thought advisable to base the entire investigation on the above assumption of infiltration through the nucellus.

Experimental Selection of Materials:

The Knudson's medium proved somewhat unsatisfactory. Difficulty was found in dissolving the mixture of salts in water, and there was a marked tendency for the salts to settle out, depositing a residue on the surface of the tissue immersed in it. There was also a tendency for the pH of the solution to fall off to a value well below the recommended 6.6. It seemed, moreover, impossible to avoid undue plasmolysis at any reasonable concentration. Accordingly, this nutrient was discarded in favour of the modified Crone's solution already described. The use of a higher concentration than the optimal 25 percent strength resulted in much plasmolysis, while lower concentrations produced a

great deal of eruption through cell turgidity.

The F.A.A. fixative first used was found to cause considerable damage and distortion of the tissues, probably due to rapid penetration. This solution was therefore replaced by the C.O.A.

The necessity for a long period of washing after staining with Erlich's haematoxylin, resulted in much damage to the delicate tissue, and for this reason a change was made to safranin and fast green, as recommended by Schopf.

Observations in Growth and Form:

Great irregularity was discovered in the date of fertilization in this species. In many instances, contents of some archegonia displayed more advanced development than others in the same ovule. Between different ovules on the same cone there was a greater variance, and a still larger discrepancy was noticed in the comparison of different cones on the same or adjacent trees. The variance between different locales was even more pronounced. This observation is not borne out by the literature on the development of Pinus. Ferguson (5) indicates a fairly rigid date for fertilization in Pinus, and Coulter and Chamberlain (3) give a definite date (July first) for the fertilization in Chicago.

The percentage of ovules which do not become fert-

ilized in this species is very high. (it may run as high as 90 percent). This necessitated the handling of abnormal numbers of ovules.

Inspections of the in-vitro tissue at intervals indicated that as the time in nutrient medium increased after a lapse of from 10 to 12 days, the cases showing maintenance of healthy appearing cells became increasingly infrequent. Accordingly, the growth periods were kept between the limits of 1 to 12 days.

Ample evidence was obtained to establish the fact that the tissue was growing while immersed in the nutrient medium. The following are the criteria on which assessment of cell vitality and growth were based:

1. The general appearance of the cells. Beyond the extent of plasmolysis to be expected, as indicated by the condition of the tissue fixed from the living state, the cytoplasm looked normal. Vacuolation and granulation also appeared indicative of living cells.

2. A visual comparison of in-vitro cells with others fixed from in-vivo tissue at comparable dates. In a high percentage of cases, this comparison was favourable.

3. The presence of mitotic figures in the in-vitro cells. Fig. 39, Plate VI, and Fig. 44, Plate VII show various mitotic figures in an embryo after 10 days of immersion in the artificial environment. Fig. 19, Plate

III shows a late telophase in a secondary embryo after ten days in the nutrient medium.

4. The appearance of developmental forms not encountered in the in-vivo situation.

In numerous in-vitro cases large embryos, sometimes approaching the cotyledenous state, appeared much nearer the archegonial end of the prothallial matrix than is ever observed in the in-vivo case. Fig. 5, Plate I shows a massive embryo near the apex. Growing in the normal environment, an embryo this large would be situated much further down the core. The foregoing statement is substantiated by numerous observations made in this investigation, and by the large assemblage of literature portraying the embryology of Pinus. The relative positions of the two above cases are illustrated in the composite diagrams 1 and 2, plate VIII. Fig. 10, Plate II, and Figs. 26 and 29, Plate IV give additional evidence of massive embryonic development in an abnormal location.

From observations made during the course of this work, and from a survey of the literature, it can be stated that in the life situation, the axes of all embryos infallibly coincide with that of the archegonium, and the embryo grows away from the archegonial apex. In many instances, secondary embryos, either from cleavage or simple ontogeny, after a period in the substituted environment, show no correlation

between their axes and that of the archegonia. Fig. 3. Plate I and Fig. 45, Plate VII show embryos whose axes are perpendicular to that of the archegonia. Fig. 6, Plate I; Fig. 13, Plate II, and Fig. 41, Plate VI depict embryos whose axes coincide with that of the archegonia. However, the direction of propagation is toward the apex, rather than away from it, representing a turn through 180 degrees. Fig. 9, Plate I displays a group of four embryos with axes and growth directions varying. Fig. 8, Plate I; Fig. 12, Plate II, and Figs. 20 and 23, Plate III, show embryos whose axes are oblique with respect to the archegonial axis. Figs. 25 and 28, Plate IV picture embryos with oblique and partially reversed polarity. The normal in-vivo situation is shown in Fig. 43, Plate VI, and Fig. 47, Plate VII, where the embryos are heading away from the archegonial apex, and have axes coinciding with that of the archegonia. Drawings 5 and 6, Plate X compare the in-vitro phenomenon with the normal in-vivo situation.

Embryos in the normal development have a characteristic shape at any age. They are elongate bodies with parallel sides, showing only a slight taper toward the base, and have a pointed apex prior to the cotyledenous stage. In the case where young embryonic development is subjected to the new environment, the form often changes radically. There is a marked tendency for such embryos to develop radially, and become more or less ball shaped. In some cases they become spherical and often it is hard or impossible to distinguish

the base of the embryo from the apex. As the embryo becomes
more mature before being placed in the nutrient medium, the
severity of the resulting rounded form progressively decreases.
However, even where the embryo has differentiated greatly, and
is nearing maturity before the insertion, there is usually a
significant rounding out noticeable. This latter case is
illustrated in composite drawings 1 and 2, Plate VIII. Figs.
26 and 29, Plate IV; Fig. 39, Plate VI; Fig. 48, Plate VII,
and Fig. 10, Plate II show this rounded appearance in the
case of well advanced embryos. Fig. 30, Plate IV shows the
form of an in-vivo embryo of comparable development. Figs.
1,2,3,4,and 7, Plate I; Figs. 20, 22, 23 Plate III; Fig. 24,
Plate IV; Figs. 32, 34 and 36, Plate V; Figs. 37, 38, 39 and
42, Plate VI and Fig. 50, Plate VII show various degrees of
this radial growth tendency in the younger embryo. In Figs.
37, 38 and 42, Plate VI, and in Fig. 1, Plate I, the apex
and base are difficult to distinguish. This tendency toward
three-dimensional growth in the individual embryo is a very
prevalent one. This type of radial growth in younger stages
of embryonic development is portrayed by composite drawings
3 and 4, Plate IX.

     In addition to this radial development in the single
embryo, there is a marked trend toward radial form in the
patterns formed by groups of embryos. In the natural state

the embryos are all axial and line up one behind the other
as seen in Fig. 43, Plate VI, and Fig. 47, Plate VII. In
the tissue cultured in the medium, this arrangement is the
exception rather than the rule. Here it is found that the
embryos spiral out in various directions, sometimes in a **sym-
metrical** manner, as spokes leave the hub of a wheel, but
usually in a more haphazard arrangement. Fig. 9, Plate I
shows this latter situation, as do Figs. 32 and 34, Plate V.
Fig. 35, Plate V shows a radial formation of very young
embryos, and Fig. 40, Plate VII shows a more symmetrical
arrangement. Since the embryos here are not all in the one
plane, only the outlines of some are visible in the photo-
graph. **Drawings 5 and 6, Plate X compare in-vitro and in-vivo.**

When grown in the usual environment, the location of
the embryos in relation to the prothallial matrix, and in
relation to one another is strictly in accordance with their
mass and shape. The longer and thicker the embryo, the
further down is its location in the gametophytic tissue.
(i.e. the greater is its distance from the archegonial apex.)
In the in-vivo state, this pattern is quite static. An
illustration of this is given in Fig. 43, Plate VI. In post
in-vitro tissue, the mass of the immature embryos apparently
bears no such relationship to its location. Often embryos
of abnormal mass and unusual shape are found extremely high

up in the archegonial end of the embryo sac. Figs. 1, 2, 3, 6, 8, and 9, Plate I; Fig. 12, Plate II; Figs. 20, 22, and 23, Plate III; Figs. 24, 25, and 28, Plate IV; Figs. 32, 33, 34, and 36, Plate V; Figs. 37, 38, 40, 41, and 42, Plate VI, and Figs. 45, 46, and 50, Plate VII illustrate instances of various embryos located very high up in the archegonial end, far removed from the position embryos of their mass would occupy in the natural growth pattern. Composite drawings 3 and 4, Plate IX, illustrate this tendency further.

In recent investigations, three-dimensional growth was discovered in the development of the archegonial contents of tissue inserted in the medium immediately following fertilization. This development has resulted in a well-defined cellular proliferation within the archegonial jacket, extending a considerable distance around the periphery of the archegonium. No such growth is evident in the central portion of the archegonium. In every case, these cells are carried throughout the series of sections and are quite distinct in appearance from the archegonial jacket cells. They appear rectangular in shape, and of very delicate complex. In some cases there is free nucleation in evidence. These nuclei are very small, and tend to appear in pairs, indicating an extremely rapid growth. In tissue removed from culture prior to this proliferation of cells, large free nuclei have been discovered. Fig. 14, Plate II shows a section of the arch-

egonial jacket lined with this cellular proliferation. Fig.
11, Plate II illustrates the appearance of the whole arch-
egonium and shows the location of this cellular growth within
the jacket. Fig. 31, Plate V depicts the proliferation in
the basal portion of this archegonium. Fig. 15, Plate II,
and Figs. 17 and 21, Plate III are various views of a second
archegonium with this same proliferation, and in these photo-
graphs there is some evidence of the free nuclear state.
Composite drawing 7, Plate XI gives a general picture of
this cellular proliferation complex. The large free nuclei
in evidence in an earlier stage of development are depicted
in drawing 8, Plate XI.

In numerous cases, the embryo in material grown in
culture displays a curvature (i.e. in the mass of the embryo
exclusive of the suspensor). This has not been observed in
any embryonic development in the natural environment. This
bending is evident in the following photographs: Figs. 2,
8 and 9, Plate I; Fig. 20, Plate III; Fig. 28, Plate IV;
Fig. 36, Plate V and Fig. 45, Plate VII.

There is considerable evidence that polyembryony is
more evident in the _in-vitro_ tissue than in the _in-vivo_. The
literature is somewhat vague on the subject of polyembryony
in _Pinus_, and the average number of embryos found in the
normal state are not reported. However, numerous observations

during the course of this investigation indicate that the number of embryos has been enhanced as a result of the culture treatment. As many as 12 embryos have been found in one embryo sac compared to a maximum of 6 discovered in in-vivo tissue. Many of the in-vitro cases display numbers of embryos in excess of 6. These numbers, of course, do not appear in any one section, and cannot, therefore, be depicted by photograph.

The ratio of cleavage polyembryony to simple is largely a matter of conjecture, but since the average number of archegonia for this species appears to be from two to four, it is quite probable that on an average cleavage is the origin in at least 50 percent of the cases in the in-vitro material.

Fig. 19, Plate III shows an embryo giving indication of impending cleavage.

Discussion of Results

The regularity in the date of fertilization for Pinus
as reported by Ferguson and Coulter and Chamberlain, does not
hold for this species. The great variance in the time of
fertilization, and therefore in the progress of post-fert-
ilization development, somewhat invalidates any comparison
of the in-vitro material with the in-vivo controls. However,
this is not a serious matter, since the embryology of Pinus
has been thoroughly investigated, and is by far the best
known for all the conifers.

While the elapsed time in culture was, in many cases,
of quite short duration, it was observed that the post-fert-
ilization development is extremely rapid, and it was considered
that the range of times used was quite adequate.

The primitive spindle theory, advanced by Bower,
endeavours to establish the premise that filamentous organ-
ization underlies all embryonic development in plants, even
in the more advanced forms. Here it has been assumed, as has
been done in the animal kingdom, that land vegetation has
evolved from some aqueous type or types. Radial development
in discoid or limited spherical forms is convenient for plants
of floating habit; a state which demands no distinction of
apex and base, as is the case in Volvox or unicellular algae.

When the plant is related to a solid substratum, polarity is
established, and with it follows, as a rule, an elongation
of the plant body into a cylindrical or spindle-like form
with a distinction of apex and base. The simplest type is
the non-septate filament, as seen in Vaucheria. The vast
majority of algae, however, become septate, forming a single
filamentous row of cells. The simple filament may persist
throughout life, as in Ulothrix or Spirogyra, but it usually
becomes branched. It very often leads to a 'fungal formation'
of an aggregation of filaments. A further modification, and
one more important in relation to the higher forms of veg-
etation, is the subdivision of the cells of the simple fil-
ament by longitudinal walls, the result of which is either
a bilaterally flattened or solid cylindrical structure.
Usually this longitudinal subdivision appears only after the
young plant has become established as a self-nourishing organ-
ism, but in some cases, the longitudinal walls may appear
earlier, so that in extreme cases, the young plant is massive
from the first. The above cases do not represent the true
embryology, since the young individual is not enclosed within
the body of the mother. However, it is generally believed,
as proposed by Bower, that the embryo of higher plants rep-
resents a young individual, corresponding to the diploid
thallus of some algae, encapsulated in the archegonium in
accordance with life on exposed land surfaces. In all types

the embryo will probably assume at first a spindle-like form, variously modified and possibly disguised. The form of the vascular plant embryo generally, is essentially that of a spindle, the stem tip being the apex, and the suspensor, when present, the organic base.

The suspensor is considered a primitive or vestigial part, the last indication of the construction of the plant body from a filament, or row of cells, often rapidly passed over, and often suppressed, as in the case of Ginkgo. This common origin agreed upon, a comparison of the hypothetical biological factors governing the development of the algae with those in the case of the advanced sporophytic generations of vascular plants is suggested by Bower.

In the algae, there is a solid substratum to which it is attached, and this is usually vertically below. There is exposure to light which is usually vertically above, and there is nutritive medium all around it. The polarity is in a roughly vertical direction. Since nutrition is from a watery medium completely surrounding the filament and equally arranged on all sides of it, the alga tends at first to a cylindrical or spindle-like form, which is often permanently retained.

Similarly, in all archegoniate plants, a definition of the polarity of the embryo is the first step in its development. The position of its apex and base is recognizable on

the appearance of the first segmental wall.

The embryo encapsulated in the archegonium has no direct relation to a solid substratum, and the possible influence of light is affected to varying degrees by the enveloping cells. In view of the fact that very little external light falls on the embryo, it is doubtful if this is a factor worthy of much consideration. The nourishing medium surrounding the free algal thallus has been substituted by the living cells of the gametophyte, rich in food materials. The tissue supplying the nutriment, as a rule, is not uniform in bulk on all sides. It is suggested that the strongest influence on the early development of the embryo is the enveloping maternal tissue (the cells immediately adjoining the proembryo) and the direction in which the nourishment of the embryo is to be derived. Since this nutrient material is, as a rule, not equally dispersed all around, it may be anticipated that, though a cylindrical or spindle-like form of the embryo is possible, it may not be constant or permanently retained.

In a great majority of archegoniate plants, the axis of the embryo coincides with that of the archegonium, but there are numerous exceptions to this. In the Bryophytes, as well as in Equisetum and Isoetes, the axis of the embryo coincides with that of the archegonium, and the embryo grows toward the neck of the archegonium. In Pteridophytes, two

types of embryo are found: those with suspensors, and those
without. In those having a suspensor, the embryo is directed
toward the base of the archegonium, and lies in the arch-
egonial axis. In instances where there is a variance between
the axis of the archegonium (and therefore axis of the
spindle), and the axis of the mature plant, this discrepancy
is usually compensated for by a curve in the shape of the
young embryo. Where no suspensor occurs, no such curve is
evident. The embryo is apparently in the same axis as that
of the mature plant, regardless of the archegonial axis.
This has led to the suggestion by Bower that the orientation
of the embryo in the axis of the archegonium is dictated by
the attachment of the suspensor, and is without reference to
the adult attitude. Where there is no such tie; or if the
encumbrance of a suspensor is abolished, the polarity of the
embryo from the first is in accordance with the growth direct-
ion of the mature sporophyte. In some suspensorless types,
however, there is a suctorial foot bent away from the axis of
the spindle into the centre of the nutritive tissue, suggest-
ing that here the axis of maturity has a less decisive
influence on the orientation of the embryo, than has the
relative position of the prothallial sources of supply. This
latter is generally conceded to be the chief factor underlying
embryo orientation.

In the leptosporangiate ferns, the polarity of the

embryo bears no fixed relationship to that of the archegonium. There is no suspensor, and the spindle direction compares favourably with the adult axis.

Marsilia has no suspensor, and the embryo is perpendicular to the archegonial axis. Isoetes has no suspensor, and again the embryo is not tied to the axis of the archegonium. Selaginella has a suspensor, and is thereby tied to the archegonial axis which varies. Here, when this axis does not coincide with that of the mature plant, the necessary adjustment is achieved by an appropriate bending in the embryo. It has been deduced that the direction of the stored food influences embryonic polarity, but later, as the embryo elongates, the response to the 'maturity' factor becomes effective, and leads to those curvatures so characteristic of Selaginella. It has also been concluded that, since in most suspensorless types an orientation in the axis of the archegonium would have created difficulty, and necessitated a compensating adjustment, the absence of a suspensor has been an advantage, and represents higher specialization. It is generally agreed that the suspensorless embryo is an advance phylogenetically.

In primitive gymnosperms, such as Zamia floridana, Coulter and Chamberlain (3), the frequent suspension of cell cleavages until many nuclei have been formed is a perplexing feature of their embryogeny, but despite an aberrant start,

the polarity is not in doubt. The embryo is directed away from the archegonial neck.

The angiosperms are similar in behaviour, and the filamentous structure is felt to underly the embryology of the sporophyte as well.

Comparison of plants in the embryonic state show that a filamentous, or spindle-like structure that Bower calls 'the primitive spindle' with polarity defined by the first embryonic cleavage is common for them all.

In animal ova it has been shown by experimentation that the planes of cleavage can be controlled by external pressure between glass plates. Another postulate, then, is that pressure exercised by the surrounding tissues may have a direct effect on polarity. If, for example, the arch-egonium exerted lateral pressure on the developing embryo, the axis of the embryo would then coincide with that of the archegonium. This pressure factor could be an important one in respect to the Bryophytes, since here the archegonia have varied orientation, and the embryo always coincides with the archegonial axis.

As already noted, it has been suggested that the function of the suspensor is to push the developing embryo deeper into the heart of the nourishing gametophytic tissue. It is also generally assumed that the absence of a suspensor represents a phylogenetic advance, in that the primitive

tendency to filamentous organization does not obtain. Attention has been drawn to the well advanced embryos occupying a position nearer the archegonial apex than is customary. At a relatively advanced stage of embryonic development, if the nucellus and gametophyte is opened at the proper location, the coiled suspensor will be seen to spring out of the embryo sac, showing it to be normally under tension. Where a severance has been made across the prothallium, it may be that this tension on the suspensor has been lessened or removed, and that the suspensor has failed to push the embryo further down in the prothallium as it continued to grow larger. This suspensorial tension could conceivably be altered considerably in the procedure, involving the insertion of the entire sporangium in the medium. The flimsy nucellus in its post-fertilization state would hardly contain the prothallium with the same rigidity as would the horny integument. A second postulate, and a more likely one, is that the suspensor has ceased to grow, once in the new environment. In this case the result would be the same. The latter suggestion is supported by the relative absence of suspensorial tissue in connection with many less mature embryos found high up in the archegonial end. In any event, it would seem that the growth of suspensorial tissue has been impeded by the treatment, and if so, there

is reason to doubt the phylogenetic significance represented by the presence or absence of a suspensor.

The filamentous form of the embryo is very pronounced in this species, and is maintained throughout the embryonic development. Insertion in the artificial environment has obviously tended to disrupt the symmetry of this so-called primitive spindle. Yet, in the <u>in-vitro</u> environment, the nutrient is more evenly dispersed on all sides of the embryo than formerly. This leaves some doubt as to the authenticity of the assumption that the spindle is retained in such forms as algae, because of the symmetrical encompassment of watery nutrient about the cell chain, and that the filamentous stage tends to be passed over quickly when this even distribution no longer exists.

Since the substituted environment creates a change in nutrient distribution, the fact that the embryonic axis is changed or loses its identity entirely under the new conditions imposed upon it, lends some credence to the theory that the position of the nutrient bulk is an important factor in the establishment of spindle polarity. There is reason to believe, therefore, that this polarity is not a phylogenetic, but rather a physiological consideration, except insofar as the allottment of nutrient tissue may be developed through heredity.

The severe rounding out in the form of young embryos developing <u>in vitro</u>, and the less marked, but similar effect

produced in the more mature embryos, indicates that the further differentiation has advanced, the less is the growth pattern subject to environment.

This rounded contour of cultured embryos, replacing the customary oblong parallel-sided shape, indicates a tendency toward three-dimensional proliferation, in contrast to the normal filamentous pattern. The fact that this radial development occurs only in the cultured tissue offers further evidence that the pattern of embryonic development is largely governed by the existing environmental conditions. Therefore, it seems reasonable to conclude that the factors underlying this growth pattern are more physical or physiological than phylogenetic, although the physiological complexes of the environment may have phylogenetic significance.

The peculiar radial or random development exemplified by groups of _in-vitro_ embryos in relation to one another, spiralling out in various directions without regard for any axis, also suggests a type of three-dimensional growth. Presumably, this phenomenon is also due to the new environment. It is possible that with 'unlimited' nutriment lying all around, the line of propagation has no significance, since no one direction presents any growth advantage. Pressure factors could be operative here, as in the cleavage experiments with animal ova. The change in pressure relationships brought about by either preparation procedure could account for this unusual growth pattern. In any event, the

phenomenon seems entirely due to physiological causes.

The curvatures observed in these embryos, reminiscent of the sort of thing seen in Selaginella, but obviously arising from some cause not attached to the latter, indicates some uncertainty as to the validity of the theory underlining adherence to the axis of the mature plant as a factor in spindle polarity. Here we have an embryo with a reduced suspensor, or with no suspensor at all, obviously not tied to the archegonial axis, as evidenced by its peculiar orientation, and yet displaying a marked curvature. This is similar to the situation in Selaginella, but here we cannot contribute the curvature to an orientation to the axis of maturity, with a suctorial foot being sent out into the heart of the nutrient supply. For whatever reason these embryos are curved, there is no suggestion of anything other than physical or physiological causation.

Bower's suggestion that the axis of the embryo is tied to that of the archegonium by virtue of the suspensor finds some support in this investigation. The in-vitro treatment has in many cases destroyed any relationship between the two axes, but it has also seemingly reduced or in some cases apparently eliminated the development of suspensorial tissue, indicating that there is some connection between suspensorial development and orientation in the archegonial

axis.

The free nucleate state observed, and the cellular proliferation within the archegonial wall is prime evidence of true radial or three-dimensional development in the embryogeny of a plant which normally shows idealistic evidence of filamentous organization. This pattern is reminiscent of the development of some of the more primitive gymnosperms, for example Zamia floridana, as illustrated by Coulter and Chamberlain. This demonstrates that three-dimensional growth can be induced at fertilization, eliminating or at least delaying the formation of any filamentous organization. It would seem, then, that filamentous growth is the result of physical or physiological causes, although these latter complexes may be passed on through heredity, and thereby have phylogenetic significance. Furthermore, if pronounced and retained filamentous organization is evidence of primitiveness, it is peculiar that the more primitive gymnosperms should show less evidence of this development than Pinus.

From the foregoing, it can be said that the issue of phylogenetic significance of the primitive spindle as a fundamental factor underlying embryonic development seems to be somewhat in doubt.

Polyembryony is common in Pinus. Since there are

several zygotes, each can undergo simple embryogeny, and produce a group of fraternal type embryos. In addition to this sexual embryo initiation, one or more zygotes by undergoing budding or cleavage at an early stage in ontogeny, can initiate asexually one or more embryos of identical type, corresponding to identical twinning in animals.

There is a great deal of similarity between the budding ontogeny in gymnosperms and in parasitic insects, as well as between other plants and animals. This would indicate that a common interpretation of origin of this asexual polyembryony might be found, and some relationship should exist between the simple and budding embryogeny in plants and animals.

The prevalent, and most widely accepted interpretation proposed by Buchholz, interprets these differently. In _Pinus_, since the suspensory column is made up of four segments longitudinally, four embryos from each zygote are possible. Buchholz represents this asexual pattern as the more primitive of the two in pine, and the forerunner of simple embryogeny. In contrast to this, it is generally accepted by investigators of animal embryology that the various forms of budding have originated independently from simple, both in distantly and closely related families. The Buchholz interpretation is based on the belief that the asexual pattern does not vary with environment, and that on a morphological basis, their

advances can be arranged in phylogenetic series. The animal embryologists, on the other hand, believe that the twinning is a conditioned response, or one prompted entirely by environmental conditions. Therefore, any phylogenetic significance that may be associated with this would, of necessity, be in relation to the hereditability of the environmental conditions necessary to bring it into expression.

The accepted viewpoint in animal budding is the result of much experimentation. It has been demonstrated that budding is a latent expression of many species which normally reproduce by simple embryogeny. It has also been demonstrated experimentally that this potentiality may be eliminated by specialization. While there has been little experimental work done with plants, R.B. Thomson suggests that budding ontogeny is also a latent feature in plants.

No experimental work has been done by Buchholz in support of this view that various forms of embryogeny are so constant under different conditions, as to have phylogenetic significance, nor has he referred to any such supporting work by other investigators.

R. B. Thomson contends that the morphological expression of any hereditary feature cannot be used to determine the course of phylogeny, until the extent of its variability under different conditions has been determined and taken into account.

As already indicated, the results of this invest-
igation indicate that the number of embryos has been enhanced
by the in-vitro programme. Since there would be no reason
for an increase in the simple, or sexual initiation; all
archegonia producing sexually in the normal environment once
fertilized, this observed increase must be a direct result
of more numerous cleavages. The fact that this asexual twin-
ning has been stimulated by the new environment gives indicat-
ion that this is a conditioned response. This experimental
evidence that cleavage embryogeny is a latent expression in
this species, casts doubt on the Buchholz belief that the
asexual initiation was a forerunner of the simple, or indeed
that there is any phylogenetic significance whatsoever assoc-
iated with the polyembryony in plants.

Conclusions and Summary

It is concluded from this investigation that 1. there is little if any phylogenetic significance in the presence of absence of a suspensor, its abolition being a physiological response. 2. The position of the nutrient bulk is one of the main, if not the chief reason for spindle polarity. 3. There is much likelihood that the suspensor is somehow instrumental in the maintenance of a fidelity between the axes of the spindle and the archegonium. 4. The reasons offered for curved embryos, as found in some Pteridophytes, are, whether correct or erroneous, quite hypothetical. 5. The fact that radial or three-dimensional growth can be initiated at fertilization, and can also be imposed on later states to a greater or lesser degree, depending on the extent of differentiation, suggests that there is reason to doubt that the primitive spindle has any phylogenetic significance in being a fundamental factor underlying embryonic development in all plants. 6. It is acknowledged that phylogenetic importance may be inferred insofar as the physical or physiological complexes bringing the phenomenon into expression are handed down, or developed through heredity. 7. It is thought that, on the basis of these observations, asexual polyembryony in Pinus has no phylogenetic significance whatsoever, but is a

physiological problem, and probably represents a latent
expression in most plants.
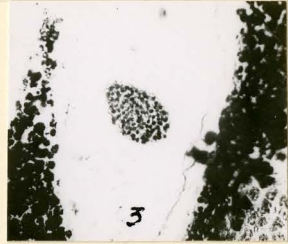
Plate I
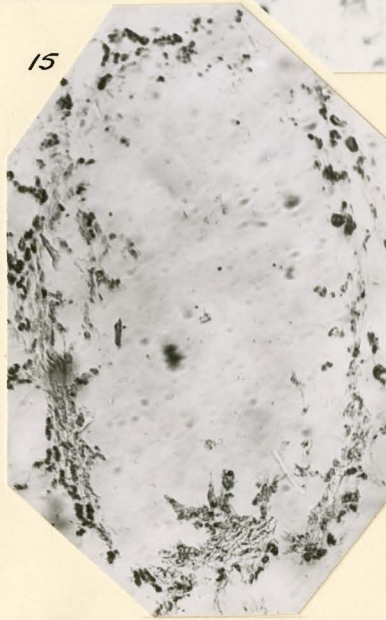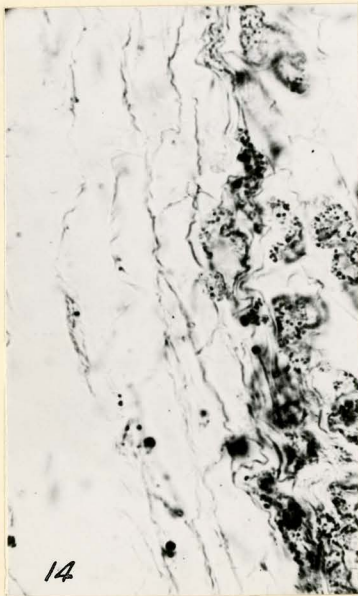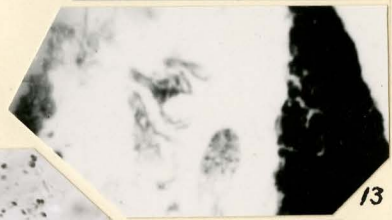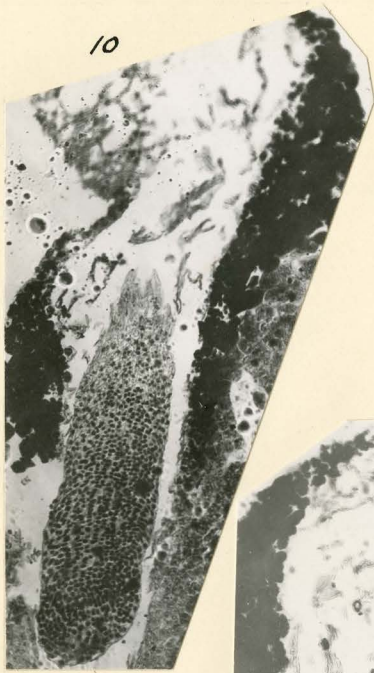
Plate II

Plate III

Fig. 17. - Section of archegonial jacket (from archegonium shown in Fig. 15) showing cell proliferation along its length, and free nucleation among the three-dimensional growth. Mag. 880X. In culture June 27-30.

Fig. 18. - Embryo showing signs of radial growth. Mag. 100X. In culture July 20-30.

Fig. 19. - Embryo with reversed polarity in the prothallial apex. Its form suggests the initiation of a cleavage near the base. Mag. 440X. In culture July 17-27.

Fig. 20. - Embryo in apex of embryo sac. It displays a slight curve and has an oblique axis. There is no evidence of suspensory tissue. Mag. 200X. In culture July 20-30.

Fig. 21. - Section of archegonium (Fig. 11) showing cell proliferation and free nucleation within the arch-egonial jacket. Mag. 880X. In culture June 27-30.

Fig. 22. - Two axial embryos showing signs of radial growth. One embryo, somewhat spherical in shape, with polarity perpendicular to the archegonial axis, and somewhat spherical in shape, situated high in the pro-thallial apex. Mag. 100X. In culture July 8-17.

Fig. 23. - Embryo with almost reversed polarity showing three-dimensional development. There is little evidence of suspensory tissue. Mag. 200X. In culture July 20-30.

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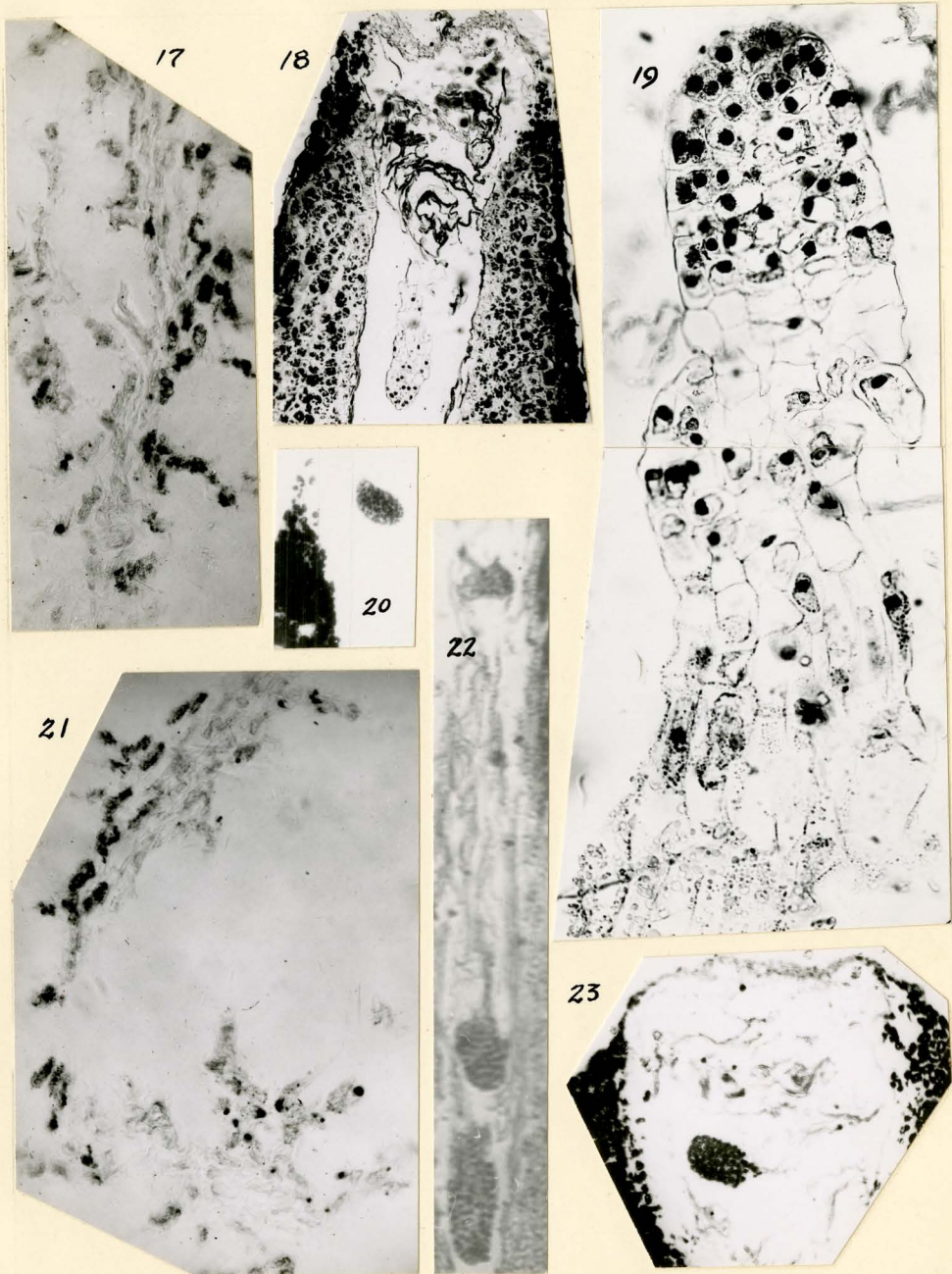
Plate III

Plate IV

Fig. 24. - Embryo in prothallial apex showing con-siderable evidence of radial development. Mag. 100X. In culture Aug. 2-10.

Fig. 25. - (Embryo shown in Fig. 19). Mag. 100X. In culture July 18-27.

Fig. 26. - Embryo situated abnormally high in the embryo sac and showing signs of radial development. Mag. 100X. In culture July 17-27.

Fig. 27. - Embryo with reversed polarity in the prothallial apex and showing signs of radial development. Mag. 440X. In culture July 14-22.

Fig. 28. - (Embryo depicted in Fig. 27.)

Fig. 29. - Well advanced embryo situated abnormally close to the prothallial apex and showing signs of limited three-dimensional growth. Mag. 100X. In culture Aug. 2-14.

Fig. 30. - Well advanced embryo showing normal form. This embryo is situated well away from the apex of the embryo sac. Mag. 200X. Fixed from the in-vivo state Aug. 8.

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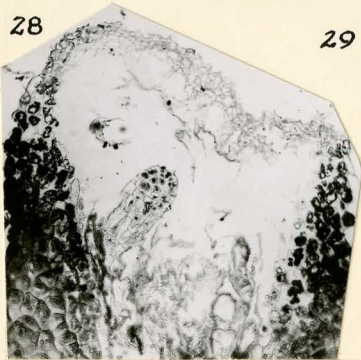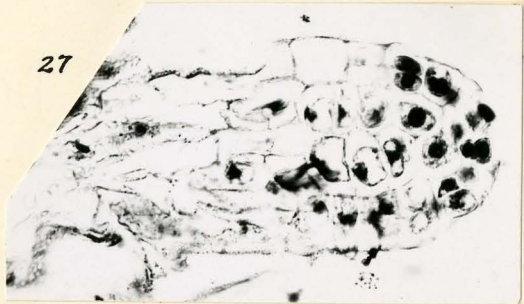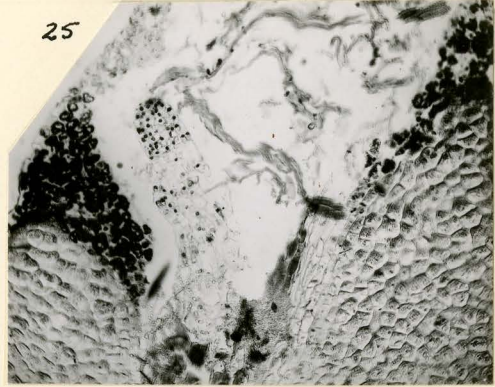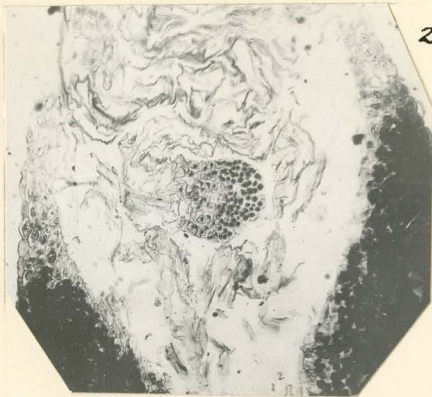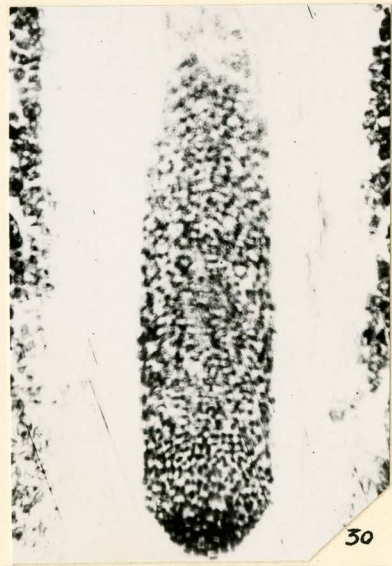
Plate IV

Plate V

Fig. 31. - Section of base of archegonium (see Fig. 11) showing radial cell proliferation inside the archegonial wall. Mag. 880X. In culture July 6-8.

Fig. 32. - Two embryos in the prothallial apex showing radial form. Apex and base are poorly defined. Mag. 200X. In culture July 24 - Aug. 5.

Fig. 33. - Embryo of rounded form with normal polarity but abnormal location. Mag. 100X. In culture July 15-27.

Fig. 34. - Two embryos situated very high in the prothallial apex, showing three-dimensional growth, and forming a radial pattern in their orientation. Mag. 100X In culture July 12-22.

Fig. 35. - Radial form as displayed by the orientation of three very young embryos, arising almost certainly from sexual initiation. Mag. 100X. In culture, July 6-10.

Fig. 36. - Curved embryo with partially reversed polarity. (see Fig. 2) Mag. 100X. In culture July 15-24.

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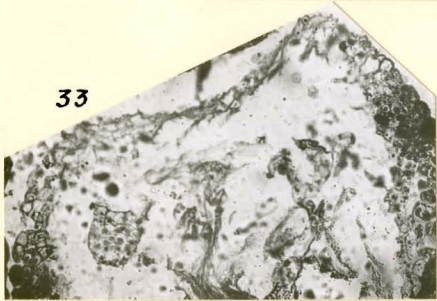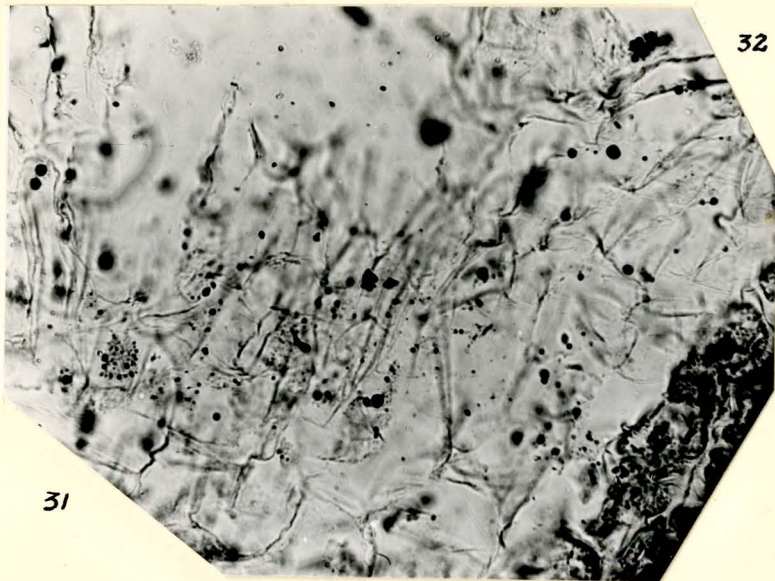
Plate V

Plate VI

Fig. 37. - Embryo showing pronounced radial development. Mag. 100X. In culture July 15-24.

Fig. 38. - Spherical embryo exemplifying three-dimensional growth. Mag. 100X. In culture July 17-29.

Fig. 39. - Well advanced embryo showing indication of radial development. Mitotic figures are visible in the embryo. Mag. 100X. In culture Aug. 10-20.

Fig. 40. - Group of embryos (outlines of some visible only) displaying symmetrical radial propagation in their orientation. Mag. 200X. In culture July 10-22.

Fig. 41. - Curved embryo with reversed polarity. Mag. 200X. In culture July 17-27.

Fig. 42. - Three-dimensional development as illustrated by spherical embryo. Base and apex are poorly defined. Mag. 200X. In culture July 15-20.

Fig. 43. - Two axial embryos showing customary orientation in the normal state. Mag. 100X. Fixed from in-vivo tissue July 20.
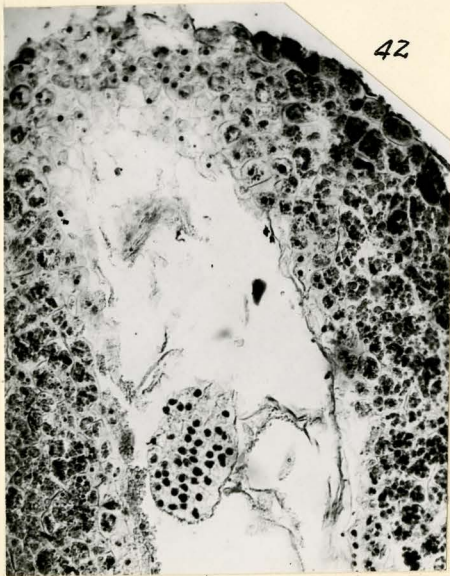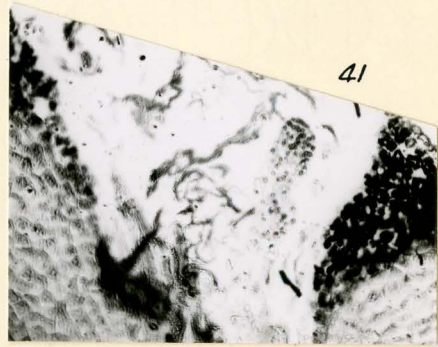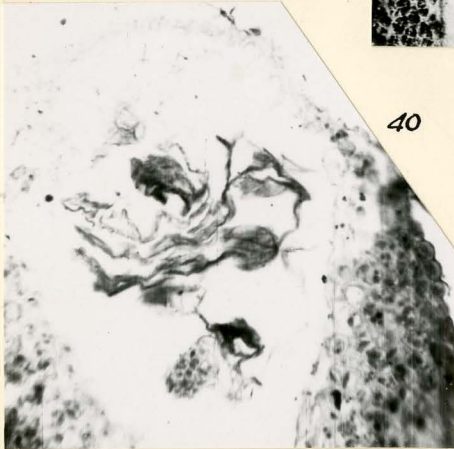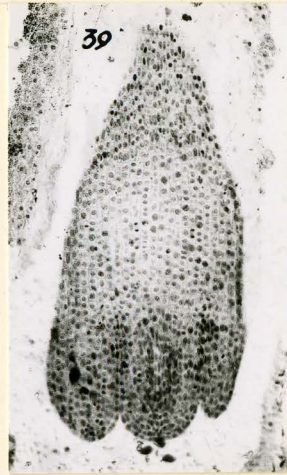
Plate VI

Plate VII

Fig. 44. - Cells from embryo showing mitotic
figures. (see Fig. 39). Mag. 880X. In culture Aug. 10-20.

Fig. 45. - Two embryos showing radial growth and
abnormal axes.

Fig. 46. - Embryo with almost reversed polarity
showing radial development. Mag. 100X. In culture July
15-24.

Fig. 47. - Axial embryos showing normal orientation
and form of developing embryos in-vivo. Mag. 100X. Fixed
from in-vivo tissue July 18.

Fig. 48. - Large embryo showing some radial growth
and situated abnormally near the prothallial apex. Mag. 200X
In culture July 28 - Aug. 4.

Fig. 49. - Embryo with abaxial orientation, showing
radial development. Mag. 440X. In culture July 22-28.

Fig. 50. - Radial development shown by spherical
embryo. Mag. 100X. In culture July 11-21.

Fig. 51. - (see Fig. 46). Mag. 440X. In culture
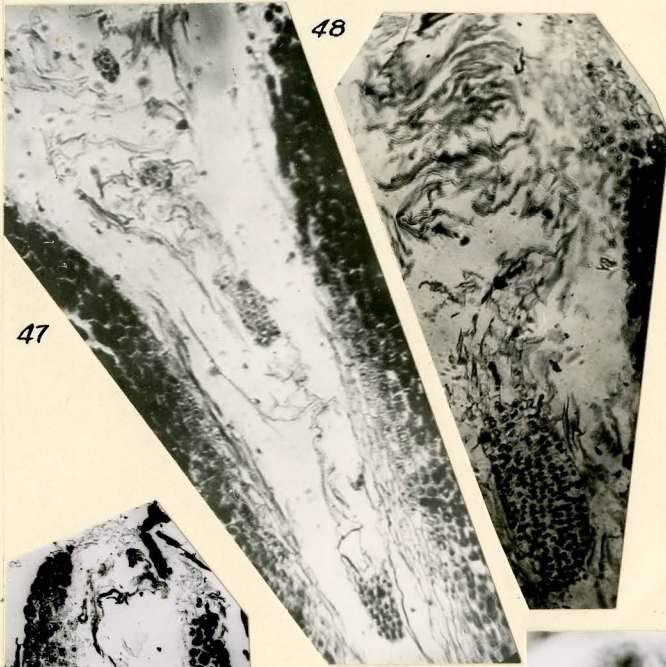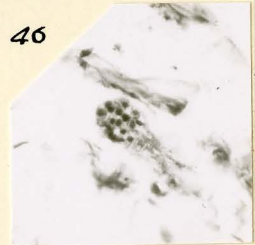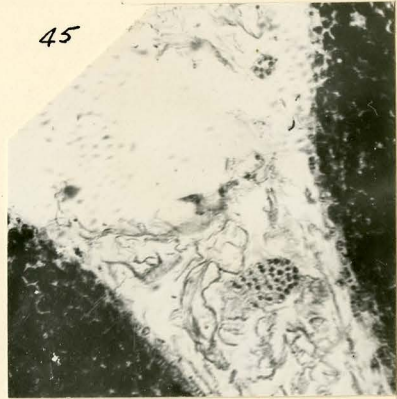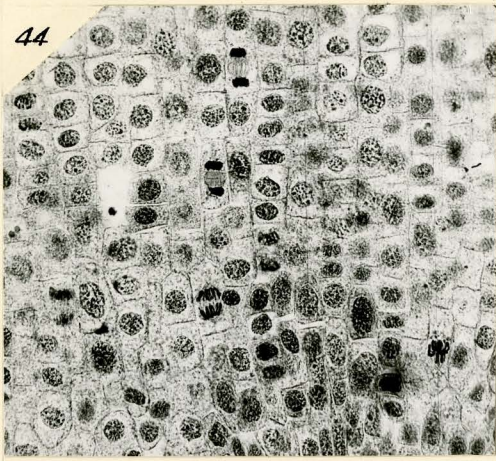July 15-24.

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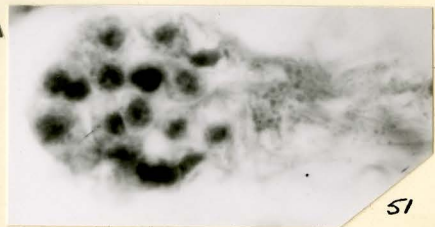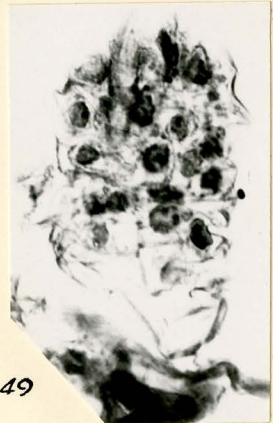
Plate VII

Plate VIII

Fig. 1. - Drawing of well advanced embryo grown in culture, situated in the prothallial apex, and showing some radial development in its form.

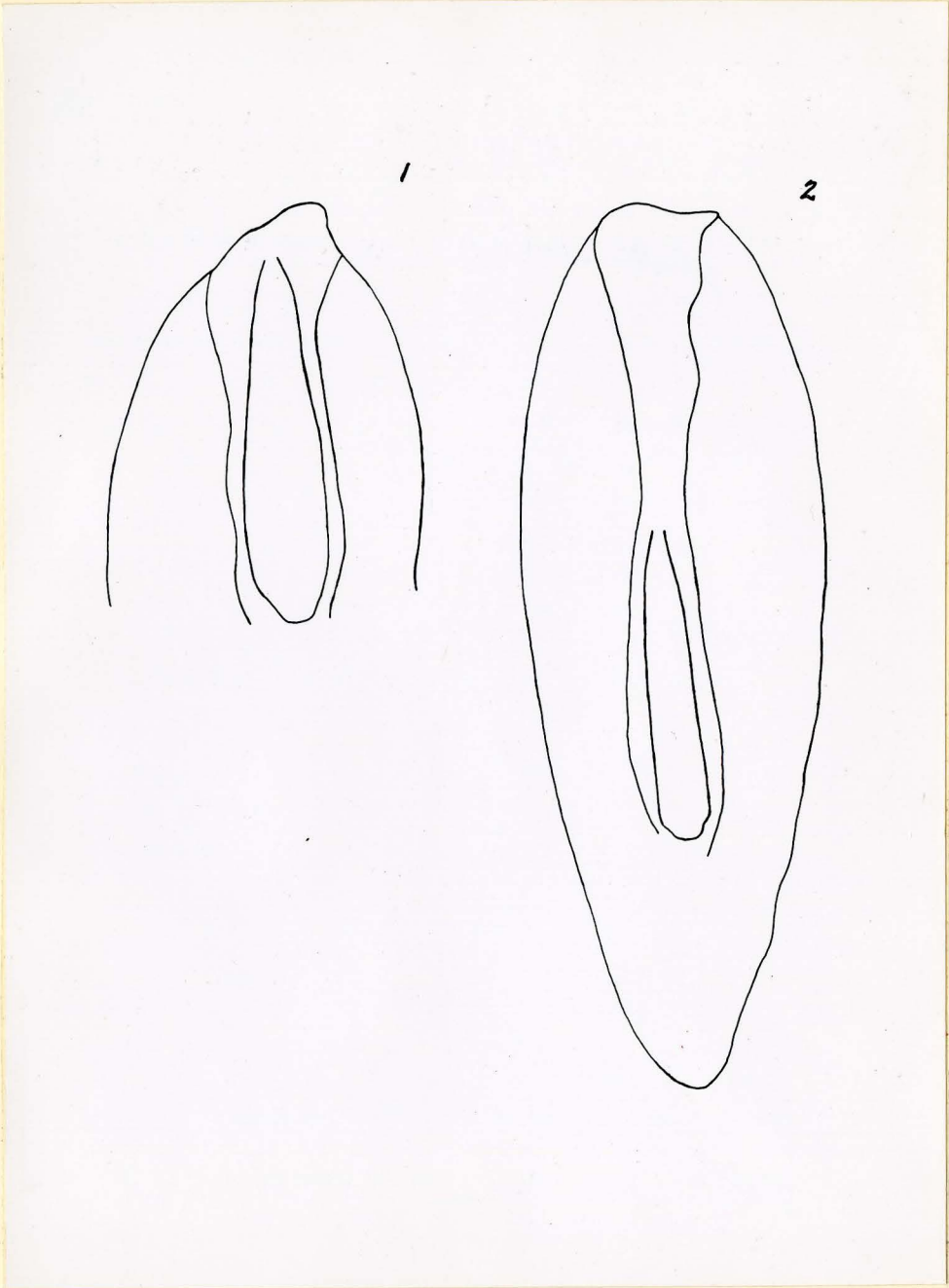Fig. 2. - Drawing of _in-vivo_ embryo showing normal form and location in the prothallium.

Plate VIII

Plate IX

Fig. 3. - Composite drawing of _in-vitro_ tissue
showing three-dimensional growth exemplified by a
spherical embryo in the apical end of the prothallium.
Another embryo shows reversed polarity.

Fig. 4. - Composite drawing of cultured tissue
showing a large embryo with reversed polarity, and some
radial development in its form. This embryo has huge
mass to be so high in the apical end of the embryo sac.
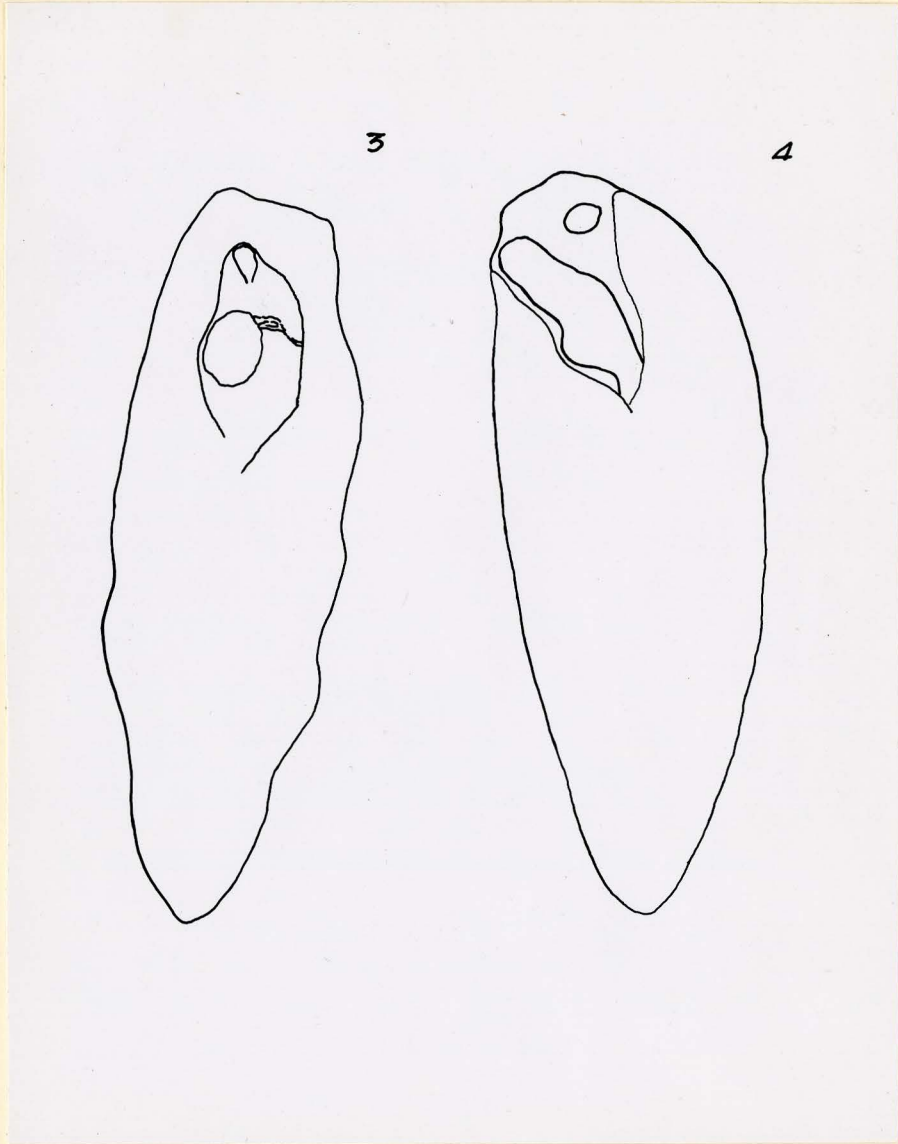A second spherical embryo is situated still higher in
the apex.

Plate IX

Plate X

Fig. 5. - Composite drawing showing a group of embryos cultured in artificial medium, displaying varied axes, as well as signs of radial development in form. Their embryonic mass is abnormally large for embryos in their respective locations.

Fig. 6. - Drawing of the normal situation in the in-vivo state, showing embryos adhering to the archegonial axis. This shows average situation in the prothallium for embryos of these respective masses.
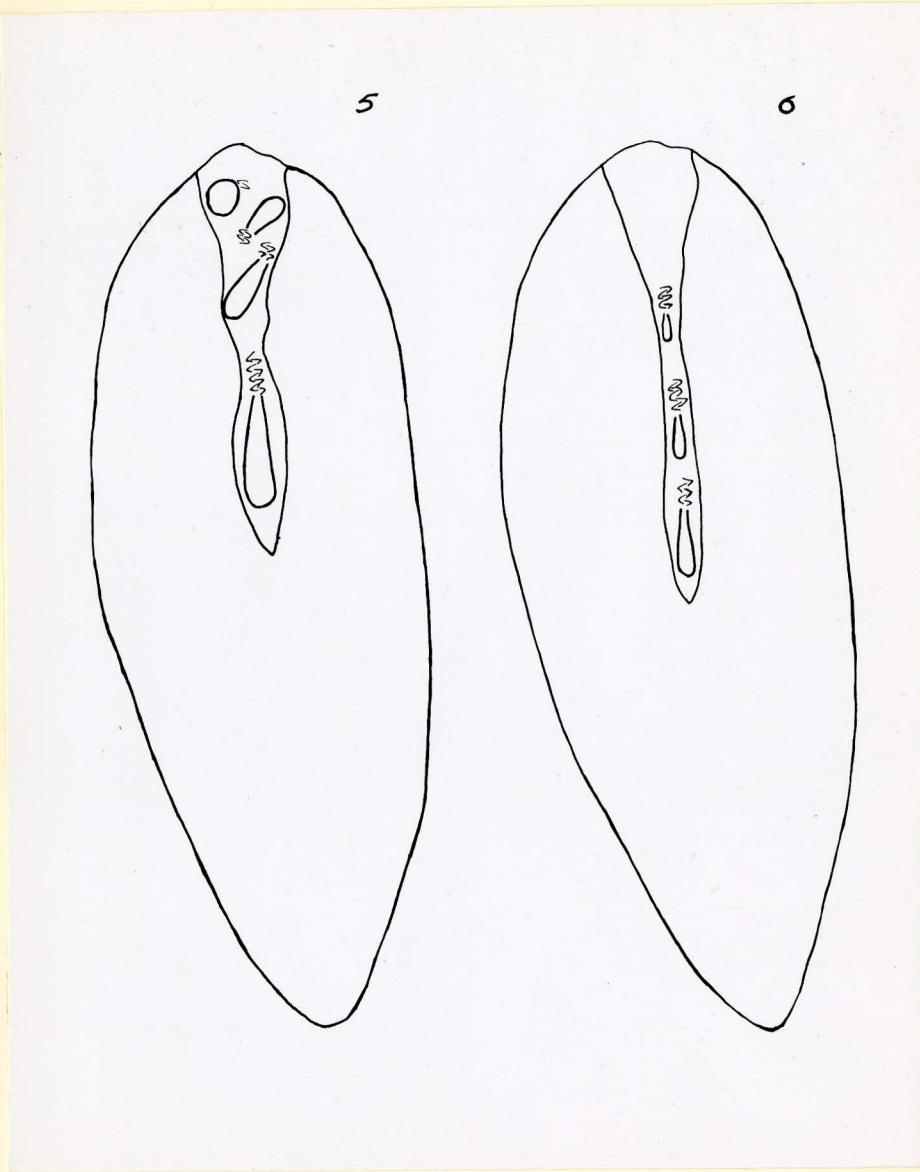
Plate X

Plate XI

Fig. 7. - Drawing of an archegonium from in-vitro
tissue, showing the location of the proliferation within
the jacket. a. - archegonial jacket of compact cells
with sturdy cell walls. b. - thin-walled, large rect-
angular cells growing inside the archegonial jacket.

Fig. 8. - Drawing of an archegonium from in-vitro
tissue at an earlier stage than Fig. 7. a. - large free
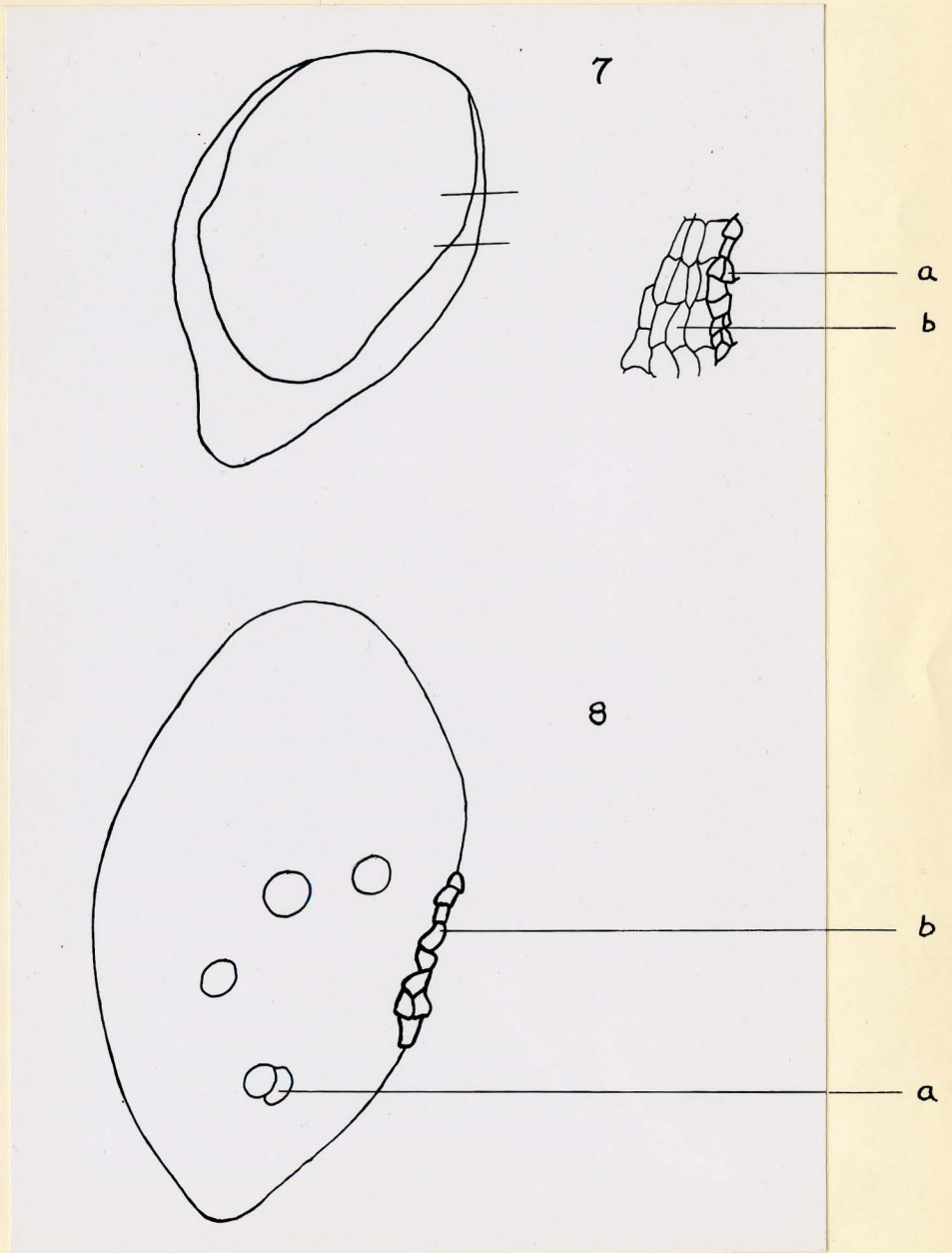nuclei. b. - a section of the archegonial jacket.

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Plate XI

Literature Cited

1. Bower, F.O. Primitive Land Plants. Macmillan, London
        (1935).

        The Primitive Spindle as a Fundamental Feature
        in the Embryology of Plants. Proc. Roy. Soc.
        Edinburgh, 43(1):1-36.

2. Buchholz, J.T. Suspensor and early embryo of Pinus.
        Bot. Gaz., 66: 185-228. (1918).

        Polyembryony among Abietineae. Bot. Gaz.
        69:153-167. (1920).

        Embryo development and Polyembryony in relation
        to the phylogeny of Conifers. Am. J. Bot. 72:
        125-145. (1920).

        Origin of Cleavage Polyembryony in Conifers.
        Bot. Gaz. 81:55-71. (1926).

3. Coulter, J.M. and Chamberlain, C.J. The embryogeny of
        Zamia. Bot. Gaz. 35:184-194. (1903).

        Morphology of Gymnosperms. University of Chicago
        Press (1917).

4. Doyle, J. and Looby, W.J. Embryogeny in Saxegothaea and
        its relation to other podocarps. Sci. Proc. Roy.
        Dublin Soc. 22:127-147. (1939).

5. Ferguson, M.C. Contributions to the Life History of
        Pinus, with special reference to sporogenesis,
        the development of the gametophytes, and fert-
        ilization. Proc. Wash. Acad. Sci. 6:1-202.
        (1904).

6. Johansen, D.A. Plant Microtechnique. McGraw Hill, New
        York. (1940).

7. Morel, G. and Wetmore, R. H. Fern Callus Tissue Culture.
        Am. J. Bot. 38:141-3. (1951).

8. Radforth, N. W. The Development in Vitro of the Pro-
          embryo of <u>Ginkgo</u>. Trans. Roy. Can. Inst.
          21:87-94. (1936).

          Robert Boyd Thomson's Morphological and
          Phylogenetical Studies bearing on
          Gymnosperm Taxonomy. Bot. Gaz. 110:39-47.
          (1948).

9. Schopf, J. M. The Embryology of Larix. University of
          Illinois Press, Urbana (1943).

10. Thomson, R. B. Polyembryony. Sexual and Asexual
          Embryo Initiation and Food Supply. Trans.
          Roy. Soc. Can. 39:143-169. (1945).

          Evolution of the Seed Habit in Plants.
          Trans. Roy. Soc, Can. 21:229-272. (1927).