EXPERIMENTAL EMBRYOLOGY

OF

CERTAIN GYMNOSPERMS

EXPERIMENTAL EXAMINATION OF GROWTH AND DIFFERENTIATION IN THE EMBRYO-GAMETOPHYTE COMPLEX OF *PINUS RESINOSA* AND *GINKGO BILOBA*

By

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TITLE: Experimental examination of growth and differentiation in the Embryo-Gametophyte Complex of Pinus resinosa Ait. and Ginkgo biloba L.

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SCOPE AND CONTENTS:

The morphogenetic changes involved with the differentiation of a unicellular fertilized egg to an organised multicellular embryo pass through a cycle of events. Manifestation of differentiation in structural diversity starts from the physiological differences in the tissue. These differences can be demonstrated through biochemical variations.

These ideas are examined in the gametophyte-embryo complex of Ginkgo biloba and Pinus resinosa.

It is shown that the embryo experiences different physiological conditions during the various phases of development. In addition, the potentialities of embryonic cells to grow and differentiate vary during these phases. Alternative developmental sequences can occur if the nutritional environment is altered.

Radiosensitivity of the embryo as measured by abortion and abnormal differentiation is shown to be dependent on the stage of embryonic development.

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PREFACE:

One of the most challenging areas of investigation in experimental embryology is the mechanism of differentiation. A great deal of knowledge about the process of differentiation has been obtained at the anatomical and histological levels. In fact, we know in great detail about embryogenesis in plants with respect to the sequence of differentiation of various tissue systems. However, the basic mechanisms of differentiation, the forces that bring about cell specialization, still remain to be elucidated.

The embryo is produced from a fertilized egg, the zygote. This single cell contains all the genetic information required to develop into an adult organism. The zygote divides and the resulting progeny cells increase in number. The daughter cells contain the same genetic information, but in spite of that, they begin to differ in characteristic ways. According to Bonner (2) the differences in morphology and function in the differentiated cells is preceded by changes in the kinds and amounts of enzymes they contain.

It is generally accepted that the enzymes are ultimately gene determined. The pattern of differentiation in an embryo, therefore, can be visualised as being controlled by the genetic constitution of the zygote. This may be true with respect to the limitations on the kinds of differentiation that may be derived. According to Wardlaw (5), "While the specific constitution and organization of the zygote will determine or limit

the kind (or kinds) of pattern that may be developed from it, the particular pattern actually developed may be more or less directly due to factors in the environment, e.g. physiological gradients, the balance of nutrients supplied, and so on." (pp 322).

In the present work these different aspects of experimental investigations are considered in four chapters as follows:
I Experimental Embryogeny of Pinus resinosa Ait.

The embryos of Pinus are suitable materials for surgical manipulation. It is possible to investigate the manner in which the embryo develops when physical limitations of containment are not in force. The first step in understanding the process of development is to see whether or not there are distinct developmental phases in the growing embryo. It has been customary for the embryologist to recognise different phases by common criteria and characteristics of visible differentiated tissues and organs. But the underlying physico-chemical criteria that lead from one phase to the other have been neglected. In the present experiments these check points have been investigated by studying differentiation in vitro of embryos at different developmental phases and comparing them with those growing in vivo.

Steward et al (4) have shown that all cells of young carrot embryos are virtually totipotent. According to them any free diploid cell may, when appropriately nurtured, behave like

a zygote, whereas the same cell situated on an organised segment such as shoot or root is restrained and limited because of its position within a differentiated cell population. In the present experiments these restraints and limitations have been investigated by cultivating different segments of differentiated embryo and attempting to break the restrictions by varying physical and chemical factors.

II Changes in the pool of free amino acids in the gametophyte of Ginkgo biloba at different stages of embryo development.

The embryo of a gymnosperm is dependent on the gametophyte for nutrition. In this chapter, the author has investigated the changes in the pool of free amino acids in the gametophytes at different stages of embryo development.

III Changes in the amount of gibberellin-like and kinetin-like substances in the developing fruits of Ginkgo biloba.

Another group of compounds which is of great importance is the growth regulating substances, e.g. gibberellins, kinins and auxins. It is important to know their distribution in vivo at different phases of development since this may throw some light on the controlling mechanisms of differentiation (4). The knowledge gained in this way may, then, be applied in vitro. The investigations under this section include the studies on the changes in the amount of gibberellin-like and kinetin-like substances in the developing fruits of Ginkgo biloba.

IV Radiosensitivity of the embryo-gametophyte complex of Pinus resinosa at different phases of development of the

embryo.

The mechanisms of differentiation may also be studied from another point of view. The Bergonie-Tribondeau Law (1) states that the radiosensitivity of cells is related directly to their reproductive capacity and inversely to their degree of differentiation. Experiments on this concept have been conducted in angiosperms with valuable results (3).

Investigations on radiosensitivity of embryos at different stages of development might throw light on the critical phases when embryonic cells demonstrate high potentiality for differentiation. On the basis of the Bergonie-Tribondeau Law, it is expected that embryos in such stages would show higher radiosensitivity.

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I Experimental Embryogeny of Pinus resinosa Ait.

Introduction:

It will be agreed that the interaction between the genetic constitution in the dependent embryo and its environment of surrounding parental tissue determines the embryonic differentiation. The inhibition of the development of a hybrid embryo is, generally, accountable to the unsuitability of the environment to the new genotype (5). In Sugar pine hybrids, this inhibitory factor is overcome by dissecting the embryos from the surrounding gametophytic tissue and transferring them to an artificial medium (14).

During the embryogeny of Pinus, the development of some of the proembryonal cells is determined at the 16-cell stage. The suspensor cells originate from the second tier of cells just above the embryo initials and normally do not divide again, while the embryo initials divide and give rise to diverse cell types (3). The specificity of origin of these cell types suggests predetermination of differentiation perhaps through differential distribution of cellular contents. This raises the question where are the differential controls located and what is the stage of development at which the differentiation of the cell is determined?

Besides these general questions, the students of Pinus embryogeny are further confronted with the phenomenon of poly-

embryony. The explanation which had received most attention was proposed by Buchholz (4). It is claimed by Buchholz that the budding form of ontogeny (cleavage polyembryony) in the Pine, which provides for the asexual initiation of 4 embryos by each zygote, is the more primitive type in living gymnosperms. This explanation was contradicted by Thomson (15) and Radforth et al (10). They have shown that budding ontogeny is a latent feature in plants. Their conclusions, therefore, call for simple, but not for budding, polyembryony as primitive if indeed phylogenetic implication is involved in the matter.

Spurr (11) has furnished information on the histogenesis and organisation in the embryo of Pinus strobus. The in vivo studies on the older embryos demonstrate the methods of development of tissue systems but do not include information on the mechanisms controlling ontogeny.

As there was no reference in the literature to any work in gymnosperms dealing with these questions, it seemed important to cultivate, in vitro, embryos of Pinus at different stages of development and to examine the sequence of differentiation in the hope that such a study would throw some light on the mechanism of development. To study the potentiality for regeneration, investigations were made on in vitro culture of segments of differentiated embryos.

Materials and Methods:

Cones of Pinus resinosa Ait. were collected locally during the months of July to August. These were dissected

along the median plane and the seeds removed. The seeds were kept for 10 minutes in 1% sodium hypochlorite solution and then rinsed in sterilised distilled water. The integument and nucellus were removed to expose the female gametophyte and brown remnants of megaspore wall. Dissection of the gametophyte was performed as described by Buchholz (3) using a dissecting microscope and using an aseptic inoculating chamber. The embryos subsequently were washed for 30 seconds in 70% ethyl alcohol and rinsed in sterilised distilled water. They were then transferred to a solid medium and kept in the dark at 27°C under aseptic conditions.

The growth medium consisted of modified Crone's solution (8) supplemented by Ginkgo gametophyte extract and was prepared as follows: 10 g. KCl., 2.5 g. Ca SO_4., 2.5 g. Mg SO_4., 2.5 g.Mg HPO_4 were thoroughly mixed. Of this mixture, .75 g. was added to 1 litre of distilled water to make half strength Crone's solution.

Gametophyte extract: Ginkgo ovules were collected on July 10, 1962, the prothallia were removed from the integument; 1 kg. of the gametophytic tissue was crushed with sterile silica and extracted with 5 l. of hot water at room temperature for two hours and then filtered. This extract was mixed with half strength Crone's solution in the proportion of 1:1. To this medium 2% sucrose was added and the pH was adjusted to 5.5 by adding phosphate buffer. The medium was then heated in a boiler and 1.5% Difco agar was added.

The medium was dispensed in 20 ml test tubes each of which was supplied with 8 ml of the medium and sealed with a cotton plug. The test tubes were then autoclaved for 30 minutes at 15 pounds pressure.

All the ovules in one cone at a certain date were not in the same embryonic stage. In order to avoid the possibility of experimental error due to culturing of embryos in different stages of development in the same experiment, the developmental stage of the embryo at the time of culture was evaluated at each collection by microscopic examination of seeds from comparable cones. For purposes of convenience, these stages were designated as I, II, III and IV (Table II) on the basis of greater degree of tissue differentiation. It was very difficult to grow the embryos in stage I (zygote) and hence the embryos at stages II, III and IV were used for these experiments. For each experiment 25 embryos were cultured.

For the study of development of excised portions, embryos at stage IV were selected. Each embryo was dissected into six pieces with transverse cuts at specific regions, viz., hypocotyl, sub-hypocotyl, root apex with few layers of columnar cells, sub-root apex, suspensors with 2-3 layers of radicle cells, and suspensors.

All the cultures were harvested at the end of six weeks. Materials for anatomical study were fixed in formalin-propionic acid-alcohol, dehydrated in tertiary butyl alcohol series and embedded in tissue mat. Sections 10μ thick were

made and stained in Heidenhain's haematoxylin; Safranin-fast green and Borax carmine-aniline blue (7).

Results:

Embryos in Stage II (Collection July 14, 1962).

Each of the controls consisted of a small mass of cells organised into a more or less spheroidal shape at a terminal portion on a thin filamentous attachment (suspensor) (Fig 1). The terminal cells were all alike in appearance. The embryos at this stage when placed in vitro, gave rise to different patterns of growth and form. In three cases the embryos grew three dimensionally into rounded masses. The cells at the distal end were larger than those at the proximal end. Cell division was confined to and localised at the distal end (Fig.2).

In sixteen cases there was profuse proliferation of the suspensorial cells and the embryos developed cylindrically (Table I). Cells at the distal and proximal ends were indistinguishable. But at the junction of the suspensor and the main mass of embryo there was one cell situated centrally which appeared different from the rest (Fig.3).

In six cases the embryos consisted of small masses of cells organised into an elongated cylindrical body with a free apex at the distal end and a basal portion continuous with the suspensor (Fig.4, 5). Cells at the distal end were equidimensional and scattered whereas at the proximal end they were

TABLE I

Summary of the results with whole embryos cultured in vitro

Stage of Development	II	III	IV
No. of Embryos Cultured.	25	25	25
Exp. Started.	15 Jul. 62.	25 Jul. 62.	5 Aug. 62.
Exp. Ended.	19 Aug. 62	29 Aug. 62.	9 Sept. 62.
No. of Normal Embryos.	6	14	22
No. of Abnormal Embryos.	19	11	3
Type of Abnormality	Proliferation of Suspensor, Rounded embryo - undifferentiated.	Slower rate of growth. Cotyledons not developed. Secondary embryos formed.	Secondary embryos formed.
No. of Embryos producing Secondary Embryo	-	7	3
Stages of Development in Secondary Embryos.	-	II	II & III
Morphology of the Secondary Embryos.	-	Spheroidal in shape. Undifferentiated.	Spheroidal in shape. Differentiated.

equidimensional but arranged in rows. Differentiation was arrested at this stage. Examination of embryos growing in vivo showed greater growth than those cultivated in vitro (Fig. 6).

Embryos in Stage III. (Collection July 24, 1962)

Embryos of the controls showed the initiation of primary root tissue. The root initials consisted of three or four cells each dividing at an oblique plane (Fig. 7).

The embryos in vitro produced both root and shoot apices (Fig. 8). The root cap was well established and was constituted of columnar cells (Fig. 9). The shoot apex had assumed a rounded structure with elevated shoulders (presumably cotyledonary primordia). Pith-like formation was initiated at the hypocotyl region in centrally located cells. These cells were bigger than those of the ground tissue. Below this region procambium strands had formed. Cells of these strands were dividing vertically and were narrow and more highly stained than the surrounding cells.

In seven embryos there was induction of budding at different parts of the suspensor and much below the rosette cells (Fig.10). In three cases, orientation of these was at an angle of 135° to the main axis of the embryo. These secondary embryos lacked a filamentous attachment. No mark of cell specialisation was noted in them (Table I).

The embryos grown in vitro showed a slower rate of growth when compared with identical embryos developed in vivo.

Those grown *in vivo* were bigger, the shoot apex had assumed a more conical shape and the cotyledons were well developed (Fig.11).

Embryos in Stage IV. (Collection August 4, 1962).

Embryos of the controls had slightly elevated shoulders at the shoot apex and a defined rounded epicotyl (Fig.12).

From this stage the embryos *in vitro* developed to maturity with a conical shoot apex and well developed cotyledons. The differentiation of procambium and pith was complete and responded to differential staining in contrasting relationship to other locations (Fig.13, 14, 15). The procambium strands in the hypocotyl-shoot axis were continuous with those of cotyledons.

Three embryos developed from suspensorial sources during this period, acquired and displayed an abnormally great variety of growth patterns. In two cases the embryos were elongated with cellular differentiation (Fig.16). In one case the root apex was initiated but the shape of the embryo still remained spheroidal (Table I). A synopsis of the characteristic development in each phase is presented in Table II.

Excised portions in Stage IV. (Collection August 7, 1962).

Hypocotyl:

This region attained growth by cell division (Fig.17).

TABLE II

Development of Embryos excised at different phases of Growth and grown in vitro

Phase of Growth	Shape of Embryo	Differentiation distinctive features
Stage II	Spheroidal	Proliferation of suspensors, cell division localized at distal end, root initial formed.
Stage III	Elongated, cylindrical.	Rounded shoot apex delineated; spheroidal embryos budded from suspensor, root and shoot apices differentiated; cotyledonary primordia, procambium, and pith formation initiated.
Stage IV	Elongated, cylindrical, cotyledons evident.	Pith and procambium well developed. Procambium from the main axis of embryo continuous into cotyledons. Suspensorial embryos differentiated.

Examination showed that cell division occurred more at the apical cut end (close to the shoot apex) than at the basal end (Fig.17, 22, 23). The epidermis was unaltered and in three cases cell expansion occurred in the cortical cells. The expansion was observed in 2 to 3 layers of cortical cells at the apical end (Fig.26). The basal cells were elongated and contained granular inclusions, a normal feature in the gametophytic cells. The segments did not proliferate into callus mass but, instead, maintained a regular growth pattern similar to the one in the parent segment (Table III).

Sub-hypocotyl:

This segment also showed differences in developmental patterns at the basal and apical ends (Fig.18, 24, 25). At the apical end the centrally located cells (pith?) produced a small compact mass of pseudo-meristem (Fig.28). The cells on the basal end maintained their pattern of growth except at the cut end where very small cells were observed. These cells were less stainable than the neighbouring ones. The inner cortical cells divided periclinally and pushed the epidermis further out. Some of these cells had expanded and had fewer inclusions (Fig.27), (Table III).

Root Apex with few layers of Columnar cells:

This segment retained its morphological characteristics (Fig.19). The columnar and peripheral cells maintained their

TABLE III

Summary of the results on excised segments cultured in vitro

Segment	No. of Segm. Cultured.	Exp. Started.	Exp. Ended.	No. of Abnom. Segm.	Abnormal Differentiation.	Change in Cortical Cells.
a) Hypocotyl	25	8-9 Aug. 62	12 Sept. 62	3	Difference in cells at two cut end; basal cells granular, slower rate of cell division.	Cell expansion.
b) Sub-hypocotyl	25	"	"	7	Very small cells at basal cut end; pseudo meristem at apical cut end.	"
c) Root apex with few layers of columnar cells	25	"	"	---	---	---
d) Sub-root apex	25	"	"	1	The column absent; pseudo apex formed.	---
e) Suspensors with 2-3 layers of radicle cells	25	"	"	9	Change in the plane of cell division of columnar cells.	---
f) Suspensors	25	"	"	4	Proliferation of suspensors; secondary embryo formed.	---

original patterns of orientation. Cells of the periphery were not derived from the central core. The columnar cells were close-packed. The epidermis was unaltered and no cell expansion was observed among the cortical cells (Fig.29), (Table III).

Sub-root Apex:

This segment from an *in vivo* source comprising a central and peripheral region showed alteration in growth behaviour. The central cells (column) generally tended to be arranged in long vertical files. This orientation was achieved through a predominance of transverse divisions.

In the segments grown *in vitro* (Fig.20), the columnar structures were absent. The new cells formed were very small and showed predominance of division at an oblique plane. The peripheral region, on the other hand, retained the radial orientation of the cells (Fig.30).

In one case, the regeneration of a pseudo-root apex occurred. A group of comparatively smaller cells at the apical end (toward the root apex) was found to be arranged on an arc created by cells dividing at a vertical plane (Fig.36). These cells were distinct from those of the peripheral region. It seemed evident that the core (column) had contributed some cells to the peripheral region (Table III).

Suspensorial Segment with 2-3 layers of Radicle Cells:

The suspensors did not proliferate much (Fig.21). The radicle cells ultimately gave rise to a meristematic region

akin to that in a root apex. These cells divided in a vertical plane instead of dividing at a transverse plane (Fig. 31). The associated filamentous structure was limited to a few elongated cells at the base of the newly formed apex (Fig. 31), (Table III).

Suspensors:

The suspensors had undergone extensive proliferation and had budded giving several secondary embryos (Fig. 32). The embryogeny was anomalous in that one of the filamentous cells produced a multinucleate structure (Fig. 33). It ultimately gave rise to a spheroidal embryo with a short filamentous attachment (Fig. 34). In another case the budded embryo had no filamentous attachment (Fig. 35), (Table III).

Discussion:

That exogenous influence is of significance in delineating and assessing development is borne out by the present experiments. It facilitates initiation of ontogeny; it may physically foreshorten the axis of polarity and lessen the time period in which polarity is being developed in early embryogenesis. It may disperse the incidence of polarity and give it a multiple prominence (budding embryogeny) and it may encourage the onset of three dimensional growth rather than of linear (filamentous) growth.

Exogenous influence is significant in controlling rates of growth as well as development. In artificial medium, cotyledons may never form though the tissue that would normally supply the initials retains activity. Finally, exogenous

influence has significance with respect to tissue differentiation. It has been shown *in vitro* that when zones differentiate, the plane of cellular division (oblique walls of procambium) or the direction of origin of new cells (*vide* source of peripheral cells) may contrast to the situation *in vivo*.

In all the experiments extract from *Ginkgo* gametophytes had been used. Steward *et al* (13) had shown that this extract had 65% of the efficiency of coconut milk factor for stimulating the growth of carrot cells *in vitro*. The same extract facilitated abnormal ontogeny only in 3/25 cases in Stage IV cultured *in vitro* while in Stage III and II it produced 11/25 and 19/25 abnormal embryos respectively. Thus it was evident that the environmental conditions exerted greater influence on the early ontogeny than on the later stages. The claims of De Maggio *et al* (6) that internal differentiation was a prerequisite for organ formation though plausible in their context might require qualification if the culture medium could be adjusted properly.

Range of adaptability response to environmental factors was associated with power to regenerate. The 'e' and 'f' segments bearing suspensors with 2-3 layers of radicle cells and suspensors respectively showed a greater potential for development and growth. The mode of budding in secondary embryo formation from the suspensor was comparable to the findings of Steward (12) in carrot cells. The 'e' segments had produced a pseudo-meristem. Thus, the results showed that

suspensor cells had greater potential for development and growth when isolated from their normal position in the embryo and cultured *in vitro* with proper supply of exogenous stimuli.

The potentiality of suspensor cells to produce varied growth patterns had been observed by earlier workers at McMaster University. Bonga (1) and Wood (17) observed pitted structures in the suspensor *in vitro*. The suspensorial embryos observed in the present work were mostly rounded structures with or without filamentous attachment. The fact that this radial development occurred only under specific nutritional environment supported the idea that the origin of the suspensor was through physiological response and not phylogenetic sequence (10). This view was contrary to the "primitive spindle" theory advanced by Bower (2). According to him, the spindle-like form of a young embryo with filamentous suspensors reflects a primitive ancestral state.

The phylogenetic implications of the results of this study as related to morphogenesis in *Pinus* are less restrictive than might have been the case had the claims of Bower (2) and Buchholz (4) held. On the other hand there is still much to be learned concerning the real limits of phylogenetic implication. For instance, totipotency, if it is an attribute of any portion of an embryo, has not yet been demonstrated. The potentialityof free cells from phloem of carrot to give rise to a plant was shown by Steward (12). Thus the potentiality to produce the whole organism by any part should be regarded as an inherent or constitutional

feature derived from the original organisation of the cell. This is in agreement with Thompson's view who "preferred to regard cells of an embryonic group as potential embryo initial rather than actual embryo initials" (9). The induction of in vitro embryo formation from the excised suspensor cells furnished further proof of totipotency of this part of the embryo.

Totipotency in this work has been applied in relation to the ability of a cell to form an embryo. The results show that this was achievable in Stages III and IV and only by the suspensor cells. It seems a possibility that the proximal apex of the embryo (suspensor) at Stage I is inhibited until Stage II has been initiated. Thereafter, it is free to proliferate as it did in the post-zygotic stage to initiate the primary embryo.

The results of the experiments reported here showed that the potentialities for growth and development were greater at Stages III and IV. Also, without attempting to apply specified chemical agents to control growth it has been shown that alternative developmental sequences occur with change innutritional environment. Whether or not the phenomenon of differentiation encountered in late development can be induced in early ontogeny (post zygotic complex) remains to be shown. According to Wetmore (16), "some of the factors involved in early embryogeny or development must be microbiochemical in nature".

The break with filamentous development and the

imposition of three dimensional growth pattern provides evidence against the argument for ontogenetic predestination in zygote. Also, where there are departures from *in vivo* ontogeny, in the *in vitro* results, response is with respect to group of cells (a zone) but not with a single initial. Thus, when re-orientation in direction of wall formation was affected, multiple protoplasts responded together (*vide* procambium, or contribution to peripheral tissue).

For the interpretation of growth pattern the emphasis, at least tentatively, must be neither on phylogenetic nor on fixed genetic attitudes in protoplasts, but on the considerable influence of the physico-chemical environment on the development of the organism.

Summary:

1. Whole embryos of *Pinus* excised at various stages of development and cultivated *in vitro* showed different degree of potential for differentiation.

2. The influence of physico-chemical environment was highest during the earliest stage of development.

3. Under *in vitro* conditions the suspensor cells of some embryos formed budded embryos while in other cases growth of the suspensor cells was arrested.

4. The growth of the suspensor or its lack *in vitro* was a function of physiological response.

5. Polyembryony was due to constitutional potentialities in the young embryonic tissue and thus all the cells of an embryonic group could be considered as "embryo initials" under appropriate environmental conditions.

6. Segments of embryos cultivated *in vitro* showed gradients in potentiality for differentiation in relation to their positions in the embryo and also within themselves in relation to their basal and apical ends.

7. Alternative developmental sequences were observed during regeneration in the segments.

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PLATE I

Fig. 1. Control embryo at Stage II. X 60.

Fig. 2. Embryo at Stage II *in vitro* - round mass, no suspensor. X 110.

Fig. 3. Embryo at Stage II *in vitro* - central cell different from the rest. X 90.

Fig. 4. Embryo at Stage II *in vitro* - cells at proximal end bigger. X 110.

Fig. 5. Embryo at Stage II *in vitro* - cells at proximal end arranged in rows. X 90.

Fig. 6. Embryo at Stage II *in vivo* - greater growth acquired. X 40.

Fig. 7. Control embryo at Stage III - root initials formed. X 110.

Fig. 8. Embryo at Stage III *in vitro*- epicotyl rounded, cotyledon primordia formed. X 25.

Fig. 9. Embryo at Stage III *in vitro*- root apex well developed with columnar and peripheral cells. X 400.

Fig. 10. Embryo at Stage III *in vitro*- two embryos budded at different places on the suspensor. X 75.

Fig. 11. Embryo at Stage III *in vitro*- cotyledons well developed, epicotyl conical. X 25.

Fig. 12. Control embryo at Stage IV - root and shoot apices well developed. X 75.

Fig. 13. Embryo at Stage IV *in vitro* - mature embryo formed. X 15.

Fig. 14. Embryo at Stage IV *in vitro* - different tissue systems developed. X 20.

PLATE I

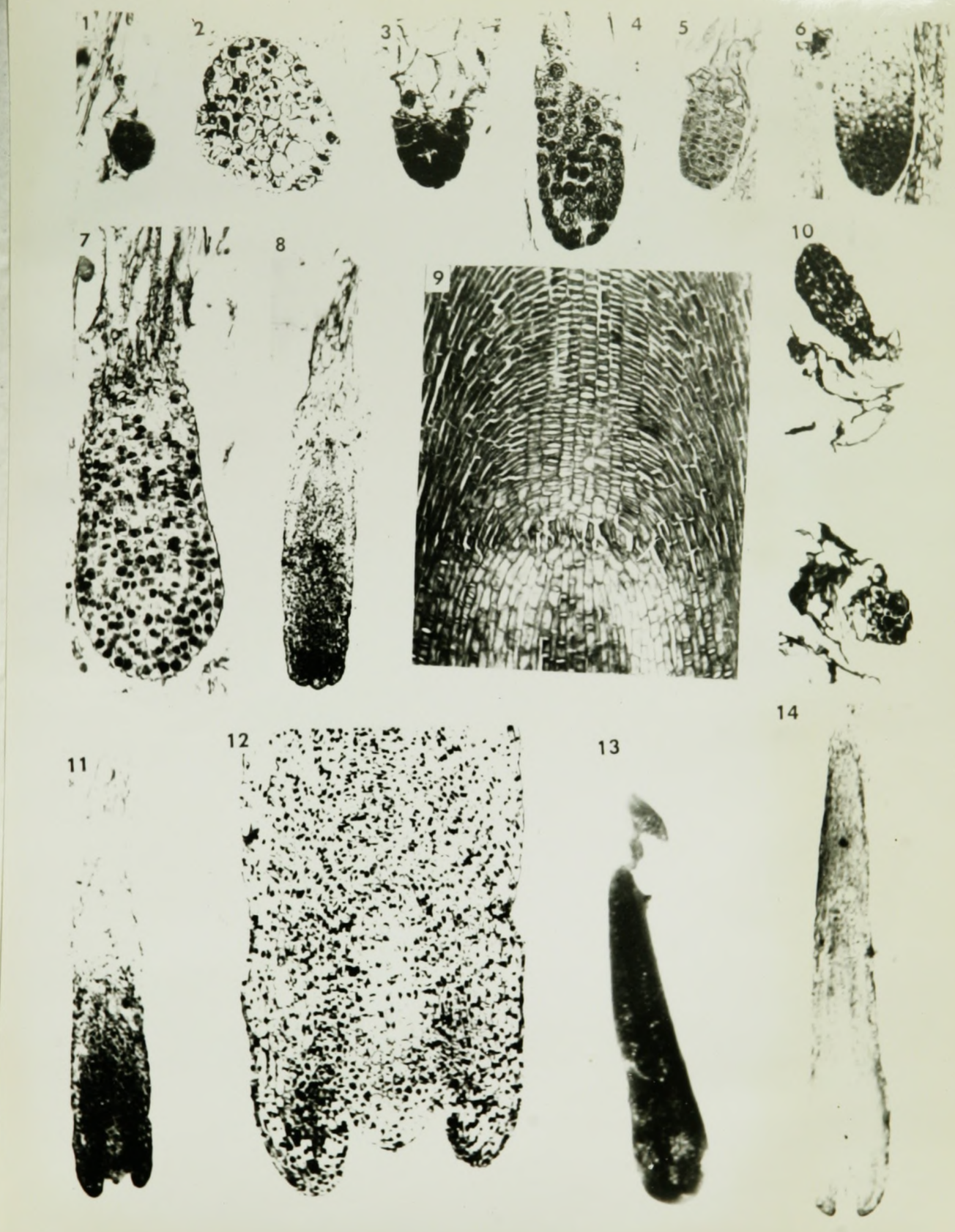


PLATE II

Fig. 15. Embryo at Stage IV *in vitro* - continuity of procambium strands into the cotyledon and pith formation. X 100.

Fig. 16. Embryo at Stage IV *in vitro* - suspensorial embryos showing differentiation of root initial and columnar cells; shape of one is still spheroidal. X 90.

Fig. 17. Hypocotyl segment grown *in vitro*. X 15.

Fig. 18. Sub-hypocotyl segment *in vitro*. X 15.

Fig. 19. Root apex segment *in vitro*. X 15.

Fig. 20. Sub-root apex segment *in vitro*. X 15.

Fig. 21. Suspensorial segment with 2 - 3 layers of radical cells *in vitro*. X 15.

Fig. 22. L. S. of hypocotyl segment at the apical end showing cell division and expanded cells. X 750.

Fig. 23. Basal end of the hypocotyl segment showing granulation in cells. X 750.

Fig. 24. L. S. of sub-hypocotyl segment at the apical end showing normal cells. X 250.

Fig. 25. Basal end of the sub-hypocotyl segment showing smaller cells. X 250.

Fig. 26. L. S. of hypocotyl segment showing expansion in cortical cells and granulation. X 475.

Fig. 27. L. S. of sub-hypocotyl segment showing intact epidermis and no expansion in cortical cells. X 475.

PLATE II

PLATE III

Fig. 28. L. S. of sub-hypocotyl segment showing pseudo-meristem formation. X 450.

Fig. 29. L. S. of root-apex segment showing normal orientation of cells. X 450.

Fig. 30. L. S. of sub-root apex segment showing absence of columnar structure. X 450.

Fig. 31. L. S. of Fig. 21 showing formation of a meristem by the radical cells. Suspensor cells limited to few long cells at the base and devoid of nuclei. X 475.

Fig. 32. L. S. of excised suspensor grown in vitro showing budded embryo. X 90.

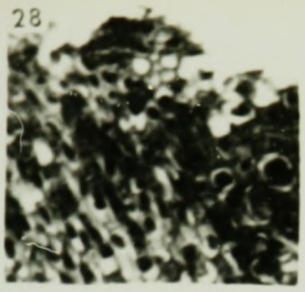
Fig. 33. L. S. of suspensorial segment showing ontogeny of budding embryo - 4 nucleated filament formed. X 750.

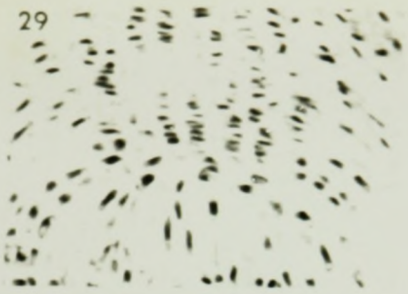
Fig. 34. L. S. of budded suspensorial embryo showing filamentous attachment. X 250.

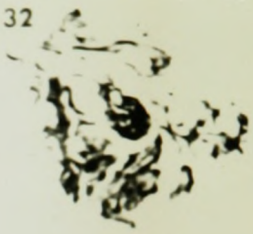
Fig. 35. L. S. of a suspensorial embryo showing absence of filamentous attachment. X 90.

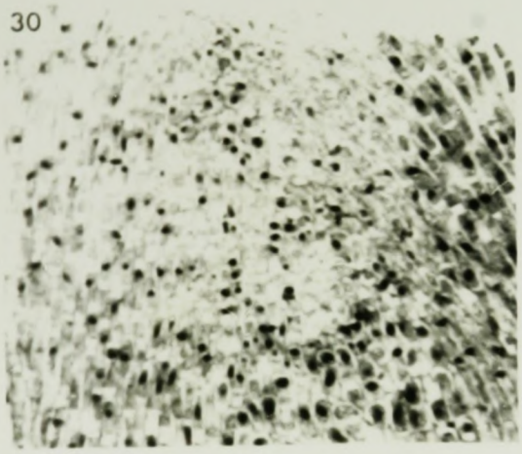
Fig. 36. L. S. of sub-root apex showing regeneration of a pseudo-root apex. The cells at the top end divided at a vertical plane. X 500.

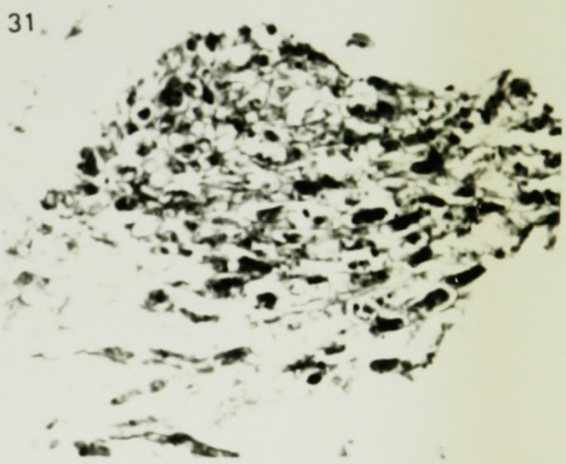
PLATE III

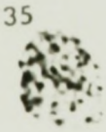


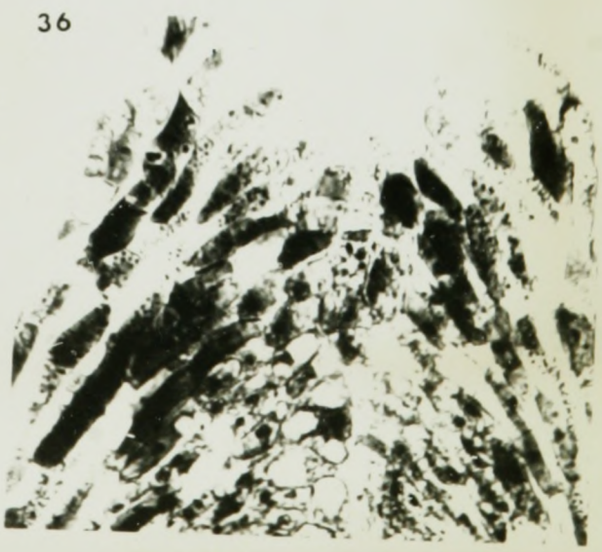


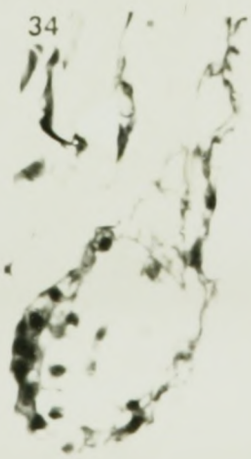












CHANGES IN THE POOL OF FREE AMINO ACIDS IN THE GAMETOPHYTE IF GINKGO BILOBA L. AT DIFFERENT STAGES OF EMBRYONIC DIFFERENTIATION

Introduction:

The female gametophyte in Ginkgo biloba furnishes nutriment for archegonia and developing embryos. Ball (1) showed that the root and shoot of the embryo in Ginkgo differ fundamentally in their nutritional requirements. The root is capable of utilising nitrate from the culture medium in producing sufficient organic nitrogen for excellent growth while the shoot requires a supplemental source of organic nitrogen. The difference in the nutritional requirements of these regions suggests its possible origin of physiological difference during early ontogeny.

After fertilisation, proembryo development proceeds through free nuclear division until about 256 nuclei. In the next phase cytokinesis occurs separating one nucleus from the other which are distributed in undifferentiated cells of equal size (15). The cells at the archegonial neck function as a meristem and ultimately give rise to root and shoot apices. Under in vitro conditions, in inorganic media, cell multiplication is localised to central cells of the proembryo while in vivo the basal cells multiply (11).

The metabolism of the developing embryo presumably depends on the supply of the substrate from the female gametophyte. Disclosure of the different kinds and amounts of metabolites in the female gametophyte constitutes a first step in understanding embryonic metabolism at different morphogenetic levels. The developing embryo-gametophyte complex of Ginkgo is especially appropriate experimental material to study these problems.

For angiosperms, Duvick (2) reports such a study on the free amino acids in the developing endosperms of maize at regular intervals during the post fertilisation period. The amino acids are the primary sources of nitrogen for the synthesis of enzymes (4) which control the process of differentiation. The present study is a preliminary investigation of the free amino acids in the female gametophyte of Ginkgo during different phases of embryonic differentiation.

Materials and Methods:

Collections of Ginkgo ovules were made from September 15 - October 15, in the years 1962 and 1963. Extractions of gametophytes up to October in both of the years were made from fresh collections while those of later months were made from stored materials kept at 14°C.

For extraction, the prothallia were removed from the stony integuments. The apex of the prothallium with 2 to 3

archegonia was dissected out and fixed in formalin-propionic-acid-alcohol (7) for later microscopical studies. The gametophytes were crushed with sterile silica in a mortar at room temperature and were extracted with 80% alcohol and filtered. The filtrate was centrifuged at 3000 rpm until the supernatant was clear and then dried on a water bath. The residue was dissolved in 1 ml. of distilled water and was stored at 10°C.

Two types of chromatograms were prepared on Whatman No. 3 MM papers treated with pyrophosphate (pH 8.9) and tartarate (pH 3.4) buffers (5,6). The chromatograms were loaded with 25 µl. of the extract along with reference spots of 10 µl. of 1% leucine and other standard amino acids. The following two systems served as the chromatographic solvents: For pyrophosphate treated papers - n-propanol/90% ethanol/pyrophosphate buffer (2:1:1). For tartarate treated papers - n-propanol/tartarate buffer (5:3). Quantitative analysis was accomplished by first removing ammonia and other volatile nitrogenous substances from the paper by spraying borate buffer in methanol, treating the spot with modified ninhydrin reagent (9) and by photometric determination. For proline, percentage of transmission was determined at 440 mµ and for other amino acids at 570 mµ. The quantity was calculated by referring to standard curves.

For microscopical studies, the fixed materials were dehydrated in tertiary butyl alcohol series and embedded in paraplast. Sections were cut at 7 µ and stained in

safranin-fastgreen and borax carmine-aniline blue (7). In order to find out the growth activity of the gametophytes, cytological studies with acetocarmine were done. Accumulation of indole derivatives in the gametophytic tissue had been studied by histochemical procedure (3).

Results:

Twelve amino acids are identified in all the extracts studied. The amounts of free amino acids are calculated per gram of wet weight of female gametophyte. The amount of total amino acids rises to a peak in the month of October when the egg is fertilised and declines to a minimum in November. It starts rising again in December reaching its peak in the month of January (Tab. I and fig. 1).

On the basis of the maximum percentage of their occurrence, five classes of free amino acids are noted viz.,

a) Less than 10% - serine, phenylalanine, valine, alanine, and leucine.

b) 10 - 20% - tryptophan, proline and threonine.

c) 20 - 30% - asparagine.

d) 30 - 40% - glutamic acid and arginine.

e) 40 - 50% - aspartic acid. (Tab. I).

The study of the trend of variation of each amino acid shows a relationship between them. Five different trends of variations are noted, viz.,

wt: µg/gm fresh wt. Table 1 % : % total amino acids

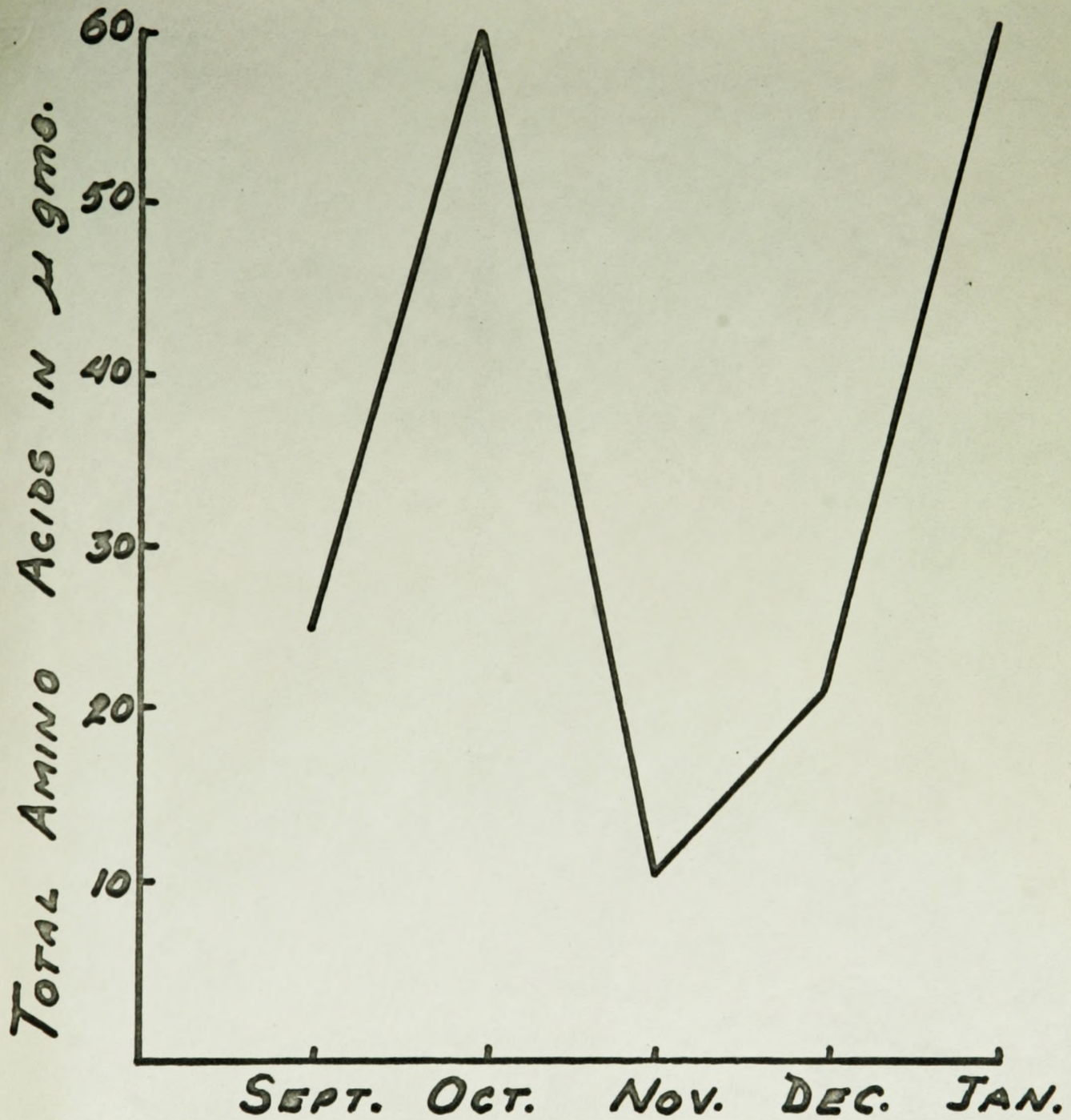
Amount of free amino acids in µg per gram of female gametophyte with their respective percentages during different phases of embryonic differentiation

Time	Embryonic Phase	Aspartic Acid		Glutamic Acid		Asparagine		Arginine		Serine		Threonine		Alanine	
1962-63		Wt.	%	Wt.	%	Wt.	%	Wt.	%	Wt.	%	Wt.	%	Wt.	%
Sept.15	No fertilization. Unfertilized egg nucleus observed.	7.9	2±.63	28.2	7.1±1.4	11.9	3±.31	37.8	9.5±.4	3.6	.9±.26	2	.5±.17	.4	.1±.05
Oct. 15	Egg is fertilized. Free nuclear division starting in proembryo.	13.6	8.2±.97	16.5	10±3.1	19.9	12±.75	32	19.3±1.32	1.6	1±.19	.83	.5±.16	9.95	6±.82
Nov. 15	Spherical proembryo. Cell wall formation completed.	48.3	5.8±.88	8.3	1±.23	4.2	.5±.22	8.3	1±.36	4.2	.5±.18	.83	.1±.036	.04	.05±.03
Dec. 15	Proembryo oblong shape. Gradient of cell size appear from apex towards base.	9	2.1±.86	30.3	7±2.14	4.3	1±.24	36.8	8.5±.78	.9	.2±.09	2.1	.5±.32	.5	.1±.03
Jan. 15	Embryonic cells at the base of archegonium organize apical meristem	3.3	2.05±.78	3.3	2.0±.22	20.4	12.5±.59	20	12.3±2.51	3.3	2±.8	10.2	6.25±.67	2.3	1.45±.56

Table 1 (continued)

Time 1962-63	Embryonic Phase	Proline Wt.	Proline %	Trypto-phan Wt.	Trypto-phan %	Valine Wt.	Valine %	Phenyl-alanine Wt.	Phenyl-alanine %	Leucine Wt.	Leucine %	Total Amino Acid	av. wt. in g.
Sept.15	No fertilization. Unfertilized egg nucleus observed.	.5±.27	2	1±.3	4	.2±.13	.79	.2±.063	.79	.2±.11	.79	25.1	.894
Oct. 15	Egg is fertilized. Free nuclear division starting in proembryo.	2.3±.74	3	.5±.23	.83	.1±.05	.16	.2±.02	.32	.18±.07	.3	60.28	1.316
Nov. 15	Spherical pro-embryo. Cell wall formation completed.	1.5±.41	12.6	1±.37	8.3	.3±.09	2.7	.18±.04	1.4	.18±.04	1.4	12.01	1.132
Dec. 15	Proembryo oblong shape. Gradient of cell size appear from apex towards base.	.9±.36	4	.7±.27	3	.8±.26	3.5	.3±.13	1.3	1±.36	4.3	23.1	.905
Jan. 15	Embryonic cells at the base of archegonium organize apical meristem	2±.69	3.3	6.75±1.47	11	5.4±.85	8.8	5±1.57	8.1	3.5±1.19	5.7	61.28	.801

FIG. 1



GRAPH SHOWING AMOUNT OF TOTAL FREE AMINO ACIDS AT DIFFERENT STAGES OF EMBRYONIC DEVELOPMENT

It should be noted that the total amino acid values used here and in Table 1 are calculated from the amounts of individual acids present. There may be, therefore, sizeable errors in these data.

1. High and low: aspartic acid and proline (Fig. 2).

2. High-low-high: asparagine and alanine (Fig. 3).

3. Low and high: threonine, valine, phenylalanine and leucine (Fig. 4).

4. Low-high-low: glutamic acid and arginine (Fig. 5).

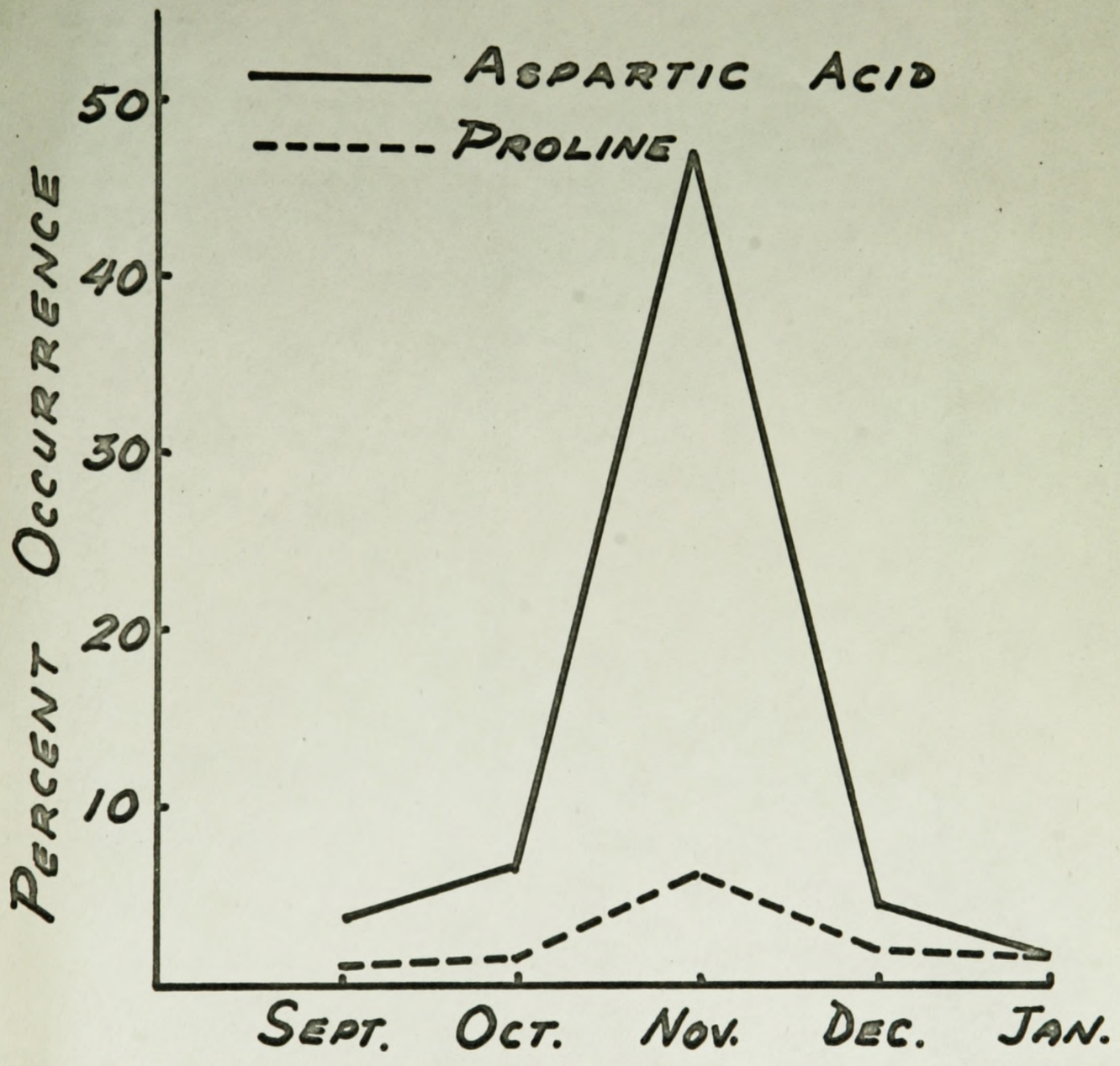
5. Low-high-low-high: serine and tryptophan (Fig. 6).

Continuous rise for aspartic acid up to November (7.9-48.3%) and then sudden decline in December (9%) is suggestive of its metabolic importance at the onset of visible polarity. Glutamic acid and arginine are highest at a time when aspartic acid is low in amount. Asparagine has very high occurrence (20.4%) near the end of January. All these observations suggest an inter-relation between these metabolites which may influence nutrition during early embryonic differentiation.

The changes in the average weight per gametophyte are represented in Fig. 7. The average weight per gametophyte is .894 g in the month of September. A marked increase of 1.316 g is noted in October. During the later months a steady decrease in weight is observed (1.132 g, .905 g and .801 g).

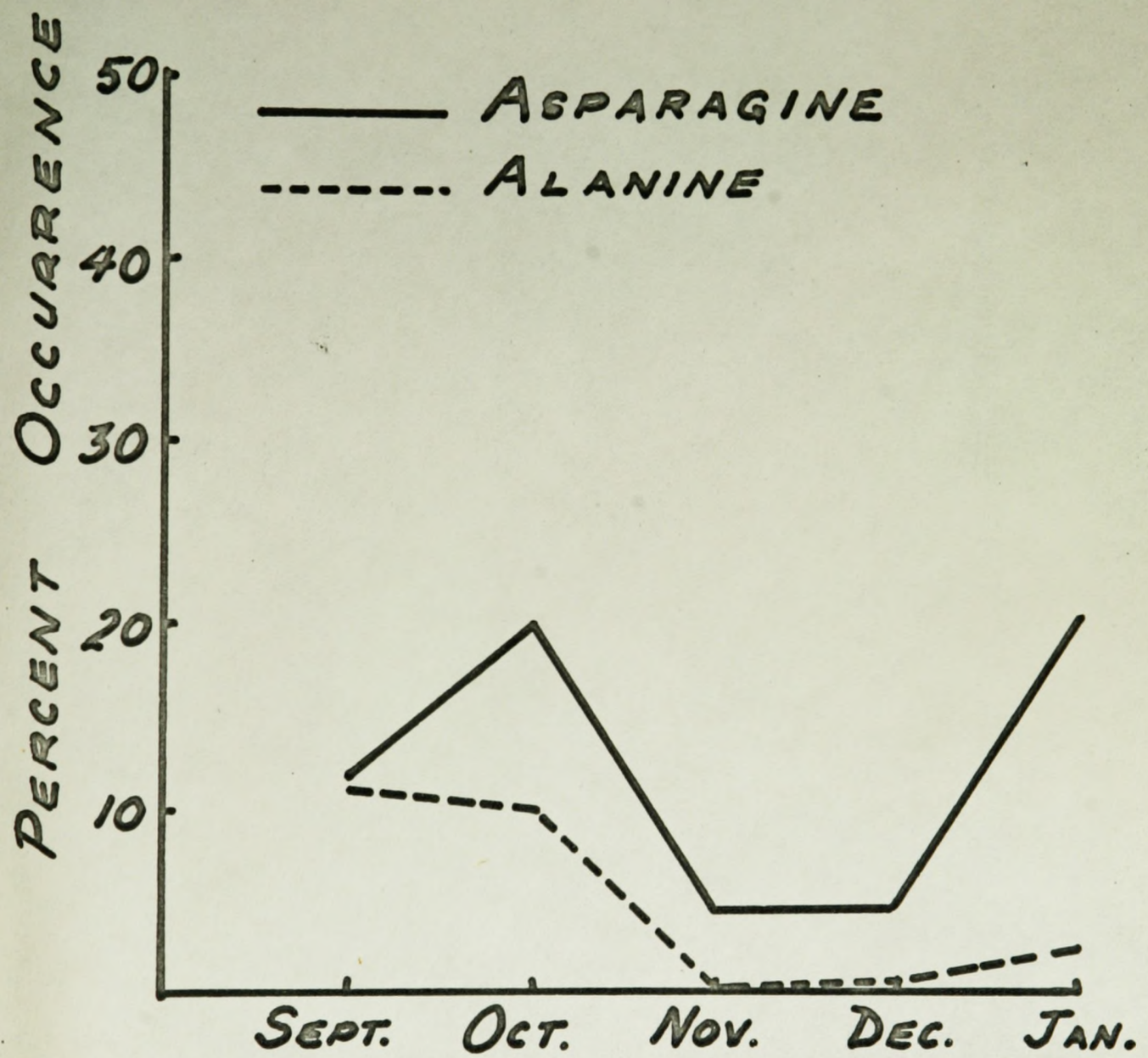
The embryological evidence shows no occurrence of fertilization in the month of September (Fig. 8, 11, 12). With the onset of fertilization and free nuclear division in the proembryo in October (Fig. 9, 13, 14) rapid increase

FIG. 2



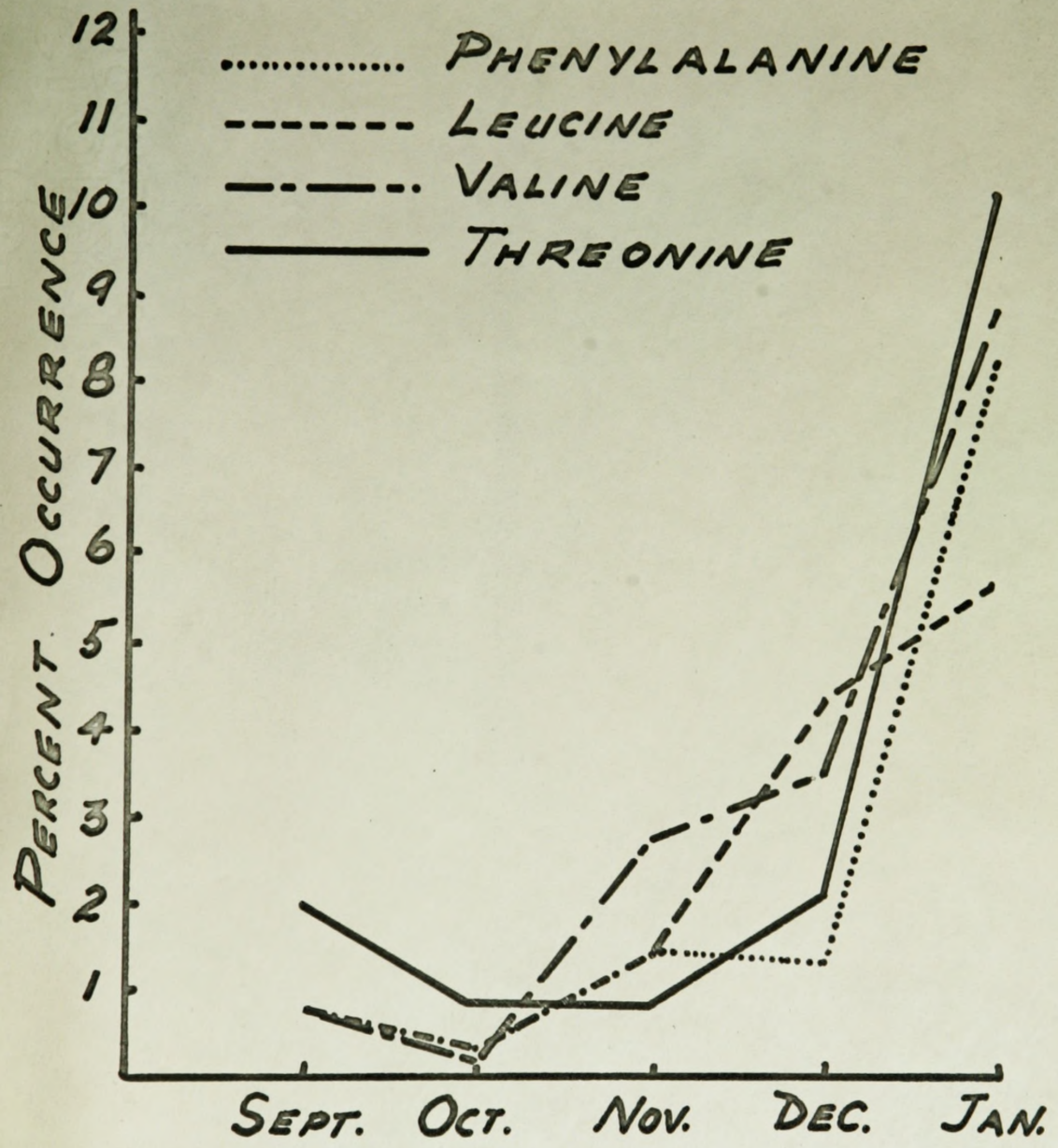
GRAPH SHOWING PERCENT OF TOTAL FREE AMINO ACIDS AT DIFFERENT STAGES OF EMBRYONIC DEVELOPMENT

FIG. 3



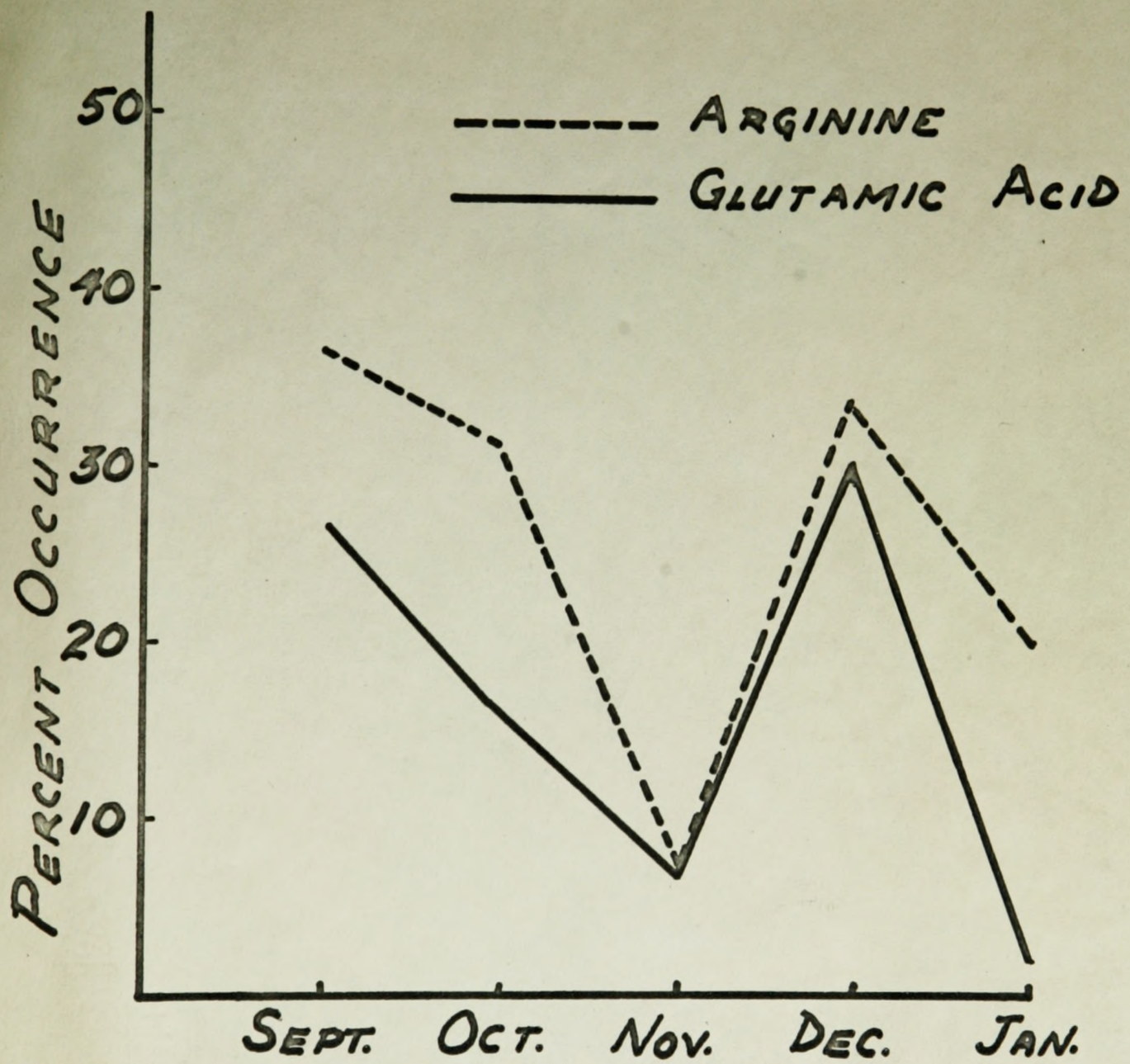
GRAPH SHOWING PERCENT OF TOTAL FREE AMINO ACIDS AT DIFFERENT STAGES OF EMBRYONIC DEVELOPMENT

FIG. 4



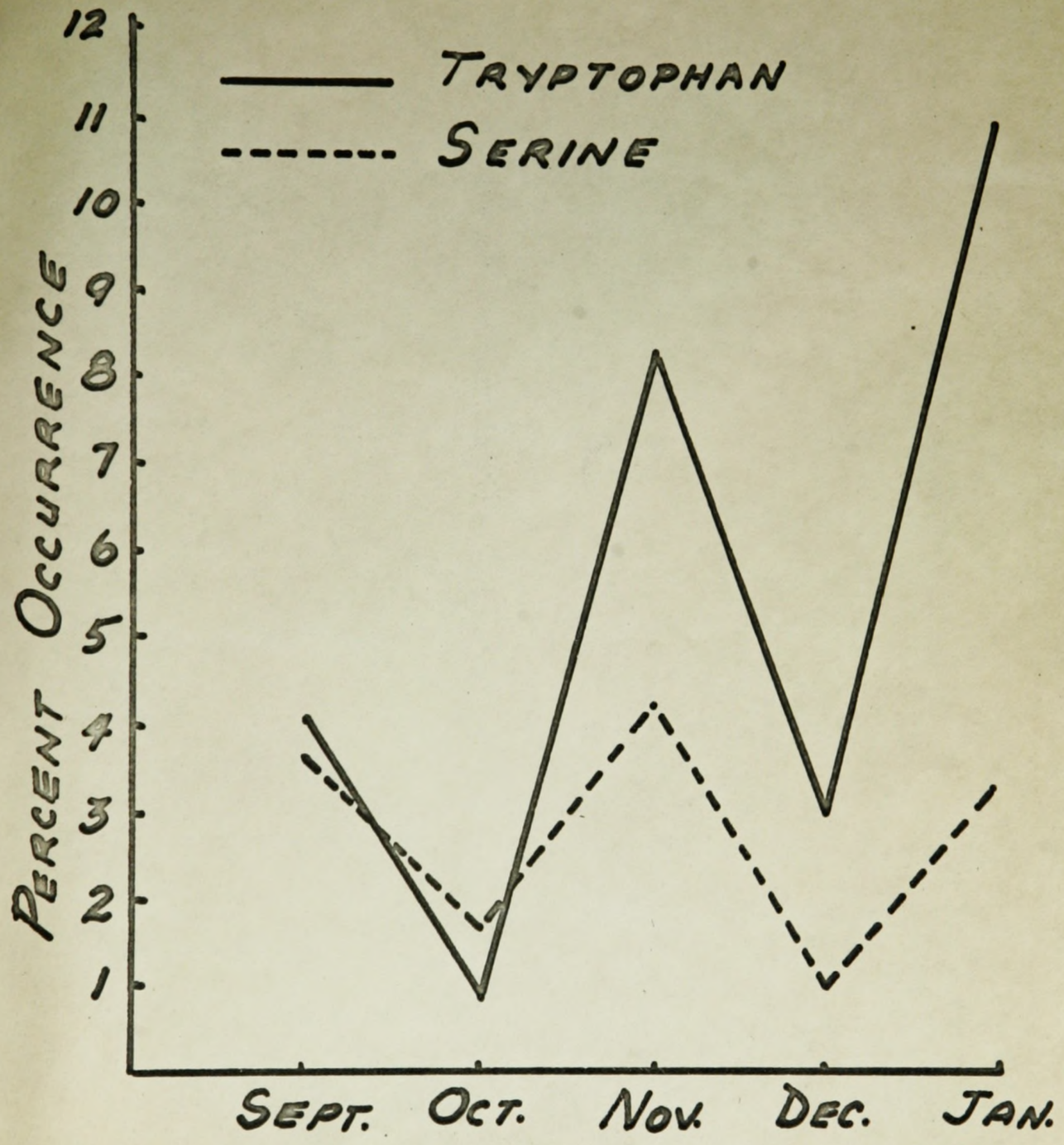
GRAPH SHOWING PERCENT OF TOTAL FREE AMINO ACIDS AT DIFFERENT STAGES OF EMBRYONIC DEVELOPMENT

FIG. 5



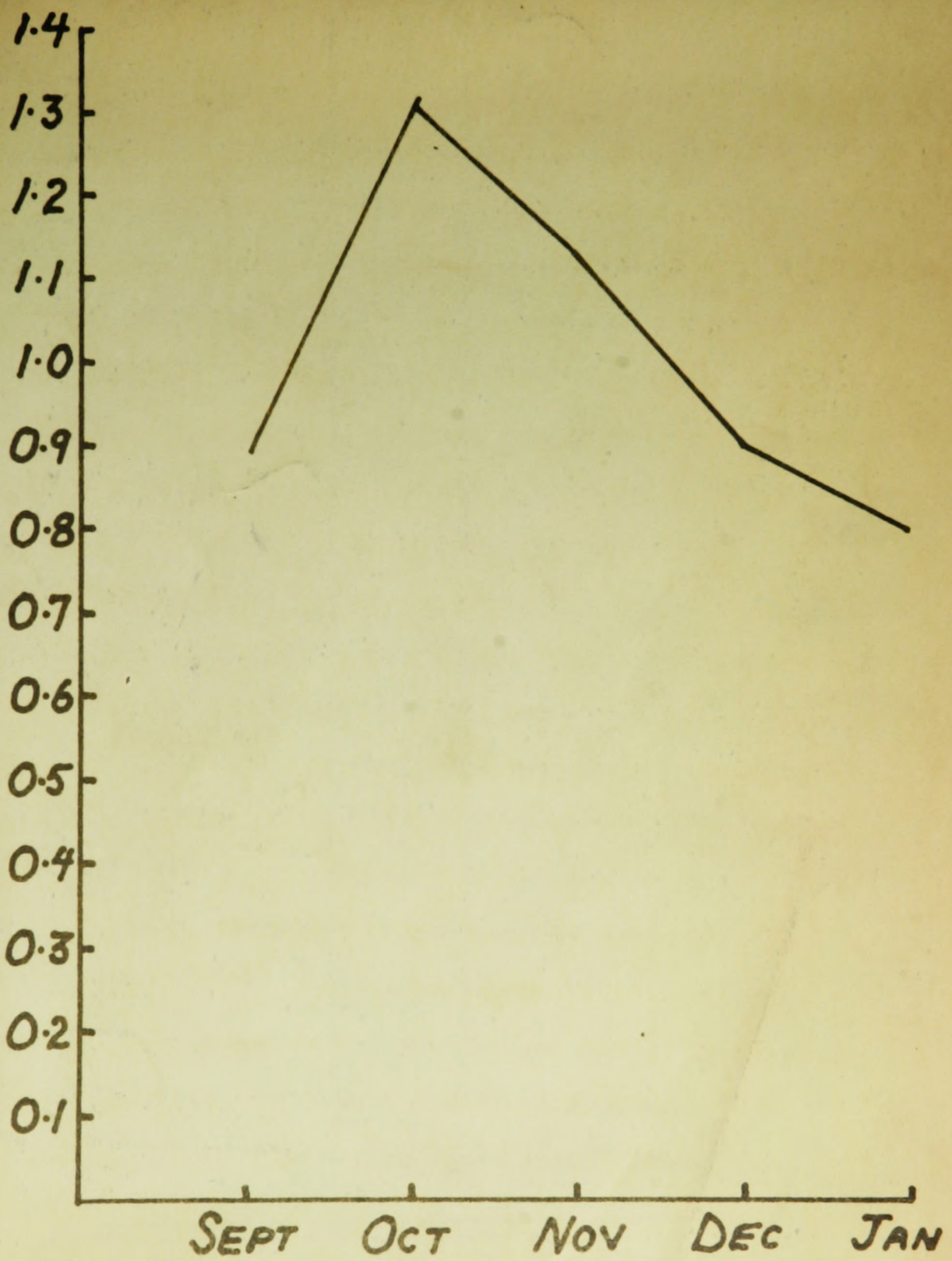
GRAPH SHOWING PERCENT OF TOTAL FREE AMINO ACIDS AT DIFFERENT STAGES OF EMBRYONIC DEVELOPMENT

FIG. 6



GRAPH SHOWING PERCENT OF TOTAL FREE AMINO ACIDS AT DIFFERENT STAGES OF EMBRYONIC DEVELOPMENT

Fig. 7



GRAPH SHOWING THE CHANGES IN WEIGHT (in g.) PER GAMETOPHYTE AT DIFFERENT STAGES OF EMBRYONIC DEVELOPMENT

in the amount of total free amino acids occurs (Fig. 1). On the basis of per cent occurrence, 4 amino acids, viz., aspartic acid, asparagine, alanine and proline show tendency for rise. In November, spherical proembryos complete cell wall formation without any visible differentiation in the cells (Fig. 10) and the total amino acid pool declines to 12.01 µg. from 60.28 µg. On the basis of per cent (relative) occurrence during this stage, a remarkable increase of 48.3% from 13.6% in the case of aspartic acid is observed. Serine, proline, tryptophan, valine, phenylalanine and leucine also show the same trend for increase.

In the month of December, the proembryo acquired an oblong form with a gradient in cell size and visible polarity was established (Fig. 15). Total amino acids increased two-fold from that of the previous month. An increasing trend (relative) as revealed by per cent analysis was shown for glutamic acid, arginine, threonine, alanine, valine and leucine.

During the next phase, the actively dividing cells at the base of the archegonium have organised an apical meristem but cotyledons have not yet been fully developed (Fig. 16). The pool of free amino acids increases greatly, for example, from 23.10 µg. to 61.28 µg. In the case of individual amino acids, 8 amino acids show increasing trend (Tab. I). The cytological studies of the gametophytic cells show that after July the cells do not undergo

division and hence are not multiplying. The histochemical test to study the presence of indole derivatives shows a greater concentration in the outermost cells and archegonia while the rest of the cells have a more or less uniform concentration as shown by uniform colouration (Fig. 17).

Discussion:

That an embryo of a gymnosperm growing in vivo gets all its nutriment from the surrounding gametophytic tissue is generally accepted (13). It is also known that for this nutrition the metabolites of high molecular weight in the gametophytic tissue must break down to simpler components through enzymatic action.

The amount of total amino acids per gametophyte is 28.5 µg during September. In October the amount increases to 45.8 µg per gametophyte. A minimum amount of 10.6 µg per gametophyte is observed in the month of November. During December the amount of total amino acids increases to 25.5 µg. A maximum amount of 76.5 µg per gametophyte is observed during the month of January.

The decrease in weight per gametophyte in the stored materials during the months of November to January may, partially, be due to dehydration of the gametophytes. However, low accumulation of amino acids (10.6 µg) per gametophyte during November can not be due to loss of water.

It is important to recognise here that the free amino acid pool is not always a measure of available supply of amino acids for new protein synthesis. Also, the increase or decrease in the amino acid pool depends on two factors, viz., the rate of production and the rate of utilisation.

Steward and Pollard (14) showed the importance of glutamyl compounds in nitrogen metabolism of plant tissues. The transfer of nitrogen to the developing embryo in barley also involves an extensive reconversion of glutamine into other amino acids (17).

In Ginkgo the concentration of free glutamic acid in the gametophyte is maximum during the month of December (30.3%). A minimum concentration of 3.3% is observed in January.

The role of tryptophan as a precursor for IAA has been shown by Wildman and Bonner (16). Leopold (8) suggests that auxin production in fruit proceeds in two phases. In the first phase it is carried out in ovarian tissue and in the later phase in the embryo.

The trends of variation for serine and tryptophan show a striking similarity. Both of them show a decline in the month of October and then rise and decline alternatively. Histochemical tests (3) in the gametophytes show higher concentration of indole derivatives in the outermost cells and the archegonium. The amount of indole

derivatives in the inner cells is uniform as shown by uniform colouration.

The minimum concentrations (relative) of aspartic acid and proline are observed at the final stages of embryogeny (December and January). A different trend is shown by alanine. Low accumulation of this amino acid occurs during the month of November when cell wall formation has been completed in the proembryo. Low accumulation (relative) of threonine, phenylalanine, valine and leucine occurs at the time of fertilization. Low accumulation (relative) of serine occurs when visible polarity is established.

Ball (1) in experiments on mature embryos of Ginkgo observes a differential nutritional requirement for root and shoot growth. The root is capable of utilising inorganic nitrogen in the form of nitrate to produce organic nitrogen whereas the shoot needs a supplemental source of organic nitrogen due to the absence of nitrate-reductase in the shoot.

The concentration (relative) of tryptophan in the gametophyte is more when the egg is unfertilized than when it is fertilized (4% and .83%). A ten-fold increase in concentration is observed during the month of November (8.3%). During the next month the concentration of tryptophan decreases (3%). The maximum concentration of 11% is observed in January when the embryonic cells

are observed to organise apical meristem.

The importance of metabolites to induce specialisation of cells has been observed by previous workers (10, 12). The changes in the pool of free amino acids in the gametophyte observed at different phases of cellular specialisation in a growing embryo in the present work suggest a close relationship of gametophyte metabolism to embryo development.

Summary:

1. Changes occur in the size and constitution of the pool of free amino acids at the time of different stages of embryo development.
2. Based on concentration the free amino acids show five different trends of variation.
3. Increase in the pool of free amino acids occurs with the onset of fertilization.
4. A second increase in the pool is observed when the apical meristem is initiated.
5. Accumulation of tryptophan is greater when the egg is unfertilized than when it is fertilized.
6. A maximum increase in the amount of tryptophan occurs when the apical meristem is organized.

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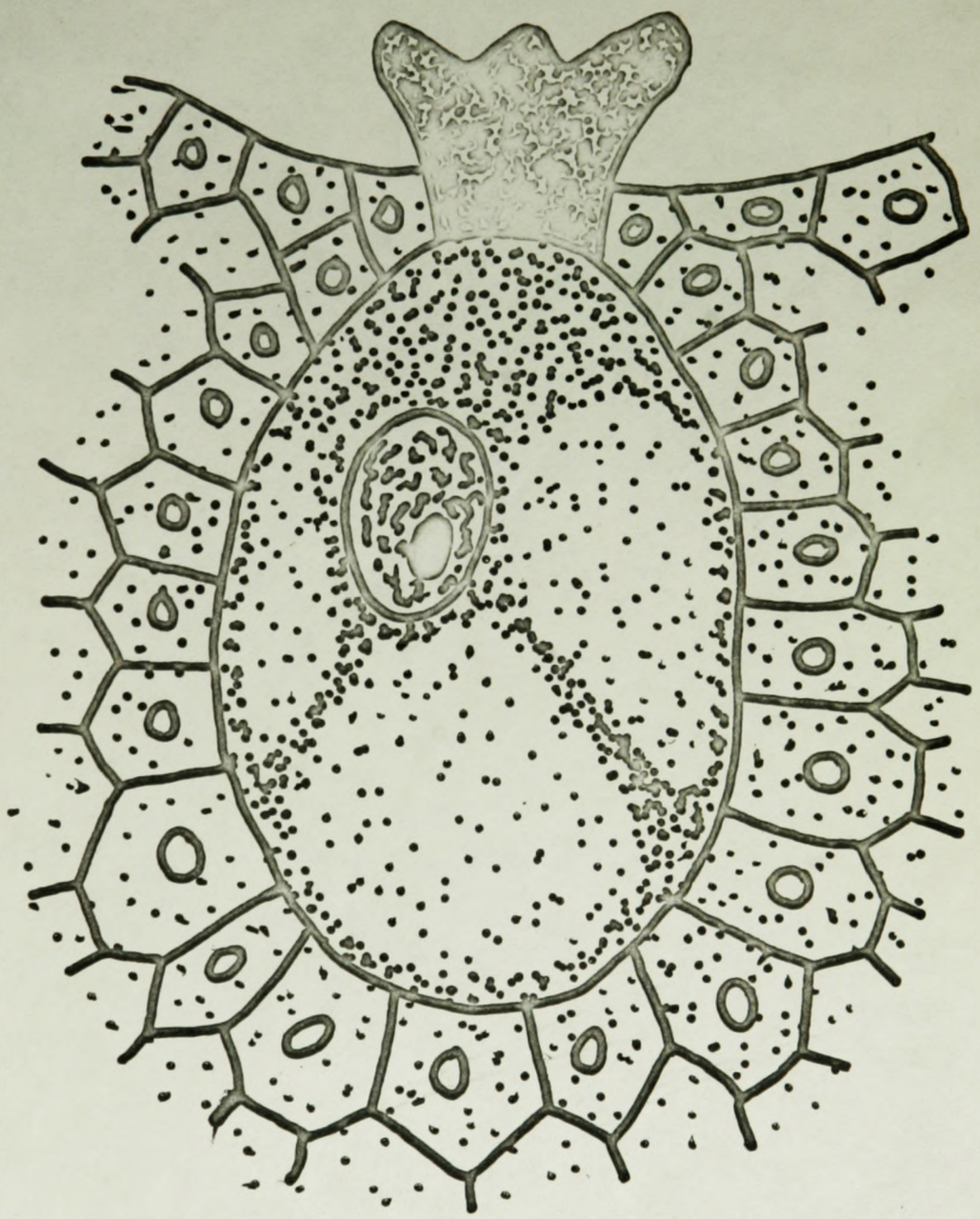
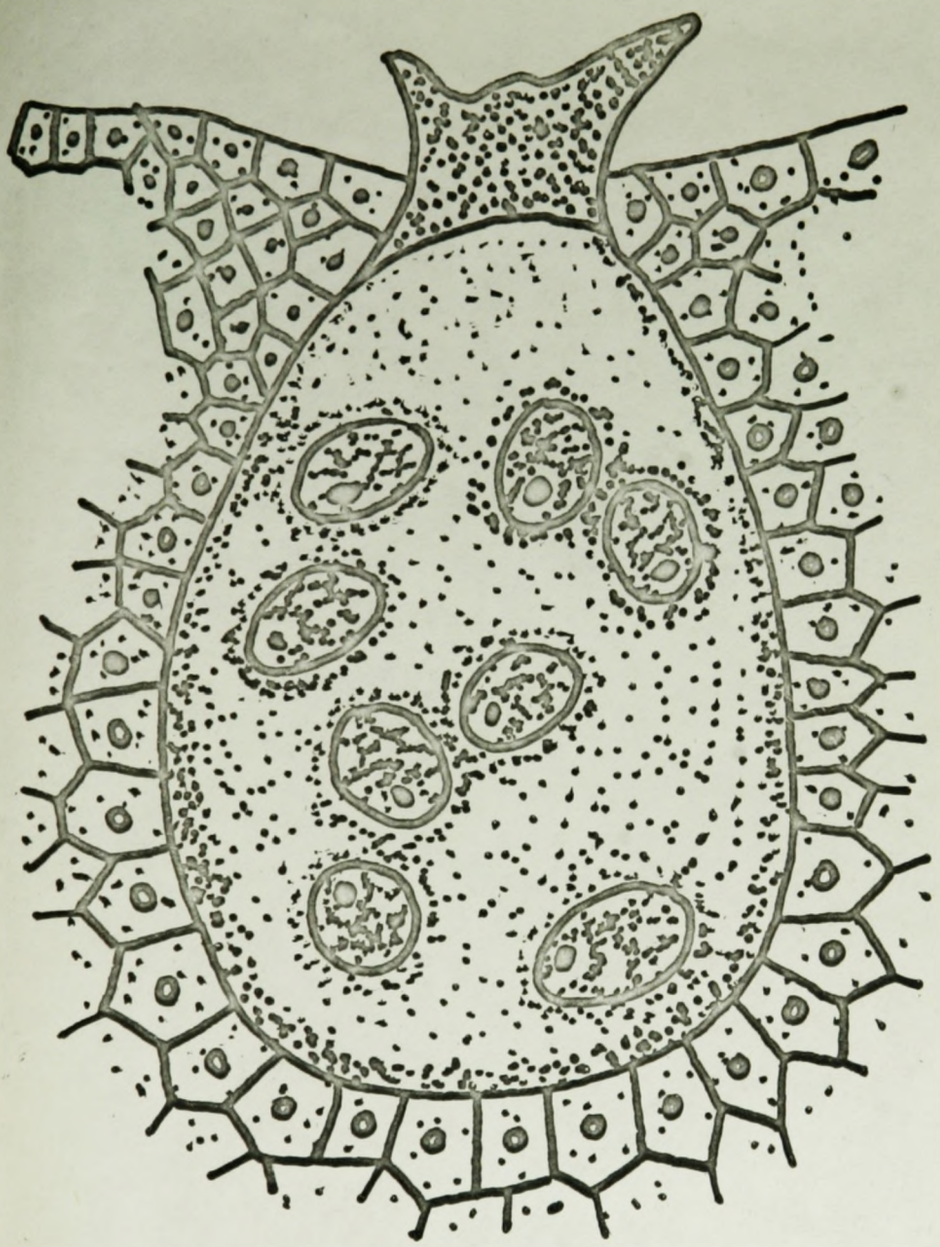
Fig. 8



Camera lucida drawing of the l.s. of an ovule in the month of September showing unfertilized egg. (Semi-diagrammatic)

X 3000

FIG. 9



Camera lucida drawing of the l.s. of an ovule in the month of October showing free nuclear division. (Semi-diagrammatic).

X 3000

PLATE I

Fig. 10. Camera lucida drawing of l.s. of a proembryo in the month of November. X 1000.

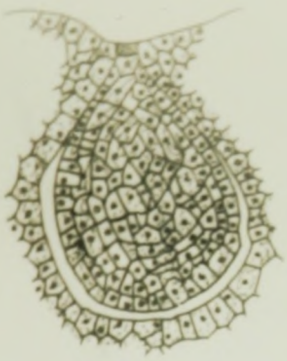
Fig. 11. L. S. showing unfertilized egg in the month of September. X 600.

Fig. 12. L. S. of the ovule in the month of September. X 600.

Fig. 13. L. S. of the ovule showing part of the proembryo in the month of October. X 520.

PLATE I

10



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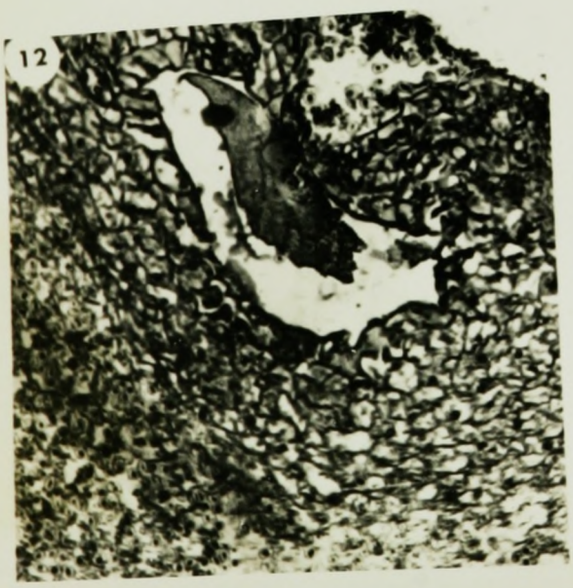

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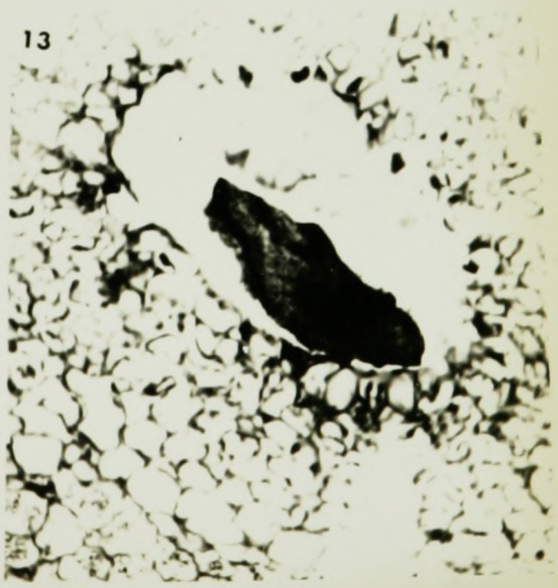


PLATE II

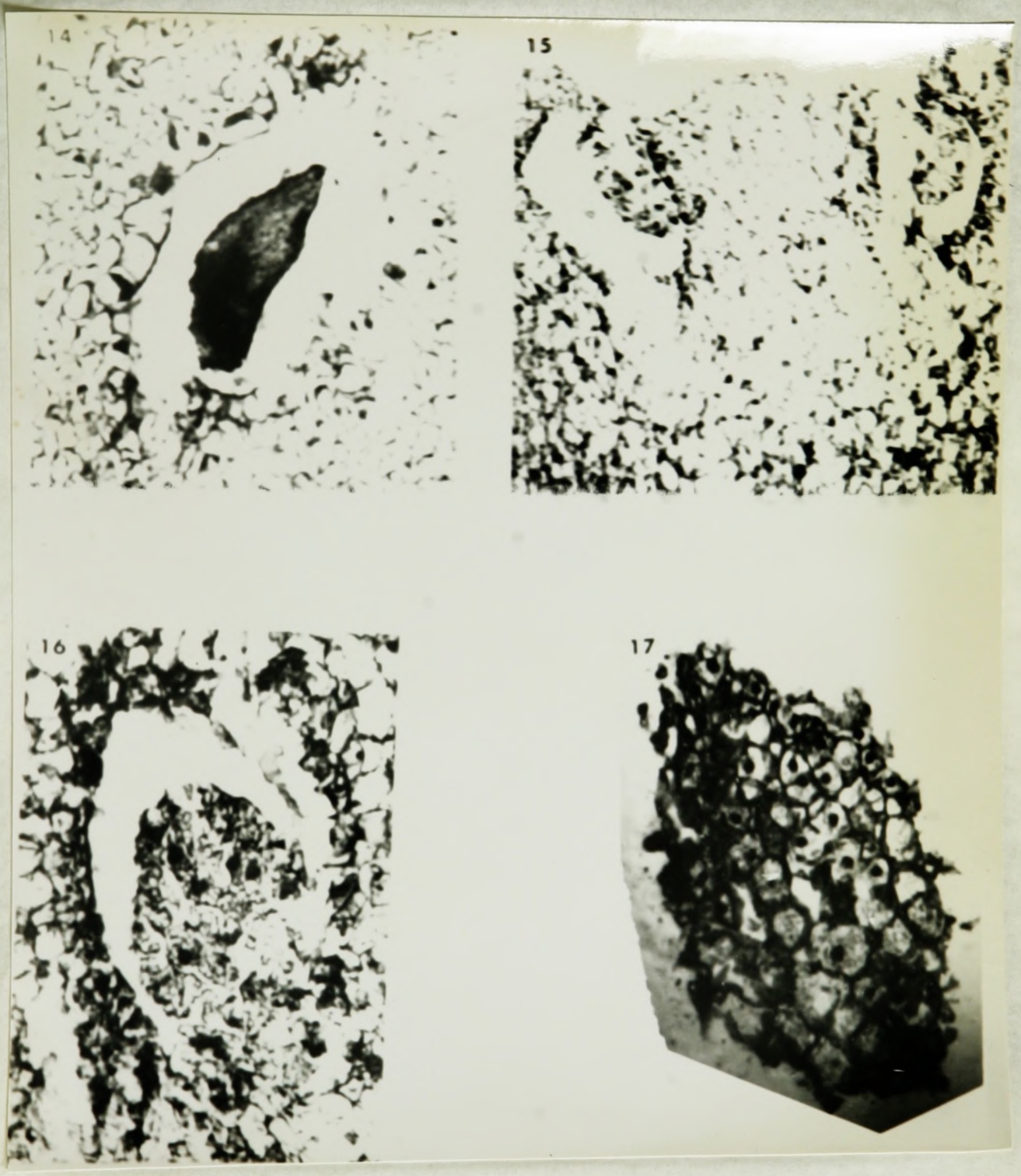
Fig. 14. Another section of the proembryo of Fig. 13. X 520.

Fig. 15. L. S. showing two embryos in the month of December. X 200.

Fig. 16. L. S. showing differentiated embryo in the month of January. X 600.

Fig. 17. L. S. showing embryonic cells stained differentially for indole derivatives in the month of January. X 800.

PLATE II



CHANGES IN THE AMOUNT OF GIBBERELLIN-LIKE AND KINETIN-LIKE SUBSTANCES IN THE DEVELOPING FRUITS OF GINKGO BILOBA

Introduction:

Growth promoting factors have been reported to occur in the developing seeds of many plants (16-25). Steward and Caplin (24) showed the presence of growth promoting factors analogous to coconut milk factors in the young gametophyte of Ginkgo biloba. They did not, however, isolate and identify the causal substances.

In the experiments described in the first chapter, embryos of Pinus resinosa were grown in the gametophytic extract of Ginkgo in vitro, with the result that budding embryos were formed from the suspensors. When these results are considered in the light of the vast literature reporting the occurrence of gibberellin-like and kinetin-like substances in many different plants (2-4, 6-8, 10-14, 27), it seemed highly probable that such substances might comprise the unknown growth factors in the gametophyte of Ginkgo. Furthermore, the studies of the changes in the amount of these substances in the developing fruits might throw light on the roles being played by these substances in the growth and development of the embryo gametophyte complex.

Materials and Methods:

Ginkgo fruits were collected locally from June 15, 1964 to October 15, 1964. The experiments were performed with fresh materials which were available up to October 15, 1964. It has been reported that gibberellin and kinetin in plant materials deteriorate even when freeze-dried and kept in cold storage for a long interval (10).

The gametophyte was seperated from the integument and nucellus by dissection. The integument and the nucellus portions were combined and freeze-dried. The gametophytes were freeze-dried seperately and percentage of dry weight was calculated. Extractions of gibberellin-like and kinetin-like substances were carried out seperately for these tissues.

Method of Extraction:

Gibberellin-like substances - Extraction was carried out according to the methods of Kends and Lang (4) with certain modifications. The freeze-dried materials were homogenized in a waring blender for 3 minutes with ice-cold methanol in a cold room (1°C) in the proportion of 20 ml. of methanol per g. of dry material. The homogenized material was extracted overnight by continuous stirring on a magneto stirrer at low speed. The extract was then filtered and the residue was extracted with the same volume of methanol for 6 hours. The combined methanol extracts

were made up to 80% with the addition of distilled water, and then shaken with petroleum ether, with not more than 250 ml. of the extract being added to the petroleum ether. The shaking was continued until the organic phase was colourless. The extract was then dried in a rotary vacuum evaporator until aqueous phase was left. This phase was then taken up in 1 M. sodium phosphate buffer (pH 8.4) in the proportion of 1 ml. buffer : 9 ml. of extract. The buffer phase was partitioned against ethyl acetate 3 times. The ethyl acetate fractions were combined, and kept for further purification and bioassay for kinetin-like substances.

The buffer phase described above was adjusted to a pH of 2.5 with 6 N. HCl. The acidic fraction was extracted by partitioning 3 times with ethyl acetate. The three ethyl acetate extracts were combined and dried over sodium sulphate and evaporated before a fan at room temperature.

Further purification of gibberellin-like substances in the extracts was done by column chromatography. A buffered celite 535 column of 20 cm. in height was prepared according to Kende and Lang (4). The dried acidic fraction was dissolved in .5 ml. of ethyl acetate saturated with the buffer (pH 6.5) and was pipetted on the top

of the column. The column was developed with dry ethyl acetate and 10 fractions of 50 ml. each were collected on an automatic fraction collector (LKB-Radirac) using 10 ml. syphon. The fractions were evaporated to dryness in front of a fan. Each fraction was eluted with water containing .05% Tween 20 and was used in the following bioassays, corn assays with d_1 and d_5 mutants and dwarf-pea (var. progress No 9) assay.

Kinetin-like substances - The ethyl acetate extract from the high pH buffer which contained kinetin-like substances was evaporated to dryness in a rotary vacuum evaporator. Purification was accomplished according to the method of Zwar and Skoog (27). The dried material was eluted with phosphate buffer at pH 7.0. Then 30% basic lead acetate was added (100 ml.) with continuous stirring and kept overnight in a refrigerator. The ppt. was filtered and washed twice with basic lead acetate. The washed ppt. was homogenized with 100 ml. of water and H_2S was bubbled for 90 minutes. The lead sulphide was filtered off via celite and the filtrate was kept. The ppt. and the celite were then washed with 100 ml. of H_2S saturated water. The previous filtrate and the later washings were combined and concentrated to 75 ml. under reduced pressure. Fifteen grams Norite charcoal, previously

washed with pyridine and heated to 100°C, was mixed with the concentrated filtrate, and filtered. The residue was washed in the following manner and 3 fractions were obtained: the charcoal was washed with 100 ml. of water, the washing was concentrated under vacuum and used for bioassay (fraction (I). Norite was then eluted twice with 50% ethanol of 50 ml. each. The elutes were concentrated and kept for bioassay (fraction II). Charcoal was again washed twice with pyridine 50 ml. each, the washings evaporated to dryness, eluted with 1 ml. of water and used for bioassay (fraction (III).

Bioassay for Gibberellin-like Substances:

Dwarf pea assay (Kende and Lang 4). Dwarf peas, var. Progress No 9 were soaked for 24 hours at room temperature and then planted in vermiculite and kept in darkness at 23°C. On the third day the seedlings were selected and transplanted to plastic boxes containing half-strength Hoagland solution. On the fourth day the plants were 17-19 mm. in height with uniform epicotyl hook. The plant extracts were dissolved in distilled water containing .05% Tween 20 and were applied to the hook in 10 λl. droplets. Four plants were used for any one assay. Test plants were kept under low intensity red light at 27°C until ninth day after sowing. Gibberellin activity was

expressed by measuring the length of pea stems between the proximal and distal nodes. For the standard curve .04 mg.-100 mg. of GA_3 /l was used in the same way and plotted as semi-log, i.e., log of concentration against actual length of the plants in mm.

Dwarf corn assay (Kende and Lang 4). Phinney's d1 and d5 corn seeds were soaked overnight and planted in vermiculite. These were kept in a growth chamber at an eight hour photo period at 27°C for five days. On the fifth day the plants were selected and transferred to plastic boxes containing half-strength Hoagland solution. The plant extract, dissolved in .05% Tween 20, was applied to the funnel produced by the first leaf in .1 ml. portion. One week after the treatment, the lengths of the first and second leaf sheaths were measured and gibberellin activity was expressed as the sum of these two lengths. A standard curve was prepared with known concentrations of GA_3 and plotted on semi-log paper. For testing each fraction, four plants were used.

Bioassay for Kinetin-like Substances:

Assay with senescing leaf tissue of mature leaves of Xanthium pensylvanicum (Osborne and McCalla 15). Cocklebur seeds were planted in the green-house in pots and grown under normal day light. Leaves were selected

when the plant had ten leaves in all. The fifth fully expanded leaf (counting back from the apex) was used for bioassay. The leaves were harvested and stored under low light with their petioles in water for 2 days. After this period 12 mm. discs were prepared from the interveinal lamina. Four discs were placed on a filter paper kept in a small petri dish and .5 ml. of extract dissolved in water was added. All the petri dishes for the bioassay were kept covered with aluminium foil and placed in a dark cabinet filled with damp filter paper. Control discs were prepared with distilled water only. After 48 hours, chlorophyll was extracted by boiling the discs in 80% ethanol, and the optical density of each solution was measured against 80% ethanol as a standard in a B and L spectrophotometer at 665 mμ and 645 mμ. For the estimation of the original value, chlorophyll was extracted from untreated discs at the onset of the experiment. A standard curve was prepared with known concentrations of kinetin and the optical density was plotted against kinetin concentration.

Assay with tobacco pith tissue-Wisconsin No 38, (Zwar & Skoog 27). The stock culture of the pith tissue was maintained in White's medium modified by Skoog, (Zwar and Skoog 27). Kinetin was omitted from all media in which extracts were being tested. Pieces from callus tissue

measuring 2X2X2 mm. were used in the assay. Three pieces were transferred aseptically to the surface of test medium contained in 125 ml. Erlenmeyer flask. The three fractions mentioned in the extraction procedure were seperately tested along with two types of controls. The basal controls contained White's medium without extract addition and the other contained .6 mg./l kinetin. The cultures were grown in diffused white light from overhead fluorescent lamps at 27° C and high relative humidity. After three weeks growth the cultures were examined and the activity in the fractions was expressed as percent of weight gained over the basal controls.

Results:

Gibberellin-like substances - The results on the concentration of GA like substances are summarized in Table I. Typical bioassays are shown in Fig. 1 and 2 for dwarf corn mutants - d_1 and d_5 and Fig. 3 for dwarf peas.

The active fractions obtained by partition chromatography on the celite column were in most cases limited to fractions 7-9. Deviations were noticed in two cases where activity was observed in the fractions 6 and 10 also. No gibberellin-like activity was found later than the 10th. fraction.

TABLE I

Summary of the results on the concentration of the GA-like substances in different parts of Ginkgo fruit collected at various stages of development.

Date of Collection	15 June	30 June	20 July	11 August	15 September	15 October
Percentage of dry wt.						
Integument-Nucellus	19.2	36.6	40.5	40.1	38.0	39.2
Gametophyte	2.5	7.8	11.0	51.2	60.0	65.0
Conc. of GA_3 Equiv. in µg/g of dry tissue						
Integument-Nucellus	.6	.005	.003	?	?	?
Gametophyte	3.5	.1	.02	.01	.01	.01
Fractions showing activity	7-9	7-10	7-9	6-9	7-9	7-9
Mitotic activity						
Integument-Nucellus	+++	+	+	-	-	-
Gametophyte	+++	++	-	-	-	-
Stage of Embryo development	Unfert-ilized egg	Unfert-ilized egg	Unfert-ilized egg	Unfert-ilized egg	Unfert-ilized egg	Proembryo formed

Active substances were observed in the integuments, nucellus and the gametophyte tissues. The highest concentration of GA_3 equivalent was noted in the gametophytes collected on 15th. June, 1964, (3.5 µg.). The gametophytes at this stage were transparent and very soft and had a very low percentage of dry weight (2.5%). The concentration of active substances was also highest in the integument nucellus tissue collected at this time, (.6 µg.).

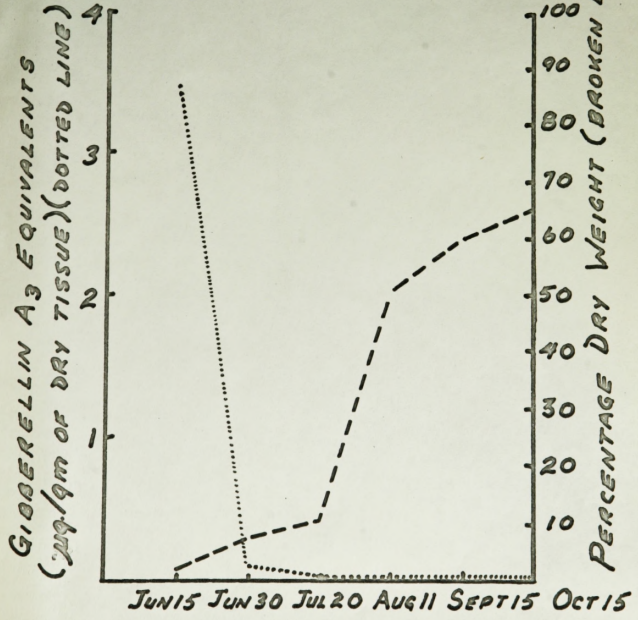
The collections of August to October had a high percentage of dry weight in the gametophyte and in the integument nucellus. The integuments were stony and the gametophytes were green in colour. The nucellus and the gametophyte had accumulated a gummy substance which burned the skin.

In the collections of August to October GA-like substances could not be identified in the nucellus-integument portion. During these months the concentration of the active substance was .01 µg./g/ of dry tissue in the gametophytes. The decreasing trend in the amount of these substances compares favourably with the increasing trend in the percentage of dry weight in the gametophyte, (Fig. 4).

During the period of 15th. June - 30th. June an increase of 5.3% in the percentage of dry weight was observed, which corresponded to a thirty-five-fold decrease

FIG. 5

CHANGES IN THE DRY WEIGHT AND IN THE AMOUNTS OF G. A-LIKE SUBSTANCES IN THE FRUITS OF GINKGO BILOBA



in the content of GA-like substances, (3.5 µg. - .1 µg.). During the next period, an increase of 3.2% in the percentage of dry weight had corresponding five-fold decrease in the content of active substances. During the next collection, a sharp increase of 40.2% in dry weight had a corresponding decrease of two-fold in the content of GA-like substances. The level of GA-like substances was found to remain constant in the gametophyte during the months of August to October although the gametophyte increased in dry weight.

Cytological evidence showed that fruits collected up to June 30th. had actively dividing cells in the gametophyte, nucellus and integument. Few dividing nucellus cells were observed after July 20th., but no dividing gametophytic cells were noticed at that time. The amount of active substance in all parts of the fruit was maximum in the collection of June 15th., when these tissues had highest rate of division.

Kinetin-like substances - Out of the three fractions obtained, the third fraction, which was obtained by elution with pyridine from 'Norite' charcoal showed an effect on the senescing leaves and tobacco pith tissue. A typical assay on tobacco pith tissue is shown in Fig. 4. The tests on kinetin-like compounds for the rest of the

experiments were done with fraction III mentioned above. The data on the senescing leaf tissue is summarized in Fig. 6. The amount of the kinetin-like substance present in the extract is expressed qualitatively by plotting the optical density against the time of extraction on a linear scale.

It was noted that there was a high concentration of the kinetin-like substance in the nucellus-integument and gametophyte in the materials collected on 15th. June, thereafter it dropped. During the later months toxic effect was noticed in the extracts. In these cases the optical density of extracted chlorophyll from the treated discs showed a decreased value than the water controls (Fig. 6).

The results on the assays of tobacco pith tissue are summarized in Table II. The activity in the extracts as measured by average fresh weight per piece of tobacco pith tissue was highest in the materials collected on June 15th. At this time the gametophyte extract had shown a four-fold higher activity than the nucellus-integument extract (897 mg. & 203 mg.). Activity in the extract was observed in the materials collected up to July 20th. Thereafter the extracts did not show any growth promoting activity on tobacco pith tissue.

FIG. 6

BIOASSAY FOR KINETIN-LIKE SUBSTANCES IN THE FRUITS OF *GINKGO BILOBA* USING SENESCING LEAF TISSUE OF *XANTHIUM PENNSYLVANICUM*

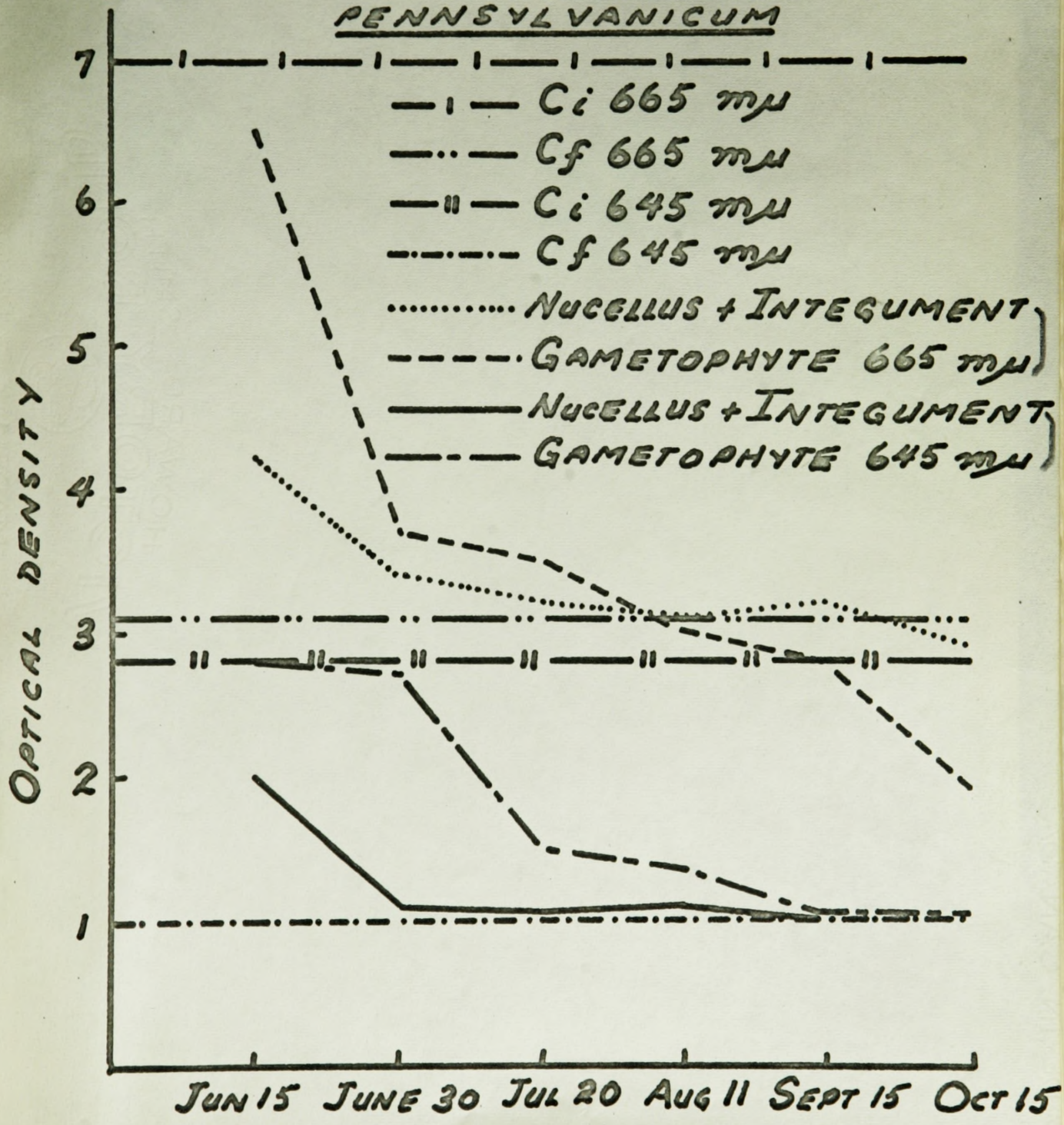


TABLE II

Promotion of growth in tobacco pith callus tissue by Fraction III isolated from Ginkgo fruits collected at various stages of development.

Date of Collection	Tissue	Fresh wt/pc in mg		Kinetin Equiv/g of dry tissue (in µg)	Fresh wt/pc with 30 µg Kinetin/culture
		Treated	Control		
June 15	Gametophyte	898	65	2	930
	Nucellus-integument	203	62	.5	1017
June 30	Gametophyte	301	53	.6	781
	Nucellus-integument	101	57	.2	765
July 20	Gametophyte	89	60	.2	819
	Nucellus-integument	107	63	.2	827
Aug. 11	Gametophyte	45	51	?	1031
	Nucellus-integument	62	59	?	1019
Sept. 15	Gametophyte	51	55	?	911
	Nucellus-integument	48	62	?	934
Oct. 15	Gametophyte	61	60	?	1011
	Nucellus-integument	52	67	?	1092

The highest concentration of kinetin equivalent was 2 µg./g. of dry gametophyte tissue in the materials collected on June 15th. The concentration in the nucellus-integument during this period was .5 µg./g. of dry tissue. In the month of June, gametophyte and nucellus-integuments had kinetin equivalent of .6 and .2 µg./g. of dry tissue respectively. In the materials collected on July 20th., both gametophyte and nucellus-integument had kinetin equivalent of .2 µg./g. of dry weight.

In the extract of nucellus-integument in the collections of September and October an inhibitory effect on tobacco pith tissue was observed. The fresh weight per piece of tissue in the control during September was 62 mg., whereas with the addition of extract fresh weight per piece of tissue was 48 mg. In the month of October the control piece of tissue weighed 67 mg. whereas the one grown in the extract, weighed 52 mg.

Discussion:

The reasons for calling the growth factors in the extracts in Ginkgo biloba as gibberellin-like and kinetin-like substances are based on the results of typical bio-assays prescribed for these two groups of substances, (Kende & Lang, 4, Osborne & McCalla, 15). Secondly, the methods

of extraction and identification are specific for these substances in pure form.

The concentrations of these two groups of growth factors were found to be highest at a time when the egg was not fertilized. It was also noted that the amount of these substances decreased in the tissue concerned when the cells had a low mitotic index. These substances could not be identified long before the egg was fertilized. This raises the question as to whether or not these substances play a genuine role in the development of the embryo of Ginkgo-biloba.

Corcoran and Phinney (2) and Ogawa (12) showed that the amount of gibberellin-like substances in the seed increased during seed development and decreased as the seed approached maturity. They also showed that larger amounts of GA-like substances were present in the endosperm than in any other tissues in the fruits. Matsubara and Ogawa (6) had shown that gibberellin-like substances were not so effective for embryo growth. They also showed that the active embryo factor from Lupinus diffusate was inactive for the bioassays of gibberellin.

Powell and Pratt (19) have shown that in Prunus persica, kinetin-like substances exist for at least three months after fertilization and larger amounts were present in the endosperm. All these findings suggest the possibility

that gibberellin-like and kinetin-like substances are necessary for the normal development of endosperm or gametophyte. According to Wardlaw, (26), "The conditions that are conducive to the accumulation of the substance seem to involve, or to be associated with, a delay in the growth of the embryo but a precocious formation of the endosperm - pp. 303." The present results show that in Ginkgo biloba the accumulation of these substances in the gametophyte and nucellus may be related to the development of these tissues and not the embryo proper.

Attempts have been made to provide quantitative analysis of the amounts of GA-like and kinetin-like substances in the present experiments. It is pertinent to note here that inspite of all the efforts of detoxifying the extracts, there is a possibility of toxic substances being present at least in the extracts of later months. The presence of inhibitors which mask the bioassay have been shown by Mowat, (10). It should also be borne in mind that the extraction, purification and bioassay techniques introduce so many variables that the results can be regarded as only semi quantitative.

The gibberellin-like substances seperated on column chromatogram appeared in most cases at R_f .7 - .9. Pure gibberellin A1 separated on the same column also appeared at R_f .7 - .9. Kende and Lang, (4) used the

same type of column for separation of their active fraction which appeared at R_f .5 - .7. They also separated pure gibberellin A_1 which appeared at R_f .8 - .9. The similarity between the R_f of the pure GA_1 and at the Ginkgo extract active in the bioassay suggests the possibility of this substance being similar to gibberellin A_1.

The active extract was further chromatogrammed on silica gel thin layer with iso-propyl ether/acetic acid (95:5) as the solvent (MacMillan & Suter, 5). The activity was located at R_f .1 which also corresponds with the R_f of GA_1. However, when the chromatogram was sprayed with 5% H_2SO_4 in ethanol and heated to 100°C for ten minutes, no characteristic fluorescent spot appeared under UV light.

Miller, (8) has shown that a kinetin-like compound from maize kernels is chemically different from kinetin although it promotes cell division in callus tissue. Zwar and Skoog, (27) showed that the extract from pea seedlings had high activity on tobacco pith tissue but was chemically different from kinetin. Therefore, it is possible that the active substance from Ginkgo biloba fruit is chemically different from kinetin.

Summary:

1. The growth factors isolated from the fruits of Ginkgo biloba were active in bioassays for gibberellin and kinetin.
2. These substances occurred in the nucellus, integument and gametophyte tissues.
3. The concentration of these two groups of growth factors were found to be highest at a time when the egg was not fertilized.
4. A higher concentration of these substances was observed in the gametophytes than in the nucellus and integuments.
5. The gametophytes had the maximum concentration of these growth factors at a time when the gametophytic cells were actively dividing.
6. The accumulation of gibberellin-like and kinetin-like substances in the gametophyte and nucellus-integument may be related to the development of these tissues and not the embryo proper.

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PLATE I

Fig. 1. Photograph of dwarf corn mutants showing a typical bioassay for Gibberellin-like substances. Fractions 7 to 9 showing activity on d_5 corns. X 1/16.

Fig. 2. Photograph showing bioassay on d_1 corns. X 1/16.

Fig. 3. Photograph showing a typical bioassay with dwarf peas. X 1/16.

Fig. 4. Photograph showing the bioassay of Kinetin-like substances on tobacco-pith callus tissue. X 1/16.

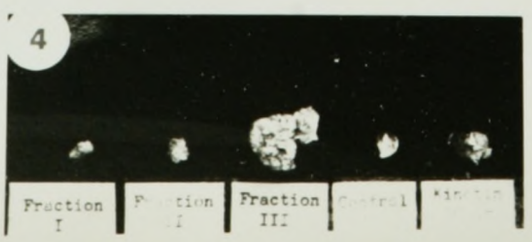
PLATE I


1 2 3 4 5 6 7 8 9 10 GA, 10 µg C

2

3


1 2 3 4 5 6 7 8 9 10 C GA3 10 µg


4
Fraction I
Fraction III

IV. Radiosensitivity of embryo-gametophyte complex of Pinus resinosa at different stages of embryonic development.

Introduction:

The potentiality of the cells of an embryo for growth and differentiation varies at different stages of development during which differentiation of specific tissue systems takes place and organs are formed. This has been shown by the *in vitro* experiments described in the first chapter.

The degree of differentiation achieved by the embryos cultured at different stages of development shows the possibility of existence of a critical differentiating phase when the embryonic cells demonstrate high potentiality for differentiation. On the basis of the Bergonie-Tribondeau Law (1906) it is expected that the cells of an embryo in such a stage would show higher radiosensitivity than in earlier or later stages. Thus a study of the effects of irradiation on the ability of a population of cells to differentiate may contribute to the understanding of the mechanisms of differentiation.

Evans and Sparrow (3) have shown the importance of nuclear volume and chromosome number as factors affecting radiosensitivity of plant growth. Mericle and Mericle (7) found that in

barley embryos an 8.4-fold change takes place in nuclear volume between the zygote and late proembryo stage. They have also shown that the younger proembryo stages having greater nuclear volumes show higher radiosensitivity. Works of these writers and of others (1,5-9,11-15) were performed to study the mechanism of radiation damage. But the present investigation attempts to elucidate the mechanism of differentiation with the help of radiobiological technique.

The workers mentioned in the preceding paragraphs have shown that the different regions of a developing embryo may vary in their radiosensitivity. The root apex is more radiosensitive than the shoot apex. Mericle and Mericle (7) have found no difference in nuclear volume between these apices of barley embryos which can explain the differential radiosensitivity. This indicates a possibility that extra-nuclear factors might affect radiosensitivity of specific cell population, e.g. root-apex. The root apex is more sensitive than the shoot apex to many exogenous stimuli such as auxins and certain other chemicals.

Materials and Methods:

The cones of Pinus resinosa were dissected and their ovules removed. The ovules were immersed in 1% sodium

hypochlorite solution for 10 minutes, rinsed with 70% ethyl alcohol and then with sterilized distilled water. The ovules were then transferred to sterilized filter paper plugs kept moist with phosphate buffer at pH 5.5 and contained in sterilized lucite vials 2½ inches in height and 3/4 inches in diameter. For preliminary investigations the ovules in lucite vials were transferred to the hot chamber of the McMaster University reactor (4) and irradiated with gamma rays from a Co^{60} source at pre-calibrated dose. For greater accuracy in dose rate and total dose the Gammacell 220 at the University of Guelph was used for all the experiments described in the present work, (Rice and Smythe 10). In all cases a dose rate of 16.2 rads/min. was used. The ovules were subjected to total doses of 250, 500, 1000, and 2000 rads. Immediately after irradiation the ovules were rinsed in sterilized distilled water and transferred to culture tubes containing solid White's medium supplemented with 15% coconut milk. These were kept in diffused light at 27°C for a period of eighteen days.

At the end of the experimental period the ovules were fixed and stained in the usual way as described in the first chapter. Cytological investigations of embryos which were at Stage III were made 48 hours after irradiation. Chromosomes were stained by the acetocarmine and Feulgen

methods. For each dose at any particular developmental stage, 25 ovules were irradiated. The developmental stages were from Stage I to Stage IV as described in the first chapter. Non-irradiated controls were analysed in the same way as the treated embryos.

The effects of radiation were assessed on the basis of two major criteria, viz., the percentage of abnormally differentiated embryos and of aborted embryos that resulted.

The number of abnormally differentiated embryos was designated on the basis of any of the following criteria:

i. Number of embryos per ovule.
ii. Frequency of embryos without suspensors.
iii. Rate of cell division in the gametophyte and embryonic cells.
iv. Cytological abnormalities.
v. Frequency of giant cells.
vi. Shape of the embryo.
vii. Abnormality in the shoot and root apices.
viii. Number of vessels formed.
ix. Abnormality in the leaf structure.

Dose response curves using two major criteria were plotted on semi-log paper. The fraction of affected embryos was always plotted on a log scale and the doses on a linear scale.

The types of abnormal differentiation investigated at different stages of development varied because, for example, leaf structure aberrations could not be studied in very young embryos. These different types will be described below in the results for each developmental stage.

The role of the nuclear factor in radiosensitivity has been studied by measuring the nuclear volume (in μ^3) of embryonic cells at different stages of development. The nuclei in early prophase of their mitotic cycle were measured in two diameters at right angles to each other at a magnification of 600X. The mean diameter obtained was used to calculate the average nuclear volume on the basis of the formula $V=4/3\pi r^3$.

Results:

Radiosensitivity at different stages of development.

Stage I: The control showing a zygote is represented in (Fig. 3). The irradiated zygotes showed a lower frequency of abnormal differentiation than embryos in the later stages. Abnormalities were noted in the formation of rounded embryos without suspensors (Fig. 4). Formation of vacuolated giant cells were also observed (Figs. 5 & 6). In three cases vessels were differentiated in the gametophyte (Fig. 7). There was no indication of cleavage polyembryony

which was a normal feature under *in vivo* conditions.

The dose response curve for abnormal differentiation after varying radiation was non linear (Fig. 1). As low a dose as 250 rads gave a significantly higher proportion of abnormal deviation than the controls (Table I).

The percentage of embryo abortion was highest at this stage (Table I). Thirteen percent of the irradiated zygotes showed no mitotic activity, and had become shrivelled (Fig. 8). The remaining embryos had more than 20 cells which were so brittle or delicate that they were distorted during preparation of the slides. The cells were vacuolated and the nuclear materials appeared diffused, (Fig. 9). The dose response curve appeared linear up to a total dose of 1000 rads (Fig. 2). At this stage of development the gametophyte cells showed unusual radiation resistance. Nuclei were intact and less damage was noticed in the cells even with the high dose of 2000 rads (Fig. 10).

Radiosensitivity assessed on the basis of total cellular effects, i.e., abnormal differentiation and embryo abortion was very high (Table I).

Stage II: The control at the time of irradiation is represented in Fig. 11. The embryos were mostly spheroidal with distorted cells (Fig. 12). In one case differentiation of root initials was observed (Fig. 13). In another case

Effects on the developing pine embryos following different doses of Co^{60} gamma radiation: relationship between total dose and stage of development of embryo irradiated.

Stage of development.	Characteristics.	Dose.	Percentage of Abnormal Differentiation.	Standard Error.	Percentage of Embryo Abortion.	Standard Error.
		0	0	± 0	0	± 0
Stage I	Zygote	250	15	± 3.58	10	± 3.0
		500	22	4.15	15	3.58
		1000	30	4.58	40	4.9
		2000	35	4.76	65	4.76
		0	2	± 1.0	0	± 0
Stage II	Polarity established	250	25	4.33	4	1.2
		500	25	4.33	5	2.18
		1000	30	4.58	15	3.58
		2000	35	4.76	30	4.58
		0	10	± 2.0	0	± 0
Stage III	Apices initiated	250	55	4.98	5	2.18
		500	61	4.88	11	3.13
		1000	67	4.70	15	3.58
		2000	78	4.15	20	4.00
		0	0	± 0	0	± 0
Stage IV	Cotyledons formed	250	20	4.00	-	-
		500	37	4.83	-	-
		1000	35	4.76	2	1.00
		2000	40	4.90	5	2.18

FIG. 1

FREQUENCY OF OCCURRENCE OF ABNORMALLY DIFFERENTIATED EMBRYOS OF *P. RESINOSA* FOLLOWING γ RADIATION AT SPECIFIC EMBRYONIC STAGES

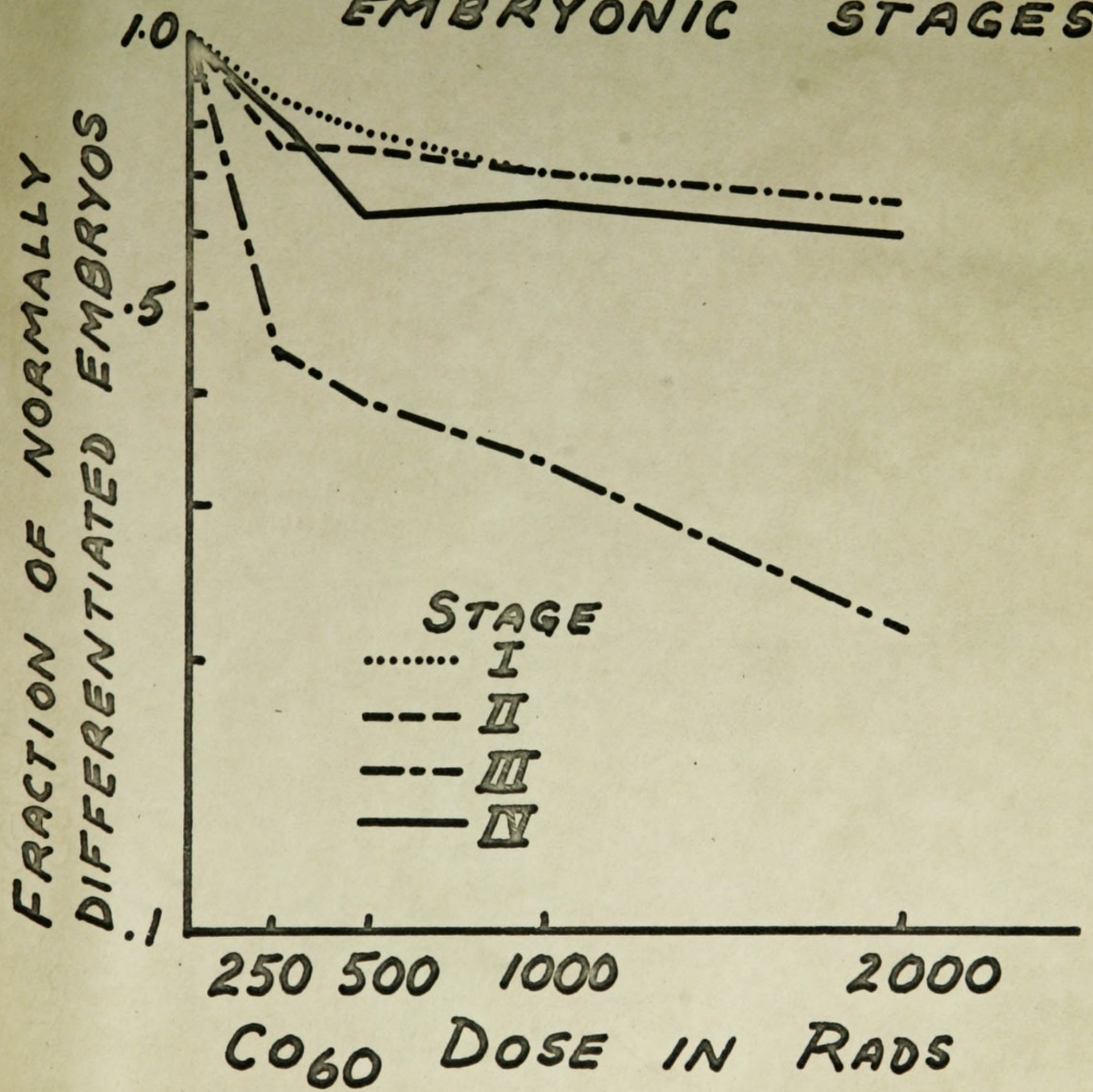
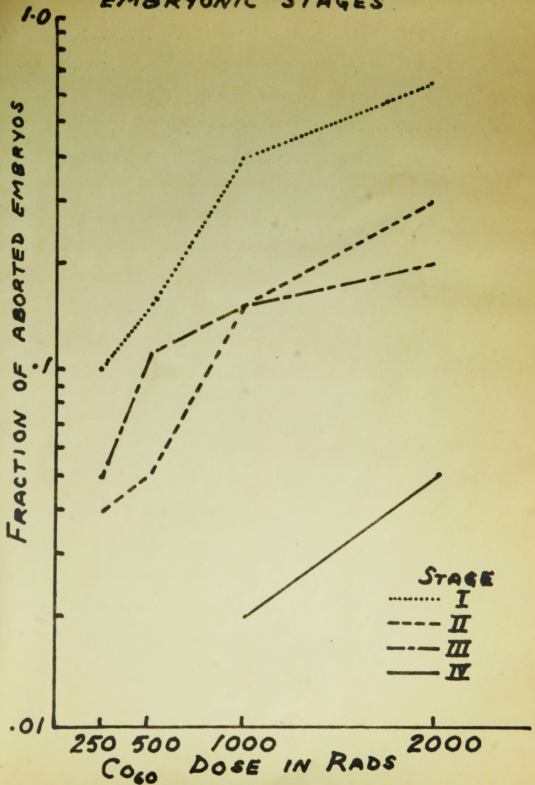


FIG. 2

FREQUENCY OF OCCURRENCE OF ABORTED EMBRYOS FOLLOWING γ RADIATION AT SPECIFIC EMBRYONIC STAGES



a spheroidal embryo-like structure was developed outside the archegonium. The mode of origin of this structure suggested apomictic development (Fig. 14). The percentage of aborted embryos was lower than the 1st. stage. No difference was noted between total doses of 250 and 500 rads (4-5%). However, with 1000 and 2000 rads significant increases in the percentage of aborted embryos were noted (15-30%).

The percentage of abnormal differentiation at different total doses were not significantly different (Table I)(Fig. 1). The dose response curve for embryo abortion was significantly different at total doses of 1000 and 2000 rads than at lower doses (Table I)(Fig. 2). Radiosensitivity evaluated on the basis of histologically detectable effects was lower than in the previous stage.

Stage III: The control at the time of irradiation is represented in (Fig. 15). The irradiated embryos had achieved oblong shape with different degrees of differentiation. In 50% of the cases cotyledon primordia were not formed (Fig. 16). Formation of cotyledon primordia was observed in 15% of the embryos. In 35% of the embryos rounded epicotyl was observed. In all the above cases most of the cells were vacuolated.

Greater damage was noted in the root apex than

in the shoot apex, (Fig17,18). The cells were smaller than the controls. The percentage of abnormal differentiation was the highest of all the stages investigated, (Table I).

The percentage of embryo abortion was lower than in the previous stages, (Table I)(Fig. 2).

Radiosensitivity assessed on the basis of total cellular effect was highest at this stage, (Table I).

Stage IV: The irradiated embryos germinated four days after irradiation. The embryos of the controls never germinated. The germinated seedlings showed stunted growth and were distorted in appearance with swollen roots, (Figs. 19, 20, 21 & 22).

The root apices were mostly damaged and abnormal, (Fig. 23). Giant cells were also observed at this region. At higher doses giant cells were also formed on the epidermis, (Fig. 24). The shoot apices were supressed, (Fig. 25). Localization of vessels were observed at the shoot region which were not continuous to other parts of the plant, (Fig. 26). The leaves (needles) on the seedlings were lacking normal vascular tissue, (Fig. 27).

The extent of damage at the root regions of the seedlings following different doses of irradiation is well represented in the following figures, viz., 500 rads, (Fig. 28), 1000 rads, (Fig. 29) and 2000 rads, (Fig. 30).

Occurrence of abnormal differentiation was lower than in the previous stage, (Table I)(Fig. 2).

The percentage of abortion at this stage was minimum. At 1000 and 2000 rads only 2% and 5% respectively of the embryos failed to germinate. Radiosensitivity measured on the basis of total cellular effects was lowest of all the stages, (Table I).

Cytological effects in the embryonic and gametophytic cells irradiated at Stage III and scored after 48 hrs.

The data are summarized in Table II. The mitotic index of the embryonic cells was more radiosensitive than that of the gametophyte cells. At 250 rads the embryonic cells showed 34% cell division. At 500 rads the embryonic cells had 30% and the gametophytic cells had twice the higher mitotic index. At 1000 rads they (gametophytic cells) showed slightly higher mitotic index and the difference was not significant.

The occurrence of polyploid cells was observed only in the case of gametophyte cells, (Fig. 31). Abnormal anaphase separation was also more prevalent in the gametophyte cells, (Figs. 32 & 33).

TABLE II

Cytological effects in the embryos and gametophyte cells at Stage III scored 48 hours after irradiation.

Dose	Type of cell	No. of cells scored.	No of cells dividing.	Fraction of cell division.	No. of polyploid cells.	No. of abnormal anaphase.	No. of dead cells.	Fraction of dead cells.	No. of cells with micronuclei.
250	Embryo	150	51	34 ± 3.9	-	-	7	4.66 ± 1.7	-
	Gametophyte	250	171	68.4 ± 2.9	-	-	3	1.2 ± .7	2
500	Embryo	200	80	30 ± 2.9	-	5	17	8.2 ± 1.9	-
	Gametophyte	250	103	41.2 ± 3.1	20	7	11	4.4 ± 1.2	17
1000	Embryo	200	21	10.5 ± 2.2	-	-	95	47.5 ± 3.5	11
	Gametophyte	250	37	14.8 ± 2.0	5	22	171	68.4 ± 2.9	-
2000	Embryo	200	4	2 ± 1.0	-	-	163	81.5 ± 2.8	-
	Gametophyte	250	10	4 ± 1.2	-	10	219	87.6 ± 2.0	-

Daughter cells showed unequal distribution of nuclear material and sometimes had a daughter cell without nucleus, (Fig. 34). In the irradiated gametophyte cells normal metaphase arrangement at equatorial plane was never observed. The chromosomes were well spread out at the periphery of the cell walls, (Fig. 35 & 36).

The formation of micronuclei was also observed more in the gametophyte cells. The incidence of dead cells in both the gametophyte and embryo was similar in proportion. The nuclei of cells irradiated at 500 rads showed 'fuzzy' appearance suggesting depolymerization of nuclear components, (Figs. 37 & 38). At 2000 rads, the cells appeared distorted and the cytoplasm became heavily stained with both acetocarmine and Feulgen methods, (Fig. 39).

Changes in nuclear volume:

The zygotes had nuclear volumes of 11700 $\mu^3 \pm$ 207. During the subsequent stage an eleven-fold decrease was noted, (Table III). The decrease in nuclear volume in later stages was not markedly significant. The high radiosensitivity at Stage III cannot be explained on the basis of nuclear volume.

However, the effects of irradiation on embryo abortion were related to the changes in nuclear volume. The nuclear volume was very high at the 1st. stage which

TABLE III

Mean nuclear volumes of prophase cells in embryos of *Pinus resinosa* at different stages of development

Stage of Development	Characteristics	Mean Nuclear Volume (in μ^3)	Standard Error
Stage I	Zygote	11700	± 207
Stage II	Polarity established	1048	± 38
Stage III	Apices initiated	920	± 27
Stage IV	Cotyledons formed	864	± 64
	Mean nuclear volumes in the apices		
Stage IV	Root apex	840	± 64
Stage IV	Shoot apex	864	± 24

corresponded to a high rate of embryo abortion (Table I). In the subsequent stages the nuclear volume decreased with concomitant decrease in the rate of embryonic abortion. At stage IV, the nuclear volume was lowest. Embryonic abortion at this stage was noticed only in the cases of 1000 and 2000 rads and the frequency was very low, e.g., 2% and 5% respectively.

Measurements of nuclear volume in root and shoot apices were taken in embryos at stage IV. In the root apex, average nuclear volume was $840 \pm 64 \mu^3$. The shoot apex showed a mean volume of $864 \pm 24 \mu^3$ (Table III).

Discussion:

It is generally accepted that radiation effects result from damage directly inflicted on nuclear material (Mortimer 9). Sparrow et al (12-14) have demonstrated that the sensitivity of various plants is proportional to nuclear volume. The data on nuclear volume in the present experiment give evidence contradictory to the above view. The highest radiosensitivity as measured by total cellular effects is observed in embryos at stage III. At this stage the average nuclear volume of the embryonic cells is twelve-fold decreased over that in stage I. However, on the basis of embryo abortion stage I is the most sensitive.

In the later stages of development, nuclear volume decreases with concommitant decrease in radio-

sensitivity for embryo abortion. These two different patterns of stage sensitivity, viz., for embryo abortion and abnormal differentiation, indicate that at least two independent developmental factors are involved. This would imply that these modifying factors are active at different stages of development - one at stage I, controlling the process of embryo abortion. The greatest difference in nuclear volume at this stage suggests the involvement of nuclear volume in this process.

However, on the basis of abnormal organogenesis in the embryo, stage III shows the highest radiosensitivity when the nuclear volume is twelve-fold decreased than in stage I. This would imply that the factor two affects through damage on cellular constituents other than the nucleus or involve nuclear factors only indirectly.

The occurrence of these two independent factors may also be explained on the basis of genetics. The first factor may be affecting the genes which are switched on at the early stage of development. The second factor may be due to effects on the genes active at stage III and are essential for determining the pattern of differentiation. The presence of such genes has been shown by Breuer and Pavan (2).

Sparrow et al (15) suggested that the basis for Bergonie-Tribondeau Law may be due to larger nuclear

volume in actively differentiating tissue. In the present studies no such relationship was observed. The occurrence of anomalous organs was highest at stage III when average nuclear volume was low. At stage I when nuclear volume was highest in occurrence abnormal differentiation was lowest.

The higher radiosensitivity of root apices than of the shoot apices is not due to a difference in the nuclear volume (present study as well as Mericle and Mericle 7). Both these regions show the same nuclear volume. The possible explanation for root sensitivity may be due to the difference in the time of development and the difference in number of cells involved. The root initials are differentiated first and from these initials both the apices are formed, the root apex being differentiated earlier than the shoot apex. The cells in the root are, therefore, comparatively older than those in the shoot. Secondly, the root areas are larger than the shoot areas. Therefore, after irradiation there have been more mitotic cycles through which root development has gone as compared to the situation in shoot development. Thus, a higher number of damaged daughter cells would be produced in the root region than in the shoot region per unit time of development following irradiation.

The incidence of high occurrence of abortion at

stage I may be explained in the following way: the one-celled zygote if subjected to lethal effects of radiation, formed daughter cells with the same genetic defects. In the embryos of later stages the individual tiers of cells have been organized. In these cases the role of the individual cell is reduced in terms of its contribution to the whole embryo. If radiation damage results in eliminating some of these cells, other cells of the embryo complex may function and abortion of the embryo is avoided. The potentiality of these cells has been shown in the 1st. chapter. Hence at these later stages of development the probability of abortion of the embryo becomes less.

Differentiated cells of various kinds cannot substitute for one another. Under these circumstances, if some of these cells are eliminated, the repair process by substitution becomes less probable in older embryos than in the very early embryos. Thus in an older embryo the unaffected cells may have to take over new functions and consequently become grossly abnormal.

Mericle and Mericle (7) suggested that the lesser occurrence of anomalous organs from irradiated barley embryos at an early stage of development is due to the imposition of "cellular homogeneity in so far as radiation induced genetic damage is concerned." At later

stages of differentiation the morphogenetic patterns of incompletely formed organs are upset and hence higher incidence of anomalous organs is observed. On the basis of this view, the stage of development showing highest incidence of abnormal differentiation may be considered as the stage in which the cells have highest potentiality for differentiation. In the present studies, embryos in stage III showed such an effect.

The enormous influence of the gametophyte on the development of the embryo is borne out by the present results. The break of dormancy in the embryos irradiated at stage IV and cultured *in vitro* is one example of such influence. Stone and Duffield (16) have shown that the gametophyte accumulates an inhibitory factor which is responsible for embryo dormancy. The germination of embryos in the present work suggests that irradiation may remove the inhibitory factor in the gametophyte. The same type of response has been observed by Melletti *et al* (6) in wheat endosperms.

It is pertinent to note here that formation of toxic metabolites due to irradiation has also been reported by Kuzin (5) and Swaminathan *et al* (17). These authors suggest that the disturbed metabolic processes in the irradiated organisms are the decisive factors for

radiation damage. On the basis of this hypothesis, all the stages of development should have shown equal degree of radiosensitivity. The present study and of others (Mericle and Mericle 7) contradict the above mentioned speculations.

Summary:

1. The changes in the nuclear volume in the embryonic cells at different stages of development are not associated with concomitant changes in radiosensitivity.

2. The higher radiosensitivity of root apices as contrasted with that for shoot apices is not due to the difference in the nuclear volume.

3. The germination of the irradiated dormant embryos suggests the removal of inhibitory factors in the gametophyte which, in the controls, keep the embryos dormant.

4. When radiosensitivity is measured only on the basis of embryo abortion, stage I shows highest radiosensitivity.

5. Sensitivity of embryos for the formation of anomalous organs is highest at stage III.

6. It is suggested that the embryonic cells at stage III have high potentiality for differentiation.

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PLATE I

Fig. 3. Control showing a zygote (Stage I) X 500.

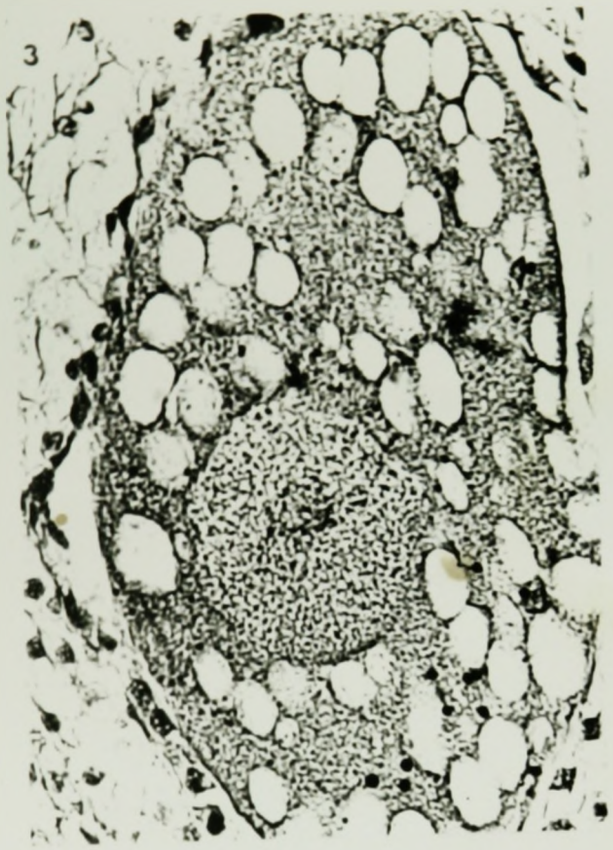
Fig. 4. Embryo at Stage I irradiated with 250 rads. L. S. showing a rounded embryo without suspensors. X 200.

Fig. 5. Embryo at Stage I irradiated with 500 rads. L. S. showing vacuolated cells with one giant cell. X 350.

Fig. 6. Embryo at Stage I irradiated with 1000 rads. L. S. showing an elongated embryo with broken cells. X 350.

Fig. 7. L. S. of the female gametophyte irradiated at Stage I with 500 rads showing vessel formation in one cell. X 150.

PLATE I



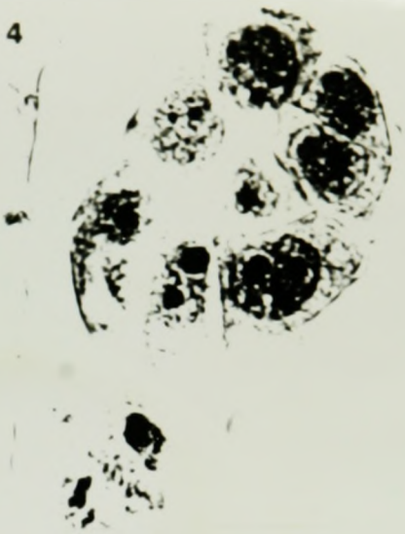


PLATE II

Fig. 8. L. S. of an irradiated zygote with 2000 rads showing shrivelling of the cytoplasm and shrinkage of the nucleus. X 500.

Fig. 9. L. S. of an embryo at Stage I irradiated with 500 rads showing distorted cell walls and diffused nuclear material. X 320.

Fig. 10. Squash preparation of gametophytic cells irradiated with 2000 rads showing intact nuclei. X 250.

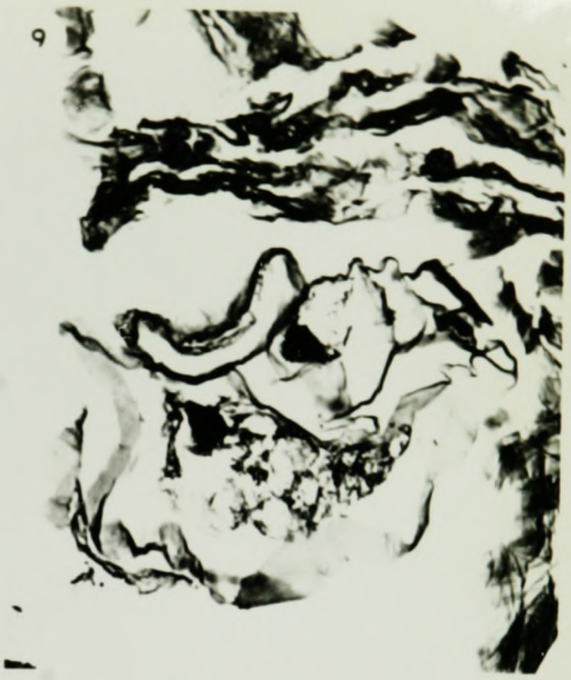
Fig. 11. L. S. of control embryo at Stage II showing suspensors. X 320.

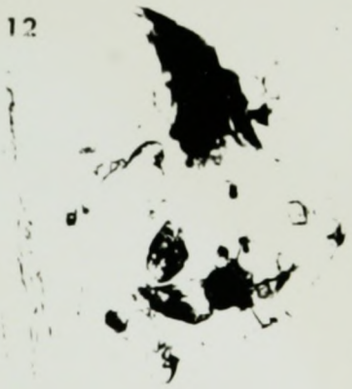
Fig. 12. L. S. of an embryo at Stage II irradiated with 2000 rads showing broken cells. X 320.

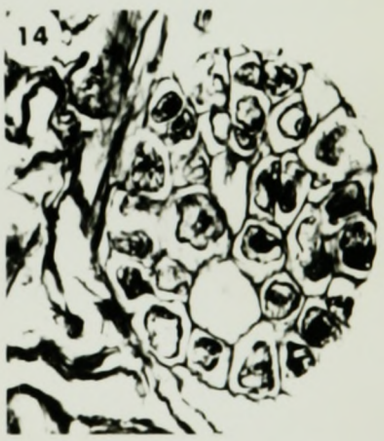
Fig. 13. L. S. of an embryo at Stage II irradiated with 250 rads showing differentiation of root initials. X 410.

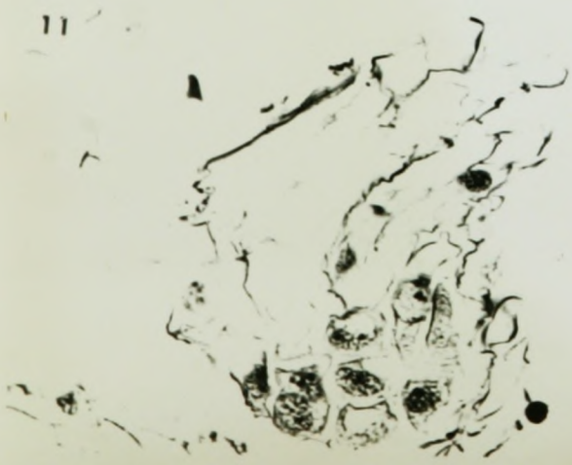
Fig. 14. L. S. of the ovule at Stage II of embryo irradiated with 250 rads showing embryo-like structure outside the archegonium. X 350.

PLATE II









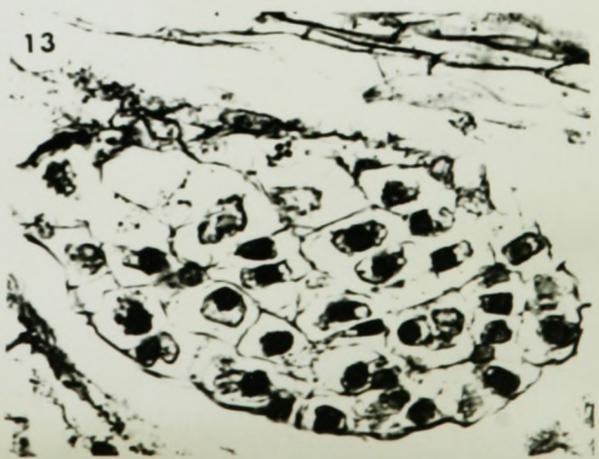


PLATE III

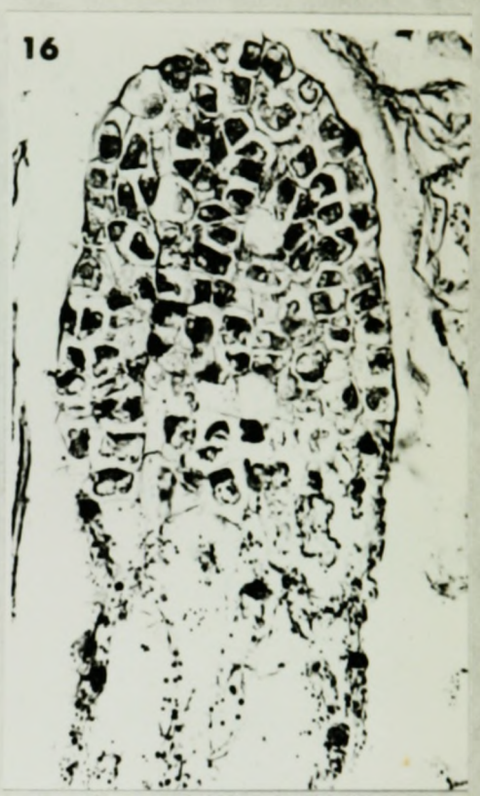
Fig. 15. L. S. of the control embryo at Stage III showing the root initials and suspensors. X 350.

Fig. 16. L. S. of an embryo at Stage III irradiated with 250 rads showing oblong shape and no cotyledon primordia. X 350.

Fig. 17. L. S. of the root apex of an embryo at Stage III irradiated with 500 rads showing no columnar structure. Cells are disorganised and vacuolated. Formation of smaller cells are frequent. X 450.

Fig. 18. L. S. of the shoot apex of the same embryo as in Fig. 17 showing cotyledonary primordia and intact cells. X 200.

PLATE III



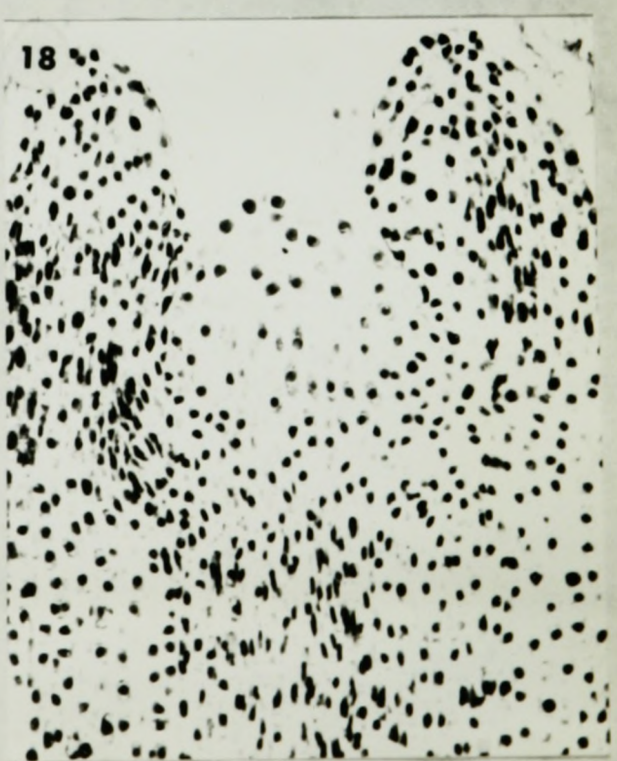


PLATE IV

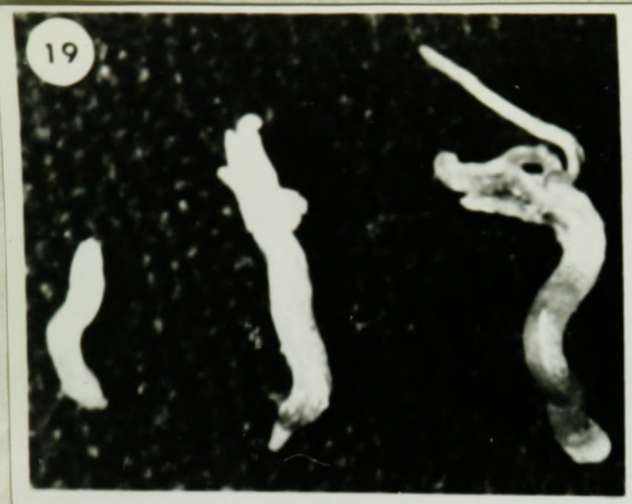
Fig. 19. Embryos at Stage IV irradiated with 250 rads showing germination and different degrees of growth rate. X 5.

Fig. 20. Embryos at Stage IV irradiated with 500 rads showing swelling of the root. The germinated embryo at the right does not show normal cotyledons. X 5.

Fig. 21. Embryos at Stage IV irradiated with 1000 rads showing different degrees of swelling of the root and abnormal cotyledons.

Fig. 22. Embryos at Stage IV irradiated with 2000 rads showing distorted seedlings. The seed at the left did not germinate. X 5.

PLATE IV



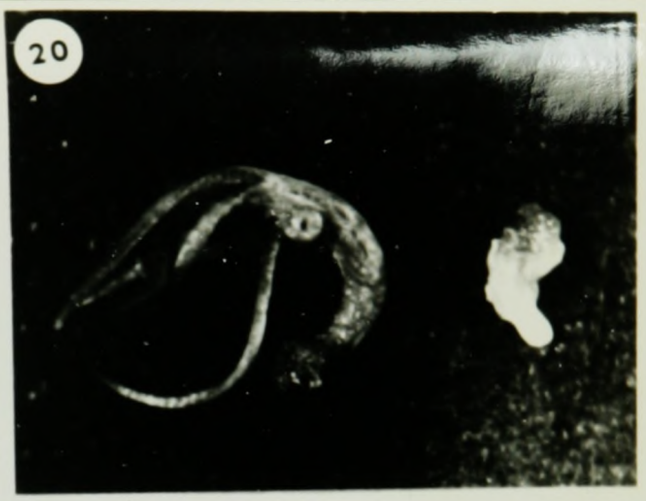


PLATE V

Fig. 23. L. S. of the root region of an embryo at Stage IV irradiated with 500 rads showing absence of columnar structure and distortion. X 120.

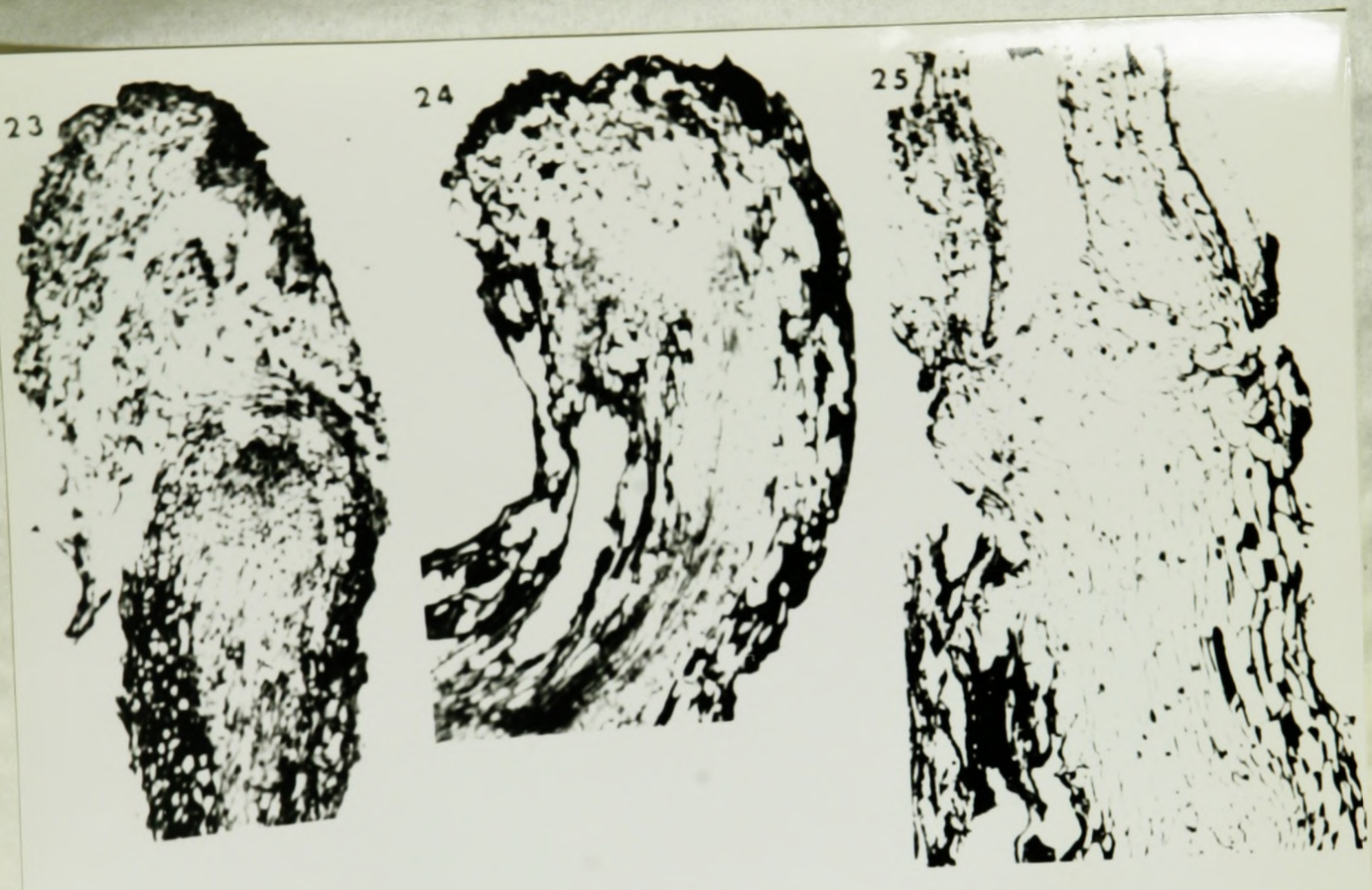
Fig. 24. L. S. of the hypocotyl region of an embryo in Stage IV irradiated with 2000 rads showing distortion and giant cell formation on the epidermis. X 120.

Fig. 25. L. S. of an embryo at Stage IV irradiated with 1000 rads showing suppression of shoot apex. X 120.

Fig. 26. L. S. of the hypocotyl of an embryo at Stage IV irradiated with 250 rads showing localization of vessels at the shoot region. X 350.

Fig. 27. L. S. of the cotyledon of an embryo at Stage IV irradiated with 500 rads showing lack of normal vascular tissue and vacuolated cells. X 300.

PLATE V



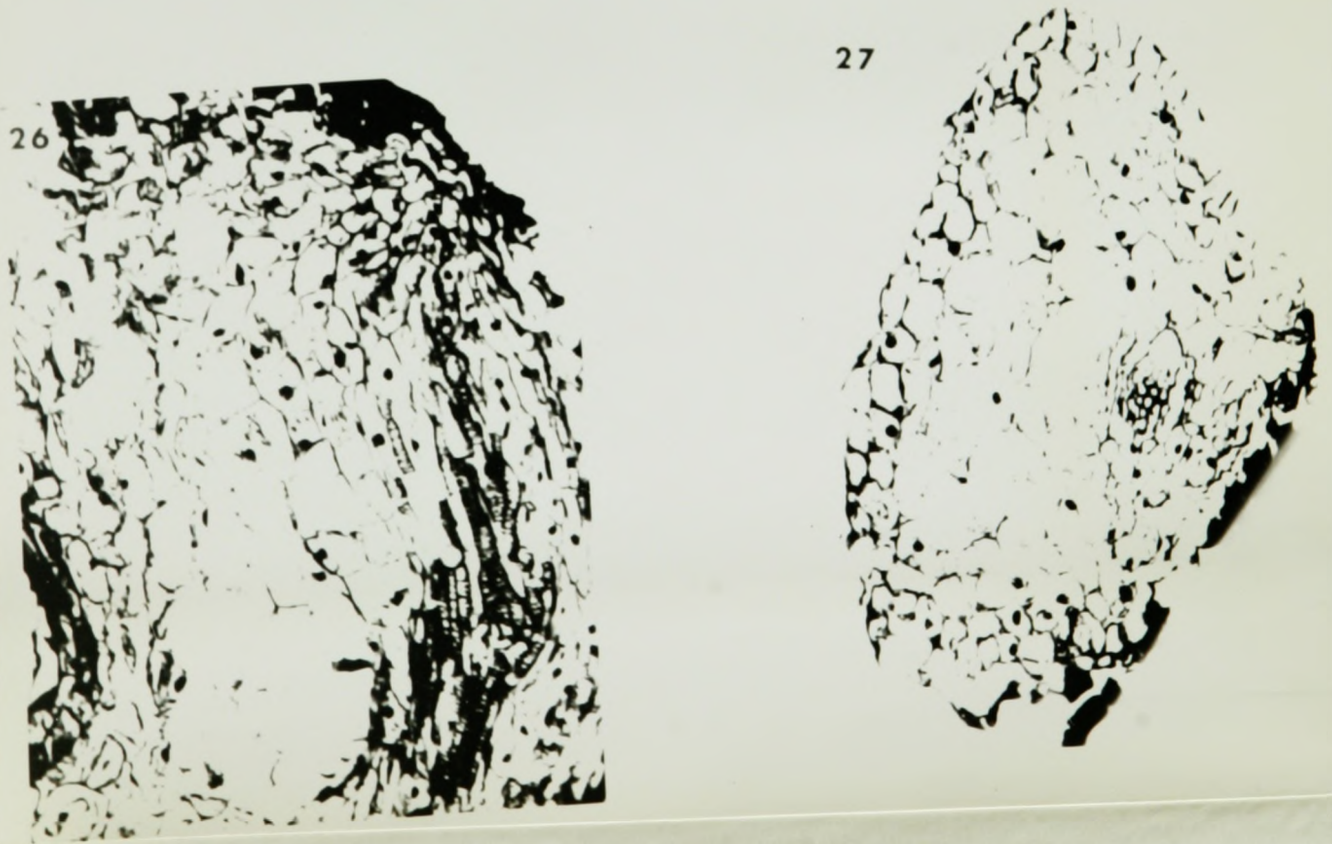


PLATE VI

Fig. 28. L. S. of the root region of an embryo at Stage IV irradiated with 500 rads showing giant cells at the epidermis. The root cap is disorganised. Vessels are localized in the middle portion of the section. X 300.

Fig. 29. L. S. of the root region of an embryo at Stage IV irradiated with 1000 rads showing a high frequency of giant cells and disorganisation. No vessel has been formed. X 300.

Fig. 30. L. S. of the root region of an embryo at Stage IV irradiated with 2000 rads showing a higher frequency of giant cells. Lesions at the bottom portion of the section is noticeable. No vessel has been formed. X 300.

PLATE VI

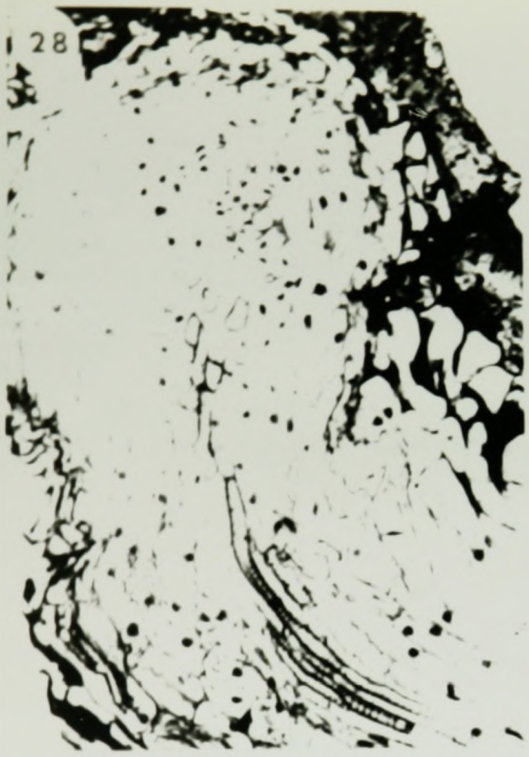


PLATE VII

Fig. 31. One gametophytic cell irradiated with 250 rads showing more than 12 chromosomes (n=12) at one end of the cell. At the other end two nuclei are seen without any cell wall in between - Feulgen stain. X 1000.

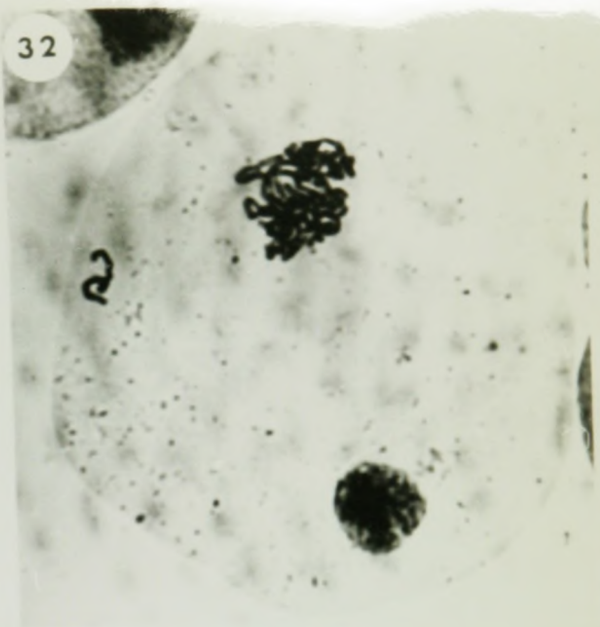
Fig. 32. Late telophase in a gametophytic cell irradiated with 500 rads. One lagging chromosome is seen at the left of the upper nucleus. X 1150.

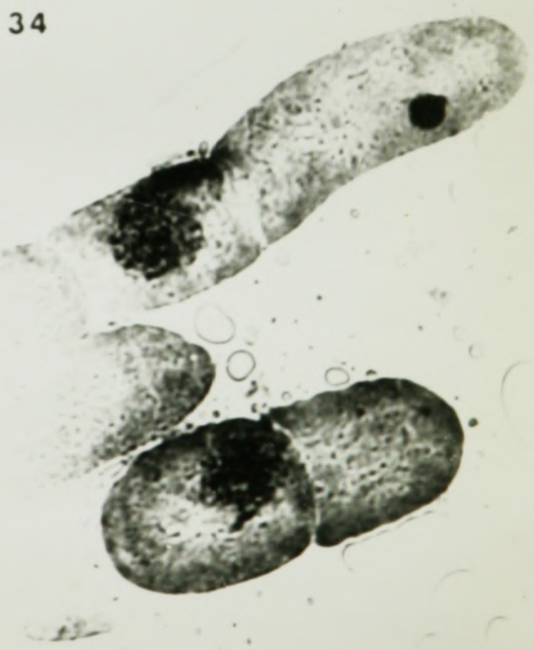
Fig. 33. Abnormal anaphase separation in a gametophytic cell irradiated with 1000 rads. X 1000.

Fig. 34. Two embryonic cells irradiated with 500 rads showing unequal distribution of nuclear materials. At the top, one of the daughter cells shows a micro-nucleus. At the bottom, one of the daughter cells shows no nucleus at all - Feulgen stain. X 500.

Fig. 35. One gametophytic cell irradiated with 250 rads showing 12 chromosomes (n=12) scattered at the periphery of the cell wall. Normal metaphase arrangement at equatorial plane is not observed. X 1150.

PLATE VII





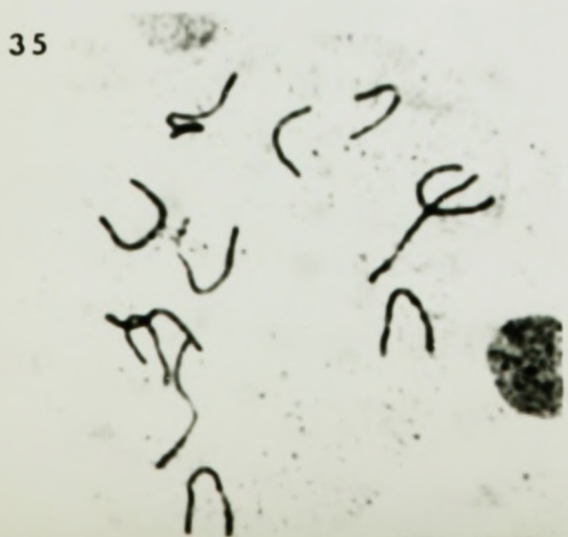


PLATE VIII

Fig. 36. Another gametophytic cell irradiated with 250 rads showing 12 chromosomes in 3 groups scattered in the cell. X 1000.

Fig. 37. Embryonic cells irradiated with 500 rads showing fuzzy appearance of nuclear material and dark staining of the cytoplasm with Feulgen stain. X 500.

Fig. 38. Embryonic cells irradiated with 500 rads showing fuzzy appearance of nuclear material and unequal distribution of cytoplasm. X 250.

Fig. 39. Gametophytic cells irradiated with 2000 rads showing distorted cells and stained cytoplasm with Feulgen stain. X 120.

PLATE VIII