DEVELOPMENTAL AND EXPERIMENTAL MORPHOLOGY OF THE OVULE AND SEED OF *ASIMINA TRILOBA* DUNAL.

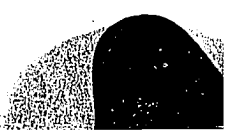
by

Robert K. Lampton

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the University of Michigan 1952

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DEVELOPMENTAL AND EXPERIMENTAL MORPHOLOGY OF THE OVULE AND SEED OF *ASIMINA TRILOBA* DUNAL.

by Robert K. Lampton

Following the success of Carl D. LaRue in culturing the endosperm of *Zea* an attempt was made to culture the endosperm tissues of dicotyledonous plants. Many seeds of dicotyledonous plants with cellular endosperms were put onto sterile nutrient agar medium and of these the endosperm of the seeds of the North American Pawpaw, *Asimina triloba* Dunal began to grow out into a mass of tissue. This tissue was subcultured and a clone of tissue built up from one seeds outgrowths. This clone of tissue was used for experiments on the growth of this tissue.

In order to definitely establish the identity of this tissue as endosperm and to compare the growth of this tissue with the normal ontogeny and growth of the endosperm tissue in the seed of this plant, a study was made of the normal growth pattern of the ovule and seed from the time the ovule is formed in the bud to the dropping of the fruits with the mature seed inside.

The ovule is anatropous, bitegumentary and produces a sub-hypodermal archesporial cell which does not divide to produce a parietal cell and a sporocyte cell. The archesporium functions directly as the megasporocyte and gives rise to a row of four megaspores. The chalazal megaspore is the one which gives rise to the embryo sac and this develops according to the normal or Polygonum type of development. It is a monosporic, eight nucleate embryo sac.

Following fertilization there is a great development of all tissues in the ovule. The primary endosperm nucleus divides almost immediately to form a row of ten to twelve cells extending from the nucellar cap to the hypostase of the ovule. Vertical cell walls are then produced in the endosperm tissue to form a long, narrow mass of tissue in the center line of the seed.

The integuments and nucellus of the seed are joined into a fusion zone in the raphe and antiraphe of the seeds and it is from this fusion zone that growth takes place as the seed grows in size. Due to cell division activity in this zone the inner integument produces an overgrowth and since this tissue is confined by the hard outer layers of the testa, the inner integument begins to wrinkle and push inward toward the center of the seed. This causes the well known ruminations of the seed which are found in all the seeds of the Annonaceae.

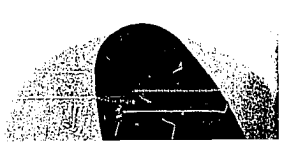
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As the ruminations advance toward the center of the seeds they crush the nucellus and the column of endosperm cells except in the region of the micropyle. When the ruminations have attained their full growth the endosperm at the micropylar end of the seed again grows up into a column of cells which rapidly grows out between the ruminations of the inner integument. The mature seed is filled with endosperm tissue which stores oil in droplets inside the cells.

Experiments performed on the endosperm tissue in vitro were designed to see what effect certain chemical substances which had been proven to be effective on the growth of other plant tissues in vitro, would have on the growth of this tissue. Other experiments were designed expressly in an effort to try to induce differentiation or regeneration in the cells of the tissue which are normally undifferentiated.

The tissue requires an acid medium of about pH 5.0 . It will not grow well on media containing unautoclaved malt or yeast extracts. Indole acetic acid inhibits the growth of the tissue but some concentrations of glutathione and casein hydrolysate give a slightly better rate of growth than the control medium. The tissue does well on media containing 2% sucrose or ½% glucose and it is capable of growing slowly on media containing starch as the carbohydrate source. Physical factors of light and cold seem to have no effect on the tissue. Chlorophyll was not produced in the tissue in the presence of light.

In one instance in which tissue was grown inside short lengths of glass tubing inserted in the bottles of nutrient agar medium there was a slight differentiation of a few of the cells in the middle of the mass.



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PREFACE AND ACKNOWLEDGMENTS

The North American Pawpaw, Asimina triloba Dunal, is a relict species of plant which belongs to the large family of Annonaceae. This family is primarily tropical in the distribution of its species. Asimina triloba is a temperate zone plant which retains many of the features of the family in its ontogeny and the normal development of its flowers and fruits is of great interest.

Having successfully grown the endosperm of this plant in vitro it was necessary that a study of the normal morphology and development of this tissue be made.

The author is greatly indebted to Professor Carl D. LaRue of the Department of Botany of the University of Michigan for his advice and leadership during the course of this investigation. To him and to all the members of his Doctoral Committee the author would like to express gratitude and appreciation for their assistance.

The wife of the author especially deserves thanks for the way in which she has encouraged him and looked after his physical needs during the course of the work. It is to her that this work is dedicated.

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INTRODUCTION AND HISTORICAL REVIEW

The mechanism of fertilization of the egg cell and the accompanying fertilization of the fusion nucleus of the embryo sac in the angiosperms is peculiar to this group of plants. The double fertilization effected by the two sperms of the pollen tube results in two distinct structures, the zygote or fertilized egg which gives rise to the embryo of the mature seed and the triple fusion nucleus which gives rise to the endosperm.

The union of male and female gametes to produce a fertilized egg cell is a general phenomenon found throughout the plant and animal kingdoms. However, the endosperm tissue which is formed after fertilization of the fusion nucleus is a unique feature found only in angiospermous plants. In the Polygonum type of embryo sac development, that is, a monosporic eight nucleate type of embryo sac, all three of the nuclei which fuse to give rise to the primary endosperm nucleus are haploid so that the resulting endosperm tissue formed from this cell is triploid.

In this type of embryo sac, the triploid endosperm nucleus is derived from two sets of maternal chromosomes (the micropylar polar nucleus and the antipodal polar nucleus) and one set of paternal chromosomes from the second sperm nucleus of the pollen tube.



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The fact that a male or sperm nucleus enters into the triple fusion has brought about much discussion as to the morphological significance of the endosperm tissue.

To date, the endosperm tissue has not been proven to function in any way except as that of a nutritive or "nurse" tissue which produces a favorable environment for the very critical early stages of the young developing sporophyte generation. Brink and Cooper (1947) state that : "The significance of the endosperm mainly lies in the fact that it plays a major role in the development and maintenance of a medium suitable for the growth of the young embryo. Indeed the endosperm appears to have no other function in many species."

Following the success of Professor Carl D. LaRue (1949) in culturing the endosperm of maize in vitro, it was decided to make an attempt to culture the endosperms of dicotyledonous plants. Consequently seeds of dicotyledonous plants which have cellular endosperms were collected, the endosperms separated from the seeds in so far as possible and placed on nutrient medium under sterile culture conditions.

Of these materials put into culture media in vitro, pieces of seeds of Asimina triloba, after several weeks, produced masses of tissue growing from the cut surfaces of the seed pieces.

From the first these tissues were considered to be endosperm tissue but since the cut pieces of seed contained other tissues than the endosperm definite proof was lacking

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that the endosperm produced the outgrowths.

Information concerning the normal developmental morphology of the ovule and the changes which it undergoes in becoming a seed were not available for this species. In order to definitely establish the identity of the tissue outgrowths and to eliminate the possibility of their being derived from seed coats or embryos, it was necessary to make a complete study of the origin of the endosperm tissue as it develops in the seeds of Asimina following fertilization of the ovule. This study has been followed through from fertilization of the primary fusion nucleus in the embryo sac to the utilization, by the germinating embryo, of the endosperm tissue in the seed.

The seeds of Asimina are of interest because they contain a peculiar wrinkling or rumination of the integument between which the endosperm grows to produce a ruminated endosperm as well. The endosperm of this plant in its capacity as a nutritive tissue, fills the large seeds and is available as a source of nutritive material when the small embryo begins its growth into a seedling plant.

When the food reserve of the endosperm has been exhausted by the seedling plant the empty shell of the seed is dropped, the plant rapidly develops green leaves and it is ready for an independent existence.

Asimina triloba, then, follows the general pattern of angiospermous plants in using the endosperm tissue simply as a nutritive source for the use of the young developing sporo-

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phyte in the seed.

Several workers in the field of plant tissue culture (Ball 1949, Curtis & Nichol 1948, Galston 1948, Levine 1950, Skoog & Tsui 1948) have reported differentiation of cells and the formation of organs from undifferentiated cultures of plant tissues caused by experimental treatments of such cultures. It was thought that the endosperm tissue of Asimina triloba would be a material with excellent properties suited to an investigation of problems of cell differentiation and organ formation. The endosperm tissue of this plant is normally undifferentiated and fairly large in extent but has the genetic potentialities of the plant present. Consequently experiments with this tissue were undertaken in an attempt to determine whether or not the endosperm tissue of Asimina is capable of producing differentiated cells and organs such as roots, stems and leaves.

Normal developmental patterns in Asimina triloba have been little investigated. Licopoli (1883) reported on the anatomy and physiology of the fruits of Annona reticulata and Asimina triloba. He found that the fruits of these two species of Annonaceae are essentially similar in their cellular components.

Herms (1907) described some stages in the embryology of Asimina triloba. He gives a sketchy description of the development of the megagametophyte from the primordium of the ovule to the first few cells of the endosperm. He also gives a summary of microspore and microsporangium formation

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relying mainly upon the figures accompanying the paper to provide the necessary description.

Locke (1936) made a detailed cytological study of the process of meiosis in the formation of the microspores in Asimina triloba and discovered that in the formation of the tetrad of microspores in this species, cytokinesis takes place through furrowing of the cytoplasm.

Bowden (1949) studied triploid mutants among diploid seedling populations of Asimina triloba and also reported the haploid chromosome number as nine.

Pollination in Asimina was described by Herman Mueller in a general treatise entitled "The Fertilization of Flowers" in 1883.

Other than Asimina several members of the family Annonaceae have been investigated to a greater or lesser degree.

Voigt (1888) published a study concerned with the development of seeds with ruminated endosperms, describing the seed development of Uvaria Lowii as a typical member of the Annonaceae. He considers the invaginations or ruminations of the seed to be produced by localized mounds of tissue arising from the fundamental tissue of the outer integument.

Lecomte (1896) described the development of the pollen grains in Xylopia aethiopica and Nicolosi-Roncati (1903) studied the development of the ovule and young seed in Annona Cherimolia.

Oes (1914) working with the ylangylang, Cananga odorata,

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studied the development of embryo sac and pollen grains. He found the archesporium is formed not hypodermally as reported by Herms for Asimina but under three or four layers of cells beneath the nucellar epidermis. He also found no parietal cell formed from a division of the primary archesporium.

Samuelsson (1914) published on some aspects of pollen grain development in Annona Cherimolia.

Schnarf (1931) compiled information from the published works of Herms, Lecomte, Nicolosi-Roncati, Oes, Samuelsson and Voigt and from unpublished work of Leopoldine Stark on Annona montana, Cananga odorata, Orophea hexandra and Unona dasymoschala into a summary of the characteristics of the family Annonaceae which was published in his "Vergleichende Embryologie der Angiospermen." In this work Schnarf draws some general conclusions regarding all the Annonaceae and takes exception to the work of Oes. Schnarf considers that the family is characterized by having a hypodermal archesporium which divides to form a parietal cell and a sporocyte cell.

Juliano (1935) working with Annona squamosa and Annona muricata found a condition similar to that reported by Oes and he took exception to the generalized statements of Schnarf regarding the Annonaceae.

In addition to his work on Asimina triloba, Bowden (1948) published chromosome numbers for 29 populations of 13 annonaceous species and one inter-specific hybrid and summarized the available cytological data for 17 species in 6 genera of this family.

Corner (1949) described the structure of 16 species in 10

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genera of Annonaceae, fruits of which were picked up on the forest floor while he was stationed at the Singapore Botanical Garden. He draws interesting morphological conclusions from his study of the development of these seeds.

The effects of pollination, development of the fruits, and hybridization between species of Annona were studied by Wester (1910, 1912, 1914, 1915).

Schroeder (1942, 1951) in California, interested in producing tropical and subtropical fruits in that region, studied hand pollination effects and the morphology of the fruit in Annona Cherimolia.

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METHODS AND MATERIALS

In the course of this investigation collections of buds, flowers and fruits of the Asimina triloba were made many times. Four natural stands of pawpaw trees provided the bulk of this material. Three of these stands were in the vicinity of Ann Arbor, Michigan and one of them is near Reading, Michigan. In addition to this material which was available and could be collected conveniently as needed, single collections were made by the author in John Bryan State Park, Greene Co., Ohio; by Dr. Rogers McVaugh near Coldwater, Michigan; in Shiremanstown, Pennsylvania by Mr. Theodore Hower; and by Mr. Walter Tulecke from still another natural stand of trees in the vicinity of Ann Arbor. The author is grateful for the assistance given by these individuals.

In addition to these collections use was made of one tree growing in the courtyard of the Natural Science Building on the campus of the University of Michigan in Ann Arbor. During the course of this work the tree did not set fruit naturally but it was used for hand-pollination studies. Excellent fruit set resulted from those blossoms which were hand-pollinated.

For studies on the development of ovules and seeds material was either collected in the field or collected from material which had been brought indoors into the laboratory and forced into bloom there.



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Material for sectioning was fixed in Randolph's fluid (Johansen 1940). This fixative is the familiar mixture known as CRAF since its ingredients are chromic acid, acetic acid, and formaldehyde. The material was fixed in this fluid from twelve to twenty four hours, dehydrated with tertiary butyl alcohol and imbedded in paraffin. Sections were cut on a Spencer rotary microtome, mounted on slides and stained with safranin together with fast green as a counter stain. In early work some slides were stained with haemotoxylin but it was found that the safranin-fast green combination gave excellent results and this stain combination was used exclusively during most of the investigation.

Materials for gross anatomical studies, such as young fruits, were preserved in formalin-acetic-alcohol.

Whole mounts of ovules in varying stages of development were mounted directly in lactic acid on microscope slides. After several days these were found to be sufficiently tranparent to allow direct observation and photography of such structures as pollen tubes entering the micropyle, the hypostase and nucellar cap, and the development of integuments and vascular strands.

From December until February stems and branches with flower buds were cut from dormant trees in the field, brought into the laboratory and placed in jars of hot water (about 60° C.). The hot water drove the air from the vascular bundles at the cut ends of the stems but cooled quickly enough so that no damage was done to the tissues of the stems. Set

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in a lighted window these stems produced well developed flowers from the buds as well as leaves. It was not possible to obtain fruits from these blossoms even though many of them were hand pollinated.

For studies in pollination, pollen was collected by hand from a natural stand of the trees in the field, brought back into the laboratory and dusted onto the stigmas of the experimental tree with a fine camels-hair brush.

In studies of experimental morphology and culture of the endosperm tissue, young developing fruits of Asimina triloba were collected in the field and brought into the laboratory. They were surface sterilized, cut open, and the seeds with endosperm tissue planted on nutrient agar medium in vitro.

Surface sterilization of the young fruits was accomplished by soaking them in a solution of sodium hypochlorite obtained as a commercial laundry bleaching preparation. Fruits were soaked in 20% clorox for a period of from fifteen to thirty minutes, rinsed in sterile distilled water and cut into slices about one half inch thick under sterile conditions.

The slices of young fruits contained pieces of the seeds which were approximately half of the seeds. Due to the varying arrangements of the seeds in the fruits it was not always possible to exactly halve the seeds but cuts were successful in making it possible to pick large pieces of seeds from the fruits and to place them onto the nutrient medium.

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The first summer that cultures were made of the endosperm tissues two different kinds of nutrient media were employed. LaRue's modification of White's basic nutrient medium in which one third to one half of the liquid consists of filtered tomato juice was used most extensively. This was used because of LaRue's success in culturing the endosperm of Zea mais on this medium (1949). The tobacco medium of Hildebrandt, Riker and Duggar (1946 b) was also used. H-R-D tobacco medium also is a modification of White's medium.

Subsequent results showed that the endosperm tissue of Asimina triloba grew better on the tobacco medium of Hildebrandt-Riker-Duggar than on the tomato juice medium of LaRue and therefore it was used exclusively for all further growth experiments with the tissue.

The second summer seed pieces were sown on three kinds of nutrient media. The Hildebrandt-Riker-Duggar medium was used as was a modification of this medium in which indole acetic acid was added in the concentration of one milligram of indole acetic acid per liter of nutrient. The third medium used was White's medium plus indole acetic acid added in the concentration of one milligram of indole acetic acid per liter of nutrient medium.

The number of seed pieces from which the endosperm tissue grew was much greater the second summer due primarily to the fact that more seed pieces were sown on nutrient medium. The number of seed pieces which produced outgrowths of

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endosperm tissue showed no significant difference for any of the three nutrient media used. Those sown on the plain H-R-D medium produced just as many endosperm tissue outgrowths as did those on the medium enriched with indole acetic acid. Later experiments to be described show that the presence of indole acetic acid in the nutrient medium upon which endosperm tissue is grown has a definite inhibitory action on the growth of the tissue. In the concentration in which indole acetic acid was present in the nutrient media on which the seed pieces were originally sown there seemed to be neither a stimulatory or an inhibiting action.

The developing fruits were collected and the seed pieces sown on nutrient medium at a time when the endosperm in the seeds was expanding rapidly to fill out the spaces between the ruminations of the seed coats. In most all cases it was the chalazal end of the seed from which the endosperm tissue grew. Growth was usually from the cut surface of the seed piece (Figure 41) but it was not unusual for the endosperm tissue to burst through the seed coats at a point where they had not been cut.

Eight of the micropylar seed pieces which were sown on nutrient medium in vitro grew small round elongated masses of tissue which were compact and grew very slowly. This was a different method of growth from that of the rapidly growing, loosely arranged mass of endosperm tissues. These slow-growing tissue masses were apparently the embryo. The

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mass was undifferentiated into radicle and cotyledons. One of these embryos is still in existence and going into its third year of growth in vitro. It has increased its size approximately three times and has a line down the middle of one end which roughly divides it into two halves but it seems to be very dilatory about forming into definite cotyledons and radicle.

Nutrient solutions for this work were made up as separate stock solutions of mineral salts and a solution of iron plus the organic constituents of thiamine and glycine. In practice, the proper amounts of the individual mineral salt solutions were pipetted into a graduated cylinder, distilled water added to bring the volume up to 990 milliliters and the resulting solution had the acidity adjusted to a pH value of 5.0. Preliminary experiments had shown this to be a good pH value for the growth of pawpaw endosperm tissue in vitro. Following the adjustment of the pH value of the solution, sucrose and agar were added in sufficient quantity to make the concentration of the mixture 2% sucrose and ½% agar.

This mixture was autoclaved at 240° C. (15 pounds of steam pressure) for fifteen minutes to dissolve the agar and to sterilize the medium. After being autoclaved and cooled somewhat, the proper amount (10 milliliters) of the iron-organic solution, which had been previously Seitz-filtered to sterilize it, was added under sterile conditions with a sterile pipette. The whole nutrient medium was then poured into previously sterilized screw capped bottles and the bottles



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with the nutrient medium were set aside to cool.

The bottles used were round capsule bottles of 25 milliliter capacity fitted with black molded screw caps. Approximately 15 milliliters of the nutrient agar medium was put into each bottle.

The nutrient medium used had this composition:

Na_2SO_4	- - -	800 mg. per liter
$CaNO_3 . 4H_2O$	- - -	400 " " "
$MgSO_4 . 7H_2O$	- - -	180 " " "
KNO_3	- - -	80 " " "
KCl	- - -	65 " " "
$NaH_2PO_4 . H_2O$	- - -	33 " " "
Ferric tartrate	- - -	40 " " "
$MnSO_4 . 4H_2O$	- - -	4.5 " " "
$ZnSO_4 . 7H_2O$	- - -	6 " " "
H_3BO_3	- - -	0.375" " "
KI	- - -	3 " " "
Glycine	- - -	3 " " "
Thiamine (HCl)	- - -	0.1 " " "
Sucrose	- - -	20 grams " "
Agar (Difco Bacto-agar)	- -	5 " " "
Distilled water	to make	1000 milliliters.

(Hildebrandt-Riker-Duggar, 1946 b)

Tissue masses were transferred from one bottle to another by means of a flat spatula made by cutting down the edges of a section lifter designed for holding paraffin microtome ribbons. The spatula was heated to a red



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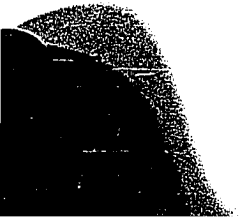
Figures of Asimina triloba.

Figure 1. A longitudinal section through a young ovary with two rows of ovular primordia. (Section slightly oblique) .

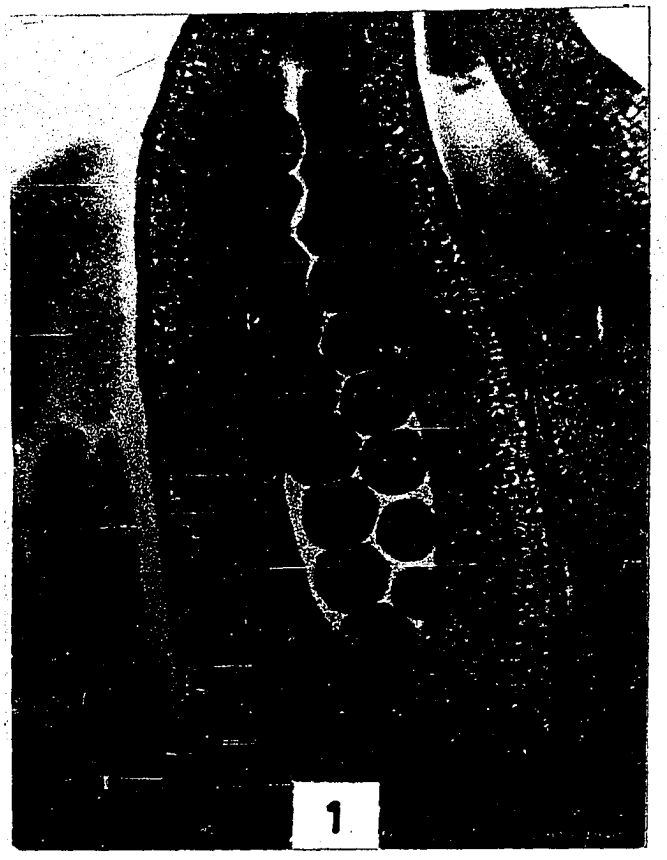
Figure 2. A longitudinal section through an ovary at a later stage than in Figure 1. The ovules show archesporial cells and the primordia of the inner integuments.

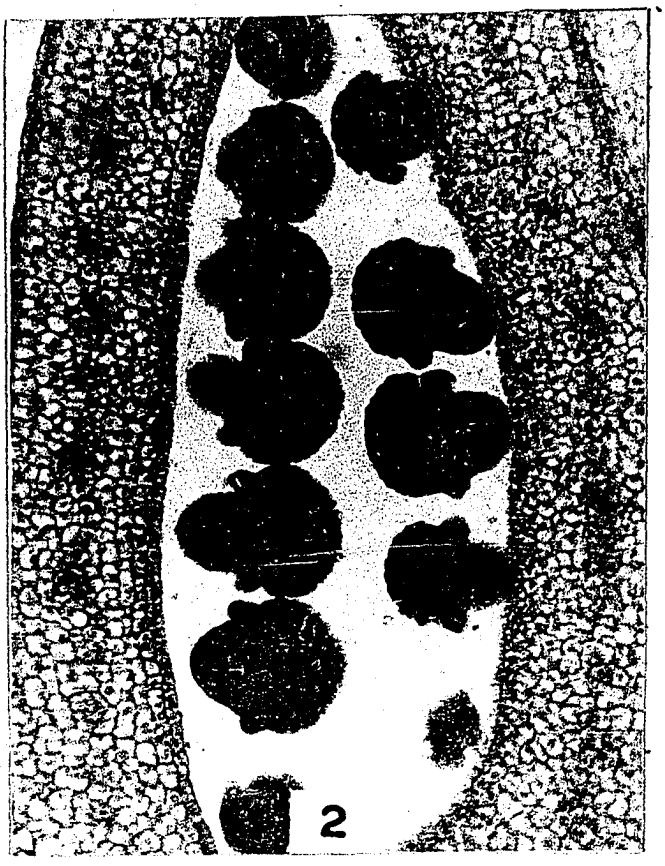
Figure 3. A cross section of an ovary which passes through the megasporocyte cell of one ovule and the funiculus of the other.

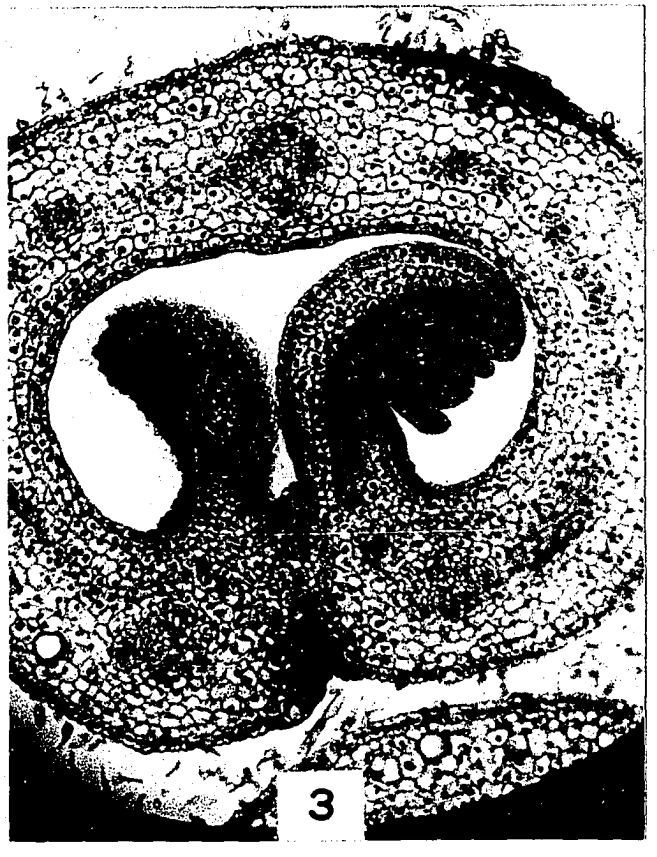
Figure 4. A median cross section through an ovule. In this ovule there may be seen the hypostase, nucellar cap, integuments, and the vascular strand of the ovule.

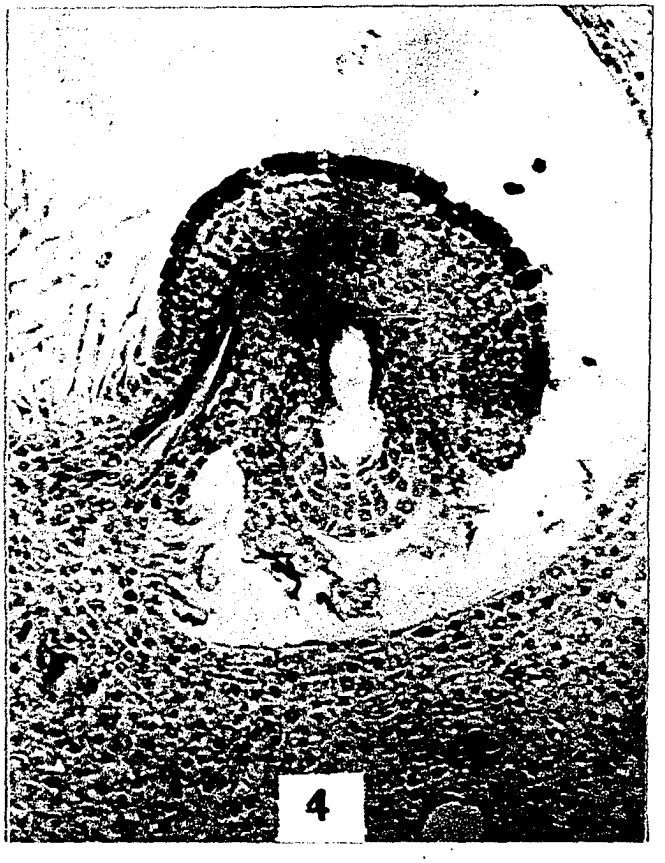


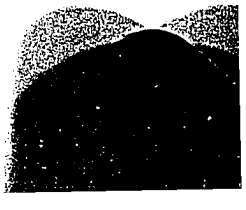
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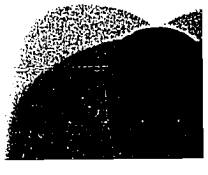






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heat in a burner flame each time it was used and then allowed to air cool before touching the tissue. Care was taken to hold the bottles containing nutrient medium and tissue pieces on their side while the caps were removed and transfers were being made. In this way contamination was kept to a minimum and it was not necessary to use a specially constructed transfer chamber for this purpose. After the technique was perfected it was very unusual to have contamination of cultures by molds or bacteria.



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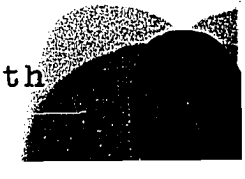
THE FAMILY ANNONACEAE

The family of plants which bears the name of Annonaceae is a very large one which is composed of about 850 species in 80 genera. This family of plants is primarily tropical and subtropical in its distribution but relict species of earlier distributions may be found in some temperate regions (Lawrence 1951). In the United States the family is represented by eight species of Asimina which range through the southeastern states and northward through the Mississippi Valley to western New York and Nebraska. One species of Annona is found in peninsular Florida.

Other species and genera of Annonaceae have been introduced and are grown in the warmer parts of this country for their fruits. Some of these are the soursop, Annona muricata L., sweetsop, Annona squamosa L., cherimoya, Annona Cherimolia Mill., custard apple, Annona reticulata L., ilama, Annona diversifolia Safford, and several species of Rollinia which have been introduced through the Miami, Florida Plant Introduction Station. The ylangylang, Cananga odorata King is grown for its perfume and volatile oil (Bailey 1935).

Of the eight species of Asimina which are native to the United States one, Asimina triloba Dunal, grows in southern Michigan where it is about at the northernmost limit of its range.

Members of this family are trees, shrubs or vines with



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aromatic wood and foliage. They are distinguished by the absence of stipules, the spirally arranged stamens with their distinctive, usually enlarged connectives, multi-pistillate gynoecium and the ruminate endosperm of the seeds (Lawrence 1951).

It is generally agreed that the Annonaceae are descended from magnoliaceous stock but they differ from the Magnoliaceae in having bisexual flowers and a gynoecium without an elongated axis. The ruminate endosperm of the Annonaceae is quite distinct from the watery, fleshy type of endosperm found in the Magnoliaceae.

The fruits of the Annonaceae are prized as food throughout the tropics but in the United States the North American Pawpaw, Asimina triloba, is not an important item of food. It is eaten and sold in some of the warmer parts of the country but in Michigan fruit set is rather erratic and the ripe fruits are more of a curiosity than a delicacy. The highly aromatic flavor of the ripe fruit is not appetizing to many people.

The large seeds of Asimina triloba are filled with a very extensive endosperm which is digested by the tiny embryo upon germination. This endosperm is filled with a large amount of oil. The high percentage of oil in the seeds would seem to be a potential source of income if it could be proven to be of any practical use.



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THE PLANT, FLOWER AND FRUIT OF *ASIMINA TRILOBA*

Asimina triloba Dunal is a shrub or small tree growing from 10 to 40 feet tall. The leaves are cuneate, obovate-oblong, acute, glabrous and 6 to 12 inches long.

Flower buds are produced on young stems of current years growth and pass the winter as small round buds covered with a dense pubescence of dark brown hairs. In April, when weather conditions have become suitable for growth, the flower buds begin to swell and rapidly develop into flowers. Flowers appear before the leaves and each flower is solitary at a node although the flowers may be arranged one above the other near the end of a branch.

Each blossom consists of three sepals which are persistent and covered with the dark brown hairs so characteristic of the bud scales, six petals arranged in two ranks of three, the outermost three being the larger, and many stamens and pistils. The petals are at first green and later turn to a reddish brown or maroon color with a yellow center. Near the base, the inner petals are wrinkled, thickened and glandular. These nectaries produce odors which attract insects to the blossoms.

The pedicel of the flower terminates in a short cylindrical receptacle upon which a cluster of 5 to 10 free carpels is borne terminally. Subtending the carpels are many stamens spirally arranged in a tight globose head with the pollen sacs tightly pressed against one another.



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Pollen grains mature soon after the opening of the flowers and the grains are shed. If pollination and fertilization are not accomplished the carpels may absciss before the whole flower drops from the branch. If pollination and fertilization are accomplished the pedicel and carpels are retained on the branch but the petals, sepals and anthers drop from the blossoms.

The carpels which are pollinated and fertilized remain attached to the branch and develop into ripe fruits. Out of five to ten carpels present in each blossom there are generally only one to four fruits in a cluster which reach maturity.

The ripe fruit is a large berry about 2½ to 5 inches long and 1½ to 2½ inches in diameter. The fruit is at first green and firm but as it ripens it becomes soft, pulpy and yellow. There are ten to twenty large flat seeds which measure from ½ to 1 inch long, ½ inch wide and ¼ inch thick, present in each fruit (Figure 25).

The endosperm is ruminate or partitioned into a number of compartments by an ingrowth of tissues from the integuments into the nucellus before the latter has been replaced by the endosperm tissue (Figure 29).

The normal growth and development of the endosperm tissue and the fact that it was possible to remove portions of endosperm tissue from developing seeds and grow it in vitro have made this tissue of the pawpaw of special interest.

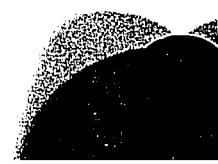
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THE GYNOECIUM

Development of the Carpel

The carpel of Asimina triloba begins as a thick U-shaped mass of tissue growing from the apex of the floral receptacle. There are from seven to ten of these in each flower. Growth continues in the carpellary primordium until the carpel resembles a thick walled, narrow tube open along one side and tapered toward the apex. The central portion of the tube is hollow and forms the loculus of the carpel. On the loculus side of the inrolled margins of the carpellary leaf there arise two rows of ovular primordia alternating with each other from side to side. As growth and development continue these ovule primordia grow longer and project toward the inside of the ovarian cavity which is itself increasing in size (Figures 1 and 2). By apical growth the tip of the carpel produces a very short style and an enlarged tip or stigma which is covered with sticky papillae of some length.

The ovary proper is simple and one celled with two parietal placentae side by side along the internal line of the ventral suture which marks the line of fusion of the inrolled margins of the carpellary leaf (Figure 3).



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Development of the Ovule

The ovules are borne in two rows, one row on each of the placentae, and the ovules arranged alternately with one another. The primordium of an ovule grows rapidly to produce a funiculus or stalk which projects into the cavity of the ovary with a slight curve toward the outside (Figure 3). It is on the side of the funiculus away from the curve, that is, on the side closest to the outside wall of the ovary that the nucellar protuberance develops.

The nucellar protuberance is a rounded mass of cells projecting from the side of the funiculus. The outer layer of cells of the protuberance are closely set together and are rectangular in outline so that a well differentiated epidermis is formed which is continuous with the epidermis covering the whole of the ovule. Immediately under the epidermis of the nucellar primordium the cells are oriented into a second layer or hypodermis. The rest of the mass of cells have no special arrangement but are packed together with somewhat angular outlines caused by mutual pressure. All the cells of the nucellus are in an embryonic condition and mitotic division figures are commonly observed in all layers of the tissues.



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Development of the Megaspores

When the nucellar protuberance has reached a height of four to six cell layers above the funiculus, one cell situated near the center of the mass and immediately below the hypodermis, grows in size and becomes larger than the surrounding cells. This cell is the primary archesporial cell (Figure 5). It increases in size and becomes angular in outline and elongated from base to apex of the protuberance (Figure 6). Due to cell division in the other cells of the nucellus the differentiated archesporial cell becomes surrounded by four layers of cells on all sides except for the massive funicular tissue at the base.

Herms (1907) states that the archesporial cell is hypodermal and divides once to produce a megasporocyte and a parietal cell. In the examination of several hundred sections of ovules I have found only one ovule in which this assertion of Herms seems to hold true (Figure 12).

At the time the archesporial cell is differentiating and growing to a larger size than its neighbors the entire nucellus tissue is growing in size through cell division. The figure which Herms gives to show the division of archesporial cell into megasporocyte and parietal cell is very unconvincing and might well be only one of the normal nucellar cells dividing.

The archesporial cell serves directly as the megasporocyte and divides twice to produce a row of four megaspores.

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Figures of Asimina triloba

Figure 5. A section through an ovule in which the archesporial cell is present.

Figure 6. A section through an ovule in which the archesporial cell has elongated to become the megasporocyte and the inner integument has begun its growth.

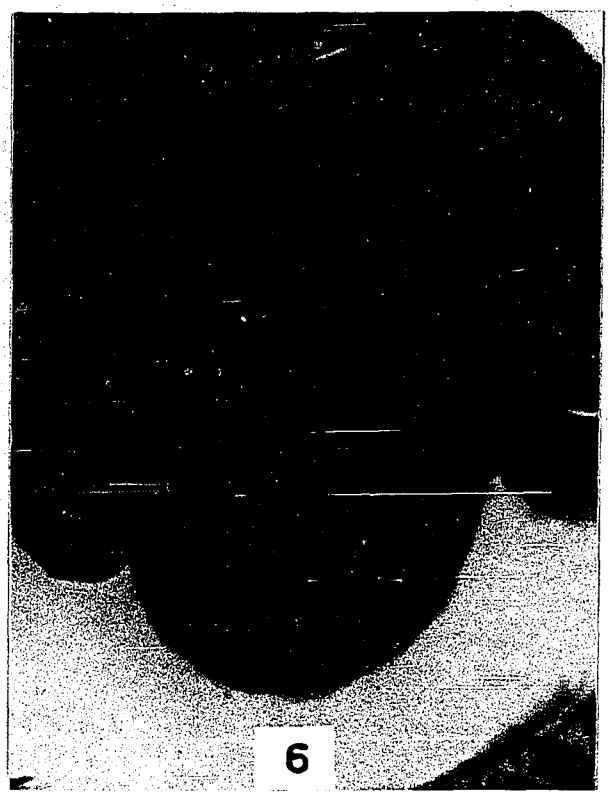
Figure 7. An anaphase figure of the first division of a megasporocyte cell.

Figure 8. Late telophase and formation of the cell plate in the first division of a megasporocyte cell.

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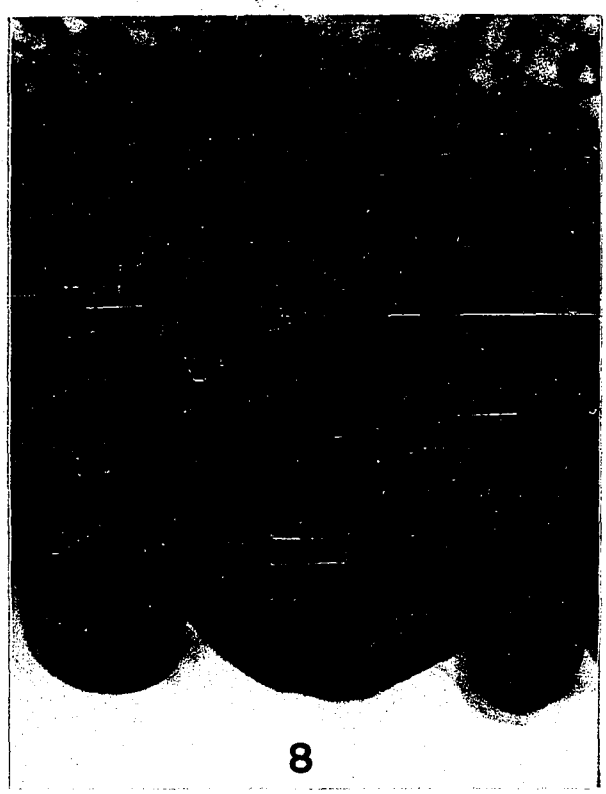
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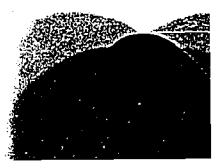
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The divisions of the megasporocyte are in a plane at right angles to the axis of the nucellus (Figures 7, 8, 11). It is assumed that one of these two divisions is a reduction division and in the material studied many of the megasporocyte cells appeared to be in the stage of synizesis prior to the first division. No attempt was made to prepare the material for cytological examination of the chromosome development.

The basal or chalazal megaspore is the functional one and as it proceeds further with its development into an embryo sac the other three megaspores disintegrate and gradually disappear.

Development of the Embryo Sac

The functional megaspore produces an embryo sac which has eight nuclei and seven cells. Its development is of the monosporic eight nucleate "Polygonum type" formed from three divisions of the functional megaspore. This type of development of the megaspore occurs in at least 70% of the angiosperms whose embryology is now known. It is the most common type of development and is sometimes spoken of as "normal type" of development. Maheshwari (1950) prefers to call this the "Polygonum type" since it was in Polygonum divaricatum that Strasburger (1879) first gave a clear and concise description of the monosporic eight nucleate type of embryo sac development.

In the production of the embryo sac of Asimina triloba



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from the functional megaspore the megaspore nucleus divides once and the daughter nuclei so produced at opposite ends of the cell, divide a second time to produce four nuclei each of which divides once more to produce a total of eight nuclei in the cell (Figure 9). Of these two groups of four nuclei, three of the four at the chalazal end of the cell, which may now be called an embryo sac or megagametophyte, invest themselves with small amounts of cytoplasm and a cell wall to become the antipodal cells (Figure 9). The antipodal cells are ephemeral and disintegrate rapidly. This is in accord with other members of the Annonaceae whose embryology has been studied. Three of the four nuclei at the micropylar end of the embryo sac also invest themselves with cytoplasm and cell walls. Two of these cells are the synergid cells which are wedge shaped and fit snugly into the micropylar end of the sac. The other cell is the egg cell which lies somewhat posterior to the synergid cells. The sister nucleus to the egg cell and the remaining nucleus from the antipodal group of four nuclei move toward the center of the sac to become the polar nuclei. These lie side by side for some time before eventually fusing to become the primary fusion nucleus which gives rise to the endosperm tissue after fertilization by the second sperm nucleus of the pollen tube (Figures 10, 14).

The complete embryo sac is vacuolate and contains particles of deeply staining reserve food materials which are of an oily nature and not starch (Figure 16).

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Figures of Asimina triloba

Figure 9. A section through an embryo sac which contains two of the three antipodal cells and four other cells which have not yet formed the egg apparatus.

Figure 10. A section through an embryo sac in which the two polar nuclei have not yet fused into the primary fusion cell. The egg apparatus is out of focus.

Figure 11. A section through an ovule in which the four megaspores are arranged in a row with the outermost three megaspores beginning to disintegrate.

Figure 12. A section through an ovule in which the archesporial cell is hypodermal in position. This section is cut at a very great angle to the main axis of the ovule.

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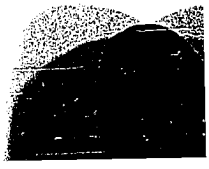
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Figures of Asimina triloba

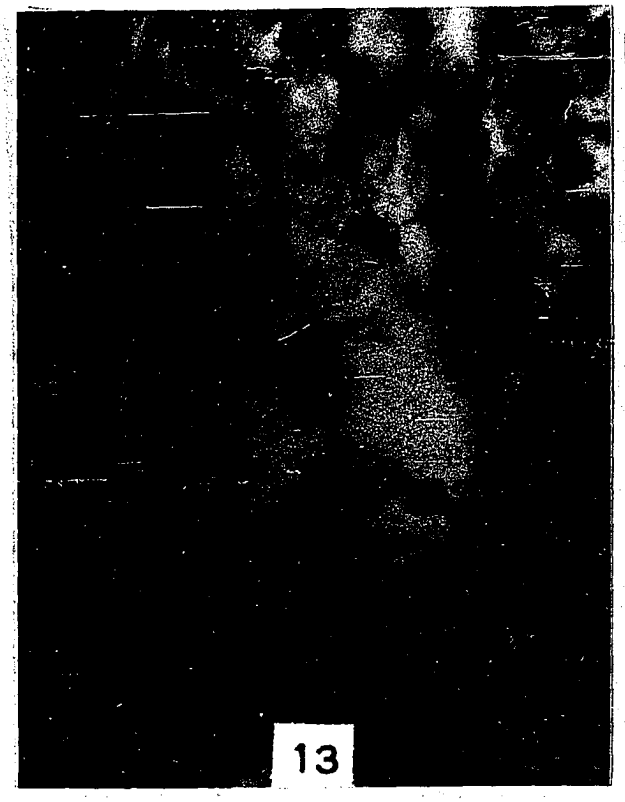
Figure 13. Micropylar end of an embryo sac with the camera focussed on the two sperms in the end of the pollen tube.

Figure 14. The same as Figure 13 but with the camera focussed on the Primary Fusion Cell and the egg cell.

Figure 15. The micropylar end of another embryo sac with the egg cell and a sperm nucleus lying side by side before fertilization.

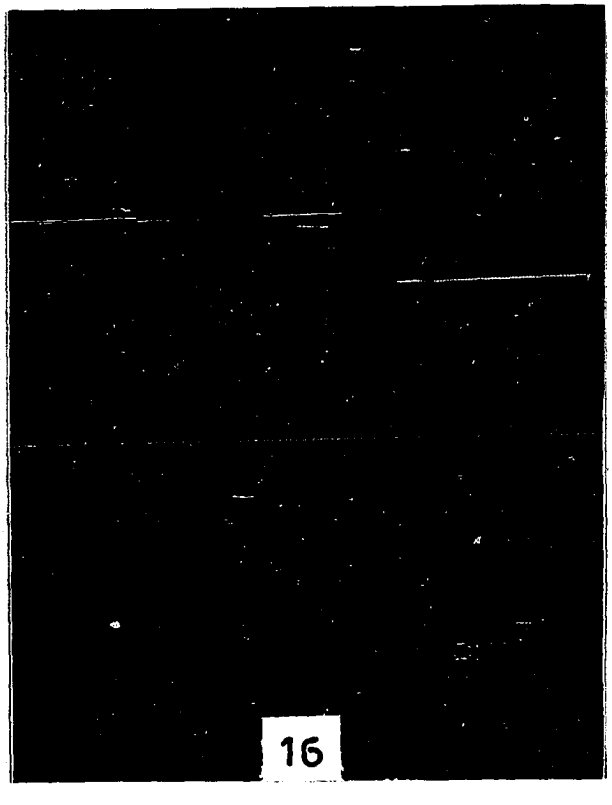
Figure 16. An embryo sac in which the reserve food materials may be seen.

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Development of the Integuments

The integuments make their appearance at the same time as the archesporial cell becomes differentiated. Around the base of the nucellar protuberance a ring of epidermal cells undergoes cell division and the cell walls are laid down in an anticlinal position. By continued cell divisions a continuous ring of tissue is raised around the nucellar protuberance. At this time the nucellar protuberance with the primordium of the integument is in a position with its axis at right angles to that of the funiculus (Figure 2).

The distal end of the funiculus at this stage is somewhat broader than it is thick with a slight concavity on the inside of the curve where the nucellus and inner integument are seated. Macroscopically, the whole of the funiculus with the nucellus and integument resembles a thumbless boxing glove with the nucellus and integument growing out from the region of the palm.

Cell division is taking place in all tissues of the ovule at this time but the region basal to the nucellus seems to be an especially active area in this respect. Because of this cell division the end of the funiculus beyond the nucellus and inner integument bends downward, increases in size but does not quite keep pace with the growth of the inner integument as it grows to cover the nucellus.

This growth of the distal end of the funiculus and corresponding downward growth of the edges of the funiculus produce



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the outer integument of the ovule (Figures 3, 4). The main axis of the funiculus becomes the raphe and antiraphe within which the vascular hoop of the seed arises (Corner 1941). The seed later becomes flattened perpendicular to this area.

By the time the integuments have completely covered the nucellus the embryo sac has its normal complement of cells, the anatropous condition of the mature ovule has been achieved and the micropyle is adjacent to the extremely short stalk of the ovule.

In the ovule which has a mature embryo sac present the outer integument consists of 8 to 10 layers of cells completely covering the ovule except for a wide opening at the micropylar end through which the inner integument projects (Figure 17).

The inner integument is only two layers of cells thick except where it projects through the wide exostome of the outer integument. At this point it flares out into a rounded mass of cells resembling a doughnut. This mass of cells is usually four cells in thickness. The flared out portion of the inner integument surrounds the micropyle proper and produces the endostome (Figure 17).

The integuments and nucellus are free from one another throughout their length except in the region of the chalaza.

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Figures of Asimina triloba

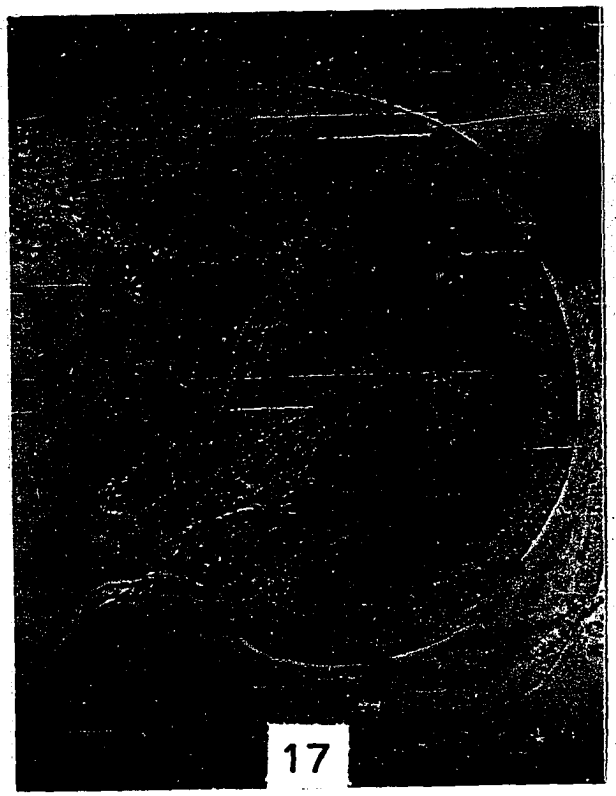
Figure 17. A young ovule cleared with lactic acid. The hypostase and nucellar cap are especially prominent. At the lower left the pollen tube may be seen entering into the micropyle.

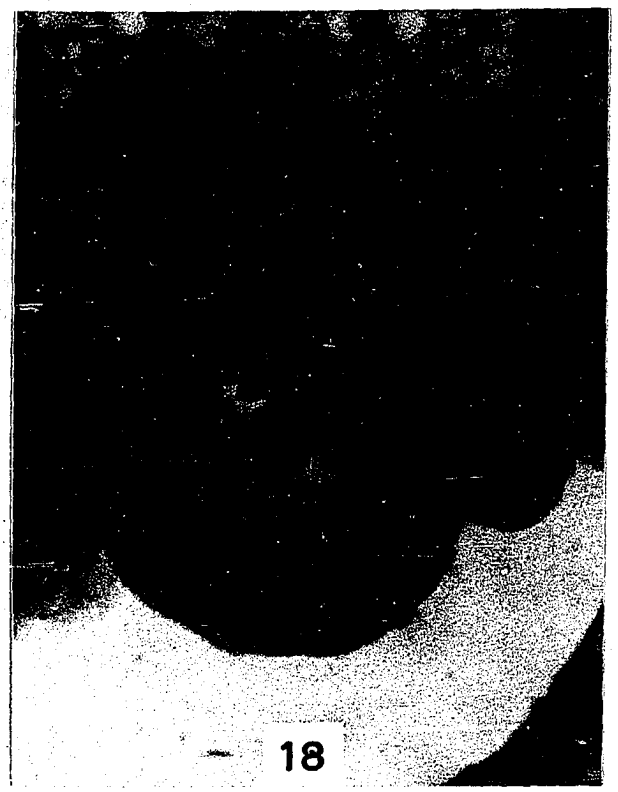
Figure 18. A section through an ovule containing two megasporocyte cells.

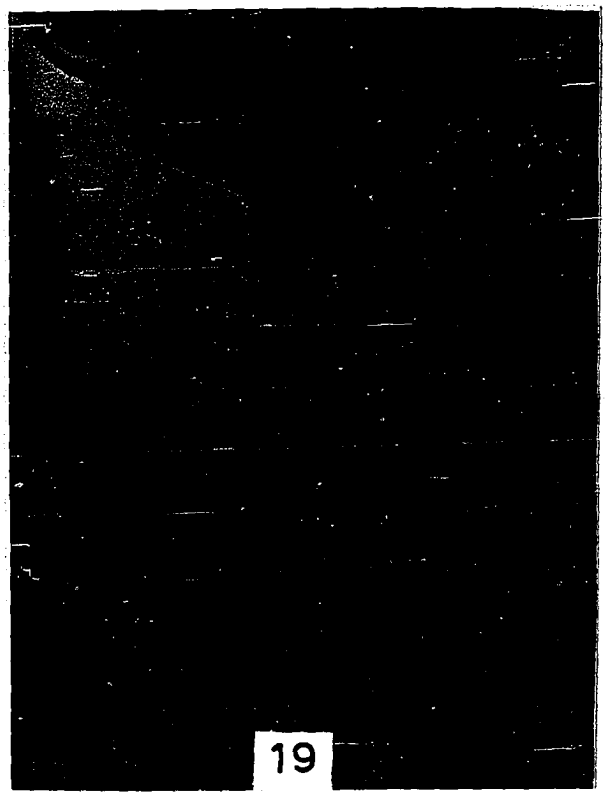
Figure 19. The convoluted end of a pollen tube growing through the micropyle of an ovule.

Figure 20. An early post fertilization stage in an ovule with the endosperm column of cells extending from nucellar cap to the hypostase.

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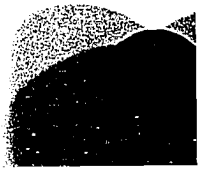


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Vascular System of the Ovule

The vascular system of the ovule up to the time of fertilization is very primitive. In the midline of the nucellar protuberance, the cells become longer and narrower. In this line of cells, which is about four cells thick, thickenings are formed in the cell walls and the cells become differentiated into tracheids. This differentiation into tracheids begins with those cells lying in contact with the vascular cells of the placenta and differentiation gradually proceeds upward until there is a row of tracheids in the funiculus from the vascular tissue of the placenta of the ovary up to and beyond the base of the nucellus in the ovule.

The vascular system of the ovule thus differentiated does not enter the nucellus or terminate near the base of the nucellus as it does in many other plants but continues beyond the chalazal end of the ovule and young seed to form a continuous hoop of vascular elements encircling the ovule. Corner (1949) has suggested the term "perichalazal" to describe this condition of the vascular system in the seeds of the Annonaceae. Greater development of the vascular system takes place subsequent to fertilization and will be discussed later in this paper.



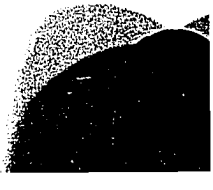
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Development of the Nucellus

It has already been stated that the nucellar protuberance is from four to six cells in depth at the time the archesporial cell makes its appearance. Increase in number and size of cells in the nucellus keeps pace with the growth of the embryo sac and integuments.

By the time the megasporocyte undergoes its first division to form the megaspores, the nucellus has become longer and rounder with a depth of about ten cells and a diameter of about the same number of cells. However, the cells are not isodiametric but are elongated which makes the nucellus as a whole longer than it is broad.

The nucellus surrounding the eight nucleate embryo sac has an aspect quite different from that of the preceding stage. The cells at the micropylar end of the nucellus have enlarged in size, produced thicker cell walls and are arranged in rows of five to six cells radiating out from the embryo sac toward the micropyle. This creates a nucellar cap at the apex of the nucellus (Figure 23). The remainder of the nucellar cells surrounding the embryo sac are smaller, without thick walls and with a thickness of only four cells on each side of the sac.



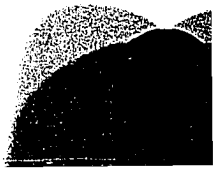
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The Hypostase

A very prominent feature of the nucellus with its mature embryo sac is the hypostase. This structure is found in a great many plants and consists of a mass of cells which are poor in cytoplasmic contents but have partially lignified or suberized walls. This irregularly outlined group of nucellar cells is found directly below the embryo sac at the level of origin of the integuments (Maheshwari 1950).

Van Tiegnem (1901) was the first to call attention to the hypostase which is found in various forms in many different kinds of plants. In his published account he states that the hypostase forms a barrier for the growing embryo sac and prevents it from pushing into the base of the ovule. Goebel (1933) said that the peculiar position of this tissue - directly above the termination of the vascular supply of the ovule - is indicative of its relationship to the water economy of the embryo sac.

In Asimina triloba the hypostase consists of a mass of cells with thickened, lignified walls at the chalazal end of the embryo sac. These lignified cells form a deep thimble shaped cup surrounding the lower one third to one half of the embryo sac. The mass of cells with lignified walls thins out toward the micropylar end of the sac but the hypostase does not disappear altogether in this region. Some strands of lignified cell walls connect the hypostase



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with the nucellar cap, completely confining the embryo sac between the two structures (Figure 17). Because of the confining action of the hypostase the chalazal end of the embryo sac is smaller in diameter than is the micropylar end (Figure 4). This gives somewhat of a bottle shape to the mature embryo sac with the neck of the bottle or flask pointing toward the chalazal region of the ovule.

In the case of Asimina triloba there is no termination of the vascular supply of the ovule at the chalaza. In view of the great development of the endosperm after fertilization, in which the end of the endosperm tube pushes into the cup of the hypostase, I accept Van Tieghem's conception of the hypostase as a barrier to prevent growth into the chalazal region.

POLLINATION AND FERTILIZATION

Pollination in Asimina triloba is effected by means of insects. The three inner petals of the flowers give off odors which attract insects of the Diptera group. Several species of flies have been observed in the open blossoms of the plants in the vicinity of Ann Arbor, Michigan. Delpino (Mueller 1883) counted seven species of Diptera visiting the plants of Asimina triloba in Italy.

In southern Michigan fruit set of the pawpaw is rather erratic. I have never seen more than three or four ripe fruits to a cluster in trees which have been insect-pollinated (Figure 27).



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A tree growing in the courtyard of the Natural Science Building on the campus of the University of Michigan in Ann Arbor was pollinated by hand with a small camels hair brush. The pollen used was obtained from another area in the vicinity of Ann Arbor. Excellent fruit set resulted from this hand pollination and in many flowers every carpel present in the blossom set fruit. This was in contrast to the other half of the tree which could not be reached for hand pollination. On this side of the tree not a single blossom set fruit. Upon returning to the natural stand of pawpaw trees from which the pollen was obtained no fruit could be observed on any of the trees. This visit was made three months after collection of the pollen and it was in this same natural stand of pawpaw trees that the insects had been observed in great numbers visiting the blossoms.

The sporadic production of fruit in nature might be due to several causes. There may be a lack of foreign pollen necessary for cross pollination though it has not been proven that cross pollination is necessary. It may be due to a lack of the necessary dipterous insects which effect the pollination process. It was observed that spiders had spun their webs across the open end of many blossoms. These webs effectively trapped any flies which were attracted to the flowers and prevented their reaching the pollen or the stigmas in the interior of the blossoms. The presence of large numbers of spiders might also have an adverse effect on the fruit set.

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The flowers of Asimina are proterogynous and it is possible that the carpels have lost their capacity to be fertilized before the pollen grains are shed in quantity sufficient to be carried by insects from one blossom to another.

The stigma of the carpel is densely covered with long papillae. Basal to the stigma the papillae follow the line of the ventral suture and gradually turn inward toward the loculus of the ovary. The ovules are borne internally amid large quantities of placental papillae which are identical with those of the stigma in form and structure. These papillae, in an unbroken succession from stigma to placenta, form a transmission tissue through which the pollen tube grows after the pollen grain has become lodged on the stigma and begun to germinate.

The pollen tubes grow downward through this transmission tissue of the stigma and ovary and enter the ovule and embryo sac through the micropyle (Figures 17, 19). Fertilization then, is porogamous. The pollen tube pushes or digests its way between the thick walled cells of the nucellar cap to reach the embryo sac. The embryo sac is penetrated, apparently one or both the synergid cells are destroyed and the end of the pollen tube curls around the egg cell to discharge its sperm nuclei (Figures 13, 15). The actual destruction of the synergid cells by the pollen tube has not been observed but if they are not destroyed at this time they break down shortly afterward since the egg

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cells have been observed many times at the very tip of the micropylar end of the embryo sac (Figures 14, 15).

One sperm nucleus fuses with the nucleus of the egg cell (Figure 15) to produce the zygote and the second sperm nucleus fuses with the primary fusion nucleus near the center of the embryo sac to produce the primary endosperm nucleus.

Fertilization provides a great stimulus to further development of the ovule and ovary and from fertilization on there is great development of all tissues present in both these structures.

POST FERTILIZATION STAGES IN THE OVULE

The zygote develops more slowly than does the endosperm. The primary endosperm cell divides almost immediately following its formation. There is formed by its divisions a row of ten to twelve cells one above the other, extending from the micropyle to the chalaza (Figure 20). Following the formation of this row of cells a vertical cell wall is produced in the endosperm at the micropylar end of the tube. Growth of the tissue is rapid and a mass of endosperm is soon formed in the shape of a long narrow rod limited at the micropylar end by the nucellar cap and at the chalazal end by the hypostase.

Along with growth of the endosperm there is a corresponding growth of the integuments and nucellus. The entire young seed is enlarging and at this time the flattened condition of the seed becomes apparent.

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The raphe and antiraphe form a vascular hoop around the greatest circumference of the seed with the flattened sides of the seed perpendicular to this vascular hoop. The vascular hoop is flat and ribbon like and pressed against the inner integument. The xylem elements consist of about six strands of vessels and tracheids arranged side by side with phloem elements toward the outside of the xylem (Figure 22). In the region of the micropyle the vascular strand in the antiraphe bifurcates to pass around the micropylar tissues and join the original vascular strand near its junction with the placenta.

The outer epidermis of the outer integument is one layer of cells thick. These cells are angular in outline, isodiametric, and have irregular thickenings of the cell walls. These cells are filled with a pigment which is present in the vacuoles of the cells since cells which have uncut walls retain this golden yellow color while cells which have had a part of the cell wall cut away during sectioning have allowed this pigment material to be lost during the course of the staining procedure. The outer epidermis covers the entire outside of the seed except for a narrow zone immediately surrounding the micropyle and placental connection of the seed.

The second layer of the outer integument is composed of a layer of cells, six to eight cells thick, which are much longer than they are broad. These cells develop into long fibers in the mature seeds and these fibers are oriented



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generally in tne long direction of the seed. Immediately below this layer of fibrous cells there is a second layer of similar fibrous cells which are oriented with their long axes at right angles to those of the top layer. This layer of fibers is about six cells thick at the narrowest portion but varies in thickness due to the fact that they grow into the spaces left by the invaginations of the inner integuments.

The inner epidermis of the outer integument is composed of a single layer of thin walled cells which are rectangular in outline. These produce a well differentiated inner epidermis with a thin cuticle.

On the edges of the seed external to the vascular system, a mass of parenchyma cells is interposed between the two layers of fibers. These parenchyma cells become stellate in shape as they grow older and eventually they break down to leave an air space between the vascular hoop and the rim of the mature seed.

The inner integument is still only two cell layers thick. The cells in both these layers are small and square in outline. The inner layer develops the same kind of cell wall thickenings and yellow pigmented vacuoles that characterize the outer epidermis of the outer integument. In the chalazal end of the developing seed the cell layers of the inner integument turn inward and then downward toward the micropyle to meet with the base of the hypostase. It appears in sections as though growth of the inner integument



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were taking place through division of the cells in the region of the hypostase. This connection of the inner integument with the hypostase is clearly shown because of the peculiar pigmented cells in the inner layer of the inner integument.

The two integuments and the nucellus are entirely free from one another in the micropyle region where the condition of these tissues remains much as it was in the ovule at the time of fertilization. Behind this micropylar region, however, growth from the chalazal regions in the seed has added to all tissues in the developing seed. The integuments are free from one another and from the nucellus except at the rim of the flattened seed where all tissues, nucellus, inner integument and outer integument, meet and join into a "fusion zone" on the outside of which is found the vascular hoop of the seed.

FORMATION OF RUMINATIONS IN THE SEED

The inner integument grows in length but not in thickness through the action of the meristematic cells in the fusion zone. This causes an overgrowth of the inner integument which becomes too large for the space which it occupies. Consequently this overgrowth causes a wrinkling of the inner integuments on both sides of the seed. The wrinkles follow the line of least resistance and push inward, crushing the softer nucellar cells and tissues before them.

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This wrinkling and folding of the inner integument causes the characteristic ruminations of the seeds in Asimina triloba. The inner layers of the outer integument then grow into the spaces left on the outer side of the invaginations but the fibrous outer layer does not follow. There is no corresponding wrinkling of the outermost layer of the seeds (Figures 25, 28).

As the invaginations of the inner integuments push their way deeper into the nucellar tissues, the nucellar epidermis, composed of regular, rectangular cells with their narrow ends toward the integument, lines the inside of the invaginations with a tissue which appears glandular in function. The cells of this tissue have prominent nuclei and dense, granular cytoplasm. There is however, a very definite cuticle on the inner layer of inner integumentary cells and a cuticle on the outer epidermis of the nucellus so that there is a distinct separation between these two layers.

The hypodermal layer of nucellar cells aligns itself on the inside of the nucellar epidermis and these hypodermal cells become large and slightly longer than broad with rounded corners. Many of these large cells appear to be secretory in function; the nucleus becomes very prominent, the cytoplasm very dense and large vacuoles are present in the cytoplasm (Figure 21).

The remainder of the nucellar cells along with the upper end of the endosperm column and the hypostase are crushed by



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the advance of the ruminations from both sides of the seed toward the center. A cross sectional view of the seed at this stage shows the ruminations with the epidermis and hypodermis of the nucellus internal to them and an indefinite line in the center of the seed composed of the crushed mass of nucellar cells.

In the micropylar end of the developing seed the ruminations of the inner integument do not completely crush that end of the nucellus and endosperm column. The endosperm encircles the tiny embryo which by now has divided once to form two cells. The outer integument has grown so much that the projecting endostome of the inner integument is being pushed together and the latter has itself begun to grow into a woody plug of tissue directly above the embryo in the seed (Figure 23).

As the young seeds continue to grow in size, the invaginations of the inner integument meet in the center and the nucellar cells which are not crushed grow slightly and fill whatever spaces are left between the ruminations.

SECOND GROWTH OF THE ENDOSPERM

The endosperm lies quiescent until the growth of the ruminations has ceased. By this time the seeds have reached the size they will have at maturity. The endosperm then begins a second growth and pushes itself through the center line of crushed nucellar cells to form a long pencil like column of tissue which then spreads out rapidly to fill the

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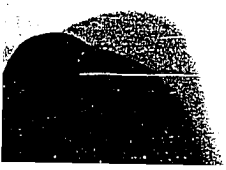
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Figure 21. A portion of a section through a young seed in which the ruminations of the inner integument are pushing the nucellus before them.

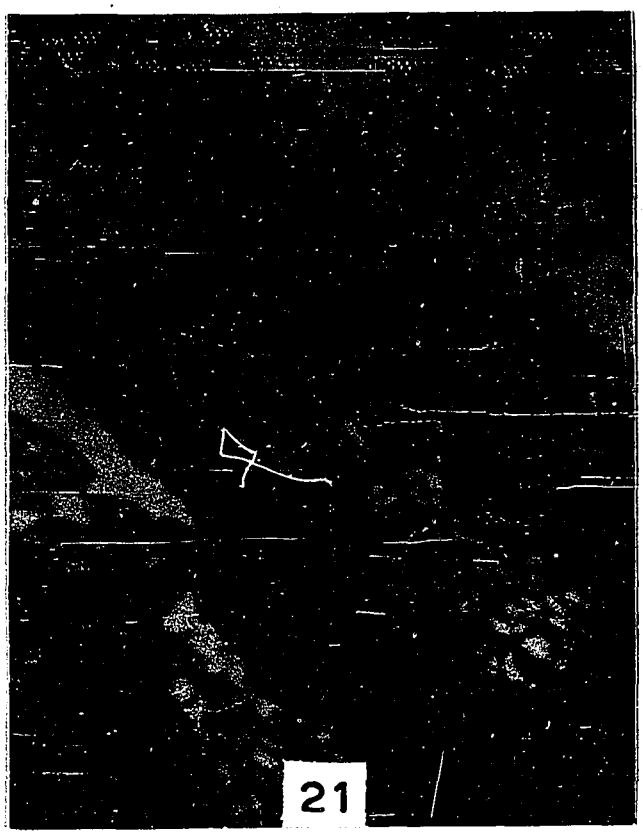
Figure 22. A portion of a longitudinal section through a young seed which shows the vascular elements of the raphe. Phloem elements are to the right of the xylem.

Figure 23. A section through the micropylar end of a developing seed with the woody plug of the endostome, the nucellar cap and the micropylar end of the endosperm tube surrounding the young embryo.

Figure 24. A mass of the endosperm tissue as grown on nutrient agar medium in vitro.



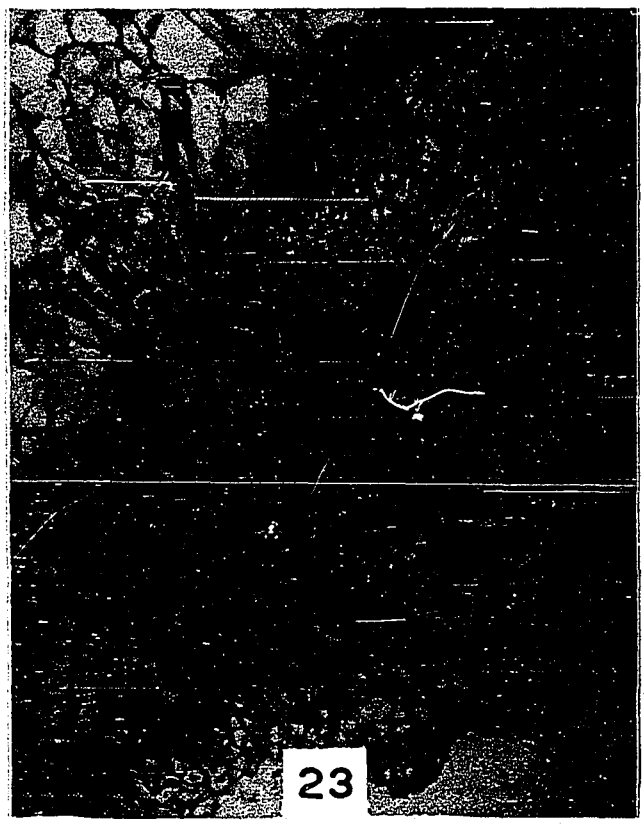
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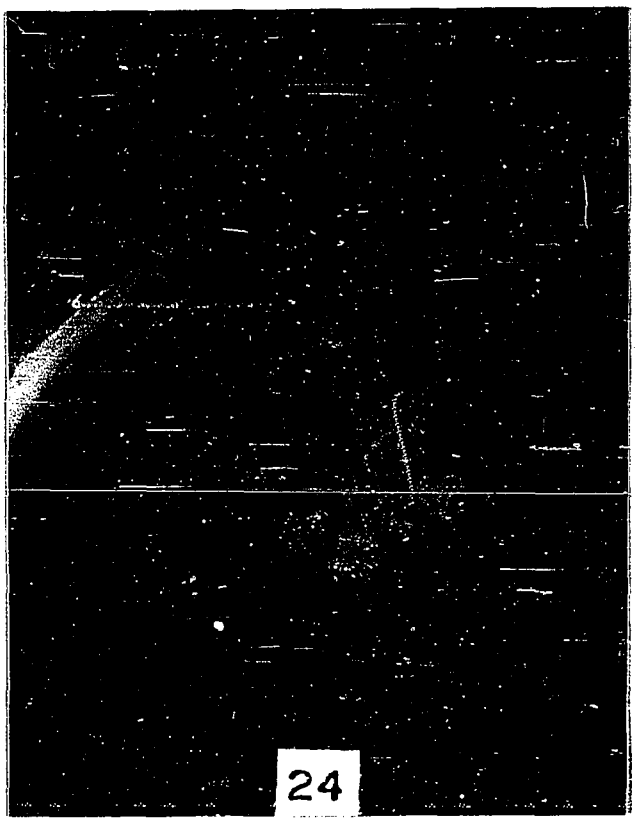
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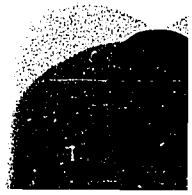
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spaces between the ruminations, digesting the nucellar tissue as it advances.

The nucellar epidermis seems to be more resistant than other nucellar tissues to the digestive action of the advancing endosperm tissue and separates the latter from the inner integument. The endosperm tissue consists of a mass of small cells, closely packed together, which store oil in droplets inside the cells.

DESCRIPTION OF SEEDS AT MATURITY

When the fruits of the pawpaw are ripe and are shed from the tree the seeds enclosed in them are from ten to twenty in number. The outer epidermis of the testa has become brown and waterproofed with a somewhat shiny, waxy covering and the two layers of fiber cells beneath the epidermis have become thickened and lignified. The parenchyma tissue on the outside of the vascular hoop has broken down to leave an air space inside the narrow rim of the seed. At the micropylar end of the seed this air space connects with the outside atmosphere through a long narrow opening which is not covered by the epidermis or fibers of the testa (Figure 26). Projecting slightly through this opening there are thickened lips of tissue formed from the inner fibrous layers of the outer integument which have grown around the original projecting portion of the inner integument. The woody plug formed from the latter lies between the lips of this inner layer of the outer integument (Figures 26, 28).

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The embryo in the mature seed is very small in comparison with the great bulk of the seed. It is about one millimeter in length and is multicellular with a well developed radicle and less well developed cotyledons. It lies in a cavity in the endosperm tissue at the micropylar end of the seed directly beneath the woody plug of the inner integument (Figures 29, 30).

The fruits of Asimina triloba are indehiscent so that unless they are eaten by man or other animals the seeds remain inside the fruits which lie on the ground after being shed. The pulpy mass of the fruit is effective in preventing the seeds from drying out before germination. The thick pulp of the fruit surrounds each individual seed in the fruit and the seeds lie, each in its own chamber on the inside of the fruit, separated from the pulp by a membranous inner epidermal skin or endocarp of the fruit. This endocarp closely invests each seed and the softer cells of the pulpy mesocarp easily come free from this inner epidermis. Superficially the endocarp surrounding each seed might be taken to be a part of the seed itself. This structure is probably responsible for the statement given in some taxonomic works (Fernald 1950) to the effect that the seeds of Asimina triloba are enclosed in an aril. Morphologically, an aril is an outgrowth from one of the integuments of a seed. I have seen no evidence of a third or even a fourth integument in these seeds even though Corner (1949) claims to have found these in other species of Annonaceae.



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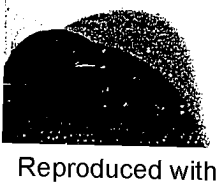
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Figure 25. The flat side of a mature seed.

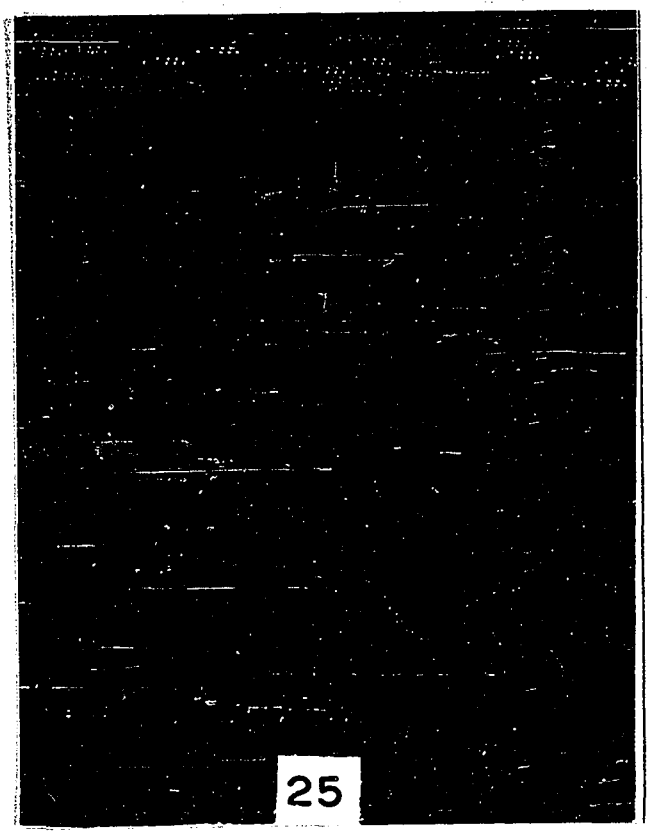
Figure 26. The same seed as shown in Figure 25 but the photograph taken looking toward the micropylar end of the seed.

Figure 27. Young developing fruits.

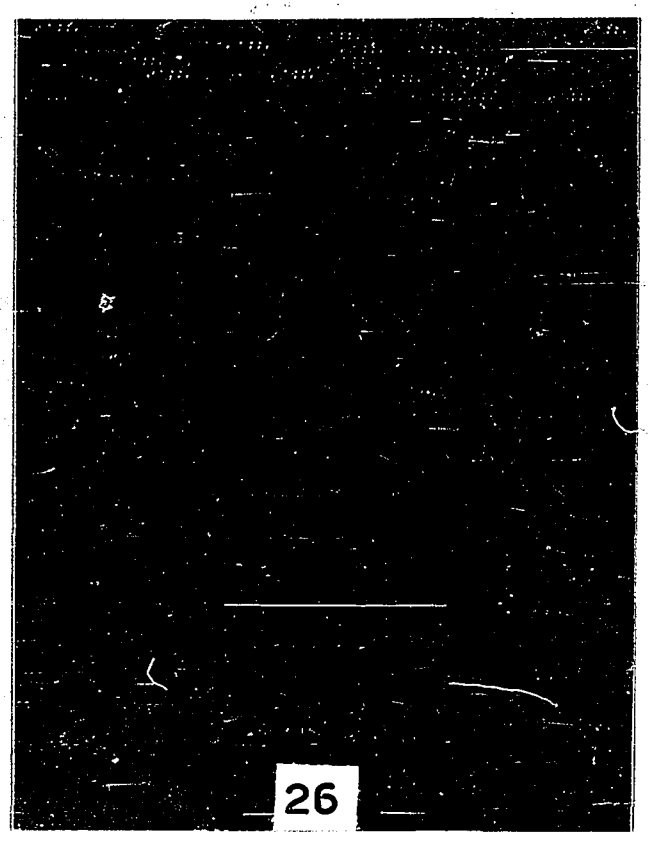
Figure 28. A seed after the endosperm has been used by the seedling and the empty seed dropped from the seedling. The outer seed coat has been split open and the two halves laid out on either side of the inner seed coat containing the cotyledons.



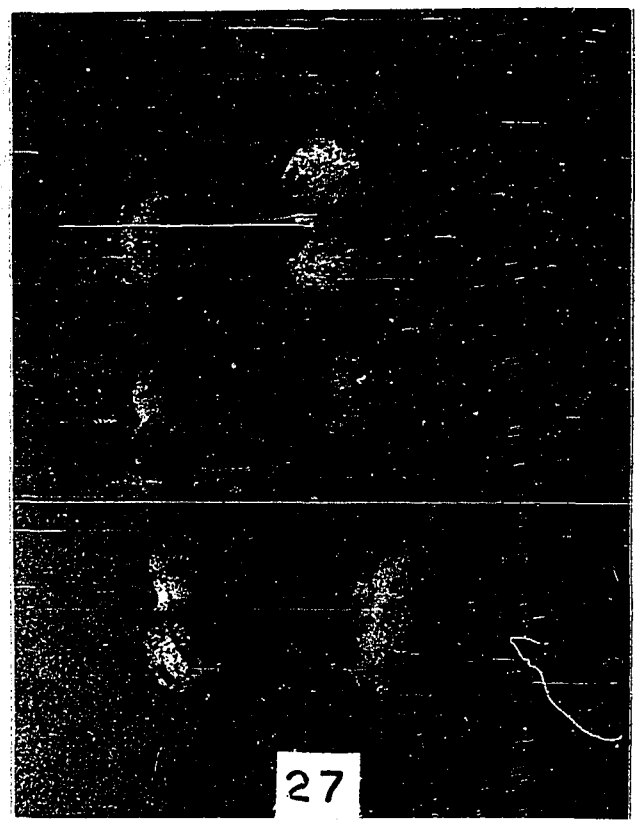
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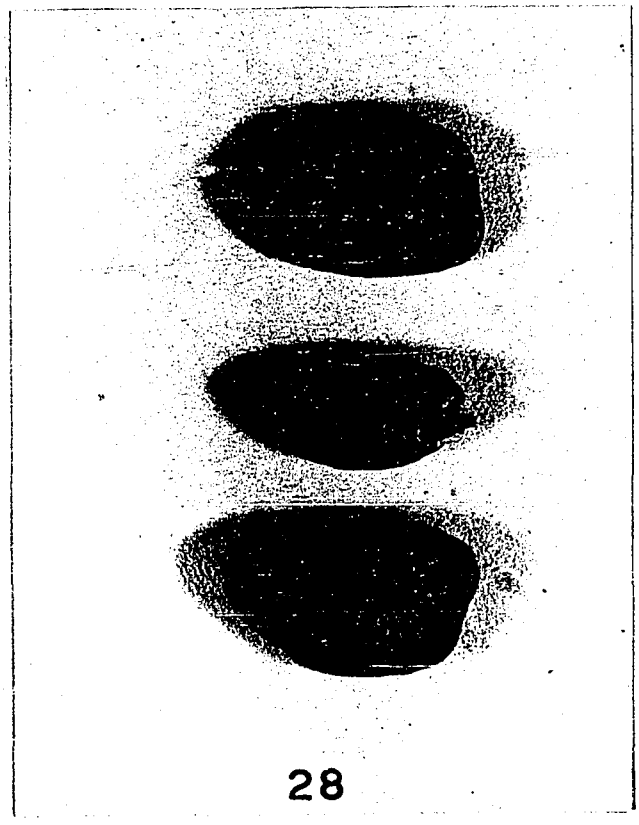
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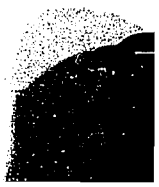
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The fruit proper of the pawpaw is not much differentiated. Technically it is a berry and it is composed of a thin exocarp derived from the outer wall tissue of the ovary, a thick mesocarp composed of a mass of parenchymatous cells which become soft and pulpy upon ripening of the fruit, and a thin inner epidermis or endocarp which closely invests the seeds inside the fruit. The vascular system of the fruit consists of a network of strands within the mesocarp tissue.

In the young ovary the ovules lie in a common cavity or loculus but after fertilization the tissues of the growing fruit push between the young seeds and the latter are soon enclosed in individual compartments and separated from the other seeds in the fruit. Licopoli (1887) has adequately described the mature fruit of Asimina triloba and the cellular entities of which it is composed.

GERMINATION OF THE SEED

The mature seed will not germinate immediately upon dropping of the fruit but the seed needs a period of dormancy before germination will occur. During this dormant period the seed must not be allowed to dry completely or the embryo will no longer be viable.

Experimentally, it was found that seeds separated from the fruits, placed in a mixture of moist sand and peat, and placed in covered glass dishes at a temperature of 5° C. from October until March, germinated readily when brought into a warm room.

Seeds contained inside fruits which were placed on a wi

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dow sill outside the laboratory in the Natural Science Building on the campus of the University of Michigan from October until March germinated as readily as did those seeds which were layered in glass dishes in the cold room.

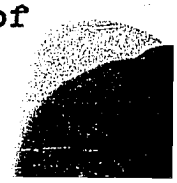
Seeds separated from the fruits and allowed to dry by standing for a period of approximately one week uncovered in the laboratory before being layered in the moist substrate, did not germinate although they had the same period of cold treatment as did those which were kept moist throughout.

Seeds which did not have a period of low temperature did not germinate. From one to two weeks after being taken from the cold room or from the outside window sill and planted in soil or vermiculite, the viable seeds began to swell slightly at the open end. The lips of the inner layer of the outer integument parted slightly because of pressure from the inside of the seed and the woody plug of the inner integument was pushed out by the radicle of the embryo emerging from the seed.

The radicle of the embryo grows rapidly and penetrates the soil as a long taproot. When it is three to four inches long branch roots arise near the upper end.

The transition region where the radicle and hypocotyl come together is well marked by a swelling and an abrupt change of color on the outside of the main axis of the seedling.

When the roots are firmly anchored in the soil the hypocotyl begins to elongate and carries the seed aloft. While the radicle is elongating the cotyledons are growing and pushing their way deeper into the endosperm toward the chalazal end of



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Figures of Asimina triloba

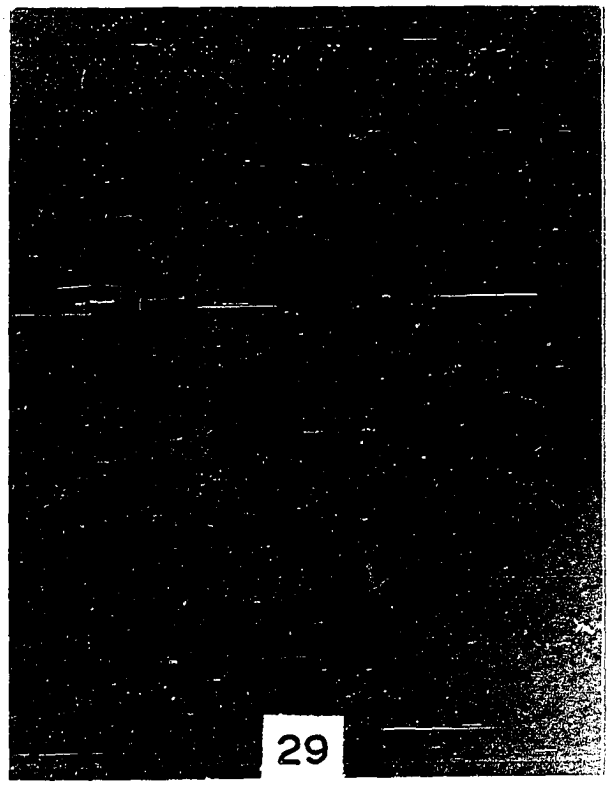
Figure 29. A seed cut longitudinally through the center in a plane at right angles to the flat side of the seed to show the ruminations of the inner integument and the very small embryo.

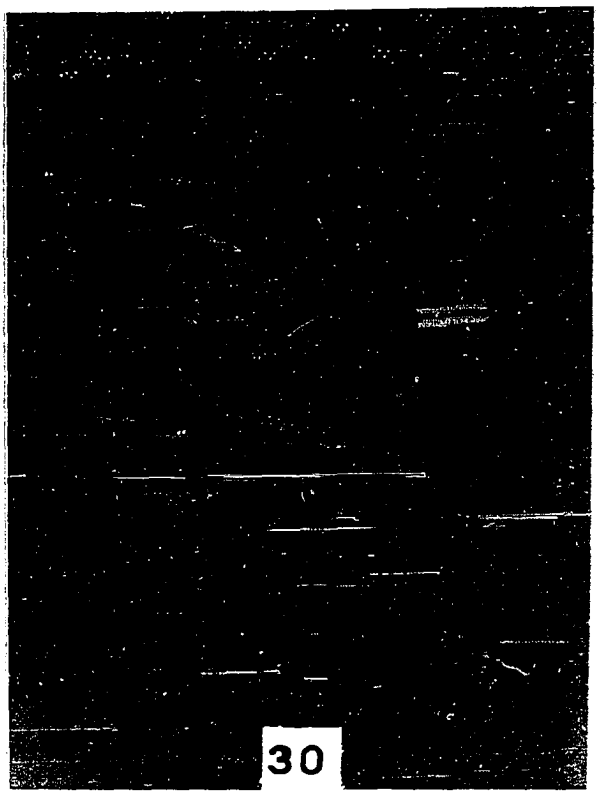
Figure 30. A seed cut longitudinally through the center in a plant parallel to the flat side of the seed to show the air space in the rim of the seed and the very small embryo.

Figure 31. A polar view of an anaphase stage in a mitotic division of a cell of the endosperm tissue grown in vitro.

Figure 32. One of a group of cells showing differentiation in the cell produced by experimental treatment of a mass of endosperm tissue grown in vitro.

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the seed. The cotyledons never leave the seed but function solely as absorptive organs to digest and absorb the nutritive materials contained within the cells of the endosperm tissue.

Several ways have been observed in which the plumule is liberated from the seed. Many of the seedlings observed retained the seed coats firmly attached to the end of the hypocotyl until the cotyledons had absorbed all the nutriment from the endosperm tissues and then dropped the seed or what remained of it, by a shrivelling and an abscission of the cotyledons at their juncture with the hypocotyl. The small plumule thus exposed by dropping of the seed rapidly developed leaves and stem to become a normal growing axis of the plant (Figures 33, 34).

Another method by which many seedlings liberated the plumule from the seed was one in which the cotyledons produced a very short growth outside the seed coats. This external portion of the cotyledons was sufficient to allow the plumule to escape from between the cotyledons and to produce its growing axis of stem and leaves while the cotyledons and seed were still attached to the seedling plant. Frequently the portion of the cotyledons which were exposed to light developed chloropnyll and expanded into leaf like structures (Figure 37).

A variation of the method just described consisted of the first or second leaf from the plumule developing while the plumule was still inside the seed. This caused the seed

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to be pushed away from the hypocotyl somewhat, through growth of the distorted leaf blade, and the remainder of the leaves and stem then grew in normal fashion. Eventually the cotyledons abscissed from the hypocotyl and the seed dropped but was held by the imprisoned tip of the leaf which was still inside the shell of the seed.

Still another way in which the leaves and stem with its growing point were liberated from the seed was a method whereby the plumule, as such, did not come free from the seed. In many seedlings there was no abscission of the cotyledons or pulling out of the cotyledons from the seed. Seedlings in which the empty shell of the seed was firmly attached to the hypocotyl quite frequently produced an axillary or adventitious bud on the hypocotyl below the attachment of the cotyledons. This adventitious bud then took over the normal function of the plumule to produce stem and leaves (Figure 38).

Some experiments were conducted in order to see what effect the removal of the seed coats and endosperm would have on the subsequent growth and development of the seedlings and cotyledons.

Seeds germinated in vermiculite in large flats were planted individually into pots of soil when the radicle was approximately one inch long. After the seedlings became established in the pot and the seed was raised above the surface of the soil, seed coats and endosperm were carefully cut away from the cotyledons. The resulting young seedlings then

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Figures of *Asimina triloba*

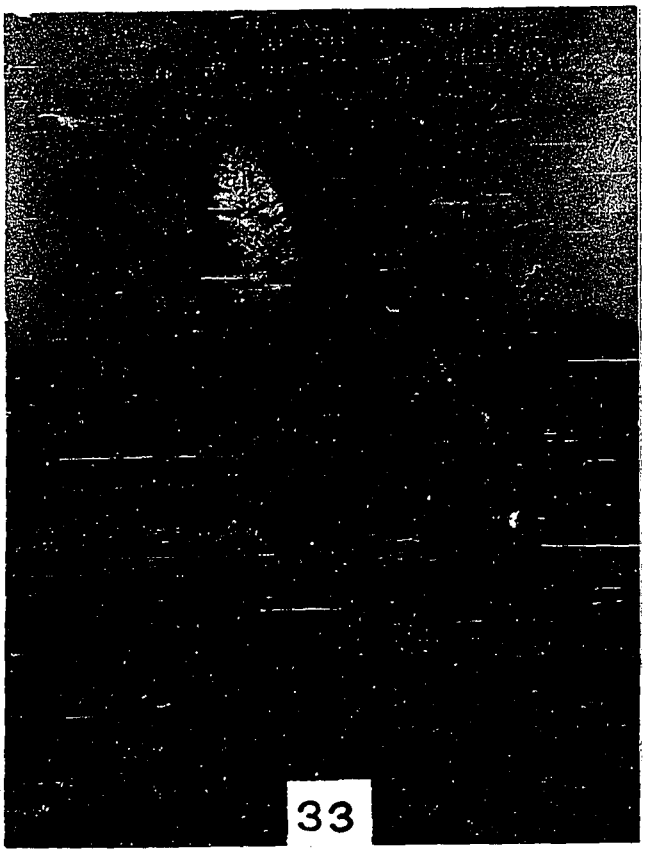
Figure 33. A seedling in which the seed has not yet been dropped from the young plant.

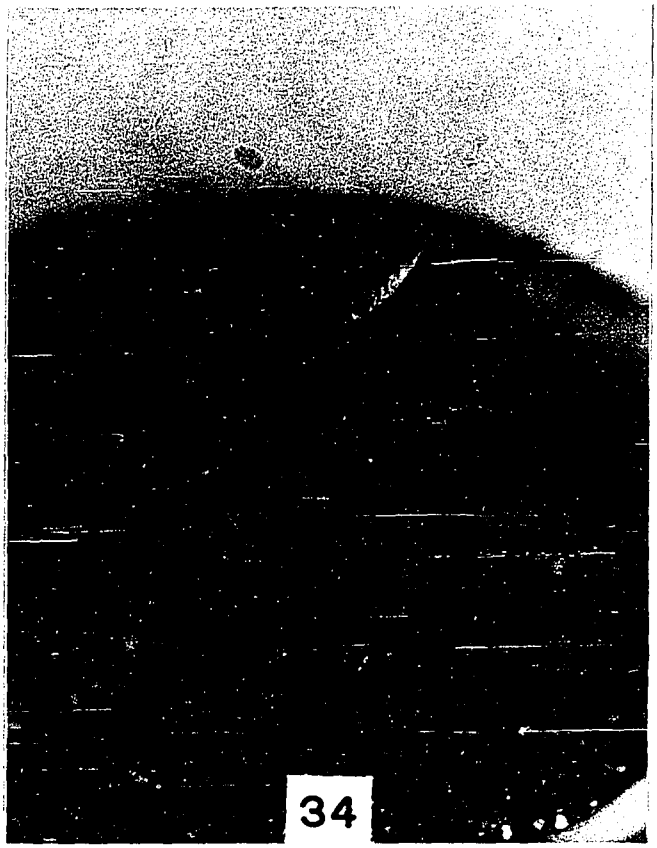
Figure 34. A seedling in which the seed has been dropped and the plumule is developing into leaves and stem.

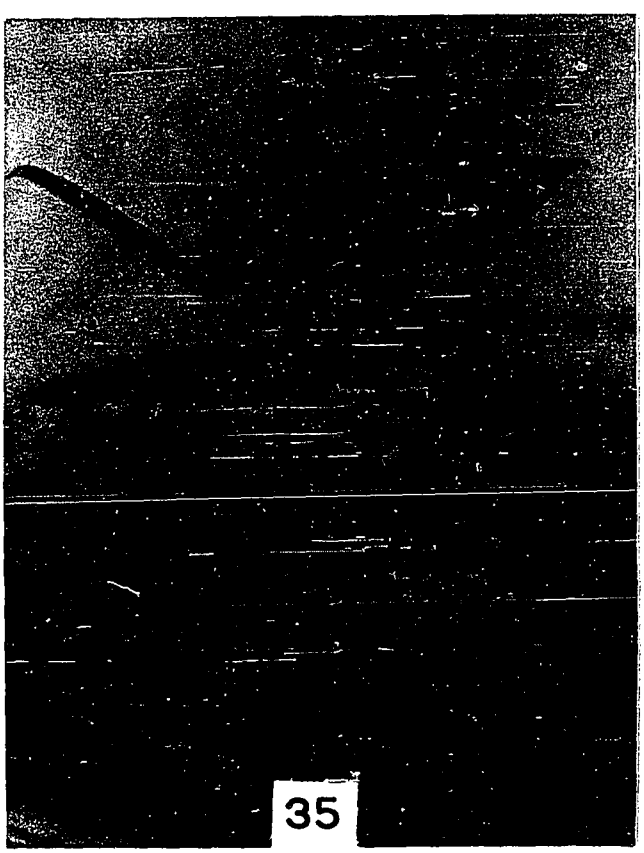
Figure 35. A seedling in which the plumule has produced several leaves and an aerial stem of some length.

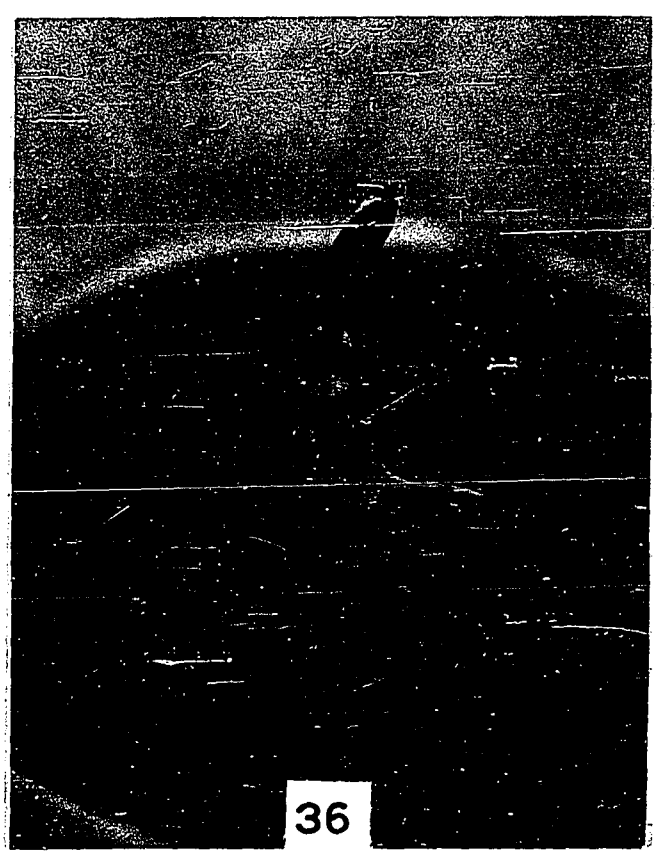
Figure 36. A seedling in which the seed coats and endosperm have been cut away to expose the cotyledons.

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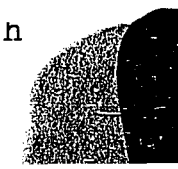
consisted of the root system, the very small plumule and the cotyledons attached to the hypocotyl. In order to protect the tender cotyledons from too rapid drying a small vial was inverted over the whole seedling for a period of three to four days or until the cotyledons had become sufficiently acclimated to life outside the seed coats. The glass vials were then removed, and the seedlings treated as any normal seedling under greenhouse conditions.

When first liberated from the seed coats and endosperm the cotyledons were pale yellow in color and of a moist appearance. After several days had elapsed the color of the cotyledons had become green and, except for an occasional browning of the extreme tip of the cotyledons, they appeared as narrow normal leaves (Figure 36).

Soon after the release of the cotyledons in these seedlings the leaf primordia of the plumules began to develop into normal leaves. No lag or deformation seemed to be present in their development and the plumule developed in normal fashion.

In the majority of the seedlings the green cotyledons gradually turned brown and shrivelled as the leaves and stems developed but in at least two instances they survived for three months or longer.

When the endosperm has been entirely consumed by the seedling and the empty seed has dropped from the plant the seed is readily split into two parts in the region of the narrow rim and the air space surrounding it. The outer seed coat comes away from the rest of the seed in two halves which



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Figures of Asimina triloba

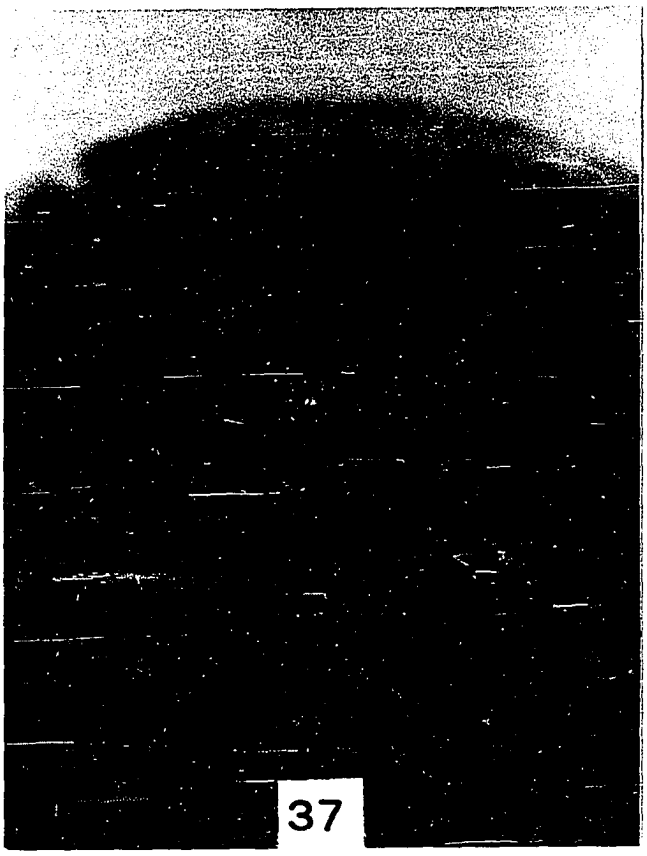
Figure 37. A seedling in which the cotyledons have grown outward from the seed sufficient to allow the plumule to expand.

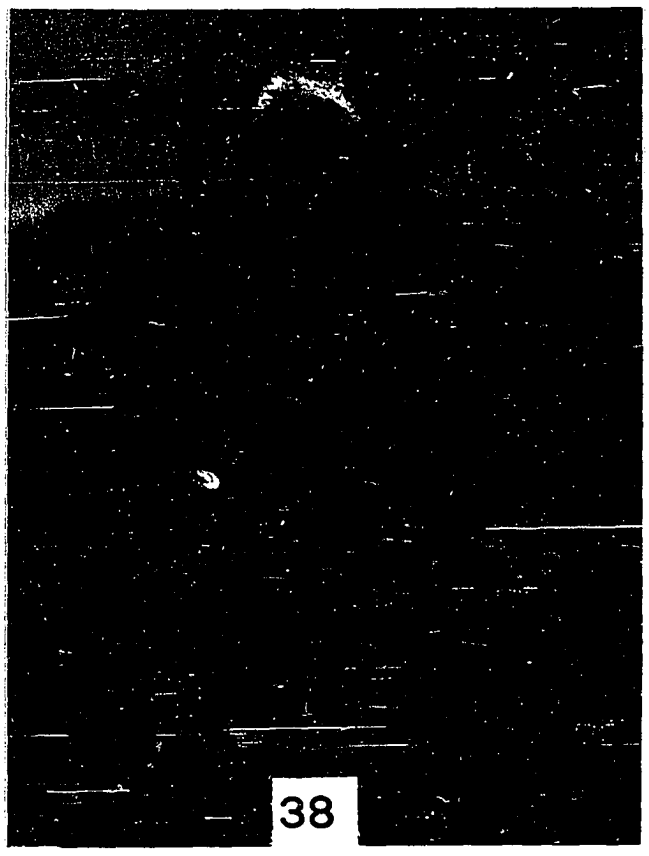
Figure 38. A seedling in which an adventitious bud has arisen below the cotyledons to take the place of the plumule imprisoned inside the seed.

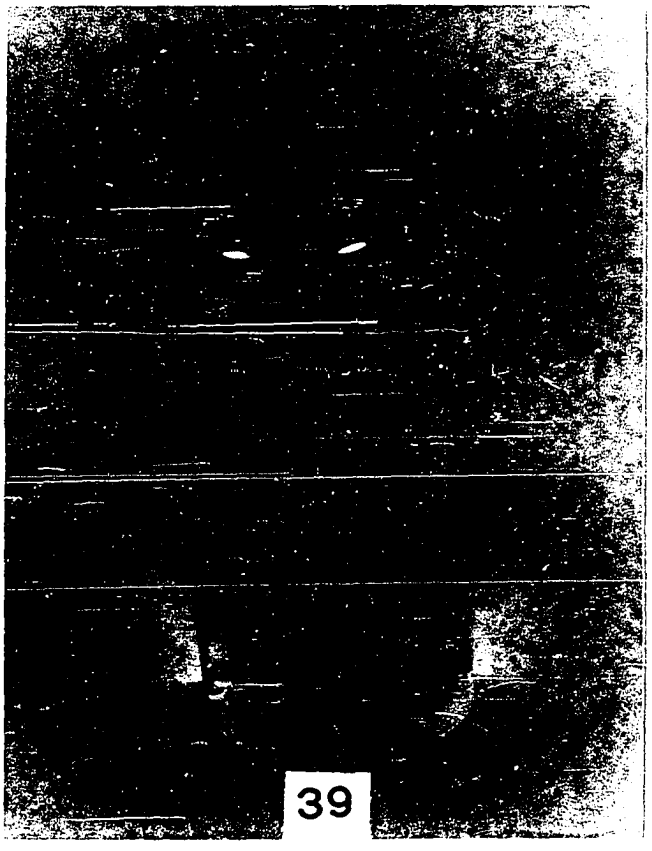
Figure 39. A mass of endosperm tissue as grown in vitro in small screw capped bottles.

Figure 40. A piece of a seed in a bottle of nutrient agar medium in which the endosperm tissue has grown out from the seed.

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show the thick lips of the exostome surrounding the woody plug of the endostome, and the projecting inner layers of the testa. The inner integuments and the shell of the endosperm plus the shrivelled cotyledons remains in one piece (Figure 28).

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EXPERIMENTAL MORPHOLOGY AND CULTURE OF THE ENDOSPERM TISSUE IN VITRO

When the theory of the cell was first formulated by Schleiden (1838) and Schwann (1839) the cell was considered to be not only the elementary unit of structure of an organism but each cell was considered to be essentially a complete organism in itself. In the words of Schwann, " Finden wir nun, dass einzelne dieser Elementarteile, die sich von den übrigen nicht unterscheiden, sich vom Organismus lostrennen und selbständig weiter wachsen können, so können wir daraus schliessen, dass auch jeder der übrigen Elementarteile, jede Zelle für sich schon die Kraft besitzt, neue Moleküle anzuziehen und zu wachsen, dass also jeder Elementarteile eine eigentümliche Kraft, ein selbständiges Leben besitzt vermöge dessen er selbständig sich zu entwickeln imstande wäre, wenn ihm bloss die äusseren Bedingungen dargeboten würden, unter welchen er im Organismus steht. " ("If, therefore we find that some of these elementary parts, which do not differ from others, are capable of being separated from the organism and of continuing to grow independently, we can conclude that each of the other elementary parts,each cell, must possess the capacity to gather new molecules to itself and to grow, that therefore each cell possesses a particular force, an independent life, as a result of which it too would be capable of developing independently if only there be provided the external conditions under which it exists in the organism. ") . Translation by White (1943) .

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Following this concept of the totipotency of the cell many attempts were made to isolate individual cells and groups of cells from the parent plant and to put them into culture to see if these individual cells and tissues could be induced to produce new plant parts or to grow into a complete new plant. To the experimental morphologist this concept is known as "regeneration" .

Gottlieb Haberlandt (1902) was one of those who took his point of departure directly from the cell theory of Schleiden and Schwann and attempted to grow isolated cells in culture solutions. His work ended in failure and he finally abandoned the problem. He did not work with meristematic cells and tissues but used mature green cells which contained chloroplasts, thinking that such cells, having a photosynthetic function, would not need a source of carbohydrate in the nutrient medium.

Kotte (1922) , a student of Haberlandt, used meristematic material and succeeded in growing excised root tips of pea and maize in a variety of nutrient materials.

Robbins (1922) also successfully cultured excised root and stem tips in a sterile nutrient medium. While Kotte had used Leibig's meat extract in the nutrient medium Robbins substituted an extract of yeast. Robbins was able to subculture his original implants but they gradually died out after about twenty weeks in culture.

White (1934) in the United States and Gautheret (1934) in France were the men who successfully developed nutrient



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media of known chemical composition which would maintain cultures of excised plant parts and keep them growing indefinitely as long as they were subcultured at periodic intervals.

The work of White and Gautheret gave a great stimulus to the work in plant tissue culture and since the publication of their work there have been many different kinds of plant tissues and plant parts which have been successfully established to a life in vitro.

The majority of these plant parts being cultured in vitro and which are being reported on in the literature are not, strictly speaking, pure cultures of tissues in the sense that they are homogeneous masses of similar cells, but are, rather, mixtures of tissues derived from well differentiated organs of plants. One of these "tissues" upon which a great deal of work has been reported was originated by White from a strain of tobacco hybrid (Nicotiana glauca X Nicotiana Langsdorfii) . Cultures of this strain were used by Skoog and Tsui (1948) in their work on the chemical control of growth and bud formation. When this strain of tissue became contaminated with a bacterium Skoog and Tsui developed their own tobacco tissue from stem internode segments of Nicotiana tabacum Wis. #38.

Other "tissue" cultures have originated from tumor tissues produced on the stems of various plants through the influence of the crown gall bacterium or from secondary tumors induced by tumor extracts or heat treatment of the

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primary tumors. Hildebrandt and Riker (1949) used tumor tissue from marigold, Paris daisy, sunflower and tobacco.

All of these so called "tissue" cultures have arisen from well differentiated tissues and consequently they have considerable differentiation of cells present in the actual mass of material which is growing in vitro. Sterling (1950) reports that in the stem segments used by Skoog and Tsui in their experiments on the regeneration of buds and roots that parenchyma of the cortex, primary phloem, secondary phloem, vascular cambium, secondary xylem, primary xylem and parenchyma of the pith are all present as well differentiated structures in the stem segments.

In this series of experiments Skoog and Tsui report that they are able to induce the production of buds and roots at will in these cultures simply through the chemical treatment which is given to the stem segments while growing in vitro on nutrient medium.

Sterling (loc. cit.) worked on the histogenesis of the roots and buds with material supplied by Skoog and Tsui and found that the neoplasms originated from well differentiated tissues in the stem segments.

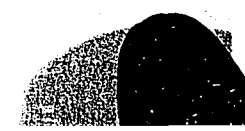
Endosperm tissues produce true tissue cultures in the sense that the tissue from which they have arisen is composed of a mass of cells which are undifferentiated into any other kind of cell or tissue. There are no vascular elements, no cambium, pericycle or epidermis, nothing but a mass of endosperm cells which resemble cells of parenchyma.

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This endosperm tissue, due to its undifferentiated condition, is excellent material for work and experimentation dealing with problems of regeneration. If each cell of a plant is capable of differentiating into a special kind of cell with specialized properties and functions depending upon the conditions in which it finds itself, then these undifferentiated cells of endosperm should be capable of producing new plant parts or even of producing a new plant if the conditions of its environment are modified in such a way as to provide the proper stimulus to the cell.

LaRue (private communication) succeeded in obtaining buds and roots from the endosperm tissue of maize in vitro but the conditions necessary for this regeneration are not yet fully understood. One factor which must be taken into consideration in this respect is "habituation" . It is well known that after being grown for long periods of time in vitro, cultures of plant parts gradually lose their capacity to regenerate and become habituated to the medium.

With these factors in mind, exploratory experiments were undertaken in an effort to see if some of the chemical substances which have been influential on the growth of other tissue cultures in vitro would have similar effects on the growth of the pawpaw endosperm tissue but with the greater hope that regeneration might be accomplished and the normally nutritive tissue could be induced to form roots, stems or leaves.

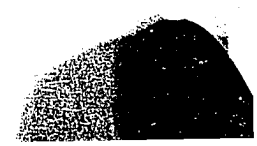


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All of the endosperm tissue which was used in this part of the investigation was derived from a single piece of a seed of Asimina triloba which was planted on nutrient agar medium on July 15, 1949. Fifteen days later this piece of seed was producing a mass of endosperm tissue as an outgrowth from the central portion of the cut seed. When the growth was large enough to be transferred from the parent piece it was removed, broken into smaller pieces with a sterile spatula and each piece planted in an individual bottle of nutrient. Similarly, when these pieces had grown large enough, they too were subdivided. In this way a clone of tissue was built up over a period of weeks and it was this clone, all derived from a single seed piece, which was used for the experimentation.

After removal of the original outgrowth the seed piece was put back into a bottle of nutrient medium and allowed to grow more of the tissue. Three different crops, so to speak, of the endosperm tissue were harvested from the original seed piece before it was fixed and sectioned to show the origin of the tissue growing from it. Microscopical examination of the seed piece showed that this repeated growth of tissue was possible because a certain mass of the endosperm tissue was buried among the ruminations of the seed coats and when the external portions were broken off, the basal part left in the seed grew out again into a mass of tissue.

Examination of this seed piece also showed that there was no direct connection of the endosperm tissue with the



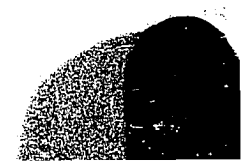
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other tissues of the seed. This is in accord with the normal development of the endosperm in which the endosperm column spreads out to fill the areas between the ruminations of the integuments.

When a sufficient quantity of tissue had been obtained by repeated subculturing of the original outgrowths, experiments were undertaken to determine the effects of chemical substances on the growth of this tissue and to see if regeneration could be induced in it.

As grown in vitro, pawpaw endosperm tissue consists of a mass of cells which are alike except for size when viewed with the microscope. A whole mount of the tissue shows cells which are rounded in outline and are composed of cell wall, cytoplasm and nucleus, with large vacuoles in the cytoplasm. The nucleus is suspended inside the cell lumen by radiating strands of cytoplasm. Such cells have the appearance of mature parenchyma cells.

Masses of tissue which have been fixed, imbedded in paraffin, sectioned and stained show some mitotic figures (Figure 31) . The chromosome number in such mitotic figures has been determined to be approximately 27. This is the normal triplod number of chromosomes for Asimina triloba as determined by Bowden (1949) . Since the endosperm tissue is derived from the triploid primary endosperm nucleus this chromosome number would be expected to be present in this tissue.



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Macroscopically the endosperm tissue is white and opaque sometimes turning to a brownish color upon aging. The growing tissue mass is of a loose texture which is apparently caused by the manner of growth of the tissue. Certain cells or groups of cells on the periphery of the mass of tissue begin to divide rapidly and this produces a nodule or ball of new cells. When this nodule of cells attains a certain size other cells or groups of cells on its periphery form new nodules. This manner of growth gives a characteristic granular appearance to the growing endosperm tissue. Young actively growing tissue has many spaces between the nodules but older tissue becomes more compact.

In experimental work with this tissue the manner of growth is an important factor in the transference and division of tissue necessary to set up an experiment. Individual bottles of nutrient medium may receive pieces of tissue which contain widely different numbers of these growing nodules of cells when a parent piece of tissue is subdivided. This causes a different rate of growth in the tissue in different bottles of nutrient medium even though the tissue pieces have come from the same parent piece of tissue.

In setting up experimental conditions the tissue used was, as far as possible, selected from young (in the sense of time after transference onto fresh medium) , actively growing tissue. Approximately equal sized pieces were used to inoculate the bottles of nutrient medium.

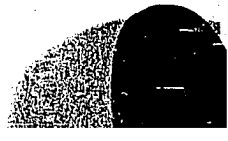
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A growth period of four weeks was chosen as the period during which experimental runs were made. Preliminary experiments had shown this amount of time to be sufficient for the tissue to recover from the shock of transplantation and division, to adapt to the conditions in the new bottle of nutrient medium and to produce new growth.

Before it was possible to determine the effects of chemical substances on the growth of the endosperm tissue in vitro it was necessary to have some idea of the way in which this tissue grew on the medium selected for the experimental work. As has been said in the section on methods and materials, this medium was the tobacco medium of Hildebrandt, Riker and Duggar (1946 b) .

An experiment was set up to determine the degree of acidity or alkalinity (pH value) which gave the best growth of the tissue on this nutrient medium. As in all the experimental work, one liter of the nutrient medium was made up from stock solutions and this one liter divided into five 200 milliliter portions. These five aliquots were each adjusted to a different hydrogen ion concentration.

After sterilization of the medium, weighed pieces of the tissue were sown on the different media with ten pieces of tissue, each in an individual bottle of nutrient, used for each different pH value. This same number of tissue replicates, each in its own bottle of nutrient medium, was used in all subsequent experiments.



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After four weeks of growth on the nutrient media the tissues were removed from the bottles and reweighed. The weights of all ten tissue pieces were averaged and the original average weight of all ten pieces of tissue were subtracted from the final average weight. These average weights were used to compute the average percentage increase in weight of the tissue.

The average percentage increases in weight of the tissues sown on nutrient media with different pH values is given in Table 1.

TABLE 1

Average percentage increases in weight of tissue on nutrient media with different pH values.

pH value 4.0	- - - -	100% increase
pH value 5.0	- - - -	95% increase
pH value 6.0	- - - -	57% increase
pH value 7.0	- - - -	71% increase
pH value 8.0	- - - -	67% increase

From these results it is apparent that under the conditions of this experiment a considerably acid medium is best suited for the growth of this tissue in vitro. The five percent difference in the growth of the tissue grown on medium with a pH of 4.0 and that grown on a medium with a pH of 5.0 is a very slight difference and since the pH of

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4.0 is very near the point at which agar will not solidify, the pH value of 5.0 was chosen as the hydrogen ion concentration of the medium which was used for all subsequent experiments with the tissue.

Due to the practical difficulties of obtaining tissue pieces of identical size, weight and number of growing nodules present, the average percentage weights used to determine differences in growth of this tissue are thought to be of a fair value. The practical values of ease in manipulation of materials under sterile conditions were thought to be of greater importance than that of obtaining highly critical growth values. In these studies a difference of twenty to twenty five percent difference in the average percentage weight of tissues is thought to be of significance in interpreting the results of experiments.

In an experiment to determine the percentage of sucrose best suited to the growth of the tissue one liter of the nutrient medium was made up and the solution divided into five equal parts. Sucrose was added to these in quantity to make concentrations of ½%, 1%, 2%, 4% and 8%. After sterilization the iron organic complex was added and the media poured into sterile bottles. Tissue pieces were sown on the media and after four weeks of growth the increases in weight calculated. The results are given in Table 2.

According to the results of this experiment 2% sucrose as commonly used in nutrient media supported the best growth of this endosperm tissue in vitro.

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In accord with these results this concentration of sucrose was used in the basic nutrient medium on which the tissue was grown to test for other factors.

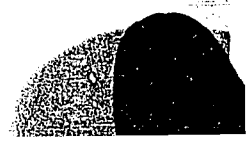
TABLE 2

Average percentage increases in weight of tissue on media with different amounts of sucrose.

½% sucrose	- - - -	44% increase
1% sucrose	- - - -	54% increase
2% sucrose	- - - -	116% increase
4% sucrose	- - - -	50% increase
8% sucrose	- - - -	1.4% increase

In an experiment to test the effects of glucose as the source of carbohydrate in the nutrient medium a series of media containing ½%, 1%, 2%, and 4% glucose were set up with the control medium containing 2% sucrose. Ten replicates of tissue were planted on each kind or medium and tissues were weighed before and after four weeks of growth. Results are shown in Table 3.

In early work with plant tissue cultures it was assumed that monosaccharides such as glucose would be the best carbohydrates to be used in the nutrient medium (Haberlandt 1902, Kotte 1922, Robbins and Bartley 1937). White (1934) found that sucrose was a more satisfactory



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carbohydrate than glucose for the growth of excised tomato roots in vitro.

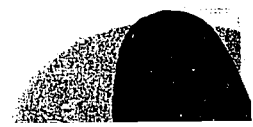
The results of this experiment show that one half of one percent of glucose in the nutrient medium gave a much greater rate of growth of this tissue than did the other concentrations of glucose or the control of 2% sucrose. There was decreasing growth with increase in concentration of the sugar in the medium.

TABLE 3

Average percentage increases in growth of tissue with different amounts of glucose.

½% glucose - - - -	110% increase
1% glucose - - - -	75% increase
2% glucose - - - -	56% increase
4% glucose - - - -	13% increase
Control (2% sucrose) - -	65% increase

Cultures of plant parts which are not green require a source of carbohydrate material in the nutrient medium on which they are grown. Many plants store carbohydrate in the form of starch and conceivably each cell is capable of converting excess carbohydrate into a storage form of some sort and then converting this storage product back into soluble carbohydrate as used in the respiration of the tissues.



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An experiment was set up using starch as the carbohydrate in the medium to see if the endosperm tissue, which normally stores excess carbohydrate in the form of oil, is capable of utilizing this form of material. The results of this experiment are given in Table 4. This experiment shows that the endosperm tissue is able to use starch as the source of its carbohydrate but that it is not nearly so good a carbohydrate as sucrose for the growth of this tissue.

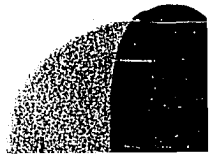
TABLE 4

Average increases in weight of tissue growing on a medium with starch as carbohydrate source.

0.5% starch - - - -	22% increase
1.0% starch - - - -	25% increase
2.0% starch - - - -	11% increase
4.0% starch - - - -	13% increase
Control (2% sucrose) - -	52% increase

Since the discovery of growth hormones in plants and their influence on the growth and differentiation of plant cells and tissues much work has been done on plant growth using the pure chemical substances similar to those found in auxins.

One of the commonest of these growth substances found



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to be influential on the growth of plants is indole acetic acid. This substance was incorporated into the basic nutrient medium in a series of concentrations in order to see the effect it would have on the growth and differentiation of the endosperm tissue. Amounts of 1, 2½, 5 and 10 milligrams of indole acetic acid per liter of medium were added to the basic solution and tissue planted on these media. After four weeks of growth results are as shown in Table 5.

TABLE 5

Average increases in weight of tissue growing on media with different amounts of indole acetic acid.

1.0 mg. IAA	- - - -	46% increase
2.5 mg. IAA	- - - -	55% increase
5.0 mg. IAA	- - - -	52% increase
10.0 mg. IAA	- - - -	46% increase
Control (no IAA)	- - - -	71% increase

Skoog and Tsui (1948) claim to be able to induce at will the formation of shoots and roots in stem segments of tobacco. Roots are induced by the addition of growth substances to the nutrient medium but under the conditions of the experiment just given, indole acetic acid does not produce root growth in the endosperm tissue but it inhibited the normal rate of growth of this tissue. The highest average percentage increase of weight of tissue grown on media with indole

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acetic acid was 55% for that medium with 2½ milligrams IAA per liter. This was much lower than the 71% average increase in the growth of the tissue on control medium.

Skoog and Tsui also report that data of H. F. Clements demonstrated a correlation between phosphate content and the ability of lateral buds of sugar cane to grow out. They used increased amounts of phosphate in some of their experiments on the chemical control of growth and bud formation in tobacco stem segments. While the results of many of their experiments were not always conclusive, "in general, they tended to show that the higher the phosphate and sugar contents of the medium the higher the concentrations of auxin required for prevention of bud formation and for inhibition of callus growth" (Skoog and Tsui 1948, p. 783).

An experiment was set up to see what effect increased amounts of phosphate would have on the endosperm tissue. Media containing 33, 66, 132, 264 and 528 milligrams of monobasic sodium phosphate per liter of nutrient were made up according to the usual procedure and pieces of tissue were sown in the bottles of media. Results after four weeks of growth on these media are shown in Table 6.

The medium containing 264 milligrams of monobasic sodium phosphate per liter showed the greatest overall increase in growth but there was no indication of any form of differentiation or the production of new plant parts.

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TABLE 6

Average percentage increases in growth of tissue with increasing amounts of sodium phosphate.

33 mg. phosphate - - - -	83% increase
66 mg. phosphate - - - -	68% increase
132 mg. phosphate - - - -	69% increase
264 mg. phosphate - - - -	111% increase
528 mg. phosphate - - - -	77% increase

It seems logical to assume that if cells of a plant are to be made to differentiate into different kinds of cells with distinct chemical and physical properties that the alteration will be brought about by conditions which will alter the metabolic processes of the cell concerned.

Sulfhydryl groups appear to be essential parts of cellular oxidative systems and glutathione is a substance which has a free and oxidizable sulfhydryl (-SH) group. This substance seems to be a universal component of the cells of both plants and animals and has been used in experiments on cell metabolism. The sulfhydryl group has been shown to be stimulating to the growth and multiplication of non-nucleated blue-green algae (Hammett and Walp 1940).

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In an attempt to see whether or not glutathione would stimulate the growth and differentiation of the endosperm tissue, nutrient media containing 5, 10, 20 and 40 milligrams of glutathione per liter were made up in the usual manner. Tissue was planted on these media and the results are given in Table 7.

TABLE 7

Average percentage increases in weight of tissue grown on media containing different amounts of glutathione.

5 mg. glutathione - - - -	109% increase
10 mg. glutathione - - - -	145% increase
20 mg. glutathione - - - -	102% increase
40 mg. glutathione - - - -	118% increase
Control (no glutathione) - -	106% increase

Under the conditions of this experiment tissue grown on nutrient medium containing 10 milligrams of glutathione per liter made a considerably greater growth than did the tissue grown on nutrient media containing other concentrations of the substance or with no glutathione. There was no evidence of the production of new plant parts from this endosperm tissue.



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Early workers in animal tissue culture succeeded in growing tissues in nutrient media which had for the principal nutrient natural extracts of animal tissues such as blood plasma or embryo juice. Those working with plant material tried to duplicate such natural materials by using such extracts as tissue juice, xylem sap, phloem exudate, liquid endosperm, etc., but all such attempts were unsuccessful. In recent years there have been several successful attempts made to grow tissue cultures in plant extracts. Robbins (1922 b) used yeast extract with excised corn root tips, Blakeslee and Satina (1944) used autoclaved malt extracts to grow embryos of Datura while Solomon (1950) found that autoclaved malt extract was inhibitory to the growth of Datura embryos. Liebur et al. (1950) found that the addition of 1 per cent casein hydrolysate to the nutrient medium inhibits germination and prolongs embryonic growth in barley embryos separated from the seeds. Van Overbeek, Conklin and Blakeslee (1941) used cocoanut milk to grow embryos of Datura.

All three of these extracts were used in experiments on the pawpaw endosperm tissue to see the reaction of this tissue to these natural extracts.

In an experiment to determine the effects of unautoclaved malt extract on the growth of the tissue it was found that malt extract is decidedly inhibitory to the growth of this tissue. Results are given in Table 8.

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TABLE 8

Effects of unautoclaved malt extract on the growth of pawpaw endosperm tissue.

0.5% malt extract - - - -	46% increase
1.0% malt extract - - - -	29% increase
2.0% malt extract - - - -	12% increase
4.0% malt extract - - - -	00% increase
Control (no malt extract) -	48% increase

Nutrient media with unautoclaved but Seitz filtered yeast extract were also made up to see what effect this substance would have on the tissue. After four weeks of growth on the yeast media, the results as given in Table 9 show that yeast extract also is inhibitory to the growth of this tissue. In no case did growth of the tissue on yeast extract medium approach that of the tissue on control medium without yeast. Furthermore the yeast had a deleterious effect on the tissue in that a dark brown pigmented substance was produced in the tissue and this pigmented substance diffused out into the agar medium. The tissue itself became dark in color and appeared to the eye to be in an unhealthy condition. The dark color of the tissue and the amount of pigmented substance diffusing into the agar were in direct proportion to the amount of yeast extract present in the medium.

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TABLE 9

Effects of unautoclaved yeast extracts on growth of the endosperm tissue.

0.5% yeast extract - - - -	68% increase
1.0% yeast extract - - - -	39% increase
2.0% yeast extract - - - -	10% increase
4.0% yeast extract - - - -	00% increase
Control (no yeast extract)-	116% increase

Casein hydrolysate is a good source of amino acids, and in the commercial acid hydrolysed product, also a source of considerable phosphate and sodium chloride (Ziebur et al. 1950). This substance in the nutrient medium was found to be beneficial to prolonged embryonic growth (loc. cit.).

A series of nutrient media containing 0.1, 0.25, 0.5 and 1.0 milligrams of casein hydrolysate per liter of medium were made up and tissue planted on the media. It was found after four weeks of growth that there was a gradual increase in weight of the tissues up to the concentration of 0.5 milligrams per liter. Doubling of this concentration of casein hydrolysate caused a falling off of growth to one half of that of the former concentration.

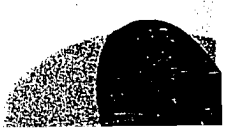
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TABLE 10

Average increases in growth of tissue with different amounts of casein hydrolysate.

0.1 mg. hydrolysate - - - -	74% increase
0.25 mg. hydrolysate - - - -	92% increase
0.5 mg. hydrolysate - - - -	110% increase
1.0 mg. hydrolysate - - - -	53% increase
Control (no hydrolysate) - -	68% increase

Many cells and tissues of plants which normally are uncolored have the capacity to produce chloroplasts and produce carbohydrate by photosynthesis when they are exposed to the light. In an effort to induce the production of chlorophyll in the pawpaw endosperm tissue ten bottles of this tissue growing on the basic nutrient medium and ten capsule dishes of the tissue on the same nutrient were placed at a distance of 12 inches from a fluorescent lighting fixture containing two twenty watt, white light tubes. An automatic time clock was arranged so that the cultures would be illuminated for a period of 18 hours out of every 24. After five months in the light chamber there was no apparent change in the tissues and the experiment was discontinued. The endosperm tissue normally is colorless and this experiment shows that there are no primordia present in the tissue which can be



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transformed into green chloroplasts under the influence of light.

Since the seeds of the pawpaw need a period of cold treatment in order for the embryos to germinate an experiment was devised to see if some changes might take place in the tissue in the cold which would cause them to in some way change their metabolism and differentiate into some other kind of specialized cells. Several bottles of stock cultures of the endosperm tissue were placed on a shelf in the 5° C. room. After four weeks these bottles of tissues were returned to the laboratory and placed in the light chamber at room temperature.

When removed from the cold room there was no apparent change in the tissues. After several days in the light chamber at room temperature the tissue began to grow like any other tissue. At the end of a four month period of time in the light chamber there was no apparent regeneration or other change in this tissue and the experiment was discontinued.

Apical meristems of stem tips seem to produce growth substances which diffuse downward through the stems and which seem to cause differentiation of cells to produce leaves and vascular tissues. Ball(1952) found that in seedlings of Lupinus albus there is strong downward differentiation from isolated shoot tips. This presumably is caused by chemical substances proceeding from the apex of the stem.

It was thought that if growing tips of stems of Asimina

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triloba could be grafted onto actively growing pieces of the endosperm tissue, differentiation might be obtained in the cells of the tissue mass. Accordingly actively growing young stem branches with young leaves and leaf primordia were picked from branches of the plant which had been brought into the laboratory and forced into bloom. These were picked off by means of sterile forceps and placed into bottles of sterile nutrient agar medium. These bottles of medium with the stem tips were placed in the light chamber and after a period of approximately two weeks it was found about 10% of these were uncontaminated by molds or bacteria. These uncontaminated stem tips were then removed from the plain agar medium and placed onto pieces of actively growing endosperm tissues in bottles of nutrient medium. The basal ends of the stem tips were placed in intimate contact with or pushed into the tissue. These cultures were returned to the light chamber and allowed to remain there.

The leaves of the stem tips so treated were of a good green color but did not grow rapidly (Figure 44). Leaf primordia present in the stem tips unfolded very slowly and grew to a height of about one half inch but after a period of two to three months the stem tips turned brown and died. Upon examination of these cultures it was found that there had been no union of the tissue of the stem tips with the endosperm tissue. There was no differentiation of cells in the tissues upon which the stem tips had been growing.

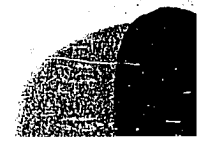


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In another experiment to see if confinement of the tissue might cause mutual pressure between cells of the tissue and hence produce regeneration, sterile bottles of nutrient medium with short pieces of glass tubing placed upright with the basal end of the tube in the agar were inoculated with small fragments of the endosperm tissue dropped into the upper end of the tubing.

Because of the smaller amount of tissue with which these were inoculated growth was not apparent as soon as it is in cultures with larger tissue inoculants. Growth did take place, however, and the tissues began to grow upward inside the glass tubing as well as growing downward through the nutrient agar in the base of the glass tube. Growth above the agar was loose and did not fill the whole diameter of the tube but the tissues which pushed downward were solid and compact.

Microscopic examination of some of the tissues in the basal portion of one of the glass tubes showed a few cells with a structure which was interpreted as being the first stages in the differentiation of the cells. The interior of the cells was filled with a mass of alveolar cytoplasm as shown in Figure 32. It is not certain whether or not these cells would have differentiated further but the remainder of the cultures are still being watched in the hope that some more apparent regeneration will take place.



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Figures of Asimina triloba

Figure 41. A piece of seed placed on nutrient agar medium with endosperm tissue growing out from the cut surface of the seed.

Figure 42. An embryo which has been dissected out from the seed and placed on nutrient culture medium.

Figure 43. A bottle of nutrient agar culture medium with a short length of glass tubing in which a fragment of endosperm tissue has been planted.

Figure 44. A sterile, growing stem tip placed on top of endosperm tissue growing in sterile nutrient medium.

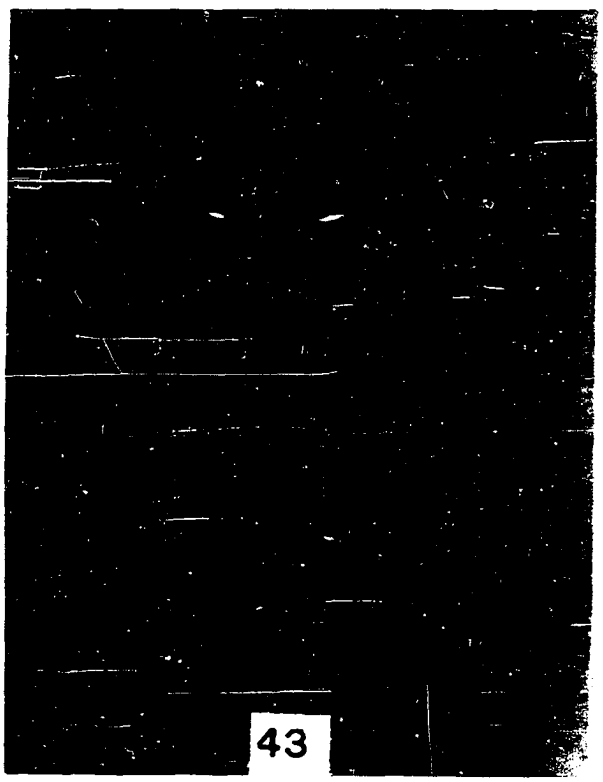
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DISCUSSION

The complete ontogeny of the ovule and seed of Asimina triloba has never been completely investigated. Herms studied the early stages in the embryology of this species but his work went no further than the stage in which the fertilized embryo sac produces an endosperm column of ten to twelve cells.

As a result of the present investigation some of the statements of Herms concerning the development of the embryo sac of this plant are questioned. His statement that the archesporial cell of Asimina is hypodermal and divided to produce a parietal cell and a sporocyte cell has not been corroborated by the author. As stated in the text of this paper, during the course of the investigation there has been seen only one section of an ovule in which the archesporial cell appears to be hypodermal and not sub-hypodermal (Figure 12) . In this section the archesporial cell is in a stage of cell division. The section itself appears to have been cut very obliquely as is shown by the appearance of the primordium of the inner integument.

In two or three instances there have been found two archesporial cells in the same ovule (Figure 18) . In all these cases the archesporial cells have been sub-hypodermal in origin but it is quite possible that the archesporial cell shown in Figure 12 which appears to be hypodermal is actually one or a pair of archesporial cells which have been produced in one ovule. The division of this archesporial

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cell might be the division to produce the megasporocyte and the parietal cell or it seems more likely, because of its size, that it is the division to produce the megaspores.

It could be argued that the primary archesporial cell divides to produce the megasporocyte cell and the parietal cell very early in its history, even before it is larger in size than the surrounding nucellar cells. Indeed Herms' figure which he uses to illustrate such a division, shows a dividing cell which is very little larger than the surrounding cells.

With present day techniques it is not yet possible to follow the development of a single cell imbedded in a mass of tissue such as one cell in the nucellus of an ovule.

An archesporium is, in the words of Maheshwari (1950), "one cell of the nucellus - (which) - becomes more conspicuous than the others owing to its larger size, denser cytoplasm, and more prominent nucleus."

In accord with this definition and the observed material accumulated in the course of this investigation, the primary archesporial cell of Asimina triloba is sub-hypodermal and functions directly as the megasporocyte cell without giving rise to a parietal cell.

Other than this the development of the embryo sac follows the normal pattern of development as in other plants which have a monosporic, eight nucleate type of embryo sac development.

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The endosperm tissue of Asimina as it is being grown in vitro is a tissue which grows rapidly and which should be very good for the investigation of problems concerned with the nutrition and differentiation of cells and organs in plants.

While there was only one instance in which any sign of differentiation appeared during the course of the experimental work, experiments are being continued and a new crop of tissue is being established in vitro.

Asimina triloba as a plant seems to be very unregenerative. During the course of this work many attempts were made to induce rooting of stems of this plant through the use of conventional methods of making cuttings from the stems as well as by the use of chemical growth substances. While the buds of dormant branches opened and produced normal flowers and leaves there was no instance of even one root appearing.

SUMMARY

The ontogeny of the ovary, ovule and seed of Asimina triloba has been investigated in all stages of its development from the dormant flower buds up to the mature fruits with seeds as they are shed from the trees.

The ovule is anatropous, bitegumentary, and the embryo sac follows the "normal" or "Polygonum" type of development similar to that of other plants which have a monosporic, eight nucleate type of embryo sac development.



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Pollination is porogamous and following fertilization there is great development of all the tissues present in the ovule.

The inner integument produces ruminations which develop from wrinkling of this structure. These wrinklings are caused by an overgrowth of the inner integument and they grow inward, pushing the nucellar and endosperm tissues before them.

Almost immediately following fertilization the endosperm develops into a long, narrow column of cells extending from the nucellar cap to the hypostase. This first endosperm column in crushed by the advance of the ruminations of the inner integument except in the region of the micropyle.

When the ruminations have attained their growth the endosperm surrounding the tiny embryo at the micropylar end of the seed again grows up into a column of endosperm tissue which rapidly spreads out to fill the spaces left among the ruminations of the integument. The endosperm in the seed is filled with oil and there is no starch present in the mature seeds.

Endosperm tissue was grown in vitro in pure culture and experiments performed on the tissue in an effort to induce differentiation in the cells or regeneration of new plant parts from this tissue.

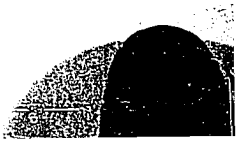
The tissue requires an acid medium of about pH 5.0 . It will not grow well on media containing unautoclaved malt or yeast extracts. Indole acetic acid inhibits the

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rate of growth of the tissue but some concentrations of glutathione and casein hydrolysate give a slightly better rate of growth. The tissue grows well on media containing 2% sucrose or ½% glucose as the carbohydrate source. It is capable of growing very slowly on media containing starch as the carbohydrate.

It was not possible to induce the formation of chlorophyll or chloroplasts in the tissue by means of light and physical factors of light and cold have no effect on the tissue.

In one instance in which tissue masses were grown enclosed in small sections of glass tubing pushed into the sterile nutrient agar medium, there was a slight differentiation of the cytoplasm of the cells in the middle of the mass of tissue.



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