DOCTORAL DISSERTATION SERIES

Publication No.: 18, 655

AUTHOR: Forrest Frederick Stevenson, Ph. D., 1956

University of Michigan

TITLE: THE BEHAVIOR OF CITRUS TISSUES AND EMBRYOS IN VITRO

University Microfilms, Ann Arbor, Michigan

THE BEHAVIOR OF CITRUS TISSUES AND EMBRYOS *IN VITRO*.

by
Forrest F. Stevenson

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in the
University of Michigan
1955

Doctoral Committee:

Professor Carl D. LaRue, Chairman
Professor Chester A. Arnold
Assistant Professor Norman E. Kemp
Professor Frederick K. Sparrow
Assistant Professor Warren H. Wagner

THE BEHAVIOR OF CITRUS TISSUES AND EMBRYOS *IN VITRO*

by Forrest F. Stevenson

The natural propensity of the citrus nucellus to form buds which develop into embryos in the embryo sac has led to an investigation directed along two lines:

1. A study of the behavior of tissues from flowers, fruits, and seeds of *Citrus* when placed in artificial culture.
2. Observations on the pattern of development and rooting ability of nucellar and gametic citrus embryos excised at all stages of growth and grown *in vitro*.

Part One describes the origins of callus tissues from the stigma, style, ovary contents and juice sacs of an unknown species of lemon, and from the seed coat and juice sacs of *Citrus auranteum*, the sour orange. The calluses which were capable of unlimited growth in tissue culture, are the first to be reported from those structures in any plant.

Calluses produced from the lemon were readily distinguishable from those obtained from *Citrus auranteum*. Those derived from different structures within a given species appeared identical.

Growth, which occurred best when cocoanut milk was added to the basic agar medium containing modified White's solution, was largely confined to regions near the margins of the calluses where new cells were laid down in files. Filamentous growth at the periphery of older cultures of *Citrus auranteum* was common.

Plastids were abundant in terminal cells of the callus masses, and cultures formed chlorophyll when exposed to bright diffuse daylight or to continuous fluorescent illumination. Differentiation of the calluses did not occur beyond the formation of tracheid-like cells. Almost all chromosome counts revealed the polyploid condition although some counts gave the normal diploid number.

Part Two describes the normal development of Citrus auranteum embryos and contrasts it with the behavior of nucellar and gametic embryos of Citrus auranteum and Citrus mitis, the Calamondin, in artificial culture. White's medium alone proved ineffective for growth of proembryos and embryos excised at the time of early cotyledonary formation, but some growth and differentiation were obtained when cocoanut milk was added to the medium. A more normal growth pattern was obtained from proembryos cultured in a liquid medium than on agar. Embryos on the surface of a solid medium often enlarged to form great spherical masses from which short cotyledon-like structures emerged after several weeks in culture. Those cultured in liquid produced green cotyledons of various sizes which were thin and leaf-like. Embryos excised at the stage of early cotyledonary formation and grown either in liquid or on agar formed three kinds of cotyledons. Some embryos possessed cotyledons which were huge, leaf-like organs with prominent oil glands. In some instances the cotyledons did not flatten, but grew out as cylindrical, horn-like structures. Cotyledons of a

third type were formed which were similar to the second type, but peltate structures arose at the tips of the elongated, cylindrical outgrowths.

All embryos failed to root until they had attained their near maximum length of ten to twelve millimeters. Embryos capable of root and shoot formation could be obtained from green fruits within five to six months after pollination, and three to four months before the fruits ripened.

ACKNOWLEDGEMENTS

The author is especially grateful for the encouragement and guidance which Dr. C. D. LaRue offered during the course of this work. The suggestions and criticisms made by the members of the doctoral committee were of great help.

Appreciation is extended to Dr. Mortimer Cohen and other members of the Citrus Experimental Station at Lake Alfred, Florida for the use of their facilities, and for providing some of the material used in this work.

TABLE OF CONTENTS

Page

ACKNOWLEDGEMENTS.....ii

TABLE OF CONTENTS.....iii

LIST OF TABLES.....v

LIST OF ILLUSTRATIONS.....vi

INTRODUCTION.....1

PART ONE: THE ORIGIN AND CULTURE OF CALLUS TISSUES.....5

Introduction.....5

The Morphology and Anatomy of the Citrus Flower and Fruit.....7

Materials and Methods.....10

Observations and Results.....13

Origins of the calluses.....13

Stigma and Style of Citrus Tibet.....13

Radial Carpel Walls and Central Axis of Citrus Tibet.....18

Juice sacs of Citrus Tibet.....20

Juice sacs of Citrus auranteum.....23

Seed Coats of Citrus auranteum.....23

Morphology and Histology of the Calluses.....24

Calluses of Citrus Tibet.....24

Calluses of Citrus auranteum.....31

Cytology of the Calluses.....32

Calluses of Citrus Tibet.....32

Page

Calluses of *Citrus auranteum*.....37

Manner and Rate of Growth of the Calluses.....38

Calluses of *Citrus Tibet*.....38

Calluses of *Citrus auranteum*.....40

Discussion.....40

PART TWO: THE CULTURE OF CITRUS EMBRYOS.....45

Introduction.....45

Materials and Methods.....45

Observations and Results.....56

The Development of *Citrus auranteum* Embryos in the Seed.....59

Proembryo.....59

Heart Stage.....60

Older Stages.....61

Maximum-sized Embryos.....62

In Vitro Development of Citrus Embryos.....65

Proembryos.....65

Proembryos in Liquid Cocoanut Media.....65

Proembryos on Agar.....74

Heart Stage.....79

Embryos Beyond the Heart Stage.....85

Older Embryos.....85

Pleiocotyly in Citrus Embryos Grown *in Vitro*..87

The Culture of Dwarfed Embryos.....92

Discussion.....93

SUMMARY.....103

LITERATURE CITED.....107

LIST OF TABLES

TABLE		Page
I	THE BASIC CULTURE MEDIUM	12
II	VARIOUS MEDIA FORMED BY MODIFICATION OF THE BASIC MEDIUM	13
III	ORIGINS OF CALLUSES DERIVED FROM CITRUS FLOWERS AND FRUITS	14
IV	INITIAL MEDIA USED IN CULTURING CITRUS EMBRYOS	54
V	ROOTING MEDIA USED IN CULTURING CITRUS EMBRYOS	54
VI	THE EFFECT OF THE VARIATION OF COCOANUT MILK ON *CITRUS AURANTEUM* EMBRYOS	55
VII	THE COMPARATIVE SIZES OF *CITRUS AURANTEUM* EMBRYOS AT THE YOUNG HEART STAGE WHEN GROWN *IN VIVO* AND *IN VITRO*	66
VIII	THE NUMBERS OF EMBRYOS THAT PRODUCED COTYLEDONS *IN VITRO* FROM EACH OF 25 EMBRYO SACS OF *CITRUS AURANTEUM*	75
IX	THE NUMBERS OF HEART EMBRYOS OF SOUR ORANGE WHICH EXHIBITED SOME DEGREE OF GROWTH ON LIQUID AND SOLID MEDIA	80
X	SIZE INCREASE OF TEN *CITRUS AURANTEUM* EMBRYOS AFTER EIGHT MONTHS ON 90% COCOANUT AGAR	85
XI	SIZE INCREASE OF TEN *CITRUS AURANTEUM* EMBRYOS AFTER EIGHT MONTHS ON WHITE'S AGAR	86
XII	THE OCCURRENCE OF PLEIOCOTYLY IN SOUR ORANGE HEART EMBRYOS ON 90% COCOANUT AGAR	88
XIII	THE OCCURRENCE OF PLEIOCOTYLY IN SOUR ORANGE EMBRYOS GROWN IN 90% LIQUID COCOANUT MEDIUM	91
XIV	INCREASE IN SIZE OF DWARFED *CITRUS AURANTEUM* EMBRYOS AFTER 100 DAYS IN MALT EXTRACT MEDIUM	93

LIST OF ILLUSTRATIONS

PLATE		Page
I	THE ORIGINS OF CALLUS FROM *CITRUS TIBET* TISSUES	16
II	THE ORIGINS OF CALLUS FROM CITRUS TISSUE	22
III	THE CELLULAR STRUCTURE OF CITRUS CALLUS	27
IV	CALLUS CULTURES OF *CITRUS*	30
V	THE CHROMOSOMES OF CITRUS CALLUS	35
VI	THE CELLULAR STRUCTURE OF NORMAL *CITRUS AURANTEUM* EMBRYOS	58
VII	CITRUS EMBRYOS	64
VIII	THE EARLY NORMAL DEVELOPMENT OF *CITRUS AURANTEUM* EMBRYOS	68
IX	THE DEVELOPMENT OF *CITRUS AURANTEUM* EMBRYOS *IN VITRO*	70
X	*CITRUS AURANTEUM* EMBRYOS *IN VITRO*	73
XI	*IN VITRO* DEVELOPMENT OF CITRUS EMBRYOS	77
XII	*IN VITRO* DEVELOPMENT OF CITRUS EMBRYOS	83
XIII	PLEIOCOTYLEDONOUS EMBRYOS GROWN *IN VITRO*	90

INTRODUCTION

The ability of most members of the genus Citrus to form pseudoembryos from the nucellus has been recognized since 1878 when Strasburger (1878) investigated polyembryony in that genus.

The initiation of an embryo from a single cell of the nucellus at a stage when the latter is fairly well developed (Strasburger, 1878) is a high expression of cellular totipotency. A tissue like this one which can respond in such an interesting manner to stimuli arising from the embryo sac seems well adapted for experimental culture in vitro.

Accordingly, during the summer of 1952, a few preliminary experiments were begun on two species of Citrus to see if it would be possible to obtain a callus from cultures of the nucellus. Although this tissue proved unsuitable, various other floral parts as well as regions of the young fruit began producing tissues from unusual places. It seemed advisable to develop these calluses because of their origins, and because it provided an excellent opportunity not only to compare calluses derived from different structures of the same plant but also to compare those derived from the same plant parts but from different species of Citrus.

A search of the literature revealed that while research on Citrus has been extensive, it has been conducted

largely on the horticultural side, no tissue culture studies having been reported for Citrus.

An interesting feature of nucellar budding in Citrus is the fact that the outgrowths which project into the embryo sac form embryos with all the structures normally associated with the gametic embryo. As many as two dozen embryos may be formed within a single sac. There they compete with each other for space and nutrition until one or two gain the ascendancy. The mature seed then contains only those embryos of mixed origins capable of subsequent germination, the other embryos remaining dwarfed in various stages of development or becoming completely squeezed out of existence.

Apart from their bizarre biological aspects, nucellar embryos have a definite commercial value. Although they mature no faster than the gametic ones, they possess certain characters which often make their seedlings superior to those obtained from the gametic embryos. Citrus fruits mature slowly, however, and nearly nine or ten months must elapse from the time of pollination until ripe seeds can be obtained for planting. This delay, while of no great inconvenience in starting seedlings for the orchard, is disadvantageous to experimental workers who are interested in the genetical and pathological aspects of Citrus.

It was decided to try culturing citrus embryos in vitro from as early stages as possible up to well developed embryos with the following objectives in mind:

1. To ascertain the youngest stage of embryo development from which it would be possible to obtain seedlings.
2. To observe the behavior of very young nucellar embryos when excised and cultured away from the formative influences of the fluids of the embryo sac.
3. To obtain a greater number of nucellar embryos per seed by culturing each embryo with adequate space and nutrition.

Although tissue culture has been used as a technique in the subsequent investigations, it will not be necessary to review its history. The reader has but to turn to the reviews of White (1936, 1946, and 1954) for a historical account of the development of plant tissue culture as a tool in science.

The term "tissue culture" itself should be defined, however, for in the culture of plants several groups of tissues or even whole organs are often cultured, and in such instances the term seems inappropriate. No definition exists which encompasses all phases of culturing plant material <u>in vitro</u>, but White (1936) has suggested a definition of plant tissue culture which with a few exceptions is still valid today: " A plant tissue culture is any preparation of one or more isolated somatic plant cells which grows and functions normally <u>in vitro</u>, without giving rise to an entire plant".

In a later paper White (1946) places plant tissue

cultures into four categories:

1. Root cultures
2. Cultures of other members such as stem tips
3. Culture of undifferentiated masses (calluses)
4. Embryo culture

The last is added, White explains, not because it conforms to his earlier definition, but because it has been so closely associated with tissue culture.

It is in the same sense that White has used the term "tissue culture" that it will be used here. Because of the dual nature of the problem, the work will be discussed in two parts. In the first will be treated the origin and culture of callus tissues, and in the second part the culture of citrus embryos.

PART ONE

THE ORIGIN AND CULTURE OF CALLUS TISSUES

Introduction

One aim of culturing plant tissues is the attainment of a mass of cells which is capable of unlimited growth *in vitro* and, as a consequence, with little cellular differentiation. This aim has been achieved and such calluses derived from a number of plants are available for the study of growth and nutritional requirements.

While it is possibly true that all parts of the plant may yield callus under certain conditions, this theory has not been translated into actual practice. Young tissues, even though still meristematic, do not always give rise to callus when placed in culture and it is impossible to predict which tissues or organs will lend themselves to successful culturing. Because plant tissue culture is still in an exploratory stage, the report of the isolation of tissues from heretofore unreported sources is still of interest.

Until recently, nearly all calluses grown in culture had been obtained from limited sources. The first *in vitro* tissues with unlimited growth to be reported were derived from the procambial strands of young stems of *Nicotiana* by White (1939b) and from the fleshy root of carrot by Gautheret

(1939) and by Nobecourt (1939). These were followed by tissues derived from fleshy roots or stems of several other plants. Morel (1944), who was the first to report the culture of a callus from a woody plant, isolated his tissue from the stem of grape.

The successful culture of a callus derived from crown gall by White and Braun (1942) provided a new source of material which could be cultured, and much work has been done with crown gall and tumorous tissues by several workers.

Within the last few years the list of tissues cultured has been extended by reports of the production of calluses from several new sources. Tissues have been derived from the prothalli of Osmunda by Morel and Wetmore (1951), from Ginkgo pollen by Tulecke (1953), and from the female gametophytes of Zamia and Cycas by LaRue (1948 and 1950). Even Haberlandt's (1902) idea of a tissue derived from a single cell seems to have been realized by Muir, Hildebrandt and Riker (1954) who report such a culture from single cells of tobacco stem callus.

The literature dealing with the culture of fruits and floral parts is not extensive. LaRue (1942) rooted flowers of many species in culture and was able to produce fruits which grew to nearly full size from flowers of Kalanchoë and Forsythia. Taylor (1948) cultured anthers of Tradescantia for the purpose of studying meiotic behavior of the pollen mother cells. The above work, however, was executed with different purposes in mind than in obtaining undifferentiated growth. Callus cultures have been reported from the endo-

sperms of three different plants: Zea mays by LaRue (1949), Asimina triloba by Lampton (1952), and Lolium perenne by Norstog (1955). Other floral structures have not been reported as sources of callus. It has been found in this investigation, however, that flowers and fruits of two species of Citrus are capable of producing calluses from a number of places. The pistil has yielded callus from the stigma, style, and portions of the ovary contents. Young fruits have given rise to tissues from seed integuments and developing juice sacs.

Because of their unusual origins, a large portion of this section will deal with the initiation of the calluses. It is desirable, therefore, to present a short description of the citrus flower and fruit.

The Morphology and Anatomy of the Citrus Flower and Fruit

The calyx of the citrus flower is composed of a number of fused sepals and surrounds the base of the large, thick petals which may vary in number from four to eight with five the usual number (Webber and Batchelor, 1948). The stamens are numerous and their filaments are fused at the bases into small groups.

A pad of thick green tissue called the disc rests on the tip of the receptacle above the points of attachment of the stamens and petals. It is upon the disc that the pistil rests. The pistil itself is large. The stigma consists of a mound of tissue whose epidermal cells are elongated for the

retention of pollen at the stigmatic surface. The style may be quite slender as in C. auranteum, the sour orange, or it may be short and thick as in certain lemons. In the latter case, the ovary is only slightly larger in diameter than the style so that externally the two are not sharply delimited.

Longitudinal sections of the stigma and style reveal that they are composed primarily of isodiametric parenchyma cells limited externally by a single layer of epidermis. Vascular strands alternating with stylar canals run the length of the stigma and style. The stylar canals which open on the stigmatic surface provide a path for the pollen tubes to travel down to the locular cavities of the ovary. The progress of the pollen tubes through the style is apparently aided by secretions from special cells lining the canals. Large oil glands of a lysigenous nature (Webber and Batchelor, 1948) appear throughout the stigmatic and stylar tissue.

The ovary is composed of a varying number of carpels which corresponds to the number of stylar canals (Tillson and Bamford, 1938). At maturity the locules are filled with juice sacs and seeds so that each carpel with its contents makes up an individual segment of the mature fruit.

A cross section of the young ovary resembles a wheel. The central axis and the radiating carpel walls form the hub and spokes respectively, and the ovary wall forms the rim of the wheel. According to the interpretation of Webber and Batchelor (1948), the ovary wall is composed of a single outer epidermis, a hypodermis consisting of several layers of

collenchyma, a wide layer of parenchymatous cells forming the mesocarp of the fruit, and an inner epidermis which is continuous with the epidermis of the radial carpel walls.

The central axis is composed of isodiametric parenchyma cells and strands of vascular tissue. The parenchyma of the mesocarp and the central axis are quite similar in appearance, and indeed, the two tissues are joined at intervals by the parenchyma of the radial carpel walls. As the fruit matures the cells of the carpel wall become elongated and finally become disorganized or become so fragile that they are easily torn when the segments of the fruits are separated. The radial carpel walls, therefore, serve as septa which divide the fruit into segments. Within each locule two vertical rows of anatropous ovules arise from the bases of the carpel walls adjacent to their points of union with the central axis.

The origin and structure of the juice sacs of Citrus have been described by several workers (Grew, 1682; Matlack, 1931; Davis, 1932; and Ford, 1942). Just before the flower opens or shortly thereafter centain parenchyma cells of the mesocarp lying just below the inner epidermis begin mitotic divisions. They produce multicellular papillae which begin to project into the locular cavities. The inner epidermis, however, keeps pace with the developing papillae so that the young juice sacs are composed of an outer layer of epidermis and internally of thin-walled parenchyma cells derived from the mesocarp.

In the Eureka lemon (Ford, 1942) nearly all cell divisions have ceased by the time the fruit has reached eight millimeters in diameter, and subsequent growth is accomplished by cellular enlargement. The parenchyma cells become largest in the center of the sac, and the cells closer to the epidermis are often smaller and less isodiametric. As the cells become filled with juice each sac becomes somewhat club-shaped and is attached to the outer carpel wall by a thin stalk.

Longitudinal sections of a young seed reveal the embryo sac surrounded by a nucellus composed of a few layers of thin parenchyma cells. Surrounding the nucellus is an inner integument and an outer integument. The inner integument is composed of a single layer of thick-walled cells adjacent to the nucellus and a few layers of thin-walled parenchyma cells without. The outer epidermis is much thicker, but its cells appear similar to the parenchyma of the inner integument. The cells of the external epidermis of the seed continue to elongate in a longitudinal direction and increase in height at the same time (Strasburger and Hillhouse, 1893). Eventually, the walls of the epidermal cells become mucilaginous to produce the slippery seeds characteristic of the genus.

Materials and Methods

Flowers and fruits were obtained from trees of Citrus auranteum, the sour orange, and an unknown species of lemon from Tibet, hereafter referred to as Citrus Tibet. Both

species are growing in the greenhouse at the University of Michigan Botanical Gardens. Citrus auranteum flowers in February or the first of March in the greenhouse, but its blossoming season approximates that of trees in the citrus belts. Citrus Tibet, while flowering intermittently throughout the year, blooms heaviest around the first of the year. Most of its flowers are staminate, and perfect flowers occur only rarely. The plant is said to be cold resistant and bears fruits well over six inches in length.

The flowers were taken either in the early bud stage or just before the opening of the buds. They were sterilized in 20% Clorox or by flaming the surface prior to cutting. The surface of the fruits were sterilized in the same manner. The rinds were cut halfway through with a sterile scalpel and then torn apart by twisting the two halves. The immature seeds were removed with sterile forceps and placed into culture in the manner described on page 23. The young juice sacs were torn out at the same time and as many as two dozen of the sacs per bottle were placed on the surface of the agar medium.

A basic medium (Table I) was used alone for some culturing, but oftener other media were used by the addition of various organic substances to the basic medium (Table II).

The pH of the media was adjusted to approximately 5.5--6.0. The cultures for the most part were kept at room temperature and in diffuse light. On other occasions, cultures were grown at approximately 22° Centigrade when observations were being made on the growth rates. Some

calluses were isolated and kept in total darkness, and others were exposed to direct sunlight or fluorescent light in order to study the effect of light on the callus.

TABLE I

THE BASIC CULTURE MEDIUM

Modified White's solution*	1000 ml.
Sucrose	20 gm.
Indole-3-acetic acid	1 mg.
Agar	8 gm.

* White's solution was prepared according to directions in the Handbook of Tissue Culture (White, 1943) except for the following modifications which have been employed in LaRue's laboratory:

a. Substitution of 10 mg./l. of ferric citrate for ferric chloride.
b. Substitution of Nitsch's (1951) trace elements for White's trace elements.
c. Increase of vitamin and glycine content 2.5 times that of White's concentration.
d. Addition of 0.25 mg./l. of calcium pantothenate.

Callus sections of 10μ in thickness were stained with safranin and fast green, or with hematoxylin and fast green according to instructions of Johansen (1940). Chromosome studies were made from smears stained with aceto-orcein (La Cour, 1941).

TABLE II

VARIOUS MEDIA FORMED BY MODIFICATION OF THE BASIC MEDIUM

1.	Basic medium plus yeast extract	2500 mg./l.
2.	" " " cocoanut milk	225 ml./l.
3.	" " " cocoanut milk	890 ml./l.
4.	" " " cocoanut milk (40 gm. sucrose)	890 ml./l.
5.	" " " adenine sulphate (no IAA)	40 mg./l.
6.	" " " adenine sulphate	1 mg./l.
7.	" " " desoxyribonucleic acid	500 mg./l.

Observations and Results

Origins of the Calluses

Most of the calluses were first isolated on the basic medium alone, although it was possible to use almost any variation of the medium and accomplish the same results with perhaps some exceptions as to the rapidity of growth.

Although flowers and fruits of both C. auranteum and C. Tibet were cultured, the two species did not behave in a similar manner. For that reason it was possible to obtain calluses from some parts in C. Tibet that were not possible from C. auranteum. Table III tabulates these callus origins and the plants producing the calluses.

Stigma and Style of C. Tibet. -- As might be anticipated, the stigmas and styles taken from younger buds re-

sponded much better to culturing than those taken from nearly mature or mature buds. Stigmas of young pistils were severed from the styles and the styles in turn were cut off just above the ovaries. Thus, the stigmas and styles were cultured as separate units.

TABLE III

ORIGINS OF CALLUSES DERIVED FROM CITRUS FLOWERS AND FRUITS

Plant Part	C. Tibet	C. auranteum
Stigma	X	
Style	X	
Radial carpel walls and central axis	X	
Seed coat		X
Juice sacs	X	X

Proliferation of tissue from both cut ends of the style and from the single cut end of the stigma usually occurred within three or four days after being placed on 90% cocoanut agar. Longitudinal sections of material made at this time revealed that no cell divisions were involved in this early increase of tissue. The cells along the cut surfaces had become greatly elongated and produced loose masses of torn and disintegrating cells. Although this growth appeared impressive at first, it soon became opaque and lusterless as the cells died. The first callus made

PLATE I

THE ORIGINS OF CALLUSES FROM *CITRUS TIBET* TISSUE

Figure 1. The stigmatic surface of a young pistil after eleven days on 90% cocoanut agar. A cambium-like layer of cells has begun to form a short distance below the epidermis. X 100.

Figure 2. The papillose epidermal cells of the stigma after six days on a 90% cocoanut agar medium. They contain inclusions which resemble aleurone grains normally found in the cotyledons. Stained with safranin. X 440.

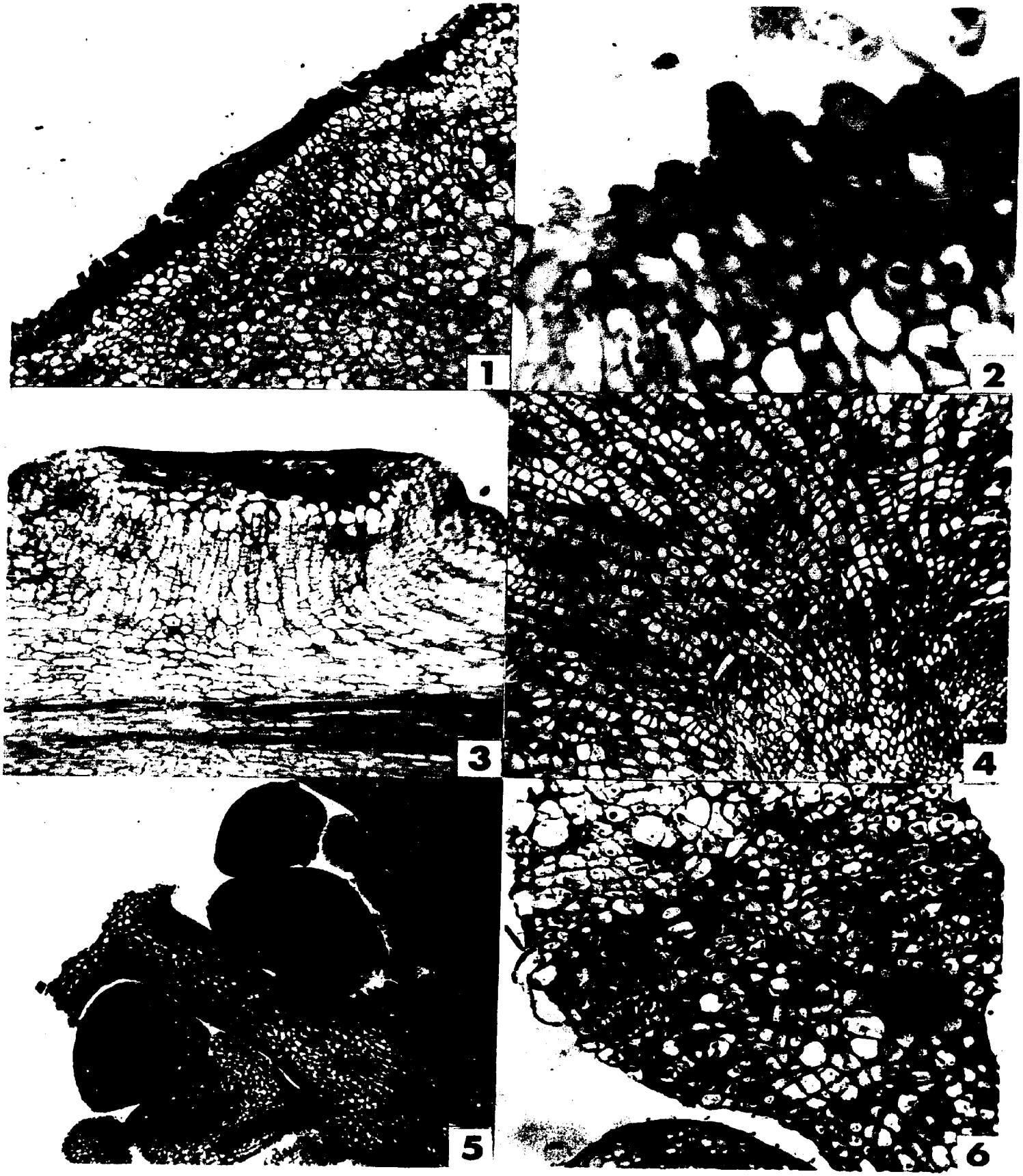
Figure 3. A portion of a style after eleven days on a 90% cocoanut agar medium. The first stage of callus formation has begun with the formation of a cambium-like layer below the epidermis, thus cutting off files of cells. X 100.

Figure 4. Young callus formed from a region of a style in the manner described in Fig. 3. The files of cells are clearly visible as they radiate from the stylar tissue at the lower right hand corner of the photograph. X 100.

Figure 5. A portion of an uncultured ovary showing ovules and a radial carpel wall. X 100.

Figure 6. A radial carpel wall similar to Fig. 5 from ovary contents cultured for fifteen days on a 90% cocoanut agar medium. Early callus formation which has begun at the wounded area of the carpel wall will finally spread to its proximal end as well. Nuclei are clearly visible. About X 200.

PLATE I



its appearance later at the cut surfaces as a tighter mass of tissue originated below the epidermis and rolled back over the surface of the style and stigma. This later growth originated from the parenchyma cells a few layers below the epidermis. Without any previous enlargement, isolated patches of cells began a systematic series of divisions to produce files of cells (Plate I, fig. 3). These meristematic regions with the plane of division occurring consistently in one direction often were cambium-like in appearance. Cell division seemed to be confined to the terminal cells in a file although mitotic divisions were not observed. As the files of cells increased in length, the parenchyma layers between them and the epidermis became crushed. Their walls stained bright red and cell contents disappeared. As growth continued below, these parenchyma cells together with the epidermis were eventually sloughed off.

Although this sequence of callus formation occurred in many places along the length of the style or under the papillose epidermis of the stigmatic surface (Plate I, fig. 1) most activity at first was confined to regions adjacent to the cut surfaces. Indeed, files of cells were usually started from the parenchyma cells lying in back of those cells which had elongated greatly in the first few days after the cultures were started. In this way the enlarged and disintegrating cells were eventually replaced by callus.

Even after the callus was well started, microscopic examination of the swollen callus mass revealed distinct

rows of cells radiating out from the points of origin (Plate I, fig. 4). As the callus became older and the cells began to enlarge, the definite rows became less distinct.

Radial Carpel Walls and Central Axis of C. Tibet. -- Cultures of the entire ovary contents were made by tearing out the radial carpel walls and central axis with ovules attached from the dissected ovaries of unopened buds and placing the entire ovary contents on agar (Plate I, fig. 5). In some instances cultures were made of ovules without any other tissue present. The first indications of activity occurred within three or four days as the ovules and the free ends of the carpel walls began to enlarge. At the end of a week on 90% cocoanut agar, the pale yellow mass possessed a definitely nodular appearance. Eventually, the nodules became less marked as true callus production began.

Microscopic examination of the ovary contents at the end of two weeks revealed that callus was originating from the radial carpel walls, central axis, and ovules. The injury sustained by the carpel walls as they were torn from the ovary apparently furnished the stimulus for callus production, for it was in the torn areas that cellular enlargement and division first occurred (Plate I, fig. 6). The nuclei of the parenchyma cells in these areas became large and stained darkly while many of the cells enlarged to double their original size and often became binucleate. Mitotic divisions were common, and occasionally both nuclei in a binucleate cell could be seen dividing simultaneously.

This pattern of cellular enlargement and division, while beginning at the free ends of the carpel walls, eventually spread to all the parenchyma cells of the walls. The great increase in cellular dimensions together with proliferation of the parenchyma caused the walls to become fan-shaped. The epidermal cells which were narrowly rectangular often became elongated at right angles to their former axes, but beyond this they did not enter into callus formation.

The early divisions of the parenchyma cells of the radial carpel walls were oriented in no one particular plane, but later divisions of the cells in the periphery of the mass were mostly of a tangential nature. The files of cells produced as a consequence marked the beginning of the true callus, for it was from the cells in this region that subsequent growth occurred.

Callus formation was not confined to the radial carpel walls alone. Certain portions of the central axis were also observed in a meristematic state apparently induced by injury at the time of culturing. The cells of the axis are similar to those of the radial carpel walls and the formation of callus proceeded in the same manner as described previously (Plate II, fig. 1).

The ovules that had become separated from the placenta and were lying free on the surface of the agar showed earlier activity than those still attached. The great increase in size of the ovules noted previously resulted from cellular enlargement and division of the cells in the outer integument

(Plate II, fig. 6). Cellular activity marked by prominent nuclei and mitotic figures began in the region adjacent to the micropyle and moved downward. The cells of the inner integument occasionally showed some degree of enlargement. Although isolated patches of callus could be seen forming at the margins of the outer integument, further callus production halted shortly thereafter. Several cultures of isolated ovules were made, but aside from general enlargement which occurred over a several week period, the cultures went no farther.

Juice Sacs of C. Tibet. -- Immature juice sacs approximately five millimeters long were taken from a fruit about six centimeters long and placed on 90% cocoanut agar. Within a few days proliferations could be noticed in several regions over the surface of the sacs. Growth was especially active at the basal end of the sacs probably because of wounding (Plate II, fig. 2). In wounded areas some degree of cellular enlargement preceded callus formation, but for the most part, callus originated in a nodular fashion. Within each nodule cell divisions occurred both radially and tangentially so that no definite files of cells were formed similar to those in the stylar tissue. In unwounded areas, the parenchyma cells immediately below the epidermis formed similar nodules of callus without definite cellular orientation (Plate II, fig. 3). As the nodules increased in size, they often crowded against one another until their individual identities were lost in the common mass of callus.

PLATE II

THE ORIGINS OF CALLUSES FROM CITRUS TISSUE

Figure 1. A portion of the central axis from the contents of a C. Tibet ovary after fifteen days in culture. Callus formation has begun from a wounded spot of the axis. X 100.

Figure 2. The wounded end of an immature juice sac of C. Tibet after seventeen days on 90% cocoanut agar. Callus growth has begun at the margins of the enlargement. X 100.

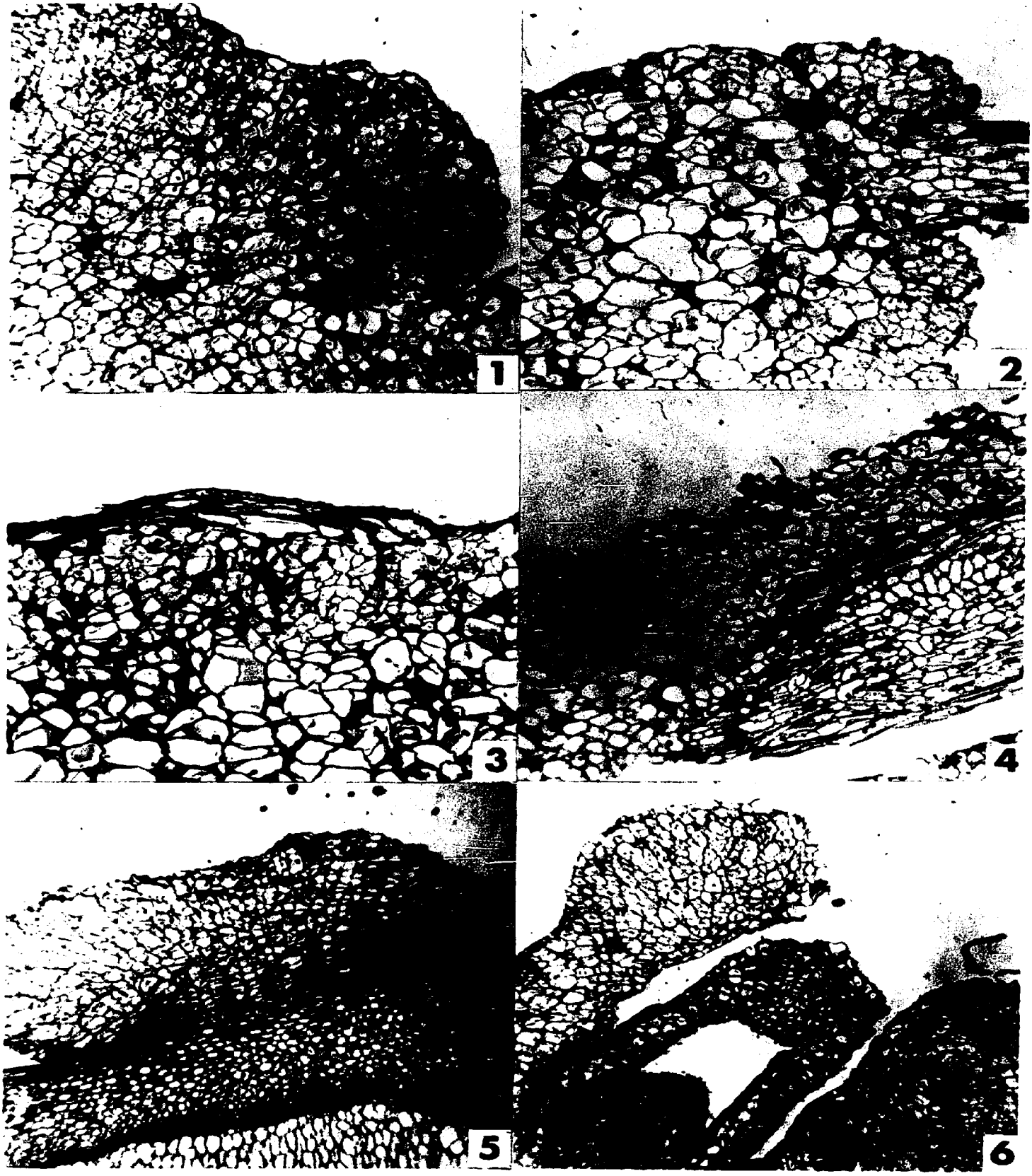
Figure 3. A portion of an immature juice sac of C. Tibet in which nodular regions of early callus formation have arisen from the cells immediately below the epidermis. X 100.

Figure 4. Callus originating from a juice sac of C. auranteum. Inclusions similar to those shown in Plate I, fig. 2 are visible in the cells of the juice sac. X 100.

Figure 5. A portion of the outer integument of a young C. auranteum seed after twelve days on 25% cocoanut agar. A definite cambium layer has formed rows of cells constituting the young callus. X 100.

Figure 6. Ovules from an unfertilized flower of C. Tibet after fifteen days in culture. Considerable callus has formed at the upper ends of the outer integument. X 100.

PLATE II



Juice Sacs of C. auranteum. -- Immature juice sacs about four millimeters long were taken from fruits approximately 35-40 millimeters in diameter before the sacs were filled with juice. Callus formation from the sacs approximated that described for C. Tibet. The parenchyma cells just below the epidermis seemed to be the most active (Plate II, fig. 4), and the centermost cells often disintegrated in older cultures. Growth was most vigorous at the torn ends of the sacs, but callus did not form from here exclusively. In some instances definite cambium-like meristematic regions were formed from the parenchyma just below the epidermis of sacs which had been in culture several weeks. These places ran for extended distances along the length of the sacs, but gave rise to little callus.

Seed Coats of C. auranteum. -- Most of the seeds were cultured at the stage of early embryo development when the seed length was approximately five millimeters, and the fruits from which they were taken were 35-40 millimeters in diameter. Cultures were started by placing five or six seeds per bottle on the surface of the agar medium. After a few days in culture scattered regions of growth became apparent. This was followed by general proliferation extending over nearly all parts of the seed. Much of this was caused by extreme elongation of certain cells of the outer integument located immediately adjacent to the inner integument. Their elongation pushed the more external cells of the integument against the outer epidermis of the seed, thus rupturing the

latter. Although this elongation of cells often caused large regions of activity, callus never arose from these regions. Cells of the outer integument lying just below the epidermis were responsible for callus formation. Certain areas often assumed a cambium-like appearance (Plate II, fig. 5) as files of cells were produced. The epidermis eventually ruptured as the cells continued to divide. The older cells nearer the periphery enlarged to varying sizes thus causing the rows of cells to radiate somewhat. Mitotic figures were not present in these places and it could not be determined with exactness whether the terminal cells or basal cells were dividing. The increase in size of the cells closer to the surface while the basal cells remained small indicated that the latter were still dividing. As Plate II, fig. 5 reveals, this early callus stage was very suggestive of a lenticel.

Morphology and Histology of the Calluses

Calluses of C. Tibet. -- Although callus tissues were derived from three distinct regions in this species, and the manner of origin differed in slight details, the mature calluses were so similar in appearance as to seem identical. Regardless of the origins, therefore, a description of any one callus is apparently true for the others.

With the exception of the callus derived from the juice sacs, all calluses have been cultured for slightly over two years and have survived repeated subculturing. The callus derived from the juice sacs has been in culture one year and it, too, has been subcultured several times.

A healthy callus culture appeared translucent at the surface and possessed a very slight yellow color, but varying accumulations of dead cells over the surface tended to give it a frosted appearance. As the culture aged, the accumulation of dead cells turned the entire callus white (Plate IV, fig. 1). The oldest portion of the callus which lay on the agar surface usually became an opaque brownish-yellow color within two or three weeks after subculturing. Unlike many calluses which are friable, this callus was so firm and tough that it was necessary to employ a sharp scalpel to cut off pieces for subculturing.

Pieces of callus subcultured on fresh agar showed resumption of growth within a week as irregular or small nodular patches of new growth arose over the surface. The new growth regions could be distinguished from the older tissues by their translucent appearance, but even in their early stages these places appeared faintly frosted at their surfaces.

Sectioned pieces of callus showed that the portions next to the agar were composed of dead cells. The cells toward the periphery contained nuclei which stained poorly but were still living. Their orientation suggested that they had been laid down in files, but the varying degrees of enlargement undergone by the cells nearly obliterated the files.

In general, the cells which made up the callus could be roughly classified into four types according to appearance:

1. Parenchymatous cells with thin walls. These cells made up the bulk of the callus (Plate III, fig. 2).

PLATE III

THE CELLULAR STRUCTURE OF CITRUS CALLUS

Figure 1. Internal structure of a spherical body from C. auranteum callus similar to that in Plate IV, fig. 6. The outer margin of the body is cambial in nature, while the cells toward the center of the sphere (right) have broken down. X 100.

Figure 2. Internal structure of C. Tibet callus derived from the ovary contents showing how a new region of growth arises from a limited portion of the callus. The new growth (a) has fanned out to cover the surface of the older callus (b). Note the enlarged space caused by the disappearance of the cell contents of an enlarged cell (c). X 100.

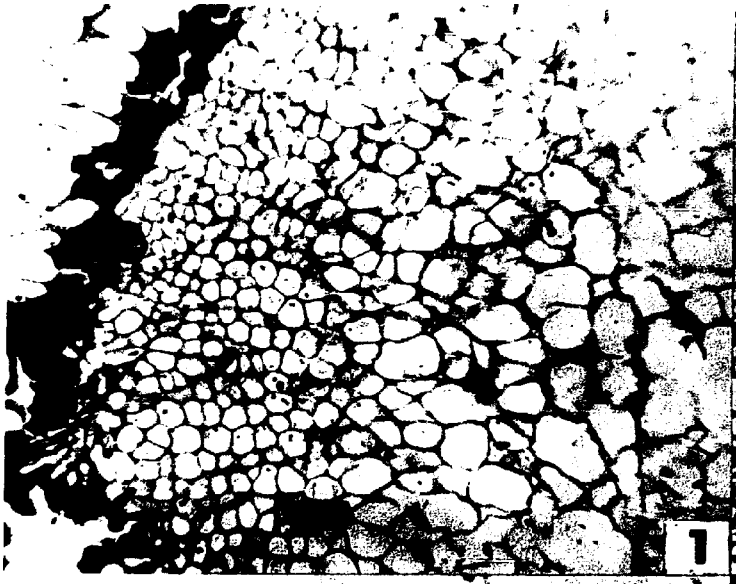
Figure 3. The marginal cells from a callus derived from the juice sacs of C. auranteum. The cell walls are thicker than those of C. Tibet. Note the arrangement of the cells into definite files. X 100.

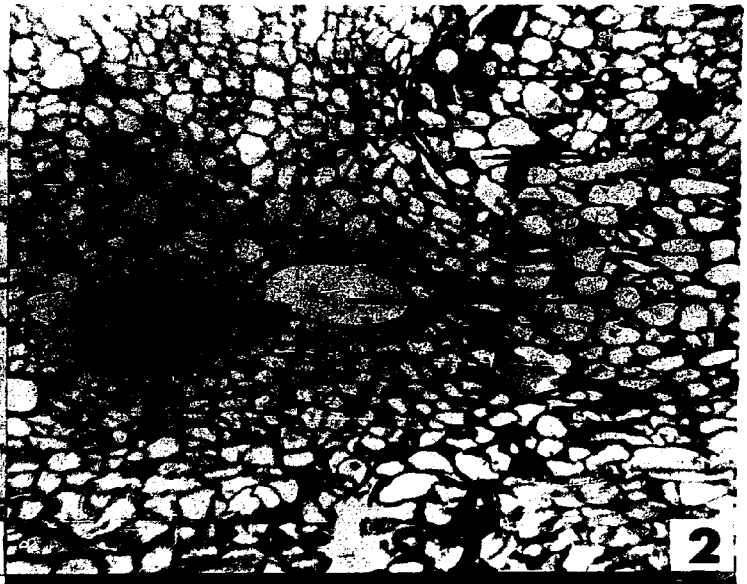
Figure 4. A group of cells with tracheary thickenings in C. Tibet callus. X 440.

Figure 5. Two terminal filaments from C. auranteum callus derived from the seed coat. X 100.

Figure 6. A group of meristematic cells from the margin of C. Tibet callus. Stained with aceto-orcein. X 100.

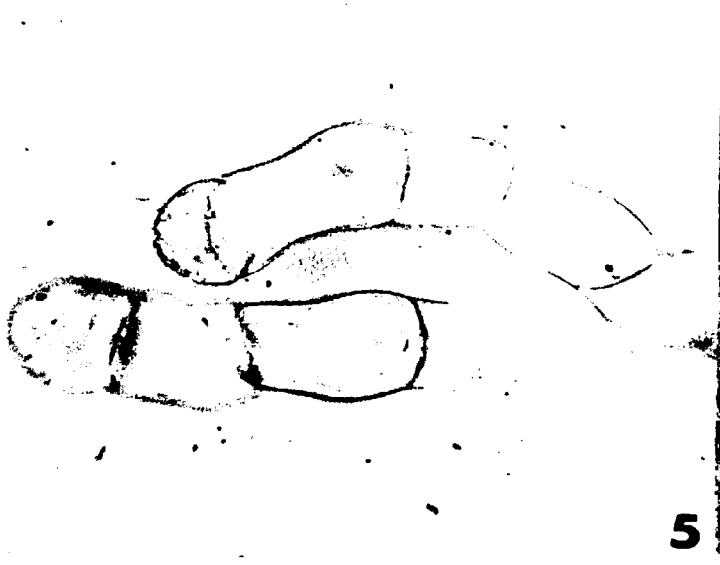
PLATE III

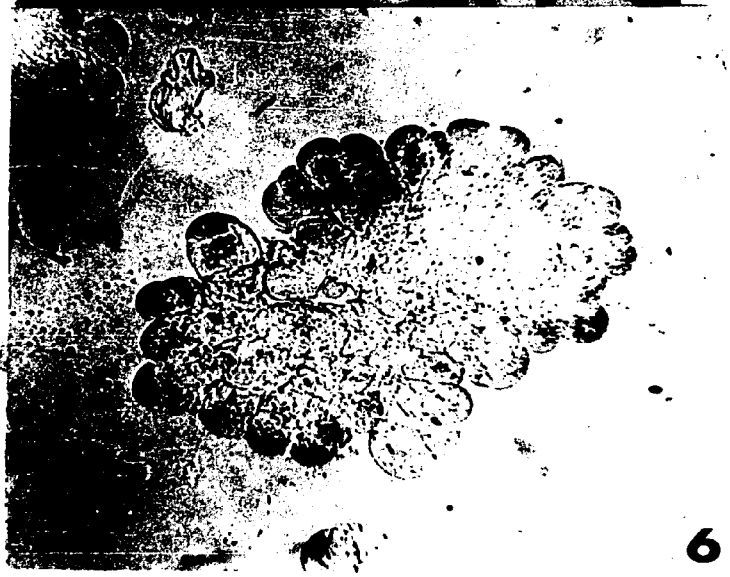

1


2


3


4


5


6

Although somewhat variable in shape, their lengths were almost that of their widths, or if elongated their lengths were not greater than twice their widths. Protoplasts were not always present, but if present they stained only slightly.

2. Greatly elongated ovoid or rectangular parenchymatous cells with lengths often several times their widths (Plate III, fig 2). As these cells enlarged their contents disappeared until at maturity only the walls remained. Their occurrence throughout the callus created many small air spaces. Oftener than not two or three of these cells occurred consecutively in a file of cells. As the cross walls separating them broke down, larger cavities were formed. Occasionally, air spaces were also formed in the callus by a failure of fan-shaped areas of new growth to fill in all spaces they enclosed.

3. Nearly spherical meristematic cells with dense cytoplasm (Plate III, fig. 6). These cells were formed at the periphery of the callus, but seldom showed up on paraffin sections because of their delicate nature. They could be found only by removing marginal cells from fresh callus and making water mounts. Oddly enough, the cells were difficult to locate while they were still meristematic. Terminal cells were usually found after they were no longer dividing and when their contents stained poorly if at all.

4. Cells whose walls had undergone reticulate thickening (Plate III, fig. 4). These tracheid-like cells appeared irregularly in isolated clusters among the other cells, but were not oriented in any particular pattern which

PLATE IV

CALLUS CULTURES OF *CITRUS*

Figure 1. An old culture of *C. Tibet* callus. The central portion is dead and the outer portion is covered with white cells.

Figure 2. An old culture of *C. auranteum*. Here, too, the central portion is dead while the outer cells are organized into filaments.

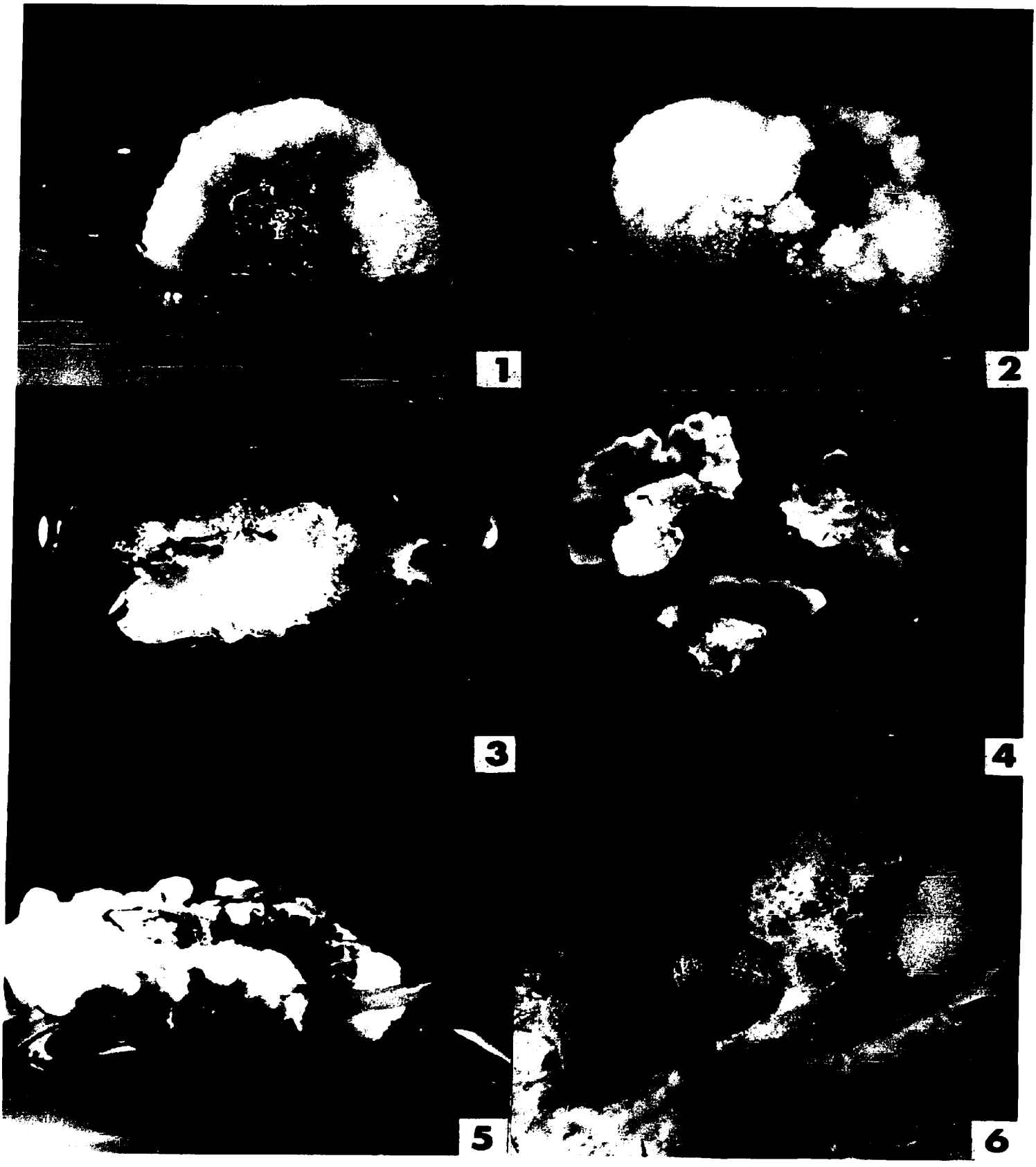
Figure 3. A sectioned portion of stylar callus of *C. Tibet* stained with iodine to show starch distribution.

Figure 4. Sections of *C. Tibet* callus derived from the ovary contents and stained with tetrazolium chloride to show living portions of the callus. The dark regions are living cells. Note the peripheral layer of non-living cells.

Figure 5. A young culture of *C. auranteum* callus derived from the juice sacs. One sac has elongated greatly after callus formation.

Figure 6. A hollow spherical enlargement which arose on a few occasions in callus cultures derived from *C. auranteum* juice sacs. See Plate IV, fig. 1.

PLATE IV



could have been interpreted as a vascular system. The frequency with which these cells were encountered while searching for chromosomes in the marginal cells of the callus indicated that they had differentiated at an early stage from the meristematic cells in that region.

Calluses of C. auranteum. -- In an active state, the calluses of C. auranteum resembled those of C. Tibet to such an extent that the two could not always be told apart with a hand lens. As the calluses from the orange became older, a very noticeable difference in the peripheral cells became evident. They, too, became white and possessed a frosted look, but they became arranged into branched filaments of varying lengths (Plate IV, fig. 2).

Although the calluses of C. auranteum were derived from two sources, they appeared similar with a few exceptions. Callus cultures only recently initiated from juice sacs and perhaps four to six months old often formed what first appeared to be roots which projected from the callus mass (Plate IV, fig. 5). Sectioning revealed, however, that these root-like outgrowths were in reality extremely elongated juice sacs. When callus formation occurred only at the torn or basal end of the sacs, the remainder of the sacs lay free on the agar surface. Elongation of the sacs then occurred by a general enlargement of the parenchyma of the sacs. Since no meristematic zones were formed, elongation did not continue.

In two cases, older callus cultures derived from juice sacs produced spherical structures which arose from the

callus mass (Plate IV, fig. 6). They appeared hollow when examined with the hand lens, and sectioned material proved this to be the case. The structure in Plate IV, fig. 6 was of such a delicate nature that it collapsed and disintegrated in the killing and fixing solution. A smaller body of a similar nature when sectioned revealed that a definite meristematic layer had formed below the surface of the sphere as shown in Plate III, fig. 1. Growth of the meristematic layer increased the diameter of the sphere at the expense of the innermost cells which disintegrated.

Cell types of the callus were similar to those described for the callus of C. Tibet. The cells with reticulate thickening seemed to be similar, but the parenchymatous cells were smaller and with thicker walls (Plate III, fig. 3). Their thicker walls often prevented the cells from appearing so irregularly shaped in sectioned material as the cells did in the callus of C. Tibet. The most striking feature of the peripheral cells was the elongation of these cells to form the filaments previously mentioned (Plate III, fig. 5). The walls of these cells were often covered with papillae or wart-like outgrowths.

Cytology of the Calluses

Calluses of C. Tibet. -- One unusual feature of the calluses was the absence of meristematic regions in paraffin sections. Although nuclei were present in many of the cells near the margins of the callus, the cells were not dividing.

Consequently, the location of chromosomes in the callus was difficult and smears made of material taken from the periphery often revealed mature cells with heavy cellulose walls. Eventually, places of limited meristematic activity were located by using a solution of 2, 3, 5, Triphenyl-2H-Tetrazolium Chloride to stain living cells. Its use will be described in greater detail in a later section.

Since the species of C. Tibet was unknown, the chromosomal number was ascertained by smears of pollen mother cells made as they were undergoing meiosis. Like most members of the genus (Frost, 1925; Krug, 1943) the haploid number proved to be nine (Plate V, fig. 1). Chromosome counts in the callus revealed the polyploid condition to be common (Plate V, figs. 2 and 3). The highest number in which an accurate count could be made was 72, an octoploid. Other polyploids were counted, but outside of these, no other chromosomal aberrations were observed. Plate V, figs. 4 and 5 show other phases of mitosis in the callus cells.

Leucoplasts were present in the younger tissue and were abundant in the meristematic cells (Plate V, fig. 2). Although the callus was colorless when grown in darkness or in weak diffuse light, chloroplasts formed readily when actively growing cultures were exposed to bright diffuse light or to constant illumination from fluorescent lamps. When exposed to continuous light for almost a week, the cultures usually became yellowish-green as chloroplasts began to form. Callus at this stage when embedded in paraffin

PLATE V

THE CHROMOSOMES OF CITRUS CALLUS

Figure 1. Microspore mother cells undergoing meiosis in the anther of a C. Tibet flower. From slides such as this, the chromosome number of C. Tibet was determined to be n = 9. X 440.

Figure 2. A group of 36 chromosomes, a tetraploid, from the callus of C. Tibet. The white circular area at the edge of the chromosomal mass is a leucoplast. X 970.

Figure 3. An octoploidal number of chromosomes (72) from callus of C. Tibet. X 970.

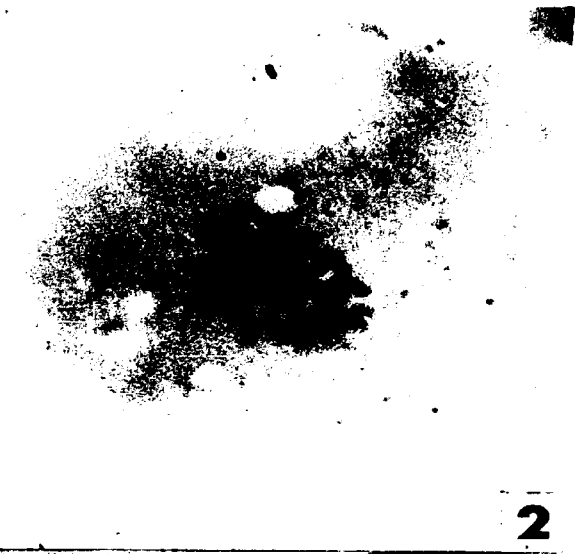
Figure 4. An anaphase in C. Tibet callus. X 970.

Figure 5. A metaphase in C. Tibet callus. X 970

Figure 6. A polyploid number of chromosomes from C. auranteum callus derived from the seed coat. An exact count was not made, but the number exceeds the normal 2n = 18. X 970.

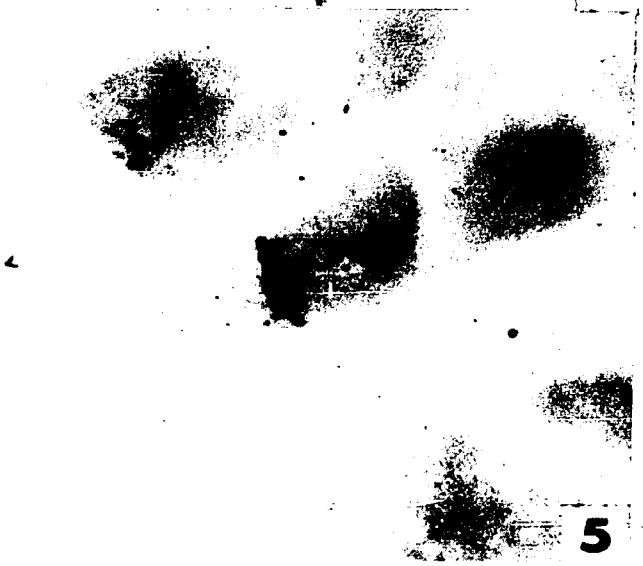
PLATE V

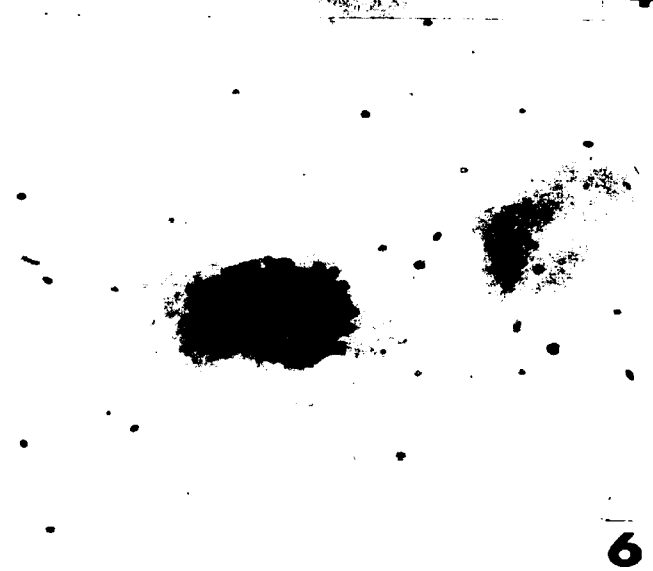

1


2


3


4


5


6

and sectioned failed to show plastids in most of the cells, although a few of the most superficial cells did contain them. Apparently plastids formed in those cells which were youngest and somewhat meristematic. Cultures exposed to light for a month showed chlorophyll confined to the tissue that had been produced from the original inoculum within the last month. The inoculum itself appeared brown and dead. Marginal cells removed from the greenest places and examined under the microscope proved to be dead and colorless. Chlorophyll production was confined to a band of cells which lay just beneath an outermost layer of dead or dying cells. Green tissues when placed back in the darkness lost their color within three or four weeks. Fresh sections of green callus showed an abundance of starch when placed in an iodine solution. The starch was limited to the outermost layers of tissues and never extended into the older brown portions. Starch formation, however, could occur in darkness as well as in light, for callus which had never been exposed to light from the time of its inception usually possessed starch in varying amounts. Plate IV, fig. 3 reveals the pattern of starch distribution in callus which had not been subcultured for nearly two months.

As stated in Part Two of this paper, the cotyledons of citrus embryos contain an abundance of oil and aleurone grains with globules. Oil droplets do not appear in material embedded and sectioned in paraffin, but the aleurone grains remain and stain heavily with safranin, and the globules

stain red in the presence of a saturated alcoholic solution of picric acid and eosin. Bodies similar in appearance to the aleurone grains of the cotyledons were often found in the cells of the callus and occasionally in the cells of floral organs, seed coats, or juice sacs after having been in culture several days or weeks (Plate I, fig. 2). These bodies stained in the same manner as described for the aleurone grains and therefore were considered to be of the same nature.

Calluses of C. auranteum. -- The chromosomes of C. auranteum calluses were even more difficult to locate than those of C. Tibet. A few meristematic cells were located, however, in which chromosome numbers were counted above the eighteen which Frost (1926) reported as the diploid number for C. auranteum. Although exact counts were not made, several polyploids were observed (Plate V, fig. 6). As in the callus of C. Tibet, polyploidy seemed to be common. Plastid formation occurred in the younger cells at the margins of the callus mass and also occurred in those cells undergoing mitosis. The callus responded to light in the same manner as did that of C. Tibet callus. The cells of young filaments contained chloroplasts, too, and under the microscope resembled algal threads. Eventually the cells of the filaments became colorless and stood out in contrast against the lower green cells. Carbohydrates were stored in the form of starch in the living portions of the callus, and aleurone grains were present.

Manner and Rate of Growth of the Calluses

Calluses of C. Tibet. -- The use of 2, 3, 5, Triphenyl-2H-Tetrazolium Chloride or "TTC" as a means of identifying living regions in the calluses was of much help in determining the more active patches of growth. TTC in the oxidized state is colorless, and when reduced forms a bright red substance called formazin. As Mattson, Jensen, and Dutcher (1947) pointed out, it can be used as a test for living cells since actively metabolizing cells reduce TTC quickly.

Thin free-hand sections of callus cut at right angles to the agar were placed in a 1% solution of TTC for varying lengths of time. At first the sections were placed in the dark since Brown (1954) and Atkinson, Melvin, and Fox (1950) showed that light could reduce TTC to formazin. Subsequent tests, however, proved that such precautions were unnecessary since diffuse light had little effect on it. Not only the color reaction but the time element was indicative of the most active zones of callus. Within five minutes isolated regions became bright red while other places either remained uncolored or stained after several hours. As Plate IV, fig. 4 shows, a colorless peripheral band was always present in the sections although the photograph exaggerates its width to some extent.

The exact nature of growth is somewhat of a mystery, although sections stained with safranin and fast green revealed something about the origin of new outgrowths from the

older sections of callus. Zones of new growth started just below the surface of the callus and consisted of narrow regions of only a few cells in width. As they pushed to the surface, the outgrowths became fan-shaped due to enlargement of the cells (Plate III, fig. 2). Some degree of orientation of the cells into files could be detected radiating out from the points of initial growth despite the changes in the size of the cells. Since definite meristematic regions were not seen in these places, it is probable that all cells within a file underwent mitotic divisions for a limited time before differentiation or death occurred. The presence of dead cells over the surface of actively growing regions indicated that cell division was not entirely confined to the terminal cells of the files.

All calluses were grown on the surface of agar media, because all of the attempts to grow shake cultures or cultures in aerated liquid media resulted in death to the calluses. Repeated attempts to plot growth curves based on the percentage weight increase after 28 days over the original weight were unsuccessful. The mosiac of living and dead tissue regions in the callus as revealed by the TTC technique precluded the possibility of obtaining equal sizes of callus with the same amounts of active tissue. Individual pieces of callus did not always grow at the same rate. This difficulty was also experienced by Caplin (1947) who tried to ascertain the growth rate of cubes of tobacco callus. Although the results were erratic, enough weights over a period of nine

months proved what could be observed by the eye. The basic medium alone produced extremely slow growth. Yeast extract added to the medium improved the rate of growth, but 90% cocoanut milk added to the basic medium gave the best growth of all. A maximum wet weight increase of 150% to 200% could be expected within a 28-day period. Not only did the cocoanut milk provide the fastest rate of growth, but tissues could be cultured for a longer period of time between transfers.

Calluses of C. auranteum. -- Calluses of C. auranteum, unlike those of C. Tibet exhibited a strong tendency toward a cambium-like growth. While the older sections of the calluses often showed little orientation of the cells into rows, definite files were found near the periphery (Plate III, fig. 3). Despite the appearance of a definite layer of meristematic tissue, all cells within a file must have been capable of continued division since the filaments terminating the files were often branched. Like the callus of C. Tibet, the cultures grew fastest when 90% cocoanut milk was added to the basic medium, but its fastest growth when measured over a 28-day period was usually less than half as fast as the growth of C. Tibet callus.

Discussion

One of the most interesting aspects of the isolation of callus tissues from different parts of the flowers and fruits is the similarity of the calluses within the same species. As diversified as the origins seem to be, all calluses originated from the not yet fully differentiated

parenchymatous cells of immature organs. Moreover, these parenchymatous cells of the flower in turn originated from the same apical meristem at the same time. The calluses derived from the stigma and style appeared no different from the tissue derived from the carpel walls because they were not different. The tissue derived from the juice sacs may have been expected to vary from that produced from the floral parts since the sacs originated from the cells of the mesocarp long after the floral organs were formed. That this callus from the sacs did not differ from the others is an indication of the ability of the parenchyma cells to form new cells which have meristematic characters.

The differences between the callus tissues of C. Tibet and those of C. auranteum were not great even though it was possible to distinguish between the two. The two groups represent different degrees of expression of the same tendencies or characteristics. For example, both groups show the same tendency toward a cambial growth though the calluses of C. auranteum show this to a greater degree. The filamentous growth of C. auranteum callus is duplicated on a lesser scale in older C. Tibet callus as the dead outer cells form in loose groups in which some are short files only a few cells in length. Other characters of the two groups of calluses are in reality very similar. The appearances of the citrus calluses suggest that:

1. The calluses from the same species appeared identical even though they were derived from

various plant structures.

2. The calluses from different species were readily distinguishable, however, regardless of the structures producing them.

The filamentous habit observed in the sour orange calluses is not unusual. It has been reported in various calluses which have been formed under normal conditions. Blackwell (1913) reported on the warted condition of the cell walls in callus filaments derived from Althaea. Barker (1952) observed filamentous growth in callus derived from Tilia. Commenting on the cause of filamentous growth, Barker (1952) expressed the opinion that it was caused by growth of isolated files of cells separated from one another by regions in which growth had ceased. Inasmuch as filamentous growth of prominent proportions was observed in older citrus cultures, his conclusion is probably correct.

The resumption of growth of the juice sacs in the sour orange to form enlarged dry sacs is reminiscent of the condition known as granulation which occurs in tree-ripened fruits. Gramulated juice sacs become dry and hard with a grayish-white color and appear to be associated with luxuriant growth in humid places (Webber and Batchelor, 1948). The elongated sacs encountered in culture may be due to the abundance of callus growth which has formed at one end of the juice sacs. The callus would act as an absorptive organ to supply an unusually large amount of nutrition to the remaining cells of the sac. Granulation of sacs in fruits grown under

conditions favorable for maximum growth indicates that a nutritional factor may be responsible in this case as well.

The polyploid condition has been found to occur not only in the calluses of Citrus but also in those from other genera of plants (Tulecke, 1952 and Norstog, 1955). The octoploid condition reported in C. Tibet callus probably occurred as the result of two successive nuclear divisions within the cell but without the formation of cell walls between the groups of daughter chromosomes.

Although the calluses failed to differentiate beyond the formation of cells with tracheal thickenings, they showed a rather high degree of organization. In calluses of a friable nature the cells tend to remain somewhat independent of one another with each cell dividing independently to produce clusters of daughter cells which soon separate. The citrus calluses show a greater degree of unity between the individual cells, for in the meristematic regions several files of cells divide more or less simultaneously to produce a tissue whose cells have some manner of definite orientation.

The number of callus cultures derived from plants other than Citrus which are capable of differentiating roots and shoots is not large. While polarity has not been demonstrated in callus, there is a possibility that the polarity exhibited by the axis of the whole plant may not be entirely lacking in the cells of the calluses derived from those organs of the axis. If this is true, root and shoot formation in calluses could be expected to occur with greater frequency

in those obtained from stems or roots than in cultures derived from other regions of the plant such as flowers and fruits.

The most important aspect of this work still lies in the future. The isolation of tissue from various organs of Citrus points to the possible future role of tissue culture in the citrus industry. The demand for vegetatively produced embryos by citrus growers has been emphasized elsewhere in this paper. As Maheshwari (1950) has pointed out, the ability to induce or suppress the formation of nucellar embryos at the will of the grower would be of much value commercially. The production of a tissue which could form clonal plants in vitro should be of much interest. At the beginning of this work it was felt that a tissue derived from the nucellus would hold the best promise of differentiation of roots and shoots because of the behavior of that tissue in the normal seed. This is probably not true, however. That buds should arise from the nucellus is possibly due not to any peculiarity of that tissue per se, but rather, perhaps, to its proximity to the embryo sac. A tissue derived from another source such as the seed integuments might accordingly hold as much promise of embryo differentiation as the tissue derived from the nucellus.

It is true that the production of young plants from undifferentiated tissue in the manner described above must await the development of techniques which will allow controlled differentiation from calluses in vitro. The work of White (1939) and Skoog (1944), and Skoog and Cheng Tsui (1948) with tobacco stem callus, however, indicates that this may no longer be a barrier within the foreseeable future.

PART TWO

THE CULTURE OF CITRUS EMBRYOS

Introduction

Polyembryony or the production of more than one embryo in a seed has been reported in many genera of angiosperms besides Citrus. According to Leroy (1947) cases of polyembryony have been reported in 250 species. The literature on the subject has been reviewed by several workers (Ernst, 1918; Schnarf, 1929; Webber, 1940; and Maheshwari, 1952).

The various methods of origins of plural embryos may be summarized as follows:

1. Two embryo sacs are formed in one ovule and a gametic embryo develops in each sac. Poa (Nielson, 1946)
2. In addition to the egg various other nuclei in the embryo sac give rise to embryos. Lilium (Cooper, 1943)
3. The zygote cleaves into two separate embryos. Erythronium (Jeffrey, 1895)
4. Adventitious embryos arise from tissues adjacent to the embryo sac. Mangifera (Juliano, 1934); Garcinia (Horn, 1940)

Polyembryony has no economic significance in most

genera of plants, and its occurrence in those plants is of academic interest only. On the other hand, in a genus of such economic value as Citrus where polyembryony is the rule rather than the exception, it becomes of the greatest importance. The literature on citrus culture is at the same time the literature on polyembryony in that genus.

Strasburger (1878) described in detail the origin of plural embryos in C. auranteum and showed that they arose from nucellar buds projecting into the embryo sac. Toxopeus (1930) confirmed Strasburger's observations, and Osawa (1912) gave a similar interpretation to the origin of embryos in Poncirus, the trifoliate orange. Since then, nucellar budding has been reported in nearly all members of Citrus and in its near relative, Fortunella, the kumquat. Although nucellar budding is by far the commonest means of plural embryo production, Frost (1926) reported the appearance of twin embryos in a citrus hybrid, and explained their appearance as due to cleavage of the zygote or early proembryo. Bacchi (1943) reported a third type of origin of plural embryos in Citrus through the occurrence of two embryo sacs in the same ovule of a variety of grapefruit.

The definite roles of pollination and fertilization in nucellar budding are not clearly understood. Strasburger (1878), after controlled pollination experiments with the orange, reported that, although fruits may be produced without pollination, seeds would not form in them. Toxopeus (1930) found that seeds would not form without pollen in

three varieties of Citrus. Wong (1939) and Frost (1938a) reported similar results with pollination experiments in various other varieties.

Webber and Batchelor (1948) described several dubious instances of nucellar budding without pollination, but concluded that with a few possible exceptions pollination is usually necessary for the production of nucellar embryos, and that no evidence exists to prove the role of fertilization in their production.

A singular characteristic of nucellar embryos in Citrus is their close resemblance to the sexually produced embryos. In their early stages of development only the position of the gametic embryos near the micropyle together with their possession of a suspensor enables one to distinguish them.

The number of viable embryos in mature citrus seeds varies with the species and even with the individual plants. While some fruits normally produce seeds with only one embryo each (Torres, 1936) others may possess seeds with ten or twelve viable embryos per seed (Webber, 1932a).

Unfortunately, in a mature seed, not only is it impossible to tell which of the surviving embryos is the gametic one, but it is also impossible to tell whether the gametic embryo has survived at all. As Frost (1926) pointed out, the gametic embryo is more often eliminated when nucellar embryos are especially abundant.

Since the nucellar embryos are of sporophytic origin,

chromosome counts are of no help in identifying them. Frost (1925) found, however, that in certain lots of hybrid seedlings three different types could be distinguished: those with hybrid characters, embryos with maternal characters, and those that showed no resemblance to either parent. The embryos which differed from either parent proved to be tetraploids. Frost (1925a and 1938b) postulated that "islands" of tetraploid tissue of chimeral origin may arise in the developing nucellus to give rise to such embryos, or that chromosomal doubling may occur at the time of nucellar formation so that the entire nucellus is composed of tetraploid tissue.

The vegetatively produced and uniform nucellar embryo bearing characteristics identical with the maternal plant may differ greatly from the gametic embryo in which the characters are variable as is normally expected of sexually produced embryos. With embryos arising from different origins, one might anticipate advantages and disadvantages from such a condition. Because of the uncertain origins of the embryos when plants are crossed to form hybrids, the taxonomy of Citrus has been confused to a large extent (Swingle, 1914). Webber (1900) pointed out the disadvantages of having to wait several years for seedlings to reach flowering size before the two types of seedlings could be told apart. Hybrid production is impeded when the percentage of adventive embryos is high (Toxopeus, 1930), and Traub and Robinson (1937) have commented on the fact that polyembryony has thrown the genetics of Citrus into disorder. The use of pollen of Poncirus in making

crosses has been of limited assistance in this field. Webber and Swingle (1904) created the citrange by reciprocal crosses of Poncirus and Citrus sinensis, the sweet orange. In instances where pollen from Poncirus was used the seedling derived from the gametic embryo possessed three leaflets and could be distinguished from the nucellar seedlings at an early stage. Where conditions will allow, this method is used as a tool by many citrus breeders to obtain identified nucellar embryos (Cook, 1938).

The advantages gained by commercial growers through the polyembryonic condition in Citrus probably outweigh the disadvantages. Webber (1932a) and others described the usefulness of nucellar embryos as a source of uniform rootstocks. Swingle (1932b) has suggested that nucellar embryos apparently undergo a beneficial rejuvenescence as they form under the influences of the embryo sac, and to this rejuvenescence he gave the name, "neophyosis". The rejuvenescence is shown by the more vigorous vegetative growth of the nucellar seedlings accompanied by an increase in thorniness. Although Traub and Robinson (1937) expressed doubt as to the existence of neophyosis, other workers (Hodgson and Cameron, 1938; Frost, 1938a; and Cook, 1938) pointed out definite signs of rejuvenation in older clones. Frost (1938a) interpreted the increase in thorniness as additional evidence of a more vigorous vegetative growth since the thorns in Citrus are in reality dwarfed branches. Hodgson and Cameron (1938) and Frost (1938a) also noticed a decrease in seediness of the fruit in addition to

a delay in flowering in rejuvenated clones. These characters are also indicative of prolonged vegetative growth.

Several attempts have been made to induce nucellar embryo formation in plants. Haberlandt (1921 and 1922) attempted with some success to produce adventive embryos in Oenothera by wounding the ovules or the ovary. His theory of "necrohormones" was based on the premise that adventive embryos were produced from nucellar tissue at the expense of neighboring cells that disintegrated. The dying cells were thought to liberate the necrohormones which in turn would induce nucellar budding.

Van Overbeek, Conklin and Blakeslee (1941a) were able to induce warty outgrowths from the nucellus of Datura by injecting a solution of naphthaleneacetic acid or indolebutyric acid derivatives into the ovary.

Fagerlind (1946) produced young nucellar embryos in Hosta by application of indoleacetic acid in lanolin on the stigmas of the pistils. The young embryos were unable to continue their development, however, because of the lack of endosperm.

Although the desirability of controlling the production of nucellar embryos in Citrus has long been recognized, little has been done in that direction. Traub (1936) was able to reduce the number of nucellar embryos per seed by reducing the nutrition of the developing fruits. Young fruits were covered with cloth and many of the leaves were removed from the branches bearing the fruit. The pale yellow fruits produced seeds with

nucellar embryos 51% to 100% below the expected number.

In vitro cultures of citrus embryos have not been reported, although Maheshwari (1950) states, ". . . it would be interesting to know how the nucellar embryos would behave if they are removed from the ovule at a comparatively early stage of development and grown in artificial media."

While the technique of culturing nucellar embryos in an artificial medium may be new, gametic embryos of various plants have been cultured on a variety of media. The earliest workers, Hannig (1904) and Stingl (1907) achieved success with fairly young embryos by growing them on nutrient gelatin and cereal endosperms, respectively. Dietrich (1924) cultured embryos one-third their mature size on Knop's solution and sugar.

The successful culture of smaller embryos than those employed by the above workers was achieved through the addition of organic substances to the media. LaRue (1936), by adding indoleacetic acid to the media, was able to produce seedlings from embryos 0.5 millimeter in length. *Datura* embryos at the heart stage were grown by Van Overbeek, Conklin and Blakeslee (1941b and 1942) who discovered an "embryo factor" in cocoanut milk. McLean (1946) and Sanders (1950) were able to grow *Datura* embryos equally well when malt extract was added to the media.

Laibach (1929) was the first worker to realize the practical application of *in vitro* cultures of plant embryos. His successful cultures of young hybrid embryos of *Linum*

taken from defective seeds gave impetus to further work in culturing embryos obtained from what had previously been considered unsuccessful hybrid crosses.

Tukey (1933) used embryo culture as a means of obtaining embryos from cherry seeds before they aborted and pointed out that plants which produced viable embryos but later aborted could not be considered sterile if these embryos could be grown to mature plants by altering their environment. Tukey (1944) also found that a quick method of determining the germinability of embryos of stone fruits was possible by artificially culturing the embryos, thus saving time by eliminating the after-ripening process.

Materials and Methods

One of the greatest difficulties in obtaining material was the seasonal aspect of *Citrus*. This was especially true in the case of extremely young embryos. They appeared in abundance for a few days, but developed quickly into advanced stages. Young fruits of *C. mitis*, the Calamondin, which served as a source for proembryos, were obtained from the Citrus Experimental Station at Lake Alfred, Florida. *Citrus auranteum* fruits with embryos of all stages were obtained from the greenhouse at the University of Michigan Botanical Gardens.

After the fruits of suitable ages were surface-sterilized, the peel was cut nearly in two with a sterile scalpel and then torn apart with the fingers. The seeds were dropped into sterile bottles and stored until the moment of dissection.

Since the division of the zygote and the initiation of nucellar embryos occur late in the development of the seed and growth of the fruit and seeds continues from the time of pollination, it was possible to obtain proembryos from seeds three or four millimeters long which could be easily dissected. The seed integuments readily separated from the enlarged nucellus so that the nucellar tip could be easily exposed with forceps and a small scalpel.

Because of the small size of the young embryos, dissection was done under a dissecting microscope and the embryos were measured with an ocular micrometer. In many instances the entire tip of the nucellus bearing several embryos was dropped into culture, but this method was unsatisfactory since it was impossible to ascertain the number and stages of development of the embryos in the nucellar tip. The technique used for culturing most embryos consisted of cutting open the tip of the nucellus, removing the young embryos with a sterile needle and placing them in culture.

Because of the limited supply of embryos and the great variability in stages of development of the embryos in any one sac, no attempt was made to select sizes. Embryos in all stages of development were cultured as they were found.

The embryos were cultured in twenty milliliter glass bottles in a variety of media, both liquid and solid. When growing in liquid it was possible to measure the developing embryos intermittently through the glass sides of the bottles, but those on agar could not be measured by such a method with-

out removing the tops of the bottles and risking contamination.

When the embryos seemed to reach their maximum size in the initial cultures, each was transferred to one of several kinds of solid media in an attempt to induce rooting. All culture media used contained White's solution but modified in the manner outlined in Table I. The various other media used are listed in Tables IV and V.

TABLE IV

INITIAL MEDIA USED IN CULTURING CITRUS EMBRYOS

Medium	Modified White's solution and 1 mg./l. indole-3-acetic acid plus--
1.	2% sucrose
2.	2% sucrose, 0.8% agar
3.	4% sucrose
4.	2% sucrose, 25% cocoanut milk
5.	2% sucrose, 25% cocoanut milk, 0.8% agar
6.	2% sucrose, 90% cocoanut milk
7.	2% sucrose, 90% cocoanut milk, 0.8% agar
8.	2% sucrose, 0.5% malt extract

TABLE V

ROOTING MEDIA USED IN CULTURING CITRUS EMBRYOS

Medium	Modified White's solution and 0.8% agar plus--
1.	1 mg./l. indole-3-acetic acid
2.	2% sucrose, 0.1 mg./l. indole-3-acetic acid
3.	0.5% sucrose, 0.1 mg./l. indole-3-acetic acid
4.	2% sucrose, 1 mg./l. indole-3-acetic acid
5.	0.5% sucrose, 1 mg./l. indole-3-acetic acid
6.	2% sucrose, 0.1 mg./l. indolebutyric acid
7.	0.5% sucrose, 0.1 mg./l. indolebutyric acid
8.	2% sucrose, 1 mg./l. indolebutyric acid
9.	0.5% sucrose, 1 mg./l. indolebutyric acid

One difficulty encountered in the culture of citrus embryos has been similar to that shared by other workers who have employed cocoanut milk for culturing embryos, namely, the variability of results due to the inability to standardize cocoanut milk (Table VI). During the summer months cocoanuts all but disappear from the markets. Those that are found are usually low in milk content and of an inferior grade. Even in the best grades of cocoanuts the quality of the milk varies in the amount of "embryo factor" present. For these reasons no appreciable difference could be noticed between the media with 25% cocoanut milk and media with 90% cocoanut milk.

TABLE VI

THE EFFECT OF THE VARIATION OF COCOANUT MILK* ON CITRUS AURANTEUM EMBRYOS

Lot number	Stage of embryos at time of culturing	
	Proembryo	Heart
I		
No. cultured	45	14
No. formed cotyledons	24	11
II		
No. cultured	52	29
No. formed cotyledons	0	5
III		
No. cultured	64	1
No. formed cotyledons	0	0
IV		
No. cultured	50	31
No. formed cotyledons	11	16
V		
No. cultured	36	17
No. formed cotyledons	1	2

*90% cocoanut milk with modified White's solution, 2% sucrose and 1 mg./l. Indole-3-acetic acid.

The pH of the liquid endosperm in the embryo sac of the sour orange was tested with pH paper and was found to be about pH 5.6. Consequently, the media were adjusted to that degree of acidity. All embryo cultures were kept at room temperature and in diffuse light.

The internal structure of the embryos was studied from 10μ sections stained with safranin and fast green or with Foster's tannic acid-iron chloride method (Foster 1934).

Observations and Results

In order to give a correct interpretation to the behavior of citrus embryos *in vitro*, it is necessary to understand the sequence of normal development of the embryos from the proembryo stage to maturity as it occurs in the seed. While Strasburger (1878) described the origin of nucellar embryos in detail, his description ends at the proembryo stage. His illustrations show no vascular development, and Osawa (1912) goes no farther for *Poncirus*. On the other hand, Cossmann (1940) has described the anatomy of the sour orange seedling, and Hayward and Long (1942) have given similar descriptions for the root and seedling of the Valencia orange. There has been, therefore, no investigation of the initiation and development of vascular tissue in the citrus embryo.

The following description of embryonic development is based on observations made of *C. auranteum* embryos. While the initiation of the nucellar embryos has been reported previously by the workers stated above, it is repeated here for

PLATE VI

THE CELLULAR STRUCTURE OF NORMAL *CITRUS AURANTEUM* EMBRYOS

Figure 1. A gametic proembryo in the embryo sac. Cells on either side of the proembryo show possible nucellar activity. X 440.

Figure 2. A nucellar embryo growing out into the embryo sac and still attached to the nucellus. X 440.

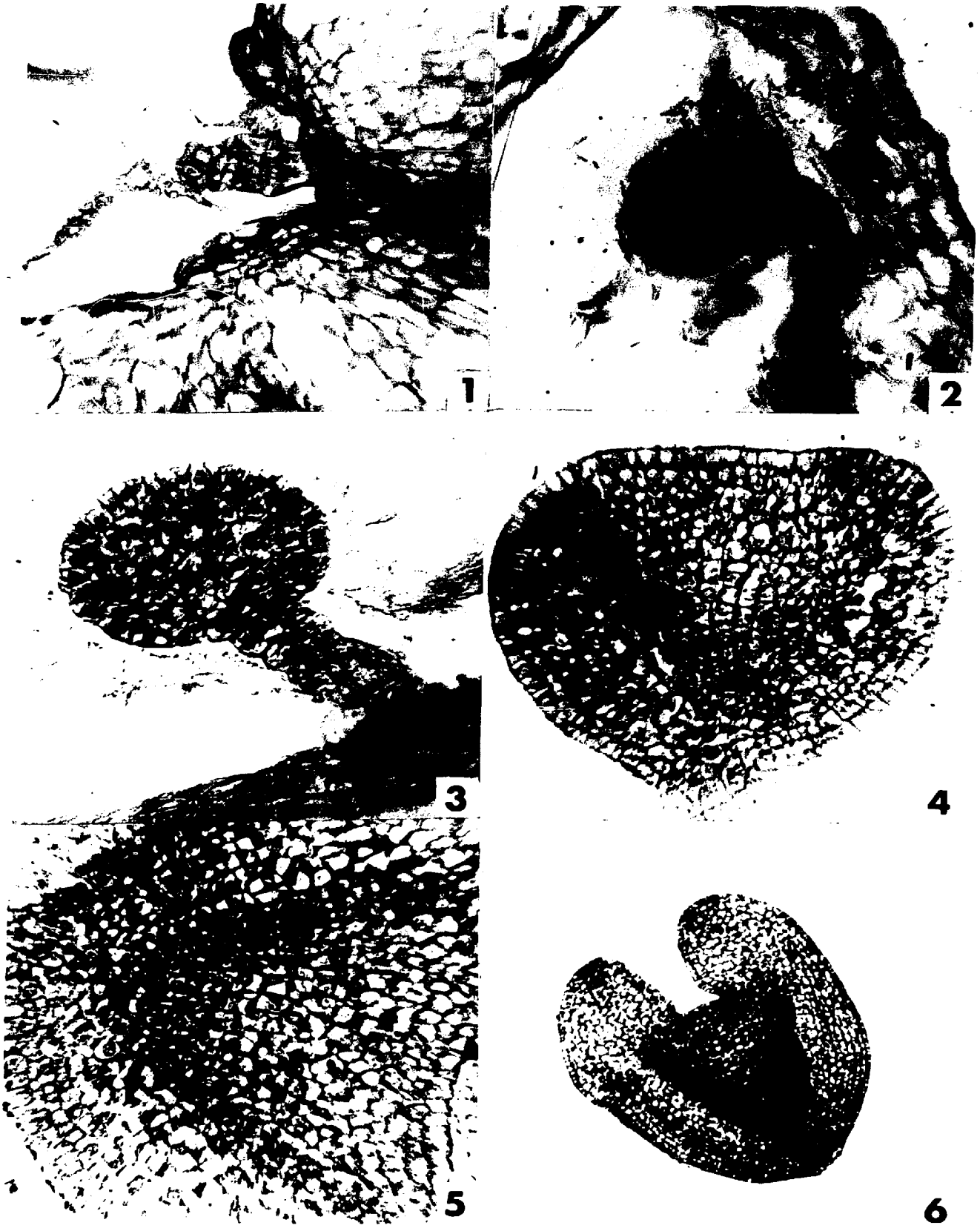
Figure 3. A nucellar proembryo surrounded by endosperm. Note the long stalk by which the embryo is attached to the nucellus. The micropylar tip of the embryo sac is to the left. Cultures were made of proembryos at this stage. Diameter of embryo is about 0.16 mm.

Figure 4. A late proembryo in which the apical portion has become flattened. Externally, the layer of cells which will form the epidermis of the embryo has been cut off from the rest of the cells. Internally, some degree of cellular differentiation marks the beginning of the procambium. Diameter about 0.19 mm.

Figure 5. Early procambium formation in an embryo at the early heart stage. Diameter of embryo about 0.22 mm.

Figure 6. A heart embryo with well developed procambium in the hypocotyl. Eventually the procambium will extend up into the developing cotyledons. Length of embryo 0.42 mm.

PLATE VI



clarity and continuity.

The Development of Citrus auranteum Embryos in the Seed

Proembryo. -- The sour orange blooms in the greenhouse around the middle of February. Although the fruits and seeds enlarge considerably, microscopic examination of the embryo sacs fail to reveal the presence of embryos until the end of May when the seeds are approximately four millimeters long. Then the few-celled gametic embryos can be seen lying near the micropyle (Plate VI, fig. 1).

Certain isolated cells of the nucellus adjacent to the embryo sac and near the micropylar end stain darkly and stand out from the neighboring cells. These cells begin a series of mitotic divisions and soon a small mass of cells projects into the embryo sac (Plate VI, fig. 2). At an early stage the nucellar embryos are longer than wide since the number of transverse divisions exceeds the periclinal ones. Later, the number of transverse divisions and periclinal divisions seems to be about equal, for the embryo becomes globose and approximately 0.16 millimeter in diameter. The embryo is attached to the nucellus by a stalk consisting of several cells in width and of varying lengths (Plate VI, fig. 3). Further periclinal divisions of the peripheral cells of the embryo produce an outer layer of cells which will become the epidermis of the embryo.

At this stage the nucellar embryos resemble the gametic embryo to such a degree that it is extremely diffi-

cult to distinguish between them. Schnarf (1929) stated that the gametic embryo could be recognized frequently by its position in the sac, and oftener by the possession of a suspensor. While this is true in many cases, the gametic embryo is frequently crowded out of position by several nucellar embryos forming around it. Moreover, the stalks by which the latter are attached to the nucellus often resemble the suspensor (Plate VI, fig. 3). Only rarely can the nucellar embryos be found still attached to the parent tissue in sectioned material. Inasmuch as all the embryos in a sac appear identical, the gametic and nucellar embryos probably share the same sequence of development except in their earliest stages.

The embryo loses its globoid shape as the region of the embryo opposite the suspensor begins to flatten. According to Miller and Wetmore (1945) who report on the similar stage in Phlox embryos, the number of periclinal divisions exceeds those of a transverse nature. This is undoubtedly true in citrus proembryos. Accompanying the flattening of the apex, certain cells in the center of the embryo begin to take a little deeper stain and stand out from the neighboring cells to form a cone of tissue whose apex is oriented toward the suspensor or toward the stalk if the embryo is of nucellar origin (Plate VI, fig. 4). This is the first evidence of procambial tissue forming in the embryo.

Heart Stage. -- When the embryo is about 0.2 millimeter in width, the cotyledonary primordia make their appearance.

They originate from two regions of the flattened region of the embryo. Because of the increase in number of cells in these regions, the embryo begins to assume the familiar heart shape typical of dicotyledonous embryos. Internally, it has became apparent that the young procambial tissue of the hypoctoyl is not composed of a solid core of tissue, but instead the elongated cells are arranged in a "V" pattern with the free arms of the "V" ending below the cotyledonary primordia (Plate VI, fig. 5). As the cotyledons develop and the embryo becomes more typically heart-shaped, the procambial strands keep pace with the growing cotyledons and some degree of cellular activity begins in the lower end of the hypocotyl at this stage (Plate VI, fig. 6).

Older Stages. -- By the time the embryo has reached 0.5 millimeter in length, the young cotyledons begin to flatten dorsiventrally thus marking the end of the heart stage. Growth of the cotyledons exceeds that of the hypocotyl so that the latter remains much smaller than the elongating and thickening cotyledons. Procambial tissue becomes more pronounced in the cotyledons and finally becomes branched by the time the embryo has become two millimeters in length. A slight mound of tissue at the apex of the hypocotyl can now be recognized as the primordium of the plumule, but there is no difference in the staining capacity of the cells in this region from those of the hypocotyl.

Protoxylem differentiation begins in the cotyledons when the embryo is approximately five millimeters long. Its initial occurrence is of a sporadic nature with a few spiraled

or ringed elements occurring in isolated patches (Plate VII, fig. 1). Cross sections of cotyledons show the xylem occurring adaxially, but phloem differentiation is difficult to detect if it occurs at all. Cross sections of the hypocotyl, however, reveal slight orientation of some of the cells of the procambial cylinder into circular patches. These cells still appear parenchymatous, but are probably protophloem mother cells. No xylem is present in the hypocotyl.

When the cotyledons are seven millimeters in length, their cells and hypocotyl contain oil droplets and inclusions which Netolitzki (1926) describes as aleurone grains with globoids. The root cap is fairly well developed at this time and xylem differentiation has proceeded still farther in the cotyledons.

Maximum-sized Embryos. -- The embryo at ten or twelve millimeters has attained its maximum size. Because there is usually more than one embryo in a single seed, the cotyledons are rarely symmetrical. One cotyledon is usually larger than the other and the smaller one may contain only a few aleurone grains. Except for larger size, the hypocotyl has undergone little differentiation beyond that of the seven millimeter embryo. The shoot primordium still remains as an inconspicuous mound at the apex of the axis, and it remains as such until germination. Within the cotyledons aleurone grains and oil droplets occur in abundance, but xylem differentiation is still incomplete, for places still occur where vessels and tracheids are lacking.

PLATE VII

CITRUS EMBRYOS

Figure 1. Scattered xylem formation in the procambial strands in the cotyledons of a normal embryo of C. auranteum. X 100.

Figure 2. A hypocotyl of a normal C. auranteum embryo six millimeters long showing the procambial cylinder. Some degree of cellular activity has occurred at the lower tip of the hypocotyl. X 100.

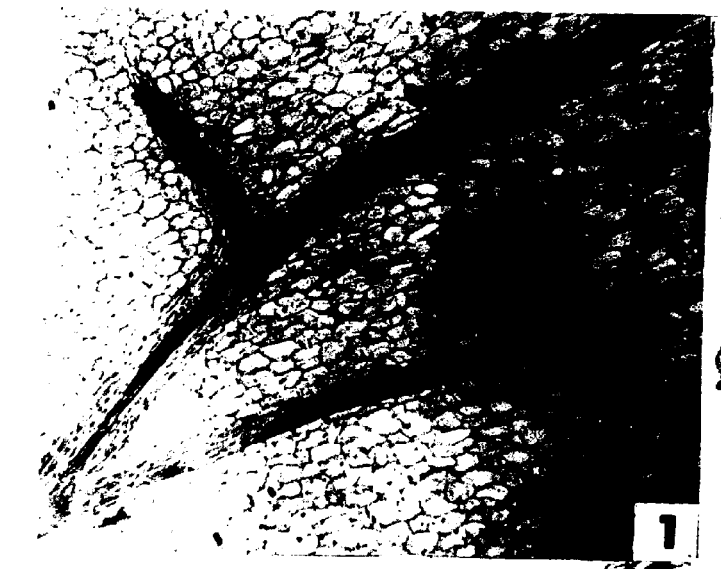
Figure 3. A C. auranteum embryo cultured at the proembryo stage. A procambial cylinder has formed in the hypocotyl and strands extend into the cotyledons. Activity at the basal end of the hypocotyl suggests some degree of root cap formation. X 100.

Figure 4. A cross section of the hypocotyl of a C. auranteum embryo cultured at the proembryo stage. The cylindrical nature of the procambium is evident. No xylem or phloem differentiation is visible. X 100.

Figure 5. In vitro culture of a Calamondin nucellus to which embryos are still attached. Although cultured at the proembryo stage, the embryos have reached the heart stage. Cultured in 25% cocoanut medium.

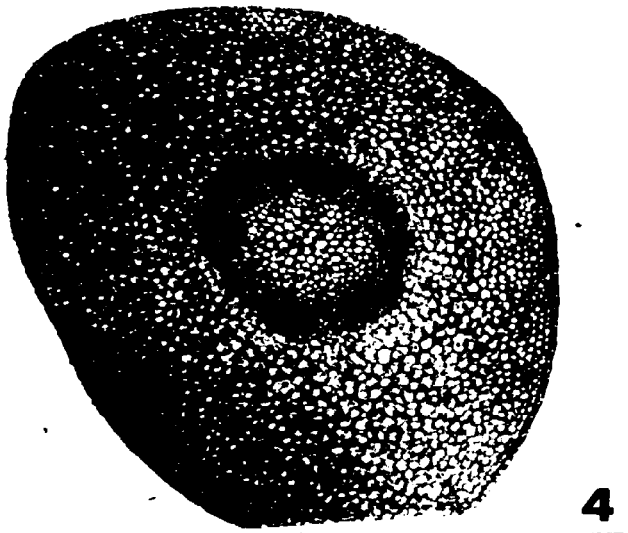
Figure 6. Seven Calamondin embryos derived from the culture shown in Fig. 5. Length of the largest embryo is about eight millimeters.

PLATE VII


1


2


3


4


5


6

In Vitro Development of Citrus Embryos

Proembryos. -- These include embryos ranging in diameters from 0.15 millimeter to 0.19 millimeter. The latter size includes those embryos which have begun to flatten but are not heart-shaped. From the description in the previous section, it can be seen that internal differentiation is limited or lacking at this stage.

Media containing only White's solution, sucrose, and indole-3-acetic acid proved inadequate for the growth of proembryos even though nucellar tissue was included in the cultures. With the addition of cocoanut milk in percentages of either 25% or 90%, however, some degree of differentiation of the proembryos often occurred. A more normal growth pattern was obtained by using a liquid medium in which the embryos were completely immersed rather than growing them on the surface of agar. Since the growth patterns were frequently different for the two types of media, their behavior in the liquid and on solid media will be discussed separately.

Proembryos in Liquid Cocoanut Media. -- Little difference could be noticed in the development of Calamondin embryos cultured while still attached to the nucellus from excised embryos, although there are no data to bear this out. It was not possible to obtain exact counts or measurements of embryos in the nucellar tips without tearing them loose from their points of attachment. While the embryos often remained attached to the nucellus during their earliest development in culture (Plate VII, fig. 5), they became detached at the heart stage

and lay on the bottom of the bottle. Their subsequent development seemed to parallel those embryos without nucellar tissue included.

Calamondin embryos developed chlorophyll while in the proembryo stage in the seed, but sour orange embryos, on the other hand, were creamy-white at the time of excision and became green after a week in diffuse light. The earliest indications of activity occurred within a few days after the embryos were placed in culture as the embryos began a general increase in size. While nearly spherical at the beginning, they became irregular in shape as they elongated somewhat (Plate X, fig. 1). By the time the cotyledonary primordia arose the embryos were much larger than normal young heart embryos (Table VII).

TABLE VII

THE COMPARATIVE SIZES OF *CITRUS AURANTEUM* EMBRYOS AT THE YOUNG HEART STAGE WHEN GROWN *IN VIVO* AND *IN VITRO**

in vivo	*in vitro*
0.23 mm.	0.42 mm.
0.23	0.49
0.23	0.65
0.23	0.68
0.23	0.68
0.23	0.76
0.23	0.76
0.27	0.76
0.27	0.76
0.27	0.84
0.27	0.87
0.27	0.95
0.30	1.14
0.30	1.25
Av. 0.25	0.78

* Cultured in 90% cocoanut milk.

PLATE VIII

THE EARLY NORMAL DEVELOPMENT OF CITRUS AURANTEUM EMBRYOS

Figures 1-3. Proembryo stage. With the increase in size the proembryo loses its spheroid shape as the apex becomes flattened.

Figures 4-5. Heart stage. Cotyledonary primordia arise from two regions of the flattened apex of the proembryo.

Figures 6-7-8. Older stages. The elongation of the cotyledons is accompanied by dorsiventral flattening. The cotyledons remain thick, however.

PLATE VIII

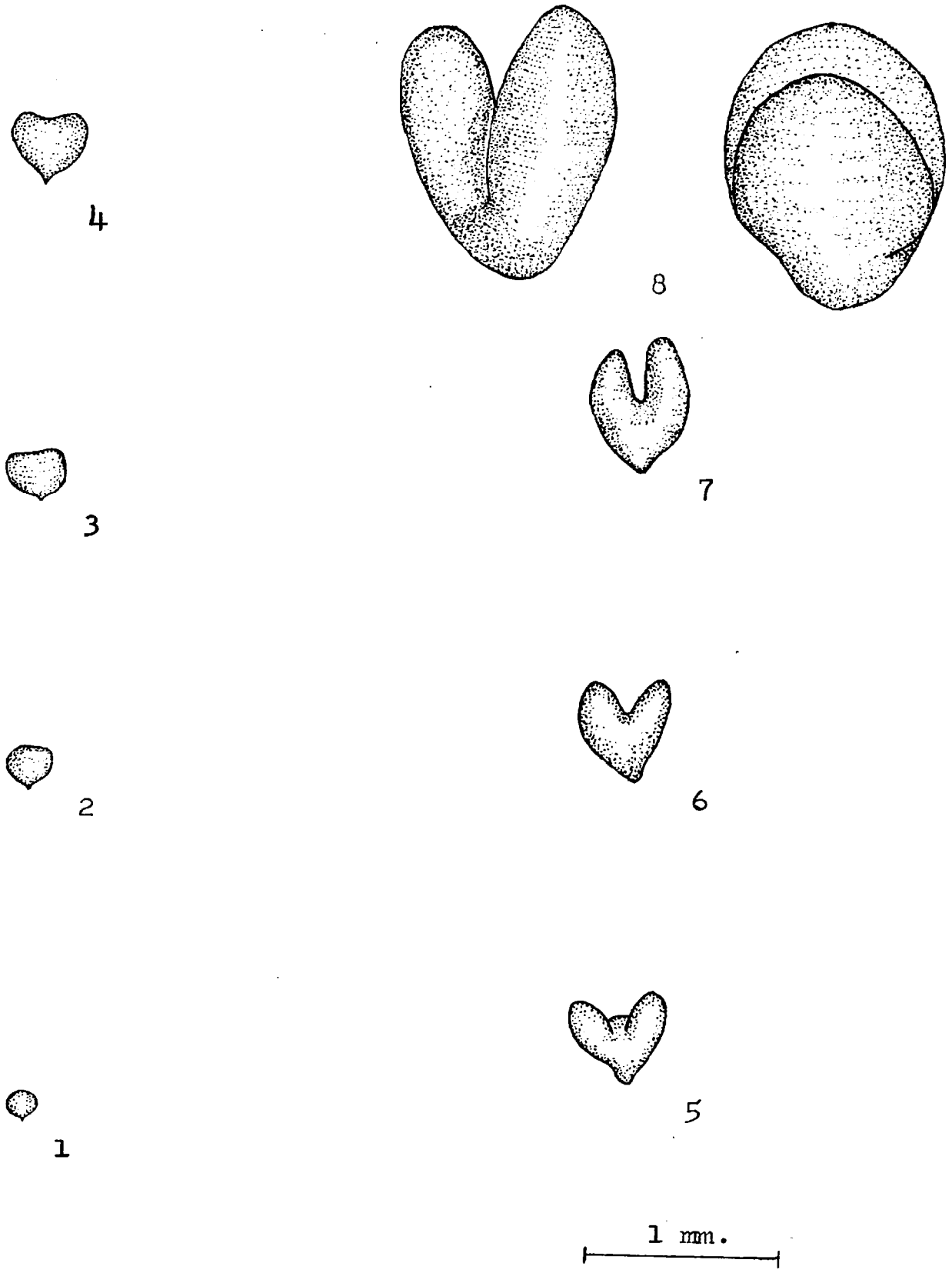


PLATE IX

THE DEVELOPMENT OF *CITRUS AURANTEUM* EMBRYOS *IN VITRO*

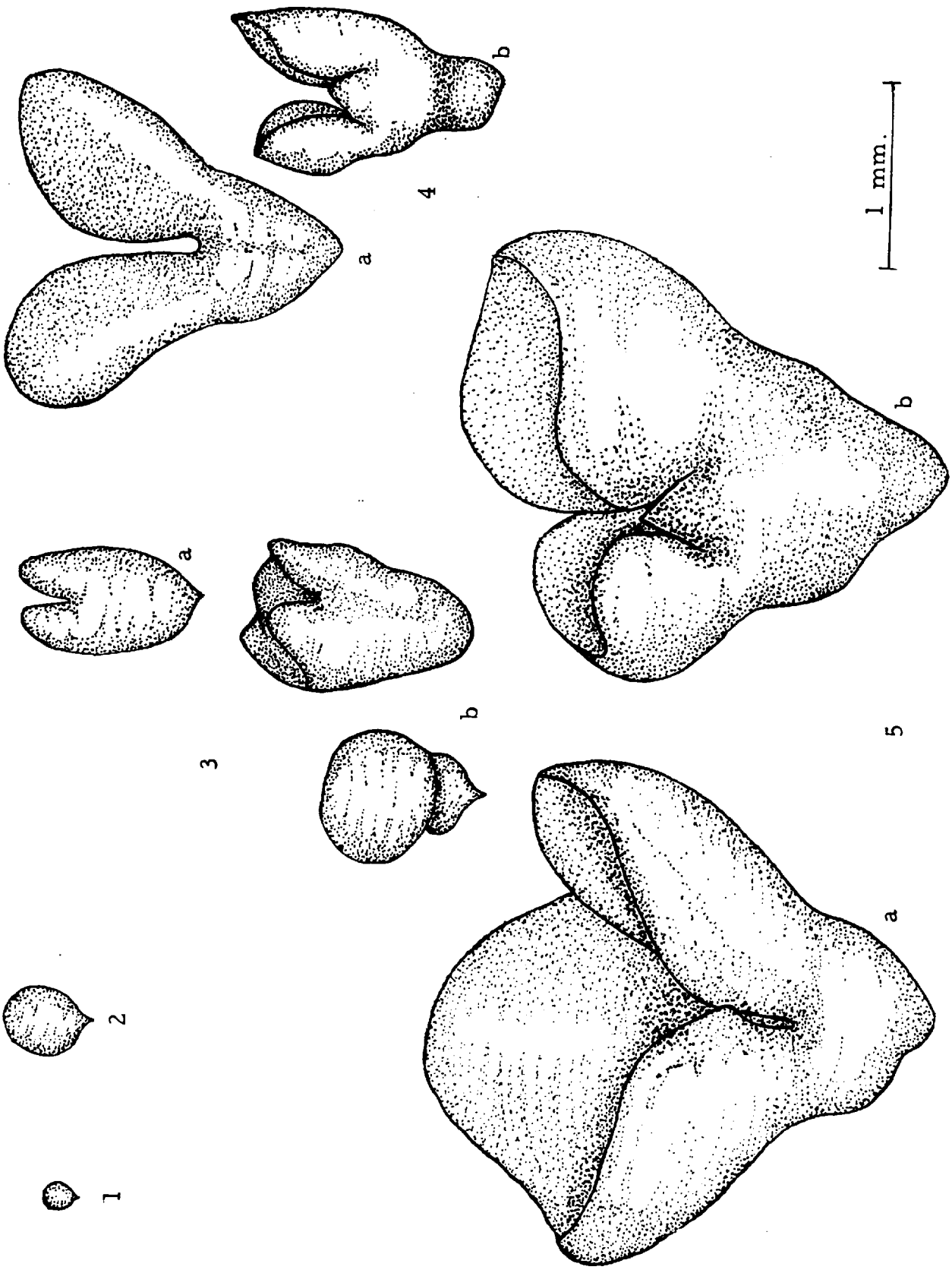
Figures 1-2. Proembryo stage. The embryo has increased greatly in diameter within a few days in culture. No flattening of the apex has occurred, but on the contrary, the embryo may elongate somewhat.

Figure 3. Heart stage. a. An elongated embryo has formed cotyledonary primordia, but the embryo is much larger at the heart stage than at the same stage shown in Plate VIII, fig. 5. b. A proembryo has become swollen at the apex causing a constriction in the future hypocotyl of the embryo.

Figure 4. Older stage. a. An enlarged hypocotyl bears two thin, flattened cotyledons. b. An embryo with a constriction in the hypocotyl reveals the leaf-like nature of the cotyledons.

Figure 5. Maximum-sized embryos. a. The thin cotyledons have increased in length over the hypocotyl. b. An embryo with an enlarged hypocotyl and short cotyledons. The constriction of the hypocotyl is still visible.

PLATE IX



From this stage on, most of the growth of the embryos seemed to be limited to the cotyledons, but in Calamondin the hypocotyls often continued to elongate (Plate VII, fig. 6).

Cotyledonary development *in vitro* proceeded in a manner atypical for normal development because cotyledons arose from thin cotyledonary primordia in the heart stage, and they retained their thin, leaf-like appearance throughout their entire period of growth (Plate X, figs. 2 and 3). A comparison of the various stages of development of embryos grown in culture as illustrated in Plate IX with the early stages of normal development of embryos in the seed (Plate VIII), reveals the differences in development of the two groups of embryos beyond the heart stage.

By the end of a month, the embryos had attained full growth. Sour orange embryos rarely exceeded three or four millimeters in length, but Calamondin embryos occasionally attained a length of eight millimeters before growth ceased (Plate VII, fig. 6). Longitudinal sections of sour orange embryos made after growth had ceased revealed an extensive development of the procambium in the hypocotyl and cotyledons. Xylem units were frequently found in the cotyledons. Root cap and aleurone grains were not present (Plate VII, figs. 3 and 4).

When it became apparent that the embryos would grow no more, they were transferred to the rooting media in order to remove them from the influence of the cocoanut milk which could hinder rooting. With the exception of an extreme elongation of

PLATE X

CITRUS AURANTEUM EMBRYOS IN VITRO

Figure 1. Three proembryos from the same sac after fourteen days in 90% cocoanut medium. Diameter of largest about 0.5 mm.

Figure 2. The three embryos shown in Fig. 1 after twenty-two days in culture. Cotyledons are starting. The embryo in the middle shows three young cotyledons. Length about 1.5 mm.

Figure 3. The same three embryos after twenty-nine days in culture. One of the three embryos possesses three cotyledons while the other two are normal. Length about 2 mm.

Figure 4. A tricotyledonous embryo excised at the heart stage after twenty-five days in culture. Length about 1.75 mm.

Figure 5. An embryo cultured at the proembryo stage after thirty days in culture. The cotyledonary primordium has failed to separate on one side thus forming the "collar" type of cotyledon described in the text. Length 2 mm.

Figure 6. An embryo with two normal cotyledons and two smaller cotyledons (arrows). The second pair arose at a later stage than the first two cotyledons. The tips of the smaller cotyledons above the arrows are projecting out of water thus causing some distortion to the photograph. Cultured at the heart stage.

PLATE X



the hypocotyl of one Calamondin embryo (Plate XII, fig. 6) they showed no tendency to root.

Because of the inability to distinguish the nucellar embryos from the gametic ones at the time of excision, a question arose as to whether any of the embryos that developed cotyledons in vitro were of nucellar origin. A tabulation of the number of proembryos per embryo sac developing cotyledons in vitro (Table VIII) indicated that many of them must have been of nucellar origin.

Proembryos on agar. -- Proembryos of sour orange when placed on the surface of solid cocoanut media often developed poorly if at all. Those that did develop often enlarged to several times their original diameters before putting out thick, stubby cotyledons.

An interesting type of growth occurred on several occasions when the nucellar tips containing proembryos were placed on 25% cocoanut agar. The embryos increased greatly in size until they formed a greenish-white mass of irregularly-shaped bodies (Plate XI, fig. 1) which eventually turned brown. At the end of nearly eight weeks small white outgrowths were seen arising from several places on each enlarged embryo. While the outgrowths often arose in pairs, they occasionally arose as single structures. They became cotyledon-like in appearance, but did not develop more than two or three millimeters in length (Plate XI, fig. 2). Sectioned material showed much lobing of parenchymatous tissue with strands of elongated darkly stained cells resembling the procambial cells

of normal embryos.

TABLE VIII

NUMBERS OF EMBRYOS THAT PRODUCED COTYLEDONS IN VITRO FROM EACH OF 25 EMBRYO SACS OF CITRUS AURANTEUM

Embryo sac	No. cultured		Embryos with cotyledons
1	2 (1)*	(1)**	0
2	4 (4)	(0)	1
3	6 (5)	(1)	2
4	3 (3)	(0)	3
5	3 (3)	(0)	0
6	2 (1)	(1)	1
7	4 (3)	(1)	3
8	8 (4)	(4)	3
9	2 (1)	(1)	0
10	5 (1)	(4)	0
11	2 (1)	(1)	1
12	3 (3)	(0)	3
13	3 (3)	(0)	1
14	6 (2)	(4)	1
15	1 (1)	(0)	1
16	7 (3)	(4)	3
17	4 (2)	(2)	1
18	5 (2)	(3)	1
19	2 (2)	(0)	2
20	6 (4)	(2)	3
21	1 (1)	(0)	1
22	4 (1)	(3)	1
23	9 (3)	(6)	2
24	5 (1)	(4)	1
25	7 (4)	(3)	4

* Cultured in 90% liquid cocoanut medium.
** Cultured in plain White's solution. None of the embryos grew in this medium.

Several attempts were made to induce embryo formation from the nucelli of young seeds by culturing nucellar tips at a stage before embryos could be detected with the dissecting microscope. Although many kinds of media were employed, no

PLATE XI

IN VITRO DEVELOPMENT OF CITRUS EMBRYOS

Figure 1. A group of greatly enlarged C. auranteum proembryos on the surface of 25% cocoanut agar medium

Figure 2. A group of C. auranteum embryos similar to those in Fig. 1, but after several weeks in culture. White cotyledonoid structures have emerged from the brown embryos.

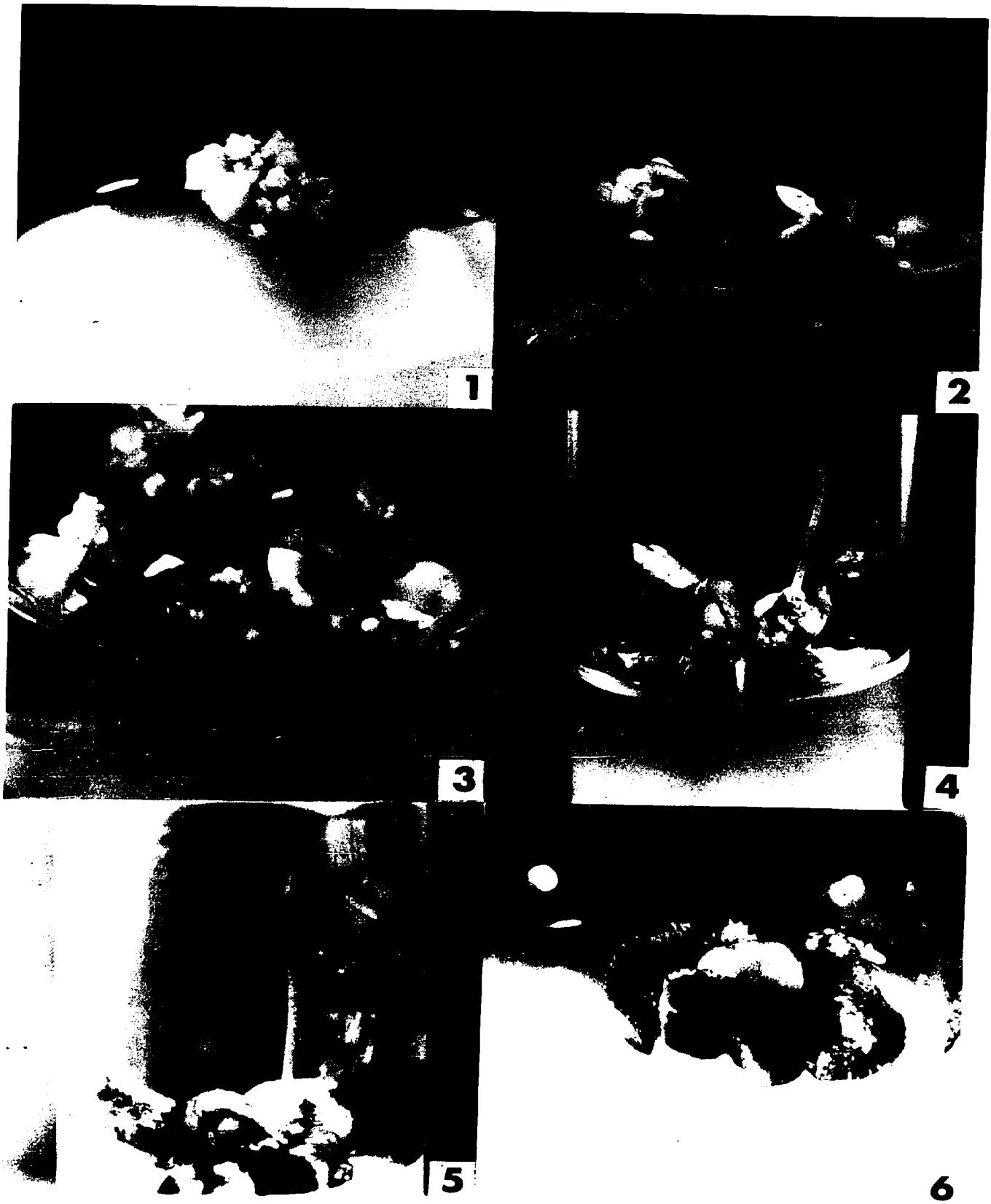
Figure 3. Halves of an unknown species of orange fruits on the surface of an adenine sulphate agar medium. Oddly shaped embryos have arisen from seeds which were less than one millimeter in size.

Figure 4. A group of the embryos derived from the source described in Fig. 3. Some root formation has occurred, and one embryo with an enlarged green cotyledon has formed a shoot.

Figure 5. The same as Fig. 4, but after the shoot has formed three leaves. Some degree of callusing has occurred at the base of the shoot.

Figure 6. A C. auranteum embryo on a rooting medium after having been cultured at the heart stage in 90% liquid cocoanut medium. Several large convoluted, green cotyledons have been formed. Oil glands are visible over the surface of the cotyledons.

PLATE XI



embryos appeared. During the summer of 1953, however, embryo formation was induced from very young seeds of orange fruits of unknown species which were received from the campus of Stanford University. Since the fruits were only six millimeters in diameter and the seeds were less than a millimeter in length, they were unsuitable as a source of dissectable embryos. Some of the fruits were killed and fixed and the rest were surface-sterilized, cut in two and placed on agar containing adenine sulphate in order to induce callus formation. No callus formed, but at the end of seven weeks, tiny, snow-white projections were observed arising from the locules of the small fruit halves. These projections increased in size and number until the bottles were literally filled with greatly lobed and convoluted structures which resembled cotyledons to a great extent.

Only after most of the growth had stopped and the material was separated, could an accurate interpretation be placed on what had occurred. Rapidly growing embryos had arisen from the seeds, and, growing faster than the other embryos, their misshapened cotyledons and hypocotyls had pulled up younger embryos from the locules. Because of pressure some embryos were stuck tightly together and could be separated only by prying them apart with needles. While the larger embryos increased in size and put out convoluted cotyledons, most of the smaller embryos enlarged to irregular shapes and cotyledonoid structures similar to those described for proembryos on agar arose from their surfaces (Plate XI,

fig. 3). A few embryos, on the other hand, were well-shaped and approached normality.

The masses of embryos were transferred several times in an attempt to induce the formation of new embryos from the original ones. One group of embryos which had been grown for several months on 90% cocoanut agar showed chlorophyll formation in the cotyledons while several of the embryos developed elongated hypocotyls (Plate XI, fig. 4). Upon transferring to a medium containing 0.5% sucrose and 0.1 mg./l. of indole-3-acetic acid, one embryo with a bright green cotyledon sent out a shoot bearing three leaves (Plate XI, figs. 4 and 5). The embryo at that time was lying on a mass of other embryos and appeared to have no roots. It would probably not have survived transplanting to soil.

Sections made from uncultured fruits which had been killed and fixed revealed that the tiny seeds contained no multicellular embryos at the time of culturing. Although the ages of the fruits were unknown, their size suggested that they were possibly no more than a month old. The embryos which arose after the fruits were placed in culture, therefore, must have originated from the zygote or from the single cells of the nucellus. While it cannot be positively demonstrated that some of these embryos did come from the nucellus, a few young embryos were found whose bases were joined together by tissue resembling young seed coats. This suggests the origin of more than one embryo from a seed.

Heart Stage. -- Young C. auranteum embryos with coty-

ledonary primordia just forming grew only with the addition of cocoanut milk to the media. Table IX indicates that the embryos did a little better on the agar than in liquid, but the difference is not great. Occasionally, heart embryos on agar formed mammoth mitten-shaped structures which finally gave rise to small cotyledonoid outgrowths as described for proembryos on agar. Usually the cotyledonary primordia enlarged and grew mainly in one plane to form thin, leaf-like cotyledons very little larger than those formed by the proembryos. Internal differentiation proceeded about as far as that described for the proembryos with procambial strands and a few xylem units in the cotyledons.

TABLE IX

THE NUMBERS OF HEART EMBRYOS OF SOUR ORANGE WHICH EXHIBITED SOME DEGREE OF GROWTH ON LIQUID* AND SOLID** MEDIA

	Liquid	Agar
Number cultured	45	50
Number that formed cotyledons	27	39
Percentage of total number cultured	60	78

* 90% liquid cocoanut medium
** 90% agar cocoanut medium

Taking larger sized embryos to be cultured did not always insure a more developed stage of growth by the embryos in vitro. For the most part, there was no correlation between

the initial size of the embryo at time of culturing and the final size attained by the time the embryo was placed on the rooting medium. Yet, in several instances, larger heart embryos showed the most promise of yielding embryos of a size comparable to that attained by fully developed ones in the seed. The enlarged embryos fell into three categories according to the method of cotyledonary development:

1. Embryos with giant, leaf-like cotyledons (Plate XI, fig. 6). Three instances of these arose from embryos grown in a 90% liquid cocoanut medium; two instances from embryos in a 25% liquid cocoanut medium; and two instances from embryos on a 90% cocoanut agar medium. At the time of culturing, the embryos were heart-shaped structures approximately 0.4 millimeter in length. As the cotyledons greatly enlarged they became so branched and lobed that it was impossible to identify separate cotyledons. The cotyledons became bright green with oil glands scattered over their surfaces. With two exceptions the hypocotyls remained inconspicuous or nearly concealed by the large cotyledons. No shoots or roots arose from these embryos.

2. Embryos with cylindrical cotyledons (Plate XII, figs. 3 and 4). Four examples of this type were found in a 25% liquid cocoanut medium from embryos cultured at the late heart stage with lengths approaching 0.5 millimeter. The cotyledonary primordia, instead of becoming flattened, grew out cylindrically with pointed tips. As growth continued they often became reflexed and showed a tendency to coil. In all four instances a large leafy appendage arose at the apex of

PLATE XII

IN VITRO DEVELOPMENT OF CITRUS EMBRYOS

Figure 1. A C. auranteum embryo cultured in a 25% cocoanut milk medium at the heart stage. The cotyledons have remained cylindrical, and a peltate structure has formed at the tip of one cotyledon. A small amount of callus has formed at the base of the hypocotyl.

Figure 2. A cluster of cylindrical cotyledons with peltate tips. A group of C. auranteum embryos at the heart stage were cultured in a nucellar tip. It is not known whether the cotyledons have originated from one embryo or several.

Figure 3. A C. auranteum embryo with two cylindrical cotyledons, and a cotyledon which is leaf-like. After nearly seventeen months in culture, a second leafy cotyledon appeared opposite the first and a plumule developed in between the two.

Figure 4. A C. auranteum embryo with cylindrical cotyledons and a leafy appendage which may be a third cotyledon. Cultured at the heart stage in 25% cocoanut medium.

Figure 5. A C. auranteum embryo originally cultured in a 90% liquid cocoanut medium at the heart stage. Two smaller cotyledons appeared shortly after the formation of the one larger cotyledon. Limited root formation occurred after being placed on a rooting medium.

Figure 6. Two Calamondin embryos cultured at the proembryo stage in 25% cocoanut medium. Some degree of rooting occurred from the embryo on the left.

PLATE XII



the axis of the embryo or at a short distance above the points of attachment of the cylindrical cotyledons. The embryo shown in Plate XII, fig. 3 had been in culture nearly twenty months and had developed a second leafy outgrowth opposite the first when a small plumule arose between the two. One of the four embryos when placed on a medium containing 1 mg./l. of indolebutyric acid developed a short root. It was transferred to sand and watered with White's solution, but it did not survive. The other embryos gave no signs of rooting.

Longitudinal sections made of the embryo shown in Plate XII, fig. 4 after several weeks on agar containing 1 mg./l. of indolebutyric acid revealed xylem elements running the entire length of the axis and continuing into the leafy outgrowth and into the cylindrical cotyledons. Oil glands were present below the epidermis in both axis and leaf. Scattered inclusions which stained bright red with safranin were present in the epidermal cells and in those cells lying a few layers beneath the epidermis. They resembled aleurone grains found in larger embryos.

3. Embryos with cylindrical cotyledons having peltate tips (Plate XII, figs. 1 and 2). Two embryos grown in a 25% liquid cocoanut medium showed this condition. The cylindrical outgrowths appeared from the cotyledonary primordia as described previously, but after attaining maximum growth they produced peltate pads of tissue at their tips. There was no evidence of leafy outgrowths of the type described for the previous embryos.

Embryos Beyond the Heart Stage. -- Embryos with sizes ranging from 0.55 millimeter to 3 millimeters were grown on agar media containing either the basic medium alone, or with 90% cocoanut milk added. All of the embryos acquired chlorophyll after a few days in culture and increased in size in varying degrees. As Tables X and XI show, the embryos increased in size to a greater extent on the medium containing cocoanut milk than on the basic medium alone. None of the embryos produced roots, but hypocotyls occasionally elongated to a slight extent.

TABLE X

SIZE INCREASE OF TEN CITRUS AURANTEUM EMBRYOS AFTER EIGHT MONTHS ON 90% COCOANUT AGAR

Initial length	Final length	Percentage increase
0.68 mm.	2.39 mm.	251
0.95	3.42	260
1.06	1.86	75
1.14	3.26	186
1.14	4.44	289
1.52	4.71	210
1.59	4.71	196
2.09	5.89	182
2.28	4.56	100
2.66	6.08	129

Older Embryos. -- When embryos of the sour orange reached lengths of four to six millimeters, the first indications of rooting occurred. They occasionally formed short roots which pushed down into the agar as far as twelve to fifteen millimeters. The formation of roots, however, was sporadic and independent of any rooting medium. There was no

shoot formation at these stages and development went no farther than this.

TABLE XI

SIZE INCREASE OF TEN CITRUS AURANTEUM EMBRYOS AFTER EIGHT MONTHS ON WHITE'S AGAR

Initial length	Final length	Percentage increase
0.95 mm.	1.29 mm.	36
0.95	1.67	76
1.02	1.52	49
1.59	2.62	65
1.82	3.34	84
1.90	4.21	122
2.20	3.99	81
2.35	3.04	31
2.35	3.04	31
2.35	5.51	134

Embryos seven to eight millimeters in length rooted in almost all instances, but shoot formation occurred only occasionally after rooting. When the embryos did form shoots, they were transferred to pots of sand and watered with White's solution. Some of them lived for several weeks but all eventually died. It is possible, however, that had they been given special care in a greenhouse some of them may have survived.

Embryos of maximum size (ten to twelve millimeters), but taken from immature fruits, did well when first rooted on any of the various agar media, transferred to sand after shoot formation for a short period and then planted in soil.

The sour orange fruits in the Botanical Gardens began to show some indications of yellowing at their bases around

the middle of October. At this time the fruits were around sixty millimeters in diameter and pollination had occurred eight months earlier. By the middle of November and when the fruits were seventy millimeters in diameter they were nearly ripe and showed only small patches of green.

It was possible to obtain maximum-sized embryos which were capable of root and shoot formation long before the fruits had ripened. While embryo size and fruit size are not closely correlated, it was possible to obtain embryos seven millimeters in length from fruits forty-eight millimeters in diameter within four months after pollination, and a few maximum-sized embryos from fruits fifty-seven millimeters in diameter within five months after pollination. Fruits six months old, although still green contained many embryos capable of root and shoot formation.

Pleiocotyly in Citrus Embryos Grown in Vitro

Although the term "pleiocotyly" refers to the production of extra cotyledons, monocotyly as well as polycotyly has been included under this heading by several workers including Haskell (1954) who reviewed the subject. The term is used here in this same sense.

Tricotyly is not uncommon in normal citrus embryos. In handling 283 sour orange embryos beyond the early heart stage, the writer found only six cases of tricotyly or about 2% of the total number. While embryos in mature seeds may be found with only one cotyledon, they are caused by suppression of one cotyledon due to extreme crowding as several embryos

grow in the same seed.

An extremely high degree of variation in cotyledon number occurred in young embryos grown *in vitro*. This was especially true of the stages from the proembryo through the heart stage (Tables XII and XIII).

Interesting forms sometimes arose from proembryos through a failure of the cotyledonary primordia to form two growing points at the apex of the embryo. Instead of assuming the familiar heart shape, the embryo produced a complete ring of meristematic tissue around its apex. Further growth produced a saucer-like outgrowth ringing the upper rim of the hypocotyl (Plate XIII, fig. 1). Occasionally, the meristematic region was not a complete ring, but was interrupted at one point by a small point of non-proliferating tissue. The outgrowth resulting from this meristematic arrangement was collar-like (Plate X, fig. 5; Plate XIII, fig. 2).

TABLE XII

THE OCCURRENCE OF PLEIOCOTYLY IN SOUR ORANGE HEART EMBRYOS GROWN ON 90% COCOANUT AGAR

"Saucers"	6
"Collars"	5
Tricots	1
Polycots	1
Normal	26
Total	39
Percentage of pleiocotyly	33%

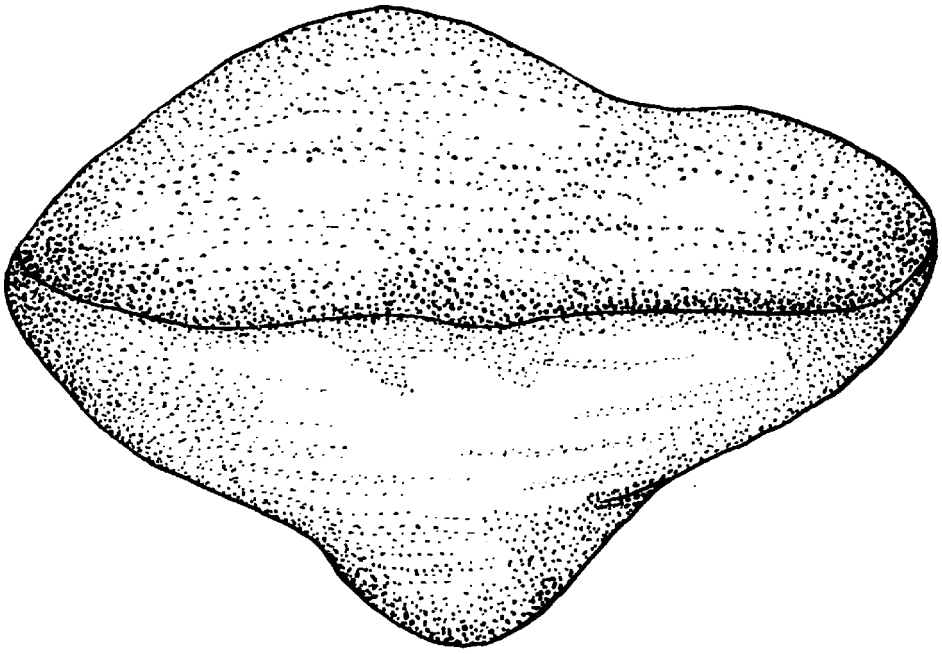
PLATE XIII

PLEIOCOTYLEDONOUS EMBRYOS GROWN IN VITRO

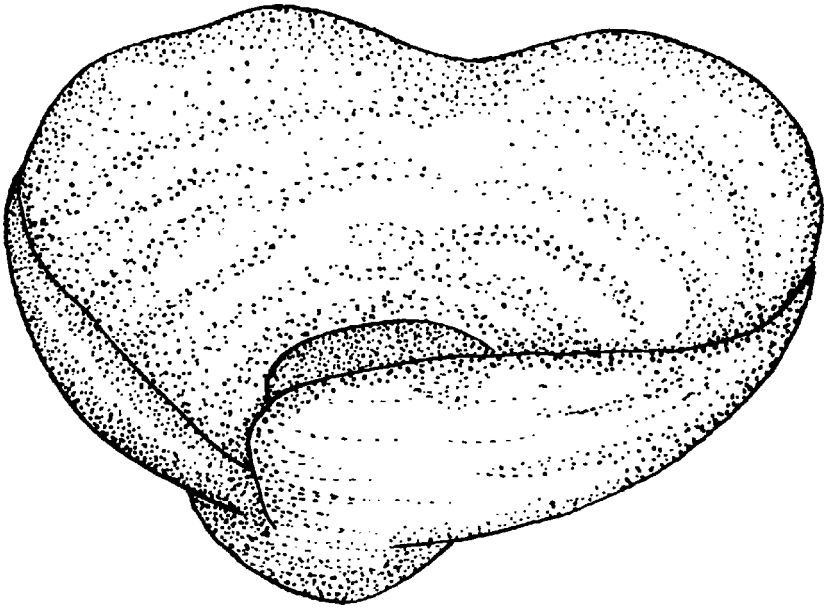
Figure 1. An embryo with a "saucer" type of cotyledon caused by the continuation of growth of the cotyledonary primordial ring at the apex of the proembryo.

Figure 2. An embryo with a "collar" type of cotyledon.

PLATE XIII



1



2

1 mm.

TABLE XIII

THE OCCURRENCE OF PLEIOCOTYLY IN SOUR ORANGE EMBRYOS GROWN IN 90% LIQUID COCOANUT MEDIUM

Type of pleiocotyly	Stage at time of culturing	
	Proembryo	Heart
"Saucers"	1	0
"Collars"	2	2
Tricots	5	5
Polycots	3	7
Normal	15	23
Total	26	37
Percentage of pleiocotyly	42.3%	37.8%

Even embryos cultured at the young heart stage when the two normal cotyledonary primordia were just being initiated sometimes produced "saucers" or "collars". The meristematic ring formed after the embryos were placed in culture and incorporated the two meristematic regions already present so that their identities were lost.

Embryos with four cotyledons commonly occurred by the formation of a second pair of cotyledonary primordia at right angles to the first pair. When all four primordia arose simultaneously as in the case of some proembryos, all cotyledons were approximately the same size. Often, the second pair of cotyledons arose well after the first pair. This was especially true of late heart embryos when the second set of primordia usually arose after several weeks in culture. The second pair arising so long after the first pair consisted only of small fingers of tissue (Plate X, fig. 6).

Tricotyly (Plate X, fig. 4; Plate XII, fig. 5) resulted from the development of only one member of the second pair of cotyledonary primordia. Again the eventual size attained by the extra cotyledon depended on the time of its initiation.

The Culture of Dwarfed Embryos

Because of the competition between the embryos of a single seed for space and nutrition, increasingly fewer numbers of embryos per seed can be found as the seeds mature. In larger seeds containing embryos capable of germination, it was still possible to find dwarfed embryos several millimeters in length which would possibly not have survived to maturity of the seed.

It was thought that inasmuch as these dwarfed embryos were older than the embryos of comparable size which were cultured earlier, they might be induced to complete their growth or at least root prematurely if removed from the seed and given adequate space and nutrition. Accordingly, dwarfed embryos of varying sizes were cultured on all the various solid media listed in Table V. Some of the embryos were placed in 90% liquid cocoanut medium, and some were placed in 0.5% liquid malt extract solution in an attempt to increase their sizes.

All embryos regardless of media became bright green and increased in size. The embryos in the liquid malt extract medium remained green and healthy looking longer than those cultured in liquid cocoanut milk. While the embryos in the

malt extract solution increased somewhat in size (Table XIV), they did not become large enough to root. With the exception of a slight elongation of the hypocotyls of some of the larger embryos on solid media, no significant changes occurred. They behaved similarly to younger embryos of comparable sizes.

TABLE XIV

INCREASE IN SIZE OF DWARFED CITRUS AURANTEUM EMBRYOS AFTER 100 DAYS IN MALT EXTRACT MEDIUM

Length at time of culturing	Size after 100 days	Percentage increase
1.63 mm.	3.00 mm.	90
1.74	3.45	98
1.97	3.57	82
2.20	4.02	83
2.28	3.99	75
2.47	3.80	54
2.58	4.56	73
2.96	4.86	64
3.11	4.67	50
3.30	5.70	73

Discussion

The behavior of citrus proembryos *in vitro* may offer a partial solution to the question of the relationship between the fluid of the embryo sac and the young nucellar buds. Swingle (1932a and 1927) has postulated, as indicated above, that the acquisition of embryonic characters by nucellar embryos during their ontogeny is due to influences arising not from the nucellar tissue, but from the embryo sac apparatus. He suggested that the action of the embryo sac on the nucellus was just the reverse of the condition he had found in the date

palm (1928). The variety of pollen used in pollination of the date affects the size of the fruit and is apparently caused by hormonal action of the embryo and endosperm on the ovular tissue.

Although it is probably not valid to compare genera so widely separated phylogenetically as Garcinia and Citrus, nevertheless it is interesting to note the behavior of the adventive embryos of Garcinia mangostana, the mangosteen, as described by Sprecher (1919) and Horn (1940). The embryos arise from buds from the inner integument of the seed as the nucellus and embryo sac disintegrate. The adventive embryos of mangosteen forming without stimuli from the embryo sac do not possess cotyledons and hypocotyls as do nucellar embryos of Citrus.

The limited differentiation of citrus proembryos when grown in cocoanut milk indicates that the latter partially fulfilled the requirements for organ differentiation which is normally supplied in the seed. Inasmuch as cocoanut milk is a liquid endosperm, one may postulate that it is the contents of the embryo sac which furnish the substances necessary for growth of the embryos. If this is true, Swingle's theory of the formative influences of the embryo sac gains support. That cocoanut milk does not entirely replace the endosperm of Citrus is not surprising, for the successful use of cocoanut milk in culturing Datura proembryos as reported by Van Overbeek, Conklin, and Blakeslee (1941b) does not extend to embryos of all genera and species. Workers such as Smith (1944) and

Hall (1948) have found cocoanut milk to be of no benefit in the culture of Lycopersicum and Solanum embryos respectively.

It should be borne in mind, however, that nucellar embryo formation in the embryo sac is localized at the micropylar end, and only rarely do nucellar buds form at other places. If the fluid of the embryo sac is solely responsible for embryo differentiation, this localization is difficult to explain. It is possible that the influences governing embryo formation may originate from sources other than the nucellus and embryo sac. The seed coats themselves may be cited as possible sources.

The more normal growth of young embryos in a liquid medium over that on an agar surface may perhaps be expected since the primary stages of embryonic development occur in the seed while the embryos are bathed in a liquid endosperm. It is not until the cotyledons are well started that the citrus endosperm becomes a solid tissue.

The formation of masses of tissue from proembryos and early heart embryos when grown on agar and the later appearance of cotyledonoid structures from the masses are reminiscent of the work of Sanders (1950). She found that Datura embryos cultured in liquid media budded small embryos from the parent embryos. The formation of cotyledonoid structures from the masses of tissue may indicate a similar tendency in citrus embryos.

It has been known since the time of Dietrich (1924) that most immature embryos when placed in vitro usually omit

further embryonic development and grow directly into young seedlings. It is surprising, therefore, to find that citrus embryos do not root much below full size on any medium. Van Overbeek, Conklin, and Blakeslee (1942) pointed out the inhibitory effect of cocoanut milk on rooting, but were able to overcome this difficulty by removing Datura embryos from the cocoanut medium as soon as differentiation of cotyledons occurred and rooting them on a medium free of cocoanut milk. Although this procedure was followed for citrus embryos, rooting still did not occur.

Tukey (1934 and 1938) and others have been able to induce rooting of young embryos larger than the heart stage on media containing merely inorganic salts and sugar. Tukey (1934) also found that salt concentrations were not critical in embryo culture, and that similar results could be obtained even when the concentrations were increased tenfold. It seems unlikely, therefore, that premature rooting of older citrus embryos was prevented because a critical concentration of inorganic salts was needed.

A possible explanation of the failure of the embryos to root may lie in the nature of food reserves stored in the fleshy cotyledons. As stated earlier, oil and aleurone grains did not appear in the cotyledons of normal embryos until the embryos had attained a length of six or seven millimeters. This was the size at which some evidence of rooting occurred. Since the cotyledons of cultured embryos remained thin and did not contain oil and protein reserves, this may indicate

an inability of the embryos to store food while under these particular conditions *in vitro*. On the other hand, the accumulation of food reserves at a certain stage may be only a visible manifestation of certain physiological processes which must occur before rooting is possible.

The high percentage of pleiocotyly in cultured citrus embryos is significant when interpreted in the light of what has been discussed in the literature. The natural occurrence of pleiocotyly in dicotyledonous plants is not uncommon. Instances of aberrant monocotyly and tricotyly have been reported for a number of dicotyledonous plants.

Holtorp (1944) attempting to ascertain if tricotyly is heritable, crossed tricots of *Brassica* and was able to produce an F_2 generation with an increased number of tricots. From this the author concluded that dicotyly was adaptive and maintained by natural selection. Straub (1948) also concluded that tricotyly was heritable, but also noted that more tricots were produced in the autumn than were produced earlier in the summer. He showed that tricotyly increased when some of the leaves were removed from the plants during the summer at the time when the fruits were beginning to develop.

Saunders (1928), working with *Matthiola incana*, found that syncotyly and tricotyly often occurred in large sowings. Syncotyly was more prevalent than tricotyly, bu' was more prevalent in some years than in others. She expressed the belief that syncotyly was the result of a "certain misfitting of the developing embryo to the mold of the embryo sac."

The occasional appearance of citrus tricots perhaps could be explained in terms of inheritance, but the number of tricots and polycots from proembryos and young heart embryos in vitro, where nearly 50% of the number which were cultured possessed supernumerary cotyledons (Table XIII), suggests that their appearance may be due to abnormal environmental conditions. Admittedly, the culture medium is a poor substitute for the fluid of the embryo sac, and the substitution of one for the other may well result in a nutritional imbalance that could be expressed in the appearance of the embryos.

Coulter and Land (1914) after studying embryos and seedlings in the genus Agapanthus, concluded that in both monocotyledons and dicotyledons the number of cotyledons produced by an embryo is dependent upon the number of growing points or cotyledonary primordia which arise from the peripheral cotyledonary zone at the apex of the embryo. Therefore, monocotyly was not the result of fusion or suppression of cotyledons, but resulted from the continuation of one growing point from the cotyledonary zone.

Regardless of whether fusion or suppression of the cotyledonary primordia was involved, the formation of "collars" and "saucers" from citrus embryos cultured at the proembryo or heart stage appeared to be due to a general meristematic condition in the cotyledonary zone.

Haskell (1954) pointed out the difficulty of interpreting the origin of supernumerary cotyledons by stating, "From comparison of healthy normal dicots and tricots one cannot tell whether there has been splitting during ontogeny

or the rudiments arose and developed independently." While this was certainly true in the case of the development of polycots from citrus proembryos *in vitro*, it was not true when extra cotyledons developed well after the formation of the first pair of cotyledons. The young cotyledons which developed from proembryos had a tendency to be notched at the tips, and it was difficult to tell how many cotyledons were forming or the manner of origin. The slight notching of these cotyledons may suggest either a fusion or splitting of the cotyledonary primordia, but the independent origin of the primordia so clearly illustrated in older embryos strongly suggests a similar origin of the extra cotyledons from the proembryos.

Because of the variety of shapes that the cotyledons assumed *in vitro*, there may be some question as to whether they may be termed cotyledons at all, but the structures which developed from proembryos, for the most part, arose in the manner as did cotyledons of embryos produced in the seed. Their internal structure was basically similar to those of normal immature cotyledons. In those embryos placed in culture at the heart stage, the cotyledonoid structures developed from cotyledonary primordia which were formed before culturing.

The various forms that citrus cotyledons assumed in culture are not readily explained. The cotyledons of embryos formed in the seed are fleshy storage organs of hypogeous habit. The leaf-like form so often adopted by the cotyledons *in vitro* is then an abnormal one. Citrus proembryos, like all

dicotyledonous proembryos, are radially symmetrical. With the initiation of the cotyledonary primordia, the symmetry changes from radial to bilateral. Even in the early heart stage, however, much of the radial symmetry of the cotyledonary primordia is still retained, for the primordia of the cotyledons are somewhat cylindrical. As the cotyledons develop they undergo a dorsiventral flattening. The cylindrical, horn-like cotyledons produced on several occasions arose from cotyledonary primordia already initiated at the time of culturing. Subsequent growth of the cotyledons was of such a nature as to retain whatever radial symmetry remained of the primordia. Why this should occur is difficult to say. The leafy outgrowths associated with the cylindrical cotyledons as shown in Plate XII, figs. 3 and 4 may be interpreted as supernumerary cotyledons which have arisen later but have enlarged to leaf-like proportions. The leafy outgrowth shown in Plate XII, fig. 4 seems to be located in the wrong position for a cotyledon, yet there is a question of what term is to be applied to the portion above the cotyledons to which the outgrowth is attached. The region above the cotyledons does not seem to be a shoot, although the leafy outgrowth does resemble the first true leaf to some extent. The first leaves, however, occur in pairs and there was no indication of a second outgrowth. The organ in question was possibly a third cotyledon which arose at a slightly different level than the original cotyledons. Subsequent elongation of the mesocotyl may have occurred to carry the third cotyledon to its elevated position.

The peltate structures which occurred at the tips of the cylindrical cotyledons are especially hard to explain. The peltations apparently represent a belated expression of laminar formation of the cotyledons. Candolle (1868 and 1899) expressed the view that the leaf was merely a branch, and that a leaf with a cylindrical petiole and peltate lamina was a more complete expression of the branch tendency than the ordinary leaf with its dorsal and ventral aspects. This view has been upheld and expanded by Arber (1930 and 1941) to support her theory that the plant consists of only one basic unit, the shoot. In her stimulating, yet speculative paper, Arber (1941) considered the leaf as a "partial shoot with tendencies toward a whole shoot", and pointed out examples to illustrate this tendency. Chief among these were (1) the tendency of branches and leaves to overtop the growth of the original shoot, and (2) the adoption of radial symmetry by the leaf with the retention of apical growth. Viewed in this light, the peltate cotyledons of the citrus embryos could be considered as an expression of the shoot tendency. The behavior of the cotyledons of unifoliate species of Streptocarpus illustrates that cotyledons can assume the functions of the plumule when the latter is not present. As described by Hill (1938) no plumule is present in seedlings of certain species of Streptocarpus. Although two cotyledons are formed, one dies and the remaining cotyledon forms the leaf and flowering shoot from its meristematic base. The view expressed at the beginning of this phase of discussion as to the interpretation of the peltate structures

at the tips of the cylindrical cotyledons seems more tenable, however, and the peltate form is only the consequence of the radial symmetry possessed by the more basal portions of the cotyledons.

Much can be gained through future work in culturing citrus embryos, and nucellar embryos in general. The few citrus workers with whom the author has talked have indicated an interest in the earliest possible age that seedlings could be obtained from green fruits. Although it has been shown that seedlings may be obtained within five to six months after pollination, additional work is needed to explore the possibility of obtaining seedlings at a still earlier age.

The culture of nucellar embryos, which has been neglected up to the present time, presents the opportunity of exploring the relative totipotency of the cell. The behavior of nucellar embryos of Citrus, both in normal development and in tissue culture, suggests that the pattern of development characteristic of embryos may not be inherent only in the zygote, but in all cells if they are subjected to conditions to which the citrus nucellus is exposed in the seed.

SUMMARY

1. Tissues capable of unlimited growth have been isolated from *in vitro* cultures of the stigma, style, ovary contents, and juice sacs of an unknown species of lemon, "*Citrus* Tibet", and from the seed coats and juice sacs of *Citrus auranteum*. This is the first reported instance of callus tissues arising from these structures in any plant.

2. Most tissues were first isolated and grown on a basic agar medium consisting of a modified White's solution, 2% sucrose, and 1 mg./l. of indole-3-acetic acid. Growth was accelerated by the addition of yeast extract to the basic medium, but the fastest growth was obtained on the basic medium to which 90% cocoanut milk was added.

3. The tight organization of the cells into files together with the tough cellulose walls gave the calluses a firm quality which required a sharp scalpel to divide the tissue for subculturing.

4. Growth occurred largely from cambium-like files of cells located at the periphery of the calluses, but older terminal cells became highly vacuolated or dead and formed layers of snow-white cells over the surface of the calluses.

5. Polyploidy was common, but no other nuclear aberrations were noted.
6. The tissues which normally possessed leucoplasts in the marginal cells, formed chloroplasts in cultures exposed to continuous bright light for several weeks. Chlorophyll could be made to form or disappear in the cultures at will by alternately storing them in darkness and exposing them to bright light.
7. Despite the cellular organization of the tissues, differentiation did not occur beyond the formation of isolated clusters of tracheid-like cells throughout the calluses.
8. Calluses derived from fruits of C. auranteum differed from those originated from flowers and fruits of the lemon by a more definite arrangement of the cells into files and by filamentous growth at the surface of older cultures.
9. The over-all similarities of the calluses together with the differences between them suggest that (a) the calluses from the same species appeared identical even though they were derived from various plant structures, and that (b) the calluses from different species were readily distinguishable, however, regardless of the structures producing them.
10. In vitro cultures of nucellar and gametic citrus embryos were made at all stages of development and their growth was compared with normal embryo development in the seed.

11. Proembryos failed to grow in a liquid medium of plain White's solution, sucrose, and indole-3-acetic acid even though nucellar tissue was often included in the cultures. Limited organ differentiation occurred, however, when an embryo factor was introduced into the medium through cocoanut milk.

12. Proembryos grown on the surface of the above cocoanut medium solidified with agar often formed enlarged spherical embryos from which cotyledonoid structures arose after several weeks in culture.

13. Embryos of C. auranteum cultured at the heart stage occasionally enlarged to nearly full size, but the cotyledons either (a) remained thin and leaf-like, or (b) grew out as cylindrical horn-like outgrowths.

14. The horn-like outgrowths from the cotyledonary primordia formed peltate structures at their tips on two occasions. Their significance was discussed in the light of Arber's theory of the leaf as a partial shoot.

15. Pleiocotyly was common among embryos grown in vitro. The origin of abnormal cotyledons in some of these embryos suggested a continuation of growth of the whole primordial cotyledonary ring at the apex of the embryo.

16. Citrus embryos, unlike embryos of many other genera, failed to root on a variety of media until they had reached their near maximum size of ten to twelve millimeters. Embryos attained this size in immature fruits within five to six months after pollination.

17. The possible future role of tissue culture in the citrus industry has been considered in the light of callus cultures as a potential source of asexual plants while the culture of immature nucellar embryos offers a means of (a) obtaining seedlings for experimental work at an earlier age than waiting for fruits to ripen, and (b) gaining a better knowledge of the causative factors underlying the initiation and growth of nucellar embryos.

LITERATURE CITED

Arber, Agnes 1930. Root and shoot in the angiosperms. New Phytol. 29: 297-315.

_____. 1941. The interpretation of leaf and root in the angiosperms. Biol. Reviews 16: 82-105.

Atkinson, Elsie, Melvin, S., and Fox, S. W. 1950. Some properties of 2, 3, 5, triphenyltetrazolium chloride and several iodo derivaties. Science 111: 385-387.

Bacchi, O. 1943. Cytological observations in *Citrus*. III Megasporogenesis, fertilization and polyembryony. Bot. Gaz. 105: 221-225.

Barker, W. G. 1952. An *in vitro* study of the proliferative capacity in the woody stele of *Tilia americana* L. Thesis. University of Michigan, Ann Arbor.

Blackwell, Winifred 1913. The histology of the callus tissue in *Althaea rosea*. New Phytol. 12: 305-310.

Brown, Walter V. 1954. A preliminary study of the staining of plant cells by tetrazolium chloride. Bull. Torrey Bot. Club 81: 127-136.

Candolle, C. de 1868. Théorie de la feuille. Arch. Sci. Phys. Nat. Geneve 32: 31-64.

_____. 1899. Sur les feuilles peltées. Bull. Soc. Bot. Geneve 9: 1-51.

Caplin, S. M. 1947. Growth and morphology of tobacco tissue cultures *in vitro*. Bot. Gaz. 108: 379-393.

Cook, R. C. 1938. A note on embryo rejuvenation. Jour. Hered. 29: 419-422.

Cooper, D. C. 1943. Haploid-diploid twin embryos in *Lilium* and *Nicotiana*. Amer. Jour. Bot. 30: 408-413.

Cossmann, K. F. 1940. Citrus roots: Their anatomy, osmotic pressure and periodicity of growth. Palestine Jour. Botany. Rehovot Ser. 3: 65-103.

Coulter, J. M., and Land, W. J. G. 1914. The origin of monocotyledony. Bot. Gaz. 57: 509-518.

Davis, W. B. 1932. Deposits of oil in the juice sacs of citrus fruits. Amer. Jour. Bot. 19: 101-105.

Dietrich, K. 1924. Über Kultur von Embryonen ausserhalb des Samens. Flora 117: 379-417.

Ernst, A. 1918. Bastardierung als Ursache der Apogamie im Pflanzenreiche. Jena.

Fagerlind, F. 1946. Hormonale Substanzen als Ursache der Frucht und Embryobildung bei pseudogamen Hosta-biotypen. Svensk. Bot. Tidskr. 40: 230-234.

Ford, E. S. 1942. Anatomy and histology of the Eureka lemon. Bot Gaz. 104: 288-305.

Foster, A. S. 1934. The use of tannic acid and iron chloride for staining cell walls in meristematic tissue. Stain Tech. 9: 91-92.

Frost, H. B. 1925a. Tetraploidy in Citrus. National Acad. Sci. Proc. 11: 535-537.

_____. 1925b. The chromosomes of Citrus. Wash. (D. C.) Acad. Sci. Jour. 15: 1-3.

_____. 1926. Polyembryony, heterozygosis and chimeras in Citrus. Hilgardia 1: 365-402.

_____. 1938a. Nucellar embryony and juvenile characters in clonal varieties of Citrus. Jour. Hered. 29: 423-432.

_____. 1938b. Genetics and cytology of Citrus. Current Science (Bangalore) Special Number Genetics: 24-27.

Gautheret, R. J. 1939. Sur la possibilité de réaliser la culture indéfinie des tissus de tubercule de carrotte. Comp. Rend. l'Acad. Sci. Paris 208: 118-120.

Grew, Nehemiah. 1682. The anatomy of plants with an idea of a philosophical history of plants and several other lectures, read before the Royal Society. Rawlins, London.

Haberlandt, G. 1902. Culturversuche mit isolierten Pflanzen-zellen. Sitzungsber. Akad. Wiss. Wien Math-Naturw. Kl. III: 69-92.

Haberlandt, G. 1921. Über experimentelle Erzeugung von Adventivembryonen bei Oenothera lamarckiana. Sitzungsber. Preussisch Akad. Wiss. Berlin 40: 695-725.

_____. 1922. Über Zellteilungshormone und ihre Beziehungen zur Wundheilung, Befruchtung, Parthenogenesis und Adventiveembryonie. Biol. Zentbl. 42: 145-172.

Hall, C. B. 1948. Culture of Solanum nigrum embryos. Amer. Soc. Hort. Sci. Proc. 52: 343-346.

Hannig, E. 1904. Zur Physiologie pflanzer Embryonen. I. Ueber die Cultur von Cruciferen Embryonen ausserhalb des Embryosacks. Bot. Ztg. 62: 45-80

Haskell, Gordon 1954. Pleiocotyly and differentiation within angiosperms. Phytomorphology 4: 140-151.

Hayward, H. E., and Long, E. M. 1942. The anatomy of the seedling and roots of the Valencia orange. U. S. Dept. Agr. Tech. Bull. 786.

Hill, A. W. 1938. The monocotyledonous seedlings of certain dicotyledons. With special reference to the Gesneriaceae. Ann. Bot. Lond. n. s. 2: 127-143.

Hodgson, R. W., and Cameron, S. H. 1938. Effects of reproduction by nucellar embryony on clonal characteristics in Citrus. Jour. Hered. 29: 417-419.

Holtorp, H. E. 1944. Tricotyledony. Nature 153: 13-14.

Horn, C. L. 1940. Existence of only one variety of cultivated mangosteen explained by asexually formed "seed". Science 92: 237-238.

Jeffrey, E. C. 1895. Polyembryony in Erythronium americanum. Ann. Bot. 9: 537-541.

Johansen, D. A. 1940. Plant Microtechnique. McGraw-Hill Book Co. New York.

Juliano, J. B. 1934. Origin of embryos in the strawberry mango. Philippine Jour. Sci. 54: 553-556.

Krug, C. A. 1943. Chromosome numbers in the subfamily Aurantioideae with special reference to the genus Citrus. Bot. Gaz. 104: 602-611.

La Cour, L. 1941. Aceto-orcein: a new stain-fixative for chromosomes. Stain-Tech. 16: 169-174.

Laibach, F. 1929. Ectogenesis in plants: methods and genetic possibilities of propagating embryos otherwise dying in the seed. Jour. Hered. 20: 201-208.

Lampton, R. K. 1952. Developmental and experimental morphology of the ovule and seed of Asimina triloba Dunal. Thesis, University of Michigan, Ann Arbor.

LaRue, C. D. 1936. The growth of plant embryos in culture. Bull. Torrey Bot. Club 63: 365-382.

_____. 1942. The rooting of flowers in sterile culture. Bull. Torrey Bot. Club 69: 332-341.

_____. 1948. Regeneration in the megagametophyte of Zamia floridana. Bull. Torrey Bot. Club 75: 597-603.

_____. 1949. Cultures of the endosperm of maize. (abstract). Amer. Jour. Bot. 36: 798.

_____. 1950. Regeneration in Cycas. Amer. Jour. Bot. 37: 664.

Leroy. J. F. 1947. La polyembryonie chez les "Citrus" Son intérêt dans la culture et amélioration. Rev. Bot. Appl. et Agr. Trop. 301-302: 483-495.

Maheshwari, P. 1950. An Introduction to the Embryology of the Angiosperms. McGraw-Hill Book Co. New York.

_____. 1952. Polyembryony in angiosperms. Paleobotanist 1: 319-329.

Matlack, M. B. 1931. The juice sac of the orange with some observations on the plastids of Citrus. Wash. Acad. Sci. Jour. 21: 437-440.

Mattson, A. M., Jensen, C. O., and Dutcher, R. A. 1947. Triphenyltetrazolium chloride as a dye for vital tissues. Science 106: 294.

McLean, S. W. 1946. Interspecific crosses involving Datura ceratocaula obtained by embryo dissection. Amer. Jour. Bot. 33: 630-638.

Miller, H. A., and Wetmore, R. H. 1945. Studies in the developmental anatomy of Phlox drummondii Hook. I. The embryo. Amer. Jour. Bot. 32: 588-599.

Morel, G. 1944. Sur la possibilité de réaliser la culture indéfinie des tissus de vigne. Compt. Rend. Acad. Sci. Paris 219: 36-37.

_____, and Wetmore, R. H. 1951. Fern callus tissue culture. Amer. Jour. Bot. 38: 141-143.

Muir, W. H., Hildebrandt, A. C., and Riker, A. J. 1954. Plant tissue cultures produced from single isolated cells. Science 119: 877-878.

Netolitzky, F. 1926. Anatomie der Angiospermen Samen. In Linsbauer, K. Handbuch der Pflanzenanatomie. Band 10 Lief: 14.

Nielsen, E. L. 1946. The origin of multiple macrogametophytes in Poa pratensis. Bot. Gaz. 108: 41-50.

Nitsch, J. P. 1951. Growth and development in vitro of excised ovaries. Amer. Jour. Bot. 38: 566-576.

Nobecourt, P. 1939. Sur la pérennité et l'augmentation de volume des cultures de tissus végétaux. Compt. Rend. Soc. Biol. Paris 130: 1270.

Norstog, K. L. 1955. The culture of excised ovules, endosperm and embryos of certain grasses. Thesis. Unversity of Michigan, Ann Arbor.

Osawa, I. 1912. Cytological and experimental studies in Citrus. Jour. Coll. Agri. Imp. Univ. Tokyo 4: 83-116.

Sanders, M. E. 1950. Development of self and hybrid Datura embryos in artificial culture. Amer. Jour. Bot. 37: 6-15.

Saunders, E. R. 1928. Matthiola. Bibl. Genet. 4: 141-169.

Schnarf, K. 1929. Embryologie der Angiospermen. Berlin.

Skoog, F. 1944. Growth and organ formation in tobacco tissue cultures. Amer. Jour. Bot. 31: 19-24.

_____, and Chen Tsui. 1948. Chemical control of growth and bud formation in tobacco stem segments and callus cultured in vitro. Amer. Jour. Bot. 35: 782-787.

Smith, P. G. 1944. Embryo culture of a tomato species hybrid. Amer. Soc. Hort. Sci. Proc. 44: 413-416.

Sprecher, M. A. 1919. Étude sur la semence et la germination du Garcinia mangostana L. Rev. Gen. Bot. 31: 513-531.

Stingl, G. 1907. Experimentelle Studie uber die Ernahrung von pflanzlichen Embryonen. Flora 97: 308-331.

Strasburger, E. 1878. Über Polyembryonie. Jenaische Ztschr. f. Naturw. 12: 647-670.

_____, and Hillhouse, W. 1893. Handbook of Practical Botany. Macmillan Co. New York.

Straub, J. 1948. Zur Genetik der Trikotylie. Z. Indukt. Absatmm. u. Vererblehre 82: 331-338.

Swingle, W. T. 1914. Citrus and related genera. In Bailey, L. H. Standard Cyclopedia of Horticulture. New York.

_____. 1927. Seed production in sterile citrus hybrids--its scientific explanation and practical significance. Mem. Hort. Soc. N. Y. 3: 19-21.

_____. 1928. Metaxenia in the date palm. Jour. Hered. 19: 257-268.

_____. 1932a. Recapitulation of seedling characters by nucellar buds developing in the sac of Citrus. VI Int. Cong. Genet. Proc. 2: 196-197.

_____. 1932b. Neophyosis or rejuvenescence of nucellar bud seedlings in Citrus. (abstract). Amer. Jour. Bot. 19: 839.

Taylor, J. H. 1948. Chromosome behavior and structure as revealed by meiosis in cultures of excised anthers. (abstract). Genetics 33: 631.

Tillson, A. H., and Bamford, R. 1938. The floral anatomy of Aurantioideae. Amer. Jour. Bot. 25: 780-793.

Torres, Juan P. 1936. Polyembryony in Citrus and the study of hybrid seedlings. Philippine Jour. Agr. 7: 37-58.

Toxopeus, H. J. 1930. De polyembryonie van Citrus en haar beteekenis voor de culture. Vereen. Landbouw Nederl. --Indie Landbouw Tijdschr. 6: 391-405.

Traub, H. P. 1936. Artificial control of nucellar embryony in Citrus. Science 83: 165-166.

_____, and Robinson, T. R. 1937. Improvement of subtropical fruit crops: Citrus. U. S. Dept. of Agr. Yearbook 749-826.

Tukey, H. B. 1933. Artificial culture of sweet cherry embryos. Jour. Hered. 24: 7-12.

_____. 1934. Artificial culture methods for isolated embryos of deciduous fruits. Amer. Soc. Hort. Sci. 32: 313-322.

_____. 1938. Growth patterns of plants developed from immature embryos in artificial culture. Bot. Gaz. 99: 630-663.

Tukey, H. B. 1944. Excised-embryo method of testing the germinability of fruit seed with particular reference to peach seed. Amer. Soc. Hort. Sci. Proc. 45: 211-219.

Tulecke, W. R. 1953. A tissue derived from the pollen of Ginkgo biloba. Science 117: 559-600.

Van Overbeek, J., Conklin, M. E., and Blakeslee, A. F. 1941a. Chemical stimulation of ovule development and its possible relation to parthenogenesis. Amer. Jour. Bot. 28: 647-656.

_____, _____, and _____. 1941b. Factors in cocoanut milk essential for growth and development of very young Datura embryos. Science 94: 350-351.

_____, _____, and _____. 1942. Cultivation in vitro of small Datura embryos. Amer. Jour. Bot. 29: 472-477.

Webber, H. J. 1900. Complications in citrus hybridization caused by polyembryony. Science 11: 308-309.

_____. 1932a. Variations in citrus seedlings and their relations to root-stock selection. Hilgardia 7: 1-79.

_____. 1932b. Economic importance of apogamy in Citrus and Mangifera. Amer. Soc. Hort. Sci. Proc. 32: 57-61.

_____. 1940. Polyembryony. Bot. Rev. 6: 575-598.

_____, and Batchelor, L. D. 1948. The Citrus Industry. Vol. I. University of California Press. Berkeley.

_____, and Swingle, W. T. 1904. New citrus creations of the Department of Agriculture. U. S. Dept. Agr. Yearbook. 221-240.

White, P. R. 1936. Plant tissue cultures I. Bot. Rev. 2: 419-437.

_____. 1939a. Controlled differentiation in a plant tissue culture. Bull. Torrey Bot. Club 66: 507-513.

_____. 1939b. Potentially unlimited growth of excised plant callus in an artificial nutrient. Amer. Jour. Bot. 26: 59-64.

_____. 1943. A Handbook of Plant Tissue Culture. Jaques Cattell Press. Lancaster, Penn.

White, P. R. 1946. Plant tissue cultures II. Bot. Rev. 12: 521-529.

_____. 1954. The Cultivation of Animal and Plant Cells. Ronald Press Co. New York.

_____, and Braun, A. C. 1942. A cancerous neoplasm of plants. Autonomous bacteria-free crown-gall tissue. Cancer Research 2: 597-617.

Wong, Cheong-Yin. 1939. The influence of pollination on seed development in certain varieties of Citrus. Amer. Soc. Hort. Sci. Proc. 37: 161-164.