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A Detailed Study of Adventive Embryogenesis in the Rutaceae

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This dissertation is dedicated to my son, Oluyomi Bolarinwa

and

to my wife, Olufunmilayo Omobola.

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ABSTRACT OF THE DISSERTATION

A Detailed Study of Adventive Embryogenesis in the Rutaceae

by

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Doctor of Philosophy Graduate Program in Botany
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Professor Toshio Murashige, Chairman

This research explored the mechanism underlying the regulation of adventive embryogenesis in plants, using members of the Rutaceae in tests.

A histological comparison of Ethrog citron, a monoembryonic cultivar, and Ponkan mandarin, a polyembryonic cultivar, disclosed no information relevant to the research objective. Embryogenesis in vivo in the monoembryonic cultivar followed the pattern typical of most dicot plants. No nucellar embryogenesis occurred in the citron. The zygote of the polyembryonic mandarin was not seen to divide. Meristematic activity began in the nucellus prior to zygote division and resulted in adventive embryogenesis all round the embryo sac. No embryos formed from the nucellus epidermis. The multiplication of embryos in the mandarin occurred largely by budding of existing embryos, rather than

continued differentiation among nucellus cells.

The histogenic pattern of polyembryogeny in Ponkan mandarin nucellus culture was the same as that in vivo. Nucellar embryogenesis in vitro in the monoembryonic citron cultivar was confined to the micropylar region. Callus formation was not a prerequisite for adventive embryogenesis in either cultivar. Occasionally, a suspensor was observed in nucellar embryos.

Fruits obtained during the fall and winter months provided best nucellus explants and those of spring months poorest. Best explants were obtained when a transverse incision was made in the nucellus at some distance from the micropylar region for the purpose of removing embryos. Maximum embryogenesis occurred when the nucellus explant was placed in culture with its chalazal end embedded in agar. Light was not necessary for embryogenesis in vitro.

Nucellar embryos were obtained in ovules excised from flower buds of several members of the Rutaceae, without prior pollination.

Test of effects of exogenous substances on nucellus cultures was conducted with the monoembryonic cultivars, Ethrog citron and Ponderosa lemon, and the moderately polyembryonic cultivar Eureka lemon. Embryos were obtained in an agar medium containing only Murashige-Skoog salts and sucrose. No addition of hormonal substances or extracts of natural complexes showed significant benefit.

Of 31 members of the Rutaceae examined, all except one produced adventive embryos in their nucellus cultures. The members differed only in degree of expression of polyembryogeny in vitro.

The micropylar half of a nucellus was clearly more prone to undergo nucellar embryogenesis than the chalazal half. Reciprocal grafts of micropylar halves of polyembryonic and monoembryonic cultivars resulted in no influence on embryogenesis by the one type on the other. In grafts made between the micropylar half of the nucellus of polyembryonic cultivars and the chalazal half of a monoembryonic cultivar, embryogenesis in the normally polyembryonic cultivar was markedly suppressed. This suggested that an embryogenic suppressant was involved in the difference observed in degree of polyembryony among the Rutaceae and between the micropylar and chalazal regions of a nucellus.

The embryogenic suppressant was graft transmissible and diffusable through agar. Its effect was irreversible. This substance(s) also suppressed embryogenesis in callus cultures of the wild carrot.

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I. INTRODUCTION

The seed-bearing plant produces a special structure, the ovule, in which the new offspring develops and through which it is dispersed. The ovule matures into the seed. The most common situation is one in which only one embryo develops per ovule, i.e., monoembryony. There are a number of plant families, however, in which there are seeds with more than one embryo, i.e., they are polyembryonic. The polyembryonic situation can arise through either sexual or asexual means. The more common sexual methods resulting in polyembryos occur by way of twinning of the zygotic embryo and multiple fertilization involving several eggs or embryo sacs per ovule. The concern of this investigation is, however, not embryogenesis through sexual means. It is restricted to asexual embryogenesis; even then, it considers only those instances in which typically embryogenetic events are evident. For example, Yarborough (1932, 1936) described foliar embryos in Bryophyllum and Sedum. These are not correctly embryos[1]; they are, instead, buds which arise in the leaf margins of these plants. Their histogenic and morphologic characteristics are not those of an embryo. Accordingly, this research will not include a consideration of foliar embryos.

The term adventive embryogenesis is used in this investigation to denote all forms of asexual embryogenesis. Under in vitro culture conditions it is evident that asexual embryogenesis can occur from somatic cells of virtually any plant organ. However, under natural

conditions adventive embryogenesis appears to be more restricted to the cells of certain tissues or organs, particularly nucellus, integuments and other tissues of the ovule. The term apomixis has been used to denote this process of asexual embryogenesis as it occurs in the ovule, and four major categories have been recognized (Battaglia, 1963; Gustafsson, 1946-1947; Johansen, 1950; Maheshwari and Sachar, 1963; Nygren, 1954).

The aim of this investigation has been to explore in some detail the process of adventive embryogenesis in plants, with special reference to the asexual embryogeny which occurs in the nucellus of the subfamily Aurantioidae of the Rutaceae. The process has been studied under both in vitro and in vivo situations. It is hoped that the information obtained from this study of the Rutaceae will be applicable in interpreting adventive embryogenetic phenomena in other plants. This research has been accomplished in three parts: (1) observation of histogenesis in monoembryonic and polyembryonic Citrus ovules in vivo and in vitro, (2) assessment of requirements for nucellar embryogenesis in vitro, and (3) determination of a possible regulatory mechanism underlying adventive embryogenesis. In the first part a comparison was made of the development of the nucellus, between the normally monoembryonic Ethrog citron (Citrus medica L. 'Ethrog') and the typically polyembryonic Ponkan mandarin (C. reticulata Blanco 'Ponkan'). In the second, the influence of diverse nutritional and environmental factors on adventive embryogenesis in nucellus cultures was determined. In the third and final part, it is shown that a basic regulatory mechanism in adventive embryogenesis involves a suppressive agent, rather than an embryogenic

stimulant.

The phenomenon of polyembryony, specifically multiple embryos of somatic cell origin, has much significance in agriculture. In the citrus industry it has provided clonal rootstocks (Webber, 1903, 1931, 1932; Toxopeus, 1930; Swingle, 1932, 1933, 1948; Chandi, 1935; Naik, 1947; Hofmeyr and Oberholzer, 1948; Johnston, 1948; Batchelor and Cameron, 1950; Oberholzer and Hofmeyr, 1955; Majsuradze, 1959, 1962; Hodgson, 1961; Cameron and Frost, 1968). Furthermore, citrus plants derived from nucellar embryos have been free of most citrus viruses and related pathogens, even if the mother tree may have been infected (Cameron et al., 1957; Weathers et al., 1957; Rangan et al., 1968, 1969; Bitters et al., 1970). There are disadvantages, particularly in plant hybridization. In the highly polyembryonic citrus, for example, the zygotic embryos are unable to survive the competition with the asexual embryos; hybrids are therefore not easy to obtain when maternal parents of highly polyembryonic characters are used in the breeding program (Webber, 1900, 1931, 1940). Another disadvantage experienced with citrus trees of nucellar origin has been their prolonged juvenility (Webber, 1931; Hodgson and Cameron, 1938; Cameron et al., 1957). Juvenile citrus plants are excessively vegetative, extremely thorny, and produce fruits of poor quality.

II. REVIEW OF LITERATURE

Since adventive embryogenesis leading to polyembryony under natural conditions in most plants occurs in the nucellus, the term nucellar embryony has been most often used. It was coined by Ernst (1918). Other terms have also been used, including extra and false embryony (Frost and Soost, 1968), sporophytic budding (Winkler, 1934), somatic embryony (Halperin, 1970), and nucellar budding (Strasburger, 1878).

Historical Background

Polyembryony in seed-bearing plants is not a recent discovery. The first observation is credited to Leeuwenhoek, who in 1719 found two embryos in some seeds of the orange (Coulter and Chamberlain, 1903; Maheshwari, 1950). Reports of similar occurrences during the next 150 years were surveyed and summarized by Braun (1859), who recorded 63 plant species which showed multiple seedlings arising from germinating seeds. Among the 63 species were *Mangifera indica* and several of *Citrus*.

Strasburger (1878) was first to observe that the multiple embryos in a polyembryonic seed arose from the nucellus. Strasburger studied the phenomenon in *Citrus aurantium*, *Coelobogyne ilicifolia*, *Funkia ovata*, and *Northoscordum fragrans*. He noted that the cells in the micropylar region became rich in cytoplasmic contents, divided particularly actively and ultimately formed several embryos that pushed their way into the embryo sac. Strasburger's observations have since been confirmed

repeatedly in the Rutaceae by many investigators (Biermann, 1896; Osawa, 1912; Frost, 1926, 1938; Toxopeus, 1930; Webber, 1931; Mauritzon, 1935; Chakravarty, 1936; Bacchi, 1943; Vasiljcova, 1951; Furusato, 1952, 1953; Johri and Ahuja, 1956, 1957; Yang, 1968).

Although nucellar embryony is most prevalent in the Rutaceae, its occurrence in other families has also been known for nearly as long. Schacht is credited for having first reported it in the Anacardiaceae in 1859 (Arndt, 1935). Strasburger (1878) and Cook (1907) disclosed that the extra embryos in the Anacardiaceae arose in the nucellus. This disclosure of the Anacardiaceae has been confirmed by numerous investigators (Belling, 1908; Webber, 1931; Juliano et al., 1932; Juliano, 1934, 1937; Cook, 1938; Darlington and Janaki-Ammal, 1945; Gurgel, 1952; Sachar and Chopra, 1957; Sturrock, 1968). In the Cactaceae polyembryony was first reported by Braun (1859), and its nucellar origin was disclosed by Ganong (1898) and confirmed by Montemartini (1899), Hull (1915), Schnarf (1929), Archibald (1939), and Maheshwari and Chopra (1955). Cook (1907) was first to report polyembryony in the Myrtaceae. He suggested that the multiple embryos in Eugenia jambos seeds arose from the nucellus. Cook's suggestion was confirmed subsequently by Tiwary (1926), Pijl (1934), Johnson (1936), and Gurgel (1952).

Nucellar embryogeny is not confined to dicotyledonous plants. It has been reported in a number of monocotyledonous species. Strasburger (1878) described nucellar embryogenesis in the Liliaceae, specifically Funkia ovata and Northoscordum fragrans. Nucellar embryony in other genera and species of the Liliaceae, as well as in other monocotyledonous families, has been reported since (Jeffrey, 1895; Tretjakow, 1895;

Hegelmaier, 1897, 1903; Haberlandt, 1925; Halperin, 1970). In the Orchidaceae, nucellar embryos were first seen by Strasburger (1878) and subsequently confirmed by Leavitt (1900) and others (Suessenguth, 1923; Afzelius, 1928, 1932; Swamy, 1946, 1948).

Extent of Occurrence of Adventive Embryogenesis Among Spermatophytes

Adventive Embryogenesis in Vivo

Natural occurrence of adventive embryogenesis has been reported in at least 27 families of seed-bearing plants. These families and their genera and species, together with the plant tissue or organ in which adventive embryogenesis occurs, are listed in Table 1. Over 85% of the reported cases indicated nucellus origin of the adventive embryos. The other major sources of asexual embryos are the integuments and the suspensor. As revealed through the literature, the Rutaceae contains the greatest number of genera and species which show nucellar embryony. In the Celastraceae, all of the reported cases have been limited to one genus, Euonymus, and all arose from the inner integument. In contrast, all diversities with respect to tissue of origin has been observed in the Liliaceae and Orchidaceae. Generally, nucellar embryony has appeared to be confined to the tissue in the micropylar region and only few cases of adventive embryogeny in the chalazal region of the nucellus have been reported.

Adventive Embryogenesis in Vitro

The number of families which have the capacity to manifest embryogenesis from somatic cells can be extended substantially when

TABLE 1

Reports of adventive embryogenesis in vivo in seed bearing plants

Plant	Tissue or Organ Source of Embryos	Remarks	Reference
Acanthaceae			
Dichanthium annulatum	Nucellus	Occurred only when syngamy failed	Reddy & d'Cruz, 1969
Dipteracanthus patulus	Suspensor		Maheshwari & Negi, 1955
Alismataceae			
Limnocharis emarginata	"		Hall, 1902
Anacardiaceae			
Mangifera indica	Nucellus	Micropylar region only	Arndt, 1935; Belling, 1908; Darlington & Janaki-Ammal, 1945; Gurgel, 1952; Juliano, 1934, 1937, 1960; Juliano *et al.*, 1932, 1960; Mendiola, 1926; Sachar & Chopra, 1957; Schurhoff & Jüssen, 1925; Strasburger, 1878; Sturrock, 1968; Webber, 1931
"	"	Broken irregular pieces of variable sizes of nucellus developed into seedlings	Cook, 1907

Table 1 (continued)

Plant	Tissue or Organ Source of Embryos	Remarks	Reference
Mangifera indica (cont.)	Nucellus	All around embryo sac in Strawberry variety	Juliano, 1934
Odorata odorata	"	All cells abutting the embryo sac	Sachar & Chopra, 1957
Amaryllidaceae *Cyanella capensis*	"		de Vos, 1950
Araceae *Spathiphyllum partinii*	"		Schurhoff & Jüssen, 1925
Betulaceae *Almus rugosa*	Nucellus	Could occur without fertilization; all round embryo sac	Woodworth, 1929, 1930
Bombacaceae *Bombacopsis glabra*	"	Embryos formed from single cells after zygote divided, but adventive embryos outgrew zygotic embryo	Duncan, 1970
Pachira oleagineae	"		Baker, 1960
Burseraceae *Garuga pinnata*	"		Ghosh, 1970

Table 1 (continued)

Plant	Tissue or Organ Source of Embryos	Remarks	Reference
Buxaceae			
Sarcococca hookeriana	Nucellus		Orr, 1923
pruniformis	"		Wiger, 1930, 1935
ruscifolia	"		Simonet & Miedyrzecki, 1932
Cactaceae			
Opuntia aurantiaca	"	Spontaneous adventive embryogenesis; egg degenerates & nucellus becomes massive	Archibald, 1939; Schnarf, 1929
dillenii	"	Pollination & fertilization required; endosperm formed, but no zygotic embryo	Maheshwari & Chopra, 1955
ficus-indica	"		Montematini, 1899
leucantha	"		Montematini, 1899
rafinesquii	"		Hull, 1915
vulgaris	"	Micropylar half only; endosperm essential; no zygotic embryo	Ganong, 1898

Table 1 (continued)

Plant	Tissue or Organ Source of Embryos	Remarks	Reference
Campanulaceae			
Isotoma longiflora	Suspensor		Kausik & Subramanyam, 1946
Lobelia syphilitica	"		Crété, 1938
Capparidaceae			
Capparis frondosa	"		Mauritzon, 1934
Celastraceae			
Euonymus europa	Inner integument	Pollination necessary, but not fertilization	Naumova, 1970
macroptera	"	"	Naumova, 1970
planipes	"	"	"
Curcubitaceae			
Momordica charantia	Nucellus		Agrawal & Singh, 1957
Euphorbiaceae			
Alchornea ilicifolia	"	Pollination not necessary	Strasburger, 1968
Euphorbia			

Table 1 (continued)

Plant	Tissue or Organ Source of Embryos	Remarks	Reference
Graminae			
Tripsacum dactyloides	Nucellus		Farquharson, 1955
Sorghum bicolor	"	Aposporic	Hanna *et al.*, 1970
Juglandaceae			
Pterocarya	"	Involves cleavage of nucellus	Bouman & Boesewinkel, 1969
Leguminosae			
Trifolium ataphaxis	"		Fedortschuk, 1935, 1944
pratense	"		Fedortschuk, 1935
Liliaceae			
Allium odorum	Nucellus		Hegelmaier, 1897
"	Nucellus & integument		Haberlandt, 1925; Tretjakow, 1895
Erythronium americanum	Suspensor & nucellus		Guerin, 1930; Jeffrey, 1895
dens-canis	Suspensor		Guerin, 1930; Hofmeister, 1861

Table 1 (continued)

Plant	Tissue or Organ Source of Embryos	Remarks	Reference
Funkia			
ovata (*Hosta*)	Nucellus		Fagerlind, 1946; Strasburger, 1878
Nothoscordum			
fragrans	"		Darlington & Janaki-Ammal, 1945; Halperin, 1970; Strasburger, 1878
Smilacina			
racemosa	"		MacAllister, 1913
Trillium			
undulatum	"	Only in chalazal region	Swamy, 1948
Tulipa			
gesneriana	Suspensor & nucellus		Beth, 1938; Ernst, 1901
Loranthaceae			
Loranthus			
europaeus	Embryo	Splitting	Braun, 1859
Malpighiaceae			
Malpighia			
sp.	Nucellus		Subra Rao, 1940
Myrtaceae			
Eugenia			
sp.	"		Gurgel, 1952
grandis	"		Johnson, 1936

Table 1 (continued)

Plant	Tissue or Organ Source of Embryos	Remarks	Reference
Myrtaceae			
Eugenia (cont.)			
hookeri	Nucellus		Tiwary, 1926
jambolana	"	In binucleate cells around embryo sac	Tiwary, 1926
jambos	"		Tiwary, 1926
"	Integuments & nucellus	Initiation occurred before pollination; no further developed unless pollinated	Pijl, 1934
"	Nucellus	Forms in germinating seeds	Cook, 1967
Nymphacaceae			
Nyphae			
advena	Suspensor		Cook, 1902
Orchidaceae			
Coelobogyne			
ilicifolia	Nucellus		Strasburger, 1878
Gymnadenia			
conopsea	"		"
Leuxine			
sp.	Integuments nucellus suspensor		Swamy, 1949

Table 1 (continued)

Plant	Tissue or Organ Source of Embryos	Remarks	Reference
Nigrittela nigra	Nucellus	Initiated without pollination	Afzelius, 1928, 1932
Spiranthes sp.	Integument, nucellus & suspensor	"	Swamy, 1948, 1949
Spiranthes cernua	Inner integument	Initiation occurs without pollination, adventive and zygotic embryos occur in different ovules	Leavitt, 1900
Zeuxine sulcata	Nucellus	Occurs by budding	Swamy, 1946, 1949
Zygopetalum mackayi	Integuments & nucellus	Initiated without pollination at micropylar and chalazal regions	Darlington & Janaki-Ammal, 1945; Suessenguth, 1923
Ranunculaceae			
Eranthis hiemalis	Embryo	2,4-D, 2,4,5-T & NAA induction of embryo budding without callus	Haccius, 1955, 1957
Rosaceae			
Alchemilla pastoralis	Nucellus	Only in chalazal region	Murbeck, 1902

Table 1 (continued)

Plant	Tissue or Organ Source of Embryos	Remarks	Reference
Potentilla reptans	Inner integument & nucellus		Souèges, 1935
Rutaceae			
Aegle marmelos	Nucellus		Chakravarty, 1936; Johri & Ahuja, 1956, 1957
Citrus aurantium	"		Bacchi, 1943; Biermann, 1896; Braun, 1859; Osawa, 1912; Strasburger, 1878
bigaradia	"		Osawa, 1912
decumana	"		Braun, 1859
grandis	"		Ensign, 1919
limon	"		Motial, 1963; Sokol-'skaja, 1938
limomum	"		Biermann, 1859
maderaspatana	"		Chandra & Shanker, 1965
medica	"		Toxopeus, 1930, 1933
mitis	"		"

Table 1 (continued)

Plant	Tissue or Organ Source of Embryos	Remarks	Reference
Citrus (cont.)			
natsudaidai	Nucellus		Iwamasa et al., 1970; Yang, 1968
nobilis	"		Osawa, 1912; Toxopeus, 1930
paradisi	"		Bacchi, 1943; Wright, 1937
pennivesiculata	"		Chandra & Shanker, 1965
pseudoparadisi	"	Degree of polyembryony increased by ringing of bark	Furusato & Ohta, 1969
reticulata	"		Sokol'skaja, 1938
"	Suspensor & embryo	Monoembryonic variety Clementine; zygotic origin only	Cameron & Garber, 1968; Ozsan, 1964
sinensis	Nucellus	No pollination necessary	Oppenheimer, 1935; Swingle, 1927; Webber, 1894, 1900, 1903, 1905
"	"		Frost, 1926; Hodgson & Cameron, 1938; Mamporija, 1957; Sokol'skaja,1938
tamurana	"		Miwa, 1952
unshiu	"		Biermann, 1896

Table 1 (continued)

Plant	Tissue or Organ Source of Embryos	Remarks	Reference
Citrus (cont.) *vulgaris*	Nucellus		Biermann, 1896
Various unidentified species and hybrids	Nucellus		Anandam & Singh, 1969; Furusato, 1960, 1968; Furusato & Ohta, 1969; Gurgel, 1951, 1952; Ikeda, 1906; Kapanadze, 1968, 1970, 1971; Majsuradze, 1966, 1968; Nasharty, 1945; Traub, 1936; Wong, 1939
Esenbeckia castanocarpum	"		Braun, 1859
jussieui	"		Braun, 1859
Fortunella sp.	"		Frost, 1926; Furusato & Ohta, 1969; Webber, 1900
Murraya exotica koenigii	"		Chakravarty, 1935, 1936
Poncirus trifoliata	"		Biermann, 1859; Frost, 1926; Iwamasa *et al.*, 1970; Osawa, 1912

Table 1 (continued)

Plant	Tissue or Organ Source of Embryos	Remarks	Reference
Ptelea trifoliata	Nucellus		Mauritzon, 1935
Triphasia auratifolia	"		Braun, 1859; Mauritzon, 1935
Xanthoxylum alata	"		Mauritzon, 1935
bungei	"		Longo, 1908; Mauritzon, 1935; Schnarf, 1929
sp.	"	Spontaneous embryogenesis and endosperm formation	Desai, 1962

observations are carried out under tissue culture conditions. The reports of adventive embryogenesis in cell, tissue or organ cultures of spermatophytes are summarized in Table 2. The total number of families shown to manifest somatic embryogenesis in vitro is not the significant aspect of this tabulation, since only 23 families have been recorded. While a few of the families shown in Table 2 appeared earlier in Table 1, 15 of the 23 families in Table 2 represent plant families which have not been reported to manifest adventive embryogenesis in vivo.

Adventive embryogenesis in vitro has been reported to result from diverse plant parts, including embryo, stem, leaf, mesophyll, root, cotyledon, hypocotyl, mesocotyl, flower, anther, microspore, ovule, endosperm, nucellus, megagametophyte, pericarp, epidermis, and phloem. In some plants embryo initiation has been reported to occur directly from the explant, whereas in others it has been described as occurring in the callus which arises from the tissue or organ. For the most part, adventive embryogenesis in vitro has occurred under relatively primitive culture conditions. Embryogenesis has occurred in either a chemically definable medium of very simple composition or in an undefinable medium containing endosperm fluids, yeast extract and other complex addenda. In either case, little has been established regarding the regulatory basis of adventive embryogenesis. The numerous reports do indicate that totipotentiality is widespread among plants.

TABLE 2

Reports of adventive embryogenesis in vitro in seed bearing plants

Plant	Tissue or Organ Source of Embryos	Remarks	Reference
Asclepiadaceae			
Tylophora indica	Stem	Via callus, no stimulation required; embryogenesis both in liquid and agar	Rao & Narayanaswamy, 1972
"	"	Via callus from young vines; coconut endosperm required	Rao *et al.*, 1970
Compositae			
Cichorium endiva	Embryo	Mature embryo callus, embryo in liquid medium, not in agar	Vasil & Hildebrandt, 1964; 1966
Convolvulaceae			
Cuscuta reflexa	Epidermis	Seedlings produced callus, then embryos	Maheshwari & Baldev, 1962
Crassulaceae			
Kalanchoe pinnata	Leaf	Marginal meristem, via callus	Wadhi & Mohan-Ram, 1964
Cruciferae			
Cheiranthus cheiri	Seedling	Callus from whole seedling, embryogenesis unaffected by subculturing	Khanna & Staba, 1970

Table 2 (continued)

Plant	Tissue or Organ Source of Embryos	Remarks	Reference
Cruciferae (cont.)			
Sinapsis alba	Hypocotyl	Via callus, regulated by coconut endosperm, 2,4-D, Kinetin, NAA, and Yeast extract	Bajaj & Bopp, 1972
Cucurbitaceae			
Curbita pepo	Hypocotyl & cotyledon	Via callus, endogenous control implicated	Jelaska, 1972
"	Pericarp	Via callus	Schroeder, 1969
Cupressaceae			
Biota orientalis	Embryo	Direct on cotyledon	Konar & Oberoi, 1965
Cycadaceae			
Cycas sp.	Megagametophyte	Haploid and diploid explants; via callus	Norstog & Rhamstine, 1967
Zamia sp.	"		"
Euphorbiaceae			
Ricinus communis	Endosperm	Via callus, only proembryo formed	Satsangi & Mohan-Ram, 1965
Graminae			
Bromus inermis	Mesocotyl	Via callus	Constanbel *et al.*1971; Gamborg *et al.*, 1970

Table 2 (continued)

Plant	Tissue or Organ Source of Embryos	Remarks	Reference
Graminae (cont.)			
Hordeum			
vulgare	Embryo	Kinetin stimulation; no callus	Norstog, 1970
Ilicinae			
Ilex			
aquifolium	"	No callus	Hu & Sussex, 1971
cornuta	"	Via callus	"
opaca			
Leguminosae			
Lotus			
caucasious	Anther	Via callus of somatic cells; no haploids	Minoru & Grant, 1971
corniculatus	"	"	"
Phaseolus			
vulgaris	Stem	Via callus, only proembryo on agar	Bergmann, 1960
Liliaceae			
Asparagus			
officinalis	Hypocotyl	Via callus; IAA - kinetin control; kinetin at low concentration	Wilmar and Hellendorn, 1968
Loranthaceae			
Nuytsia			
floribunda	Embryo	Via callus	Nag & Johri, 1969

Table 2 (continued)

Plant	Tissue or Organ Source of Embryos	Remarks	Reference
Loranthaceae (cont.)			
Dendrophthoe falcata	Embryo	Via callus, from globular zygotic embryo	Johri & Bajaj, 1962, 1963, 1965
Malvaceae			
Gossypium hirsutum	Embryo & suspensor	In cultured ovules, no callus	Joshi, 1962
Moraceae			
Ficus afganistanica	Nucellus & egg apparatus	Stimulation by foreign pollen and penicillin	Zdruikovskaya-Rikhter, 1970
Orchidaceae			
Cymbidium sp.	Shoot tip	Via protocorm	Morel, 1960, 1964
sp.	Embryo	Cell suspension culture, coconut endosperm & NAA beneficial	Steward *et al.*, 1971
Ophrys sp.	"	Via protocorm	Champagnat & Morel, 1972
Ranunculaceae			
Ranunculus sceleratus	Anther	Direct embryogenesis and via callus of proliferated connectives	Konar & Nataraja, 1965, 1969; Nataraja & Konar, 1970

Table 2 (continued)

Plant	Tissue or Organ Source of Embryos	Remarks	Reference
Ranunculus sceleratus	Embryo	Direct in achenes of cultured ovaries; coconut endosperm and casein hydrolysate beneficial	Sachar & Guha, 1962
"	Epidermis	Direct embryogenesis on stems of in vitro developed seedlings	Konar & Nataraja, 1965b, 1965e; Konar *et al*, 1972
"	Flower	Via callus from sepals, petals carpels and seeds; no direct embryo from natural seedlings	Nataraja & Konar, 1970
"	Flower bud	Via callus	Konar & Nataraja, 1965a
"	"	Direct embryogenesis	Thomas *et al*., 1972
"	"	Via callus in suspension cultures	Konar & Nataraja, 1965c
"	Flower primordium	Via callus; sequence of development followed	Konar & Nataraja, 1965, 1969
"	Radicle	Direct embryogenesis, seedlings produced in vitro, no callus	Konar & Nataraja, 1965e
Rubiaceae *Coffea canephora*	Stem	Via callus	Staritsky, 1970

Table 2 (continued)

Plant	Tissue or Organ Source of Embryos	Remarks	Reference
Rutaceae			
Citrus			
aurantifolia	Nucellus	Monoembryonic variety through direct embryogenesis, no callus; malt extract beneficial	Bitters et al., 1970
"	"	Polyembryonic explant used; only micropylar halves, longitudinal section produced callus & some embryos	Sabharwal, 1962
aurantifolia	"	Via callus	Singh, 1963
grandis	"	Monoembryonic species direct embryogenesis, no callus, malt extract beneficial	Rangan et al., 1968, 1969; Bitters et al., 1970
kharna	"	Via callus	Singh, 1963
limon	"	Monoembryonic, variety Ponderosa	Rangan et al., 1968, 1969; Bitters et al., 1970
"	"	Monoembryonic, variety Meyer	Bitters et al., 1970
microcarpa	Ovule & nucellus	Via pseudobulbils explants with proembryos used, casein hydrolysate beneficial	Maheshwari & Rangaswamy, 1958; Rangaswamy, 1958
"	"	Adverse effects of gibberellin, coconut endosperm & malt extract	Rangaswamy, 1961

Table 2 (continued)

Plant	Tissue or Organ Source of Embryos	Remarks	Reference
microcarpa	Ovule	Via callus & pseudobulbils, explants from pollinated flowers	Johri, 1961
paradisi	Ovule and nucellus		Kochba *et al.*, 1972
Citrus reticulata	Nucellus	Monoembryonic, variety Algerian; direct embryogenesis	Bitters *et al.*, 1970
"	"	Polyembryonic variety, via callus and pseudo bulbil	Sabharwal, 1963; Singh, 1963
" x *C. sinensis*	"	Monoembryonic hybrid Temple tangor; direct embryogenesis	Rangan *et al.*, 1968, 1969; Bitters *et al.*, 1970
sinensis	Ovule	Ovules from unpollinated flower buds; no callus; polyembryonic variety Robertson	Bitters *et al.*, 1970
"	"	Polyembryonic fruits harvested 8 weeks after anthesis, unpollinated; via callus; malt extract and adenine sulfate beneficial; Washington Navel	Button & Bornman, 1971
"	Ovule and nucellus	1-8 week-old parthenocarpic fruits; via callus, malt extract beneficial; polyembryonic variety	Kochba *et al.*, 1971

Table 2 (continued)

Plant	Tissue or Organ Source of Embryos	Remarks	Reference
sinensis	Nucellus	Via callus; 1st report other than in *Citrus*	Singh, 1963
Papeda *Khasi*	"	Monoembryonic species; direct embryogenesis	Bitters, *et al.*, 1970
Poncirus *trifoliata*	"	Via callus; polyembryonic species	Singh, 1963
Santalaceae			
Santalum *album*	Seed	Via callus due to yeast extract kinetin and 2,4-D; & embryogenesis due to casein hydrolysate & coconut endosperm	Rao, 1965; Rao and Rangaswamy, 1972
Solanaceae			
Atropa *belladona*	Root	Via callus	Konar, *et al.*, 1972
"	"	Via callus; NH_4^+ beneficial for adventive embryogenesis	Thomas & Street, 1972
Datura *innoxia*	Anther	First anther culture; spontaneous embryogenesis; casein hydrolysate, IAA kinetin and coconut endosperm beneficial	Guha & Maheshwari, 1964

Table 2 (continued)

Plant	Tissue or Organ Source of Embryos	Remarks	Reference
Datura (cont.)			
innoxia	Embryo	Via embryonic mass of tissue malt extract beneficial	Sanders, 1950
"	Microspore	Anther culture; coconut endosperm beneficial	Guha & Maheshwari, 1966, 1967
metel	"	Anther culture; diploid & haploid embryos	Narayanaswany & Chandy, 1972
meteloides	"	"	Kohlenbach & Geier, 1972
wrightii	"	"	"
Nicotiana			
tabaccum	Microspore	Anther culture	Davreux et al., 1971; Minoru & Grant, 1971; Nakata & Tanaka, 1968; Nitsch, 1969; Nitsch & Nitsch, 1969; Sunderland & Wick, 1971; Vazart, 1971
"	Epidermis & subepidermis	Via callus; additional embryos arise directly in the tissues of in vitro produced adventive embryos	Haccius & Lakshmanan, 1969

Table 2 (continued)

Plant	Tissue or Organ Source of Embryos	Remarks	Reference
Nicotiana tabaccum	Germinating seed	Via callus; isolated single cell form embryo in microculture; IAA & kinetin beneficial	Vasil & Hildebrandt, 1967
"	Mesophyll	Via isolated protoplast and callus	Takebe et al., 1971
"	Stem	Via callus; isolated single cell on agar	Bergmann, 1959, 1960
"	"	Via callus	Davreux et al., 1971
melongena	Hypocotyl & root	Via callus	Yamada et al., 1967
nigrum	Microspore	Anther culture; haploid callus & plants	Harn, 1971
tuberosum	"	Via callus from anther culture	Kohlenbach & Geier, 1972
Umbeliferae			
Ammi majus	Zygote	Via callus in ovule explants from pollinated flowers	Sehgal, 1972
Anethum graveolens	Embryo	Via callus; casein hydrolysate and yeast extract beneficial	Johri & Sehgal, 1963
"	"	No callus; in ovary culture	"

Table 2 (continued)

Plant	Tissue or Organ Source of Embryos	Remarks	Reference
Anethum (cont.) graveolens	Embryo	Via callus in ovary cultures	Sehgal, 1969
"	Hypocotyl	Via callus of in vitro produced seedlings, casein hydrolysate & yeast extract beneficial	"
Apuim graveolens	Phloem	Via callus	Reinert et al., 1966
Datura ceratocaula	Embryo	Via callus; malt extract beneficial	McLean, 1946
Daucus carota	"	Via callus; adventive embryos formed in vitro; ethylene prevents organogenesis	Wochock & Wetherell, 1971
"	Leaf	Via callus from petioles and peduncles; specific cells develop into embryos	Halperin & Wetherell, 1964
"	"	Via callus from petioles; low auxin cone or antiauxins beneficial	Newcomb & Wetherell, 1971
"	"	Via callus	Musiyaka et al., 1972
"	Phloem	Via callus; coconut endosperm and cell isolation prequisite	Steward et al., 1958

Table 2 (continued)

Plant	Tissue or Organ Source of Embryos	Remarks	Reference
Daucus (cont.) carota	Phloem	Via callus	Reinert, 1959
"	"	Via callus; embryogenesis induced chemically through substances like coconut endosperm	Reinert, 1961
"	"	Via callus; NH_4^+ requirement satisfied by kinetin	Halperin & Wetherell, 1965
"	"	Via callus; coconut endosperm and nitrogen compds beneficial	Reinert, 1963, 1967; Reinert et al., 1967
"	"	Via callus; no embryogenesis on agar with coconut endosperm or without IAA; casein hydrolysate reduces period for embryogenesis; explant of domestic carrot, 17 year old in culture	Sussex & Frei, 1968
"	"	Via callus; auxin-nitrogen balance and all forms of N-compds are beneficial	Reinert & Tazawa, 1969
"	"	Via callus; single cell traced through embryogenesis	Backs-Husemann & Reinert, 1970
"	Root	Via callus; structure developed occurs in two steps, each step regulated by specific metabolite, e.g., protein	Butenko & Yokovleva, 1962

Table 2 (continued)

Plant	Tissue or Organ Source of Embryos	Remarks	Reference
Daucus (cont.) carota	Root	Via callus	Halperin, 1966; Halperin & Wetherell, 1963, 1964; Linser & Newmann, 1968; Wetherell & Halperin, 1963
"	"	Via callus; NaCl & Na_2SO_4 inhibit organogenesis but not embryogenesis	Butenko et al., 1967
"	"	Via callus; adventive embryogenesis in vitro is very different from that in vivo in sequence of events & behavior of seedlings	Nakajima & Yamaguchi, 1967
"	"	Via callus; number of embryos formed is proportional to glucose concentration	Homes, 1968
"	"	Via callus; auxin-kinetin produce abnormal embryos	Ibrahim, 1969
"	"	Via callus; M-S and White's media compared with and without NH_4^+ or NO_3^- addenda	Tazawa & Reinert, 1969
"	"	Via callus; high osmoticum and high nitrogen compds beneficial	Ammarito & Steward, 1971

Table 2 (continued)

Plant	Tissue or Organ Source of Embryos	Remarks	Reference
Daucus (cont.) carota	Stem	No callus; from hypocotyl epidermis of in vitro produced seedlings	Kato, 1968; Kato & Takeuchi, 1963
"	"	Via callus, hypocotyl sections; glucose regulates number of embryo formation	Homes, 1968
"	"	Via callus of hypocotyl section; NH_4^+ stimulates better than NO_3^-; low auxin not required for embryogenesis, but 2-NOA is best auxin	Norreel & Nitsch, 1968
Foreniculum vulgare	"	Via callus; better differentiation basal medium, but further growth requires coconut endosperm	Maheshwari & Gupta, 1965
"	Embryo	Via callus, yeast extract and coconut endosperm beneficial	Sehgal, 1964
Petroseluim hortense	Leaf	Via callus from petioles; embryogenesis only with adenine sulfate; coconut endosperm not essential	Vasil & Hildebrandt, 1966
Sium suave	Embryo	Via callus; high osmotica with high nitrogen beneficial	Ammarito & Steward, 1971

Histogenesis Associated with Adventive Embryogenesis

Histogenesis in Vivo

Rutaceae. Strasburger (1878) was first to describe the sequence of histogenetic events associated with nucellar embryogenesis in the Rutaceae. In Citrus aurantium he observed that the nucellus cells in the micropylar region, and especially the more deeply seated cells, became richer in cytoplasmic material and stained more intensely than the other cells of the ovule. These deeply staining cells were also isodiametric. They divided rapidly and produced cell clusters which eventually invaded the embryo sac. From these cell clusters arose the adventive embryos by a process which Strasburger described as nucellar budding. Subsequent to Strasburger, Biermann (1896) described in less detail the same process in C. vulgaris Risso. Osawa (1912) presented a more elaborate account of the same events from observations of C. aurantium, C. nobilis and Poncirus trifoliata. Still later investigations have described nucellar embryogenesis in further genera of the Rutaceae, e.g., Xanthoxyllum (Longo, 1908; Schnarf, 1929; Mauritzon, 1935; Desai, 1962b), Fortunella (Webber, 1900; Frost, 1926; Furusato and Ohta, 1969), Murraya (Chakravarty, 1935, 1936), Aegle (Chakravarty, 1936; Johri and Ahuja, 1956, 1957), and Ptelea and Triphasia (Mauritzon, 1935). In each case the histogenetic pattern of embryogenesis has been identical to that described by Strasburger. Additional reports from studies of the Rutaceae by Bacchi (1943), Yang (1968), Iwamasa et al. (1970), and Kapanadze (1971) have only served a confirmatory purpose. There has yet to be a report describing nucellar embryogenesis in the chalazal region of a Rutaceous ovule.

In passing, mention is made of polyembryony associated with the sexually derived embryo. In the Rutaceae, for the most part these polyembryos of sexual origin occur in the normally monoembryonic types and arise by budding or cleavage of the zygotic embryo (Swingle, 1900; Frost, 1926, 1943; Traub et al., 1937; Ozsan, 1964; Ozsan and Cameron, 1963; Cameron and Garber, 1968).

Other families. Strasburger's (1878) report showed that nucellar embryogenesis in other plant families occurred by histogenic processes similar to that in the Rutaceae. His description of nucellar embryogeny in the Anacardiaceae, Euphorbiaceae and Liliaceae differed from that of the Rutaceae only by the smaller numbers of asexual embryos produced per ovule in the former families.

Ganong's work (1898) describing similar histogenetic patterns of nucellar embryogenesis in the Cactaceae has been confirmed by other investigations (Montemartini, 1899; Hall, 1915; Schnarf, 1929; Maheshwari and Chopra, 1955). According to Ganong, nucellar embryogenesis in the Cactaceae included cells of the more superficial layers, as well as the deep-seated layers of the nucellus. Ganong referred to the more superficial nucellus layers as nucellar walls. Tiwary (1926) observed adventive embryogenesis occurring also in superficial layers of the nucellus of Eugenia and he referred to such locations as nucellar pads.

Murbeck (1902) was first to describe the confinement of adventive embryogenesis to the chalazal region of the nucellus of Rosaceae. Subsequently, Suessenguth (1923) and Swamy (1948) reported on similar restricted nucellar embryony in the chalazal region of the ovules of the Orchidaceae and Liliaceae, respectively.

The occurrence of adventive embryos in the micropylar and chalazal regions as well as the lateral flanks of the nucellus has been reported in two families: Anacardiaceae (Juliano, 1934; Sachar and Chopra, 1957) and Betulaceae (Woodworth, 1929, 1930).

Maheshwari and Rangaswamy (1958) described multiple embryo formation in Mangifera indica through cleavage of the nucellus; this cleavage of the nucellus occurred at the time when the nucellus was still in its primordial stage of development within the flower bud. This observation has now been confirmed by Bouman and Boesewinkel (1969).

In some families, adventive embryogenesis is known to occur in tissues of the ovule other than the nucellus. Tretjakow (1895) published one of the earliest reports of adventive embryogenesis occurring in the integument of Allium odorum. Leavitt (1900) noted that the inner integument served as a source of embryogenic tissue in Spiranthes cernua. Integumentary embryony has now been confirmed through observations of many plants, including A. odorum (Haberlandt, 1925), Eugenia jambos (Pijl, 1934), Potentilla reptans (Souege, 1935), S. cernua (Swamy, 1948), and Euonymus sp. (Naumova, 1970). The histological events in integumentary embryony are apparently comparable to those of nucellar embryony.

While embryogenesis resulting in polyembryos from suspensor cells is more correctly one of multiplication of the sexual rather than the somatic embryo, it is nevertheless an adventitious method of embryogenesis and is worthy of mention. The first report of multiple embryos arising from the suspensor can be attributed to Jeffery (1895). According to Jeffery sometimes the suspensor of an Erythromium americanum embryo remains meristematic and develops into a parenchymatous

mass from which numerous embryos can bud off. In other species, adventive embryos have been reported to occur by budding directly from the suspensor, without prior formation of a parenchymatous mass (Hall, 1902; Crete, 1938; Swamy, 1943; Mohan Ram, 1959).

Histogenesis in Vitro

Rutaceae. Rangaswamy (Rangaswamy, 1958, 1959; Maheshwari and Rangaswamy, 1959) was first to culture successfully the excised nucellus. He cultured the micropylar half of the nucellus of Citrus microcarpa, a usually polyembryonic cultivar. The proembryos which had already been initiated in the explant developed into a multitude of seedlings. Rangaswamy noted that the cells first proliferated into structures which he termed pseudobulbils and from which embryos subsequently arose. Sabharwal (1961, 1963) extended Rangaswamy's work to other polyembryonic Citrus cultivars: C. reticulata and C. aurantifolia. According to Sabharwal, the embryos arising in vitro were initiated from cells of existing proembryos or embryos, and not from previously uninvolved nucellus cells. This observation by Sabharwal has been substantiated by other investigators from work with still other polyembryonic members of the Rutaceae (Singh, 1963; Button and Bornman, 1971; Kochba et al., 1972). Callus formation and pseudobulbil initiation were always reported to precede embryo differentiation.

Rangan et al., (1968) were first to describe the initiation of embryos in nucellus explants isolated from normally monoembryonic Citrus: C. limon L. Burm 'Ponderosa' and C. reticulata x C. sinensis.(Templetangor). In contrast to the observations made on cultures of naturally polyembryonic materials, the embryos in the nucellus of monoembryonic

Citrus arose directly from the nucellus and without an intermediary callus or pseudobulbil stage. This work with in vitro cultures of the nucellus of naturally monoembryonic Citrus has since been extended to diverse other monoembryonic species and varieties including C. aurantifolia 'Bearsslime,' C. grandis, C. limon 'Meyer,' C. reticulata 'Algerian' (Rangan et al., 1969; Bitters et al., 1970).

Other families. In the last 15 years there have been reports of adventive embryogenesis in tissue and organ cultures of diverse plants. The most thoroughly studied of these has been the embryogenesis in tissue cultures of Daucus carota . Steward et al. (1958) were first to observe the D. carota phenomenon, and many have subsequently conducted a variety of experiments with the system (Bergmann, 1959, 1960; Reinert, 1959; Kato and Takeuchi, 1963; Steward et al., 1963, 1964; Konar and Nataraja, 1964, 1965c, 1969; Halperin, 1964, 1966; Halperin and Wetherell, 1964, 1965; Vasil and Hildebrandt, 1966a, 1966b, 1967; Nakajima and Yamaguchi, 1967; Backs-Husemann and Reinert, 1970; Rao and Narayanaswamy, 1970, 1972; Constabel et al., 1971; Takebe et al., 1971; Konar et al., 1972a, 1972b).

According to Steward et al.,(1958, 1963, 1964), embryogenesis in carrot tissue cultures occurs by a sequence of events which simulates very closely that of sexual embryogenesis in vivo; a single isolated cell, mimicking the zygote, undergoes ontogenesis to give rise to an embryo. This interpretation of Steward and his associates has not been supported by the facts, however. Carefully carried out observations by numerous other investigators, e.g., Halperin (1964, 1966), Halperin and Wetherell (1964, 1965) and Backs-Husemann and Reinert (1970), have shown

consistently that adventive embryogenesis in carrot tissue cultures occurs through an intermediary callus stage. A mass of parenchymatous cells is formed first, and one to many embryos are initiated subsequently within this mass. There has been no evidence of embryogenesis occurring directly from a single cell. The histogenetic process as evident in D. carota has also been reported in adventive embryogenesis in tissue cultures of other species (Table 2, plants described as undergoing embryogenesis through callus). The embryos which arise in the callus mass manifest some of the key stages which are normally observed in embryogenesis associated with the zygote. One is able to observe globular, heart-shaped and torpedo-shaped stages, and the mature embryo which develops from the callus possesses cotyledons, plumule and radicle, and sometimes even a suspensor. Nevertheless, the histogenic and organogenic events, particularly in the initial stages, are not the same. In zygotic embryogenesis it is possible to follow the cell division sequence and to identify the cells which ultimately make up the proembryo; in adventive embryogenesis in callus cultures, on the other hand, there is no predictable sequence of cell divisions and it is difficult to trace the original cells of specific embryos (Ball and Joshi, 1965; Nakajima and Yamaguchi, 1967; Konar and Nataraja, 1969; Nataraja and Konar, 1970).

In some plants embryogenesis in vitro has been reported to occur directly from somatic cells without the intermediary of a callus. In such instances the embryos have been observed to arise directly from the freshly excised tissue. Multiple embryos have occurred in embryo cultures of many plants, presumably by a process of budding from the existing embryo, e.g., Cymbidium sp. (Morel, 1960, 1964; Steward et al.,

1971), Biota orientalis (Konar and Oberoi, 1965), Hordeum vulgaris (Norstog, 1970), Ophrys sp. (Champagnat and Morel, 1972), and Ilex aquilifolium (Hu and Sussex, 1971). In other cases embryos have been reported to arise from the epidermis, particularly of the seedling stem, e.g., Cuscuta reflexa (Maheshwari and Baldev, 1962), Ranunculus sceleratus (Konar and Nataraja, 1965b, 1965c, 1965e; Konar et al., 1972a; Thomas et al., 1972b), Daucus carota (Kato, 1968), and Nicotiana tabacum (Haccuis and Lakshmanan, 1969). A few cases of embryogenesis through budding in cultures of ovule, ovary and flower bud have also been reported, e.g., Gossypium hirsutum (Joshi, 1962), Anethum graveolens (Johri and Sehgal, 1963) and Ranunculus sceleratus (Thomas et al., 1972a,b).

In view of their major agricultural significance, particularly in hybridization and mutation breeding, the haploid embryos which arise directly from microspores in anther cultures have attracted much interest in recent years. Embryogenesis from microspores was first reported by Guha and Maheshwari (1964) in Datura innoxia anther cultures. Anther cultures resulting in haploid embryos have now been confirmed in Datura innoxia (Guha and Maheshwari, 1966, 1967) and further reported in D. metel (Narayawaswany and Chandy, 1972); D. meteloides (Kolenbach and Geier, 1972); Lotus sp. (Minoru and Grant, 1972) and Nicotiana tabacum (Nakata and Tanaka, 1968; Nitsch, 1969; Nitsch and Nitsch, 1969; Devreux et al., 1971; Sunderland and Wicks, 1971; Vazart, 1971). In each of these instances the microspores appeared to behave as zygotes; and instead of differentiating into pollen grains they proceeded through a series of cell divisions and organization to produce embryos, one per

microspore.

Concepts Underlying Adventive Embryogenesis

Historical Information

Haberlandt, 1894-1902, was first to propose the concept of totipotentiality in plant cells (White, 1931; Krikorian and Berquam, 1969). According to Haberlandt, any plant cell has the capacity to reproduce the whole plant if provided the appropriate environment. He attempted to demonstrate this in a series of unsuccessful experiments with isolated cells in vitro. Haberlandt was unable to cause isolated plant cells to divide in vitro, let alone to develop into complete plants. His cells were placed in a culture solution containing inorganic salts, glucose and asparagine. Although he failed to demonstrate totipotentiality, Haberlandt proposed a fundamental physiological basis for adventive embryogenesis. According to him (1921a, 1921b, 1922a, 1922b), in nucellar embryony the degenerating cells of the ovule, particularly the degenerating nucellus cells, produced a substance necrohormone which stimulated the other nucellus cells into embryogenesis. As evidence, he cited the adventive embryos which arose in ovules of Oenothera lamarkiana following injury of the ovules, either by pricking them with sharp objects or by squeezing them. Numerous attempts have been made since to repeat Haberlandt's Oenothera experiment; whereas there were two reports of successes (Hedemann, 1931; Balasubramaniam, 1932), all others described failures (Nemec, 1935; Doak, 1937; Beth, 1938; Ivanov, 1938). Nevertheless, it has been generally believed that Haberlandt's proposal of an embryo initiating

substance is logical and may still be proven correct.

Nucellar Embryogenesis in the Rutaceae

Much of the information regarding nucellar embryony can be attributed to investigations of the Rutaceae, as shown by reviews of Cameron and Frost (1968) and Frost and Soost (1968). In the Rutaceae nucellar embryony has been demonstrated to be genetically determined and regulated by a single dominant gene (Nagai and Takinawa, 1928; Frost, 1938; Webber, 1940; Nasharty, 1945; Illies, 1959; Parlevliet and Cameron, 1959; Furusato, 1961; Ozsan and Cameron, 1963; Majsuradze, 1966; Iwamasa et al., 1967; Kapanadze, 1967, 1968, 1970; Cameron and Frost, 1968; Furusato. et al., 1968). The monoembryonic species or variety is homozygous for the recessive allele, whereas the polyembryonic could be either homozygous or heterozygous with respect to the dominant allele. Swingle (1927) and Frost (1938) proposed that nucellar embryony was nature's way of invigorating the clone. Kapanadze (1967, 1968, 1970) suggested that nucellar embryony was a relic of an adaptive device against drought. Indeed, seasonal fluctuation in the rate of nucellar embryogeny has been reported by Moreira, et al. (1947) and Ueno, et al. (1969).

There have been several reports implicating pollination and fertilization as prerequisites to nucellar embryogenesis (Ikeda, 1906; Frost, 1926; Toxopeus, 1930, 1936; Torres, 1938; Wong, 1939; Furusato, 1952, 1953; Mamporija, 1957, 1968; Majsurdze, 1961, 1968; Furusato et al., 1967, 1969). The validity of these reports is subject to question, inasmuch as other reports have indicated seed set without pollination

(Webber, 1894, 1905, 1930; Swingle, 1927; Oppenheimer, 1935; Wright, 1937; Frost, 1938).

In vitro culture studies have shown that pollination or fertilization is unnecessary for nucellar embryo formation (Bitters et al., 1970; Button and Bornman, 1971; Kochba et al., 1972). Cultures of either ovules or nucellus excised from unpollinated flowers have been reported to give rise to embryos.

According to the observations of Rangaswamy (1958, 1959, 1961), Maheshwari and Ranganswamy (1959), Sabharwal (1961, 1963), and Singh (1963) nucellus cultures of polyembryonic Citrus gave rise to adventive embryos only when the explants contained proembryos, suggesting that the proembryos may be somehow essential to adventive embryogenesis. This suggestion is unwarranted because Rangan et al. (1968, 1969) and Bitters et al. (1970) have shown that nucellus explants from normally monoembryonic Citrus produced multiple embryos in the absence of existing proembryos.

The nutritional experiments with Citrus nucellus cultures have suggested beneficial roles of such nutrient medium addenda as malt extract (Bitters et al., 1970), casein hydrolysate (Rangaswamy, 1958, 1959, 1961; Maheshwari and Rangaswamy, 1959) and adenine (Button and Bornman, 1971). The basis for their effects remains undetermined.

Adventive Embryogenesis in Other Families

In the Anacardiaceae nucellar embryony has also been believed to be genetically determined (Sachar and Chopra, 1957); polyembryony appears to be regulated by a dominant gene. Pollination and fertilization may be necessary, perhaps by influencing nucellar embryogenesis indirectly.

Pollination and fertilization affect zygote formation and endosperm development, and these in turn may determine adventive embryo development (Juliano, 1934, 1937; Sachar and Chopra, 1957). According to Juliano (1937) climatic factors may also influence the genetic expression with respect to nucellar embryogenesis; the study by Sachar and Chopra (1957) refutes this suggestion, however. An interesting observation of the mango by Sturrock (1961) has been the higher concentration of growth stimulating substances and lower concentration of inhibiting substances in extracts of fruits in polyembryonic than in monoembryonic varieties.

In the Cactaceae the question regarding the requirement of pollination and fertilization remains debated. In Opuntia aurantiaca nucellar embryony has been reported to occur spontaneously and in the absence of fertilization (Archibald, 1939). In all other species of the Cactaceae an abundance of free nuclear endosperm has been prerequisite to nucellar embryogenesis (Ganong, 1898; Maheshwari and Chopra, 1955).

Much of the information with respect to the regulation of plant embryo initiation and development has been derived through cell and organ culture investigations. Totipotentiality in plant cells has been demonstrated in cell cultures and complete plants have been obtained from single isolated cells (Vasil and Hildebrandt, 1965; Vasil and Vasil, 1972).

Steward et al. (1963) emphasized physical separation of a cell from its neighbors as being a key to the expression of embryogenic potency. This view is not supported by overwhelming evidence, however (Konar and Nataraja, 1969; Halperin, 1970; Vasil and Vasil, 1972). In

fact, according to Halperin (1970), there are interconnections between embryogenic and nonembryogenic nucellus cells of Northoscordum fragrans through plasmodesma. Similarly, Konar et al. (1972a) reported that embryogenic initials in the stem epidermis of Ranunculus sceleratus possess numerous plasmodesma, connecting such cells cytoplasmically with the neighboring cells. The plasmodesma are severed only after the embryo initiation is completed. There has been no evidence of an embryo arising from a single isolated somatic cell. All adventive embryos have been observed to originate in clusters of cells as in callus, or tissue or organ explants.

Nutritional investigations in vitro have not demonstrated significant roles of currently known plant hormones. In Daucus carota tissue cultures, whereas low levels of exogenous auxin have been found beneficial for embryo initiation in some cases (Kato and Takeuchi, 1966; Sussex and Frei, 1968; Rao et al., 1970), for the most part embryogenesis, particularly embryo initiation, has not been stimulated by auxin in the nutrient medium (Reinert, 1959; Halperin, 1964, 1967; Halperin and Wetherell, 1965; Newcomb and Wetherell, 1970). Sussex and Frei (1968), Reinert and Tazawa (1969), and Rao and Narayanaswamy (1972) have all implicated an auxin-nitrogen balance as underlying adventive embryogenesis in vitro. Other investigators have suggested a significance of auxin-cytokinin balance (Guha and Maheshwari, 1964; Vasil and Hildebrandt, 1966, 1967; Wilmar and Hellemdorn, 1968). Still others have reported stimulation of adventive embryogenesis in tissue cultures of some plants by such substances as adenine sulfate (Vasil and Hildebrandt, 1966) and Kinetin (Norstog, 1970).

Another embryogenic prerequisite proposed by Steward et al. (1958, 1963, 1966) has been a nutrient milieu comparable to that found in the embryo sac. Steward and his associates emphasized repeatedly the need to include in nutrient media such addenda as coconut endosperm and other natural complexes. Critical tests with diverse species, including carrot, (Kato and Takeuchi, 1963, 1966; Halperin, 1966, 1967; Vasil and Hildebrandt, 1966a, 1966b; Yamada, 1967; Sussex and Frei, 1968; Rao and Rangaswamy, 1971) have demonstrated consistently that adventive embryogenesis in tissue cultures could occur equally well in chemically definable nutrient media lacking coconut endosperm. Sometimes, depending on the basal nutrient solution used and particularly on the salt content of the solution, benefits may be obtained from supplements of coconut endosperm fluid (Morel, 1960, 1964; Reinert, 1961, 1963, 1967; Guha and Maheshwari, 1964, 1966, 1967; Rao, 1965; Konar and Nataraja, 1969; Rao et al., 1970; Bajaj and Bopp, 1972).

Halperin and Wetherell (1965) suggested a critical role of reduced-nitrogen compounds in carrot embryogenesis in vitro. Neither is this point supported by the evidence, however. For example, embryogenesis has been observed in nutrient media containing only nitrate nitrogen (Reinert et al., 1966; Konar and Nataraja, 1969). Nevertheless, reduced nitrogenous substances, including amino acids, amides and NH_4-Nitrogen, have consistently stimulated embryo initiation in tissue cultures of diverse plants (Reinert, 1963, 1967; Johri and Seghal, 1963; Guha and Maheshwari, 1964; Rao, 1965; Kato and Takeuchi, 1966; Linser and Neumann, 1968; Norreel and Nitsch, 1968; Reinert and Tazawa, 1969; Tazawa and Reinert, 1969; Halperin, 1970; Rao and Rangaswamy, 1972; Thomas and Street, 1972).

III. MATERIALS AND METHODS

Histological Studies

Histological studies were carried out with *Citrus medica* L. 'Ethrog' (Ethrog citron), a naturally monoembryonic cultivar, and *C. reticulata* Blanco 'Ponkan' (Ponkan mandarin), a polyembryonic variety.

Sampling in Vivo Materials

Two trees of the Ethrog citron, grown at the South Coast Field Station of the University of California, were selected for the study. Two hundred and fifty flower buds were tagged just prior to anthesis. They were examined every day, and those that had opened and showed pollen grains deposited on their stigmas had their tags dated. Similarly, two trees of Ponkan mandarin grown on the Riverside campus of the University were selected and tags were placed on 600 flower buds. During the course of daily inspection those that had opened and showed pollen grains deposited on their stigmas had their tags dated.

Ten flower buds of each cultivar were collected at the time of tagging. The ovules of five buds were excised and placed in fixative; the other five flower buds were fixed intact. Five fruit samples per cultivar were collected bi-weekly during the first four weeks, and weekly after that until the sixteenth week. The ovules from each fruit were removed and fixed.

The solution, designated as FAA, used in all fixations was that of

Sass (1968) and contained the following: 95% ethanol, 50ml; glacial acetic acid, 5ml; 37-40% formaldehyde, 10ml; and distilled water, 35ml. All fixed materials were stored at room temperature.

Sampling in Vitro Materials

Nucellus explants to be placed in tissue culture were obtained from fruits sampled 100 days after anthesis for Ethrog citron and 53 days after anthesis for Ponkan mandarin. In the citron the zygotic embryos were at the late heart to torpedo-shaped stage; the embryos were removed and discarded. The Ponkan mandarin ovules still showed no visible embryos, zygotic or adventive, at the time nucellus explants were being obtained. One hundred and twenty cultures were established for each cultivar. The details of the nucellus excision procedure and culture conditions are described in the section below, Assessment of Requirement for Adventive Embryogenesis in Rutaceous Nucellus Culture.

The basal medium as shown in Table 3 was utilized and culture environmental conditions which are standard to this laboratory (27 $\pm$ 1°C constant, 1000 lux Gro Lux light 16 hours per day) were adhered to. Ten nucellus samples were fixed in FAA after 3, 7, 14, 17, 21, 24, 28, 31, 35, 38, and 42 days in culture. Ten samples of freshly excised nucellus were also fixed for each cultivar.

Preparation of Histological Sections

Dehydration of fixed samples was carried out via the tertiary butyl alcohol (TBA) series of Johansen (1940). They were cleared in xylene and embedded in paraplast. Sections 10 µ thick were stained with Harris' haematoxylin, following the procedure described for progressive

haemalum by Sass (1968).

Assessment of Requirements for Adventive Embryogenesis in Rutaceous Nucellus Culture

Species and Varieties Employed

For most experiments nucellus cultures of Citrus medica L. 'Ethrog' (Ethrog citron), a monoembryonic cultivar, C. limon L. Burm 'Ponderosa' (Ponderosa lemon), also monoembryonic; and C. limon L. Burm 'Eureka' (Eureka lemon), a moderately polyembryonic cultivar, were utilized.

These three cultivars were employed for a number of reasons. First, they flowered and fruited the year round, providing a supply of experimental material of suitable developmental stage at virtually all times. Second, the monoembryonic cultivars were preferred because they ensured that the study included a consideration of the embryo initiation process; some previous studies with polyembryonic Citrus have been clouded by embryogenetic development associated with existing proembryos and embryos. Third, the moderately polyembryonic Eureka lemon was chosen also for another reason; this cultivar already manifested nucellar embryogenesis in vivo and might enable studies of stimulation and inhibition of embryogenesis simultaneously in vitro.

In a few experiments C. reticulata Blanco 'Ponkan' (Ponkan mandarin), a highly polyembryonic variety, was used. In certain experiments in which they are specified, naturally polyembryonic types were also employed: C. hystrix D.C. 'Kuffre' lime, C. sinensis L. Osbeck 'Koethen,' C. sinensis L. Osbeck 'Ruvel,' C. sinensis L. Osbeck 'Surprise

Navel,' C. sinensis L. Osbeck 'Wallace seedless Valencia,' and Fortunella crassifolia Swing. 'Meiwa.' The Ethrog citron and Eureka lemon samples were obtained from the South Coast Field Station, whereas samples of all others were obtained from the Riverside campus of the University of California.

Characteristics and Handling of Explant Materials

Isolation of nucellus explants. Fruit samples were obtained from each of the cultivars periodically to observe the developmental stage of the embryos, zygotic as well as nucellar, in order to determine the culturability of the nucellus. Of the monoembryonic types, only fruits with visible embryos in the heart-shaped stage were utilized as a source of nucellus explants. With the naturally polyembryonic types fruits of comparable age were also utilized; however, they differed from the monoembryonic type in that the ovules contained more than one visible embryo.

The fruits were washed and their receptacular and stylar ends were severed. They were then immersed in dilute laundry bleach (Purex diluted 10-fold and containing a few drops of Tween-20 emulsifier) for 15 minutes. Without rinsing and following asceptic procedure, the fruits were cut open to expose the ovules. The ovules were removed and placed in sterile petri dishes. Surgical steps were carried with the aid of a dissecting microscope, using 10-20X magnification. The ovule was kept in position with the aid of a pair of microforceps. Using a sharp surgeon's scalpel, a shallow incision was made through the integuments, without injuring the nucellus, along the length of the ovule from the chalazal to the micropylar end. The integuments were lifted away, exposing the nucellus. The embryos became visible as shiny, pale green

structures, embedded in the micropylar region of the nucellus. A small incision perpendicular to the longitudinal axis was made in the micropylar region, and the embryos were expelled from the nucellus through the incision. The embryos were collected, counted and discarded. The nucellus was then transferred to culture, with its chalazal end partially embedded in the nutrient medium. One nucellus explant was planted per culture. At least 20 cultures were used per treatment of any experiment.

In vivo developmental stage of explant. To investigate the influence of fruit age on the behavior in vitro of nucellus explants, 120 Ethrog citron flowers were self-pollinated and suitably identified with tags. Fruit samples were harvested weekly beginning with the eleventh week after pollination and their nucellus isolates placed in culture in basal nutrient medium. Periodic observations disclosed that fruit samples obtained earlier contained no visible embryos and could not be used for the experiment.

To determine the behavior in vitro of nucellus from unpollinated material, whole ovules were isolated at the flower bud stage and placed in culture. The cultivars used specifically for this test were Eureka and Ponderosa lemons, Ethrog citron, Ponkan mandarin, Koethen sweet Ruvel, Surprise Navel, Wallace seedless valencia orange and Meiwa kumquat. In this experiment four ovules were used per culture and 20 cultures were employed per cultivar.

Seasonal factor. To determine seasonal variability in behavior of nucellus explants, three batches of the same basal nutrient medium were prepared every other month and nucellus explants obtained from Ethrog

citron and Eureka lemon fruits sampled every other month were cultured in them. The experiment was repeated a second season.

Fruit storage effects. Ethrog citron and Eureka lemon fruits, harvested at a suitable developmental stage, were placed in polyethylene plastic bags and stored at 5°C to determine the effects of their prior cold storage on the behavior of the nucellar explants in vitro. The nucellar explants were obtained and placed in culture 1, 2, 4, 6, and 8 weeks after storage. Nucellus explants from freshly harvested fruits were used as controls.

Effects of incision. A comparison was made of transverse, single longitudinal, and multiple (6) longitudinal incisions of the micropylar region to determine their influence on the behavior in culture of the excised nucellus. The transverse incision was made away from the micropylar tip and at a distance where the enlarged zygotic embryo had extended. The longitudinal incisions, on the other hand, were made from the tip to a distance to which the zygotic embryo had extended. This test was done because it was believed that the nature and degree of injury to the nucellus might influence the rate of adventive embryogenesis. Nucellus explants of Ethrog citron and Ponderosa lemon were used for this study.

Method of implantation. Still another initial experiment was carried out with Ethrog citron, Ponderosa lemon and Ponkan mandarin nucellus explants to determine the effects, if any, of the method of their implantation in the nutrient medium. In one treatment the micropylar region of the nucellus was embedded in the medium, leaving only the chalazal region exposed. In another, the chalazal region was

embedded in the medium, leaving only the micropylar region exposed. Finally, in a third treatment the nucellus was simply placed horizontally on the agar medium.

Length of culture passage. The optimum length of the culture passage was determined with nucellus explants of Ethrog citron, Kuffre lime and Ponkan mandarin. The rates of adventive embryo formation were recorded at weekly intervals over a period of 24 weeks.

Nutrient Medium Characteristics

Physical quality of medium. A comparison was made of agar and liquid media as they influenced the behavior in vitro of Ethrog Citron nucellus isolates. In one treatment where liquid medium was used the explant was supported on the surface of a folded filter-paper platform (hereafter identified as Heller support). The base of the platform was kept immersed in the nutrient solution; this allowed movement of nutrient solution to the nucellus by wick action. The platform also enabled exposure of the explant to air. In other treatments involving liquid nutrient the explants were immersed in the solution and left stationary, or agitated either gently at 1 rpm on a rotating culture apparatus (New Brunswick tissue culture roller drum Model TC-4), or vigorously at 300 rpm on a gyrotory shaker (New Brunswick Model G-10).

A similar comparison was made between agar and liquid media in cultures of Eureka lemon and Meiwa kumquat nucellus. The experiment with these, however, considered only liquid medium in which Heller support was used. Each treatment in tests of liquid and agar employed 40 cultures.

Medium composition. The composition of the basal nutrient medium utilized in cultures of nucellus explants of the Rutaceae is shown in Table 3. As evident in the table, the medium contained the Murashige and Skoog inorganic salts, 5% Sucrose, White's B-vitamin and glycine mixture in double strength, 100 mg/l myo-inositol, 500 mg/l of Bacto malt extract, and 1% Bacto agar. The pH of the medium was set at 5.7 prior to autoclave sterilization, using 1N HCl or NaOH. The agar medium was distributed at a rate of 25 ml per tube, utilizing 25 x 150 mm pyrex culture tubes. The nutrient tubes were capped with polypropylene closures. The medium was sterilized by autoclaving at 121°C for 15 minutes. Gibberellin-containing solutions, when used, were added to presterilized agar medium following filter sterilization of gibberellin stocks.

In attempts to improve the nutrient medium for increased survival of explants and enhanced rate of adventive embryogenesis, alterations in the concentrations of the basal constituents as well as further addenda were tried. In a series of initial experiments the basal constituents were divided into four groups: (1) agar (1% Bacto agar), (2) sucrose (5% sucrose), (3) salts (Murashige and Skoog formulation), and (4) organics (White's B-vitamins and glycine at double strength, 100 mg/l myo-inositol, and 500 mg/l Bacto malt extract). These groups, individually and in combinations, were tested with Ethrog citron, Eureka lemon, Koethen sweet orange, and Ponkan mandarin nucellus cultures. A second set of experiments considered modifications in the concentration of some of the individual components, e.g., sucrose, myo-inositol, and Bacto malt extract. This set of experiments was carried out with Ethrog

TABLE 3

Composition of the basal nutrient medium employed in nucellus cultures of Rutaceae

Ingredient	mg/l Medium
Inorganic Salts (Murashige and Skoog, 1962)	
NH_4NO_3	1650.0
KNO_3	1900.0
$CaCl_2 \cdot 2H_2O$	440.0
$MgSO_4 \cdot 7H_2O$	370.0
KH_2PO_4	170.0
Na_2-EDTA	37.3
$FeSO_4 \cdot 7H_2O$	27.85
$MnSO_4 \cdot H_2O$	16.9
$ZnSO_4 \cdot 7H_2O$	8.6
H_3BO_3	6.2
KI	0.83
$Na_2MoO_4 \cdot 2H_2O$	0.25
$CuSO_4 \cdot 5H_2O$	0.025
$CoCl_2 \cdot 6H_2O$	0.025
Organic Constituents	
Sucrose	50,000
Vitamins	
Thiamine·HCl	0.2
Pyridoxin·HCl	1.0

Table 3 (continued)

Ingredient	mg/l Medium
Vitamins (cont.)	
Nicotinic acid	1.0
Glycine	4.0
Inositol	100.0
Complex Addenda	
Difco Bacto agar	10,000.0
Difco Bacto malt extract	500.0

citron, Eureka lemon and sometimes also Ponderosa lemon nucellus. The vitamins were not tested individually but only as a combined group. A third series of experiments considered the effects of additions to the basal nutrient medium of varying concentrations of the auxins IAA (Indole-3-acetic acid), NAA (Naphthalene-1-acetic acid) and 2,4-D (2,4-Dichlorophenoxyacetic acid), the cytokinin, kinetin, Gibberellin A_3, adenine sulfate, thymidine, casein hydrolysate, Bacto yeast extract, and orange juice. In one experiment the influence of juice vesicles obtained from the naturally polyembryonic Valencia and Washington Navel oranges was also tested as a nutrient medium addendum. The third series was carried out with Ethrog citron, Eureka lemon and sometimes Ponderosa lemon cultures.

The Culture Environment

Illumination characteristics. Utilizing the basal nutrient medium, the behavior in vitro of Ethrog citron and Eureka lemon nucellus was determined when cultured under Gro Lux light intensities of 0, 300, 1000, and 30,000 lux and a photoperiod of 16 hours.

Following the light intensity test and using a Gro Lux intensity of 1000 lux, the influence of the daily light period on Ethrog citron and Eureka lemon nucellus cultures was determined. The light periods tested were 0, 4, 8, 12, 16, 20, and 24 hours. The temperature in both above experiments was constant at 27°C.

Temperature effects. Nucellus cultures of Ethrog citron and Eureka lemon were exposed to nycto-temperatures of 12, 17, 22, and 27°C. The temperature of the light period was 27°C. Light was provided 16 hours daily from Gro Lux lamps at an intensity of 1000 lux.

Physiological Mechanism Underlying Adventive Embryogenesis in the Rutaceae

Embryogenic Potential Among the Rutaceae

As a first step towards elucidating the physiological mechanism underlying adventive embryogenesis in the Rutaceae, the embryogenic potential of the excised nucellus of 32 members of the Rutaceae was determined. The investigation included 8 genera, 24 species, 9 varieties, and 3 hybrids. Eleven of them belong to the monoembryonic category and the remainder were naturally polyembryonic. Each was tested by utilizing 40 explants. Fruit samples were obtained from either the South Coast Field Station or the Riverside campus of the University. The investigation was conducted in 1971 and repeated in 1972. The basal nutrient medium shown in Table 3 was used throughout, also the standard light and temperature conditions, i.e., 16 hours daily illumination with 1000 lux intensity Gro Lux light and constant 27°C. The nucellus explants were obtained as described previously in the section of Assessment of Requirements for Adventive Embryogenesis in Rutaceous Nucellus Culture. The numbers of embryos removed from explants prior to their culture were recorded. After eight weeks the cultures were examined. The counting of embryos was aided by a dissecting microscope, with observations being carried out at 10-20X magnification. Seedlings arising in vitro were counted as embryos.

Differential Behavior of Micropylar Half, Chalazal Half and Whole Nucellus

Eighty nucellus explants with embryos removed were obtained for

each of Ponkan mandarin and Ethrog citron. Forty of each were cultured as whole nucellus, whereas the other 40 were cut transversely into halves and cultured separately as micropylar and chalazal halves. The basal nutrient medium and standard cultural conditions with respect to light and temperature were utilized. After eight weeks the embryogenetic characteristics were compared among whole nucellus and micropylar and chalazal halves.

Interaction Between Polyembryonic and Monoembryonic Nucellus

One hundred nucellus explants of Ethrog citron, and 60 each of normally polyembryonic Cleopatra mandarin (C. reticulata Blanco) and normally polyembryonic Rough lemon (C. jambhiri Lush.) were obtained each free of embryos, and cut transversely into halves. The chalazal halves of all three were discarded. Twenty micropylar halves of the nucellus of each Citrus were cultured individually. Another 20 each of Cleopatra mandarin and Rough lemon were grafted on to micropylar halves of the nucellus of Ethrog citron and placed in culture with the Ethrog citron nucellus being embedded in the nutrient agar. Twenty each of the remaining 40 micropylar halves of the nucellus of Ethrog citron were grafted onto micropylar halves of the nucellus of Cleopatra mandarin and Rough lemon and placed in culture with the Cleopatra mandarin and Rough lemon tissues embedded in nutrient agar. All grafts involved tissue contacts at the transverse cut surfaces. Basal nutrient medium and standard culture conditions were used. After eight weeks the embryo yield of each micropylar half was measured to disclose possible interactions between monoembryonic and polyembryonic nucellus.

Suppression of Polyembryony by Monoembryonic Citrus

As the research progressed it became apparent that the micropylar region of a nucellus was more prone to undergo embryogenesis than the chalazal region. It also appeared that this difference was due to a difference in distribution of a possible embryogenetic suppressor, the content of the suppressor being higher in the chalazal half than in the micropylar half. The next step considered a possible difference in suppressor concentrations in the nucellus of monoembryonic and polyembryonic types of the Rutaceae.

Forty micropylar halves of the nucellus of Rough lemon and Koethen sweet orange, both highly polyembryonic, were grafted onto an equal number of chalazal halves of the nucellus of Ethrog citron, a strongly monoembryonic citrus. Grafts were achieved by joining the transverse cut surfaces together. The grafted tissues were placed in culture with only the Citron half being embedded in the agar medium. Forty more micropylar halves of the nucellus of each of Rough lemon and Koethen sweet orange were cultured in separate tubes for reference. Basal nutrient medium and standard culture conditions were used. After eight weeks the yields of embryos in each of the micropylar halves were determined.

Evidence of Diffusable Embryogenic Suppressor

To test the possible involvement of a diffusable repressive substance in *Citrus* nucellar embryony, micropylar halves of polyembryonic Ponkan mandarin nucellus were cultured on nutrient agar, surrounded by varying numbers of the chalazal halves of Ethrog citron ovules. The

citron ovules were placed in a radius 1 cm away from the Ponkan mandarin explant. For this test, instead of culture tubes, 50-ml Delong culture flasks were employed in order to provide a larger culture surface. Each flask contained 25 ml of medium and was capped with stainless steel closures. The basal medium and standard light and temperature conditions were employed. The yield of embryos per Ponkan mandarin nucellus was determined after eight weeks.

Tests of Leachability of Suppressant

Nucellus explants of Ethrog citron were cultured in liquid nutrient solution as well as agar medium for prescribed periods and transferred to fresh agar medium to examine the extractability of any suppressive substance. The experiment involving liquid medium employed 200 explants and with the medium agitated constantly at 1 rpm. One hundred of the explants consisted of whole nucellus and another hundred were only micropylar halves. After 0, 1, 5, 10, and 30 days the nucellus pieces were transferred to agar medium, 20 on each date. On each transfer date, fresh nutrient solution replaced the liquid of untransferred tissues.

The leaching in agar involved the same number and kinds of nucellus explants as those of the liquid test above. Handling of the tissues was also the same, with these exceptions: the transfers to fresh agar medium were made after 0, 5, 10, and 15 days, and no fresh nutrient agar was used to replace the medium of untransferred nucellar pieces on any of the prescribed transfer dates.

The basal nutrient medium was used throughout this experiment

except that the agar was excluded in the liquid medium and the pH of liquid media was set initially at 5.0. Also, only 5 ml of liquid nutrient solution were used per tube; agar medium was dispensed at a rate of 25 ml per tube. Standard light and temperature conditions were employed. In both experiments, data of embryogenesis were obtained twice: (1) eight weeks from the start of the experiment and (2) eight weeks following transfer of nucellus pieces to fresh nutrient agar medium.

Permanence of the Suppressive Effect

This experiment was carried out in a manner comparable to that in which diffusability of the embryogenic suppressant was being determined. The micropylar halves of Ponkan mandarin nucellus were placed in culture on nutrient agar with each explant being surrounded by a ring of 10 chalazal halves of citron ovules. Twenty flasks of such cultures were prepared. As reference, micropylar halves of Ponkan mandarin nucellus were cultured in the absence of any association with citron ovules. After eight weeks of culture in basal nutrient medium and under standardized light and temperature conditions, the Ponkan mandarin tissues were examined for embryo yields. Recultures into fresh medium were then made of the mandarin tissue from the two treatments, but now both were grown in the absence of any citron tissue. Ten Ponkan mandarin embryos from the reference cultures were recultured in each of 20 tubes. Aliquots of approximately 1 cm^3 of callus from the citron-associated treatment were recultured in each also of 20 tubes. This procedure of gathering data and reculturing was repeated four times every eight weeks.

Embryogenic Inhibitor in Integuments

To assess the possibility of an embryogenic inhibitor being present also in the integuments, the behavior of nucellus explants, with and without integuments, was studied. For this test Ethrog citron, Eureka lemon and Cleopatra mandarin were used. In the two types of explants any embryos present were first removed, counted, and then discarded. Nucellus explants with integuments were simply those in which the integuments have been left attached; the other explant was obtained by the standard isolation procedure. The data with respect to embryo yields were obtained after eight weeks.

Action of Citrus Suppressant on Daucus carota Callus

In assessing the extent to which any physiological mechanism which regulates adventive embryogenesis in the Rutaceae may be applicable to plants in general, the anti-embryogenic effect observed in citron ovules was tested on callus cultures of Daucus carota, variety 'Queen Anne's Lace.' The callus of the wild carrot was obtained from seedling hypocotyl sections and established on a nutrient medium containing Murashige and Skoog salts, 3% sucrose, White's vitamins, 100 mg/l inositol, 0.1 mg/l 2,4-D, and 0.6% agar. Stock cultures of the callus were maintained in darkness.

To test the suppressive effects of citron ovules, 1 cm^3 samples of the carrot callus were placed on nutrient agar and cultured while surrounded by varying numbers of the chalazal halves of Ethrog citron ovules. The procedure was the same as that for determining the diffusability of the embryogenic suppressant in Citrus. The nutrient

medium was the same as that for establishing the carrot callus, but without 2,4-D. Reference cultures contained carrot callus cultures with no citron tissues. The cultures were left under conditions designated as standard for Citrus nucellus culture, and the data were collected after four weeks. Seedlings were counted along with embryos.

IV. RESULTS

Histological Observations

In Vivo Development of the Ovule

In the flower buds just preceding anthesis the ovules of both Ethrog citron (monoembryonic) and Ponkan mandarin (polyembryonic) appeared similar in gross histology. Those of the citron can be seen in Fig. 1-a and b,, and those of the mandarin are shown in Fig. 2-a. They were oval to round in shape and anatropous. The ovules of both were bitegmic, i.e., two integuments, and crassinucellate, i.e., nucellus with many cell layers in the micropylar region (see Fig. 1-c). The crassinucellate condition indicated a substantial parietal tissue, which in turn reflected a development of the nucellus epidermis at the micropylar region after the formation of the megaspore mother cell. The outer integument was comprised of cells which were larger and more vacuolated than those of the inner integument. The outer integument also was composed of 4-6 cell layers and as thick as ten layers near the funiculus. The inner integument was composed of smaller, more intensely staining cells and was 2-3 cell layers thick.

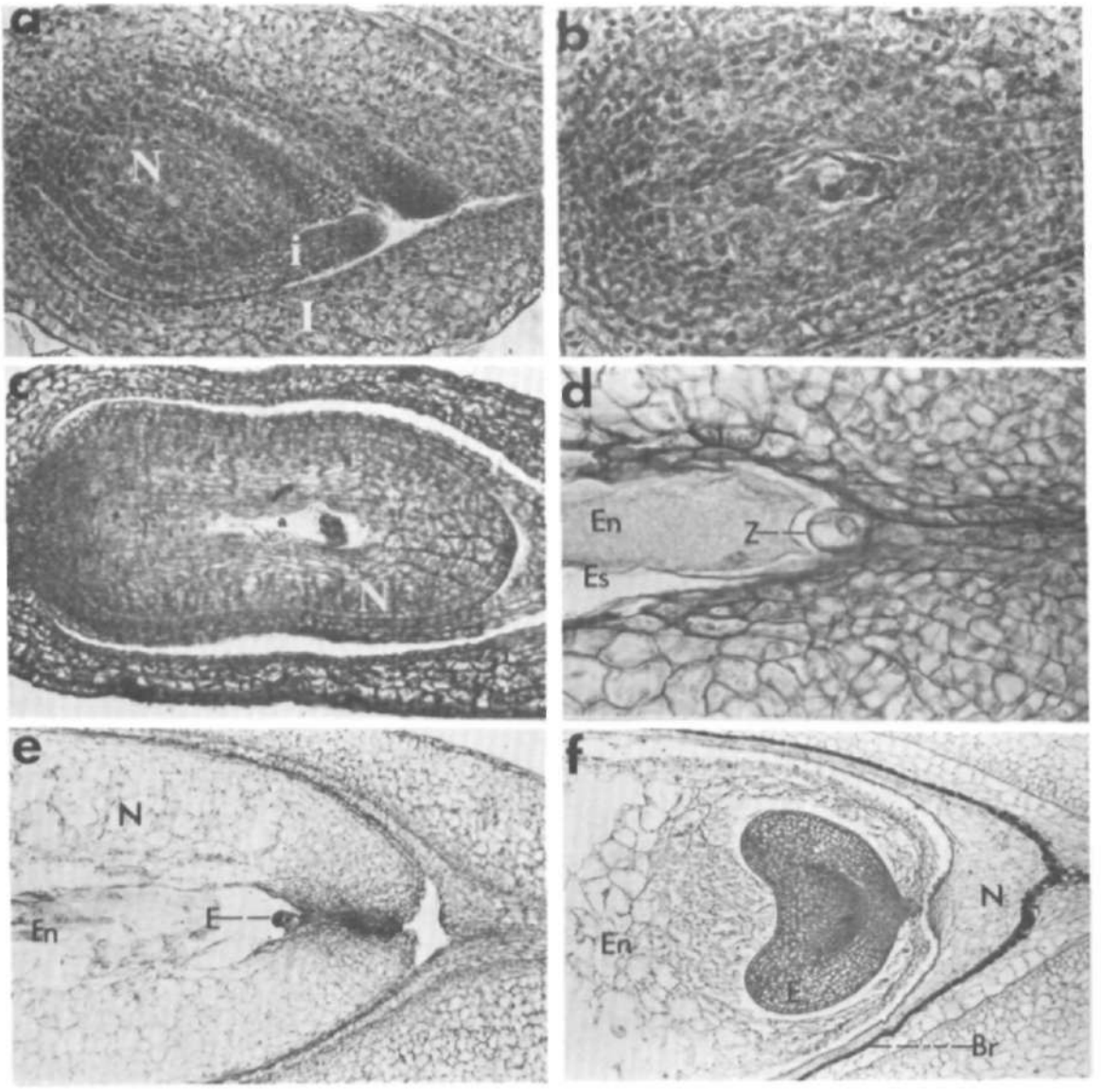
The nucellus in both cultures was a central mass of densely staining cells. Two parts could be identified in each nucellus: an outer region just beneath the integument, composed of one or more layers of cells with more deeply staining cells, and a more substantial inner region consisting of many layers of slightly more vacuolated cells. The

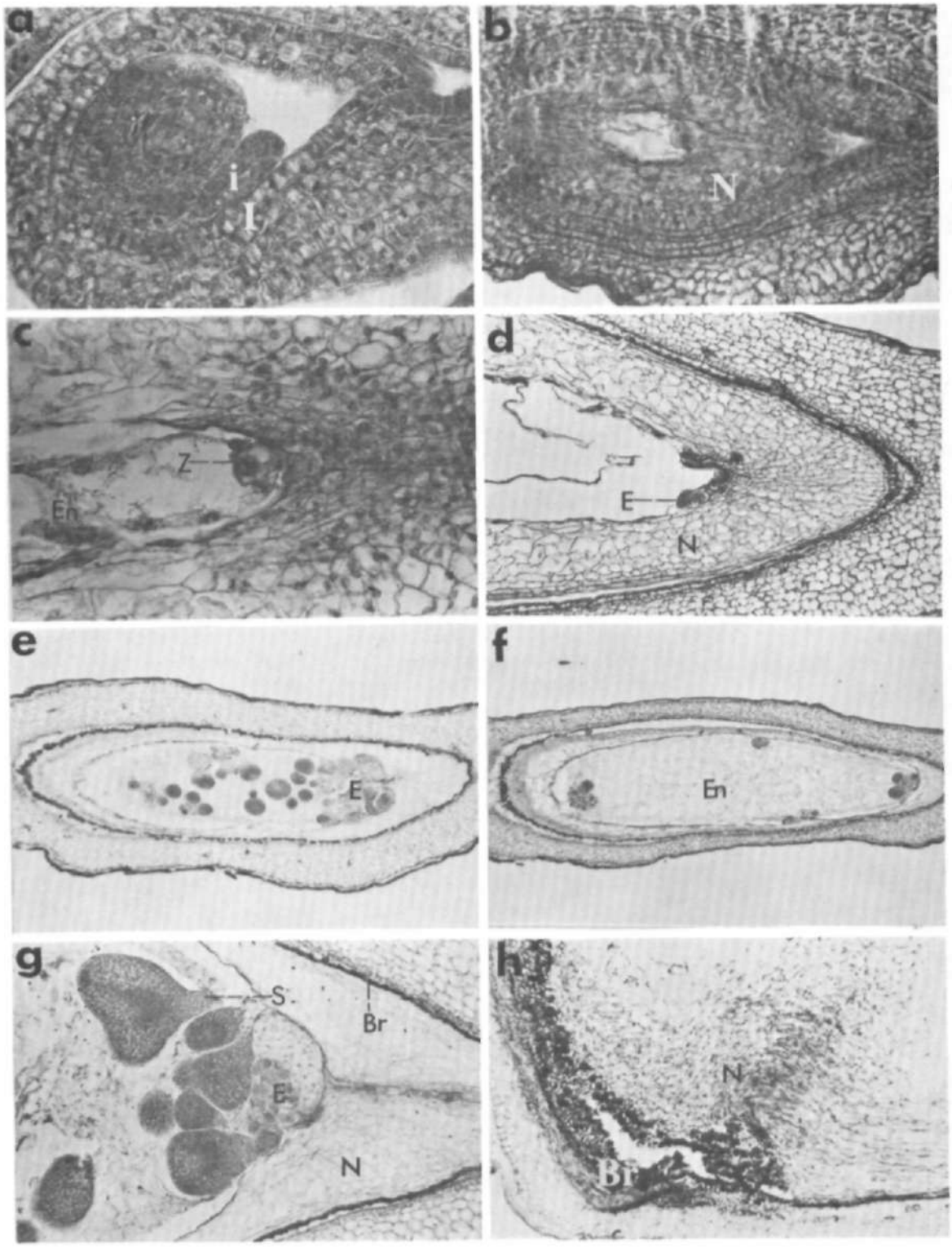
outer portion of the nucellus has been referred to by morphologists as the nucellus epidermis. The nucellus was demarcated clearly from the inner integument, except in the chalazal region where it appeared fused with the integumentary tissue.

The embryo sac was centrally located within the nucellus of each species, although perhaps more often nearer the micropylar than the chalazal end. Prior to pollination the ovule of the Ponkan mandarin appeared smaller and rounder than those of the citron. The cells of the various tissues of the ovule of the Ponkan mandarin also appeared larger than those of the citron. The inner integument of the mandarin seemed to have developed more slowly relative to the outer integument than of the citron. The nucellus of the citron ovule was composed of many more cells and cell layers and was generally larger than that of the mandarin.

Following pollination, the ovules of both species enlarged progressively, elongated considerably, and appeared to flatten on two surfaces and ridged on the others, especially along the funiculus.

Ultimately the Ethrog citron ovule attained a larger size and became plumper, smoother, slimier, and less compressed laterally than the mandarin ovule. The mandarin ovule became more corrugated. The integuments grew substantially, principally by cell enlargement. The differentiation of vascular strands occurred only in the outer integument; moreover, vascular differentiation terminated in the chalazal region and did not extend into the nucellus. What appeared to be a third integument differentiated from the innermost layer of the inner integument. The differentiation began about 70 days following anthesis for the citron and 2-3 weeks later in the mandarin. This tissue was


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comprised of cells which could be readily distinguished by their contents of highly refractive, brown substances. Because of its color morphologists have referred to this tissue as the Brown layer. The differentiation of the brown layer in the citron began in the micropylar region, and from there it continued progressively to the chalazal region. In contrast the brown layer of the mandarin differentiated near but not at the micropylar region, and from there it progressed first towards the micropyle and then to the chalazal region. The brown layer did not fill the micropylar region until embryogenesis had begun in both species. In the citron ovule it can be seen very distinctly in Fig. 1-f and in the Ponkan mandarin in Figs. 2-d to 2-h.

The nucellus of both species underwent considerable enlargement. The constituent cells became more elongated and more highly vacuolated. There appeared to be additional layers of cells in the nucellus of both species. The nucellus epidermis became more readily distinguishable from the inner nucellus tissue. The more centrally located cells of the nucellus had elongated considerably and many had undergone disintegration, particularly those surrounding the embryo sac. Degeneration of the centrally located cells appeared to continue indefinitely and to spread outward as the ovule progressed in development.

Not much was seen of the endosperm in either species, but it remained in the free nuclear condition for a very long time after anthesis. In the citron, cellular endosperm made its appearance when the zygotic embryo had attained the proembryo stage. Once started, the endosperm became cellular very rapidly, starting at the micropylar region and progressing toward the chalazal region. The endosperm

reached maximum development at the heart-shaped stage. At this stage the endosperm cells were highly irregular in shape and size. They were also vacuolated, particularly the most centrally located endosperm cells.

In Ponkan mandarin, the endosperm became cellular only after many nucellar embryos had already formed and had attained considerable development. This occurred much later than in the citron. The final characteristics of the endosperm and its constituent cells were essentially the same as those of the citron. Cellular endosperms can be seen in Figs. 1-f and 2-f.

The citron zygote remained dormant for as long as 60 days after pollination (Fig. 1-d). A fully developed embryo was attained when the fruit was about 140 days old. No adventive embryogenesis was observed in the citron, except one rare case where multiple embryos were obtained through budding of the suspensor. The proembryo stage can be seen in Fig. 1-d, and that of the late heart-shaped stage in Fig. 1-f.

It was not possible to observe embryogenesis from the zygote of Ponkan mandarin, although the dormant zygote could be seen even 60 days after pollination (Fig. 2-c). Division of the zygote could not be observed; instead meristematic activity could be observed among the nucellus cells which were in contact with the embryo sac, particularly those in the micropylar region (Fig. 2-d). Very quickly these meristematic activities gave rise to clusters of embryos (Figs. 2-e through g).

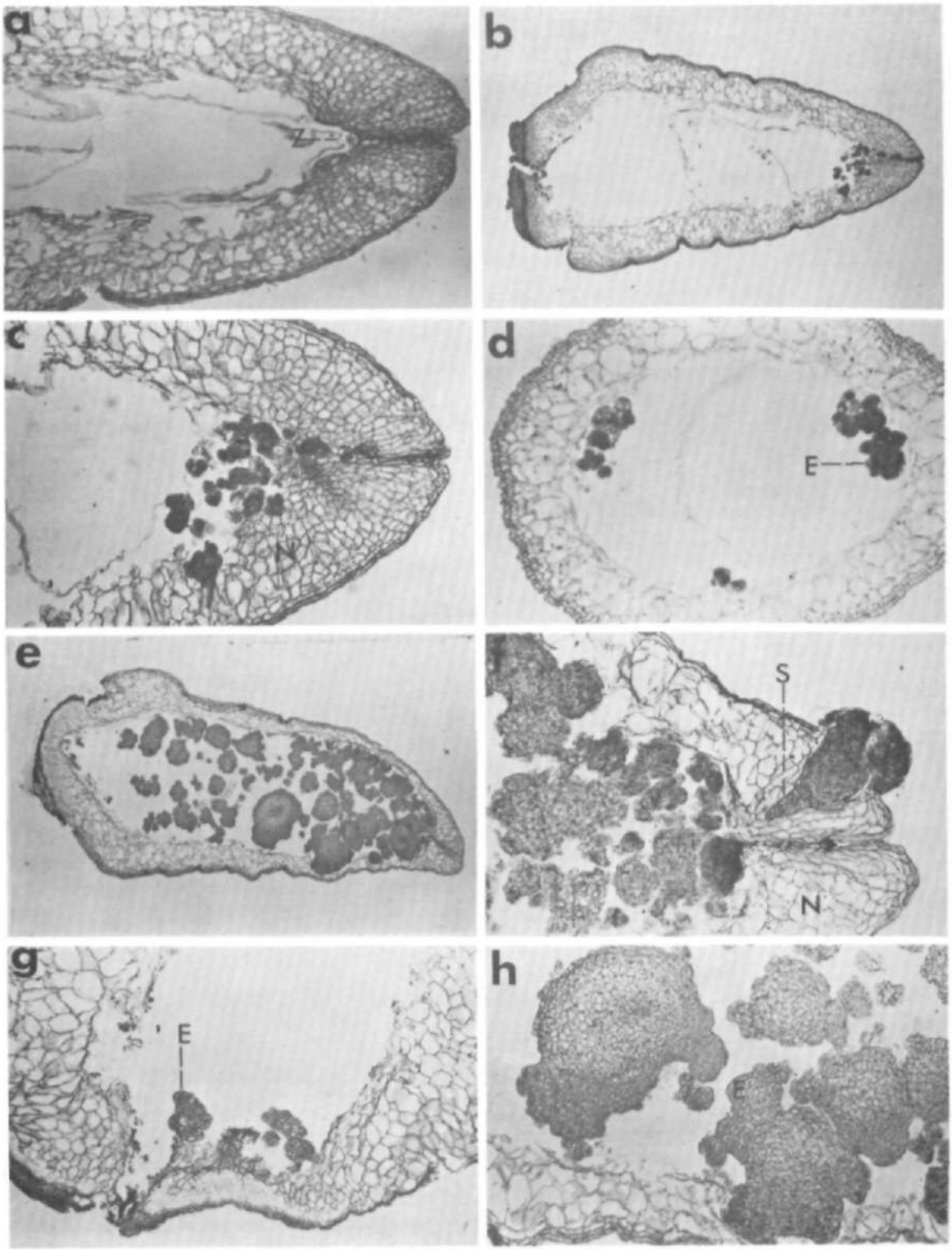
In many of the ovules examined adventive embryos were observed in the micropylar and chalazal regions, as well as along the flanks of the nucellus (see Figs. 2-e and f). The embryos appeared to arise generally

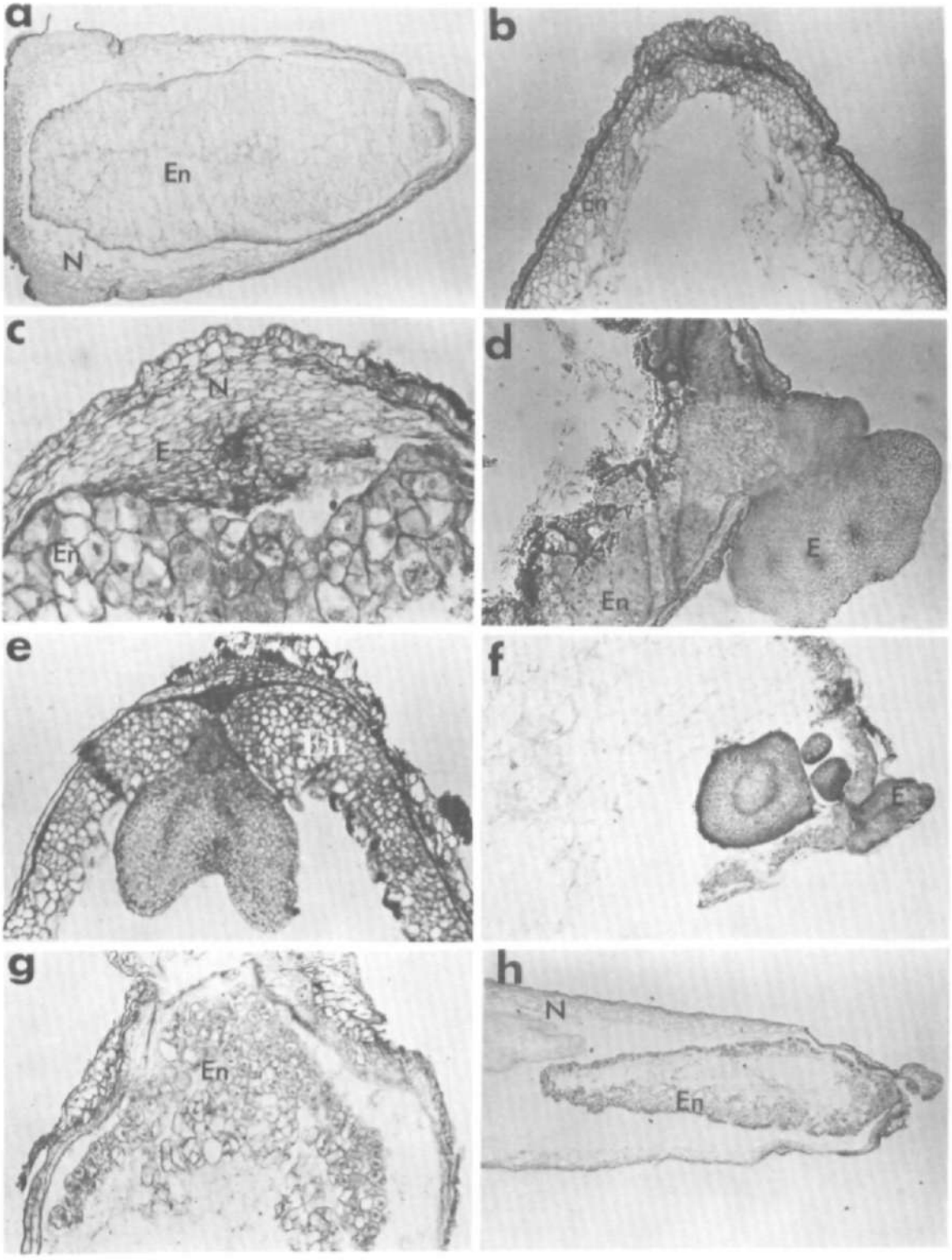
only from those nucellus cells which were most proximally located with respect to the embryo sac. Numerous embryos were produced per ovule. Many of the adventive embryos appeared to have arisen by budding from existing nucellar embryos. The budding process seemed to go on indefinitely, at least up to 150 days after pollination. No case of monoembryony among Ponkan mandarin ovules was encountered in this investigation.

One interesting phenomenon observed in the Ponkan mandarin nucellus, but not in citron, was the marked proliferation of the nucellus at the chalazal end (Fig. 2-h). The proliferation produced a substantial mass of parenchyma cells which seemed to grow into the embryo sac, displacing some of the endosperm and often dividing the endosperm into micropylar and chalazal halves. The proliferation began some time after many of the nucellar embryos had attained complete development. The significance of this phenomenon was not explored.

Behavior of the Nucellus in Vitro

The citron nucellus, at the time of its excision for culture, contained a heart-shaped to torpedo-shaped zygotic embryo. This embryo was discarded. The ovule at that time was about 100 days from pollination. In contrast, the Ponkan mandarin nucellus contained a dormant zygote but no embryo, zygotic or nucellar, at excision time. The mandarin fruit was 50-60 days old since pollination. The histological characteristics of the nucellus explants of the two species can be observed in Figs. 3-a, 4-a and 4-b. The citron nucellus contained a well-developed endosperm (Figs. 4-a, 4-b) whereas the Ponkan nucellus


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did not (Fig. 3-a). The citron nucellus formed a thinner envelope around the embryo sac than did the mandarin.

No signs of growth were seen in the nucellus of either species until seven days in culture. Gross observation showed that the Ponkan mandarin nucellus developed a dense white growth at the micropylar region and the development progressed towards the chalazal end. After three weeks in culture there appeared a loose mass of tiny white clumps of tissue, seemingly expelled from the nucellus. Soon green specks also were evident; these were the small nucellar embryos. Some of the embryos enlarged and proceeded to germinate in the test tube. Others appeared to proliferate into further embryos.

In the citron nucellus the only sign of activity was one of increase in size and swelling for the first 2 - 2-1/2 weeks. By the third week, a tiny speck of green tissue could be seen in the extreme micropylar tip of some of the explants. The green speck often developed into an embryo after further time in culture. In contrast to the Ponkan mandarin nucellus, the citron nucellus produced only one to few embryos and showed little further proliferation.

Histological examination of the cultures showed that in some Ponkan mandarin nucellus explants adventive embryogenesis had already begun within three days in culture. Meristematic activity among interior cells in the micropylar region of the nucellus gave rise to numerous embryos (Figs. 3-b, 3-c). Few if any of the cells of the nucellus epidermis and others close to the epidermis appeared to be involved directly in the embryogenic process (see especially Fig. 3-d). Within three weeks of culture the nucellus appeared virtually completely

filled with a multitude of embryos (Fig. 3-e). As already observed with the Ponkan ovules during in vivo development, nucellar embryogenesis was not confined to the micropylar region, but could be observed also in the chalazal end (Fig. 3-g). While widespread meristematic activity among nucellus cells was responsible for the initially arising embryos, it appeared that a major fraction of the total population of embryos resulted from budding among the embryos (Fig. 3-e, 3-f, 3-h). The term budding is used in this investigation to denote tissue outgrowths from existing embryos and which eventually differentiated into further embryos. What appeared on gross observations, expulsion of tissues and embryos in three-week-old cultures, probably indicated bursting of the nucellus in response to an internal pressure from the mass of embryos. Some outgrowths of embryos through the nucellus also occurred (Fig. 3-f).

A suspensor-like structure was observed in some of the nucellar embryos (Fig. 3-f); however, this was not a common feature.

Histological examination of the cultured citron nucellus disclosed that nucellar embryogenesis began on about the seventh day. The meristematic activity could be seen in the micropylar region of the nucellus primarily among cells neighboring the embryo sac (Fig. 4-c). In differing from the Ponkan mandarin, nucellar embryogeny was confined to the micropylar regions and even then only few embryos formed. While the newly-formed embryo sometimes developed within the embryo sac (Fig. 4-e), it mostly grew in an external direction (Fig. 4-d, 4-f). The embryos seemed to grow through the micropyle and eventually appear exterior to the nucellus.

The endosperm in the citron explant continued to undergo division and growth in vitro (Fig. 4-c, 4-e, 4-g and 4-h). Little if any evidence of endosperm development was seen in the Ponkan mandarin.

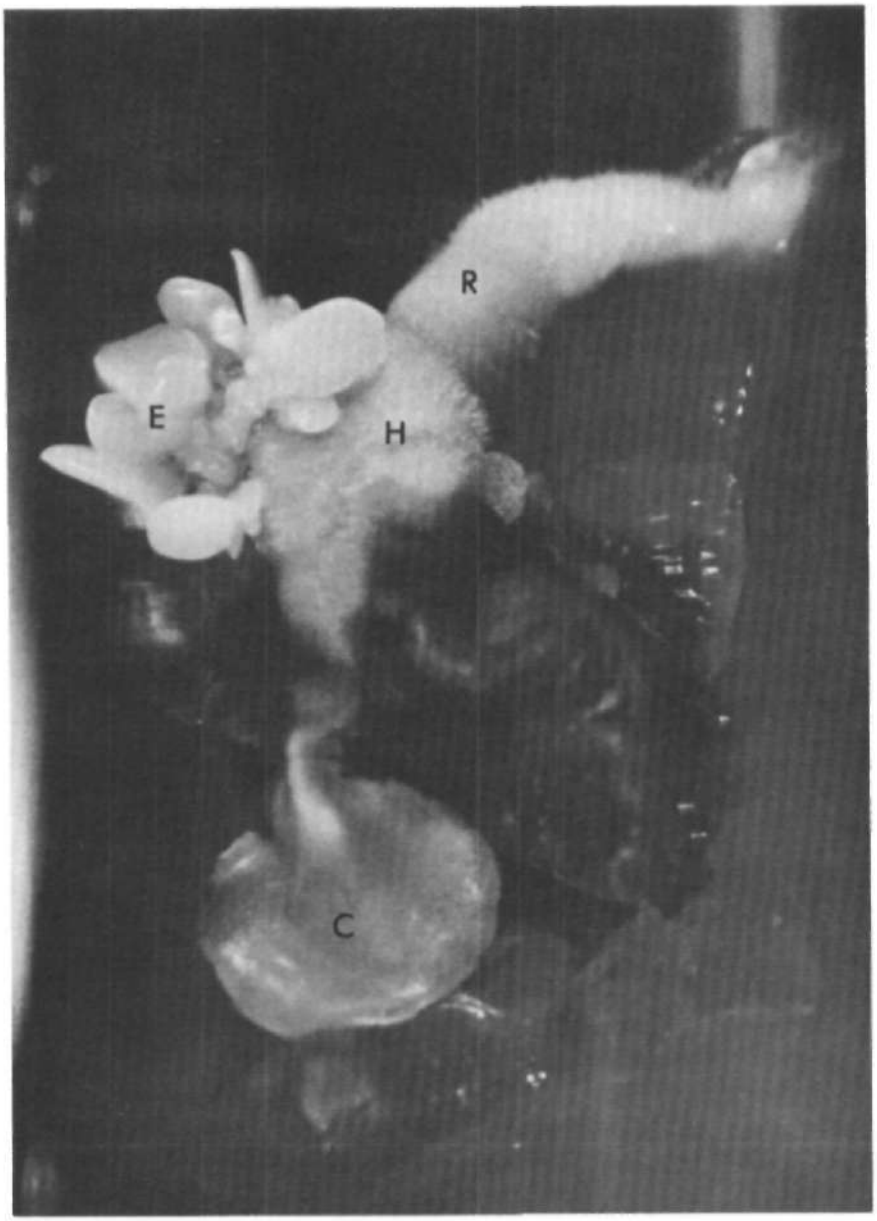
Multiplication of embryos by budding was also evident in citron nucellus cultures (Figs. 5, 6 and 7). In Fig. 5 can be seen embryos arising by budding in the radicle of an existing embryo, Fig. 6 shows embryo multiplication by budding of the hypocotyl, and Fig. 7 shows budding taking place in the cotyledon.

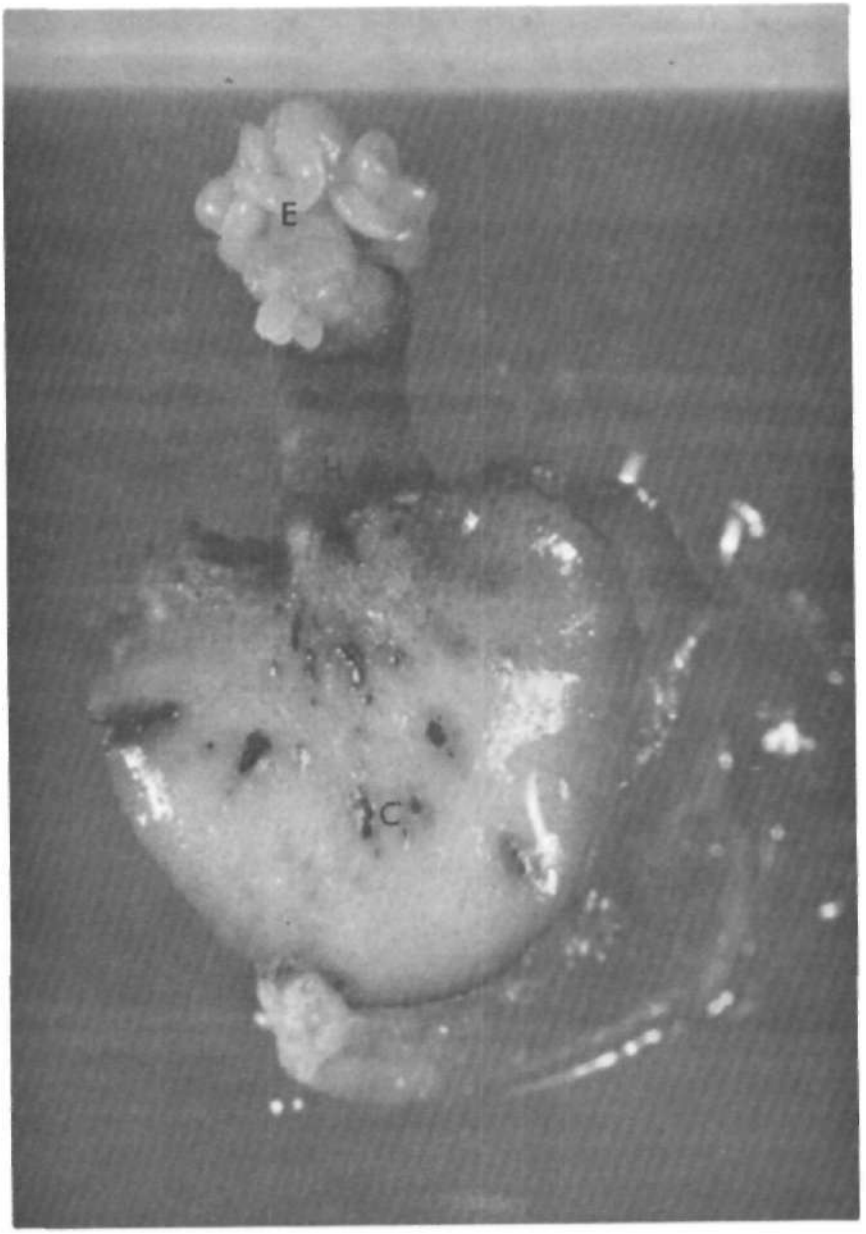
Assessment of Requirement for Adventive Embryogenesis in Rutaceous Nucellus Cultures

Characteristics and Handling of Explant Materials

Influence of fruit age. The experiment with Ethrog citron disclosed that fruit age at the time of nucellus excision is not a critical factor governing the potential of the nucellus of the monoembryonic citrus to produce adventive embryos in vitro (Table 4). Survival of nucellus explants in culture was virtually the same for all tissues isolated from fruits ranging in age from 77 to 133 days after pollination, but declined in older fruits. Of the nucellus explants that survived, all produced embryos, regardless of the age of the fruit from which explants were obtained. Moreover, there was little variation in the number of embryos produced by each surviving nucellus: each nucellus produced an average of approximately two embryos.

Some of the naturally polyembryonic Rutaceae were capable of adventive embryogenesis in vitro using nucellus explants from very young fruits. Ovule explants from flower buds of some of the Rutaceous plants


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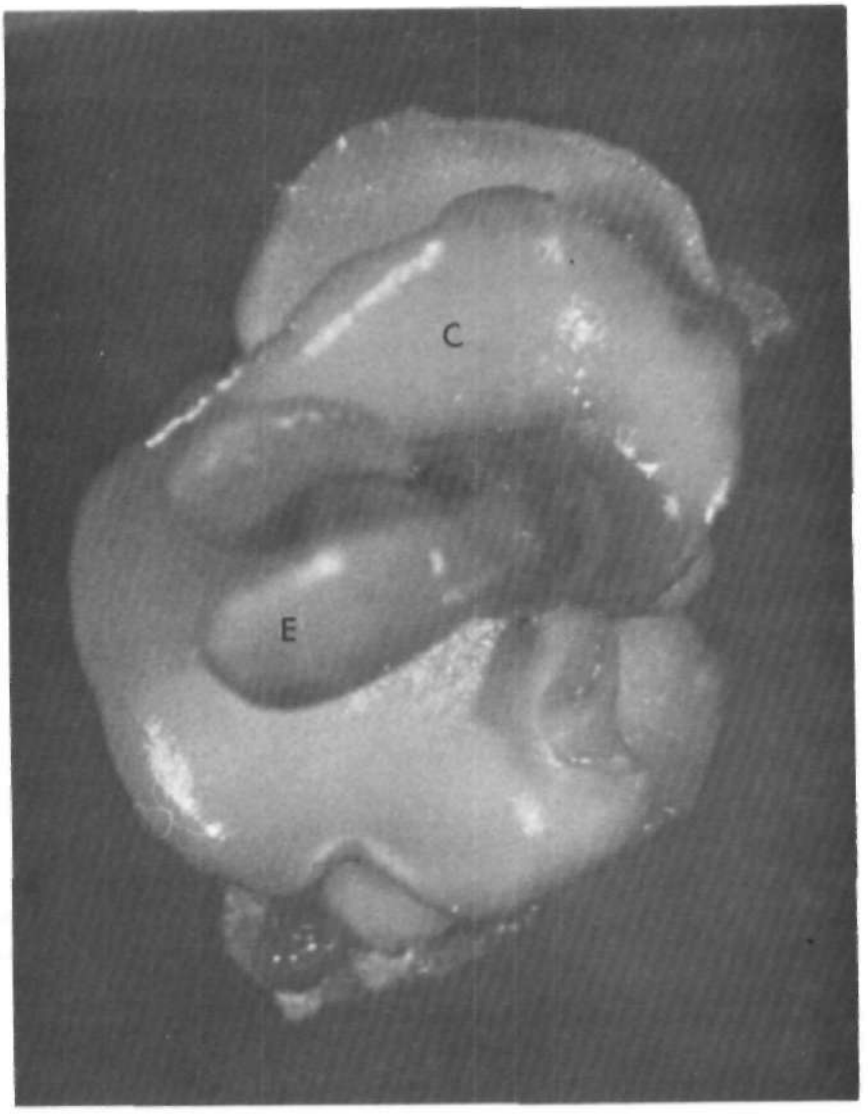

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TABLE 4

Influence of fruit age on behavior of nucellus cultures of *Citrus medica* L. 'Ethrog'.

Fruit Age (days since Pollination)	% Surviving Explants	% Surviving Cultures with Adventive Embryos	No. Adventive Embryos Per Surviving Nucellus
77	62 (47-79)	100 (91-100)	1.8 ± 0.3
84	68 (52-83)	100 (91-100)	1.5 ± 0.3
91	75 (60-91)	100 (91-100)	2.1 ± 0.4
98	72 (59-88)	100 (91-100)	1.9 ± 0.3
105	78 (63-94)	100 (91-100)	1.8 ± 0.3
112	70 (55-85)	100 (91-100)	2.0 ± 0.4
119	58 (40-75)	100 (91-100)	2.2 ± 0.3
126	75 (60-91)	100 (91-100)	1.4 ± 0.3
133	50 (36-69)	100 (91-100)	2.3 ± 0.2
140	38 (22-56)	100 (91-100)	1.9 ± 0.3
147	25 (10-43)	100 (91-100)	2.2 ± 0.3

95% confidence limits in parenthesis.

were also capable of surviving in vitro and producing adventive embryos (Table 5). The monoembryonic Ethrog citron and the naturally moderately polyembryonic Eureka lemon showed no adventive embryogenesis. The naturally highly polyembryonic types, i.e., Meiwa kumquat, Ponkan mandarin, and Wallace seedless Valencia, showed a small but significant proportion of the surviving ovules producing embryos. The number of embryos produced per explant was very low. Similar observations have been made with C. sinensis L. Osbeck 'Koethen,' C. sinensis L. Osbeck 'Ruvel' Roubidoux, C. sinensis L. Osbeck 'Surprise Navel.'

Seasonal factor. Since flowering and fruiting in most Rutaceous plants are confined to specific periods of the year, the season in which explants are obtained for culture is fixed. Nevertheless, some species and varieties, e.g., Ethrog citron and Eureka lemon, flower and fruit the year round, and it is possible to obtain nucellus explants at any time. In employing nucellus cultures of such species or variety, it may be important to first assess the behavior of the explants as influenced by the season in which they are obtained.

The highest frequency of adventive embryogenesis in vitro occurred during the fall and winter months, i.e., September through January (Fig. 8). The incidence of embryo formation in the nucellus cultures was poorest during the spring months. Summer explants gave an intermediate frequency of embryogenesis.

The potential for embryo formation in a given nucellus explant was the same throughout the year, however. Allowing for experimental errors, any nucellus explant that survived culture and produced embryos gave rise to the usual number of embryos. As seen in Fig. 9, the Eureka

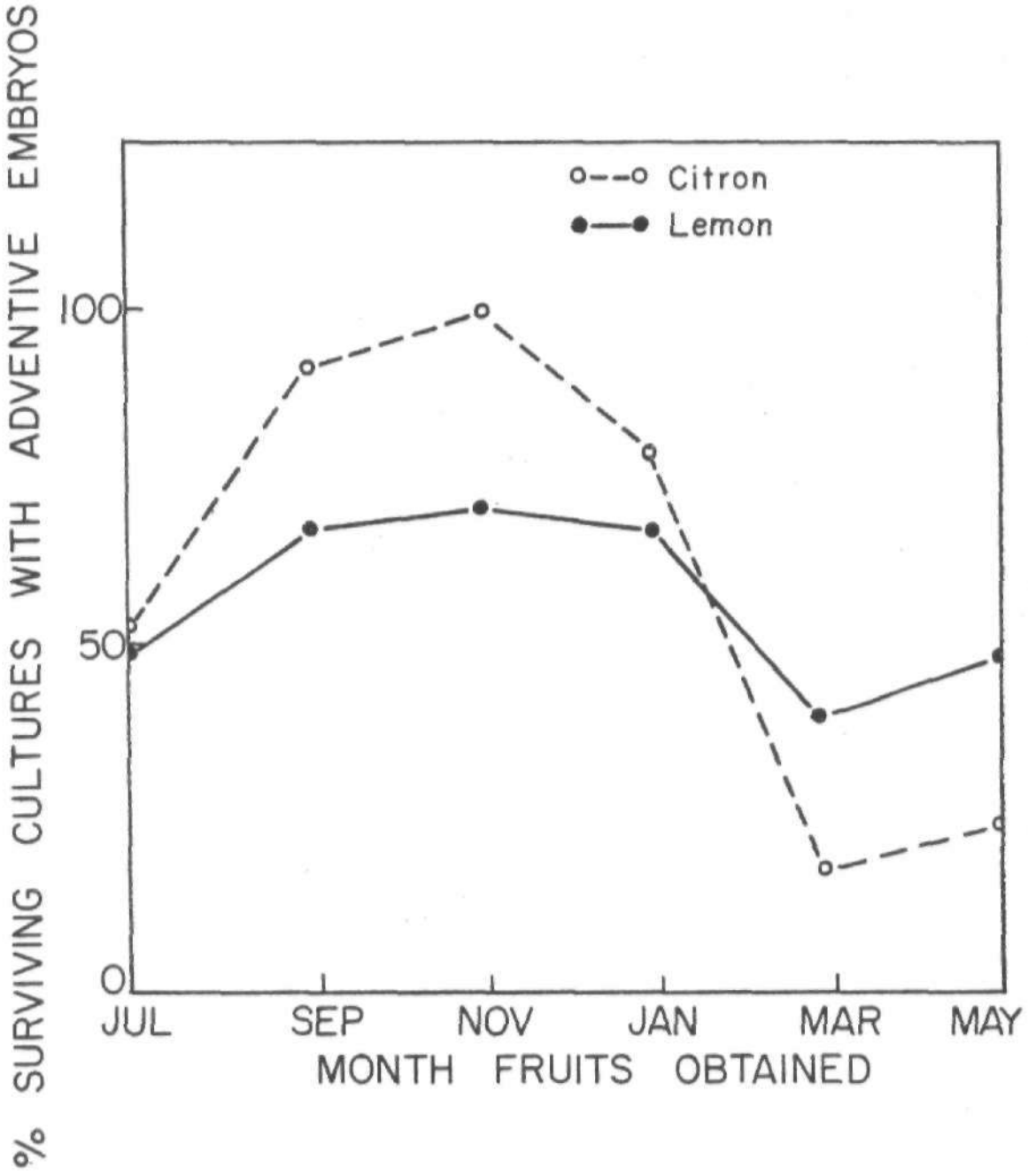
TABLE 5

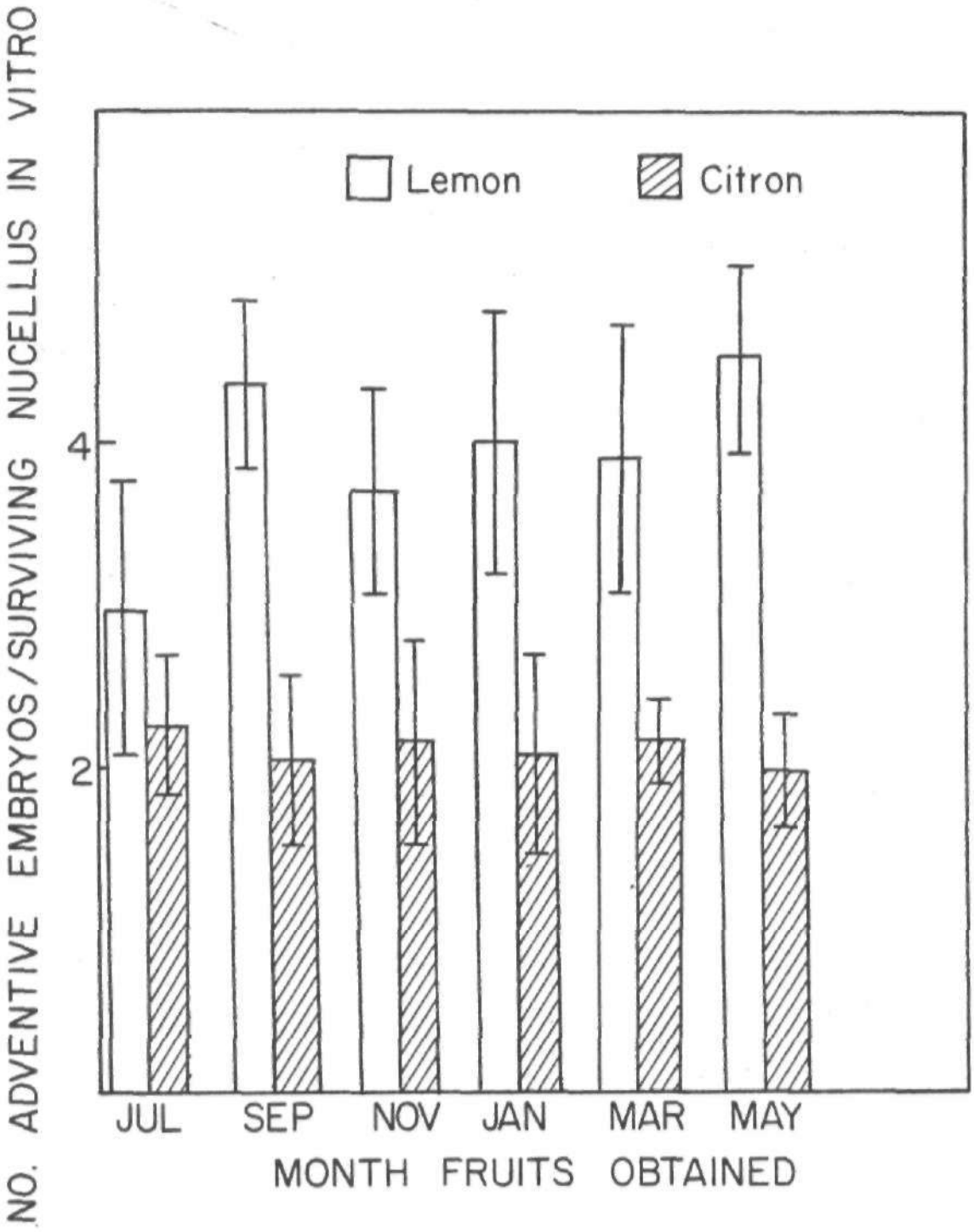
Adventive embryogenesis in ovules excised from flower buds of some *Citrus* species and *Fortunella crassifolia* Swing. 'Meiwa'

	Species[1]				
	A	B	E	G	I
% Surviving explants in vitro	45 (22-71)	40 (20-65)	15 (4-36)	40 (20-65)	50 (29-71)
% Surviving cultures with callus only	40 (20-65)	20 (7-42)	5 (0-28)	40 (20-65)	50 (29-71)
% Surviving cultures with adventive embryos	5 (0-28)	20 (7-42)	10 (1-30)	0 (0-15)	0 (0-15)
No. adventive embryos/ surviving culture	0.1 ± 0.1	0.9 ± 0.2	0.5 ± 0.2	0	0
No. adventive embryos/ embryo producing culture	2.5 ± 0.6	2.5 ± 0.8	1.6 ± 0.3	0	0

[1] A = *Fortunella crassifolia* Swing 'Meiwa kumquat'; B = *Citrus reticulata* Blanco 'Ponkan mandarin'; E = *Citrus sinensis* L. Osbeck 'Wallace seedless'; G = *Citrus limon* L. Burm 'Eureka'; and I = *Citrus medica* L. 'Ethrog'.

95% confidence limits in parenthesis.


% SURVIVING CULTURES WITH ADVENTIVE EMBRYOS
100
50
0
Citron
Lemon
JUL
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MONTH FRUITS OBTAINED


NO. ADVENTIVE EMBRYOS/SURVIVING NUCELLUS IN VITRO
Lemon
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MONTH FRUITS OBTAINED

lemon nucellus produced an average of about four embryos per explant, whereas the citron nucellus averaged about two embryos.

Effects of fruit storage. It was possible to store Ethrog citron and Eureka lemon fruits under refrigeration for at least four weeks without adversely affecting the nucellus explants obtained. Survival in vitro of nucellus explants was unaffected until fruits had been stored six or eight weeks (Table 6). The potential for embryo initiation was undiminished in nucellus explants from all stored fruits. All surviving cultures produced embryos in vitro. Each surviving citron nucellus produced about two embryos per explant, and each Eureka lemon explant produced three to four embryos.

Method of incision. Table 7 shows the influence of incision methods, prior to removing the zygotic embryo, on the behavior of the nucellus in vitro of Ethrog citron and Ponderosa lemon. Both are normally monoembryonic cultivars. A single transverse incision in the micropylar region gave the highest rate of survival of explants. Longitudinal incisions at the micropylar region, whether only one or many incisions were made, caused drastic reductions in the frequency of surviving explants. Perhaps to be expected, the multiple incision method was worse than the single longitudinal incision. Both cultivars showed comparable effects.

Any nucellus explant that survived, however, showed potential for embryogenesis and each produced virtually the same number of embryos.

Method of implantation. The method by which the nucellus explant was placed in the nutrient medium showed a dramatic effect on the developmental behavior of the nucellus (Table 8). Survival of the

TABLE 6

Influence of prior cold storage of *Citrus medica* L. 'Ethrog' and *C. limon* L. Burm 'Eureka' fruits on subsequent behavior of nucellus explants. Storage temperature, 5°C.

Storage Period (Weeks)	% Surviving Explants		% Surviving Cultures with Adventive Embryos		No. Adventive Embryos per Surviving Nucellus	
	Ethrog Citron	Eureka Lemon	Ethrog Citron	Eureka Lemon	Ethrog Citron	Eureka Lemon
0	60 (44-77)	63 (47-79)	100 (91-100)	100 (91-100)	2.1 ± 0.3	3.7 ± 0.6
1	63 (47-79)	73 (59-88)	100 (91-100)	100 (91-100)	1.9 ± 0.4	3.8 ± 0.5
2	50 (36-69)	63 (47-79)	100 (91-100)	100 (91-100)	2.2 ± 0.4	3.4 ± 0.4
4	58 (40-75)	65 (50-81)	100 (91-100)	100 (91-100)	2.0 ± 0.3	3.6 ± 0.6
6	32 (14-53)	57 (40-75)	100 (91-100)	100 (91-100)	2.0 ± 0.2	2.9 ± 0.3
8	10 (2-25)	25 (10-43)	100 (91-100)	100 (91-100)	1.6 ± 0.3	2.2 ± 0.4

95% confidence limits in parenthesis.

TABLE 7

Influence of incision method in removing zygotic embryo on subsequent behavior of nucellus cultures of *Citrus medica* L. 'Ethrog' and *C. limon* L. Burm 'Ponderosa'

	Single Transverse Incision		Single Longitudinal Incision		Multiple Longitudinal Incisions	
	Ethrog citron	Ponderosa lemon	Ethrog citron	Ponderosa lemon	Ethrog citron	Ponderosa lemon
% Surviving explants	80 (58-93)	70 (47-86)	40 (20-65)	30 (14-54)	10 (1-30)	10 (1-30)
% Surviving cultures with adventive embryos	100 (85-100)	100 (85-100)	100 (85-100)	100 (85-100)	100 (85-100)	100 (85-100)
No adventive embryos per surviving nucellus	1.8 ± 0.4	1.6 ± 0.4	1.4 ± 0.3	1.2 ± 0.3	1.5 ± 0.3	1.5 ± 0.5

95% confidence limits in parenthesis.

TABLE 8

Morphogenic behavior of nucellus cultures of Citrus medica L. 'Ethrog' and C. limon L. Burm 'Ponderosa' as influenced by method of implantation in nutrient medium

	Micropylar End in Medium		Chalazal End in Medium		Horizontal Placement	
	Ethrog citron	Ponderosa lemon	Ethrog citron	Ponderosa lemon	Ethrog citron	Ponderosa lemon
% Surviving explants	100 (85-100)	80 (58-93)	85 (64-96)	65 (41-86)	100 (85-100)	80 (58-93)
% Surviving cultures with adventive embryos	15 (4-36)	10 (1-30)	100 (85-100)	100 (85-100)	25 (10-47)	20 (7-42)
% Surviving with callus only	85 (64-96)	70 (47-86)	0 (0-15)	0 (0-15)	75 (53-90)	60 (35-80)
Days to half-maximum production embryos	49	52	24	42	42	47
No. adventive embryos per surviving nucellus	0.2 ± 0.1	0.2 ± 0.1	1.8 ± 0.3	1.7 ± 0.3	0.6 ± 0.1	0.7 ± 0.1

95% confidence limits in parenthesis.

nucellus of Ethrog citron and Ponderosa lemon was unaffected, whether the explant was implanted with its micropylar end in the medium, or with the chalazal end in the medium, or simply horizontally on the surface of the agar medium. But the potential and the rate of embryogenesis were determined by the method of positioning of the explant (Fig. 10). When the explant was placed with its micropylar end in the medium or simply horizontally, the usual result was development of unorganized callus; also, appearance of embryo was delayed considerably and the number of embryos per explant was reduced markedly. Nucellus cultures of Ethrog citron and Ponderosa lemon, in which the chalazal end of the explant was embedded in the medium and the micropylar end left exposed, produced no callus but only embryos; the embryos were apparent within 4-6 weeks and each explant produced nearly two embryos.

The experiment was repeated with a highly polyembryonic cultivar, Ponkan mandarin, with virtually the same results (Table 9). However, survival was reduced drastically when the explant was placed with its micropylar end in the medium. All surviving cultures of Ponkan mandarin produced embryos and none resulted in callus only. The time of appearance of embryos was delayed when the explant was placed with its micropylar end in the medium or simply horizontally on the medium. The number of adventive embryos produced per surviving nucellus was drastically reduced when the explant was placed with its micropylar end in agar; the horizontal placement gave an intermediate rate of embryogenesis.

Clearly these experiments showed that embryogenesis in nucellus cultures was influenced, determined in some cases, by the method of placement of explants in the nutrient medium. Manifestation of the

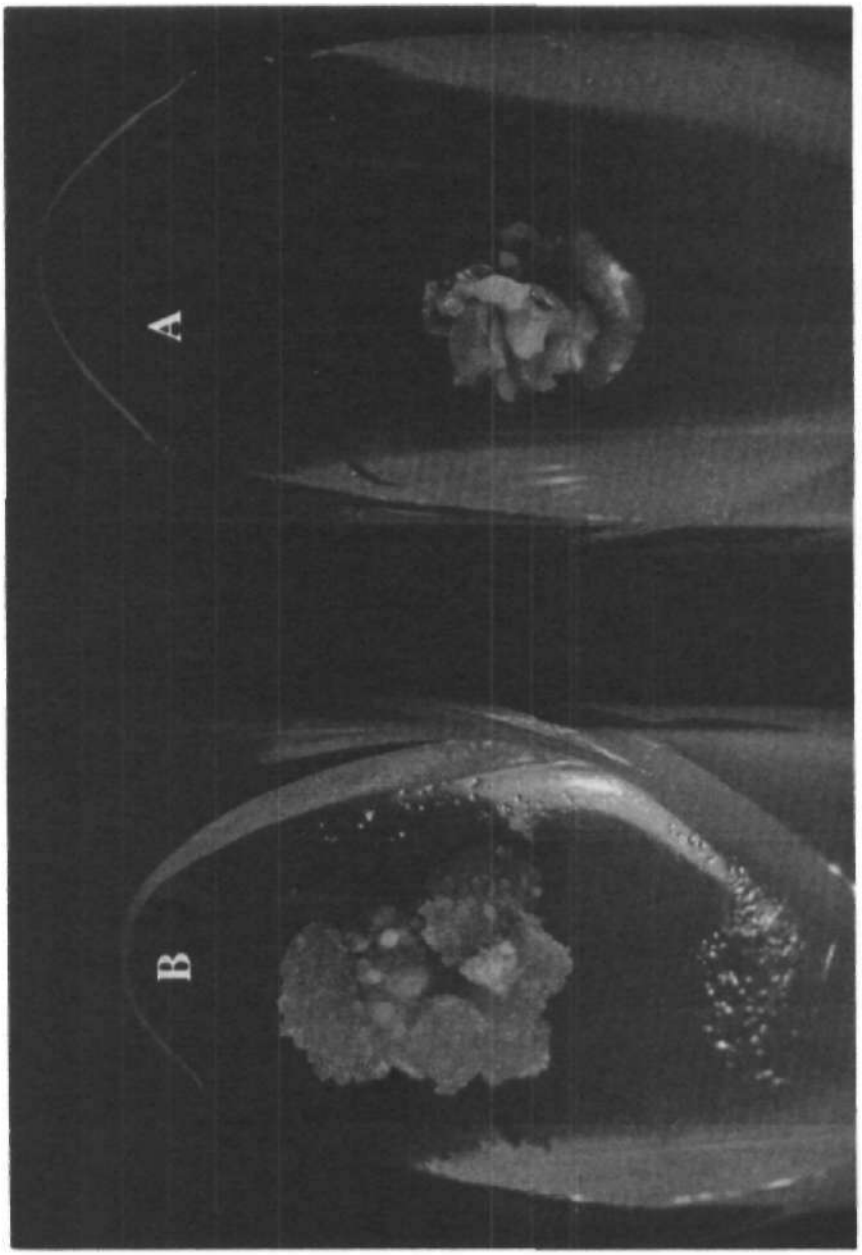

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TABLE 9

Morphogenic behavior of nucellus cultures of Citrus reticulata Blanco. 'Ponkan' as influenced by method of implantation in nutrient medium.

	Micropylar End in Medium	Chalaza End in Medium	Horizontal Placement
% Surviving explants	35 (14-59)	100 (85-100)	75 (53-90)
% Surviving cultures with adventive embryos	100 (85-100)	100 (85-100)	100 (85-100)
% Surviving cultures with callus only	0 (0-15)	0 (0-15)	0 (0-15)
Days to half maximum production of embryos	35	18	24
No. adventive embryos per surviving nucellus	2.5 ± 0.6	7.4 ± 1.4	4.5 ± 1.3

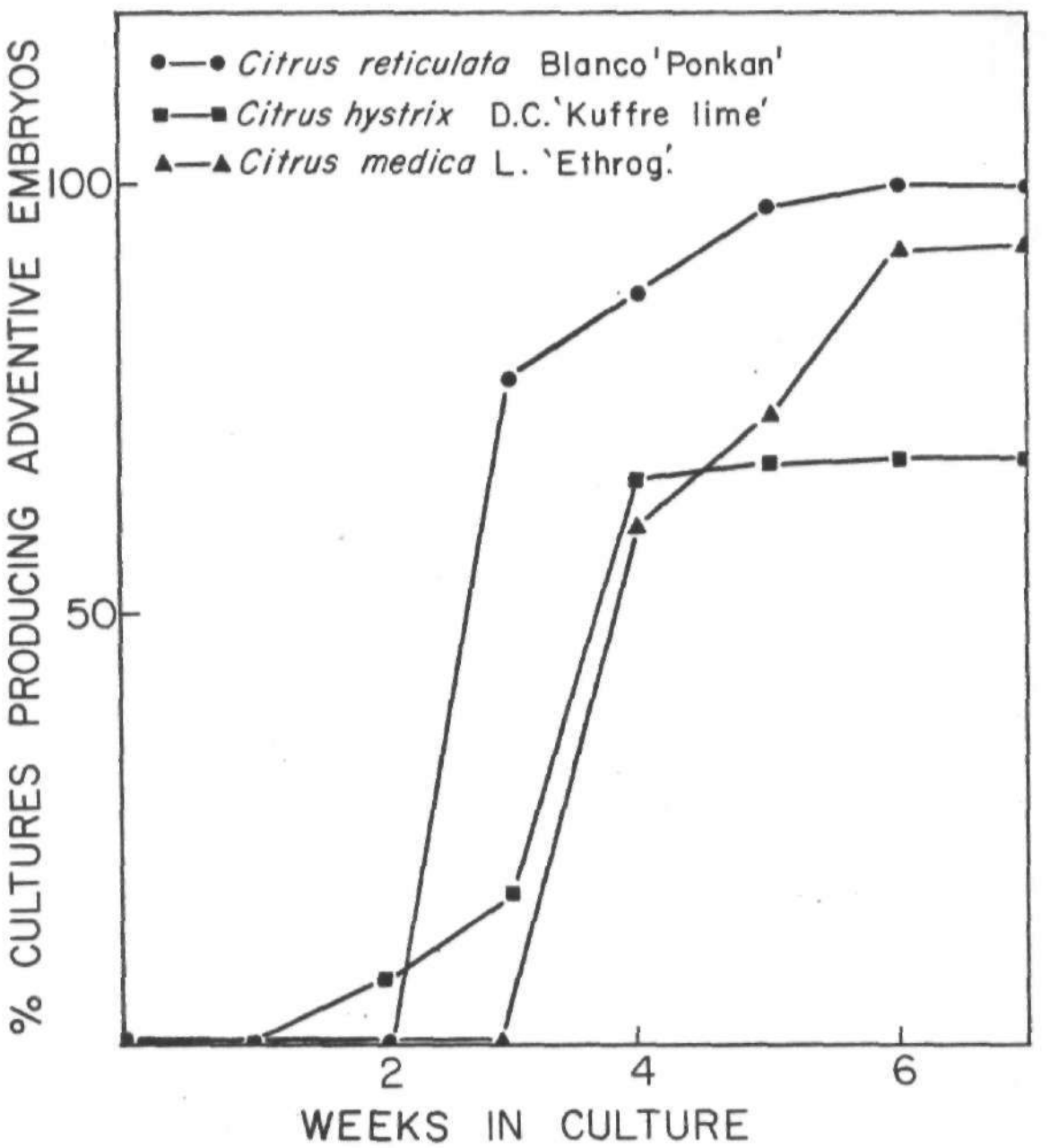
95% confidence limits in parenthesis.

maximum embryogenic potential occurred only when the explant was placed with its chalazal region embedded in the nutrient agar and its micropylar end left exposed to the atmosphere.

Length of culture passage. The minimum passage period required for meaningful embryogenesis to occur depended on the cultivar (Fig. 11). Embryo producing cultures of Kuffre lime were evident after two weeks, and maximum frequency of embryo producing cultures after four weeks. There was no further increase with longer passage periods, although perhaps the longer periods were necessary for the growth of the embryos. Ponkan mandarin showed no embryo producing culture for two weeks; however, a high percentage of embryo producing cultures was obtained after three weeks, reaching virtually 100% in five weeks. Embryogenesis in vitro was not apparent in Ethrog citron nucellus even after three weeks. Subsequently, there was a progressive and rapid increase in the frequency of embryo-producing cultures; maximum frequency was reached in six weeks. According to these data, then, experiments utilizing Kuffre lime could be terminated after four weeks, those of Ponkan mandarin after five weeks, and those of citron after six weeks.

Nutrient Medium Characteristics

Liquid vs. agar. Successful embryogenesis in Daucus carota, Chicorium endiva, Asparagus officinalis and other plants evidently occurred best in gently agitated liquid nutrient medium (Steward, et al., 1958, 1963, 1966; Vasil and Hildebrandt, 1966; Wilmar and Hellendoorn, 1968). Previous work with citrus nucellus cultures had been


●—● Citrus reticulata Blanco 'Ponkan'
■—■ Citrus hystrix D.C. 'Kuffre lime'
▲—▲ Citrus medica L. 'Ethrog'
% CULTURES PRODUCING ADVENTIVE EMBRYOS
100
50
2
4
6
WEEKS IN CULTURE

carried out entirely with agar gel media. A comparison of the two media was presently made with Ethrog citron, a monoembryonic; Eureka lemon, a moderately polyembryonic; and Meiwa kumquat, a highly polyembryonic cultivar. The agar medium was the basal medium. Nucellus cultures in liquid nutrient media involved (1) stationary culture tube with the explant completely submerged, (2) stationary culture tube with explant placed on Heller support, (3) culture tube rotated at 1 rpm on a revolving drum, (4) liquid in flasks and agitated at 300 rpm on a gyratory shaker. Survival of citron nucellus explants was highest on agar medium and in liquid medium agitated vigorously at 300 rpm (Table 10); stationary liquid cultures with or without Heller support gave the poorest survival frequency. The polyembryonic types, Eureka lemon and Meiwa kumquat, showed the same survival frequencies in either agar medium or stationary liquid medium with Heller support. As expected, the survival frequency was highest in the polyembryonic Meiwa kumquat and lowest in the monoembryonic Ethrog citron.

Interestingly, manifestation of embryogenesis in surviving citron nucellus cultures was best in agar medium and in liquid medium rotated at 1 rpm; the vigorously agitated liquid which gave a high survival frequency showed no manifestation of the embryogenic potential among surviving cultures (Table 11). The stationary liquid medium with no support showed consistently poor embryogenesis. Eureka lemon and Meiwa kumquat nucellus cultures showed no difference between agar and liquid medium with Heller support as far as frequency of surviving cultures producing embryos was concerned.

Table 12 shows the number of embryos produced per surviving

TABLE 10

Influence of certain physical characteristics of the nutrient medium on survival of nucellus cultures of Citrus medica L. 'Ethrog', C. limon L. Burm 'Eureka' and Fortunella crassifolia Swing. 'Meiwa'

Medium Characteristics	% Surviving Explants Ethrog citron	Eureka lemon	Meiwa kumquat
Agar	80 (58-93)	85 (64-96)	100 (85-100)
Liquid			
Stationary No support	30 (14-54)		
Heller support	45 (22-71)	65 (41-86)	100 (85-100)
Rotated, 1 RPM	60 (35-80)	-	-
Agitated, 300 RPM	100 (85-100)	-	-

95% confidence limits in parenthesis.

TABLE 11

Influence of certain physical characteristics of the nutrient medium on frequency of adventive embryogenesis in nucellus cultures of *Citrus medica* L. 'Ethrog', *C. limon* L. Burm 'Eureka' and *Fortunella crassifolia* Swing. 'Meiwa'

Medium Characteristics	% Surviving Cultures with Adventive Embryos		
	Ethrog citron	Eureka lemon	Meiwa kumquat
Agar	100 (85-100)	100 (85-100)	100 (85-100)
Liquid			
Stationary			
No support	5 (0-23)	-	-
Heller support	55 (29-78)	80 (58-93)	100 (85-100)
Rotated, 1 RPM	100 (85-100)	-	-
Agitated, 300 RPM	0 (0-15)	-	-

TABLE 12

Influence of certain physical characteristics of the nutrient medium on number of adventive embryos produced per cultured nucellus of *Citrus medica* L. 'Ethrog', *C. limon* L. Burm 'Eureka' and *Fortunella crassifolia* Swing. 'Meiwa'

Medium Characteristics	No. Adventive Embryos Per Surviving Nucellus		
	Ethrog Citron	Eureka Lemon	Meiwa Kumquat
Agar	2.5 ± 0.3	4.2 ± 0.7	7.9 ± 0.9
Liquid			
Stationary			
No support	1.3 ± 0.1	---	---
Heller Support	2.1 ± 0.4	4.4 ± 0.9	11.6 ± 1.1
Rotated 1 RPM	2.0 ± 0.2	---	---
Agitated 300 RPM	0.0	---	---

nucellus in relation to agar and liquid media. The nucellus of Ethrog citron showed again the highest number of embryos in an agar medium; no embryo was obtained in the vigorously agitated medium. The stationary liquid medium enabled only half as many citron nucellar embryos to be produced as the agar medium. The liquid medium with Heller support and that rotated gently at 1 rpm showed the same moderate rates of embryo formation. Eureka lemon showed equal rates of embryo formation in the surviving nucellus cultures in agar medium and liquid medium provided with Heller support. The Meiwa kumquat cultures showed a surprisingly higher rate of embryo formation among surviving nucellus cultured in a liquid medium containing Heller support than in agar medium.

In summary, the consistently best cultures, considering incidence of explant survival, frequency of embryonic cultures among survivors and number of embryos formed per surviving culture, were obtained on an agar nutrient medium. Liquid media, whether maintained stationary or agitated at various rates, were for the most part inferior to agar.

Medium composition

Evaluation of four components. In the initial series of experiments, aimed at improving the nutrient medium composition, the basal constituents were divided into four categories and their effects separately, and in combinations were examined. The data from this series are shown in Tables 13, 14 and 15. None of the nucellus explants survived culture in a medium composed of agar alone; agar and salts; agar and organics (Malt extract, B-vitamins, inositol, and glycine); agar and sucrose; or agar, sucrose, and organics (Table 13). All four cultivars examined showed the same effects. Survival of 100% of Ponkan

TABLE 13

Influence of nutrient medium constituent group on percentage survival of *Citrus* nucellus explants in vitro

Medium Constituent Group	% Surviving Explants			
	Ponkan Mandarin	Koethen Sweet Orange	Eureka Lemon	Ethrog Citron
Agar	0 (0-15)	0 (0-15)	0 (0-15)	0 (0-15)
Salts	0 (0-15)	0 (0-15)	0 (0-15)	0 (0-15)
Organics	0 (0-15)	0 (0-15)	0 (0-15)	0 (0-15)
Sucrose	0 (0-15)	0 (0-15)	0 (0-15)	0 (0-15)
Organics + Sucrose	0 (0-15)	0 (0-15)	0 (0-15)	0 (0-15)
Salts + Organics	100 (85-100)	100 (85-100)	0 (0-15)	0 (0-15)
Salts + Sucrose	100 (85-100)	100 (85-100)	20 (7-42)	20 (7-42)
Complete medium	100 (85-100)	100 (85-100)	40 (20-65)	75 (53-90)

Agar = agar alone, 1.0% concentration

Salts = 1.0% agar + MS salts

Organic = 1.0% agar + 500 mg/l malt extract, 100 mg/l inositol, thiamin.HCl 0.2 mg/l, Nicotinic acid, 1.0 mg/l, Glycine 4.0 mg/l and prydoxin.HCl 0.2 mg/l

Sucrose = 1.0% agar + 5% sucrose

95% confidence limits in parenthesis

TABLE 14

Influence of nutrient medium constituent group on frequency of nucellus cultures with adventive embryos

Medium Constituent Group	Ponkan Mandarin	Koethen Sweet Orange	Eureka Lemon	Ethrog Citron
Agar	0 (0-15)	0 (0-15)	0 (0-15)	0 (0-15)
Salts	0 (0-15)	0 (0-15)	0 (0-15)	0 (0-15)
Organics	0 (0-15)	0 (0-15)	0 (0-15)	0 (0-15)
Sucrose	0 (0-15)	0 (0-15)	0 (0-15)	0 (0-15)
Organic + Sucrose	0 (0-15)	0 (0-15)	0 (0-15)	0 (0-15)
Salt + Organic	100 (85-100)	100 (85-100)	0 (0-15)	0 (0-15)
Salts + Sucrose	100 (85-100)	100 (85-100)	100 (85-100)	100 (85-100)
Complete medium	100 (85-100)	100 (85-100)	100 (85-100)	100 (85-100)

Agar = agar alone, 1.0% concentration

Salts = 1.0% agar + MS salts

Organics = 1.0% agar + 500 mg/l Malt extract, 100 mg/l inositol, thiamin.HCl 0.2 mg/l, Nicotinic acid, 1.0 mg/l, Glycine 4.0 mg/l and pyridoxin.HCl 0.2 mg/l

Sucrose = 1.0% agar + 5% sucrose

95% confidence limits in parenthesis

TABLE 15

Influence of nutrient medium constituent group on number of adventive embryos produced per cultured nucellus

Medium Constituent Group	Number adventive embryos per surviving culture: Ponkan Mandarin	Koethen Sweet	Eureka Lemon	Ethrog Citron
Agar	0	0	0	0
Salts	0	0	0	0
Organics	0	0	0	0
Sucrose	0	0	0	0
Organics + Sucrose	0	0	0	0
Salts + Organics	18.2 ± 2.0	6.4 ± 0.9	0	0
Salts + Sucrose	12.4 ± 1.7	5.0 ± 0.9	3.0 ± 0.9	1.0 ± 0.1
Complete medium	23.0 ± 2.2	14.3 ± 1.1	5.5 ± 0.6	2.1 ± 0.3

Agar = agar alone, 1.0% concentration

Salts = 1.0% agar + MS salts

Organics = 1.0% agar + 500 mg/l Malt extract, 100 mg/l inositol, thiamin.HCl 0.2 mg/l, Nicotinic acid 1.0 mg/l, Glycine 4.0 mg/l and pyridoxin.HCl 0.2 mg/l

Sucrose = 1.0% agar + 5% sucrose

mandarin and Koethen sweet orange nucellus explants, both highly polyembryonic cultivars occurred in media containing agar, salts and organics; agar, salts and sucrose; and the complete set of ingredients. Survival of the moderately polyembryonic Eureka lemon and the monoembryonic Ethrog citron was best in the complete nutrient medium, although a low but significant percentage of cultures were obtained in a medium composed of agar, salts and sucrose fractions.

All media combinations which enabled survival of the explants also were adequate for the initiation of nucellar embryos among the surviving cultures. Every surviving culture of the four cultivars produced embryos in a medium combination of either agar, salts and organic fractions; agar salts and sucrose; or agar, salts, sucrose and organic fractions (Table 14).

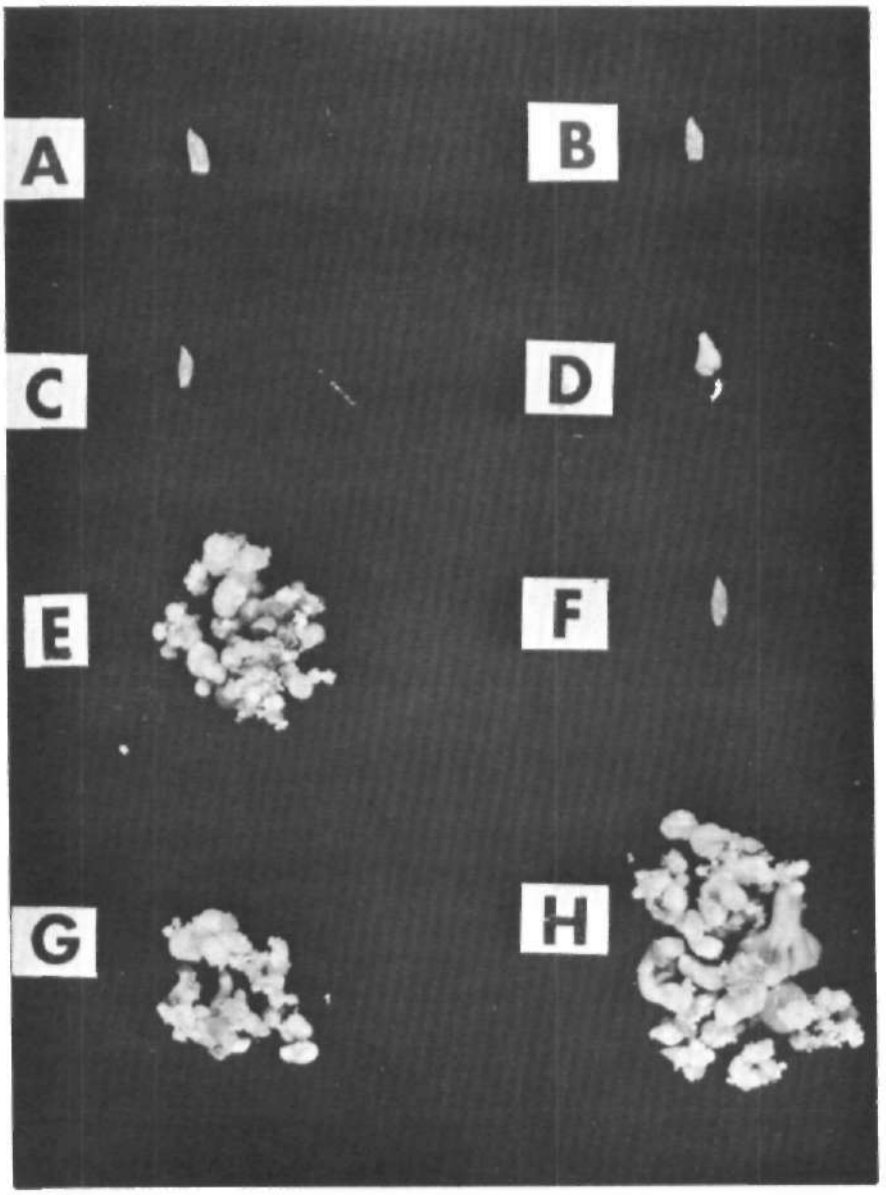
The difference among the three medium combinations in which survival occurred was in the number of embryos produced per surviving nucellus. As expected, the highest numbers of embryos were produced in the complete medium (Table 15). With a highly polyembryonic cultivar, particularly Ponkan mandarin, the next best medium was one in which agar, salts and organic fraction were combined. The moderately polyembryonic Eureka lemon and the monoembryonic Ethrog citron, on the other hand, showed the agar, salt and sugar combination to be better than the agar, salt and organic fraction combination.

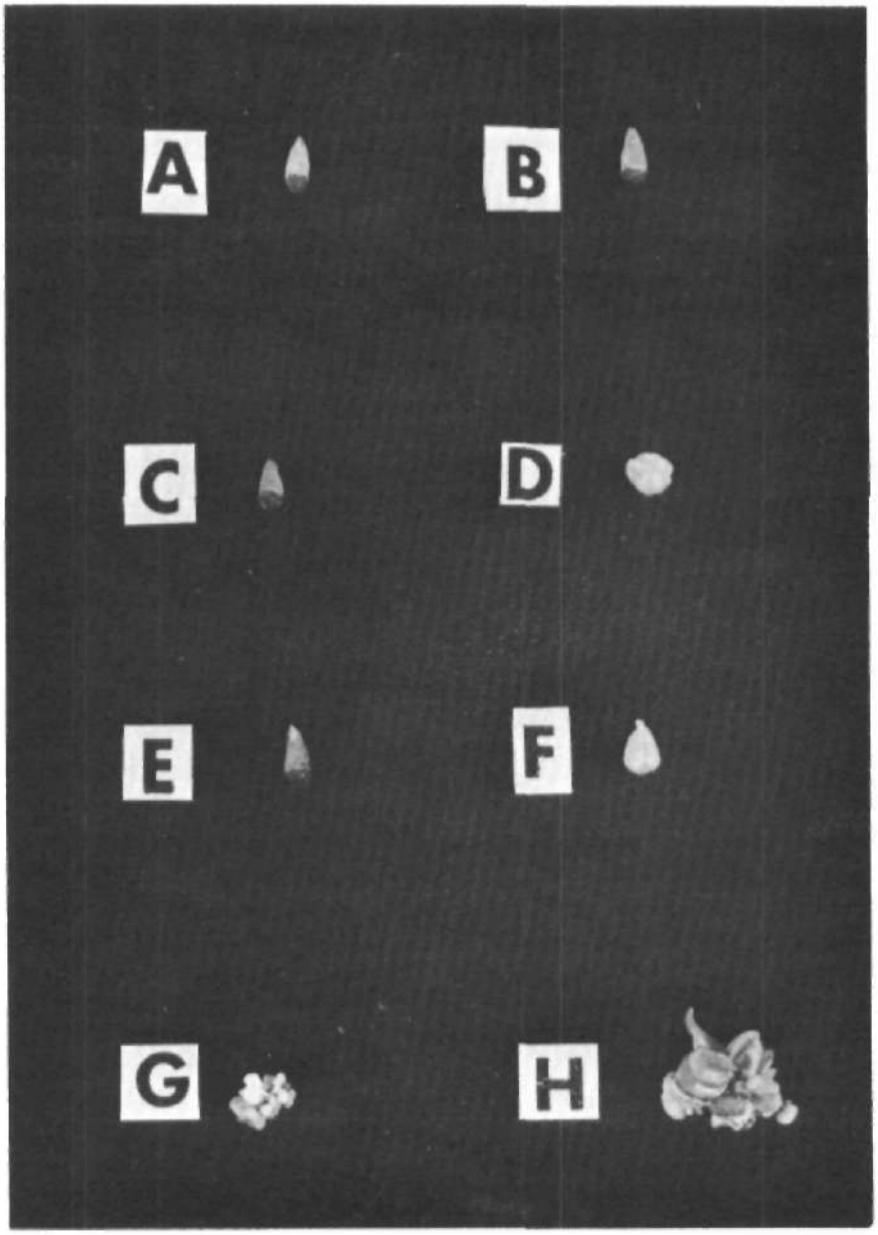
Summarizing the above observations, survival of nucellus explants as well as the initiation of adventive embryos required only that the medium contain agar, Murashige and Skoog salts, and sucrose. For the nucellus of the highly polyembryonic cultivars the sucrose could be

replaced by combination of malt extract, B-vitamins, inositol, and glycine. Representatives of cultures showing the effects of these constituent groups on the excised nucellus of Ponkan mandarin Eureka lemon and Ethrog citron can be seen in Figs. 12, 13, and 14, respectively.

Sucrose. The above experiments showed clearly that sucrose, when provided together with inorganic salts and agar, was a key ingredient in the culture of the Rutaceous nucellus. Accordingly, this constituent was investigated in some detail. First, a range of sucrose concentrations, from 0 to 20%, in the basal nutrient medium was examined. The data from one experiment with Ethrog citron, a monoembryonic, and Eureka lemon, a moderately polyembryonic cultivar, are shown in Table 16. For both cultivars maximum survival required a sucrose concentration of 5%. A concentration of 10% or higher was excessive. The sucrose concentration did not influence the frequency of surviving cultures showing adventive embryo formation. On the other hand, the number of embryos per surviving nucellus was clearly dependent on the sucrose concentration; the maximum number of embryos, for both Ethrog citron and Eureka lemon, was obtained in a 5% sucrose medium. Higher or lower concentrations progressively reduced the number of embryos per nucellus.

The sucrose requirement was not altered by enriching the basal medium with kinetin and adenine sulfate. The data in Table 17 show the response to sucrose in the enriched medium by two highly monoembryonic cultivars, Ethrog citron and Ponderosa lemon. As the data show, sucrose was an essential ingredient, inasmuch as no culture survived without its inclusion. Maximum benefit, considering survival frequency, incidence


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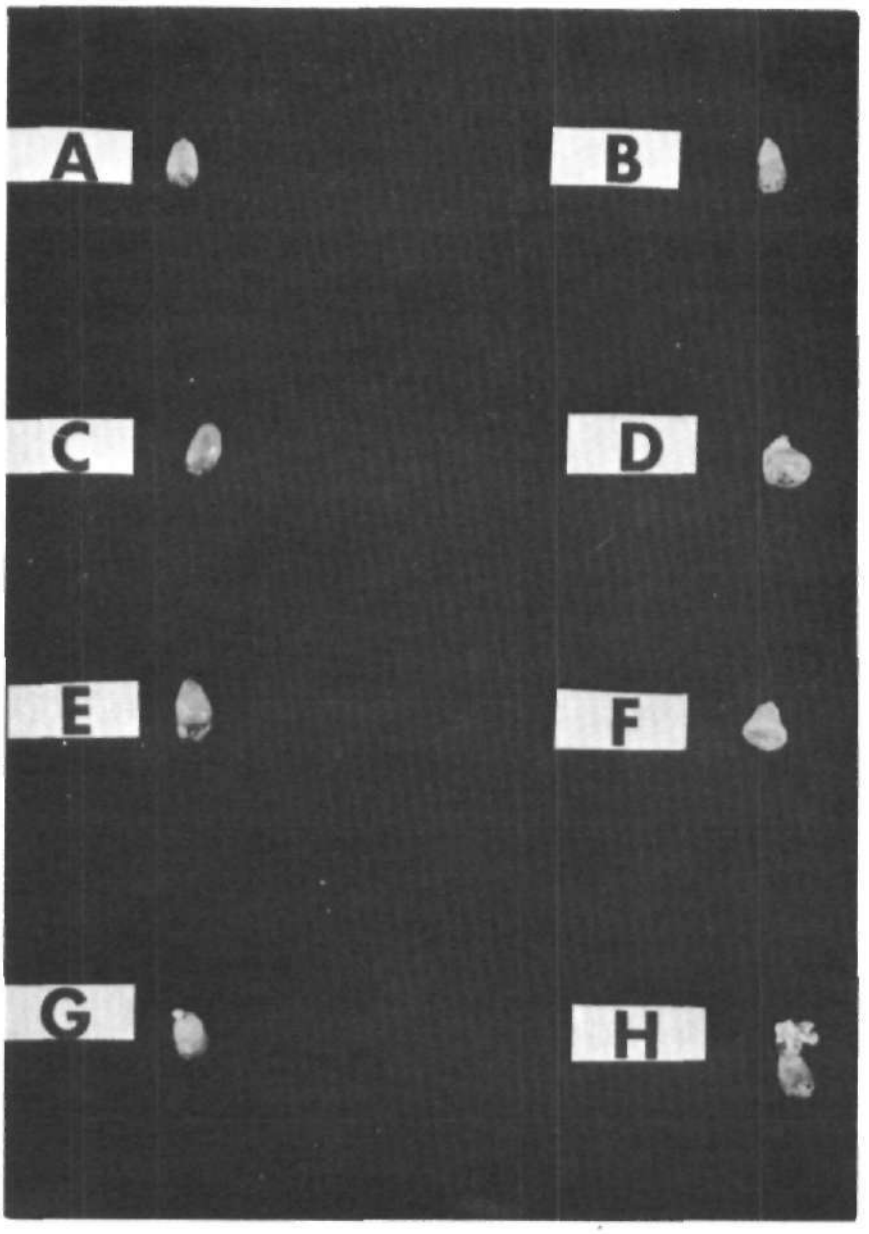

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TABLE 16

Effect of sucrose concentration on behavior of nucellus cultures of *Citrus medica* L. 'Ethrog' and *C. limon* L. Burm 'Eureka'

% Sucrose	% Surviving Explant		% Surviving Cultures with Adventive Embryos		No. Adventive Embryos Per Surviving Nucellus	
	Ethrog Citron	Eureka Lemon	Ethrog Citron	Eureka Lemon	Ethrog Citron	Eureka Lemon
0.0	10 (1-30)	20 (7-24)	100 (85-100)	100 (85-100)	1.0 ± 0.1	1.5 ± 0.5
1.0	20 (7-42)	30 (14-54)	100 (85-100)	100 (85-100)	1.0 ± 0.1	3.7 ± 0.5
5.0	80 (58-93)	70 (47-86)	100 (85-100)	100 (85-100)	1.9 ± 0.3	4.7 ± 0.4
7.5	75 (58-90)	60 (85-100)	100 (85-100)	100 (85-100)	1.7 ± 0.3	3.8 ± 0.6
10.0	60 (35-80)	60 (35-80)	100 (85-100)	85 (64-96)	1.3 ± 0.5	3.1 ± 0.5
12.5	40 (20-65)	50 (29-71)	100 (85-100)	60 (35-80)	1.2 ± 0.1	1.2 ± 0.6
15.0	35 (14-59)	45 (22-71)	100 (85-100)	100 (85-100)	1.6 ± 0.4	1.4 ± 0.8
20.0	15 (20-65)	40 (20-65)	100 (85-100)	100 (85-100)	1.0 ± 0.3	1.8 ± 0.8

95% confidence limits in parenthesis.

TABLE 17

Effect of sucrose concentration on behavior of nucellus cultures of *Citrus medica* L. 'Ethrog' and *C. limon* L. Burm 'Ponderosa'. Basal medium modified to include kinetin 0.1 mg/l and adenine sulfate 80 mg/l

% Sucrose	% Surviving Explants		% Surviving Cultures with Adventive Embryos		No. Adventive Embryos Per Surviving Nucellus	
	Ethrog Citron	Ponderosa Lemon	Ethrog Citron	Ponderosa Lemon	Ethrog Citron	Ponderosa Lemon
0	0 (0-15)	0 (0-15)	0 (0-15)	0 (0-15)	0.0	0.0
1	15 (4-36)	10 (1-30)	100 (85-100)	100 (85-100)	1.1 ± 0.3	0.8 ± 0.1
5	60 (35-80)	55 (29-78)	100 (85-100)	100 (85-100)	2.1 ± 0.4	1.7 ± 0.3
10	50 (29-71)	45 (22-71)	100 (85-100)	100 (85-100)	1.3 ± 0.3	1.0 ± 0.1

95% confidence limits in parenthesis.

of surviving cultures producing embryos and number of embryos per surviving nucellus, was obtained with a 5% sucrose concentration.

The sucrose concentration also influenced the size of the embryos produced in vitro. In Table 18 is shown the relationships between sucrose concentration and rate of embryo initiation, as well as total weight of embryos produced by each surviving nucellus. The 5% sucrose concentration not only produced the maximum number of embryos per Ethrog citron or Eureka lemon nucellus, but it also yielded the highest total weight of embryos per surviving nucellus. Rough calculations will show that the weight per embryo was also highest with the 5% sucrose treatment.

Organic fraction. For convenience the combination of B-vitamins, inositol and malt extract has been designated as the "organic fraction." The influence of varied levels of this combination was examined with Ethrog citron and Eureka lemon nucellus culture (Table 19).

There was no significant effect of the combined fraction on frequency of explant survival, frequency of embryo producing cultures and the number of embryos produced per nucellus.

The B-vitamins as a combined subgroup were next examined. For simplicity the text only compared two vitamin formulations, differentiating between them quantitatively but not qualitatively (Table 20). Again no difference between treatments was obtained.

The myo-inositol requirement was examined by testing a range of concentrations, from 0 to 3000 mg/l, in nucellus cultures of Ethrog citron and Eureka lemon (Table 21). No significant difference was

TABLE 18

Effect of sucrose concentration on fresh weight of nucellar embryos produced per nucellus in vitro

% Sucrose	No. Adventive Embryos Per Surviving Nucellus		Fresh Weight of Adventive Embryos/Nucellus (mg)	
	Ethrog Citron	Eureka Lemon	Ethrog Citron	Eureka Lemon
0	1.0 ± 0.1	1.5 ± 0.5	3.0 ± 0.3	7.0 ± 2.0
5	1.9 ± 0.3	4.7 ± 0.4	45.0 ± 10.7	85.0 ± 14.3
10	1.3 ± 0.5	3.1 ± 0.5	17.0 ± 3.4	35.0 ± 8.2
15	1.6 ± 0.4	1.4 ± 0.8	12.0 ± 2.2	15.0 ± 2.6
20	1.0 ± 0.3	1.8 ± 0.8	4.0 ± 0.7	5.0 ± 0.9

TABLE 19

Influence of variation in organic fraction (Vitamins, Inositol and Malt extract)

content on the behavior in vitro of *Citrus medica* L. 'Ethrog' and

***C. limon* L. Burm 'Eureka' nucellus.**

Concentration of organic fraction (as multiples of basal level)	% Surviving Explants		% Surviving Cultures with Adventive Embryos		No. Adventive Embryos Per Surviving Nucellus	
	Ethrog Citron	Eureka Lemon	Ethrog Citron	Eureka Lemon	Ethrog Citron	Eureka Lemon
0.0	35 (14-59)	50 (29-71)	100 (85-100)	100 (85-100)	2.0 ± 0.3	2.2 ± 0.6
0.1 x	65 (41-86)	75 (53-90)	100 (85-100)	100 (85-100)	1.6 ± 0.3	2.1 ± 0.7
1.0 x	70 (47-86)	75 (53-90)	100 (85-100)	100 (85-100)	1.9 ± 0.3	2.6 ± 0.7
3.0 x	80 (58-93)	80 (58-93)	100 (85-100)	100 (85-100)	2.0 ± 0.3	2.6 ± 0.9
10.0 x	75 (53-90)	75 (53-90)	100 (85-100)	100 (85-100)	2.0 ± 0.4	2.4 ± 0.7

95% confidence limits in parenthesis.

TABLE 20

Influence of vitamin mixtures on nucellus cultures of *Citrus medica* L. 'Ethrog' and *C. limon* L. Burm 'Eureka'

Vitamin Mixtures(1)	% Surviving Explants		% Surviving Cultures with Adventive Embryos		No. Adventive Embryos Per Surviving Nucellus	
	Ethrog Citron	Eureka Lemon	Ethrog Citron	Eureka Lemon	Ethrog Citron	Eureka Lemon
Murashige/Tucker (1969)	45 (30-62)	80 (65-97)	94 (84-99)	100 (91-100)	1.5 ± 0.2	3.5 ± 0.5
Whites (1943)	38 (22-56)	75 (60-91)	100 (91-100)	100 (91-100)	1.9 ± 0.4	4.2 ± 0.5

(1) Murashige/Tucker (1969) = Thiamin.HCl, 10 mg/l; Nicotinic acid, 5 mg/l; Pyridoxin.HCl 10 mg/l and Glycine 2 mg/l

Whites (1943) = Thiamin.HCl, 0.25 mg/l; Nicotinic acid 1.25 mg/l; Pyridoxin.HCl 0.025 mg/l and Glycine 7.5 mg/l

95% confidence limits in parenthesis.

TABLE 21

Influence of myo-inositol concentration on nucellus cultures of *Citrus medica* L. 'Ethrog' and *C. limon* L. Burm. 'Eureka'

Inositol concentration (mg/l)	% Surviving Explants		% Surviving Cultures with Adventive Embryos		No. Adventive Embryos Per Surviving Nucellus	
	Ethrog Citron	Eureka Lemon	Ethrog Citron	Eureka Lemon	Ethrog Citron	Eureka Lemon
0	35 (14-59)	60 (35-80)	100 (85-100)	100 (85-100)	1.6 ± 0.4	2.4 ± 0.4
30	55 (29-78)	60 (35-80)	100 (85-100)	100 (85-100)	2.0 ± 0.3	3.3 ± 0.4
100	40 (20-65)	65 (41-86)	100 (85-100)	100 (85-100)	2.3 ± 0.5	4.3 ± 0.6
300	65 (41-86)	60 (35-80)	100 (85-100)	100 (85-100)	2.3 ± 0.4	4.1 ± 0.5
1000	60 (35-80)	65 (41-86)	100 (85-100)	100 (85-100)	1.8 ± 0.3	3.8 ± 0.4
3000	80 (58-93)	70 (47-86)	100 (85-100)	100 (85-100)	2.2 ± 0.4	3.2 ± 0.3

95% confidence limits in parenthesis.

observed in the frequency of explant survival nor in the incidence of embryo producing cultures. However, a significant increase in the number of embryos produced per surviving nucellus, particularly of Eureka lemon, was obtained with an inositol concentration of 100 mg/l. No additional benefit was obtained from higher concentrations.

Finally the Bacto malt extract was examined, using Ethrog citron and Eureka lemon nucellus cultures. The malt extract concentration ranged from 0 to 10,000 mg/l. In spite of the wide range of concentrations no significant effect, beneficial or adverse, was obtained (Table 22).

The malt extract effect was re-examined with a medium enriched with kinetin, adenine sulfate and NAA. The concentrations of these three substances were found to be satisfactory, at least non-toxic, in other tests. Again no effect could be observed from malt extract (Table 23).

Summarizing the effects of the "organic fraction," the only constituent which gave significant benefit, specifically in the number of embryos produced per nucellus, was myo-inositol. The Bacto malt extract was clearly unnecessary. The B-vitamins, more correctly White's organic supplement mixture, since it includes the amino acid glycine, also showed no beneficial effect; however, perhaps tests of these compounds were not presently carried out adequately.

Effects of further addenda. The possibility of improvements in the nutrient medium by the inclusion of additional substances, particularly the hormonal compounds and others known to have morphogenetic regulatory effects, was considered. To this end three auxins,

TABLE 22

Influence of Bacto malt extract on nucellus cultures of *Citrus medica* L. 'Ethrog' and *C. limon* L. Burm 'Eureka'

Malt Extract Concentration (mg/l)	% Surviving Explants		% Surviving Cultures with Adventive Embryos		No. Adventive Embryos Per Surviving Nucellus	
	Ethrog Citron	Eureka Lemon	Ethrog Citron	Eureka Lemon	Ethrog Citron	Eureka Lemon
0	50 (29-71)	55 (29-78)	100 (85-100)	100 (85-100)	1.8 ± 0.4	3.6 ± 0.6
300	55 (29-78)	70 (47-86)	100 (85-100)	100 (85-100)	2.0 ± 0.4	3.3 ± 0.7
500	75 (53-90)	60 (35-80)	95 (77-100)	100 (85-100)	1.9 ± 0.3	3.7 ± 0.5
1000	60 (35-80)	90 (70-99)	100 (85-100)	100 (85-100)	2.1 ± 0.2	4.2 ± 0.3
3000	70 (47-86)	60 (35-80)	90 (70-99)	100 (85-100)	2.2 ± 0.5	4.0 ± 0.4
10000	40 (20-65)	40 (20-65)	75 (53-90)	100 (85-100)	1.9 ± 0.5	4.0 ± 0.7

95% confidence limits in parenthesis.

TABLE 23

Influence of Bacto malt extract on nucellus cultures of *Citrus medica* L. 'Ethrog' and *C. limon* L. Burm 'Ponderosa'. Basal medium modified to include kinetin, 0.1 mg/l; adenine sulfate, 80 mg/l and naphthalene-1-acid 0.3 mg/l

Malt Extract Concentration (mg/l)	% Surviving Explants		% Surviving Cultures with Adventive Embryos		No. Adventive Embryos Per Surviving Nucellus	
	Ethrog Citron	Ponderosa Lemon	Ethrog Citron	Ponderosa Lemon	Ethrog Citron	Ponderosa Lemon
0	50 (29-71)	45 (22-71)	100 (85-100)	100 (85-100)	2.1 ± 0.3	1.6 ± 0.2
10	45 (22-71)	50 (29-71)	100 (85-100)	100 (85-100)	1.9 ± 0.4	1.9 ± 0.3
50	55 (29-78)	45 (22-71)	100 (85-100)	100 (85-100)	2.2 ± 0.2	2.0 ± 0.3
100	40 (20-65)	55 (29-78)	100 (85-100)	100 (85-100)	2.2 ± 0.3	1.6 ± 0.2
500	55 (29-78)	55 (29-78)	100 (85-100)	100 (85-100)	2.0 ± 0.3	1.9 ± 0.3
1000	50 (29-71)	40 (20-65)	100 (85-100)	100 (85-100)	1.7 ± 0.4	1.5 ± 0.4

95% confidence limits in parenthesis.

IAA, NAA and 2,4-d; cytokinin Kinetin; gibberellin GA_3; nitrogen bases adenine sulfate and thymidine; casein hydrolysate; yeast extract; orange juice; and orange juice sacs were assayed.

Auxins. The auxin effect was examined first by comparing the actions of IAA, NAA and 2,4-D. Each was examined in the concentrations 0, 0.01, 0.03, 0.1, 1.0 mg/l, employing Ethrog citron nucellus cultures. In this experiment the basal medium was enriched with kinetin and adenine sulfate. The data are shown in Tables 24 - 26. Frequency of explant survival was unaffected by any of the three auxins (Table 24). In contrast, the incidence of surviving cultures which produced embryos was significantly repressed by all three auxins in concentrations of 0.3 mg/l and higher; IAA and 2,4-D were repressive even in 0.1 mg/l (Table 25). The number of embryos produced per surviving nucellus was unaffected by the addition of IAA and NAA. In contrast, 2,4-D in a concentration of 0.3 mg/l or higher suppressed embryo formation significantly (Table 26). No embryo was obtained in a 2,4-D concentration of 1.0 mg/l. Although not evident in the table, increasing concentrations of 2,4-D, while repressing embryogenesis, stimulated callus formation.

The 2,4-D effect was examined further with the highly polyembryonic cultivar, Ponkan mandarin (Fig. 15). The results were the same. The 2,4-D simply repressed embryogenesis in all concentrations tested. Representative cultures can be seen in Fig. 15.

Additional experiments were carried out using a range of concentrations of NAA, 2,4-D and combinations of the two auxins. The tests were carried out with moderately polyembryonic Eureka lemon, and the

TABLE 24

Influence of auxins on survival of nucellus cultures of *Citrus medica* L. 'Ethrog.' Basal medium modified to include kinetin 0.1 mg/l and adenine sulfate 800 mg/l

Auxin Concentration (mg/l)	% Surviving Explants		
	IAA	NAA	2,4-D
0.0	70 (47-86)	75 (53-90)	70 (47-86)
0.01	70 (47-86)	70 (47-86)	85 (64-96)
0.3	65 (41-86)	90 (70-99)	100 (85-100)
0.1	90 (70-99)	90 (70-99)	100 (85-100)
0.3	100 (85-100)	100 (85-100)	100 (85-100)
1.0	100 (85-100)	100 (85-100)	100 (85-100)

IAA = Indole-3-acetic acid

NAA = Naphthalene-1-acetic acid

2,4-D = 2,4-Dichlorophenoxy acetic acid

95% confidence limits in parenthesis

TABLE 25

Influence of auxins on frequency of adventive embryogenesis in nucellus cultures of Citrus medica L. 'Ethrog.' Basal medium modified to include kinetin 0.1 mg/l and adenine sulfate 80 mg/l

Auxin Concentration (mg/l)	% of Surviving Cultures with Adventive Embryos		
	IAA	NAA	2,4-D
0.0	100 (85-100)	100 (85-100)	100 (85-100)
0.01	100 (85-100)	100 (85-100)	65 (41-86)
0.03	100 (85-100)	100 (85-100)	50 (29-71)
0.1	60 (35-80)	85 (64-96)	25 (10-47)
0.3	55 (29-78)	65 (41-86)	10 (1-30)
1.0	55 (29-78)	60 (35-80)	0 (0-15)

IAA = Indole-3-acetic acid

NAA = Naphthalene-1-acetic acid

2,4-D = 2,4-Dichlorophenoxyacetic acid

95% confidence limits in parenthesis.

TABLE 26

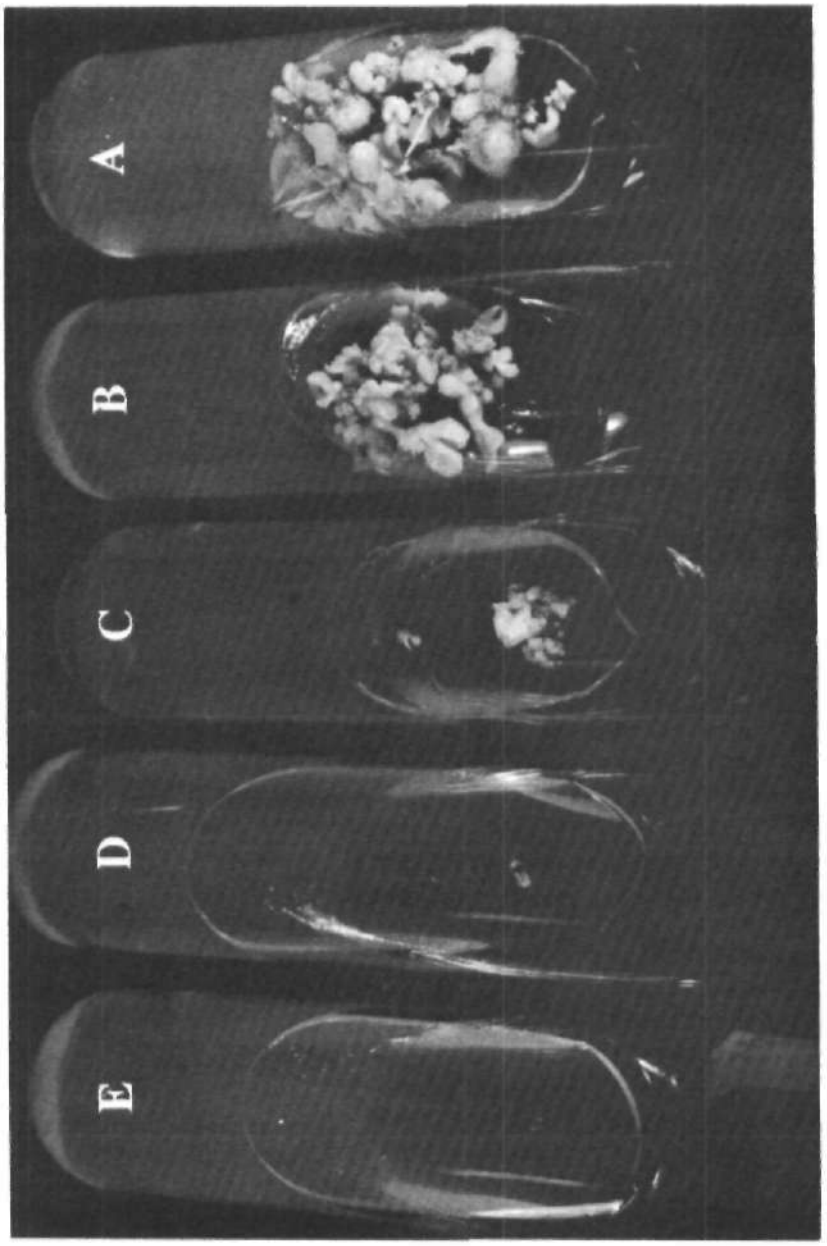
Influence of auxins on number of adventive embryos produced per cultured nucellus of *Citrus medica* L. 'Ethrog.' Basal medium modified to include kinetin 0.1 mg/l and adenine sulfate 80 mg/l

Auxin Concentration (mg/l)	No. Adventive Embryos Produced Per Surviving Nucellus		
	IAA	NAA	2,4-D
0.0	1.9 ± 0.6	2.1 ± 0.4	2.2 ± 0.3
0.01	2.0 ± 0.5	2.4 ± 0.4	2.3 ± 0.3
0.03	2.2 ± 0.4	2.7 ± 0.3	1.2 ± 0.3
0.1	1.8 ± 0.3	1.8 ± 0.2	0.9 ± 0.2
0.3	2.1 ± 0.5	2.0 ± 0.5	0.4 ± 0.1
1.0	1.9 ± 0.3	1.5 ± 0.3	0.0

IAA = Indole-3-acetic acid

NAA = Naphthalene-1-acetic acid

2,4-D = 2,4-Dichlorophenoxyacetic acid


A
B
C
D
E

strongly monoembryonic Ponderosa lemon and Ethrog citron. The results were consistent; there was no enhancement, but only repression of nucellar embryogenesis in vitro.

Cytokinin. The cytokinin kinetin was tested with Ethrog citron and Eureka lemon (Table 27). No benefit was obtained at any concentration of kinetin. There was also no adverse effect on citron explants, even with a 10 mg/l concentration, but adverse effect was apparent for Eureka lemon at this concentration. The kinetin effect was repeated twice, employing Ethrog citron and Ponderosa lemon. Even with enrichments of basal medium with adenine sulfate or adenine sulfate plus an extremely low concentration of an auxin, no benefit from kinetin was obtained. In one experiment, however, a kinetin concentration of 3.0 mg/l and higher was lethal to both Ethrog citron and Ponderosa lemon nucellus.

Gibberellin. Addition of filter-sterilized GA_3 solutions to the basal medium did not improve nucellus cultures of either Ethrog citron or Eureka lemon (Table 28). The gibberellin did not affect survival frequency nor percent of surviving cultures with adventive embryos when tested in the concentration range 0-10 mg/l. However, a concentration of 10 mg/l repressed the number of embryos produced per surviving nucellus drastically, at least in the case of Eureka lemon. Perhaps repression occurred in even lower concentrations, but the result could not be evaluated accurately because of a high degree of inconsistency encountered in the experiment. Gibberellin, while possibly repressing embryogenesis, nevertheless induced premature germination of embryos which formed in its presence and caused excessive elongation of

TABLE 27

Influence of kinetin concentration on nucellus cultures of *Citrus medica* L. 'Ethrog' and *C. limon* L. Burm 'Eureka'

Kinetin Concentration (mg/l)	% Surviving Explants		% Surviving Cultures with Adventive Embryos		No. Adventive Embryos Per Surviving Nucellus	
	Ethrog Citron	Eureka Lemon	Ethrog Citron	Eureka Lemon	Ethrog Citron	Eureka Lemon
0.0	75 (53-90)	60 (35-80)	100 (85-100)	100 (85-100)	2.1 ± 0.4	3.6 ± 0.7
0.01	70 (47-86)	80 (58-93)	100 (85-100)	100 (85-100)	2.0 ± 0.3	2.5 ± 0.5
0.1	75 (53-90)	85 (64-96)	100 (85-100)	100 (85-100)	1.9 ± 0.5	2.7 ± 0.4
1.0	55 (29-71)	45 (22-71)	100 (85-100)	100 (85-100)	2.0 ± 0.3	4.4 ± 1.8
10.0	30 (14-54)	25 (10-47)	100 (85-100)	100 (85-100)	1.9 ± 0.4	1.8 ± 0.4

95% confidence limits in parenthesis

TABLE 28

Influence of gibberellic acid (GA_3) concentration on nucellus cultures of *Citrus medica* L. 'Ethrog' and *C. limon* L. Burm 'Eureka'

GA_3 Concentration (mg/l)	% Surviving Explants		% Surviving Cultures with Adventive Embryos		No. Adventive Embryos Per Surviving Nucellus	
	Ethrog Citron	Eureka Lemon	Ethrog Citron	Eureka Lemon	Ethrog Citron	Eureka Lemon
0.0	30 (14-54)	65 (41-86)	100 (85-100)	100 (85-100)	2.4 ± 0.4	5.3 ± 1.2
0.001	5 (0-23)	65 (41-86)	100 (85-100)	100 (85-100)	1.0 ± 0.1	4.3 ± 1.1
0.01	20 (7-42)	55 (29-78)	100 (85-100)	100 (85-100)	1.3 ± 0.3	3.7 ± 0.8
0.10	15 (4-36)	55 (29-78)	100 (85-100)	100 (85-100)	1.6 ± 0.3	3.3 ± 0.6
1.0	10 (1-30)	65 (41-86)	100 (85-100)	100 (85-100)	2.0 ± 0.1	2.8 ± 0.4
10.0	30 (14-54)	60 (35-80)	100 (85-100)	100 (85-100)	1.3 ± 0.3	3.2 ± 0.3

95% confidence limits in parenthesis.

seedlings (Fig. 16).

Nitrogen bases. In view of the stimulating effect of adenine or adenine sulfate on shoot-bud initiation in many plant tissue cultures, the possible beneficial effect of adenine sulfate on adventive embryogenesis in Ethrog citron and Eureka lemon nucellus cultures was examined (Table 29). No advantage was gained by adding adenine sulfate to the medium in a concentration as high as 160 mg/l, although perhaps an 80 mg/l concentration increased the number of embryos produced in Eureka lemon nucellus. A re-run of this test with Ethrog citron and Ponderosa lemon explants, and with the basal medium enriched with very low concentrations of kinetin and NAA also disclosed no benefit from adenine sulfate.

The pyrimidine derivative thymidine was tested in Ethrog citron nucellus cultures although for no morphogenetically motivated reasons. The test was carried out by employing a basal medium enriched with NAA, kinetin and adenine sulfate. As evident in Table 30, no significant improvement was obtained by the addition of thymidine.

Casein hydrolysate. Many reports have indicated enhancement of embryogenesis in tissue cultures of diverse plants by casein hydrolysate. Accordingly, this complex, which contains largely amino acids, was tested on Ethrog citron and Eureka lemon nucellus cultures. The data from one experiment are shown in Table 31. Whereas the 5000 mg/l concentration was apparently excessive, particularly when examined in terms frequency of explant survival, casein hydrolysate in concentrations of 50 and 500 mg/l evidently enhanced Eureka lemon embryogenesis in vitro. This was clearly the case.

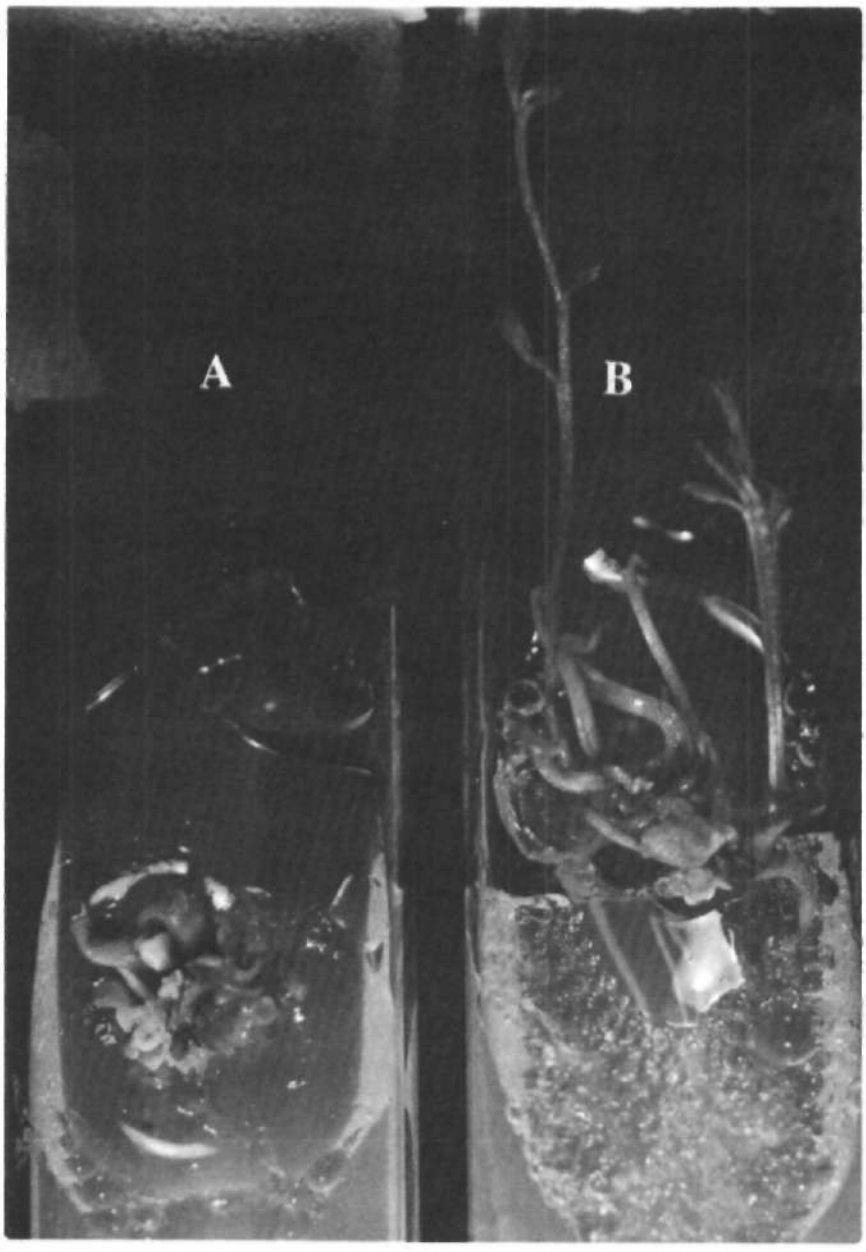

A
B

TABLE 29

Influence of adenine sulfate concentration on nucellus cultures of *Citrus medica* L. 'Ethrog' and *C. limon* L. Burm 'Eureka'

Adenine Sulfate Concentration (mg/l)	% Surviving Explants		% Surviving Cultures with Adventive Embryos		No. Adventive Embryos Per Surviving Nucellus	
	Ethrog Citron	Eureka Lemon	Ethrog Citron	Eureka Lemon	Ethrog Citron	Eureka Lemon
0	85 (64-96)	75 (53-90)	100 (85-100)	87 (68-96)	2.1 ± 0.3	2.6 ± 0.6
40	80 (58-93)	85 (64-96)	100 (85-100)	100 (85-100)	1.9 ± 0.4	3.8 ± 0.7
80	75 (53-90)	60 (35-80)	100 (85-100)	100 (85-100)	2.2 ± 0.5	4.2 ± 0.8
120	65 (41-86)	70 (47-86)	100 (85-100)	100 (85-100)	2.1 ± 0.3	2.8 ± 0.9
160	80 (58-93)	75 (53-90)	100 (85-100)	100 (85-100)	1.8 ± 0.4	2.9 ± 0.4

95% confidence limits in parenthesis.

TABLE 30

Influence of thymidine on nucellus cultures of Citrus medica L. 'Ethrog.' Basal medium modified to include kinetin, 0.1; NAA, 0.03 adenine sulfate 80 mg/l

Thymidine Concentration (mg/l)	% Surviving Explant	% Surviving Cultures with Adventive Embryos	No. Adventive Embryos Per Surviving Nucellus
0.0	50 (29-71)	80 (58-93)	2.2 ± 0.4
0.1	80 (58-93)	63 (41-86)	1.9 ± 0.3
1.0	40 (20-65)	75 (53-90)	2.0 ± 0.5
10.0	70 (47-86)	72 (50-88)	1.4 ± 0.7
100.0	10 (1-30)	100 (85-100)	3.0 ± 0.6
150.0	80 (58-93)	50 (29-71)	1.6 ± 0.5

95% confidence limits in parenthesis.

TABLE 31

Influence of casein hydrolysate (enzyme hydrolysed) concentration on nucellus cultures of *Citrus medica* L. 'Ethrog' and *C. limon* L. Burm 'Eureka'

Casein Hydrolysate Concentration (mg/l)	% Surviving Explants		% Surviving Cultures with Adventive Embryos		No. Adventive Embryos Per Surviving Nucellus	
	Ethrog Citron	Eureka Lemon	Ethrog Citron	Eureka Lemon	Ethrog Citron	Eureka Lemon
0	80 (58-93)	80 (58-93)	100 (85-100)	100 (85-100)	1.5 ± 0.2	1.8 ± 0.3
5	70 (47-86)	80 (58-93)	100 (85-100)	100 (85-100)	1.9 ± 0.3	1.8 ± 0.3
50	90 (70-99)	70 (47-86)	100 (85-100)	100 (85-100)	1.4 ± 0.3	3.1 ± 0.6
500	95 (77-100)	70 (47-86)	100 (85-100)	100 (85-100)	2.0 ± 0.4	3.0 ± 0.4
5000	50 (29-71)	40 (20-65)	100 (85-100)	100 (85-100)	1.8 ± 0.3	1.6 ± 0.3

95% confidence limits in parenthesis.

A re-examination of the casein hydrolysate effect with Ethrog citron and Ponderosa lemon confirmed the toxic effect of the 5000 mg/l concentration, but not the embryogenic enhancement by the lower concentrations.

Yeast extract. Some investigators have used yeast extract as a standard constituent in tissue culture media intended for studies of embryogenesis. In this investigation Bacto yeast extract was tested in nucellus cultures of Ethrog citron and Eureka lemon in a concentration range 0-5000 mg/l (Table 32). There was a trend towards decreased survival of nucellus explants with increase in the medium's content of yeast extract. While not yet confirmed, Eureka lemon nucellus cultures were perhaps stimulated to produce more embryos per explant by a 500 mg/l addendum. Otherwise, there was no substantial effect of yeast extract on embryogenesis.

Orange juice. Orange juice has been described (Murashige and Tucker, 1969) as being beneficial, if not critical, in callus cultures of Citrus, especially citron. The possibility of an enhancement of embryogenesis in nucellus cultures of Ethrog citron was thus examined. Orange juice in concentrations ranging up to 20% by volume did not alter the survival frequency of explants (Table 33). Concentrations of 15 and 20%, however, repressed significantly the manifestation of embryogenesis in the surviving cultures. Both the frequency of cultures producing embryos and the number of embryos per culture were reduced in these two concentrations. These were not pH effects, since all media were set at pH 5.7 initially.

TABLE 32

Influence of yeast extract concentration on nucellus cultures of *Citrus medica* L. 'Ethrog' and *C. limon* L. Burm 'Eureka.' Basal medium modified to include kinetin, 0.1 mg/l; adenine sulfate, 80 mg/l; and naphthalene-1-acetic acid 0.03 mg/l

Yeast Extract Concentration (mg/l)	% Surviving Explants		% Surviving Cultures with Adventive Embryos		No. Adventive Embryos Per Surviving Nucellus	
	Ethrog Citron	Eureka Lemon	Ethrog Citron	Eureka Lemon	Ethrog Citron	Eureka Lemon
0	50 (29-71)	60 (35-80)	100 (85-100)	100 (85-100)	1.8 ± 0.6	2.6 ± 0.4
5	30 (14-54)	20 (7-42)	100 (85-100)	100 (85-100)	2.0 ± 0.1	2.0 ± 0.3
50	30 (14-54)	10 (1-30)	100 (85-100)	100 (85-100)	2.0 ± 0.6	2.0 ± 0.2
500	30 (14-54)	10 (1-30)	100 (85-100)	100 (85-100)	2.6 ± 0.7	4.0 ± 0.9
5000	10 (1-30)	20 (7-42)	100 (85-100)	100 (85-100)	1.0 ± 0.1	3.5 ± 1.0

95% confidence limits in parenthesis.

TABLE 33

Effect of orange juice on nucellus cultures of *Citrus medica* L. 'Ethrog.' Basal medium modified to include kinetin, 0.1 mg/1, adenine sulfate 80 mg/1

% Orange Juice	% Surviving Explants	% Surviving Cultures with Adventive Embryos	No. Adventive Embryos Per Surviving Nucellus
0	60 (35-80)	100 (85-100)	1.5 ± 0.3
1	70 (47-86)	100 (85-100)	1.4 ± 0.4
5	60 (35-80)	100 (85-100)	1.5 ± 0.3
10	70 (47-86)	100 (85-100)	1.7 ± 0.3
15	80 (53-93)	62 (29-71)	0.6 ± 0.4
20	70 (47-86)	57 (20-65)	0.7 ± 0.2

95% confidence limits in parenthesis.

As an added experiment the effects of freshly excised juice sacs placed alongside the nucellus in vitro were also examined. The juice sacs were obtained from immature and mature fruits of valencia and Washington Navel oranges. Ethrog citron nucellus explants cultured under such conditions showed no effect by the juice vesicles from mature fruits, but significant repression of embryogenesis by the tissue from immature fruits. The number of embryos per nucellus was reduced considerably by the juice sacs from immature orange fruits.

This series of experiments, which considered possible improvement of the nutrient medium by use of additional ingredients, did not produce results to justify changes in the basal medium. The auxins IAA, NAA, 2,4-D and Kinetin were without benefit on nucellar embryogenesis in vitro, while gibberellin produced adverse effects. The nitrogen base compounds adenine sulfate and thymidine also showed no significant advantage in the medium. Casein hydrolysate appeared to enhance nucellar embryogenesis when included in the concentration range 50-500 mg/l in one experiment, although the observation could not be confirmed in a second experiment. Yeast extract as well as orange juice were also shown not to be of value as addenda to the citrus nucellus culture medium.

The Culture Environment

Illumination requirements. Survival of Ethrog citron and Eureka lemon of nucellus explants was unaffected by light intensity (Table 34). Similarly, there was no difference among surviving cultures in the frequency with which embryos were produced. Perhaps the number of

TABLE 34

Influence of light intensity on nucellus cultures of *Citrus medica* L. 'Ethrog' and *C. limon* L. Burm 'Eureka'. (16 hours light)

Light Intensity (lux)	% Surviving Explant		% Surviving Culture with Adventive Embryos		No. Adventive Embryos Per Surviving Nucellus	
	Ethrog Citron	Eureka Lemon	Ethrog Citron	Eureka Lemon	Ethrog Citron	Eureka Lemon
0	65 (41-86)	60 (35-80)	100 (85-100)	100 (85-100)	2.1 ± 0.5	4.4 ± 0.6
300	50 (29-71)	65 (41-86)	100 (85-100)	100 (85-100)	2.0 ± 0.4	3.3 ± 0.7
1000	65 (41-66)	55 (29-78)	100 (85-100)	100 (85-100)	1.8 ± 0.7	4.5 ± 0.7
3000	50 (29-71)	50 (29-71)	100 (85-100)	100 (85-100)	1.6 ± 0.5	2.8 ± 0.9

95% confidence limits in parenthesis.

embryos produced per nucellus was repressed by the highest, 3000 lux, light intensity, especially in Eureka lemon cultures. Clearly, constant darkness was entirely satisfactory as an environment for nucellar embryogenesis in vitro.

Daylength in vitro was also without effect (Table 35). Both Ethrog citron and Eureka lemon nucellus cultures showed no significant difference in the frequency of explant survival, the incidence of surviving cultures producing embryos, or in the number of embryos produced per surviving nucellus, in response to variation in the length of the daily light period. This experiment confirmed that light was unnecessary for nucellar embryogenesis in vitro.

Temperature influence. Temperature studies in this investigation were limited to an examination of the effects of lowering of the night temperatures. Lowering the nycto-temperature to 22, 17 and 12°C did not alter the behavior of the nucellus cultures of Ethrog citron and Eureka lemon (Table 36).

In summary, the experiments aimed at exploring the culture environment requirements disclosed that a constant 27°C incubation temperature was satisfactory, and that light was not necessary for embryogenesis in vitro.

Physiological Mechanism Underlying Adventive Embryogenesis in the Rutaceae

Embryogenic Potential in the Rutaceae

The potential for nucellar embryogenesis in several members of the Rutaceae, as determined through cultures of their nucellus in vitro was

TABLE 35

Influence of the length of the daily light period on nucellus cultures of Citrus medica L. 'Ethrog' and C. limon L. Burm 'Eureka'. Light intensity, 1000 lux

Light Period per Day (Hours)	% Surviving Explants		% Surviving Cultures with Adventive Embryos		No. Adventive Embryos Per Surviving Nucellus	
	Ethrog Citron	Eureka Lemon	Ethrog Citron	Eureka Lemon	Ethrog Citron	Eureka Lemon
0	75 (53-90)	85 (64-96)	100 (85-100)	95 (77-100)	2.2 ± 0.3	4.7 ± 0.6
4	80 (58-93)	90 (70-99)	100 (85-100)	85 (64-96)	2.2 ± 0.4	5.0 ± 0.7
8	55 (29-78)	95 (77-100)	100 (85-100)	90 (70-99)	1.9 ± 0.5	3.8 ± 0.8
12	65 (41-86)	70 (47-86)	100 (85-100)	100 (85-100)	2.5 ± 0.5	4.4 ± 0.4
16	70 (47-86)	80 (58-93)	100 (85-100)	100 (85-100)	2.3 ± 0.3	4.3 ± 0.3
20	80 (58-100)	70 (47-86)	100 (85-100)	100 (85-100)	1.8 ± 0.3	4.0 ± 0.9
24	85 (29-78)	60 (35-80)	100 (85-100)	100 (85-100)	2.1 ± 0.2	4.1 ± 0.6

95% confidence limits in parenthesis.

TABLE 36

Influence of lower nycto-temperatures on nucellus cultures of *Citrus medica* L.'Ethrog' and *C. limon* L. Burm 'Eureka'. Temperature of the light period (16 hours daily and 1000 lux Gro-lux lamp) was 27°C

Night Temperature °C	% Surviving Explants		% Surviving Explants with Adventive Embryos		% Surviving Cultures Per Surviving Nucellus	
	Ethrog Citron	Eureka Lemon	Ethrog Citron	Eureka Lemon	Ethrog Citron	Eureka Lemon
27	85 (64-96)	75 (53-90)	100 (85-100)	100 (85-100)	2.1 ± 0.3	4.7 ± 0.6
22	85 (64-96)	80 (58-93)	100 (85-100)	100 (85-100)	2.0 ± 0.4	5.3 ± 0.8
17	75 (53-90)	70 (47-86)	100 (85-100)	100 (85-100)	2.2 ± 0.5	4.2 ± 0.7
12	80 (58-93)	75 (53-90)	100 (85-100)	100 (85-100)	2.0 ± 0.2	3.6 ± 0.3

95% confidence limits in parenthesis.

determined (Table 37, Fig. 17). According to their behavior in vitro, the diverse genera, species, varieties, and hybrids of the Rutaceae could not be accurately classified as either polyembryonic or monoembryonic. Except for Aeglopsis chevalieri Swing., all members of the subfamily Aurantioidae in the Rutaceae examined showed polyembryogenesis in nucellus cultures. The significant fact, however, is that the various members of the Rutaceae differed in the degree to which their potentials for adventive embryogenesis were manifested. There was some correlation between the in vivo classification and the in vitro behavior among the Rutaceae. Those classed as being highly polyembryonic showed highly polyembryonic nucellus cultures, whereas many of those classed as consistently monoembryonic revealed low rates of adventive embryogenesis in vitro. For example, Ponkan mandarin and Koethen Sweet orange were also highly polyembryonic in vitro, while Ethrog citron and Ponderosa lemon were poorly polyembryonic. Some of the Rutaceaeous members, which in the natural environment are characterized by low rates of polyembryogenesis, showed degrees of embryogenesis in vitro which overlapped with or were surpassed by certain normally monoembryonic types. For example, Citrus paradisi Macf., a naturally polyembryonic type, produced the same or slightly fewer embryos in vitro than C. ichangensis, a monoembryonic type.

The appearance of the cultures, including certain morphogenic manifestations, also varied considerably among the Rutaceae (Fig. 18). The nucellus of some members enlarged only slightly, whereas others increased their tissue mass by producing callus. Still others produced embryos which developed little beyond the embryonic stage, and in some

TABLE 37

The potential for nucellar embryogenesis among Citrus and relatives as evaluated through in vitro culture

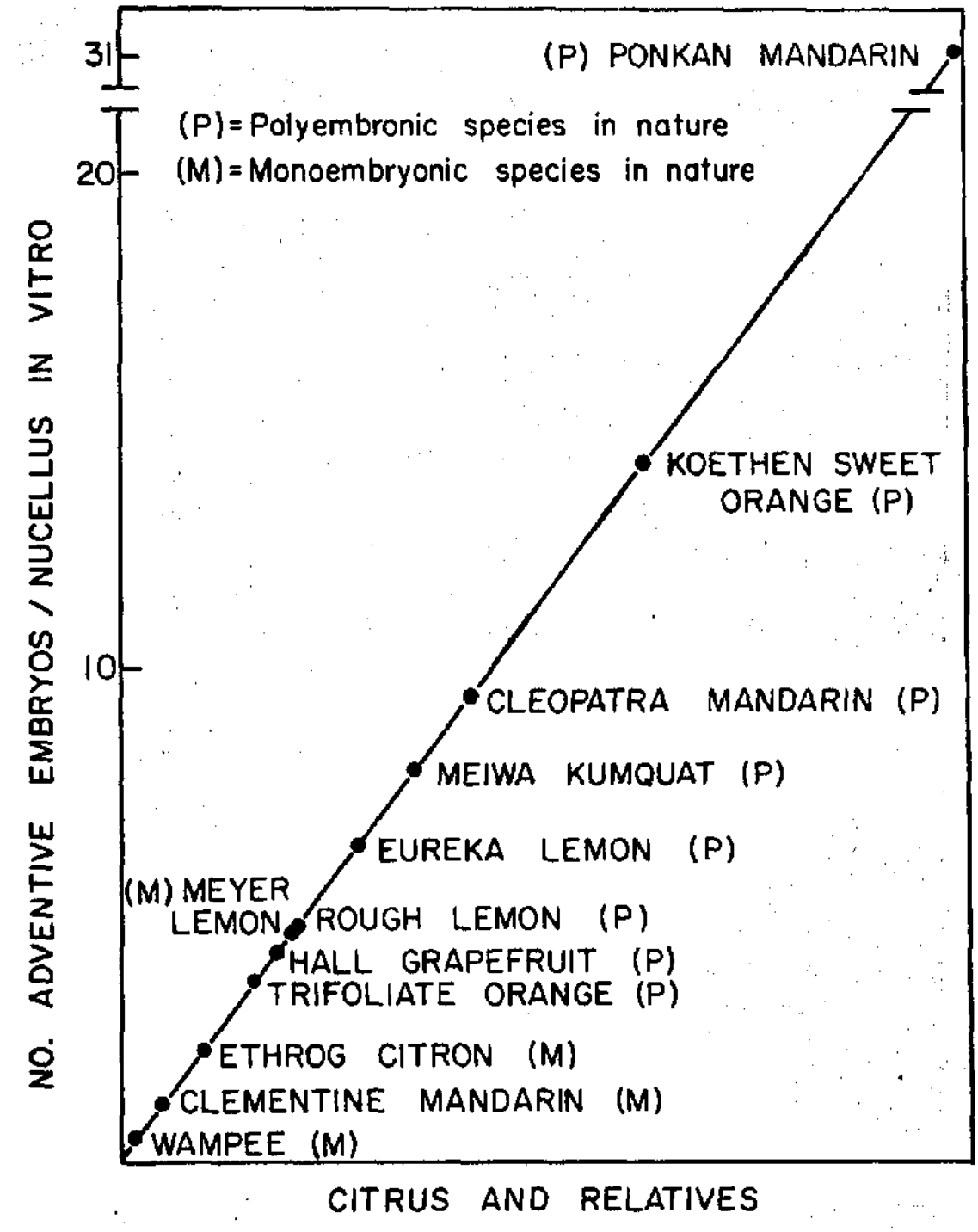
Species or Cultivar[1]	No. of Adventive Embryos Per Nucellus	
	In Vitro	(Total in Vivo & in Vitro)[2]
Citrus reticulata Blanco 'Ponkan mandarin' (P)	31.4	47.5
C. sinensis L. Osbeck 'Koethen Sweet' (P)	14.0	21.9
C. reticulata Blanco 'Cleopatra mandarin' (P)	9.1	13.4
Fortunella crassifolia Swingle 'Meiwa kumquat' (P)	7.5	16.8
C. limonia Osbeck 'Rangpur lime' (P)	7.4	11.5
Troyer Citrange Webber and Swingle (P)	6.4	14.2
C. limon L. Burm 'Eureka lemon' (P)	5.8	6.0
C. aurantium L. 'Sour Orange' (P)	5.5	7.0
C. Kharna Raf. 'Kharna' (P)	5.2	8.2
C. sinensis L. Osbeck 'Valencia Orange' (P)	4.7	17.1
Faustrime 'Finger lime' (P)	4.6	5.1
C. hystrix D.C. 'Kuffre lime' (P)	4.5	8.4
C. jambhiri Lush 'Rough lemon' (P)	4.3	9.0
C. limon L. Burm 'Meyer lemon' (M)	4.2	4.2
Eremocitrus glauca (Lindle) Swing. 'Australian Desert lime' (P)	4.2	12.6
C. ichangensis Swing. 'Ichang Papeda' (M)	4.0	4.0
C. paradisi Macf. 'Grape fruit' (P)	3.6	9.7
C. madurensis Lour. 'Calamondin' (P)	3.6	4.7

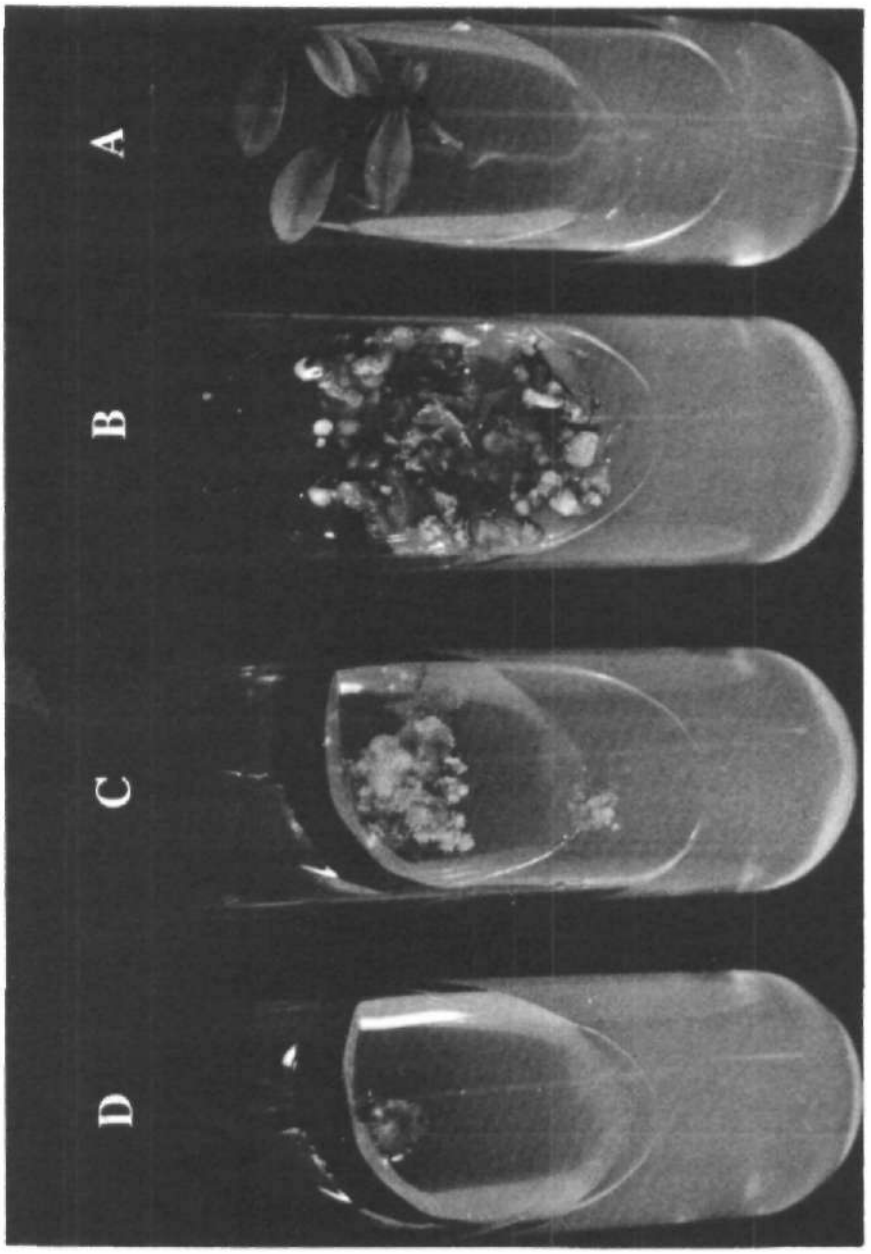
Table 37 (continued)

Species or Cultivar[1]	No. of Adventive Embryos Per Nucellus: In Vitro	No. of Adventive Embryos Per Nucellus: (Total in Vivo & in Vitro)[2]
Triphasia trifoliata Burm. P. Wils;		
'Lime berry' (P)	3.5	11.0
Poncirus trifoliata L. 'Trifoliate Orange' (P)	3.2	7.6
C. auratifolia (Christm) Swing 'Lime' (P)	3.2	6.8
C. medica L. 'Ethrog Citron' (M)	2.5	2.5
C. medica L. 'Citron of Commerce' (M)	2.1	2.1
C. limon (L.) Burm. 'Ponderosa lemon' (M)	1.7	1.7
C. grandis (L.) Osbeck 'Shaddock' (M)	1.5	1.5
C. reticulara x C. sinensis 'Temple Tangor' (M)	1.5	1.5
Microcitrus warburgiana F. M. Bail 'Wild lime' (M)	1.0	1.0
C. reticulata Blanco 'Algerian tangerine' (M)	1.0	1.0
C. lansium (Lour) Skeels, 'Wampee' (M)	0.4	0.4
M. australasica F. Muell 'Red Finger lime' (M)	0.4	2.1
Aeglopsis chevalieri Swing 'Powder flask fruit' (M)	0.0	0.0

[1](P) = Naturally polyembryonic, (M) = naturally monoembryonic type.

[2]Sum of nucellar embryos initially removed from explant and those obtained in vitro.


31
20
10
(P) PONKAN MANDARIN
(P)=Polyembronic species in nature
(M)=Monoembryonic species in nature
KOETHEN SWEET ORANGE (P)
CLEOPATRA MANDARIN (P)
MEIWA KUMQUAT (P)
EUREKA LEMON (P)
(M) MEYER LEMON
ROUGH LEMON (P)
HALL GRAPEFRUIT (P)
TRIFOLIATE ORANGE (P)
ETHROG CITRON (M)
CLEMENTINE MANDARIN (M)
WAMPEE (M)
NO. ADVENTIVE EMBRYOS / NUCELLUS IN VITRO
CITRUS AND RELATIVES


A
B
C
D

the embryos germinated prematurely in vitro.

Differential Behavior of Micropylar Half, Chalazal Half and Whole Nucellus

Most previous investigations indicated that nucellar embryogenesis in vivo among diverse plant families was confined to the micropylar region. This would suggest that either the micropylar region was being embryogenically stimulated by some promotive substance or the other regions of the nucellus were being repressed by some inhibitory agent. Accordingly, a comparison was made of the embryogenic behavior in vitro of the micropylar and chalazal halves of the nucellus. Cultures of whole nucellus were included as reference. The results of experiments with Ponkan mandarin and Ethrog citron are presented in Table 38.

The chalazal half of the nucellus of either the highly polyembryonic Ponkan mandarin or the monoembryonic Ethrog citron was distinctly poorer embryogenetically in vitro than the micropylar half. The frequency of surviving cultures producing embryos and the number of embryos produced per surviving nucellus were markedly higher in cultures of the micropylar halves than those of the chalazal halves. In fact, none of the chalazal halves of Ethrog citron survived culture.

Perhaps most significant was the fact that the micropylar halves produced just as many embryos per explant as the whole nucellus. In the highly polyembryonic Ponkan mandarin, the micropylar half yielded more embryos than the whole nucellus. Representative cultures taken from another but similar experiment with Ponkan mandarin nucellus cultures can be seen in Fig. 19. The same experiments were repeated with Koethen

TABLE 38

In vitro adventive embryogenic potentials of micropylar and chalazal regions and whole nucellus of Citrus reticulata Blanco 'Ponkan' and C. medica L. 'Ethrog'*

	Ponkan Mandarin			Ethrog Citron		
	Micropylar Half	Whole Nucellus	Chalazal Half	Micropylar Half	Whole Nucellus	Chalazal Half
% Surviving explants	100 (91-100)	90 (77-98)	40 (24-58)	85 (71-97)	65 (50-81)	0
% Surviving cultures with adventive embryos	100 (91-100)	100 (91-100)	60 (44-77)	100 (91-100)	100 (91-100)	0
No. adventive embryos per surviving nucellus	19.5 ± 4.2	14.1 ± 3.2	3.7 ± 1.2	2.1 ± 0.4	2.0 ± 0.4	0

95% confidence limits in parenthesis.

*Other species tried with the same relationship between ends include:

1. Citrus sinensis L. Osbeck 'Koethen'
2. Fortunella crassifolia Swing. 'Meiwa'
3. C. limon L. Burm 'Eureka'

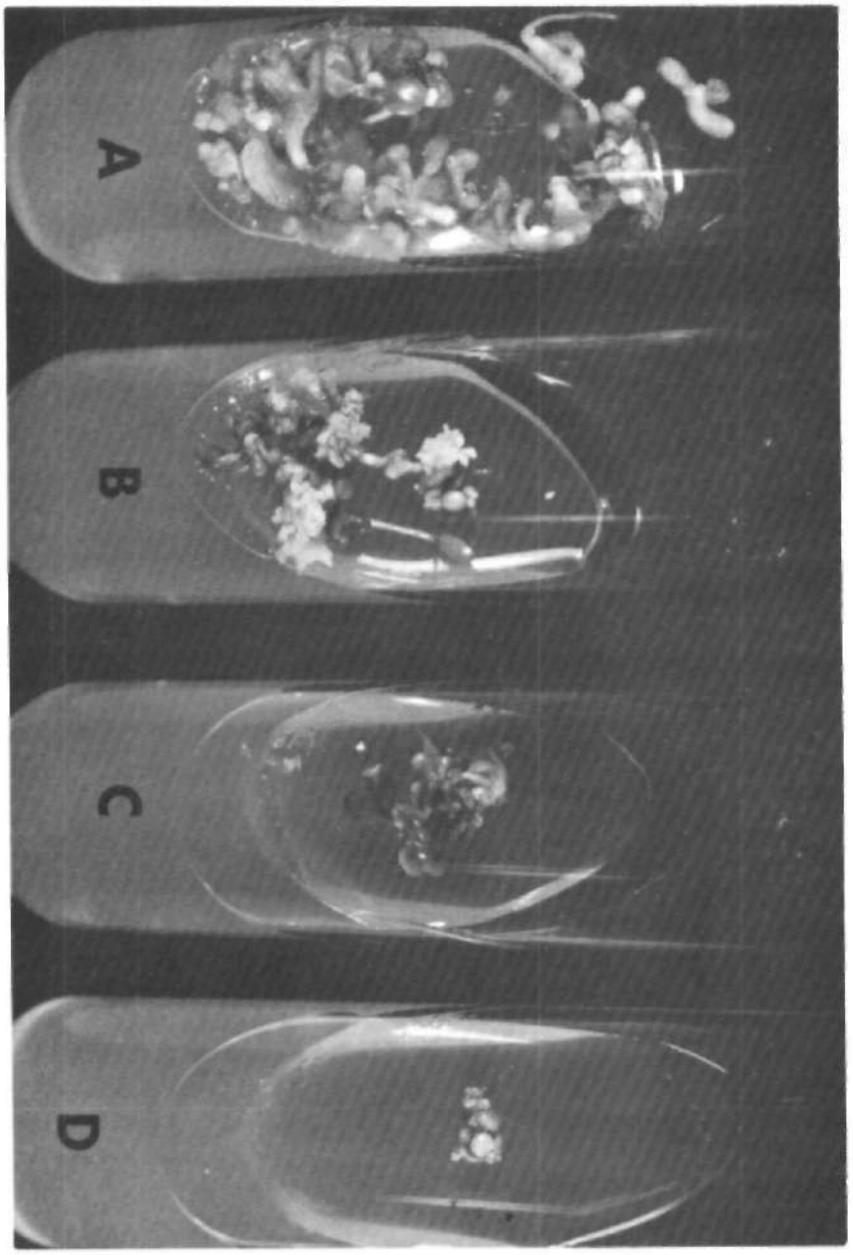
Figure 19. Adventive embryogenic potentials of the nucellus explants of Ponkan mandarin cultured in vitro.

A = Micropylar half

B = Whole nucellus cut into halves and re-grafted

C = Whole uncut nucellus

D = Chalazal half


A
B
C
D

Sweet orange, Meiwa kumquat and Eureka lemon with essentially the same differential results between the two ends of the nucellus.

Interaction Between Polyembryonic and Monoembryonic Nucellus

The findings from the above experiments showed clearly that the micropylar portion of the nucellus was more prone to undergo embryogenesis in vitro than its chalazal region. But the question remained whether this difference was due to a difference in content of an embryogenic stimulant or of an embryogenic suppressant. The alternatives were examined.

In one experiment the micropylar halves of the nucellus of monoembryonic Ethrog citron and those of the polyembryonic cultivars, Rough lemon and Cleopatra mandarin were grafted together. The grafted material was then placed in nutrient medium with the citron tissue being embedded in the medium in some cases and the polyembryonic tissues in others. The embryogenic behavior of only the exposed tissue was determined. Ungrafted micropylar halves of the three cultivars were also cultured for reference. The data from this experiment can be seen in Table 39.

The term numerator species (Table 39) refers to the half exposed above the nutrient medium; although not shown, the term denominator is then applicable to the micropylar half which was embedded in the agar. According to the data there was no influence on the embryogenic behavior of the micropylar half of the nucellus of the monoembryonic citron by the micropylar half of either Rough lemon or Cleopatra mandarin. Similarly, there was no influence by the micropylar half of the Ethrog

TABLE 39

Behavior of grafts involving micropylar halves of nucellus from monoembryonic Citrus medica L. Ethrog and polyembryonic Citrus species: Citrus jambhiri Lush. and C. reticulata Blanco 'Cleopatra'

	Ungrafted Tissues		
	Ethrog Citron	Rough Lemon	Cleopatra Mandarin
% Surviving explants	85 (64-96)	100 (85-100)	100 (85-100)
% Surviving cultures with adventive embryos	100 (85-100)	100 (85-100)	100 (85-100
No. adventive embryos per surviving nucellus	1.6 ± 0.3	8.4 ± 2.3	12.6 ± 3.2

	Grafted Tissues*			
	Ethrog Citron Rough Lemon	Rough Lemon Ethrog Citron	Ethrog Citron Cleopatra Mandarin	Cleopatra Mandarin Ethrog Citron
% Surviving explants	75 (53-90)	100 (85-100)	80 (58-93)	100 (85-100)
% Surviving cultures with adventive embryos	100 (85-100)	100 (85-100)	100 (85-100)	100 (85-100)
No. adventive embryos per surviving nucellus	1.4 ± 0.2	7.8 ± 2.4	1.8 ± 0.3	12.8 ± 3.3

*Values of numerator species.

95% confidence limits in parenthesis.

citron on the behavior of the micropylar halves of the two polyembryonic Citrus. The data thus appeared to negate the embryogenic stimulant alternative.

Suppression of Polyembryony by Monoembryonic Citrus

The next set of experiments examined the possibility that the chalazal half, particularly that of the nucellus of a monoembryonic cultivar, might contain a substance(s) which inhibited embryo formation. The tests involved grafting of the micropylar halves of the nucellus of polyembryonic Rough lemon and Koethen Sweet orange to the chalazal half of monoembryonic Ethrog citron. The grafted material was placed in nutrient agar with the citron portion being embedded in the medium.

The data in Table 40 showed very clearly that the chalazal half of the citron nucellus repressed severely the embryogenic characteristics of both Rough lemon and Koethen sweet orange. In both varieties the number of adventive embryos produced per nucellus was reduced to less than half of the reference cultures. It is important to note that the grafting procedure did not alter the frequency of survival of the Rough lemon and Koethen sweet orange nucellus sections.

From this experiment it seemed clear that the difference between micropylar and chalazal regions of a nucellus and between polyembryonic and monoembryonic cultivars was associated with a difference in content of an agent which suppressed embryogenesis. Furthermore, the suppressive agent appeared to be transmitted through the tissue graft.

Representative cultures showing the repressive effect of citron nucellus on the micropylar half of Rough lemon nucellus can be seen in

TABLE 40

Inhibition of nucellus embryogenesis in micropylar halves of polyembryonic species (Citrus jambhiri Lush. and C. sinensis L. Osbeck 'Koethen') following grafts onto chalazal half of monoembryonic species (C. medica L. 'Ethrog')

	Rough Lemon		Koethen Sweet Orange	
	Ungrafted	Grafted	Ungrafted	Grafted
% Surviving explants	85 (64-96)	65 (41-86)	100 (85-100)	75 (53-90)
% Surviving cultures with adventive embryos	100 (85-100)	100 (85-100)	100 (85-100)	100 (85-100)
No. adventive embryo per surviving nucellus	8.4 ± 3.1	3.1 ± 1.1	11.0 ± 3.5	4.1 ± 1.9

95% confidence limits in parenthesis.

Fig. 20C. This figure also shows the lack of embryogenic stimulation of the micropylar half of the citron nucellus by the micropylar half of the Rough lemon nucellus (Fig. 20A).

Evidence of Diffusable Embryogenic Suppressor

In view of the graft transmissability of the embryogenic suppressant, the next series of tests examined its diffusability in nutrient agar. The embryogenically responding tissue used for this experiment was Ponkan mandarin nucellus; only the micropylar halves were cultured. The source of suppressant was the chalazal half of Ethrog citron ovules. The Ponkan mandarin explant was placed on nutrient agar and in the center of the culture flask. The explant was then surrounded by varying numbers of chalazal halves of citron ovules, hoping that the suppressive agent in the citron tissue would reach the mandarin tissue by diffusion through the agar and thereby effect an inhibition.

Embryogenesis in the Ponkan nucellus diminished progressively upon being cultured together with an increasing number of citron chalazal explants (Fig. 21). In the presence of three or more citron explants, the Ponkan mandarin nucellus no longer produced embryos, only unorganized callus. A generally inhibitory action on all forms of growth of the mandarin tissue also resulted as the number of associated citron ovule sections was increased.

In a second experiment (Fig. 22) the number of embryos produced per nucellus was decreased progressively and drastically as the number of associated citron ovules was increased. One citron ovule was sufficient to reduce embryo production to nearly half; 10 ovules repressed it

Figure 20. Interaction between nucellus fraction explants of Ethrog citron and Rough lemon.

A = Grafted micropylar halves of Ethrog citron and Rough lemon;

B = Micropylar half of Ethrog citron only;

C = Micropylar half of Rough lemon grafted to chalazal half of Ethrog citron;

D = Micropylar half of Rough lemon only.

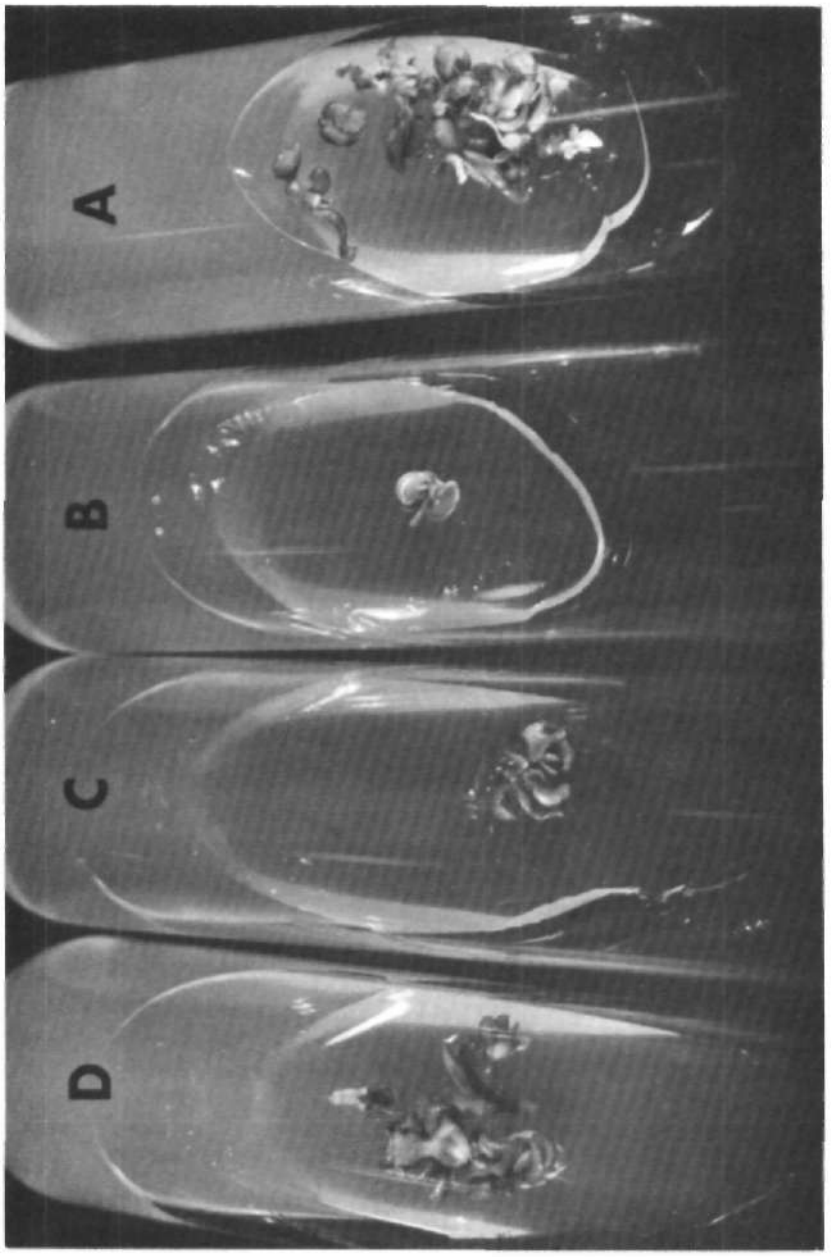

A
B
C
D

Figure 21. Effect of varying number of citron ovules on the micropylar half nucellus explants of Ponkan mandarin cultured in vitro.

A = No citron ovules (control)

B = 1 citron ovule

C = 3 citron ovule

D = 10 citron ovule

E = 20 citron ovule

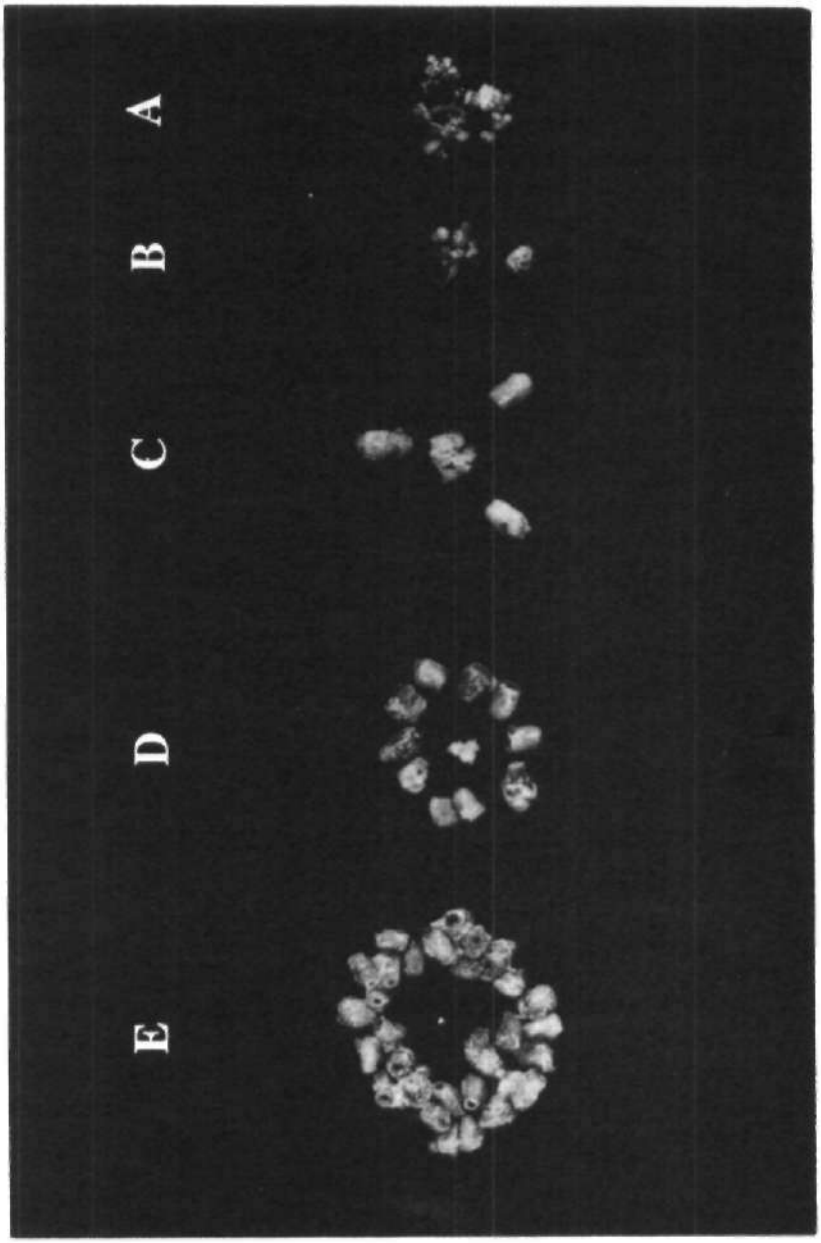
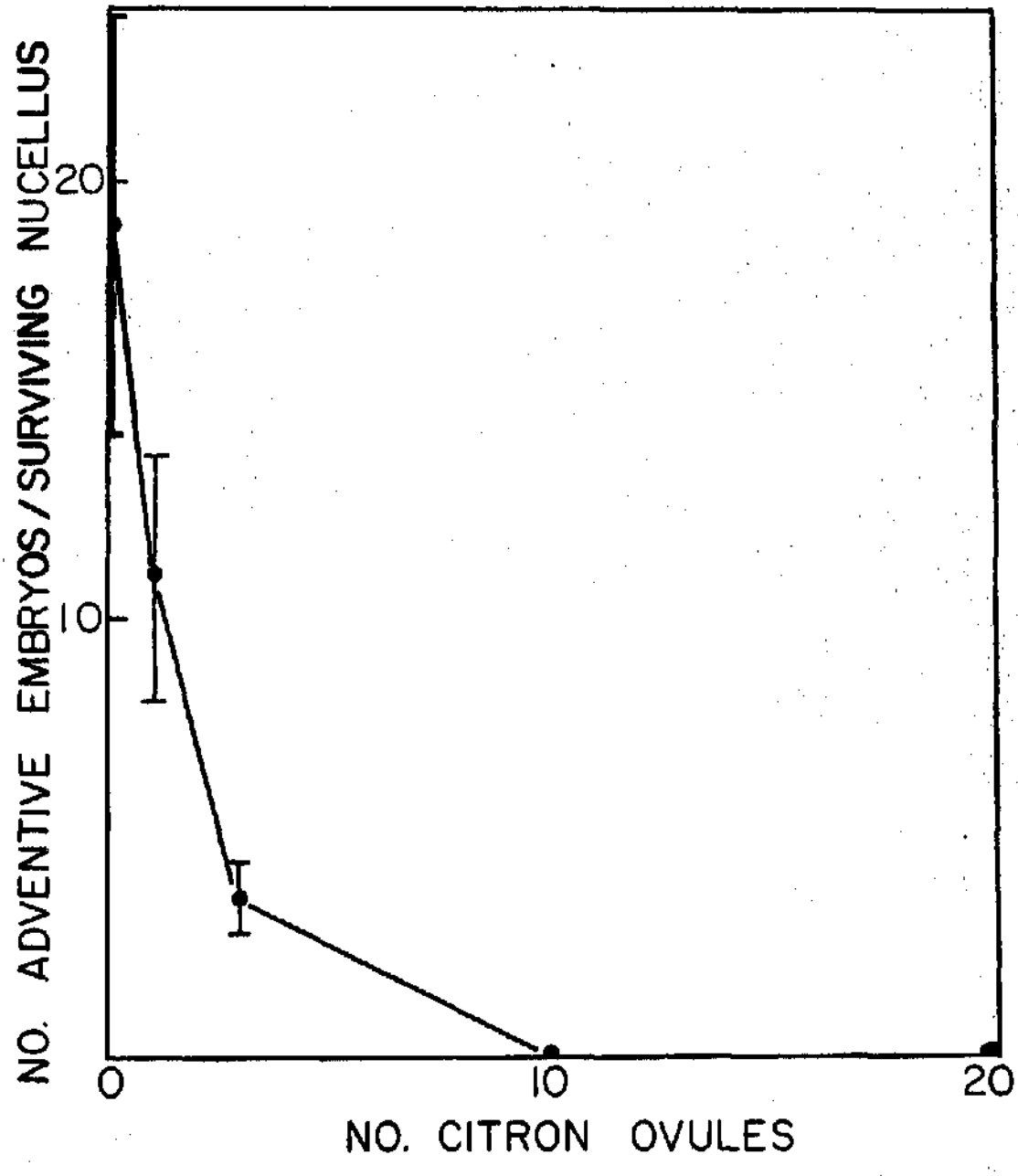

A
B
C
D
E

Figure 22. Effect of varying number of citron ovules on adventive embryogenesis in nucellus explants of Ponkan mandarin.


NO. ADVENTIVE EMBRYOS / SURVIVING NUCELLUS
20
10
0
10
20
NO. CITRON OVULES

completely.

These experiments indicated clearly that the ovular tissues of the normally monoembryonic citrus contained a diffusable substance in significant quantity and which possessed a repressive action on adventive embryogenesis.

Test of Leachability of Suppressant

It was not possible to overcome the embryogenically suppressed state of the Ethrog citron nucellus. Preculturing nucellus explants for prescribed periods in liquid (Table 41) or agar (Table 42) and subsequently transferring them into fresh nutrient agar did not improve the embryogenic behavior. No change in frequency of surviving explants occurred in whole nucellus explants on agar but the incidence of surviving cultures producing embryos or the number of embryos produced per surviving nucellus was not improved. These data indicated that either (1) the suppressive agent was not completely removable by leaching or (2) the agent was removed by leaching but its effect was irreversible.

Permanence of the Suppressive Effect

Repeated reculturing in media free of suppressive agent did not enable the embryogenically repressed tissue to regain its more natural embryogenic tendency compared to the reference cultures. Pertinent data can be seen in Fig. 23. When Ponkan mandarin nucellus was cultured in the same flask with 10 citron ovules per nucellus, embryogenesis in the mandarin nucellus was suppressed completely. Only callus arose. Reculturing of this callus in fresh media and in the absence of citron tissue did not result in rates of embryogenesis which were comparable to

TABLE 41

Effect of varying leaching periods in liquid on the ability of Citrus medica L. 'Ethrog' nucellar isolates to produce adventive embryos in vitro

Leaching in Liquid					
	Whole Nucellus				
Period of leaching (days)	0	1	5	10	30
% Surviving explants	68 (52-83)	53 (38-70)	42 (28-62)	38 (22-56)	55 (39-73)
% Surviving cultures with adventive embryos	100 (91-100)	100 (91-100)	100 (91-100)	100 (91-100)	100 (91-100)
No. adventive embryos per surviving nucellus	2.3 ± 0.6	2.2 ± 0.4	2.0 ± 0.4	2.1 ± 0.4	2.2 ± 0.7
	Micropylar Half Only				
Period of leaching (days)	0	1	5	10	30
% Surviving explants	81 (66-97)	72 (59-88)	60 (44-77)	70 (55-85)	80 (65-97)
% Surviving cultures with adventive embryos	100 (91-100)	100 (91-100)	100 (91-100)	100 (91-100)	100 (91-100)
No. adventive embryos per surviving nucellus	2.0 ± 0.2	2.6 ± 0.3	2.5 ± 0.1	2.7 ± 0.2	2.8 ± 0.2

95% confidence limits in parenthesis.

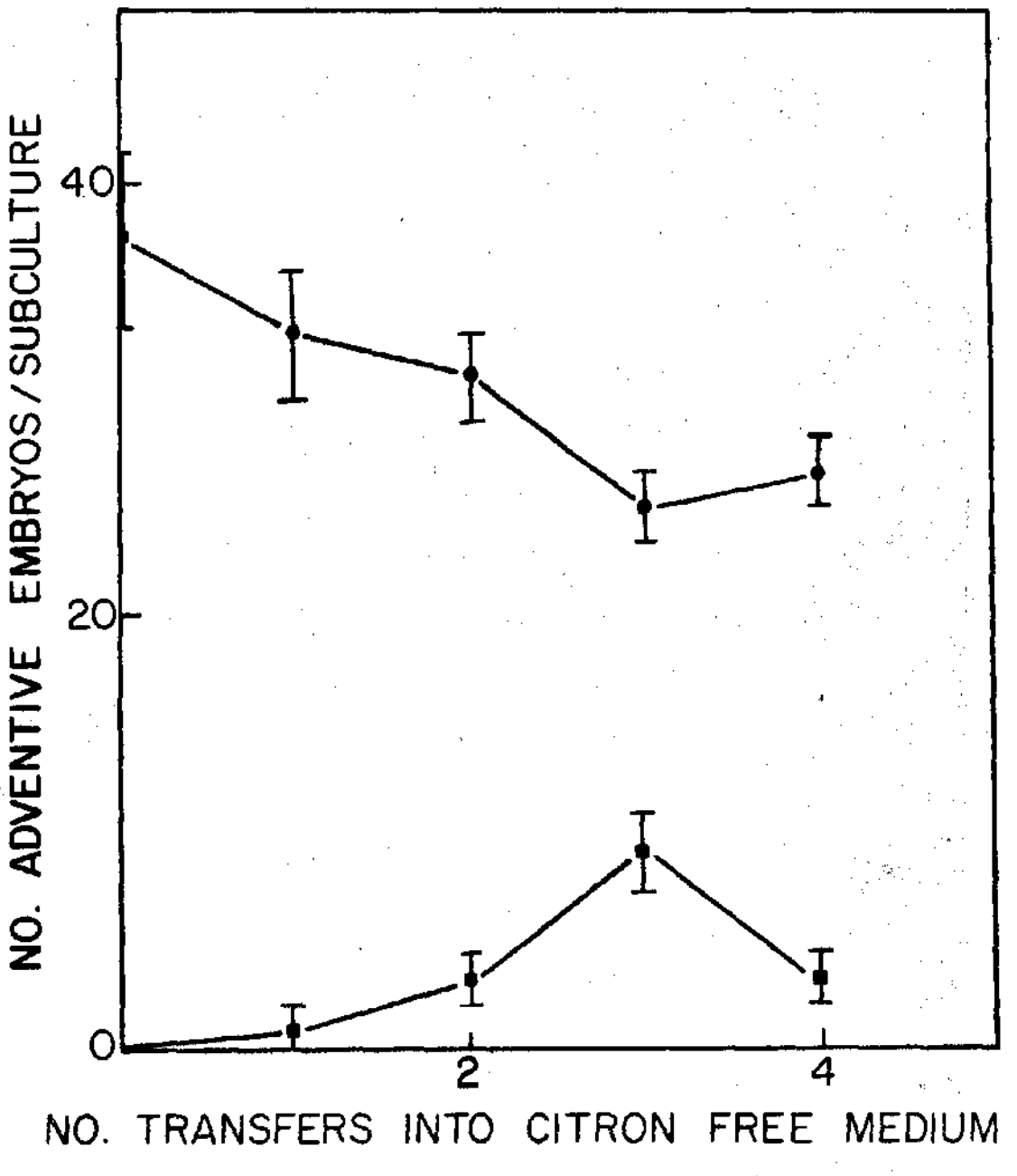
TABLE 42

Effect of varying leaching periods in agar on the ability of C. medica L. 'Ethrog' nucellar isolates to produce Adventive Embryos in vitro on agar medium

	Leaching in Agar			
	Whole Nucellus			
Periods of leaching (days)	0	5	10	15
% of Survival	28 (22-56)	33 (14-53)	35 (20-56)	50 (36-69)
% of Surviving nucellus with adventive embryos	100 (91-100)	100 (91-100)	100 (91-100)	100 (91-100)
No. of adventive embryos per surviving nucellus	1.7 ± 0.6	1.5 ± 0.7	1.5 ± 0.7	1.4 ± 0.8
	Micropylar Half Only			
Periods of leaching (days)	0	5	10	15
% of Survival	53 (38-70)	48 (33-67)	38 (22-56)	43 (28-62)
% of surviving nucellus with adventive embryos	100 (91-100)	100 (91-100)	100 (91-100)	100 (91-100)
No. of adventive embryos per surviving nucellus	1.9 ± 0.2	1.3 ± 0.3	1.6 ± 0.3	1.7 ± 0.2

95% confidence limits in parenthesis.

Figure 23. Reculture of Ponkan mandarin callus for recovery from treatment with Ethrog citron ovules.


NO. ADVENTIVE EMBRYOS / SUBCULTURE
40
20
0
2
4
NO. TRANSFERS INTO CITRON FREE MEDIUM

those of reference cultures. There was some increase in embryogenesis during the third reculture, but this diminished in the next. Recultures of the reference mandarin tissue showed a decline in embryogenesis, but the rate of embryogenesis in this tissue did not at any time fall to the level of the repressed tissue. Samples of the recultures of Ponkan mandarin tissues, both reference and embryogenically repressed, can be seen in Fig. 24.

These data showed that the repressive action of the substance in citron ovules was for all practical purposes irreversible. A nucellus which had been exposed to the suppressive agent could no longer manifest embryogenesis to the extent it was originally capable, even after a substantial period of culture away from the agent.

Embryogenic Inhibitor in Integuments

The fact that the repressive action of citron tissue could be observed with ovule sections suggested that the suppressant might also be present in the integument. This possibility was examined in several experiments. The data from those with Ethrog citron, Eureka lemon and Cleopatra mandarin can be found in Table 43, and representative cultures with Meiwa kumquat can be seen in Fig. 25. In each of the experiments a comparison was made of the in vitro behavior of explants comprised of nucellus alone or of nucellus with the integuments still attached. As apparent in Table 43, the three citrus cultivars showed virtually the same trends. Survival of citron and lemon explants was better when integuments were present; the mandarin showed no difference. However, the frequency of surviving cultures which produced embryos was reduced

Figure 24. Permanence of the embryogenic suppression in Ponkan mandarin tissue due to the Ethrog citron suppressor factor after four transfers into a medium free of citron ovules.

A = Reference culture

B = Affected culture

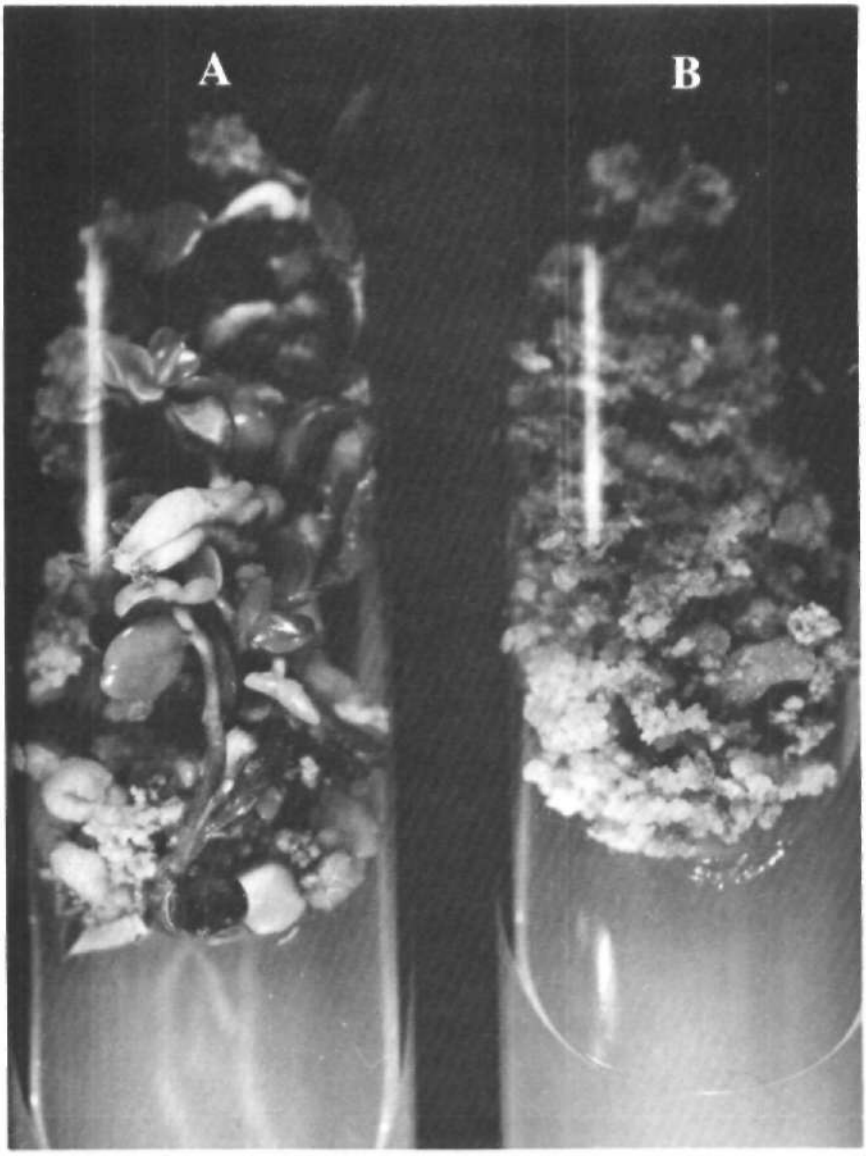

A
B

TABLE 43

Influence of integuments on nucellar embryogenesis in Citrus[1]

	Ethrog Citron		Eureka Lemon		Cleopatra Mandarin	
	Nucellus Alone	With Integuments	Nucellus Alone	With Integuments	Nucellus Alone	With Integuments
% Surviving explants	55 (29-78)	100 (85-100)	55 (29-78)	100 (85-100)	100 (85-100)	100 (85-100)
% Surviving cultures with adventive embryos	100 (85-100)	2.5 (0-19)	85 (64-96)	10 (1-30)	100 (85-100)	74 (53-90)
% All cultures with adventive embryos	55 (29-78)	2.5 (0-19)	55 (29-78)	10 (1-30)	100 (95-100)	73 (53-90)
% All cultures with callus only	0 (0-15)	97.5 (85-100)	10 (1-30)	90 (70-99)	0 (0-15)	27 (10-47)
No. adventive embryos per surviving nucellus	1.3 ± 0.4	0.05 ± 0.01	2.8 ± 0.6	0.1 ± 0.1	11.0 ± 3.6	2.4 ± 0.9

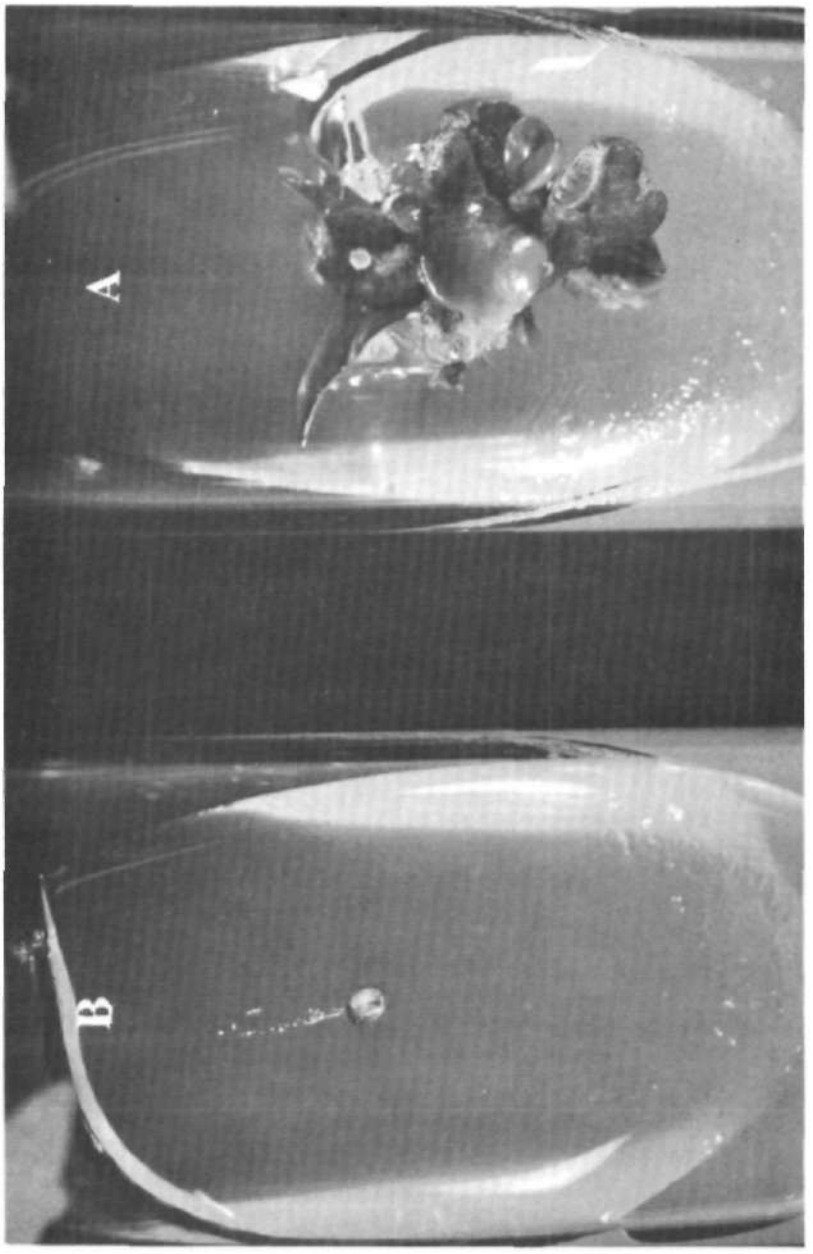
[1]Species used; Citrus medica L. 'Ethrog', C. limon L. Burm 'Eureka', C. reticulata Blanco 'Cleopatra'.

95% confidence limits in parenthesis.

Figure 25. Effect of ovule integuments of Meiwa kunquat on adventive embryogenesis in vitro.

A = Nucellus explants from ovules of two-week-old fruit (post anthesis)

B = Ovule explant from two-week-old fruits (post anthesis)


A
B

drastically, particularly in citron and lemon, in the presence of integuments. Callus production was increased substantially and the number of embryos produced per surviving tissue was repressed markedly by the integuments. These data thus revealed that the embryogenic inhibitor was also present in the integuments.

Action of Citrus Suppressant on Daucus carota Callus

The repressive action of the agent in citrus was not restricted to the Rutaceae. Embryogenesis in the callus of Daucus carota, variety 'Queen Anne's Lace,' was reduced noticeably when the tissue was cultured together with citron ovule sections (Fig. 26). The number of embryos produced per wild carrot callus culture decreased progressively as the number of citron ovule sections with which the tissue was associated was increased. Only half as many embryos were obtained in cultures containing three citron ovules and virtually all embryogenesis disappeared with 20 ovules.

The effect of the citron substance was not limited to embryogenesis alone. Growth of the carrot tissue as well as of plants which arose from the carrot embryos was depressed significantly (Fig. 27).

Figure 26. Effect of varying number of citron ovules on adventive embryogenesis of Daucus carota in vitro.

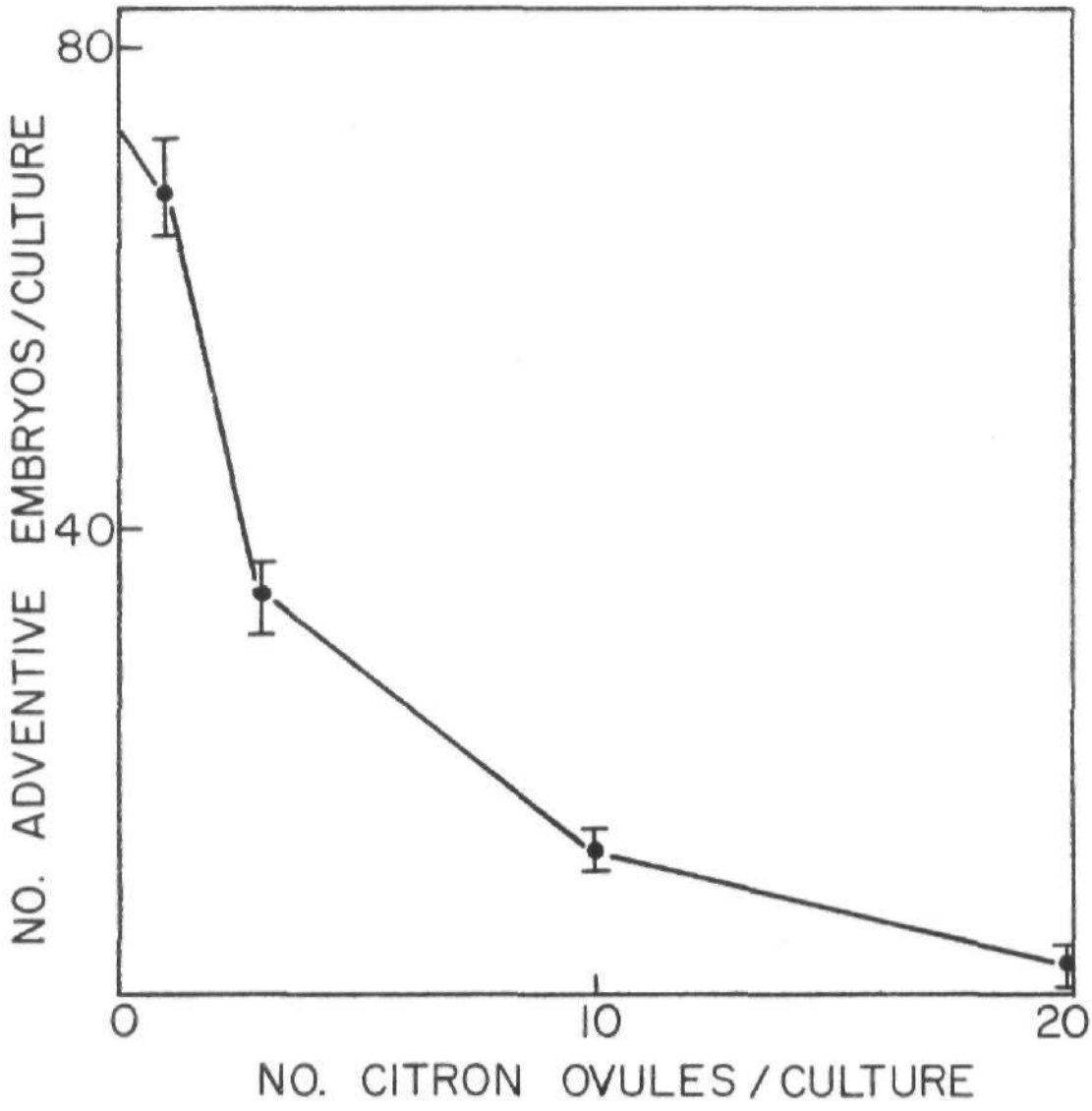
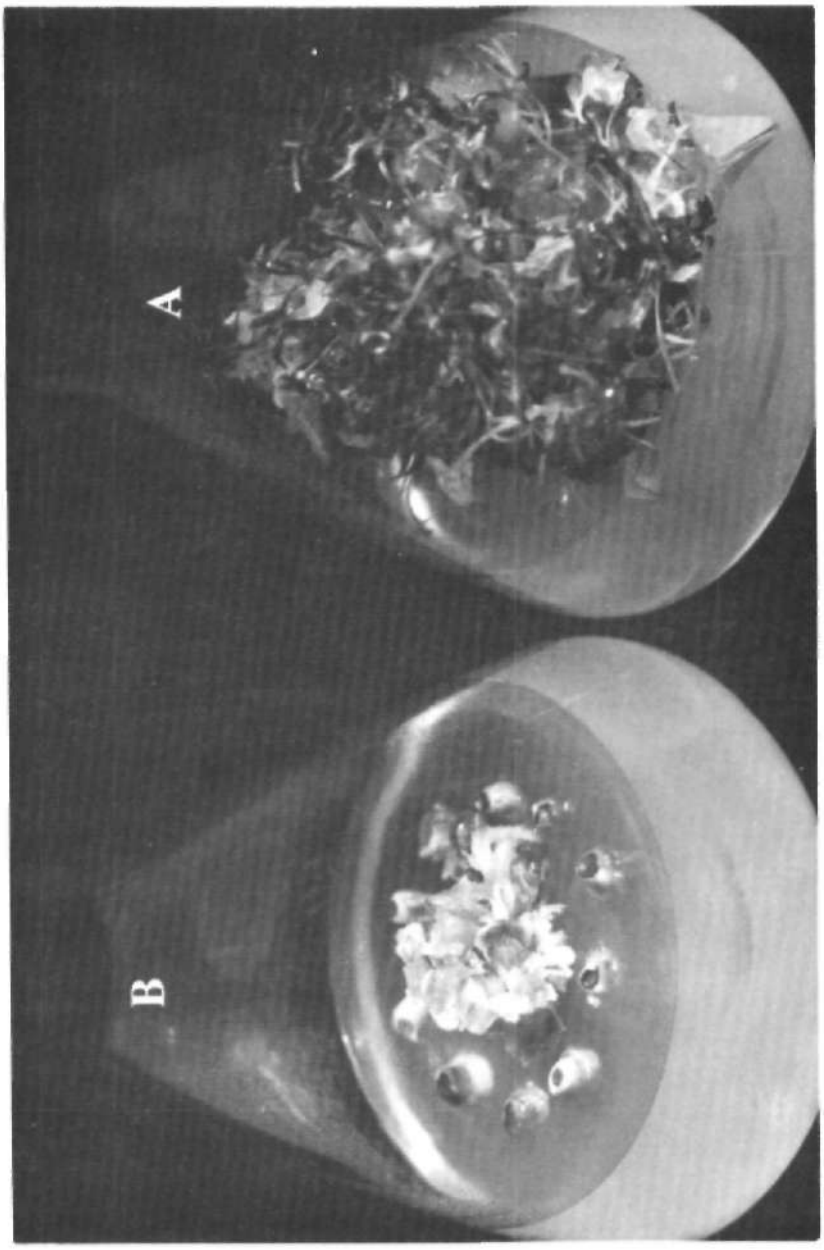

80
40
0
10
20
NO. ADVENTIVE EMBRYOS/CULTURE
NO. CITRON OVULES / CULTURE

Figure 27. Influence of citron ovules on adventive embryogenesis of Daucus carota.

A = Untreated reference culture

B = From callus cultures treated with 10 citron ovules


A
B

V. DISCUSSION

An ultimate goal is the elucidation of the process of adventive embryogenesis in plants, particularly with respect to the physiological mechanisms which underlie the process. A survey of the literature, from which some of the data have been summarized in Tables 1 and 2, show that embryogenesis by means other than through development from the zygote is widespread among a large number of seed bearing plants. At least 27 families have been reported to contain genera or species which manifest adventive embryogenesis as a natural part of their life activities. Tissue culture studies have shown that another 15 families contain members which will undergo somatic embryogenesis in vitro.

This investigation has been conducted with plants which are members of the Rutaceae, hoping that the information obtained can be applied to plants in general. The literature has shown that adventive embryogenesis, particularly embryo development from the nucellus, is most preponderant in the subfamily Aurantioideae of Rutaceae. Moreover, nucellar embryony has been the predominant type of adventive embryogenesis encountered among plants investigated.

This investigation has been conducted in three stages. First, a study has been made of the histogenetic events which are associated with the development of tissues in the ovules of a highly polyembryonic cultivar, Citrus reticulata Blanco 'Ponkan,' and of a monoembryonic cultivar, C. medica L. 'Ethrog,' hoping that there are observable

differences in their developmental patterns and which may serve as clues for further investigation of the mechanism underlying nucellar embryogenesis. The study has been carried out with materials which were in ontogenesis in vivo as well as with materials which were undergoing development in vitro from excised tissues. A second stage has attempted to examine the mechanism by studying the response of excised nucellus in vitro to diverse morphogenetically active as well as nutritional substances. The experiments have been intended to reveal substances which either trigger, e.g., in experiments with nucellus of monoembryonic cultivars, or enhance, e.g., in tests with moderately polyembryonic cultivars, the embryogenic process. A subsequent study might then explore the mechanism of action of such substances. The final stage has been more directly aimed at disclosing the regulatory mechanism underlying nucellar embryogenesis. It also has been carried out with tissue cultures; however, it has considered the role of endogenous rather than exogenous substances. The experiments have examined correlative effects between tissue regions, i.e., micropylar vs chalazal halves of the nucellus, as well as between tissues of embryogenically contrasting cultivars, i.e., nucellus of polyembryonic co-cultured with that of a monoembryonic cultivar.

The histogenetic study has revealed some differences in the ontogeny in vivo of the ovule tissues of monoembryonic and polyembryonic Citrus. However, there has been nothing apparent in the histogenetic patterns which provided a direction for further investigations with underlying mechanisms as objective. Both monoembryonic Ethrog citron and polyembryonic Ponkan mandarin nucellus tissues are crassinucellate

and with two distinct parts, an outer epidermis and a more massive inner portion. Following pollination the ovules of both cultivars undergo vascularization in the outer integument; the vascular differentiation terminates in the chalazal region and fails to establish continuity with the nucellus. A brown layer differentiates from the inner integument. This layer consists of cells containing highly refractive and brown substances, and it eventually envelopes the nucellus completely. The Ponkan mandarin and the Ethrog citron differ slightly in the pattern of brown layer differentiation; in the citron the brown layer is initiated in the micropylar end and its differentiation progresses towards the chalazal end, whereas in the mandarin the layer appeared initially along the flanks of the micropyle and from there it continued its differentiation towards the micropyle first and then towards the chalazal end. The significance of this brown layer has escaped elucidation, although it may be suggested that it serves as barrier against invasion of the nucellus and embryo sac by viruses and other pathogens. The nucellus of both Citrus undergoes enlargement for some time. The centrally located cells of the nucellus degenerate rapidly as the embryo sac develops. The endosperm of both Citrus remains free nuclear for a long time, even after the zygote has begun embryogenesis.

The zygote in both cultivars remains dormant for a substantial period after flower pollination. That of the citron begins division and embryogenesis about 60 days after pollination, and the pattern of embryogenesis is much like that seen in most other dicotyledonous plants. There has been no embryonic anomally observed in the citron, except for one rare case of budding in the suspensor.

The zygote of the Ponkan mandarin has not been observed to undergo division. The nucellus begins embryogenic activity before the zygote divides and any embryogenic activity in the zygote may be clouded from view by nucellar embryogeny. As described in the literature (Strasburger, 1878; Biermann, 1896; Osawa, 1912; Frost, 1926, 1938; Toxopeus, 1930; Webber, 1931; Mauritzon, 1935; Chakravarty, 1936; Bacchi, 1943; Vasiljcova, 1951; Furusato, 1952, 1953; Johri and Ahuja, 1956, 1957; Desai, 1962; Yang, 1968), the predominant meristematic activity leading to nucellar embryo formation is in the micropyle region, although it is not restricted to this region. Embryo initiation has been observed along the lateral flanks of the nucellus as well as in the chalazal region. Others have observed a few instances of embryogenic activity only in the chalazal region of the nucellus of plants in other families (Murbeck, 1902; Suessenguth, 1923; Juliano, 1937; Swamy, 1946, 1948). Initiation of the embryo occurs primarily in the inner nucellus cells immediately adjacent to the embryo sac. As other investigators have observed, embryogenesis is not associated with the nucellar epidermis or other more superficially located cells of the nucellus.

Multiplication of the asexually derived embryos in the Ponkan mandarin ovule apparently occurs largely through budding of existing embryos, rather than through continued differentiation among nucellus cells. Although this is the first recorded observation in the Rutaceae, others have previously reported the same budding process of embryo multiplication in some other families, e.g., Orchidaceae (Swamy, 1946). The term budding is used in this investigation to denote embryogenesis which occurs as offshoots from existing embryos. The budding process

probably occurs through localized meristematic activity in a number of peripheral regions of existing embryos. Protruding spheres of cells are produced. When a sphere has attained a certain volume organization begins, leading to embryo formation.

Substantial proliferation of the nucellus in the chalazal region appears to be a common occurrence in the Ponkan mandarin ovule. The nucellus enlarges greatly, filling the chalazal region of the embryo sac. The significance of this phenomenon has not been explored.

When cultured in vitro the Ponkan mandarin nucellus begins meristematic activity in the micropylar region within three days from excision. The histogenetic pattern leading to polyembryogeny in this mandarin is the same as that in vivo. In citron nucellus cultures the meristematic activity begins on the seventh day of culture. All nucellar embryogenesis in the citron is confined to the micropylar region. Adventive multiplication of embryos also occurs by budding among existing embryos. The extent of manifestation of polyembryogenesis in the monoembryonic cultivar is far less than that of the polyembryonic, even in vitro. Whereas a seemingly infinite number of embryos is produced in Ponkan mandarin nucellus cultures, only one to few embryos are obtained per citron nucellus in vitro. The process of embryo multiplication in vitro by budding has been observed in many plants and diverse plant parts, e.g., Maheshwari and Baldev (1962), Konar and Nataraja (1965a,b,d,e), Kato and Takeuchi (1966), Haccius and Lakshmanan (1969), and Konar *et al*. (1972a,b). These observations together with the one made presently in *Citrus* are additional evidence against the notion of Steward *et al*. (1958, 1963, 1964, 1966), and

supported by Button and Bornman (1971a,b) that embryogenesis requires physical isolation of cells into single-cell units.

A suspensor has been observed associated with some nucellar embryos, but this has not been a general characteristic of the asexually derived embryos.

Callus formation is not a necessary intermediate step in the adventive embryogenesis of either Ethrog citron or Ponkan mandarin nucellus, in vitro and in vivo. This is contrary to the reports of some others who have also investigated with citrus nucellus cultures (Rangaswamy, 1958, 1959, 1961; Maheswari and Rangaswamy, 1959; Sabharwal, 1961, 1963). The process of embryogenesis from nucellus cells in vitro is not characterized by an orderly and a predictable pattern of cell divisions. This has been noted earlier in investigations with some other families (Ball and Joshi, 1965; Yamada et al., 1967; Konar et al., 1972). Very high rates of meristematic activity in local areas within the nucellus produce clusters of small, densely cytoplasmic, and isodiametric cells. These clusters are probably comparable to the meristemoids of Torrey (1966, 1968). Eventually, cell division in the clusters takes a more coordinated pattern, and the organized growth leads ultimately to a structure which can be recognized as an embryo.

Tests of exogenous substances in nucellus cultures have not served as a satisfactory approach towards elucidating the regulatory mechanism underlying adventive embryogenesis. Embryogenesis in vitro can be manifested in nutrient media of very simple and physiologically unrevealing compositions. Nucellar embryos are obtainable in an agar medium containing solely Murashige and Skoog salts and sucrose. It is

possible to substitute for the sucrose to some extent with a mixture of malt extract, inositol and certain B-vitamins. The key constituent of this substituent mixture is probably inositol, since neither malt extract nor the B-vitamins have been shown to be necessary for embryogenesis in vitro. There has been no improvement by enriching the medium with the auxins IAA, NAA and 2,4-D; the cytokinin kinetin; the gibberellin GA_3; or the nitrogen base relatives adenine sulfate and thymidine. Casein hydrolysate and yeast extract are also without beneficial effects on nucellar embryogenesis in vitro. Orange juice might stimulate callus growth (Murashige and Tucker, 1969), but not embryogenesis.

With respect to some basic parameters in nucellus culturing, this investigation has disclosed some useful information. Within a given range, the age of a fruit from which nucellus explants are obtained is not critical in determining the behavior of nucellus cultures. Citron nucellus cultures showing equal rates of embryogenesis have been obtained with explants from fruits which were 77 to 133 days since flower pollination.

Pollination is not essential for nucellar embryogenesis to occur in vitro, at least with the normally polyembryonic cultivars of the Rutaceae. The present findings of embryogenesis in vitro in ovules excised from flower buds of Fortunella crassifolia Swing. 'Meiwa', Citrus reticulata Blanco 'Ponkan', C. sinensis L. Osbeck 'Koethen', C. sinensis L. Osbeck 'Ruvel', C. sinensis L. Osbeck 'Surprise Navel', and C. sinensis L. Osbeck 'Wallace seedless Valencia' are irrefutable evidence. A previous report by Bitters et al. (1970) noted that some of the Robertson Navel Orange nucellus explants from unpollinated flower

buds also produced embryos in vitro. Button and Bornman (1971a,b) purported to show that they were able to obtain nucellar embryos in vitro of Washington Navel orange from unpollinated material; however, their conclusion was premature because they used developing fruits as the source of nucellus explants, and they were unable to present uncontestable evidence substantiating their claim that the fruits developed from unpollinated flowers.

Although flowering and fruiting in most members of the Rutaceae are seasonal, there are cultivars, e.g., lemons and citrons, which flower and fruit the year round. According to this investigation, the seasonal factor should be considered in obtaining fruits to be used as sources of nucellus explants of such cultivars. Best explants are obtained during the fall and winter months, whereas the poorest are encountered during the spring.

Fruit samples to be used as source of nucellus explants may be kept under refrigeration for as long as four weeks without diminishing the embryogenesis in tissue cultures.

In removing existing embryos from nucellus isolates during the preparation of nucellus explants, it is important that injury to the embryogenic micropylar region is kept to a minimum. Best results are obtained by making a transverse incision at some distance from the micropylar tip. Incisions through the tip reduce severely the survival of explants in vitro.

One of the more interesting findings has been the significant role in embryogenesis played by the method of placement of the nucellus explant in an agar medium. While horizontal placement on the surface of

the agar is satisfactory, maximum embryogenesis occurs when the nucellus is placed with its chalazal portion embedded in the agar and its micropylar region exposed. When the micropylar end is embedded in nutrient agar, embryogenesis is reduced considerably in the polyembryonic cultivars and only callus results in the monoembryonic citrus.

Satisfactory embryogenesis in vitro requires that the cultures are maintained for an optimum passage period. Too short a period does not enable maximum expression of the embryogenic potential; the embryos need time for initiation as well as for enlargement. Too long a passage period leads to adverse effects associated with deterioration of the nutrient medium. It is not possible to suggest a passage length which will be applicable to all of the Rutaceae, since the behavior in culture varies significantly from one cultivar to another. For example, a four-week passage period may be adequate for Kuffre lime, whereas six weeks may be the minimum for Ethrog citron.

This research has also disclosed that agar-gelled nutrient media are superior to liquid formulations. Gentle agitation may be helpful when liquid media are used; nevertheless, the results are not expected to equal that of agar media. Embryogenesis in nucellus cultures of the Rutaceae does not require light. Maximum rate of embryogenesis in the test cultivars, Ethrog citron and Eureka lemon, has occurred even in total darkness. Temperature also has not been a critical factor in Citrus nucellus embryogenesis in vitro. A constant 27°C temperature has been satisfactory.

The third and final stage of this investigation has been most fruitful in revealing clues regarding the physiological mechanism

underlying the regulation of adventive embryogenesis. As a first step the polyembryogenic potential of several members of the Rutaceae has been surveyed by examining the behavior of their nucellus cultures. The examination included 20 members which have been classified as being polyembryonic on the basis of the behavior of their nucellus in vivo, and 11 which have been classed as monoembryonic (Nandi et al., 1945; Gurgel et al., 1951; Py, 1951; Bernaji, 1954; Chapot and Praloran, 1955; Ueno et al., 1967). The present survey has disclosed that under in vitro conditions, with the exception of Aeglopsis chevalieri Swing., all members of the Rutaceae examined are polyembryonic. The significant differences among the Rutaceae have been only in degree of nucellar embryogenesis in vitro. Some, such as the Ponkan mandarin and the sweet oranges, have been shown to be highly polyembryonic, whereas others, e.g., Ethrog citron and Ponderosa lemon, are characterized by very low ratea. Still others, including Eureka lemon, have disclosed more intermediate rates of nucellar embryogenesis in vitro. The type of cultures produced by various members of the Rutaceae has also shown variations. Some produce predominantly unorganized callus, others form embryos which remain as embryos indefinitely in culture, and still others yield embryos which germinate quickly into seedlings.

A number of experiments have been carried out in attempts to elucidate the basis for the differences in the embryogenic behavior of various members of the Rutaceae. First, the reason for the largely localized embryogenic activity within the nucellus of most cultivars has been determined. A comparison of micropylar and chalazal halves of several cultivars has shown that (1) the micropylar half is clearly more

prone to undergo embryogenesis than the chalazal half, (2) a halved nucellus comprised of the micropylar region produces more embryos than the whole nucellus, and (3) the halved nucellus composed of the chalazal portion is less likely to survive culture or generate embryos than the whole nucellus. These observations suggest, on one hand, that the micropylar region of a nucellus contains a significantly higher concentration of a stimulant of embryogenesis than the chalazal region. On the other hand, they may indicate that the chalazal portion contains a strongly inhibiting substance(s) which suppresses embryogenesis.

The first alternative has been considered by examining interactions in embryogenesis in vitro between micropylar halves of the nucellus of polyembryonic and monoembryonic cultivars. The interaction involved grafts of the micropylar halves of the two types of cultivars. It has not been possible to observe any influence on the embryogenic behavior, stimulatory or inhibitory, of the nucellus tissue of one type, monoembryonic or polyembryonic, by the nucellus of the other type. It has thus been disclosed that the different rates of embryogenesis among the Rutaceae, as well as in the micropylar and chalazal region of a nucellus, is attributable not to variations in the content of an embryogenic stimulator.

When grafts are made between the micropylar half of the nucellus of polyembryonic cultivars and the chalazal half of a strongly monoembryonic cultivar, it is observed that embryogenesis in the normally polyembryonic nucellus is markedly suppressed. This reveals quite conclusively that an embryogenic suppressant is the key to the differences in the degree of polyembryogenesis among the Rutaceae.

Further studies have shown that the suppressive agent is transmitted through grafts and diffuses through agar, suggesting a substance(s) of relatively low molecular weight.

Whether it is possible to remove the suppressive agent from the affected nucellus has not been established. Embryogenesis in an inhibited tissue is not improved even after long periods of leaching.

Evidently, action of the agent renders the affected tissue relatively permanently suppressed. Reculturing of the embryogenically suppressed tissue does not result in restoration of the original embryogenetic characteristics, even after several transfers to media free of source of the agent.

The anti-embryogenic phenomenon observed in the Rutaceae evidently reflects the more general situation of plants. The substance(s) in citron tissue represses also embryogenesis in carrot callus cultures. In the carrot the inhibitor also suppresses growth of seedlings which arise from somatic embryos.

The finding of an anti-embryogenic effect may be fundamental in the elucidation of the regulatory mechanism underlying plant embryo initiation. Haberlandt's (1922a,b) necrohormone concept infers a specific embryo initiator. While this concept has been generally accepted, supporting evidence has been lacking. In contrast, the result of another investigation by Sturrock (1961) has provided data which indirectly support the present finding. According to Sturrock, the fruits of normally monoembryonic Mangifera indica yield extracts containing higher amounts of plant growth inhibiting substances than fruits of the polyembryonic types.

It may be proposed, based on the present investigation with the Rutaceae, that adventive embryogenesis is an inherent potential of all cells. However, early in the formative stage of a plant, perhaps as early as after the first division of the zygote, an inhibitor of embryo initiation is produced. This substance, by way of genetic repression, renders somatic cells incapable of expressing their embryo initiating potentials. Indeed, repression of embryo initiation among somatic cells is fundamental to the normal development of a plant. Otherwise, development will be chaotic. Adventive embryogenesis can be re-manifested later in ontogeny, e.g., at flowering time, should gene derepression occur and there is no significant source of embryogenic suppressor. This may be the case of the nucellus of the normally polyembryonic plants.

One of the tasks for the immediate future is that of identifying the substance(s) responsible for repressing embryo initiation in the Rutaceae. This may be followed by a study of its mode of action. The extent to which the repressive mechanism is operative among various plant families is also worthy of investigation.

VI. SUMMARY

The primary objective of this research was to explore the mechanism of regulation of adventive embryogenesis in spermatophytes, using the Rutaceae as the test family. The research was carried out in three stages. First a comparison was made, between polyembryonic and monoembryonic citrus, with respect to the histogenesis associated with embryogenesis in the nucellus in vivo and in vitro. Second, the response of cultured nucellus to exogenous substances was studied. Finally, studies were conducted of correlative effects between tissue regions and between contrasting cultivars.

The histological investigation was carried out with Citrus medica L. 'Ethrog,' a monoembryonic cultivar, and C. reticulata Blanco 'Ponkan,' a polyembryonic cultivar. No information relevant to the pursuit of this research's objective was obtained, although interesting phenomena and histogenic differences between the two cultivars were observed. The zygote in both cultivars remained dormant for a long time after pollination. That of the citron began division and embryogenesis about 60 days after pollination. Embryogenesis in the monoembryonic cultivar followed the pattern typical of most dicot plants. The zygote of the polyembryonic mandarin was not seen to divide. The nucellus began embryogenic activity while the zygote was still dormant. The predominant meristematic activity was in the micropylar region, although not restricted to this region. The activity was confined to nucellus cells

immediately adjacent to the embryo sac; no embryogenic activity was seen in the nucellus epidermis. Multiplication of the asexual embryo in the Ponkan mandarin occurred largely by budding of existing embryos, rather than through continued differentiation among nucellus cells.

The histogenetic pattern leading to polyembryogeny in vitro in the mandarin was the same as that in vivo. Meristematic activity, largely in the micropylar region of the nucellus and among cells immediately adjacent to the embryo sac, began within three days in culture. Multiplication of embryos in vitro also occurred largely by budding of existing embryos. The citron nucellus cultures showed meristematic activity beginning with the seventh day in culture. All embryogenesis in this monoembryonic cultivar was confined to the micropylar region. It also achieved embryo multiplication by the budding process. Callus formation was not a necessary intermediate step of adventive embryogenesis in either cultivar. A suspensor was observed in some nucellar embryos, but this was not a consistent feature.

The process of embryogenesis from nucellus cells in vivo or in vitro was not characterized by an orderly and predictable pattern of cell divisions. Meristematic activity in local areas within the nucellus produced clusters of small, densely cytoplasmic and isodiametric cells. Eventually cell division in these clusters followed a more coordinated pattern, and organized growth ultimately resulted in embryos. The budding process appeared to occur through localized meristematic activity in a number of peripheral regions of existing embryos. Protruding spheres of cells were produced. Enlargement of these spheres and organization led to additional embryos.

Tests of exogenous substances in nucellus cultures were carried out largely with the monoembryonic cultures C. medica L. 'Ethrog' and C. limon L. Burm 'Ponderosa' and the moderately polyembryonic C. limon Burm 'Eureka.' Other materials were also used in special cases. Embryogenesis in citrus nucellus in vitro was manifested in nutrient media of very simple compositions. Embryos were obtained in an agar medium containing only Murashige and Skoog salts and sucrose. There was no advantage gained from additions of IAA, NAA, 2,4-D, kinetin, GA_3, adenine sulphate and thymidine. Similarly, no significant benefit was obtained from casein hydrolysate, malt extract, yeast extract, and orange juice. This series of experiments was also unrevealing insofar as clues to the mechanism of embryogenic regulation were concerned.

Some interesting information regarding tissue culture parameters were obtained, however. Within a wide specified range, no difference could be observed among nucellus explants obtained from fruits of different ages. Fruits obtained during the fall and winter months provided best nucellus explants and those of spring months, poorest. The embryogenic characteristics of nucellus explants were not diminished following refrigerated storage of their source fruits. The kind of incision made in the nucellus for the purpose of removing embryos contained within affected the survival and embryogenic characteristics of the explant. Best results were obtained by a transverse incision made at some distance from the micropylar region. Maximum embryogenesis occurred when the nucellus explant was placed in culture with its chalazal end embedded in the agar and its micropylar end exposed. The nucellus of the monoembryonic Citrus produced only callus when the

micropylar end was embedded in the agar. The optimum length of the culture passage period varied with the cultivar and ranged from four to six weeks. Light was not necessary for embryogenesis to occur in vitro. A constant 27°C was also satisfactory as incubation temperature.

Substantial additional evidence was obtained indicating that pollination was not a prerequisite to nucellar embryogenesis in vitro. Nucellar embryos were obtained in ovules excised from flower buds of Citrus reticulata Blanco 'Ponkan,' C. sinensis L. Osbeck 'Koethen,' C. sinensis L. Osbeck 'Ruvel,' C. sinensis L. Osbeck 'Surprise Navel,' C. sinensis L. Osbeck 'Wallace seedless Valencia,' Fortunella crassifolia Swingle 'Meiwa.'

A survey was conducted of the in vitro embryogenic potential of several members of the Rutaceae, including 20 which were classified as being polyembryonic in vivo and 11 as monoembryonic. Their nucellus cultures revealed that, with the exception of one species, all members of the Aurantoidae of the Rutaceae were polyembryonic. However, they differed in the degree to which polyembryogeny was expressed.

Experiments comparing micropylar and chalazal halves of the nucellus showed that (1) the micropylar half was clearly more prone to undergo embryogenesis than the chalazal half, (2) a halved nucellus comprised of the micropylar region produced more embryos than the whole nucellus, and (3) the halved nucellus comprised of the chalazal portion was less likely to survive culture or to generate embryos than the whole nucellus.

Reciprocal grafts of micropylar halves of polyembryonic and monoembryonic cultivars resulted in no influence on embryogenesis by the one

type on the other. This observation suggested that the greater embryonic tendency of the micropylar half of the nucellus, particularly as evident in the polyembryonic type, was not related to contents or actions of a stimulant of embryogenesis.

When grafts were made between the micropylar half of the nucellus of polyembryonic cultivars and the chalazal half of a strongly monoembryonic cultivar, embryogenesis in the normally polyembryonic cultivar was markedly suppressed. This suggested that an embryogenic suppressant was a key to the difference observed in degree of polyembryony among the Rutaceae and between the micropylar and chalazal regions of a nucellus.

The substance was graft transmissible and diffusible through agar.

The effect of the suppressive substance was, for all practical purposes, irreversible. Embryogenesis in an inhibited tissue was not improved by long periods of leaching. Furthermore, reculturing of the embryogenically suppressed tissue through several transfers in media free of the suppressive agent did not result in restoration of the original embryogenic characteristic.

The inhibitory substance contained in the ovules of C. medica L. 'Ethrog' was also effective in suppressing embryogenesis in callus cultures of Daucus carota. This observation suggested that the physiological mechanism observed in the Rutaceae may be of general occurrence among plant families.

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