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Somatic Embryogenesis in Loblolly Pine (*Pinus taeda* L.)

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Somatic Embryogenesis in Woody Plants

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1. Introduction

1.1 Brief background on somatic embryogenesis

Somatic embryogenesis, the formation and development of embryos from somatic (vegetative) tissues under in vitro conditions, was first discovered in carrot (Steward et al., 1958) and in conifers with *Picea abies* in 1985 (Hakman et al., 1985). The first report of somatic embryogenesis with *Pinus taeda* was in 1987 (Gupta & Durzan, 1987a).

1.2 Purpose statement

Here we review progress to date on somatic embryogenesis in loblolly pine, one of the most economically important tree species in the world. We have also made several comparisons of how somatic embryogenesis in loblolly pine is either similar to or different than other conifers.

1.3 Origin of species name "loblolly"

Early English settlers in the Southeastern United States first called the species *Pinus taeda* "loblolly", an American dialect word for mudhole, because the sites in which they grew appeared as murky as the gruel the pioneering travelers ate on the sailing vessels at sea (Walker, 1990). This porridge was called loblolly in old England. It is possible that at the time the name loblolly was used to refer to several closely related Southern pines native to the region, including: *P. elliottii* (slash pine), *P. palustris* (longleaf pine), and *P. serotina* (pond pine). The English botanist William Bartram, noted that along with the wetland sites after which loblolly pine drew its name, there were vast stands of this species on higher, drier lands as well. This adaptation to a wide variety of site conditions is in fact one of the attributes that has made loblolly pine so important commercially. The native distribution of loblolly pine extends as far west as eastern Texas and as far north as Delaware near the east coast of the U. S. (Fig. 1).

Fig 1

1.4 Economic importance

About 1.6 billion tree seedlings, including coniferous and hardwood

species, are currently produced per year in the United States for reforestation (Moulton et al., 1993). These data, compiled yearly by the U.S. Forest Service, do not list seedling production by species. Boyer & South (1984) found that in 1980 approximately 966 million loblolly pine seedlings were produced in the Southeastern U.S.; representing 60 percent of the trees seedlings produced in the U.S. that year (Table 1). Thus, loblolly pine is a very important, if not the most important, tree species economically in the U.S.

Tab 1

2. Zygotic embryogenesis in loblolly pine

Understanding zygotic embryogenesis in loblolly pine is relevant to somatic embryogenesis for several reasons, including the following. First, somatic embryo development in conifers, as in other plants, is very similar to zygotic embryo development. Not only do somatic embryos of loblolly pine appear morphologically similar to their zygotic embryo counterparts, but their ontogeny is similar. This suggests that similar patterns of gene expression and regulation occur in somatic and zygotic embryogenesis. Secondly, initiation of somatic embryogenesis in loblolly pine occurs from very early stage zygotic embryos, and therefore it is useful to understand the early stages of zygotic embryogeny (Fig. 2).

Fig 2

Multiple fertilization, common in conifers, occurs in *Pinus taeda* (Fig. 2A), resulting in multiple viable zygotic embryos formed in individual seeds (Buchholz 1920). In addition, each zygotic embryo can cleave into four individual embryos (Fig. 2B). One of the multiple zygotic embryos develops more rapidly and outgrows or dominates the other (subordinate) zygotic embryos (Fig. 2C). The occurrence of multiple immature zygotic embryos is significant for culture initiation in *Pinus taeda* because researchers frequently use the whole immature megagametophytes as explants to initiate somatic embryogenesis (Fig. 2D).

In addition to the importance of understanding the early morphological stages of zygotic embryogenesis, another approach to the improvement of somatic embryogenesis is to study the biochemistry and physiology of zygotic

embryos during development. Kapik et al. (1993, 1994) have begun to support this approach by monitoring the abscisic (ABA) levels present in megagametophyte and embryo tissue during development. They found the highest levels of ABA were present during early embryo development. Gates & Greenwood (1991) measured the osmotic potential of gametophytic supernatant during embryo development in *Pinus resinosa*. Osmotic potential increased slightly (became less negative) between the zygote and proembryo stages of embryo development and remained constant thereafter. They also analyzed gametophytic supernatant for hexose sugar and lipid contents. Hexose sugar content increased gradually during development while lipids increased the most during precotyledonary to early cotyledonary development.

3. Culture Initiation

3.1 Explant

In *Picea* species the most frequently used explant for embryogenic culture initiation has been isolated immature or mature zygotic embryos (Tautorus et al., 1991). In *Pinus taeda* and other *Pinus* species this has not been the case. Smith et al. (1985) first used megagametophyte explants containing developing zygotic embryos with *Pinus radiata* to initiate embryogenic cultures. Since it is easier and faster to use megagametophyte explants, as compared to isolated immature zygotic embryos, they have become the preferred explant for embryogenic culture initiation for *Pinus taeda* (Gupta & Durzan 1987a; Becwar et al., 1990) and other *Pinus* species (Gupta & Durzan 1986; Becwar et al., 1988; Finer et al., 1989; Chandler et al., 1989; Laine & David 1990; Nagmani et al., 1993; Jones et al., 1993) The megagametophyte surrounds the embryo and supplies nutrients to the developing zygotic embryos. The specific effect of the megagametophyte on culture initiation has not been determined. It most likely provides nutrients and/or endogenous phytohormones that are sub-optimal in the culture medium. An advantage of using megagametophyte explants, as compared to isolated dominant zygotic embryos, is that the culture initiation process is much faster and easier. This is especially significant with loblolly pine and other *Pinus* species where culture initiation frequencies are exceedingly low (e.g., 1 to 5% initiation typical). In contrast, among *Picea* species embryogenic culture initiation

frequencies are relatively high; as high as 95% from immature zygotic embryos, and as high as 55% from mature zygotic embryos harvested from fully developed, dry seeds (Tautorus et al., 1991).

In addition, it should be noted that zygotic embryo extrusion often occurs within the first several weeks of culturing immature megagametophyte explants. Extruded zygotic embryos have the same appearance as somatic embryos and cannot easily be distinguished except by serial observations and obvious continued growth. Researchers have occasionally mistaken the zygotic extrusion process for initiation of somatic embryogenesis and reported high initiation rates. Frequently, extruded zygotic embryos do not initiate embryogenic tissue. Successful initiations will show proliferation of embryogenic tissue from the extruded zygotic embryos and culture increase over time. Repeated cleavage polyembryony is often visible by microscopic observation of the extruded embryogenic tissue soon after extrusion.

Isolated immature zygotic embryos have also been used to initiate embryogenic cultures in loblolly pine (Becwar et al., 1990). A disadvantage of using isolated immature zygotic embryos as explants is that much more time is required for dissection of the dominant zygotic embryo from within the megagametophyte, as compared to simply culturing the whole megagametophytes.

3.2 Optimum Window for Initiation

The optimum time for initiating embryogenic cultures from loblolly pine zygotic embryos is prior to cotyledon primordia development in the dominant zygotic embryo (Becwar et al., 1990) (Fig. 3). The embryogenic tissue frequently initiates from cell division and proliferation in the suspensor region near the interface of the suspensor cells and the embryo head. Nearly 50% of precotyledonary zygotic embryos undergo this initial cell division, and the response decreases at later stages of zygotic embryo development to near zero when zygotic embryos are predominantly cotyledonary (Fig. 3A). The time course of proliferation of embryogenic tissue is similar, proceeding from the highest levels at the precotyledonary stage to near zero as the zygotic embryos reach the cotyledonary stage (Fig. 3B). But, very few of the explants which show the initial cell division in the suspensor region continue to proliferate. For example, with explants from parent tree 7-34, about 50% of

the explants started embryogenic tissue cell division, but only 10% of the explants which showed initial cell division in the suspensor region continued to proliferate and yield vigorously growing embryogenic tissue. Thus, the initial cell division is transitory in most explants (90%). It is possible that medium components are sub-optimal or inhibitory to continued cell division which leads to proliferation of embryogenic tissue.

Fig 3 The results in Fig. 3 also show that initiation of non-embryogenic callus occurs inversely to initiation of embryogenic tissue in loblolly pine. At the optimum precotyledonary stage for initiating embryogenic tissue, very little non-embryogenic tissue initiates (Fig. 3C). Whereas, two weeks later at the mostly cotyledonary stage, greater than 75% of the explants initiate non-embryogenic callus and initiation of embryogenic tissue drops to near zero. This inverse relationship between initiation of embryogenic and non-embryogenic tissue does not occur in spruce species, where the optimum stage of initiation occurs at the cotyledonary stage of zygotic embryo development (Hakman & Fowke 1987; Becwar et al., 1988). Both embryogenic and non-embryogenic tissue are frequently initiated from the same cotyledonary stage zygotic embryo explant of spruce species. This suggests that different mechanisms of initiation are operative in loblolly and other pine species as compared to spruce species.

3.3 Origin of Embryogenic Tissue

The terms "embryonal suspensor masses" and "somatic polyembryogenesis" have been used to describe, respectively, proliferating embryogenic cultures of loblolly pine and other conifers, and the *in vitro* embryo formation process in the cultures (Gupta & Durzan 1987a). They reported multinucleate cells (coenocytes) and noted the apparent similarity of the early stages of the somatic embryogenesis process to cleavage polyembryony which occurs *in vivo* in loblolly pine and other conifers. In addition, Gupta & Durzan (1987a) suggested that there is no true callus or unorganized phase of proliferation in embryogenic cultures of loblolly pine. Rather, according to their hypothesis, somatic embryos form by a repetitive cleavage process.

Proliferation of cells in the suspensor region preceded somatic embryo formation from zygotic embryos of slash and loblolly pine (Jain et al., 1989; Becwar et al., 1991). Although the resulting embryogenic cultures of loblolly

pine did not appear as typical "callus", it did appear that some type of dedifferentiation preceded the initial formation of somatic embryos from extruded zygotic embryos. One explanation for the different interpretations of loblolly pine somatic embryogenesis is the possibility that there are several mechanisms of embryo formation *in vitro*. It is possible that initially somatic embryos form via a dedifferentiation of cells of the zygotic embryo, but that later the cleavage process that Gupta & Durzan (1987a) refer to as somatic polyembryogenesis, plays a role in embryo multiplication as the culture continues to proliferate.

Several workers have used the term "embryogenic tissue" to refer to the mass of proliferating cells and embryos that make up a conifer embryogenic culture, including loblolly pine. Originally, the general term "tissue culture" was used to refer to maintenance and growth of pieces of tissue in culture away from the source organism (Lackie & Dow 1989; Schaeffer 1990). More recently, the term is used nearly synonymously with "cell culture", referring to the maintenance of cell strains or lines *in vitro*. Since conifer embryogenic cultures contain both proliferating cells and differentiated somatic embryos (tissues) the term "embryogenic tissue" seems appropriate to use for describing embryogenic cultures of loblolly pine and other conifers. It is also consistent with the terminology used by other workers in plant tissue and cell culture, and with the general field of cell biology (Lackie & Dow, 1989).

3.4 Genetic Origin and Homogeneity of Embryogenic Tissue

Isozyme analysis has been used to genotype embryogenic tissue initiated from multiple zygotic embryos contained within seeds of loblolly pine (Becwar et al., 1991). The study showed that: (1) somatic embryogenesis can be initiated from zygotic embryos other than the dominant embryo which develops to maturity, and (2) one cannot rule out the possibility that genetically heterogeneous cultures are produced when initiating cultures from immature megagametophyte explants containing multiple zygotic embryos.

Further studies revealed that at least 27% of the cultures derived from zygotic embryos extruded from immature megagametophyte explants of loblolly pine were derived from the non-dominant zygotic embryos (Becwar et al., unpublished). In these studies the explants were from control crosses of

parents with known isozyme markers at the 6-phosphogluconate dehydrogenase (6PGD1) gene locus. In cases where somatic embryos were derived from embryogenic tissue which originated from the dominant zygotic embryo, the 6PGD1 markers were the same between dominant zygotic and somatic embryos. In cases where somatic embryos were derived from subordinate zygotic embryos which had a different marker allele, differences in the isoenzyme banding patterns were found between dominant zygotic and somatic embryos. This technique which used a single genetic marker could detect some, but not necessarily all, cases of genetic polymorphism between dominant zygotic and somatic embryos. It could not detect cases of polymorphism where somatic embryos were derived from a non-dominant zygotic embryo which had the same marker allele as the dominant zygotic embryo. Therefore, it is likely that even more than 27% of the cultures were derived from non-dominant zygotic embryos. Further studies are needed to: (1) determine if somatic embryo plants derived from non-dominant zygotic embryos are of similar quality and vigor as those derived from dominant zygotic embryos, and (2) verify that somatic embryos derived from embryogenic tissue extruded from megagametophytes are genetically homogeneous.

In terms of comparisons to spruce somatic embryogenesis; origin of embryogenic tissue from non-dominant zygotic embryos is not a relevant issue for researchers working with spruce species where cultures are readily initiated from excised dominant zygotic embryos. Evidence to date suggests that spruce embryogenic cultures produce genetically homogeneous somatic embryos. Eastman et al. (1991) tested fifteen different enzymes, representing a minimum of 25 loci, for isozyme variation among 1500 somatic embryos derived from three parental genotypes. No variation in isozyme banding patterns was found among the interior spruce somatic embryos within culture genotypes, or between somatic embryos and parental genotypes, suggesting a lack of somaclonal variation during somatic embryogenesis of spruce. In another study of genetic integrity of black spruce somatic embryos, ten RAPD markers were screened and no variation was found among somatic embryos derived from individual zygotic embryos (Isabel et al., 1993).

4. Culture Maintenance

As with other conifers, embryogenic cultures of loblolly pine can be

maintained in a proliferating state either on semi-solid (gelled) medium, or in liquid as suspension cultures. Semi-solid grown cultures of loblolly pine have been maintained at the Institute of Paper Science and Technology for over 6 years. Frequently it is difficult to maintain highly embryogenic cultures of loblolly pine over extended periods of time on semi-solid medium. The cultures apparently undergo an aging process and often show a decline in embryogenic potential. Gupta & Durzan (1987a) first reported growing embryogenic cultures of loblolly pine in liquid. Liquid suspensions containing early-staged embryos have the advantages of higher multiplication rates, improved observation of embryos, decreased labor requirements, and reduced variation of embryos available for experimentation.

In addition to maintenance of cultures in a proliferating state, the potential for long-term storage of embryogenic cultures of loblolly pine in liquid nitrogen (cryopreservation at -196°C) has been demonstrated (Gupta et al., 1987). At the ultra-low temperatures of cryostorage, cultures are held in a quiescent state which should halt or greatly reduce aging of cultures that can occur when they are grown and maintained for extended periods of time on semi-solid or liquid medium. Larch and *Pinus caribaea* plants from cryostored and non-cryostored somatic embryos appeared similar when established in a seedling nursery (Klimaszewaska et al., 1992) and greenhouse (Laine et al., 1992) respectively.

5. Cryopreservation

Cryopreservation will become an important part of tree improvement programs of loblolly pine and other conifers which breed and test clonal material produced through somatic embryogenesis prior to commercial use. The optimum age for identification of superior individuals within a family of loblolly pine was estimated to be 6 to 10 years (Balocchi et al., 1994). Their study also suggested that selections for clonal propagation systems can be effectively made at 6 to 7 years. However, while embryogenic cultures may be maintained for long periods of time (i.e., 6 to 7 years) labor costs to do so are high. Furthermore, prolonged maintenance of cultures may lead to selection of less desirable variants or culture aging over time resulting in decreased regeneration capacity. Cryopreservation provides the opportunity to create liquid nitrogen clonal banks which contain valuable clones with the

potential to increase forest yields and improve raw material uniformity and quality. These high value clones could be retrieved whenever needed for commercial production.

6. Embryo Development and Maturation

Gupta & Durzan (1987a) first reported development of cotyledonary stage somatic embryos from embryogenic cultures of loblolly pine. Cultures were initiated and embryos developed on a modified 1/2 strength MS medium supplemented with glutamine, casein hydrolysate, 2,4-D, kinetin, and BAP. Hormones were then reduced by a factor of 10, resulting in globular embryo formation. Embryos were further elongated and developed to a cotyledonary stage on a filter paper support containing liquid medium without growth regulators.

Since then, several embryo development improvements have been reported for loblolly pine in a series of United States Patents. Gupta & Pullman (1990) reported improved cotyledonary embryo development of loblolly pine through the use of a multistaged culturing process where the maintenance medium contained an osmotic level raised from 158 to 240 Mm/Kg with myo-inositol as an osmoticant. The shift in osmotic level resulted in the further development of early-staged embryos of 10 cells or less to approximately 100 cells. These advanced embryos continued growth in the presence of abscisic acid while the earlier-staged embryos did not continue growth. Gupta et al. (1993) briefly discuss this improvement resulting in enlarged embryo head size.

Maltose, either alone or in combination with glucose, was reported as a superior carbon source compared to sucrose for development of cotyledonary-stage somatic embryos of loblolly pine (Uddin et al., 1990; Uddin, 1993).

Pullman & Gupta (1991) reported further improved loblolly pine embryo development using a combination of activated charcoal and abscisic acid (ABA). Eighty mg/l of ABA combined with 2.0 g/l of activated charcoal was optimal. When the same concept was tested with Norway spruce (*Picea abies*) and Douglas-fir (*Pseudotsuga menziesii*) 23 of 26 and 17 of 22 genotypes, respectively, responded with the production of cotyledonary embryos. In addition to the increased number of genotypes forming cotyledonary embryos, these embryos exhibited improved apical dome regions (more similar to zygotic embryos),

greater yields of cotyledonary embryos per ml of settled cells, were able to withstand several months of cold storage at 4-5°C, and upon germination showed improved vigor. Desiccation tolerance to less than ten percent water content with 80 to 90 percent germination was also achieved with Norway spruce embryos produced with a combination of activated charcoal and ABA (Gupta et al., 1993). The improved cotyledonary embryo yield and morphology was thought to be due to a slow decline in available ABA due to the adsorption of ABA by activated charcoal. A later U.S. Patent continued with the same line of thought and improved embryo yield and development by decreasing stepwise the ABA levels in media without activated charcoal (Gupta & Pullman 1993).

One of the major challenges ahead is in the area of embryo maturation. Few, if any, somatic embryo systems produce embryos similar in biochemistry or vigor to zygotic embryos. Many embryogenic systems appear to produce somatic embryos which are capable of germination and plant establishment, but which do not fully mature resulting in slow germination and initial growth.

7. Germination and Growth in the Field

Gupta & Durzan (1987a) first reported germination and continued growth of a somatic seedlings of loblolly pine under non-axenic conditions. From this study one somatic seedling from a single culture genotype was transferred to soil (Pullman & Gupta, 1991). This first somatic seedling of loblolly pine is growing at the Weyerhaeuser Company Technical Center in Centralia, Washington and is shown in Fig. 7 of Gupta & Durzan (1991).

Researchers at the Westvaco Corporation Forest Science Laboratory in Summerville, South Carolina have established three separate field plantings of somatic embryo plants (somatic seedlings) of loblolly pine (Fig. 4). A total of 583 somatic seedlings derived from 77 different genotypes of loblolly pine have been successfully established in the field during 1991, 1992 and 1993. The somatic seedlings were derived from embryogenic cultures initiated from immature zygotic embryos of 14 different open pollinated families and 5 different controlled crosses of loblolly pine. To date, survival of somatic seedlings that have been in the field for at least one year has been similar to conventional seedlings; 98% for the 1991 and 1992 field tests. The somatic seedlings appear phenotypically similar to conventional seedlings planted at

the same time (Fig. 4).

Fig 4

8. Conclusion

Loblolly pine is one of the most commercially important conifer species grown in North and South America today. Private and public breeding programs have worked to improve this species for over forty years. Clonal propagation through somatic embryogenesis provides a method to rapidly capture the gains in yield and raw material quality that have resulted from breeding programs and are likely to result from genetic engineering of conifers. In addition, conifer embryogenic systems offer a unique potential for large scale production of highly valuable genotypes.

Mass production of improved conifers by somatic embryogenesis is rapidly becoming a model for clonal propagation of high value plants. Embryogenic culture establishment, storage in liquid nitrogen culture banks, rapid early-stage embryo multiplication, embryo maturation, and somatic seedling establishment in the field have all been demonstrated for several conifers including loblolly pine. Based on the progress since the first report of somatic embryogenesis in loblolly pine in 1987, the large-scale production of this species appears to have a bright future.

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Index Words: somatic embryogenesis, *Pinus taeda*, loblolly pine, tissue culture, embryo maturation, megagametophyte

Table 1. Production of forest tree seedlings in U.S. in 1980. (From Boyer & South, 1984)

Region	Species	Number (millions)	Percent
Southeast U. S.	loblolly pine	966	60
	slash pine	167	11
	other pines	117	7
	other species	35	2
Total in Southeast		1,285	80
Total outside Southeast		329	20
Grand total in U. S.		1,614	100

Fig. Captions

Fig. 1. Native distribution of loblolly pine (*Pinus taeda* L.) in the United States (From Little, 1971; used with permission).

Fig. 2. Schematic diagrams of in vivo zygotic embryogenesis (A, B and C) and in vitro initiation of embryogenic tissue from zygotic embryos of individual immature loblolly pine seeds (D). A: Multiple fertilization results when gametes from separate pollen grains fuse with separate egg nuclei within archegonia. B: Multiple and genetically different zygotic embryos (shown as shaded or non-shaded) develop; simple polyembryony. In addition, each genetically different embryo cleaves to form four individual zygotic embryos; cleavage polyembryony. C: One of the zygotic embryos develops more vigorously (dominates) in comparison to the other (subordinate) zygotic embryos. Elongation of suspensor cells push the embryonal region of the dominant embryo toward the chalazal end of the megagametophyte and a corrosion cavity is formed. D: Culture of immature megagametophytes at the developmental stage shown in C results in initiation of embryogenic tissue from extruded zygotic embryos. Frequently, the dominant zygotic embryo remains intact at the chalazal end of the corrosion cavity and it is not extruded. Less frequently, subordinate zygotic embryos remain intact at the micropylar end of the corrosion cavity.

Fig. 3. Time course of three morphogenic responses of isolated immature zygotic embryo explants of loblolly pine. A: Initiation of cell division in suspensor region. B: Proliferation of embryogenic tissue. C: Proliferation of non-embryogenic callus. The stage of dominant zygotic embryo development: Precotyledonary (100%), Mostly Precotyledonary (>50%), Mostly Cotyledonary (>50%). Explants from open pollinated parent trees 7-34 (solid bars) and 11-9 (open bars). Culture conditions as described in Becwar et al., 1990.

Fig. 4. Somatic embryo plants (somatic seedlings) and conventional seedlings of loblolly pine planted April 1991 near Summerville, South Carolina. A: Three somatic seedlings derived from one embryogenic cell line. B: Three conventional seedlings from one open pollinated parent tree. The parent tree from which the embryogenic cell line originated was different than the parent tree of conventional seedlings. The photos were taken on February 17, 1994. The height bar is 2 m, with divisions every 25 cm.

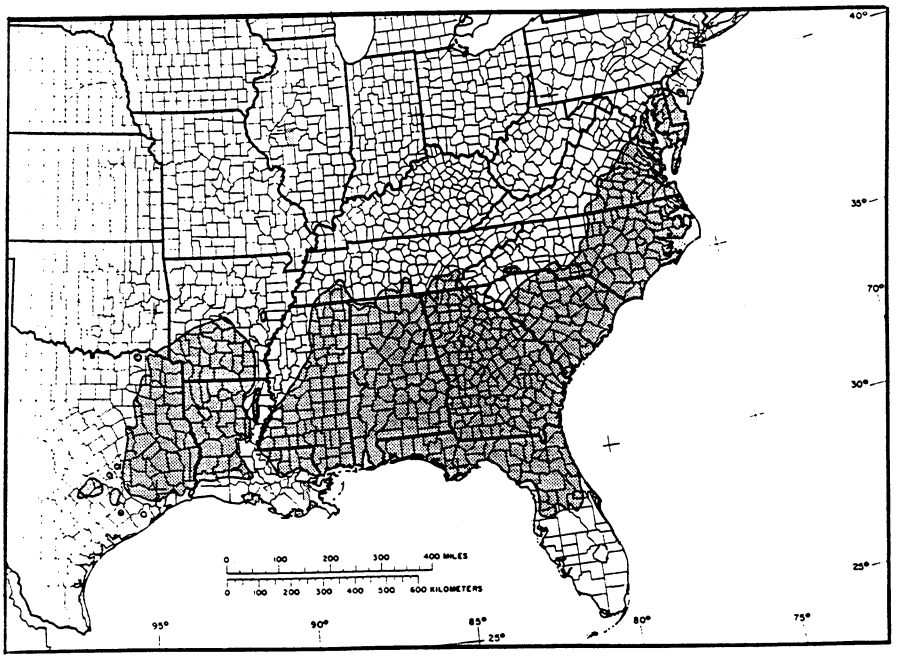
40°
35°
70°
30°
25°
0 100 200 300 400 MILES
0 100 200 300 400 500 600 KILOMETERS
95°
90°
85°
25°
80°
75°

Figure 1.

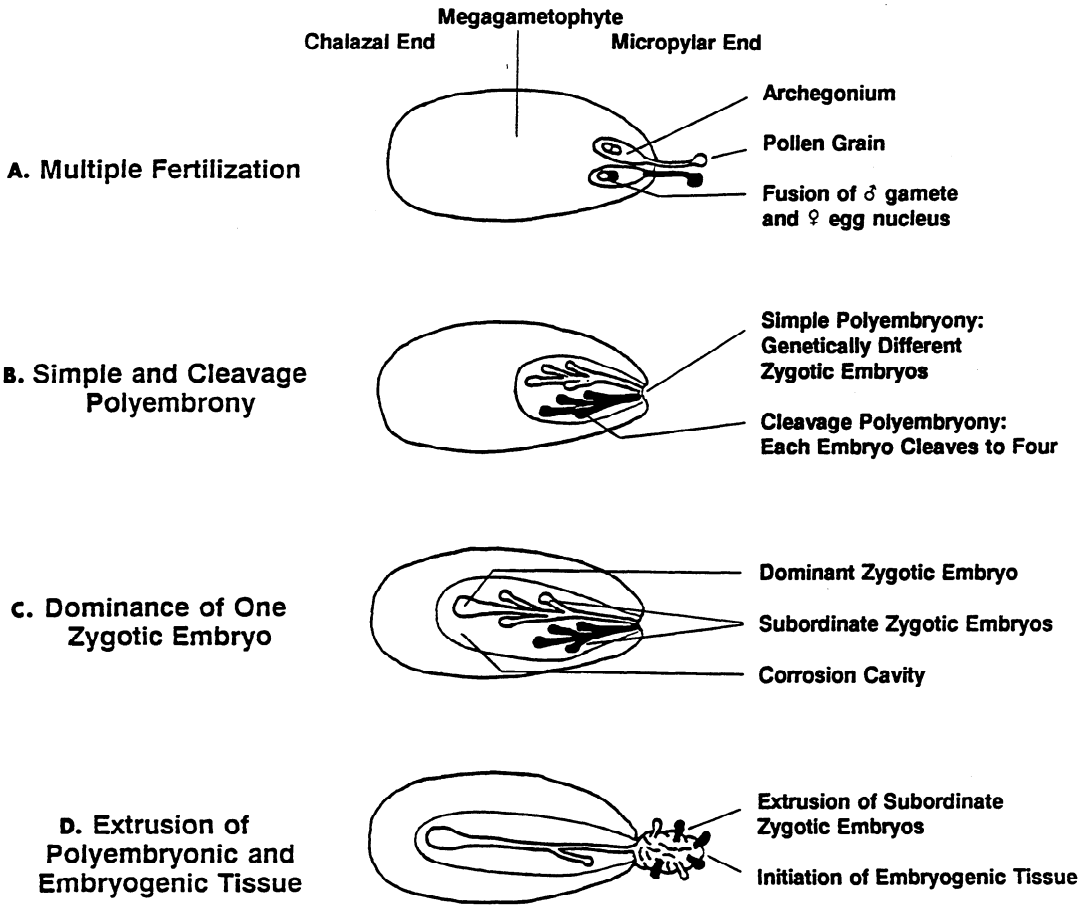
Megagametophyte
Chalazal End
Micropylar End
A. Multiple Fertilization
Archegonium
Pollen Grain
Fusion of ♂ gamete and ♀ egg nucleus
B. Simple and Cleavage Polyembrony
Simple Polyembryony: Genetically Different Zygotic Embryos
Cleavage Polyembryony: Each Embryo Cleaves to Four
C. Dominance of One Zygotic Embryo
Dominant Zygotic Embryo
Subordinate Zygotic Embryos
Corrosion Cavity
D. Extrusion of Polyembryonic and Embryogenic Tissue
Extrusion of Subordinate Zygotic Embryos
Initiation of Embryogenic Tissue

Figure 2.

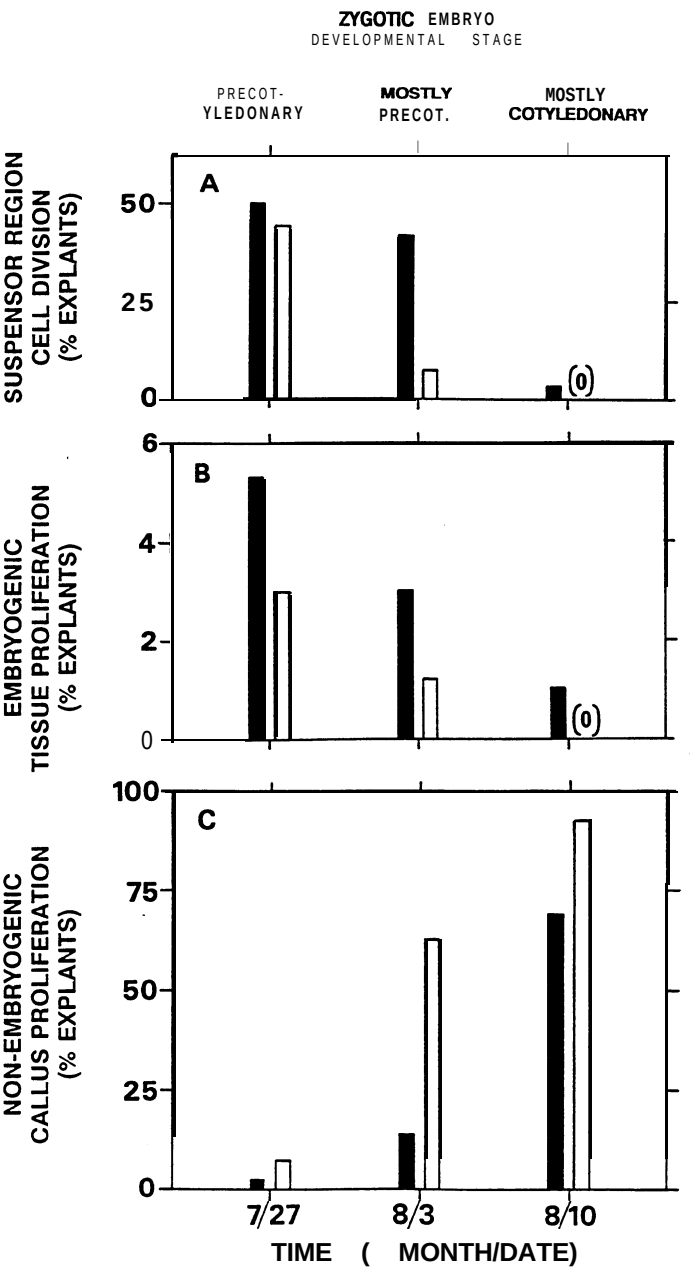
ZYGOTIC EMBRYO
DEVELOPMENTAL STAGE
PRECOT-
YLEDONARY
MOSTLY
PRECOT.
MOSTLY
COTYLEDONARY
SUSPENSOR REGION
CELL DIVISION
(% EXPLANTS)
A
50
25
0
(0)
EMBRYOGENIC
TISSUE PROLIFERATION
(% EXPLANTS)
B
6
4
2
0
(0)
NON-EMBRYOGENIC
CALLUS PROLIFERATION
(% EXPLANTS)
C
100
75
50
25
0
7/27
8/3
8/10
TIME (MONTH/DATE)

Figure 3.

Figure 4.