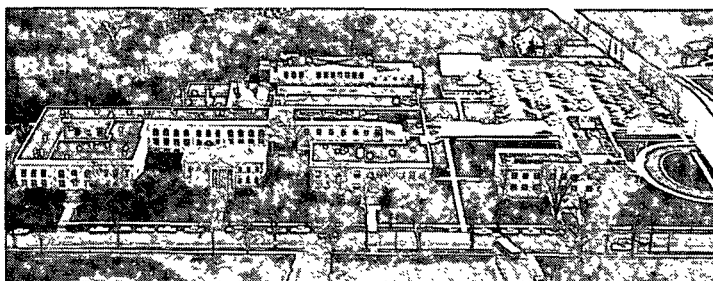


*Pas*



**THE INSTITUTE OF PAPER CHEMISTRY, APPLETON, WISCONSIN**

**IPC TECHNICAL PAPER SERIES  
NUMBER 204**

**PATTERNS AND PROTOCOLS FOR SOMATIC  
EMBRYOGENESIS IN WOODY SPECIES**

**STEVEN WANN**

**OCTOBER, 1986**

**Patterns and Protocols for Somatic Embryogenesis in Woody Species**

**Steven Wann**

**This manuscript is based on results obtained in IPC Project 3223,  
The Mass Production of Conifer Hybrids, and is to be submitted  
for consideration for publication in the Annual Review of Plant Breeding**

**Copyright, 1986 by The Institute of Paper Chemistry**

**For Members Only**

**NOTICE & DISCLAIMER**

The Institute of Paper Chemistry (IPC) has provided a high standard of professional service and has exerted its best efforts within the time and funds available for this project. The information and conclusions are advisory and are intended only for the internal use by any company who may receive this report. Each company must decide for itself the best approach to solving any problems it may have and how, or whether, this reported information should be considered in its approach.

IPC does not recommend particular products, procedures, materials, or services. These are included only in the interest of completeness within a laboratory context and budgetary constraint. Actual products, procedures, materials, and services used may differ and are peculiar to the operations of each company.

In no event shall IPC or its employees and agents have any obligation or liability for damages, including, but not limited to, consequential damages, arising out of or in connection with any company's use of, or inability to use, the reported information. IPC provides no warranty or guaranty of results.

# PATTERNS AND PROTOCOLS FOR SOMATIC EMBRYOGENESIS IN WOODY SPECIES

Steven Wann

## INTRODUCTION

Somatic embryogenesis is the process by which somatic cells develop into entire plants through a series of stages characteristic of zygotic embryo development. This process was first discovered in carrot but was quickly extended to many other plant families besides Umbelliferae. Today, somatic embryogenesis is almost considered an intrinsic characteristic of higher plants, representing the epitomy of totipotentiality. Accordingly, somatic embryogenesis has been reported in a variety of woody perennials (Table 1), the majority of which are trees. This list spans the wide array of trees utilized by man as sources of food, fuel, and fiber that includes the monocotyledonous palms, dicotyledenous (Citrus) and coniferous gymnosperms. Although the capacity for somatic embryogenesis is a property trees share with other plants, the context of woody perennials provides certain challenges and opportunities that set the process in trees apart from grasses and herbaceous plants. Not altogether unexpectedly, these challenges and opportunities arise from the very nature of trees - they are large organisms with long life cycles. For example, many trees have large inflorescence or seeds that undergo development over periods lasting several months. This feature facilitates the isolation and subsequent culture of many plant parts (nucellus, ovule, and immature embryo) that would be extremely difficult to culture in many nonwoody species. On the other hand, the explants described above are only available at discrete time intervals throughout the year. Furthermore, because this explant material is only available from sexually mature trees, it must be brought in from the field, with all the attendant problems of sterilization and physiological variation due to climate and site.

Table 1. Taxa of woody species in which somatic embryogenesis has been reported.

Angiosperms

Monocots	Dicots
Palmae	Magnoliaceae (yellow poplar)
(date, oil palm, coconut	Santalaceae (sandalwood)
palmetto)	Juglandaceae (walnut spp.)
Muscaceae	Betulaceae (filbert)
(plantain)	Sterculiaceae (cacao)
	Leguminosae (E. Indian walnut)
	Rosaceae (apple, pear, loquat)
	Vitaceae (grape)
	Rutaceae ( <u>Citrus</u> spp.)
	Rubiaceae ( <u>coffee</u> )
	Aquifoliaceae (holly)
	Hamaelidaceae (sweet gum)
	Scrophulariaceae ( <u>Paulownia</u> )
	Saxifragaceae (red currant)
	Myrataceae (rose, malay apple)
	Caricaceae (papaya)
	Anacardiaceae (mango)

Gymnosperms

Coniferophyta	Cycadophyta
Pinaceae	Cycadaceae ( <u>Zamia</u> )
Picea (Norway spruce)	
Larix (European larch)	
Pinus (sugar pine)	

This review is intended to represent a comprehensive compilation of published procedures that were successfully used to obtain somatic embryogenesis in woody species. Within the last 10 years, the list of species has expanded to the extent that some means of categorization would be useful. This review makes several attempts at categorizing. Obviously, taxonomic categorization is a rational start, but perhaps more importantly an attempt is made to collect species with respect to the pattern and protocol of somatic embryogenesis. Categorization with respect to the pattern and protocol of embryogenesis should

assist in the development of a generalized methodology for extending somatic embryogenesis to other species.

The pattern of embryogenesis identifies the contribution of the physiological state of the explant to the process of embryogenesis. The physiological state of the explant will to a large extent define the cultural protocol that is required to achieve somatic embryogenesis. Nevertheless, within a pattern a variety of methods and culture procedures have been used to achieve somatic embryogenesis. Thus, organizing the embryogenic process with respect to protocol adds the necessary detail to the pattern that transforms the theory into the practice of embryogenesis.

#### **Category 1: Patterns of Somatic Embryogenesis**

The basic approach to the control of morphogenesis in vitro has been through the manipulation of the physical, nutritional and hormonal environment. While this approach has led to outstanding success in the development of propagation systems in a wide range of species, it should be appreciated that success in any particular species is often limited to a single type of starting tissue. In many species the goals of the in vitro propagation program are such that the choice of explant material is of little consequence. However, in most woody species, propagation of proven genotypes is highly desirable. In these cases explant material is restricted to tissues that can be reliably considered to express the proven genotype. Unfortunately at the present time, embryogenesis in many woody species can only be achieved from embryonic material. The cloning of such unproven tissues is of little practical value and serves to highlight

the pressing need for extending somatic embryogenesis to a wider range of tissue explants.

The observation that the internal state of the explant is critical to the successful expression of somatic embryogenesis suggests that the in vitro system "permits" as well as "induces" the development of embryoids or embryogenic tissue. In accordance with a recently developed working hypothesis (Sharp et al., 1982) both permissive and inductive patterns of somatic embryogenesis are possible. For the purposes of this review, these two patterns will form the basis of the first category of embryogenesis in woody species.

Before consideration of Sharp's hypothesis of patterns of embryogenesis, it is important to define several terms that will be used throughout this review. Competence is defined as the capacity to express an inherent potential. Determination is the process by which the developmental potential becomes restricted to a particular pathway. Induction can take two forms. Either it is directive, meaning a change in competency, or it is permissive meaning an induction of an already determined developmental response (Amirato, 1985).

In Sharp's hypothesis there are two patterns of somatic embryogenesis. In the first, embryogenesis proceeds from cells that are already determined for embryogenesis prior to explanting. Embryogenesis from these pre-embryogenic determined cells (PEDC) requires only an in vitro environment to release them into the requisite pattern of cell division. In contrast, induced embryogenic determined cells (IEDC) require an in vitro environment not only to reenter the mitotic cycle, but also for redetermination of the formerly quiescent cells to an embryogenic state. Note that embryogenesis from both PEDCs and IEDCs are inductive - the former being permissive while the latter is directive.

Although this somewhat artificial system of classification may suffer from overgeneralization, the division of somatic embryogenesis into two patterns is useful as long as the limitations of the categorization are appreciated. An example of the utility of this classification is that it delineates a possible role for plant growth regulators as agents that contribute to the determination process, but not directly to embryogenesis. Cells undergoing embryogenesis by a PEDC pattern often require no exogenous growth regulators, and, in some cases, the initiation of embryogenic tissue is inhibited by their addition. However, in examples of PEDC where growth regulators are applied after the proliferation of embryogenic tissue the result is the formation of a callus consisting entirely of masses of proliferating proembryos. These examples are best considered PEDC cloning, and the role of growth regulators can be considered as agents to stimulate division that result in more PEDCs. In IEDC systems, a distinct dedifferentiated callus phase is required for redetermination, and the presence of growth regulators ensures callus formation with the attendant redetermination.

Direct and indirect embryogenesis are two additional terms used to describe PEDC and IEDC embryogenesis, respectively. Both terms are descriptive but useful in that they convey whether or not a callus phase is present (except in PEDC cloning), which correspondingly defines the relative timing of determination. However, the two patterns become indistinguishable when considering embryos arising from a population of proliferating embryonic cells. This can be restated in the form of a question: (1) When is an embryonic cell in an explant developmentally determined for an alternate function and (2) How can callus formation be distinguished from the early stages of embryogenesis? In many instances a glance at the type of tissue explant used easily defines the pattern of embryogenesis. Tissue associated with zygotic embryogenesis (nucellus, ovule,

proembryos) can all be considered as PEDC systems. Gradually, tissue specialization occurs in these explants such that redetermination will be required to regain competence for embryogenesis. The further removed from the state corresponding to PEDC, the longer it will take to regain embryogenic competence, and this will be reflected morphologically by an increased and/or extensive callus formation. For a cell residing near the cusp of determination for an in vivo specialized function, it becomes very difficult to distinguish between the early stages of embryogenesis or the early stages of callus formation. In situations like this (and they are not uncommon in woody species) this review will identify the pattern of embryogenesis as IEDC if a true callus phenotype is described. If embryos appear directly, without any visible callus phase, these examples will be considered PEDC.

## **Category 2: Protocol for Somatic Embryogenesis**

Auxin has historically been the class of growth regulators that have been used to effect somatic embryogenesis. In particular, the synthetic auxin 2,4-D has found the greatest applicability in obtaining embryogenesis - a full 68% of the reports of somatic embryogenesis in monocots employ 2,4-D as the only plant growth regulator (Sondahl, 1985).

The mechanisms by which 2,4-D operates to achieve embryogenesis is not well understood and is outside the scope of this review. In PEDC systems, auxin is used to clone PEDCs and in IEDC it is the mitogenic substance that achieves redetermination. In this review plant growth regulators can be considered as having two basic functions in plant tissue cultures - initiation and maintenance of embryogenesis. In embryogenesis, the sequence in which growth regulators are applied depends upon the pattern of embryogenesis. While it can easily be

appreciated that at some time all growth regulators must be removed to produce a free-living plant, this timing in application and removal is variable from species to species. In this review, the timing and sequence of application has been broken down into five basic protocols:

1. High auxin ( $\pm$  cytokinin)  $\rightarrow$  low auxin ( $\pm$  cytokinin)
2. Auxin ( $\pm$  cytokinin)  $\rightarrow$  same
3. Auxin ( $\pm$  cytokinin)  $\rightarrow$  no growth regulators
4. No auxin applied ( $\pm$  cytokinin)
5. Cytokinin ( $\pm$  auxin)  $\rightarrow$  auxin ( $\pm$  cytokinin)

In the tables that follow the protocols will be referred to by the above numbering system. For those cases in which auxins are not employed in a particular initiation protocol, the same number system will be used but lack of auxin will be denoted by a prime. Thus, 5' means initiation on cytokinin containing medium followed by transfer to auxin containing medium that may or may not contain cytokinin; 2' and 3' indicate initiation on cytokinin containing medium followed by transfer to the same medium, or no growth regulators, respectively. The "plus" or "minus" within the parentheses denotes whether the medium may contain or lack, respectively, the indicated substance.

## DISCUSSION

### Direct Somatic Embryogenesis from PEDCs

The examples shown in Table 2 are easily classified as PEDC systems due to the rapid appearance of somatic embryos directly from the explant. The absence of callus proliferation in all these examples (except English holly) would seem to preclude a subculturable system that would allow a continuous production of embryos. While this is true for most of the examples listed in Table 2, red currant, cacao, and walnut, all exhibit sustained embryo propagation by

continuous, direct embryogenesis from the adventive embryos isolated from the explant. In English holly and cacao, sustained embryogenesis also occurs but this is through a callus phase that is later derived from the adventive embryos. For this reason, sustained embryogenesis in English holly and cacao is best considered as "PEDC cloning," (see Table 3), while the production of embryos from the explant constitutes an example of PEDC in these species.

Table 2. Examples of direct somatic embryogenesis from PEDCs in woody species (Dicots).

Species	Explant	Protocol	Reference
<u>Citrus grandis</u> (pummelo) <u>C. limon</u> (lemon) <u>C. reticulata</u> x <u>C. sinensis</u> (orange)	nucellus (fertilized ovules)	3,4	Ragan <u>et al.</u> , 1968
<u>C. sinensis</u> (navel orange) <u>C. paradisi</u> (grapefruit)	nucellus (unfertilized ovules)	5	Kochba <u>et al.</u> , 1972
<u>C. sinensis</u>	unpollinated, unfertilized ovules	4	Button and Bornman, 1971
<u>Mangifera indica</u> (many)	nucellus (pollinated ovules)	2',5'	Litz <u>et al.</u> , 1982
<u>Ribes rubrum</u> (red currant)	fertilized ovules	2',3'	Zatyko <u>et al.</u> , 1975
<u>Ilex aquifolium</u> (English holly)	embryos	4	Hu and Sussex, 1971
<u>Albizzia lebeck</u> (E. Indian walnut)	hypocotyl	4	Gharyal and Maheshwari, 1981
<u>Theobroma cacao</u>	immature embryo	3	Pence <u>et al.</u> , 1980
<u>Juglans regia</u> <u>Juglans hindsii</u> (walnut)	cotyledon	3	Tulecke and McGranahan, 1985
<u>Malus domestica</u> (apple)	nucellus (fertilized ovules)	4	Eichholtz <u>et al.</u> , 1979
	leaves from seedlings	2	Liu <u>et al.</u> , 1983
<u>Pyrus spp.</u> (pear)	nucellus (fertilized ovules)	4	Janick, 1982

Table 3. Examples of PEDC "cloning" in woody species.

Species	Explant	Protocol	Reference
<u>Citrus microcarpa</u>	nucellus (fertilized ovules)	2	Ranga Swamy, 1961; Mahestawari and Ranga Swamy, 1958
<u>C. aurantifolia</u> <u>C. sinensis</u>	nucellus (unpollinated, un- fertilized ovules)	5	Mitra and Chaturvedi, 1972
<u>C. sinensis</u>	nucellus (unpollinated, un- fertilized ovules)	4	Button and Botha, 1975 Button <u>et al.</u> , 1974 Kochba and Button, 1974 Kochba and Spiegel- Roy, 1973 Spiegel-Roy and Kochba, 1973
<u>Carica papaya</u> (papaya)	pollinated, immature ovules	2' 5'	Litz and Conover, 1981, 1982 Litz and Conover, 1983
<u>Mangifera indica</u> (mango)	nucellus (fertilized ovules)	5' 2'	Litz <u>et al.</u> , 1984 Litz, 1984
<u>Eugenia jambos</u> (rose apple)	immature, adventitious embryos	5'	Litz, 1984
<u>E. malaccensis</u> (Malay apple)	(nucellar) embryos		
<u>Myrciaria cauliflora</u> (jaboticaba)	immature, adventitious (nucellar) embryos	5'	Litz, 1984
<u>Ilex aquifolium</u> (English holly)	immature embryos	4	Hu <u>et al.</u> , 1978
<u>Vitis vinifera</u> (grape) <u>V. vinifera</u> x <u>V. rupestris</u>	unfertilized ovules	5	Srinivasan and Mullins, 1980
<u>Eriobotrya japonica</u> (loquat)	fertilized ovules	1	Litz, 1985
<u>Theobroma cacao</u>	somatic embryos	2	Kononowicz <u>et al.</u> , 1984

The phenomenon of repetitive embryogenesis by direct production or "budding" of new embryos on the surface of previously differentiated embryos is quite common. Although this process is exemplary of PEDC systems as a principal means of propagation via embryogenesis, it is also found in many IEDC systems as well. In fact 20% of the reports of somatic embryogenesis in this review describe further cycles of direct embryogenesis, although the process may not be relied upon for plant propagation.

Embryo budding has been considered a manifestation of the tendency toward independent behavior exhibited by embryogenic cells. Somatic embryogenesis has long been set apart from organogenesis as being a single cell as opposed to a multicellular event (Reinert, 1977). The criteria of cell separation as reflected in callus friability has also been taken as an indication of the importance of cellular independence in embryogenic systems. Embryo budding is therefore considered as a departure from integrated behavior of a cell or group of cells that allows them once again to express their potential in an additional cycle of embryogenesis (Williams and Maheswaran, 1986). The relative number of cells that depart from a pattern of organized development within an embryo and reinitiate new embryos can be determined by the extent of attachment of the secondary embryos. For example, in cacao, subsequent rounds of somatic embryos are initiated as glandular "hairs" on the hypocotyl surface - perhaps reflecting a single-cell origin (Pence et al., 1980). In English holly, a multicellular origin can be ascertained by the fused appearance of somatic embryos with the underlying cotyledonary tissue (Hu and Sussex, 1971).

Aside from repetitive embryogenesis from adventive embryos in the above-mentioned species, all other examples listed in Table 2 do not provide a sub-culturable system under the protocols described. However, by alternate methods

PEDC cloning can be achieved in mango and several of the Citrus species. Despite the lack of a subculturable system, embryogenesis in C. grandis, C. limon, and C. reticulata x C. sinensis is of considerable value even though only a limited number of plants are produced, because these species are normally monoembryonic. Nucellar embryony in Citrus has been known since Leewenhok, and has found considerable application in Citrus horticulture by (1) providing clonal, rejuvenated plants and (2) exploiting the observation that nucellar embryos can be used to produce virus-free plants. The lack of a requirement for polyembryony and fertilization reinforces the notion that this tissue is pre-embryogenically determined and needs only a release from the constraints of the developing seed to express its potential. Pre-embryogenic determination is also implied by the types of protocols used to achieve somatic embryogenesis in these species. Only 25% of the examples in Table 2 require an auxin to stimulate embryogenesis, and 50% of the examples require no growth regulators to initiate embryogenesis.

For sustained embryogenesis, cells apparently remain in a pre-embryogenically determined state, as only one of four species that sustain embryogenesis requires auxin (cacao), one requires cytokinin (red currant), and the other two (walnut, holly) can proliferate somatic embryos for considerable periods of time (e.g., walnut, 18 months) without the application of growth regulators.

Observations such as these lend considerable support to the concept of permissive induction from an appropriate explant. Thus the state of the tissue explant becomes the dominant feature in the successful initiation and maintenance of somatic embryogenesis. All examples in Table 2 exhibit explant tissue that is only receptive toward embryogenesis for a definite period of time.

In examples of embryogenesis from nucellar tissue, this "window" of embryogenic potential can last 2-3 weeks, although the window is stringently defined. For embryogenesis from seedling or embryo explants the time period depends on the rate of maturation or germination. For example, in walnut cotyledons can be taken from immature embryos 6-11 weeks after pollination, holly embryos must be taken at the heart stage, and 10 to 15-day-old E. Indian walnut seedlings are used as explants.

In all cases (except for mango, pear, cacao, and apple nucellar embryos) somatic embryos were readily converted into plantlets. A common feature of direct somatic embryogenesis is that even if it is from single cells, the physical limitations of the explant size restrict the absolute number of embryos produced per cycle. Of course, for these systems in which no subculturable material is available the number of embryos produced per culture is small. For example Citrus spp. produce "one to several" per ovule/nucellus (Ragan et al., 1969). In E. Indian walnut, apple, holly, cacao, and walnut, the number of embryos produced per culture (or per cycle) is on the order of 1-3 dozen. In red currant, single embryos were reported in some instances to give rise to thousands of embryoids.

Although direct embryogenesis suffers from the inability to conveniently produce large numbers of embryos (except for English holly, cacao, and red currant, which eventually become PEDC cloning systems), it has the potential advantage of having the ability to produce plants rapidly without passing through a callus phase. This feature might become a distinct advantage should cell cultures induce variability that cannot be tolerated. Should it become necessary to exploit direct embryogenesis for clonal propagation, in species

employing nucellar tissue as explants the procedure could become viable due to the abundance of explant material per plant.

The conversion frequency, or the ability of somatic embryos to "germinate" and develop into plants, is highly variable for PEDC systems. In monoembryonic Citrus, though not explicitly stated, the conversion frequency seems close to 100%. In C. sinensis and C. paradisi the frequency is on the order of 30-50% (Kochba et al., 1972). In walnut and apple, the frequency drops to 10 and 5%, respectively. In red currant, although the formation of thousands of embryoids is reported, only a few could be converted into plants (Zatyko et al., 1975). Finally, the conversion frequency for E. Indian walnut was not reported, although plants were obtained. In mango, cacao, and pear no information is given on the conversion of embryos to plants.

In some cases, one factor that contributed to a reduced conversion frequency was the formation of abnormal embryos. Although the almost universal abnormality was pluricotily, this was not a factor contributing to poor conversion, as many multiple-cotyledon embryo developed shoots with a normal appearance. Only gross, macroscopic abnormalities are cited and then only as circumstantial evidence for the lack of normal growth. Some of these abnormalities include multiple apices, failure of stem elongation, faciated appearance, fused cotyledons, and neomorph formation. In most of the reports the picture emerges that the growing-on process acts as a sieve to remove at least grossly abnormal embryos that reach a significant level of development but are nevertheless incapable of normal germination. Whether or not the regeneration protocol is capable of sifting out more subtle variations remains an area of long-term investigation.

## PEDC "Cloning"

The cloning of pre-embryogenic determined cells represents a method in which direct embryogenesis can be converted into a system that will continually produce proembryos. Accordingly, what is sacrificed in terms of the direct production of a limited number of highly differentiated structures is compensated for by a large scale proliferation of embryonic primordia. However, if these primordia (i.e., proembryos) can be developed into plants, this method may become an effective way to exploit PEDC systems for plant propagation.

Cloning systems for PEDCs are characterized by a callus phase, but the term callus is really a misnomer. Instead of cultures consisting of unorganized tissue, PEDC callus consisting entirely of a proliferating mass of proembryos is highly organized. This phenotype has been described in the case of Citrus aurantifolia (Mitra and Chaturvedi, 1972), Citrus sinensis (Button et al., 1974; Mitra and Chaturvedi, 1972), Carica papaya (Litz and Conover, 1982), and Mangifera indica (Litz, 1984). In other PEDC cloning systems a tendency toward a highly organized callus is observed (Malay and rose apple, jaboticaba, and grape) but proembryonal structures tend to be dispersed in a matrix of less organized, paranchymatous tissue. English holly and cacao are also considered under PEDC cloning systems because the products of PEDC's (adventive embryos) become the second generation explants from which the embryogenic callus is derived. Regardless of the extent of organization, in all examples the callus phase is not required for redetermination because PEDCs are the cells from which the callus is initiated.

An outstanding feature of PEDC cloning is the timing required for the application of auxin to produce the proliferation of proembryonal structures. Although 10 of the 12 examples (83%) require an auxin for sustained embryogenesis,

in 6 of these examples the application of auxin after an initial proliferation of embryoids/or embryogenic tissue in the absence of auxin is necessary. Recall from an earlier discussion of permissive induction that the in vitro environment permits embryogenesis to occur, and therefore no directive inducing agent such as auxins are required. Therefore, if auxin is used only to clone PEDC, why can't it be applied at the time of culture initiation? It might be that embryogenesis proceeds more efficiently from partially organized structures (proembryos) than from individual or groups of pre-embryogenically determined cells. Given that the structure of PEDC-cloned callus resembles "budding" proembryos, the presence of partially differentiated structures provides the requisite group of cells for this process to occur. Thus, the entire process could be envisaged as (1) explanted tissue containing PEDCs are cultured under conditions that allow the formation of a limited number of proembryos and (2) the application of auxin to proembryos stimulates the large-scale proliferation of proembryos by a budding process.

Somatic embryogenesis by PEDC cloning represents examples of prolific embryogenesis in woody species. In grape, PEDC cloning produces 1200-1400 somatic embryos per ovule (Srinivasan and Mullins, 1980) and papaya and mango easily approach  $10^3$  embryos/ovule (Litz, 1985; Litz and Conover, 1983). In Citrus and English holly, somatic embryo production is similar to the amount obtained in PEDC systems (1-3 dozen/culture) except the system is now subculturable for extended periods. In all other species shown in Table 3 the level of somatic embryogenesis has not been reported.

While many of these reports indicate significant if not high yields of somatic embryos that can be continually produced at regular intervals, the conversion frequency of somatic embryos into plants is often very low or unreported.

However, once plantlets are obtained, the subsequent transfer to soil and growing on is usually readily achieved, once again implying that the conversion process acts as a sieve to eliminate variants.

In PEDC systems, there appears to be an inverse ratio between the number of embryos produced and their subsequent ability to develop into plants. For example, in C. sinensis callus, where several dozen embryos are produced per culture, the conversion frequency approaches 50% (Kochba et al., 1972). In English holly this frequency approaches 100% (Hu et al., 1979). However, in papaya, mango and grape, though not reported, it seems that only a handful of plants can be produced from literally thousands of embryos. The situation with tropical fruit trees is indeed disturbing because it has all the requisites for a large-scale propagation scheme - clonal explant material, prolific embryogenesis and an efficient liquid culture system. The only drawback is that it does not produce large numbers of viable plants. Clearly, developments in tropical fruit trees (as in palms) should be followed closely, as they represent a model system for the commercial exploitation of somatic embryogenesis in woody species.

#### **Embryogenesis in IEDC Systems**

Somatic embryogenesis from PEDCs as it is currently practiced and understood is primarily a phenomenon of nucellar tissue in temperate and tropical fruit trees. Although PEDCs have been associated with in vivo nucellar embryogenesis, the experience with monoembryogenic Citrus and mango implies that this is not a prerequisite. Nevertheless, the utilization of nucellar/ovular tissue has not been extended to any of the additional 12 families (Litz, 1985) in which nucellar polyembryony has been reported. Furthermore, nucellar tissue has not been widely tested as an explant source in the numerous remaining families in which it could be readily isolated and cultured. While PEDC systems

have been explored as a method to produce asexual embryos in only a few dicotyledenous species, IEDC systems have found a wide range of applicability in mono- and dicots as well as gymnosperms. The versatility to a large extent arises from the very definition of the IEDC system. In particular, differentiated cells must become redetermined by a successive series of mitotic divisions. Ultimately, all cells, regardless of their initial state of differentiation must arrive at a similar meristematic ground state. Of course, some cells need little in the way of mitotic events for redetermination, while others clearly are too highly differentiated (especially if secondary cell wall formation has occurred) to ever be redetermined. In between the two extremes of PEDCs and highly differentiated cells reside a wide range of cell types (e.g., hypocotyl epidermis, leaf mesophyll, apical and lateral meristems, etc.) that share the capacity for reentry into the mitotic cell cycle if given an appropriate stimulus.

In the majority of examples of IEDC, the appropriate stimulus is auxin. Out of the 31 reports of embryogenesis (Table 4) via cell cultures (callus or suspension) 30 require the application of an auxin. The predominant auxin used is 2,4-D, which is used in two-thirds of the examples. In the remaining species, the majority of the examples utilize NAA while some employ IBA and IAA. All species requiring auxin involve the initiation of cultures on auxin-containing medium. This stands in sharp contrast to PEDC or PEDC-cloning systems, where auxin is generally not used in the initiation medium.

Once embryogenic tissue is initiated, most IEDC and PEDC systems utilize auxins for the maintenance and/or expression of embryogenic potential. In IEDC systems 26 of the 30 examples utilizing auxin require its application for both initiation and maintenance of embryogenic potential.

Table 4. High frequency somatic embryogenesis from callus in woody species (IEDC).

Species	Explant	Protocol	Reference
Angiosperms - Monocots			
<u>Veitchia merrilli</u> (Christmas palm)	embryos	2	Srinivasan, 1985
<u>Chamedorea costaricana</u> <u>Howeia forsteriana</u> (Kentia palm)	immature embryos	2	Reynolds and Murashige, 1979
<u>Phoenix dactylifera</u> (date palm)	immature embryos	2	Reynolds and Murashige, 1979
	embryos	2	Tisserat, 1980
	apical tips	2	Tisserat, 1980;
	lateral buds	2	Tisserat and De Mason, 1980
	meristele inflorescence	2 2	Tisserat, 1980 Reynolds and Murashige, 1979, Tisserat, 1980
<u>Phoenix pusilla</u> <u>Erythea edulis</u> <u>Brahea armata</u> <u>Livistonia decipiens</u> (fan palm) <u>Sabal minor</u> (bush palmetto)	embryos	2	Tisserat, 1984
<u>Elaeis guineensis</u> (oil palm)	leaf	1	Blake, 1983
	root	1	Hanower and Pannetier, 1982
<u>Cocos nucifera</u> (coconut palm)	leaf	2	Pannetier and Buffard-Morel, 1982
<u>Musa paradisiaca</u> (plantain)	shoot cultures	2	Cronnauer and Krikorian, 1983

Table 4 (Contd.). High frequency somatic embryogenesis from callus in woody species (IEDC).

Species	Explant	Protocol	Reference
Angiosperms - Dicots			
<u>Corylus avellana</u> (filbert)	cotyledonary nodes	1	Perez <u>et al.</u> , 1983
	immature embryos	2	Radojevic <u>et al.</u> , 1975
<u>Coffea arabica</u> (coffee)	leaves	1	Sondahl and Sharp, 1977
		2'	Yasuda <u>et al.</u> , 1985
<u>Liriodendron tulipifera</u> (yellow poplar)	immature embryos	3	Merkle and Sommer, 1986
<u>Paulownia tomentosa</u>	embryos, fertilized ovules	2	Radojevic, 1979
<u>Santalum album</u> (sandalwood)	embryos	2	Rao, 1965
	shoot cultures	2	Rao and Bapat, 1980
		3	Lakshmi Sita <u>et al.</u> , 1979
<u>Malus pumila</u> (apple)	seedling pieces	1	Mehra and Sachdeva, 1980
<u>Pyrus communis</u> (pear)	seedling pieces	1	Mehra and Jaidka, 1980
<u>Vitis vinifera</u> (grape)	stem	1	Krul and Worley, 1977
<u>Carica stipulata</u>	peduncle	2	Litz and Conover, 1980
Gymnosperms			
<u>Larix decidua</u> (European larch)	fertilized ovules	3	Nagmani and Bonga, 1985
<u>Pinus lambertiana</u> (sugar pine)	immature and mature embryos	2	Gupta and Durzan, 1986
<u>Picea abies</u> (Norway spruce)	immature embryos	2	Hakman <u>et al.</u> , 1985 Hakman and von Arnold, 1986
	embryos	2	von Arnold and Hakman, 1986; Krogstrup, 1986
<u>Zamia pumila</u>	embryos	2	Webb <u>et al.</u> , 1983

Systems involving IEDCs are noted for the presence of a callus phase. One of the most striking features of embryogenesis of woody species is that embryogenic callus can be readily recognized as being distinctly different from nonembryogenic callus. The differences between the two callus types are of sufficient magnitude that they are manifested on a macroscopic level as differences in color, texture, and morphology. While all these types of differences can be used as markers to identify embryogenic tissue, the most convincing marker is morphology. That is, embryogenic callus of woody species can often be easily recognized because it is often loaded with somatic embryos! While variations exist between species as to level of development that somatic embryos exhibit while in the callus matrix, in all the species recorded in Table 4 embryogenic callus exhibits some aspect of organization. In angiosperms, this tends to be a friable parenchymatous matrix containing light colored nodules. In gymnosperms the single cycad, Zamia resembles angiosperm embryogenic tissue, whereas conifers exhibit a heretofore undescribed phenotype consisting of somatic embryos dispersed in an extracellular, mucilaginous matrix containing tubular callus-type cells (Hakman et al., 1985). A characteristic embryogenic phenotype is not confined to woody species but is also present in cereals, where successful embryogenesis has been dependent on the ability to recognize and segregate an embryogenic callus out of a mixed culture comprised of both embryogenic and nonembryogenic callus (Nabors et al., 1983).

Embryogenesis in IEDCs in which a clearly recognizable embryogenic callus is produced has been termed high-frequency somatic embryogenesis (HFSE; Sondahl and Sharp, 1977). This is an appropriate term due to the high density of somatic embryos per culture. In palms, this can be up to 500 embryos per single 8-week-old culture, (Tisserat, 1982), in coffee, 100 embryos/7 mm<sup>2</sup> leaf disc

(Sondahl and Sharp, 1977), in Norway spruce, 1000 embryos/g (Becwar et al., 1986), and in grape, "several hundred"/cm<sup>3</sup> (Krul and Worley, 1977).

Somatic embryos in embryogenic callus often exhibit a wide range in development within a culture. This asynchronous mode of development may reflect an escape from integrated control reminiscent of the budding process in PEDCs except that it is on a less differentiated level. The effect is that embryogenic callus becomes an asynchronous system of plant production. Unfortunately, this then becomes a rather tedious method of plant propagation, as the more highly developed embryos must be continually teased away from the less developed embryos. The establishment of suspension cultures as well as methods to synchronize remain important barriers to the large scale exploitation of HFSE.

Despite these practical limitations IEDC systems have considerable potential as a propagation method due to the extended periods over which embryogenesis can be sustained, as well as the respectable conversion frequencies that have been observed. Both date and oil palm cultures produce plantlets at nearly a 100% quantitative conversion frequency and will sustain this process for years. In oil palms, this has been commercially exploited by Unilever to establish plantations of somatic embryo clones of high yielding oil production (Blake, 1983). Although considerable success, has been achieved in palms, not all monocotyledonous species have followed this same course. Coconut and plantain are two species in which somatic embryogenesis has been obtained but in which it has not yet been possible to produce entire plants.

Dicotyledonous plants can also sustain embryogenesis for years as is the case with filbert, coffee, Citrus, and sandalwood. Although somatic embryos in dicots are not reported to convert to plants as readily as monocots, the low

frequency has been compensated for by the prolific nature of the cultures. For example, filbert embryos grow into plants at about 50% frequency; coffee, 30-40%; sandalwood, 20-30%; and yellow-poplar, 10%.

An encouraging feature of IEDC in woody species is that in the 25 species producing embryogenic callus almost one-third (8/25) of the species will produce embryogenic tissue from mature (clonal) explants. Once an embryogenic state is achieved callus from mature explants is indistinguishable from callus obtained from immature (embryonal) explants in terms of the level and duration of embryogenesis and the quality of the plants obtained.

In gymnosperms, the conversion frequency to plantlets is low (< 1%) or unreported for conifers and the cycad, Zamia pumila. In the family Pinaceae it appears that embryogenic callus exhibits the same phenotype regardless of species or explant. This phenotype is striking as previously noted and suggests that extension of somatic embryogenesis to other conifers and coniferous tissue explants might be made by using this phenotype as a physical marker to streamline treatment evaluation.

Embryogenesis from IEDC systems has been further subdivided into high- and low-frequency somatic embryogenesis (Sondahl and Sharp, 1977). By definition, the majority of the IEDC systems reported here are high frequency due to the presence of a distinct embryogenic callus. Low-frequency somatic embryogenesis (LFSE) refers to the sporadic production of embryos directly from a callus that is nondistinctive (Table 5). Somatic embryos in these cases are considered to arise as a result of a quantal cell division that produces two daughter cells with an unequal distribution of cytoplasmic contents (Sondahl, 1985). One of these cells is then embryonic, and proceeds to form an embryo directly. Due to this direct embryo formation (which required a previous redetermination event)

there is no phenotypically distinguishable embryogenic callus. Additionally, the process is termed LFSE because of its apparently random and unsustainable nature.

Table 5. Low frequency somatic embryogenesis (LFSE) in woody species.

Species	Explant	Protocol	Reference
<u>Liquidambar styraciflua</u> (sweet gum)	hypocotyl	2,3	Sommer and Brown, 1980
<u>Coffea arabica</u>	leaf	1	Sondahl and Sharp, 1977
<u>Elaeis guineensis</u> (oil palm)	leaf	2	Hanower and Pannetier, 1982

In two species (coffee and oil palm) a relationship between HFSE and LFSE is evident due to the observation that both can take place on the same culture in a sequential fashion (LFSE first) under appropriate conditions. In both coffee and oil palm the sequential application of 2,4-D followed by a somewhat lower level of NAA (coffee) or 2,4-D (palm) produces HFSE. However, sustained application of constant levels of auxin tends to favor LFSE. In both species, LFSE is a prelude to HFSE and heralds the incipient differentiation of the embryogenic callus. In sweet gum, LFSE is associated with organogenesis and the procedure for obtaining LFSE in gum is very similar to that of coffee and oil palm. In all three species LFSE-derived embryos have been successfully grown into plants.

#### **Androgenesis and Protoplast Culture of Woody Species**

Very few reports exist on somatic embryogenesis from anther cultures (Table 6) of woody species, although organogenesis from anthers has been explored extensively in Populus spp. To date, two disparate examples exist - one, in

Table 7. Embryogenesis from protoplasts of woody species.

Species	Tissue Source	PE <sup>a</sup> , %	Protocol	Comments	Reference
<u>Citrus sinensis</u>	nucellar callus	4-9	4	irradiation required for embryogenesis	Vardi <u>et al.</u> , 1975
		70	4	direct embryo formation	Kobayashi <u>et al.</u> , 1985
<u>Santalum album</u>	embryogenic cell suspensions from stem segments	--	1	colony formation precedes embryogenesis	Ozias-Akins, <u>et al.</u> , 1985

aplating efficiency.

In sandalwood, high frequency somatic embryogenesis was obtained from protoplast derived callus. Protoplasts were formed in high yields from embryogenic suspension cultures, and the protocol for obtaining plantlets, as in Citrus, was very similar to the protocol used to obtain embryos from cell cultures.

As was the case with androgenesis, protoplast culture of woody species was no different than that for herbaceous plants. Apparently, embryogenic cell cultures are a good starting material, as no reports exist of embryogenesis from protoplasts derived from intact tissues of woody species.

## CONCLUSIONS

Somatic embryogenesis in woody species is, to a first approximation, no different from embryogenesis in any other species. However, there are several features that make the process in trees unique. Such as the type of explants employed, the extended period of somatic embryo development that may involve dormancy considerations, and the distinct phenotype of embryogenic tissues. This observation can be used to develop a set of guidelines for extending somatic embryogenesis to other species:

1. Explant choice. A systematic search for PEDC explant types should be made. This should include nucellar and ovular tissue. Depending on the goal of the program, embryonal tissue and pollen should also be examined. For IEDC systems, a wide variety of explants should be tested and cultures should be examined visually for an unusual callus type that shows aspects of organization and tends to be devoid of color.

2. Protocol. For PEDCs, initiation under auxin-free conditions should be investigated. Any promising growth should be transferred to auxin containing medium. For IEDC, auxin levels used at initiation should be maintained or reduced slightly for several subculture intervals. The auxin 2,4-D should be tested.

3. Further considerations. Attempts at suspension culture or regeneration from protoplasts should not be made until an embryogenic callus system is established. Although the acquisition of embryogenic competence is usually rapid, cultures should be examined for several subculture intervals especially if rapid growth or an unusual phenotype is later produced. Embryogenic callus initiation can have a low initiation frequency, although once initiated cultures can

produce large numbers of embryos. Therefore, a considerable number of explants (and perhaps genotypes) should be investigated for a particular treatment.

Finally, in regard to forest species, this review provides considerable evidence for optimism in obtaining somatic embryogenesis in these species. In addition to the existing reports in commercially important forest species (sweet gum, larch, spruces, and pine) members of the family Betulaceae (birches) and Myratacae (Eucalyptus) are represented, so it seems likely that somatic embryogenesis can be extended to these species as well. Reasons for optimism are also apparent when it is considered that in well over half the species in which somatic embryogenesis has been reported tissue explants have been taken from mature trees. Thus, the ability to propagate proven material via somatic embryogenesis on a considerable scale appears feasible. Still open to question, however, is the clonal fidelity of plants obtained. In time this question too, will be answered as the products of these reports are planted and carried to maturity.

REFERENCES

- Amirato, P. V. (1985). Patterns of development in culture. In: Tissue Culture in Agriculture and Forestry. R. R. Henke, K. W. Hughes, M. J. Constantin, and A. Hollaender, eds., Plenum Press, New York. p. 9-29.
- Becwar, M. R.; Noland, T. L.; Wann, S. R. (1986). Somatic embryo development and plant regeneration from embryogenic Norway spruce callus. Tappi (in press).
- Blake, J. (1983). Tissue culture propagation of coconut, date, and oil palm. In: Tissue Culture of Trees. J. H. Dodds, ed., AVI Publishing Co., West Port, CN. p. 29-51.
- Button, J.; Botha, C. E. J. (1975). Enzymic maceration of Citrus callus and the regeneration of plants from single cells. J. Exp. Bot. 26:723-729.
- Button, J.; Bornman, C. H. (1971). Development of nucellar plants from unpollinated and unfertilized ovules of the Washington navel orange in vitro. J. S.Afr. Bot. 37:127-134.
- Button, J.; Kochba, J.; Bornman, C. H. (1974). Fine structure of and embryoid development from embryogenic ovular callus of 'Shamouti' range (Citrus sinensis Osb.). J. Exp. Bot. 25:446-457.
- Cronauer, S.; Krikorian, A. D. (1983). Somatic embryos from cultured tissues of triploid plantations (Musa 'AAB'). Plant Cell Rept. 2:289-291.
- Eichholtz, D. A.; Robitaille, H. A.; Hasegawa, P. M. (1979). Adventive embryony in apple. HortScience 14:669-700.
- Gharyal, P. K.; Maheshwari, S. L. (1981). In vitro differentiation of somatic embryoids in a leguminous tree - Albizia lebbeck L. Naturwissenschaften 68:379-380.
- Gupta, P. K.; Durzan, D. J. (1986). Somatic polyembryogenesis from callus of mature sugar pine embryos. Bio/Tech. 4:643-645.
- Hakman, I.; Fowke, L. C.; von Arnold, S.; Eriksson, T. (1985). The development of somatic embryos in tissue cultures initiated from immature embryos of Picea abies (Norway spruce). Plant Sci. 38:53-59.
- Hakman, I.; von Arnold, S. (1986). Plantlet regeneration through somatic embryogenesis in Picea abies (Norway spruce). J. Plant Physiol. 121: 149-158.
- Hanower, J.; Pannetier, C. (1982). In vitro propagation of the oil palm Elaeis guineensis Jacq. In: Proc. 5th Intl. Cong. Plant Tissue and Cell Culture. A. Fujiwara, ed. Japan Assoc. for Plant Tissue Culture, Tokyo. p. 745-746.

- Hu, C. Y.; Ochs, J. D.; Mancini, F. M. (1979). Further observations on Ilex embryoid production. *Z. Pflanzenphysiol.* 89:41-49.
- Hu, C. Y.; Sussex, I. M. (1971). In vitro development of embryoids on cotyledons of Ilex aquifolium. *Phytomorph.* 21:103-107.
- Janick, J. (1982). Adventive embryony in pear. *Acta Hort.* 124:37-41.
- Kobayashi, S.; Ikeda, I.; Uchimiya, H. (1985). Conditions for high frequency embryogenesis from orange (Citrus sinensis Osb.) protoplasts. *Plant Cell Tissue Organ Culture* 4:249-259.
- Kochba, J.; Button, J. (1974). The stimulation of embryogenesis and embryoid development in habituated ovular callus from the 'Shamouti' orange (Citrus sinensis) as affected by tissue age and sucrose concentration. *Z. Pflanzenphysiol.* 73:415-421.
- Kochba, J.; Spiegel-Roy, P. (1973). Effect of culture media on embryoid formation from ovular callus of 'Shamouti' orange (Citrus sinensis). *Z. Pflanzenphysiol.* 69:156-162.
- Kochba, J.; Spiegel-Roy, P.; Safran, H. (1972). Adventive plants from ovules and nucelli in Citrus. *Planta* 106:237-245.
- Kononowicz, H.; Kononowicz, A. K.; Janick, J. (1984). Asexual embryogenesis via callus of Theobroma cacao. *Z. Pflanzenphysiol.* 113:347-358.
- Krogstrup, P. (1986). Embryolike structures from cotyledons and ripe embryos of Norway spruce (Picea abies). *Can. J. For. Res.* 16:664-668.
- Krul, W. R.; Worley, J. F. (1977). Formation of adventitious embryos in callus cultures of 'Seyval,' a French hybrid grape. *J. Amer. Soc. Hort. Sci.* 102:360-363.
- Lakshmi Sita, G.; Raghava Ram, N. V.; Vaidyanathan, C. S. (1979). Differentiation of embryoids and plantlets from shoot cultures of sandalwood. *Plant Sci. Lett.* 15:265-270.
- Litz, R. E. (1985). Somatic embryogenesis in tropical fruit trees. In: Tissue Culture in Agriculture and Forestry. R. R. Henke, K. W. Hughes, M. J. Constantin, and A. Hollander, eds., Plenum Press, New York. p.
- Litz, R. E. (1984). In vitro somatic embryogenesis from callus of jaboticaba, Myrciaria cauliflora. *HortScience* 19:62-64.
- Litz, R. E. (1984). In vitro responses of adventitious embryos of two polyembryonic Eugenia species. *HortScience* 19:720-722.
- Litz, R. E. (1984). In vitro somatic embryogenesis from nucellar callus of monoembryonic mango. *HortScience* 19:715-717.
- Litz, R. E.; Conover, R. A. (1983). High-frequency somatic embryogenesis from Carica suspension cultures. *Ann. Bot.* 51:683-686.

- Litz, R. E.; Conover, R. A. (1982). In vitro somatic embryogenesis and plant regeneration from Carica papaya L. ovular callus. *Plant Sci. Lett.* 26: 153-158.
- Litz, R. E.; Conover, R. A. (1981). In vitro polyembryony in Carica papaya L. ovules. *Z. Pflanzenphysiol.* 104:285-288.
- Litz, R. E.; Conover, R. A. (1980). Somatic embryogenesis in cell cultures of Carica stipulata. *HortScience* 15:733-735.
- Litz, R. E.; Knight, R. J.; Gazit, S. (1984). In vitro somatic embryogenesis from Mangifera indica L. callus. *Scientia Hort.* 22:233-240.
- Litz, R. E.; Knight, R. J.; Shimuel, G. (1982). Somatic embryos from cultured ovules of polyembryonic Mangifera indica L. *Plant Cell Rept.* 1:264-266.
- Liu, J. R.; Sink, K. C.; Dennis, F. G. (1983). Adventive embryogenesis from leaf explants of apple seedlings. *HortScience* 18:871-873.
- Maheshwari, P.; Ranga Swamy, N. S. (1958). Polyembryony and in vitro culture of Citrus and Mangifera. *Ind. J. Hort.* 15:275-282.
- Mehra, P. N.; Jaidka, K. (1980). Experimental induction of embryogenesis in pear. In: Proc. National Symp. on Plant Tissue Culture, Genetic Manipulation, and Somatic Hybridization of Plant Cells. P. S. Rao, M. R. Heble, and M. S. Chadha, eds. Bhabha Atomic Research Centre, Bombay. p. 301-305.
- Mehra, P. N.; Sachdeva, S. (1980). In vitro plantlet formation through embryogenesis in apple. In: Proc. National Symp. on Plant Tissue Culture, Genetic Manipulation, and Somatic Hybridization of Plant Cells. P. S. Rao, M. R. Heble, and M. S. Chadha, eds. Bhabha Atomic Research Centre, Bombay. p. 295-300.
- Merkle, S. A.; Sommer, H. E. (1986). Somatic embryogenesis in tissue cultures of Liriodendron tulipifera L. *Can. J. For. Res.* 16:420-422.
- Milewska-Pawliczuk, E.; Kubicki, B. (1977). Induction of androgenesis in vitro in Malus domestica. *Acta Hort.* 78:271-276.
- Mitra, G. C.; Chaturvedi, H. C. (1972). Embryoids and complete plants from unpollinated ovaries and from ovules of in vivo-grown emasculated flower buds of Citrus spp. *Bull. Torrey Bot. Club* 99:184-189.
- Nabors, M. W.; Heyser, J. W.; Dykes, T. A.; DeMott, K. J. (1983). Long-duration, high-frequency plant regeneration from cereal tissue cultures. *Planta* 157: 385-391.
- Nagmani, R.; Bonga, J. M. (1985). Embryogenesis in immature subcultured callus of Larix decidua. *Can. J. For. Res.* 15:1088-1091.

- Ozias-Akins, P.; Rao, P. S.; Schieder, O. (1985). Plant regeneration from embryogenic suspension-derived protoplasts of sandalwood (Santalum album). In: Tissue Culture in Agriculture and Forestry. R. R. Henke, K. W. Hughes, M. J. Constantin, and A. Hollaender, eds. Plenum Press, New York. p. 338-339.
- Pannetier, C.; Buffard-Morel, J. (1982). Production of somatic embryos from leaf tissues of coconut, Cocos nucifera L. In: Proc. 5th Intl. Cong. Plant Tissue and Cell Culture. A. Fujiwara, ed. Japan Assoc. for Plant Tissue Culture, Tokyo. p. 755-756.
- Pence, V. C.; Hasegawa, P. M.; Janick, J. (1980). Initiation and development of asexual embryos of Theobroma cacao L. in vitro. Z. Pflanzenphysiol. 98:1-14.
- Perez, C.; Fernandez, B.; Rodriguez, R. (1983). In vitro plantlet regeneration through asexual embryogenesis in cotyledonary segments of Corylus avellana L. Plant Cell Rept. 2:226-228.
- Radojevic, L. (1979). Somatic embryos and plantlets from callus cultures of Paulownia tomentosa Steud. Z. Pflanzenphysiol. 91:57-62.
- Radojevic, L.; Vujicic, R.; Neskovic, M. (1975). Embryogenesis in tissue culture of Corylus avellana L. Z. Pflanzenphysiol. 77:33-41.
- Ragan, T. S.; Murashige, T.; Bitters, W. P. (1968). In vitro initiation of nucellar embryos in monoembryogenic Citrus. HortScience 3:226-227.
- Rajasekaran, K.; Mullins, M. G. (1979). Embryos and plantlets from cultured anthers of hybrid grapevines. J. Exp. Bot. 30:399-407.
- Ranga Swamy, N. S. (1961). Experimental studies on female reproductive structures of Citrus microcarpa Bunge. Phytomorph. 11:109-127.
- Rao, P. S. (1965). In vitro induction of embryonal proliferation in Santalum album L. Phytomorph. 15:175-179.
- Rao, P. S.; Bapat, V. A. (1980). Morphogenic investigations on tissue and organ cultures of sandalwood tree. In: Proc. National Symp. on Plant Tissue Culture, Genetic Manipulation and Somatic Hybridization of Plant Cells. R. S. Rao, M. R. Heble, and M. S. Chadha, eds. Bhabha Atomic Research Centre, Bombay. p. 206-215.
- Reinert, J.; Bajaj, Y. P. S.; Zbell, B. (1977). Aspects of organization - organogenesis, embryogenesis, cotydifferentiation. In: Plant Tissue and Cell Culture. H. E. Street, ed. Univ. of Calif. Press, Berkeley. p. 389-429.
- Reynolds, J. F.; Murashige, T. (1979). Asexual embryogenesis in callus of palms. In vitro 15:383-387.

- Sharp, W. R.; Evans, D. A.; Sondahl, M. R. (1982). Applications of somatic embryogenesis to crop improvement. In: Proc. 5th Intl. Cong. Plant Tissue and Cell Culture. A. Fujiwara, ed. Japan Assoc. Plant Tissue Culture, Tokyo. p. 759-762.
- Sommer, H. E.; Brown, C. L. (1980). Embryogenesis in tissue cultures of sweetgum. *For. Sci.* 26:257-260.
- Sondahl, M. R.; Nakamura, T.; Sharp, W. R. (1985). Propagation of coffee. In: Tissue Culture in Agriculture and Forestry. R. R. Henke, K. W. Hughes, M. J. Constantin, and A. Hollaender, eds. Plenum Press, New York. p. 215-233.
- Sondahl, M. R.; Sharp, W. R. (1977). High frequency induction of somatic embryos in cultured leaf explants of Coffea arabica L. *Z. Pflanzenphysiol.* 81:395-408.
- Spiegel-Roy, P.; Kochba, J. (1973). Stimulation of differentiation in orange (Citrus sinensis) ovular callus in relation to irradiation of the media. *Rad. Bot.* 13:97-103.
- Srinivasan, C.; Litz, R. E.; Barker, J.; Norstog, K. (1985). Somatic embryogenesis and plantlet formation from Christmas palm callus. *HortScience* 20:278-280.
- Srinivasan, C.; Mullins, M. G. (1980). High-frequency somatic embryo production from unfertilized ovules of grapes. *Scientia Hort.* 13:245-252.
- Tisserat, B. (1984). Palm tissue culture. In: Applications of Plant Tissue Culture Methods for Crop Improvement. D. A. Evans, W. R. Sharp, P. V. Amirato, and Y. Yamada, eds. MacMillan Press, New York. p. 505-545.
- Tisserat, B. (1982). Development of new tissue culture technology to aid in the cultivation and crop improvement of date palms. In: Proc. 1st Symp. on the Date Palm. King Faisal University, Al-Hassa, Saudi Arabia. p. 126-140.
- Tisserat, B. (1979). Propagation of date palm (Phoenix dactylifera L.). *J. Expt. Bot.* 30:1275-1283.
- Tisserat, B.; DeMason, D. A. (1980). A histological study of the development of adventitive embryos in organ cultures of Phoenix dactylifera L. *Ann. Bot.* 46:465-472.
- Tulecke, W.; McGranahan, G. (1985). Somatic embryogenesis and plant regeneration from cotyledons of walnut, Juglans nigra Regia L. *Plant Sci.* 40: 57-63.
- Vardi, A.; Spiegel-Roy, P.; Galun, E. (1975). Citrus cell culture: isolation of protoplasts, plating densities, effect of mutagens and regeneration of embryos. *Plant Sci. Lett.* 4:231-236.

- von Arnold, S.; Hakman, I. (1986). Effect of sucrose on initiation of embryogenic callus from mature zygotic embryos of Picea abies L. Karst (Norway spruce). J. Plant. Physiol. 122:261-265.
- Webb, D. T.; Rivera, M. E.; Starszak, E.; Matos, J. (1983). Callus initiation and organized development from Zamia pumila embryo explants. Ann. Bot. 51:711-717.
- Williams, E. G.; Maheswaran, G. (1986). Somatic embryogenesis: factors influencing coordinated behavior of cells as an embryogenic group. Ann. Bot. 57:443-462.
- Yasuda, T.; Fujii, Y.; Yamaguchi, T. (1985). Embryogenic callus induction from Coffea arabica leaf explants by benzyladenine. Plant Cell Physiol. 26:595-597.
- Zatyko, J. M.; Simon, I.; Szabo, C. S. (1975). Induction of polyembryony in cultivated ovules of red currant. Plant Sci. Lett. 4:281-283.