2. Historical Aspects of Somatic Embryogenesis in Woody Plants[1]

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1. Introduction

During the next few decades, the world demand for wood products is expected to rise sharply. To meet this growing demand, there will be an increasing need for mass production of improved-quality planting stock of many tree species. The conventional methods of tree improvement and selection offer only limited possibility of meeting the growing demands. Therefore, new and innovative techniques for the creation of new hybrids, early selection and testing of desirable genotypes, rapid vegetative propagation of selected genotypes, and improvement through genetic engineering, etc., must be developed to achieve these goals.

In any tree improvement program, the quality of the product and the economy of propagation are two fundamental measures of success. In addition to the need for genetically uniform stocks of selected genotypes for direct use in planting, these plants can be of enormous value to tree physiologists, pathologists, ecologists, and geneticists for testing their resistance to fungal, bacterial and environmental stress factors. Similarly, an early selection of desirable genotypes could alleviate the need for waiting periods that take several years before field testing can begin. Vegetative propagation has a clear edge over sexual means of reproduction for achieving both rapidity of propagation and assured maintenance of genetic composition of the progeny.

Commercial applications of cell and tissue culture to mass propagation are presently confined mainly to herbaceous plants and to species which can easily be propagated by traditional methods. The potential usefulness of cell and tissue culture techniques for the propagation of forest trees has long been recognized and discussed. However, it is only during the past two

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decades or so that concerted efforts have been made to adapt these methods for the propagation of commercially important tree species. Recent successes with cell and tissue culture of plants like *Pinus lambertiana*, *Pseudotsuga menziesii* and *Picea abies*, although somewhat less than perfect, have generated high hopes for the application of these techniques to mass production of several tree species. The list of plants from which it is now possible to obtain tissue cultures and to induce the formation of plantlets from callus is long and expanding. Nevertheless, the information at hand does not allow for the routine propagation of all species. It is obvious from published literature that the results can seldom be generalized and extrapolated from one species to another with regard to the nutritional requirements for growth and differentiation *in vitro*. Therefore, detailed steps for the successful propagation of a particular species must be worked out individually.

Cell and tissue culture techniques entail the growth of tissue or organ segments on suitable media that stimulate their development along one of several pathways. The most common and so far the most effective pathway is to directly produce whole plants or multiple shoots from stem segments or from excised shoot apices. Depending on hormonal composition of the growth media, these plants may be rootless or rooted. The rootless plantlets can be rooted successfully in most cases. This technique, commonly known as "micropropagation", produces sufficiently large clonal populations of plants and generates the least amount of genetic instability commonly observed in many cell and tissue culture systems. Micropropagation has been successfully used to clone a large number of plant species including fruit trees, conifers, forest trees and other commercial tree crops.

The second pathway, by far the most desirable, involves the regeneration of whole plants from callus and suspension cultures, and is known as somatic embryogenesis. This process is analogous to the development of zygotic embryos, and results in the production of a complete germling with the potential to grow into a whole plant, much as a seedling would. This technique has been successful with some non-woody horticultural plants and its feasibility has been demonstrated in several woody plants (Tautorus *et al.*, 1991). However, large scale routine success has not been achieved with many of the commercially important tree species. Properly controlled somatic embryogenesis carries a low risk of genetic instability, guarantees juvenile plants of normal growth habit, and is amenable to mass production and planting processes. Suspension cultures have been established from various tissues of several tree species, though spontaneous and induced embryogenesis in these suspensions are rare events. However, somatic embryos have been regenerated in more than 50 woody species upon transfer of tissues grown on solid media to suitable growth conditions.

A future advantage of the cell and tissue culture techniques in tree breeding lies in the production of haploid plants from microspores and female gametophytes. Such plantlets can then be induced to produce homozygous diploids by colchicine treatment. This offers the possibility of breeding pure lines. An

open-pollinated orchard containing two selected homozygous lines developed from superior individuals would allow all the genetic gain to be captured through the seed.

Research on the formation of protoplasts in tree species has remained far behind that of herbaceous plants. A prerequisite for success with protoplast regeneration and production of somatic hybrids, of course, is the availability of techniques for regeneration of plants from callus. This information is currently limited.

2. Somatic Embryogenesis in Tree Species

The first report of regeneration of adventitious buds from cambial tissue of a woody plant was with *Ulmus campestris* (Gautheret, 1940). This was followed by similar observations from the laboratory of Jacquiot (1949, 1951, 1955) with the same species as well as with *Betula*. In this case some root formation was also noted but complete plantlets were not produced. The first complete plants from tissue culture of a tree species were regenerated by Winton (1970) from leaf explants of aspen. It should be noted that the first tissue cultures of tree species were initiated from cambial explants (Gautheret, 1934, 1948, 1959; Morel, 1948), probably because these explants contained a high content of endogenous auxin and cytokinin. After the discovery of cytokinins, most other explant sources were successfully cultured in vitro for callus and plantlet production. Sommer and Wetzstein (1984) listed more than 100 species of angiospermic woody plants for which shoots or plantlets have been produced from organ/tissue cultures. It is notable from the list that a majority of cases of regeneration involve the use of juvenile tissues such as zygotic embryos and/or young seedlings. Genera with the largest number of species in which regeneration has been obtained by this method include *Eucalyptus*, *Populus*, *Citrus* and *Salix*. Since 1984, the number of hardwood species that have shown regeneration in vitro has nearly doubled.

There has been some confusion regarding the regeneration of somatic embryos from cell/tissue cultures of woody plants. Part of the ambiguity lies in the lack of a clear definition of a somatic embryo. Alternative terms such as "embryoids", "embryo-like structures", "adventitious embryos", etc., have often been used to describe the regenerants. Halperin and Wetherell (1964) emphasized the importance of bipolar organization with distinct root and shoot primordia to distinguish a somatic embryo from a shoot or a plantlet. An additional problem hindering the acceptance of a true somatic embryo relates to the origin of these structures. In some cases the growth of pre-existing zygotic or nucellar embryos, which are a direct product of polyembryonic development, has been confused with the regeneration of somatic embryos. Terms such as apomixis (Nygren, 1954), polyembryony (Webber, 1940), adventive embryony (Schroeder, 1968), nucellar embryony

(Ernst, 1918), and sporophytic embryony or embryogeny (Battaglia, 1963) have all been used to describe such naturally arising structures. Others (Vasil and Hildebrandt, 1966; Haccius and Lakshmanan, 1969) have attempted to distinguish somatic embryos produced in vitro from those of asexual origin in nature by referring to the former as embryoids. The natural occurrence of asexual embryogenesis (cf. somatic embryogenesis) in plants was summarized by Tisserat *et al.* (1979).

Rao (1965) reported the regeneration of embryo-like structures from tissue cultures of *Santalum album* that did not grow into complete plants. The direct production of somatic embryos from the cotyledons of *Ilex aquifolium* was reported by Sussex (1972); again, no mature plants were produced. Several studies from the laboratory of Radojevic described the ultrastructural aspects of somatic embryogenesis in *Corylus avellana* and *Paulownia tomentosa* (Radojevic *et al.*, 1975; Vujicic *et al.*, 1976). A pattern of membrane-bound ribosomes was found in the embryogenic cells that was similar to that seen in fertilized fern eggs and active secretory glands. Histochemical studies revealed that the embryogenic cells were rich in sugars, lipids and protein, and were also involved in starch accumulation.

Sharp *et al.* (1980, 1982) distinguished two different patterns of the origin of somatic embryos from in vitro grown explants: (1) direct production of somatic embryos from the explant cells called the pro-embryonic-determined cells (PEDC), and (2) indirect production of somatic embryos from an unorganized callus/tissue mass called the induced embryogenic-determined cells (IEDC). In the former, somatic embryos are presumed to originate from explant cells that require only an in vitro environment to be released from some suppressive condition imposed by the organization of the explant. By contrast, the IEDC pattern not only requires the release of previously differentiated state through mitotic cell divisions but also an induction of the new pattern of cell divisions to form organized embryos. Often, while the former situation requires no growth regulators, the latter may depend on a sequence of growth regulator treatments first to form callus from which the somatic embryos are regenerated.

Tissues such as nucellus, suspensors, integuments, proembryos, megagametophyte, etc., that are associated with the zygotic embryos generally produce asexual embryos through the PEDC pattern. Conversely, leaf, cotyledon and stem explants often produce somatic embryos via the formation of callus. Some examples of direct somatic embryogenesis in woody angiosperms include various species of *Citrus*, *Ilex aquifolium*, *Malus domestica*, *Mangifera indica*, *Theobroma cacao*, and *Pyrus* spp. Indirect somatic embryogenesis from juvenile and mature tissues of a number of woody plants has been seen both in monocots and dicots; some examples are *Cocos nucifera*, *Chamedorea costaricana*, *Phoenix dactylifera*, *Elaeis guineensis*, *Coffea arabica*, *Corylus avellana*, *Malus pumila*, *Paulownia tomentosa*, *Pyrus communis*, *Santalum album*, *Sapindus trifoliatus* and *Vitis vinifera*.

While the two patterns of somatic embryo development may be distinct

and many plants seem to follow either pattern, these are by no means mutually exclusive. Numerous examples exist where the PEDC continue to proliferate in a pro-embryogenic state under appropriate culture conditions. This pro-embryogenic tissue mass can be subcultured through many generations and complete somatic embryos can be generated on transfer to a different medium. Such proliferative tissues have been obtained in *Citrus sinensis*, *C. aurantifolia*, *Mangifera indica*, *Ilex aquifolium*, and *Theobroma cacao*.

Among the gymnosperms, the first reports of somatic embryogenesis in Norway spruce (*Picea abies*) appeared in 1985 (Hakman *et al.*, 1985; Chalupa, 1985). Within a year, other reports were added to the literature, expanding the list to include the genus *Pinus* (Gupta and Durzan, 1986a,b; Von Arnold and Hakman, 1986; Krogstrup, 1986). Since then, *Picea abies* has been used widely as a model experimental system for somatic embryogenesis in conifers (see Roberts *et al.*, 1993, and references therein). Using quite similar treatments, somatic embryogenesis has now been achieved in several additional conifer species.

Somatic embryogenesis in most conifers follows a similar pattern of development. With few exceptions, the embryogenic mass of cells is initiated from immature or mature zygotic embryos. The developmental stage of the zygotic embryo plays a crucial role in the production of embryogenic callus. Whereas in *Larix* the ideal material is young developing embryos (2 to 4 weeks postfertilization), fully mature embryos are suitable for various species of *Picea*. In *Pinus*, pre-cotyledonary stage embryos have generally yielded the best embryogenic tissue. Krogstrup (1986) and Attree *et al.* (1990a) demonstrated the feasibility of obtaining somatic embryos from cotyledons of 7-day-old and 12- to 30-day-old seedlings of *Picea abies* and *Picea mariana*, respectively. These observations were further extended to the use of 1-year-old seedlings of *Picea abies* (Ruaud *et al.*, 1992).

A detailed analysis of *Picea glauca* and *P. abies* revealed that embryogenic tissue originates from epidermal or subepidermal layers of the embryonic axis of zygotic embryos. The embryogenic tissue consists of elongated suspensor-like cells with small densely cytoplasmic clusters of meristematic cells at one end. Based on the similarity of this tissue with the in vivo cleavage polyembryony in conifers, Gupta and Durzan (1986a,b, 1987) suggested the term "somatic polyembryogenesis" for this tissue, which can be proliferated by routine subculture and it still retains its characteristic morphology. It is only upon transfer to a maturation medium, usually supplemented with abscisic acid (ABA), do the pro-embryogenic masses develop into complete embryos (see Dunstan *et al.*, 1991; Tautorus *et al.*, 1991; Roberts *et al.*, 1990a, 1993, and references therein). As opposed to *Picea*, where the embryogenic tissue arises from the hypocotyl zone, somatic embryogenesis in *Pinus* is initiated from the suspensor cells (Tautorus *et al.*, 1991).

In contrast to the dissected embryos from seeds, the use of seedlings offers ease in handling of explant materials, the former being highly labor intensive.

Eventually we must develop procedures to obtain embryogenic tissues from selected mature genotypes in order to take full advantage of proven genetic superiority of mature trees.

Since the first commercial application of somatic embryogenesis was realized for oil palms (Blake, 1983), few commercial plantations with other species have been established. The current status of commercial propagation of *Picea* through artificial seed production has been recently reviewed (Roberts *et al.*, 1993; see also, Lulsdorf *et al.*, 1993, and references therein). A major problem in most cases is the low frequency of maturation and germination of somatic embryos into whole plants. Exceptions include *Coffea arabica*, *Santalum album*, *Liriodendron tulipifera* and *Corylus avellana* for which more than 10 percent of the somatic embryos grew into whole plants (Sita *et al.*, 1979; Merkle and Sommer, 1986; Sondahl and Sharp, 1977; Perez *et al.*, 1983).

Numerous attempts have been made with various species of *Picea* to improve the quality and yield of mature embryos capable of germination into whole plants (Roberts *et al.*, 1993). Lack of production of a well-developed root has been a common problem in the failure of somatic embryos to develop into emblings [a term coined by Libby (1986) to distinguish the plantlets that are produced via somatic embryos from the seedlings that are produced from seeds]. Light has been shown to inhibit the germination of somatic embryos in most cases (Von Arnold and Hakman, 1988). It has been observed that a gradual desiccation under controlled relative humidity conditions substantially improves the efficiency of germination of *Picea* somatic embryos (Gray, 1989; Roberts *et al.*, 1990b).

Field trials of plants of several species of *Picea* produced from somatic embryogenesis have been reported (Becwar *et al.*, 1989; Attree *et al.*, 1990b; Webster *et al.*, 1990). Most have involved relatively small populations; the largest study involved 1200 plants showing a survival rate of more than 80 percent (Webster *et al.*, 1990). Height measurements and cold-hardiness studies showed a great similarity between the emblings and the seedlings. The overall survival rate at the end of the second season was almost 100 percent. These studies have since been expanded to include approximately 10,000 plants of *Picea glauca-engelmannii* complex and 8,000 plants of *P. sitchensis*. Due to the long life span of trees, it will take many years before the overall performance of these plants can be evaluated.

An ideal approach to increasing the efficiency of somatic embryogenesis is the use of large-scale bioreactors that can produce somatic embryos in liquid cultures. While there have been several instances of growth of embryogenic tissues in suspension cultures of gymnosperms (*Abies nordmanniana*, *Picea abies*, *Picea mariana*, *Pinus caribaea*, *Pinus strobus*, *Pseudotsuga menziesii*), sustained production of somatic embryos is not commonly observed (Hakman *et al.*, 1985; Durzan and Gupta, 1987; Gupta and Durzan, 1987; Hakman and Fowke, 1987; Finer *et al.*, 1989; Attree *et al.*, 1989a; Lainé and David, 1990; Tautorus *et al.*, 1990). To date only one case of the

production of mature somatic embryos of any conifer in large bioreactors has been reported (Tautorus *et al.*, 1992). Encapsulation of somatic embryos in a variety of gel coatings to produce artificial seeds for direct field planting has also been achieved with limited success (see Roberts *et al.*, 1993; Lulsdorf *et al.*, 1993, and references therein).

A prerequisite for sustained production of superior genotypes by somatic embryogenesis is the ability to field test the product before large-scale commercial planting. This means that successful genotypes should be maintained in an embryogenic state over several years. Cryopreservation is an ideal way to maintain and store highly embryogenic tissues for long periods (Chen and Kartha, 1987). A few attempts have already been made to demonstrate the regeneration of cryopreserved tissues of woody plants (Gupta *et al.*, 1987a,b; Kartha *et al.*, 1988; Ward, 1990; Bercetche *et al.*, 1990). In *Picea glauca*, *Picea abies* and *Pinus taeda*, the tissue remained competent of producing somatic embryos following short term storage in liquid nitrogen. The effect of long-term storage on embryo regeneration still needs to be determined.

3. Haploids and Triploids

Tulecke (1953) first demonstrated the ability of mature pollen grains of *Ginkgo biloba* to produce haploid callus. While limited morphogenesis was seen in the callus, no plantlets were produced. Subsequent work by several laboratories demonstrated the production of haploid callus in a number of gymnosperms including *Taxus brevifolia*, *Taxus baccata* (Tulecke, 1959; Rohr, 1973), *Torreya nucifera* (Tulecke and Sehgal, 1963), *Ephedra foliata* (Konar, 1963) and *Pinus resinosa* (Bonga, 1974). The first reports of haploid callus and plantlet formation from pollen cultures of angiospermic trees date to 1974 (Michellon *et al.*, 1974, for *Prunus amygdalus* and *Prunus persica*; Sato, 1974, for *Populus* spp.).

Numerous attempts have since been made to obtain haploid plants and somatic embryos from anther cultures of other woody plants. Anther cultures of *Malus* domestica yielded early-stage somatic embryos (Milewska-Pawliczuk and Kubicki, 1977) but these embryos failed to develop into plantlets. A mixed ploidy callus obtained from anther cultures of *Vitis vinifera* × *V. rupestris* produced several diploid somatic embryos which developed into plantlets with a high frequency (Rajasekaran and Mullins, 1979). Chen *et al.* (1982) reported the production of haploid somatic embryos from anther cultures of *Hevea brasiliensis*, which developed into whole plants at a relatively low frequency of 3 percent. Numerous haploid plants of *Annona squamosa* were obtained by Nair *et al.* (1983) through regeneration of multiple shoots from anther callus.

In contrast to anther/pollen culture, which has yielded positive results mostly with angiosperms, megagametophytic tissue has been used to produce haploid somatic embryos in a few gymnosperms. Haploid callus capable of

producing some roots and shoots was obtained from megagametophytic tissue of *Ginkgo biloba* by Tulecke (1965) and of *Zamia integrifolia* by Norstog (1965). Within a few years, root/shoot regeneration was reported in haploid megagametophytic tissues of *Cycas circinalis* (Norstog and Rhamstine, 1967; Huhtinen, 1972). While in most cases the frequency of regeneration of somatic embryos is extremely low, *Larix decidua* megagametophytic cultures have yielded haploid plants at a relatively high frequency. Rohr (1987) has described in details the development of haploid tissues in a number of gymnosperms.

Triploid plantlets have been produced from the endospermic tissues of some angiospermic woody plants (*Putranjiva roxburghii* – Srivastava, 1973; *Jatropha panduraefolia* and *Leptomeria acida* – Johri & Srivastava, 1973). In none of these has the growth of complete plants in the soil been reported.

4. Protoplasts

The first report on the isolation and culture of protoplasts in woody plants appeared in 1972. Rona and Grignon (1972) demonstrated the growth of protoplasts isolated from suspension cultures of *Acer pseudoplatanus*. While protoplast isolation and culture (to form callus) has been successful in a diverse group of woody angiosperms (McCown and Russell, 1987) and also a few gymnosperms (David, 1987), reports of regeneration of whole plants from single isolated protoplasts are rare. Somatic embryos were first regenerated from protoplasts of embryogenic cells of *Pinus taeda*, and *Picea glauca* (Gupta and Durzan, 1987; Attree *et al.*, 1987, 1989b). Similar results were later reported for several other conifers. The limited range of the explant source from which morphogenetically competent tissues can be obtained is a major reason for such limited success with protoplast culture in trees. The production of somatic hybrids through protoplast fusion has not been demonstrated in any tree species.

5. Major Problems and Future Perspectives

In the preceding pages, we have provided a brief review of the different stages of the research on somatic embryogenesis with tree species. Published information shows that: (1) somatic embryogenesis in some woody plants, especially conifers, has become routine; (2) somatic embryos usually can be grown into whole plantlets (albeit at low-to-moderate frequency) that can be tested for performance in the field; (3) the process of somatic embryogenesis is developmentally similar in most conifers whereas specific requirements for somatic embryo differentiation and maturation may vary among species; (4) the source of the explant plays a decisive role in the ability to produce embryogenic tissue; and (5) the developmental pattern of somatic embryos

is analogous to the development of zygotic embryos without the complexity of surrounding gametophytic tissues.

The following are some of the major problems commonly associated with tree tissue culture.

1. Most of the published work has utilized juvenile tissues, particularly zygotic embryos. Tissues from mature trees seldom seem amenable to successful propagation. There is a need for systematic physiological studies on the underlying changes in juvenility to maturity. A knowledge of the process of "phase change" could then be utilized to reverse the process in shoot apices or obtain adventitious juvenile material from mature plants. Rejuvenating treatments currently being investigated include serial grafting of buds onto juvenile rootstock, severe pruning of trees to stimulate latent juvenile meristems, spraying plants with cytokinins, serial subculture of shoot apices *in vitro*, and hormone-induced production of adventitious shoots *in vitro*.
2. Growth of regenerated plants in the greenhouse remains a formidable problem, particularly in conifers. In addition to the use of ABA for maturation of somatic embryos and physical treatments for acclimation of emblings, the usefulness of mycorrhizal associations in the formation and growth of roots should be studied.
3. Production of haploid plants from pollen and microspore cultures has been studied only in a few tree species. The potential advantages of haploid plants through the production of homozygous diploids cannot be overemphasized. As mentioned earlier, for gymnosperms, the female gametophyte should provide a good source of material for haploid plants.
4. There has been little effort to produce somatic hybrids in tree species through protoplast fusion. Although of limited value thus far, this technique has unlimited potential for the production of intraspecific and interspecific hybrids. The best approach will be to work with closely related taxa. Since many of disease/chemical resistance mechanisms reside in cytoplasmic factors, production of cybrids (a fusion product of a nucleated and an enucleated protoplast) can be advantageous in the production of resistant varieties.
5. Recent advances in the techniques of gene transfer and our current knowledge about the regulation of gene activity can be used in tree improvement programs. These techniques are primarily dependent on the availability of easily regenerating cell and tissue cultures.

6. Conclusion

A descriptive analysis of the development of both zygotic embryos and somatic embryos is now available for a number of woody plant species. A major focus of research during the past two decades has been the manipu-

lation of growth media and growth conditions. Another is the testing of a variety of explant sources to obtain somatic embryogenesis in dozens of species of woody angiosperms and gymnosperms. However, only a limited effort has been made to enhance our understanding of the biochemical and molecular basis of somatic embryogenesis; there has been even less work on addressing the question: "What makes a somatic cell become embryogenic?" Perhaps the answer must await a set of breakthroughs in routinely used model experimental systems for somatic embryogenesis such as carrot and alfalfa, or in the biochemical and molecular analysis of developmental mutants in plants like *Arabidopsis* and *Zea mays*. Ultimately, however, the molecular aspects of somatic embryogenesis in conifers will be understood only from direct work with model experimental systems of woody plants such as *Picea* and *Pinus*. The area of developmental mutation research in woody plants, particularly the conifers, is untouched. Obviously, the long lifespan and a long, sometime multiyear, routine for zygotic embryo development are major hurdles to a large-scale analysis of putative mutants. Nevertheless, guidelines provided by research on herbaceous plants should pave the way for the use of modern techniques for genetic, biochemical, and molecular analysis of somatic embryogenesis. This is a prerequisite for achieving full utilization of this approach in the production of genetically improved, economically important woody plants.

While the potential usefulness of haploids, somaclonal variation, and somatic hybridization should not be minimized, these techniques have not yet proven as beneficial as generally presumed. Moreover, genetic transformation with foreign genes has already shown its potential as a viable alternative to somaclonal variation and mutagenesis. Applications of genetic transformation technology hold greater potential for woody plants than even the herbaceous plants because the gains in genetic improvement can be realized over relatively short periods compared to routinely used techniques of hybridization, selection, and mutagenesis which require decades. This area of research depends on at least three factors that must come together for each species or genotype to be improved: (1) availability of specific genes and promoters for transfer and expression; (2) development of reliable techniques for transfer of the genes to target cells and the selection of transformed cells; and (3) regeneration of transformed cells into whole plants which can be mass-propagated, preferably by asexual means.

A variety of potentially useful genes is being characterized and cloned for transfer to agriculturally important plants, many of which also will be useful for the improvement of woody plants. Likewise, a number of different methods of genetic transformation of plants have been developed, some of which have shown applicability to woody plants as well. Stable transfer, integration, and expression of a model gene (such as NPT or GUS) have been demonstrated only in a few woody plant species, and genes regulating cellular metabolism or physiological aspects of growth and development have

been tested in still fewer cases. It is hoped that this area of research would soon realize a major spurt of activity.

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