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# Somatic Polyembryogenesis for the Multiplication of Tree Crops

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## Introduction: reconstitution, restoration and reproductive regeneration

This review will examine the unique attributes and concepts underlying the possible utility of somatic polyembryogenesis as distinct from other forms of vegetative propagation. The emphasis is on biotechnology aimed at processes leading to true-to-type plantlet regeneration. The review is based on our limited experience with coniferous and *Prunus* species.

The multiplication of tree crops can be achieved in three general ways (Sinnott, 1960): reconstitution, restoration and reproductive or vegetative propagation. In reconstitution, cells such as those associated with the zygotic proembryo, proliferate and reorganize through true-to-type ontogeny to re-form the original structures, i.e. a portion of the original embryo reconstitutes a complete whole, e.g. polyembryony. Polyembryony is the formation of more than one embryo in an ovule. An embryo is a new individual with a bipolar root and shoot promeristem arising from a single cell, i.e. vascular connection to the maternal tissue is not evident (Haccius, 1978). The single-cell origin applies equally to zygotic and non-zygotic or somatic embryos.

Restoration describes a wide range of cases where missing cells, tissues or organs are replaced through a dedifferentiation and redifferentiation that leads to renewed meristematic activity arising in the adjacent region. Restoration is largely organogenetic, e.g. rooting of cuttings. Somatic embryogenesis, with its bipolar presentation of primary meristems, may be distinguished from organogenesis where the formation of adventitious buds is often based on vascular connections with the original tissues and where usually only a single meristem (shoot, root, cambial) emerges.

Reproductive regeneration, like reconstitution, involves the separation of a cell or plant part (sporophytic or gametophytic phase), and its regeneration into a new plant either by organogenesis or embryogenesis. Reproductive

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regeneration differs from reconstitution because it is an adventitious form of vegetative propagation based on the excision of an explant (e.g. cotyledons, female gametophyte, apical meristems) with subsequent steps involving dedifferentiation, redifferentiation and transformation of one phase of the life cycle into another, e.g. somatic, gametophytic, or callus cell changing into an embryo.

Somatic polyembryogenesis is the non-adventitious formation by reconstitution of more than one embryo from a pre-existing proembryonal–suspensor mass. While this mass may be used as an explant for reproductive regeneration, the phenomenon of regeneration and multiplication can be traced to the proliferation of a proembryonal cell that reconstitues by a very true-to-type cleavage and developmental process, a multiplicity of proembryos with their accessory suspensor cells. Somatic polyembryogenesis and the multiplication of selected tree crops by this process is a relatively new phenomenon that has added a novel dimension to domestication, tree improvement, reforestation and afforestation programmes.

The need for tree improvement, new strategies for clonal forestry and capturing genetic gains, and the continual requirement for new genetic variation have been reviewed by Dogra (1983), Durzan (1980, 1985), Durzan and Campbell (1974), Cannell and Jackson (1985), Libby (1986), Libby and Rauter (1984), Porterfield, Zobel and Ledig (1975), Timmis, Abo El-Nil and Stonecypher (1987), Ranney, Wright and Layton (1987) and Allard (1960). The aim of such work is provision of élite germplasm for a multiplicity of uses independent of climate, insects, disease and vagaries of nature. Wood, fibre, food and waste products are used for structural materials, energy, chemical feedstock and nutrition. Élite germplasm is used for breeding orchards, developing ornamental variants and novelties, artificial seeds, and for afforestation and reforestation.

The use of cell cultures and somatic embryos offers the possibility of rapid, simple production and selection of élite trees and variants with specific biochemical and physiological characters. In forestry, somatic polyembryogenesis could provide novel alternative tree-improvement strategies in support of programmes that rely entirely on breeding and seed orchards. Embryogenic cell suspension cultures also provide a supply of morphogenic protoplasts for the introduction of useful genetic variation, for the removal of existing lethal genetic loads, and for the exploration of variations in the origin of the seed habit. In these ways, biotechnological innovation leads to potentially patentable, novel germplasm for a variety of end uses in product utilization and marketing cycles (*see* Durzan, 1985; Haissig, Nelson and Kidd, 1987).

The size, and the long complex life cycle of trees greatly hinders the testing of novel tree cultivars, no matter how derived. Pressure to provide trees for intensive cultivation will continue because of energy and fuel crises, endangered species, natural and man-made disasters (fires, ozone layer depletion, acid deposition, insects, disease, etc.), social customs and inexorable increases in world populations (World Resources Institute, 1986).

## Special attributes of tree breeding and improvement

### GENERAL

Tree breeding strategies for domestication and tree improvement are now dominated by the currently available natural modes of sexual and asexual reproduction (Libby, Stettler and Seitz, 1969; Zobel and Talbert, 1984). Depending on conditions, species and cultivars, improved trees may be multiplied by vegetative propagation or from seed which is usually produced by open-pollination in seed orchards. In many tree crops, inbreeding leads to inbreeding depression. Élite attributes based on the 'position' of genes in the genome are often lost in the breeding process. This valuable component includes dominance and non-additive genetic variance.

Clonal propagation, as practised by rooting, microprogation and somatic embryogenesis, captures both the additive and non-additive genetic variance. It provides, through the totipotency of explants, a vehicle to convey élite traits into populations on a massive scale. Clonal propagation is now only generally applicable to tissue explants from embryonic and juvenile sources, rather than from proven mature trees. Methods for the clonal propagation of mature trees need to be developed (e.g. Gupta and Durzan, 1987a). Cells and tissues of older trees will have to be rejuvenated and cloned for significant progress to be made in tree improvement (Boulay, 1987).

For some tree-fruit crops, breeding strategies aim to reduce seediness and to induce parthenocarpy, e.g. bananas, seedless grapes (Simmonds, 1985). In other fruit crops there is a need to increase multiple seed production to obtain a wider genetic variation in the new generation. Strategies based on protoplast, cell and tissue culture methods now offer opportunities, through protoplast fusion, for the introduction of foreign genes, to create seedless varieties, or to recover seeds—both natural and artificial—with greater value-added properties (*see* Fraley, Rogers and Horsch, 1986; Schell, 1987; Cocking, 1988).

In forestry, élite traits are selected, improved and multiplied through the use of breeding and seed orchards (Thomson, Lester and Martin, 1987). The recovery and multiplication of progeny is long term and dependent upon good seed years (Dogra, 1983). Often, in virgin forests, seed yield is constrained by wood formation, and by the years it takes trees to replenish nutrients from poor soils for seed production. Once full seeds are obtained, insects, diseases and animals must be kept away from seeds. Harvesting of reproductive cones is achieved with assistance of mechanically elevated platforms, mechanical shakers, by climbing and hand-picking, helicopters and in some cases by rifle or by stealing seeds from squirrel caches.

The reliable production of somatic embryos and artificial seeds can overcome many of these constraints. Where reproductive maturity is not reached for decades and where seed reproduction can take up to 3 years, as in sugar pine (*Pinus lambertiana*), plant regeneration methods based on protoplasts could greatly reduce the time to introduce genetic changes. Progeny testing for accurate and precise environmental adaptation, precocious

maturity and developmental aberrations could still need considerable time, space and cost. Techniques to predict field performance are thus as important as those needed to induce genetic change or to clone the desirable selectants (Russell and Libby, 1986). Moreover, germplasm conservation is needed to preserve the existing genetic variation.

## SOMATIC EMBRYOGENESIS AND GERMPLASM CONSERVATION

In California, most gene pools for conifers are highly diverse and relatively undisturbed (Miller, 1987). In Canada and the USA, pools for white pine have been severely eroded by high-grading, i.e. harvesting the best trees (Lower, 1938). One current tendency in the domestication of trees is to replace harvests or areas blackened by fires by genetically improved and sometimes non-local genotypes that are introduced through seed banks (*Figure 1*). In California, the price to restore some 200 000 acres of forest lost by fire damage in 1987 alone is estimated at 150 million US dollars.

Concerns have been raised that we are not yet sure of the impact of replacing native forests by man-made forests. We do not yet understand the resource-saving attributes of native populations that are being heavily eroded. This is especially true for the tropics (Reis, 1985), and in areas faced with mass extinction of species (Wolf, 1988).

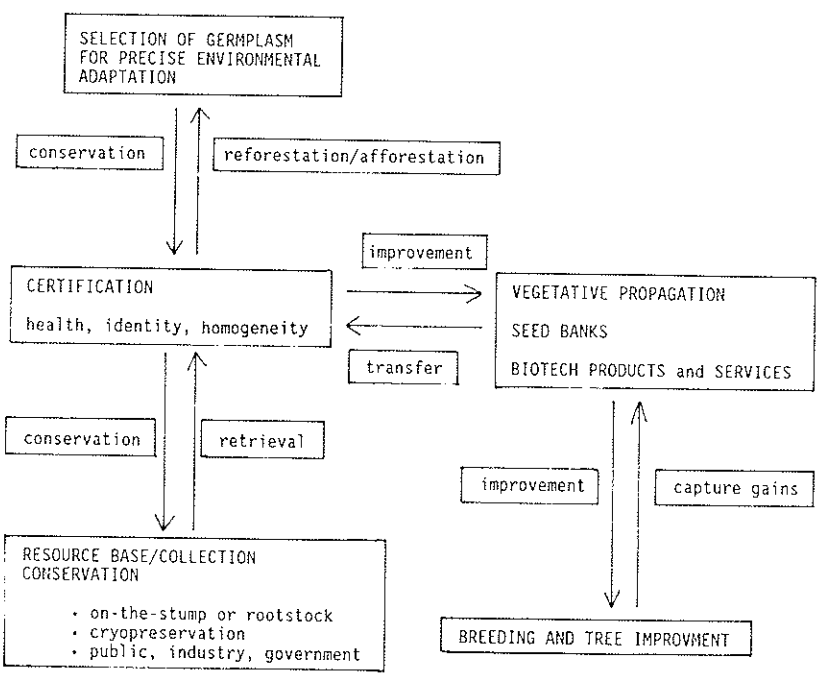


**Figure 1.** A simplified general system for germplasm conservation of woody perennials. Somatic polyembryogenesis impacts through the provision of genetically improved germplasm for reforestation and afforestation and for conservation.

Loss of existing genetic variability may come with reforestation and afforestation by creating quantal changes in species composition and ecology. In Chile, plantation of *Pinus radiata* has threatened already endangered native species. In Mendocino County, California, the coastal redwoods are, for unknown reasons, not reproducing by seeds so that seeds have to be imported (Miller, 1987). This changes the genetic mix of the next generation. Current guidelines in California restrict the movement of seeds, i.e. seeds for artificial regeneration must come from within a designated seed zone to maintain genetic continuity.

Where widespread forest fires have wiped out whole seed zones, there is little alternative but to introduce adapted or imported genotypes. The production of artificial seed by somatic embryogenesis and polyembryogenesis could help to relieve this situation through the creation of local germplasm factories where cells can be converted to plants or stored until needed in the future.

### THE CLONAL LIFE CYCLE

When we examine the life cycles of tree crops, several problems arise, related to the selection of explants in vegetative propagation (e.g. Durzan 1984b and the previous section of this chapter). Each type of explant or cutting defines a clonal idiotype or variant that is more or less totipotent. The expectation is that as clones are reconstituted, restored or regenerated from this source, subsequent gene expression, growth and development will remain reasonably true-to-type and predictable. Given the current technology, these expectations are too stringent, at least until more experience and field-testing are gained.

The cycle of vegetative propagation represents a developmental subset of the life cycle (Kester, 1983). Only certain phases of the crop's life cycle may be of interest to the propagator, so that complete reproduction of all features of the plant's life may not be of value. In 1987, more than 33 million Christmas trees derived largely from seed were sold in the USA at an average cost of 3–5 dollars per foot (0·3 m). This industry alone, which exploits mainly the juvenile and adolescent phases of the life cycle, is worth US \$1 x $10^9$ on a combined wholesale and retail level. As rooting of cuttings is difficult, it is possible that somatic embryogenesis could contribute clonal products to this market, provided that the selected attributes are significantly value added, e.g. improved tree shape, colour, xerophytic habit, fire retardant, extended cut life, etc.

## Somatic embryogenesis and polyembryogenesis

### EXPLANT CHOICE

In gymnosperms and a few angiosperms, the term 'polyembryogenesis' in clonal terms usually represents the reconstitutive multiplication of the zygote, i.e. the new generation (e.g. Singh, 1978; Norstog, 1982; Dogra, 1984) (*Table 1*). However, polyembryogenesis is of several types: *Simple polyembryony*

**Table 1.** Clonal products produced by polyembryogenesis

| | Type | Product attributes |
|---|---|---|
| 1. | Simple | Several zygotes, but one embryo from one zygote to produce groups of embryos of *fraternal type* (not clones). |
| 2. | Cleavage and/or lobing and budding | Asexual reconstitution of zygotic embryos yielding an *identical* type (clones). One or more groups of a given fraternal type are possible. Cleavage, lobing and budding are are similar except in terms of source and topological orientation of the reconstituted embryos. |
| 3. | Sporophytic or gametophytic | Adventitious embryos arise by budding from the nucellus (clones of the sporophyte) or by induced somatic cells (clones of new generation) or from transformed haploid gametophytes. The haploid embryos are not considered clones, and if they become diploid, they are not identical to the parental tree. Clonal products represent a form of reproductive regeneration (Sinnott, 1960). |

occurs when several eggs develop from one haploid megaspore and each is fertilized by a separate sperm or their development is parthenogenetic (Reiger, Michaels and Green, 1976). Where polyembryony arises from the fertilization of more than one archegonium, it is also called *polyzygotic* (Dogra, 1984). Products are genetically distinct and not clonal, at least initially. If polyembryony arises from the cleavage of proembryonal cells, it is known as *cleavage polyembryony*. The resultant non-adventitious embryos are monozygotic in origin and genetically identical (*Table 1*).

Embryogenesis also can be derived by reproductive regeneration from cells of the haploid gametophytic generation of maternal or paternal origin. *Apogamy* is a term used to describe the production of plants of sporophytic form through vegetative growth of pollen cells (Norstog, 1982). Here the genetically distinct embryos may arise by budding and/or cleavage, and are therefore 'adventitious', i.e. arising or occurring in other than the usual location. Moreover, they may reconstitute themselves by the budding and cleavage process. Embryos may also arise by parthenogenic development. Collectively, processes of this type have been called *gametophytic embryogenesis or polyembryogenesis* where applicable. During the early stages of development of the embryonal–suspensor masses in conifers, it is very difficult to find tissues of the female gametophyte that are not intercalated with multiple proembryos, at least with many of the seeds from tree improvement programmes. Somatic embryos recovered by such processes must be carefully checked cytogenetically before assuming a discrete developmental origin.

In gymnosperms, genetic variation associated with seeds is further complicated by the attributes of the proembryonal–suspensor mass, polyembryony, and by differences in the ontogeny of embryos (*see* Chamberlain, 1935; Singh, 1978; Norstog, 1982; Dogra, 1984). The latter author has reported variations in pollination mechanisms, fertilization and embryogeny that could further widen the genetic variability. Taken together, these variations or aberrations have been considered as bases for opportunistic evolutionary adaptations that increase the capacity of the species to colonize habitats that are frequently, suddenly, or periodically disturbed by drought, erosion and

changes in climate (Sorensen, 1982; Tomlinson, 1987). Recent publications on somatic embryogenesis in conifers have not recognized the polyembryonal aspects of their cultures, even though the species are known to show polyembryony (e.g. Hakman and von Arnold, 1985; Nagmani and Bonga, 1985; Krogstrup, 1986; Becwar, Noland and Wann, 1987; Hakman and Fowke, 1987; Lu and Thorpe, 1987).

For angiosperms, Johri (1982) has classified polyembryony. *Zygotic polyembryony* arises by budding and/or cleavage of the proembryo, or by differentiation of supernumary embryos from an embryonal callus. In angiosperms, there are only a few examples of cleavage polyembryony (Haccius and Bhandari, 1975). Presumably when callus arises from embryos or explants, the undifferentiated cells become transformed, or in the callus there is a carryover of proembryonal cells that have not yet become callus (Gupta and Durzan, 1986b). This aspect is rarely made clear in existing publications. *Nucellar polyembryony* is used to describe somatic embryogenesis in polyembryonic *Citrus* sp., where embryos arise by budding from nucellar cells that represent the maternal genome (Spiegel-Roy and Vardi, 1984). Johri (1982) uses the term 'embryoids' in association with polyembryony to represent structures obtained from callus, endosperm, protoplasts, etc. In all of these cases, comparisons are needed with zygotic embryo development to establish criteria based on polyembryony which is not always done. For angiosperms, Johri (1982) extends polyembryony to include 'true' and 'false' types, depending on whether the embryos arise in the same embryo sac or different embryo sacs in the same ovule, and distinguishes the spontaneous or induced production of polyembryonic types.

Gymnosperm somatic embryogenesis and polyembryogenesis can be distinguished from angiosperm processes through at least three types of post-fertilization events. The first involves a free-nuclear stage characteristic of basal plans found in zygotic proembryogenesis of gymnosperms (Singh, 1978; Dogra, 1966, 1984). *Sequoia sempervirens* does not have a free-nuclear condition (Chamberlain, 1935; Thomson, 1945). It may be merely a latent or unexpressed feature. The size of the egg and food supply may determine the number of free nuclei (Thomson, 1945). The size of the conifer cell correlates with level of DNA in the nuclear genome (Price, Sparrow and Nauman, 1973). With *Pinus banksiana* cell suspensions, kinetin supply can lead to at least two nuclei per cell (Durzan and Bennett, 1968). By contrast, 2,4-dichlorophenoxyacetic acid and other plant growth regulators, when added to the medium, contribute to nuclear fragmentation during the cell cycle. The second involves the formation of a proembryo by the internal segmentation of a cell without a free nuclear stage as occurs in many angiosperms. In gymnosperms, one typical product of a free-nuclear stage is proembryonal–suspensor mass. By contrast, in *Prunus* sp. and a few angiosperms, the product of internal cell divisions is a proembryonal cell complex (Haccius, 1978; D.J. Durzan, unpublished observations). These are equivalent systems in terms of their morphogenic potentials but not in developmental mechanisms. The third is the characteristic basal plans of development that have led to terminology that is uniquely gymnosperm (Dogra, 1980) or angiosperm (Johri, 1982).

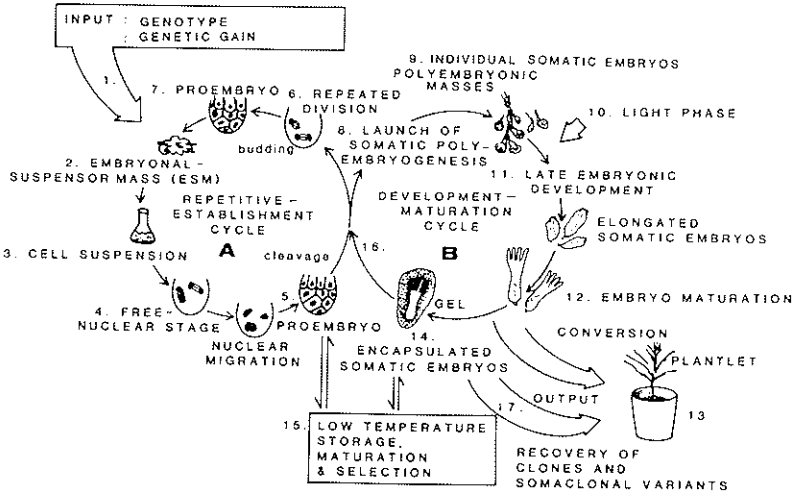


**Figure 2.** Two-cycle process for the recovery of plantlets by somatic embryogenesis and polyembryogenesis from cell suspension cultures. (A) Cultures (*ca.* 10 cc packed cell volume per 100 ml) are maintained by a repetitive establishment cycle involving the isolation (1,2) and establishment in cell suspension cultures (3) of an embryonal-suspensor mass (ESM) in the absence of callus (*see Figure 4*). The free nuclear stage (e.g. *Figure 6A*) occurs in the embryonal tube which arises mainly from other proembryonal cells and in some cases from the embryonal suspensor (Dogra, 1984). The composition of the ESM can be assayed for the distribution of embryogenic cells by examining the reactivity of cells to acetocarmine, Evans' blue and fluorescein diacetate. In a 7–10-day suspension culture cycle, the main two types of cells (proembryonal and suspensor) grow rapidly and divide with the production of a free-nuclear stage (4) Polar migration (5) of the nuclei results in the formation of the proembryo and its attached suspensor. This process repeats itself in darkness (6,7) under the conditions of cycle A (steps 1–7) (e.g. 5 μM 2,4-dichlorophenoxyacetic acid and 2 μM each of $N^6$-benzyladenine and kinetin. Other factors are identified in *Figure 3* (process control algorithm). Somatic embryos produced under these conditions multiply by cleavage and budding. Embryonal-suspensor masses that grow may show lignification in shake cultures as with loblolly pine (*Pinus taeda*) (Gupta and Durzan, 1987b) and in some cases multiple embryos may remain attached (*Figure 4*) to form a fabric of ESMs. This continues into cycle B.

For the development and maturation of complete embryos, a second cycle (B, Steps 8–17) is needed. Suspension cultures of globular proembryos will continue to divide by cleavage and by continual reinitiation of proembryos unless the culture cycle A is changed. The development of individual somatic embryos and inhibition of the cleavage process (8) can be brought about by reduction in levels of plant growth regulators and in some cases by the addition of abscisic acid. Some cell lines, particularly of élite genotypes, can produce embryos even in the absence of exogenously added plant growth regulators. These represent an interesting and special case where conditions need to be simplified to match zygotic development and to maintain process and quality control. As abscisic acid encourages the development of individual somatic embryos, exposure of cultures to white light (10) becomes advantageous. This promotes, at reduced plant growth regulator levels or (in some cases) their absence, the development, elongation and maturation of the somatic embryos (11). At this point, cultures may be plated out on a solid or semi-solid support and further media changes introduced to establish cotyledonary development, the filling of nutrient reserves and root development (12) before transfer (conversion) to soil (13,17). Somatic embryos may be encapsulated to produce artificial seeds (14) for a variety of end uses, cryopreserved (15) or reintroduced into cycle A (16).

According to Haccius (1978), it is still preferable to reserve the term 'suspensor' to describe the morphologically recognizable appendage of zygotic and non-zygotic embryos. However, for gymnosperms, we are dealing with a suspensor system of variable complexity that is characteristic of the type of embryogeny (Dogra, 1984). The terms 'proembryonal cell complex' (angiosperms) or 'proembryonal–suspensor mass' (conifers) should be used when referring to polymorphic embryogenic cell groups, as opposed to the term 'callus'. In the latter, the daughter cells grow in an unorganized fashion unless they are somehow transformed to become embryogenic, i.e. we have an 'embryogenic callus'. In this case, concerns must be expressed about genetic changes induced by substrates that may occur in clonal products arising from callus.

Usage of the term 'proembryonal–suspensor mass' is comparable with Haccius' proembryonal cell complex, but for the gymnosperms we distinguish further factors that relate to the origin of the seed habit: such factors include cell cycle variations, free-nuclear stage and the cleavage process with its variations (Thomson, 1945; Dogra, 1984). Haccius (1978) also recognizes that some suspensors have an embryogenic potency even in differentiated embryos. This potential can contribute to a second wave of regeneration.

## ORIGINS OF SOMATIC EMBRYOS BY RECONSTITUTION

Zygotic embryos arise from the fusion of the egg with a pollen cell. Through karyogamy (the fusion of nuclei) and through plasmogamy (the mixing and sorting out of cytoplasms) the zygote develops into a new plant by successive and characteristic stages of development. Where the reproductive structures become abortive, the embryo or embryonal–suspensor masses can be rescued from the ovule or seed and can be cloned by somatic embryogenesis and polyembryogenesis (*Figures 2* and *3*). A somatic embryo may reconstitute itself from a single proembryonal cell or from a group of cells (e.g. Durzan and Steward, 1968, 1970; Williams and Maheswaran, 1986).

In reproductive regeneration where the embryo may arise from a sporophyte or gametophyte, variations of the cell cycle that result in changes of ploidy (haploidy, colchiploidy, aneuploidy, amphidiploidy) need to be considered (Brown and Dryer, 1972; Schlarbaum, 1987). Unfortunately, cytogenetic studies have been minimal, with the exception of studies of haploids and triploids in a few species (Chen, 1987; Lakshmi Sita, 1987).

Depending on genetic source, aberrations in true-to-type processes may occur as embryos are rescued and regenerated. Some embryogenic cells may contain relic nuclei, i.e. nuclei from other tissues (e.g. ventral canal cell) that have somehow entered and fused with the egg (Chamberlain 1935; Singh, 1978; Dogra, 1966, 1984). Embryos derived from relic nuclei have not yet been knowingly recovered in somatic embryos. Products of this type may be tentatively called phenoclonal variants (Gupta and Durzan, 1987b).

Evidence is accumulating, especially with somatic embryos of conifers, to show that the products of cleavage embryony recapitulate synchronously in cell suspension culture the same stages of development (*Figures 4, 5* and *6*), at least

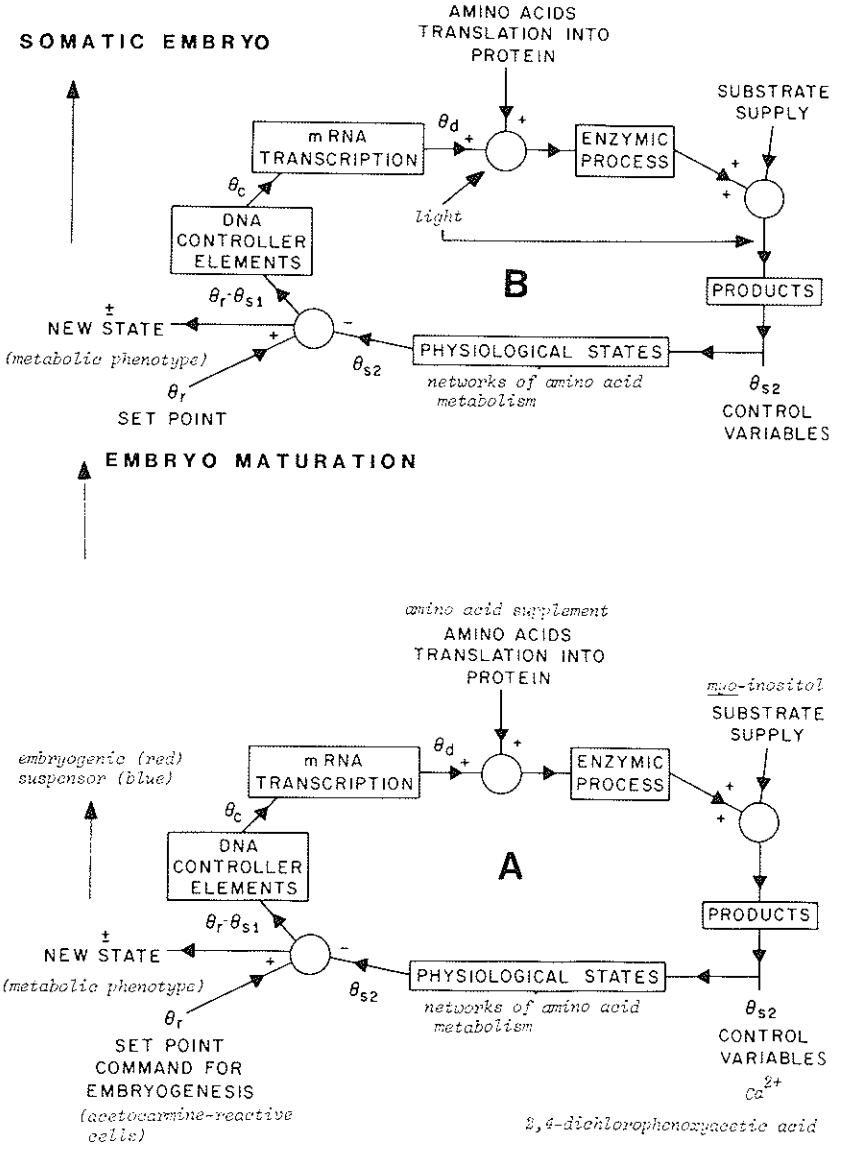


**Figure 3.** Hypothetical signal flow diagrams for the process control of somatic embryogenesis and polyembryogenesis. Proembryonal–suspensor masses in cell suspension culture are poised for embryogenesis by 2,4-D and other plant growth regulators (e.g. $N^6$-benzyladenine, kinetin) in the presence of ammonia or reduced nitrogen and often at low calcium levels. All signals are designated by θ. The set point or command signal $\theta_r$ is fed into proembryonal cells where it is compared with the current physiological state $\theta_{s1}$. The difference between the set point and the signal $\theta_{s1}$ is called the deviation or error signal. The nuclei respond and generate free nuclear-divisions which may involve amplifying, integrating or differentiating the error signal. The signal $\theta_c$ represents a RNA-polymerase complex acting on DNA which evokes mRNA production associated with the formation of embryonic proteins. Another signal, based on mRNA production $\theta_d$ moves to sites of protein synthesis. Protein synthesis is promoted by an amino acid supplement, e.g. casein hydrolysate with L-glutamine. A change in products produced by enzymic reactions and

promoted by *myo*-inositol, represented by $\theta_{\leq 2}$, helps to establish the embryogenic physiological state and the production of the associated and characteristic acetocarmine-reactive mucilaginous material. Process control is achieved by comparison of $\theta_{\leq 2}$ with the previous set point to help define the next physiological state. The current physiological state can be maintained as a repetitive cycle by keeping cells exposed to the appropriate levels of plant growth regulators and by culturing cells in darkness. The physiological state of cells in cycle A can be rapidly established by a diagnostic double-staining colour-coded test with acetocarmine and Evans' blue.Signals (±) are placed arbitrarily to indicate positive or negative flux.

Reduction in removal of plant growth regulators coupled with the addition of abscisic acid for many conifer embryos comprises a forcing signal for the initiation of embryo maturation (cycle B). We now have a new set point and error signal that moves through cycle B. However, as embryos mature, nutrient reserves in the form of starch (amyloplasts), proteins (protein bodies) and lipids (liposomes) are laid down mainly in cotyledons. These steps are facilitated by exposure of cultures to light; however, in zygotic embryo maturation all events continue to occur in darkness. The outcome is the production of an elongated embryo with cotyledons that is sometimes arrested in growth and development unless the abscisic acid is removed earlier.

Cycles A and B coincide with the same cycles in *Figures 2* and *3* and may be controlled as shown in *Figures 7* and *8*.

up to the mature embryo with cotyledons (*Figure 6E*). For example, somatic proembryos pass through stages having free nuclei (*Figures 5C, 5D, 6A*; Gupta and Durzan, 1987b; cf. Von Anderkas and Bonga, 1988). Large nuclei are surrounded by a sheath of new cytoplasm similar to the neocytoplasm that is formed upon fusion of gametes (Dogra, 1966; Camefort, 1969; Willemse, 1974). Chamberlain (1935, p. 138) points out that nuclei of the pollen and egg are filled with a substance which stains deeply with iron haematoxylin and that most of this material is not chromatin. He states that Strassburger called it metaplasm, because it was not regarded as chromatin or as protoplasm. A tiered, cellular organization and the cleavage polyembryonic process (*see Figure 6BC*) emerges from this early developmental plan.

Calli formed under culture conditions favouring somatic embryogenesis usually do not survive and are readily distinguished from proembryonal–suspensor masses by their cytochemical properties and by visual browning of cells. Cells, staining red with acetocarmine, are embryonic (*Figure 4*); cells permeable to Evans' blue are suspensors or moribund. Callus stains weakly with both dyes (*see* Durzan in Powledge (1984) for *Prunus*; Gupta and Durzan, 1987b for *Pinus*).

The regeneration of proembryos from protoplasts or cryopreserved proembryonal cells indicates that it is the isolated single proembryonal cells that restore the formation of the blue-staining suspensor cells (Gupta, Durzan and Finkle 1987; Gupta and Durzan, 1987c). In loblolly pine (*Pinus taeda*), nuclei at the free-nuclear stage stain differentially with acetocarmine and Evans' blue, not unlike nuclei in proembryonal and suspensor cells respectively. As nuclei migrate, they already carry with them the developmental programme for the reconstitution of a new proembryo.

Many variations in reconstitution and restoration are possible in reproductive regeneration (e.g. Krogstrup, 1984; Hakman *et al.*, 1985). Variation is based on the wide variety of available explants throughout the life cycle. One danger in dealing with all of the cases is the proliferation of terminology in the absence of a general understanding of the mechanisms responsible for the recovery of somatic embryos (*see* Kester, 1983).

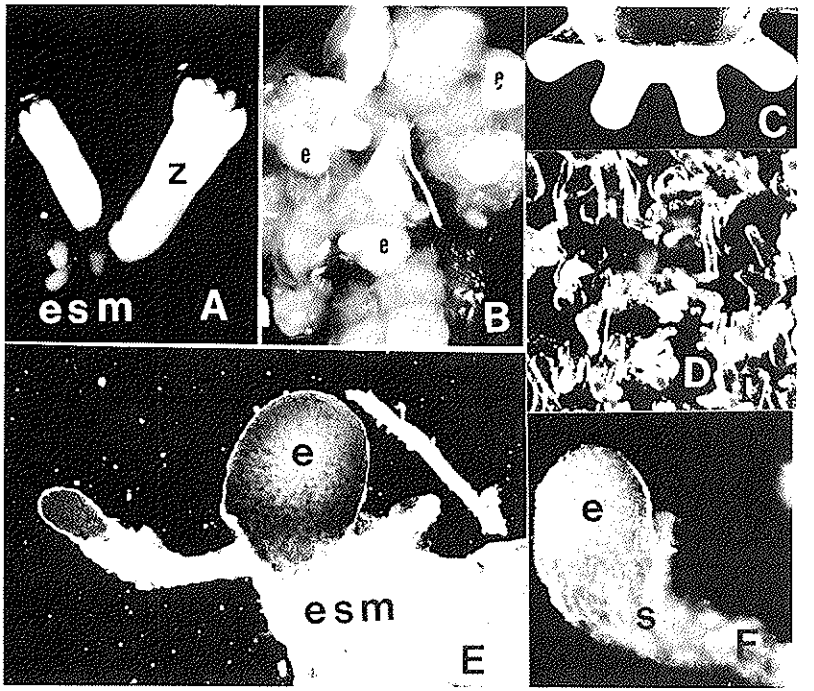


**Figure 4.** Somatic polyembryogenesis in a sugar pine (*Pinus lambertiana*) cell suspension culture. A. the embryonal-suspensor mass (esm) is isolated from seeds (e.g. sugar pine) and cultured to rescue the zygotic embryo (Z) and other fraternal or identical twins. The esm often contains proembryos which when plated on a semisolid medium will generate other somatic embryos by a budding process as in B. Somatic proembryo development is accompanied by the continued production of the esm and its mucilaginous material. C. The esm is inoculated *c.* 10 g/l in a nipple flask rotated in 1 rpm in darkness to give a uniform and relatively homogeneous cell suspension. D. The esm cell suspension consists of elongated suspensor (*ca.* 100–200 μ long) and proembryonal cells can be distinguished from callus by double-staining with acetocarmine and Evans' blue. Under these culture conditions, callus cells rapidly deteriorate so that the developmental process is surprisingly true to type and may include a free-nuclear stage. E. Staining of an esm with acetocarmine to show reactivity of the proembryo (e). F. Globular proembryo (*c.* 1 mm dia) with an attached suspensor (S) in an unstained preparation.

## INITIATION OF CLEAVAGE POLYEMBRYOGENESIS AND SECONDARY LOBING

Cleavage is a regular and highly organized splitting of the zygote to yield an identical proembryonal cell. Cleavage products are produced along a plane of symmetry from the proembryonal head to the suspensor (e.g. *Figure 6BC*). Some gymnosperms do not exhibit cleavage (Chamberlain, 1935; Johri, 1982); moreover, the phenomenon may be transitory or persistent (*see* Dogra, 1984). In angiosperms, cleavage is more difficult to observe, but budding is especially evident with *Prunus* sp. Budding is seen more as an embryonal protuberance that differentiates into a new individual.

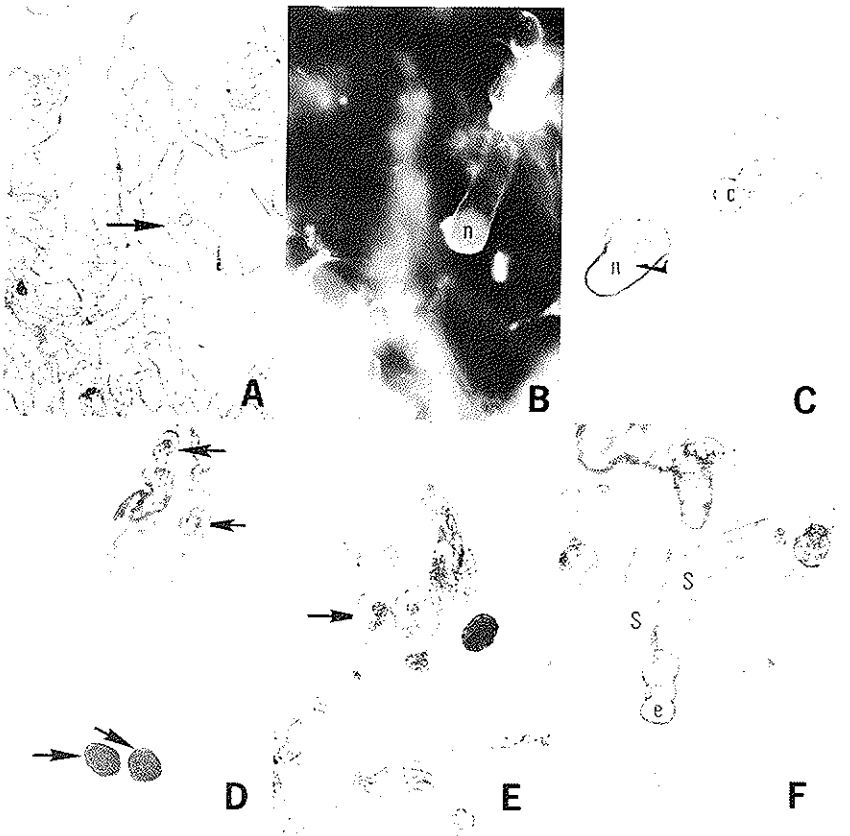


**Figure 5.** Nuclear migration of the proembryonal sheath and proembryonal tier formation in Douglas fir (*Pseudotsuga menziesii*) cell suspension cultures in cycle A (cf. *Figure 2*). (A) Light microscopic view of a proembryonal–suspensor mass with an elongated cell bearing a proembryonal sheath with neocytoplasm and a nucleus (arrow) (x40); (B) Polarized light microscopic view of (A) to show weak birefringence of the neocytoplasm (n) in the proembryonal sheath (x40); (C) Light microscopic view of migrating cytoplasm (c) and a proembryonal sheath (arrow) in a cell that is brightened by reaction with calcofluor. Reactivity with calcofluor (Stone, 1984) suggests the presence of β-glucans in the cytoplasm; (D) Two elongated, binucleated embryonal tube cells each bearing a large acetocarmine-reacted nucleus and neocytoplasm (arrows at left). Nuclei weakly reactive with Evans' blue are shown by arrows at right (x40); (E) Four-cell tier of a double-stained proembryo (arrows) derived from a large acetocarmine-reactive proembryonal sheath (x40); (F) Light microscopic view of an early unstained proembryo of Douglas fir (e) and its suspensor (s) (x40).

Dogra (1967) has used X-ray images to explore the quality of conifer seeds and the products of specific genetic crosses. Where seed development is aberrant or disturbed, as revealed by X-ray images, the polyembryonic masses can be removed from seeds and studied. His evidence reaffirms that polyembryogenesis is common and that the cleavage process is both genetically and environmentally controlled.

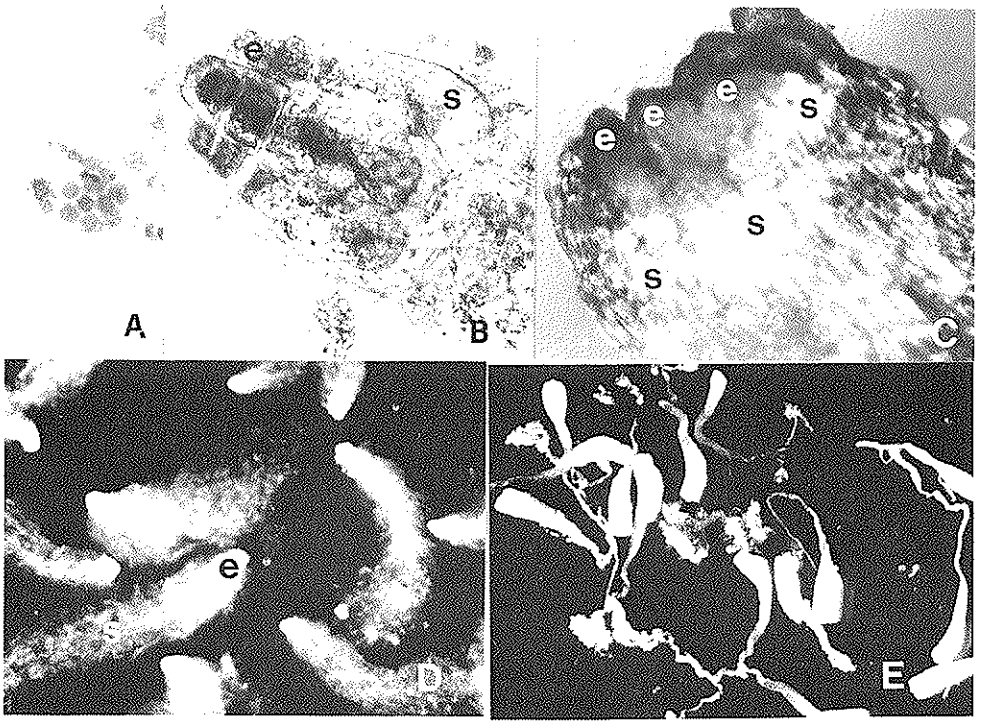


**Figure 6.** Somatic embryo development in cell suspension cultures of conifers. (A). Norway spruce cell stained with acetocarmine to reveal free nuclear stage (x60); (B) Arrangement of tiers of proembryonal cells in sugar pine (*Pinus lambertiana*) (e) as files and the associated suspensor cells (s) (x40). Each file is capable of producing a somatic embryo and the set of files represents cleavage polyembryogenesis. The preparation is stained with acetocarmine. (C) Polyembryonic mass of somatic Douglas fir proembryos (e) and their suspensor (s) that remain attached as files. Douglas fir does not normally cleave (Allen and Owens, 1972) but does so in suspension culture (Durzan and Gupta, 1988). The preparation is stained with acetocarmine and Evan's blue and viewed under polarized light to bring out the refringence associated with cell walls of the suspensors (x25). (D) When abscisic acid is added to cell suspensions of loblolly pine, the polyembryonic process in inhibited and proembryos (e) and suspensors (s) develops individually. Unstained preparation. (E) Individual Douglas fir somatic embryos during maturation. Embryos are now elongated and show signs of cotyledonary and apical meristem development. Suspensors remain attached to embryos and in some cases the esm can be seen as an irregular mass of cells that have been inhibited by the abscisic acid. Continued development and greening of embryos, now 1–2 mm long, is promoted by light.

A method is needed to arrest cleavage and to launch later stages of embryogenesis. One control factor here is the level of endogenous or added abscisic acid. When abscisic acid is added to cell suspensions of loblolly pine (*Pinus taeda*) and Norway spruce (*Picea abies*), polyembryogenesis is inhibited (Durzan and Gupta, 1987; Boulay *et al.*, 1988). Cleavage can be induced in suspension cultures of Douglas fir (*Pseudotsuga menziesii*) (Durzan and Gupta, 1987) which does not normally display polyembryogenesis *in situ*

(Chamberlain, 1935; Allen and Owens, 1972). This suggests that cleavage polyembryony is a latent feature of embryonal initials, even in a tree that normally reproduces by simple embryogeny (*Figure 6C*).

'Indeterminate cleavage polyembryony' (Buchholz, 1933) denotes that any one of the embryos derived from a single zygote has an equal opportunity for future development. Normally only one embryo comes to maturity in the seed. This has been called 'determinate cleavage polyembryony'.

Aberrations in cleavage polyembryogenesis may be a slow method for creating *in situ* new genetic variation when compared with the sexual process. However, somatic cells can now be grown on a large scale in suspension culture (Gupta and Durzan, 1987b). As the sexual and asexual reproductive processes are difficult to manipulate *in situ* by classic methods, the opportunity arises to explore the role of somatic mutations and aberrations *in vitro* and by molecular biology. Cell suspensions provide a large-scale source of morphogenic protoplasts for the production of artificial hybrids that may be able to perpetuate themselves by the cleavage or budding process.

Under the influence of synthetic plant growth regulators in cycle A of *Figure 2*, the proliferating cells produce irregular lobes or patches of proembryonal–suspensor masses. Lobing may occur in both cleavage or non-cleavage species (Dogra, 1984) and in haploid (Bonga, Von Anderkas and James, 1988) or diploid cells (Durzan and Gupta, 1988) to yield secondary embryos. In the seed, lobing may correspond to the so-called rosette embryos described by Buchholz (1929). Lobing has been observed to be both transitory or persistent (Dogra, 1984). More study is needed to define a simple minimal medium for the recovery of mutant cell lines that have significant genetic variation in the cleavage process. In this way, the capacity of cells to form multiple embryos in relation to a cleavage factor could be tested experimentally.

Cleavage can be nicely initiated by plant growth regulators in the culture medium (*Figure 3*). A few hybrids of *Pseudotsuga menziesii* and *Pinus taeda* with élite genotypes and a high degree of heterozygosity have required no plant growth regulators to initiate cleavage or budding processes (unpublished data). Medium and environment components (*Table 2*) such as carbon, nitrogen, oxygen, pH, carbon dioxide, phosphate, reduced pyridine nucleotides can be manipulated to control gene expression and cellular differentiation of plant cells just as has been done over the last four decades in fungal, bacterial and now mammalian cultures (Bailey and Ollis, 1986). Exploitation of these factors has contributed to our limited but useful knowledge of how to direct the expression of totipotency in plant cells, i.e. their inherent potential to recapitulate the life cycle (Steward, 1968).

## DIRECTED TOTIPOTENCY IN *PRUNUS*

For peach and nectarine (*Prunus persica*), cherry (*P. avium*), plums (*P. domestica*), almond (*P. amygdalus*), and apricot (*P. armeniaca*), we can now induce *in vitro* repetitive multiple embryogenesis by budding on rescued zygotic embryos that would normally abort if left on the tree (R. Bhansali, J. Driver, and D.J. Durzan, unpublished). Budding occurs at several loci, e.g.

**Table 2.** Some of the major factors which may be used to control and to indicate the status of cell suspensions in bioreactors

| | |
|---|---|
| A *Physiological factors* | |
| Carbohydrate concentration | Number of generations |
| Nitrogen concentration | Sequential exposure to and release of growth regulators |
| Cell mass concentration | Macro and micro (trace) elements such as $Mg^{2+}$, $Ca^{2+}$, $K^{+}$, $PO_4^{2-}$, $NO_3^{-}$ and $Cl^{-}$ |
| Biological | NAD $NADH_2$ |
| Contamination | ATP, ADP, AMP |
| Enzyme activities | Energy charge of cells |
| Lipid concentration | Presence of transforming factors |
| Doubling time | |
| Specific growth rate | |
| B *Physicochemical environment* | |
| Light intensity and quality ($\mu W/cm^2/nm$) | Redox potential (mV) (electro chemical) |
| Temperature (°C) | Oxygen partial pressure (mg/l) |
| Pressure (bar) | Exit gas: |
| Power input P/v (watt/l) | oxygen concentration (%) |
| Aeration rate (vvm) | carbon dioxide concentration (%) |
| Anti-foam factors | Surface tension (dyn/cm) |
| Turbidity (OD) | Density (g/ml) |
| Viscosity ($dynes/cm^2$) | Revolutions per minute ($min^{-1}$) |
| pH (mV) | |
| C *Indicators of productivity and biosynthesis* (*cf* Clarke *et al.*, 1986) | |
| pH (acid production) | Oxygen uptake, carbon dioxide evolution (respiration quotient) |
| Dissolved oxygen (oxygen transfer rate) | Carbohydrate level + feed rate, carbon dioxide evolution (yield and cell density) |
| Oxygen in exit gas, gas flow rate (oxygen uptake) | Cell mass, used substrate, yield: g/l/day/cost |
| Carbon dioxide in exit gas, gas flow rate (carbon dioxide evolution) | Specific growth rate |
| | Acoustic propagation and scattering |
| | Optical scattering |
| D *Cell quality* | |
| Division patterns and rates | Developmental trueness-to-type |
| Affinity for diagnostic stains | Selection differential (responsiveness) |
| Fluorescence | Availability of embryonic/juvenile/mature correlations |
| Nuclear magnetic resonance | Yield of morphogenic or competent protoplasts |
| Explant origin | |
| Pedigree | |

cotyledons of rescued embryos, rather than at a plane parallel to the axis of the embryo as in conifers (*Figure 6BC*). The rescued embryos represent crosses made by commercial breeders for important traits, such as early ripening and fruit quality. Rescued zygotic embryos of *Prunus* have also yielded embryo-producing cell suspension cultures. Invariably, cells competent to produce globular embryos also react diagnostically with acetocarmine, as do the gymnosperm cells, but further embryonic development and plant recovery is needed.

Petiole cells of young leaves from a mature cherry tree can be induced to produce, by reproductive regeneration, somatic embryos at low frequency in cell suspensions (Hansen and Durzan, 1986). Cells are rejuvenated and dedifferentiated in darkness to an embryonic state. By adding plant growth regulators, some cells regenerate somatic embryos (*see* Powledge, 1984, for

figures). By contrast, the populations of cells from the same culture when placed in white light behave like cells of the developing and ripening mesocarp (Durzan, 188b). Cells produce red pigments and in some cases (on supplementation of the medium with amino acids), fragrances of cherry become apparent. Fragrances are not commonly derived from fruit in the orchards. Red nodules resembling the mesocarp of cherry fruit senesce after 2–3 weeks. This process is tentatively called 'somatic parthenocarpy'. Normally, stone fruits do not set parthenocarpically (Westwood, 1978), although parthenocarpy can be induced on cherry trees in orchards, by the application of a methionine conjugate of 2,4-dichlorophenoxyacetic acid and by phthalimide (Rebeiz and Crane, 1961; Retamales and Bukovac 1986).

Somatic parthenocarpy enables the study, separation, control and exploitation of partial reactions in directed developmental processes associated with fruit production and ripening. Study can occur without the influence of seed development, yet the same cell suspension may be used for the production of somatic embryos (Durzan, 1988b).

## Process control: polyembryogenesis in cell suspension

### INTRODUCTION

Embryonal–suspensor masses of Norway spruce and loblolly pine have been maintained in cycle A (*Figure 2*) for over 4 years by subculture every 7–10 days, without the formation of callus. In the culture medium, high levels of synthetic plant growth regulators are commonly added at the start for most, but not all, genotypes. Media formulations enriched with *myo*-inositol and reduced nitrogen, are based on the composition of zygotic proembryos grown in darkness, and on experience gleaned from studying the factors listed in *Table 2*. The skotomorphogenic or 'dark' phase of development is followed by the progressive removal from the medium of stimuli fostering the cleavage (*Figure 6BC*), or budding process (e.g. *Figures 4B, 5*), and by morphogenesis in light. In some cases, abscisic acid or other factors may be required to inhibit cleavage, synchronize cultures and enhance proembryo maturation (Boulay *et al.*, 1987; Durzan and Gupta, 1987). Overexposure of proembryos to abscisic acid creates other problems later, such as arrest of embryo development or poor germination. Media are progressively shifted from highly heterotrophic to more autotrophic conditions, i.e. the composition of media is simplified by the sequential removal of plant growth regulators, free amino acids, carbohydrate supply, etc.

With polyembryogenic cell suspension cultures, we are faced with the development of scaled-up, more controllable systems for pilot studies (Durzan, 1982b). Process control over the fate of cells has become increasingly difficult due to the complexity of cell growth, differentiation and morphogenesis. The bioengineering approach with plant cells started mainly in the laboratory of H.E. Street (e.g. King, 1980), and has provided much of our present understanding.

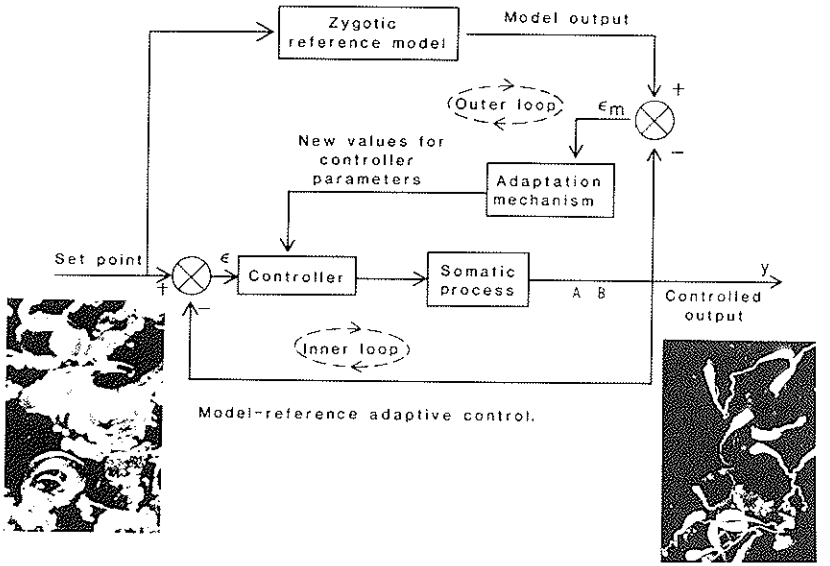


**Figure 7.** Model-reference adaptive control (MRAC) for the process control of somatic polyembryogenesis using repetitive cycle (A) and the development maturation cycle for completion of the overall process (cycle B in *Figures 2* and *3*). Em is the difference error used to estimate the mean square error. The system is based on a zygotic reference model whose data (values) feed into the overall controller for SPE. The controlled output from cycles A and B yields from cell suspensions (shown at left); somatic embryos (shown at right).

## MODELLING PROBLEMS

In suspension cultures we have to distinguish among cells that have the potential to form proembryos, suspensors and callus. Some proembryonal initials and cells have the capacity to form proembryos but are not yet ready to do so because they are engaged in the repetitive cleavage cycle A (*Figure 2*). Cell types in inocula can now be rapidly assayed for their embryogenic potential by acetocarmine reactivity so that the final yield of plantlets can be estimated for a given batch.

To answer questions about process control, we need mathematical representations of the physical, chemical and biological parameters associated with the ontogenetic sequence (*Figure 2*). Where possible, simulation of the process would be helpful (e.g. Evans, 1988). For conifers, the ontogenetic sequence starts with a defined genetic gain associated with a packed cell volume that is introduced into a bioreactor (set point in cycle A, *Figure 3*).

Maintenance of the polyembryogenic potential of cell suspensions is specified by the control variables which include plant growth regulators such as 2,4-dichlorophenoxyacetic acid, kinetin, $N^6$-benzyladenine, and, in some cases, by casein hydrolysate and low levels of calcium salts. The need for clearer ideas and precise terms regarding sensitivity of plants to growth substances has been pointed out by Firn (1986). Embryogenic cells are

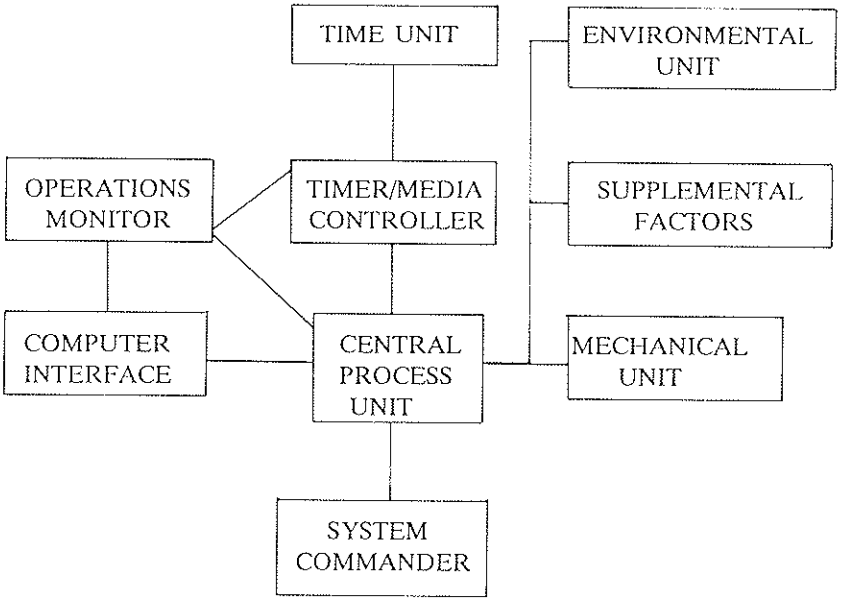


**Figure 8.** Layout for hypothetical 'process control module' with a system command control for somatic polyembryogenesis outlined in *Figure 7*, and employing a reference model as shown in *Figure 9*.

maintained in cycle A in the presence of plant growth regulators by subculture every 7–10 days.

When proembryos are reconstituted (cycle A, *Figure 2*), a new set point (cycle B, *Figure 3*) is reached by reducing levels of plant growth regulators. The addition of abscisic acid inhibits the cleavage process (Boulay *et al.*, 1988). The proembryos, now primed for embryogenesis, begin to differentiate and complete this phase of development through conditions established in cycle B. A mathematical model of this system (A and B) is a prerequisite to the design of its controller (*Figures 7* and *8*).

## DESIGNS OF THE CONTROLLER

The controller design is the element of the process that receives information from measurements in the culture system (e.g. *Table 2*, *Figure 9*). It takes the appropriate control actions to adjust values of the manipulated variables. Until now, this function has been performed by the investigator responsible for the cultures. For automation of somatic embryogenesis, we must ask how information taken from measurements may be used to adjust values of the manipulated variables. This constitutes the control laws which are implemented automatically and is the focus of current research (Clarke *et al.*, 1986).

Moreover, we are faced with the design of an 'inferential' control system because the critical controlled variables (e.g. *Table 2*) are not yet fully

understood and hence cannot be easily measured. One approach is to measure some performance output and attempt to regulate the value of the unmeasured control objective at a desired level. In the inferential approach, the control objective is not measured directly. It can only be estimated from some measured output and checked by visual examination as follows:

$$\text{Control objective} = f(\text{measured output})$$

If we do not have a mathematical representation of the true-to-type developmental process or zygotic basal plan, such a relationship in turn is not possible. Once values of the control objective are identified and estimated, they can be compared with the desired value (set point) and the controller can be activated for action by a feedback control. So far, we have only a rudimentary idea of the measured input that is needed for cycles A and B (*Figures 2, 3* and *7*; *Table 2*).

A set of fundamental, dependent quantities, the values of which will describe the natural states of developing cells, and a set of equations, are needed to describe how the natural states of somatic cells change with time (steps 1 to 17, *Figure 2*). These quantities must relate to a reference model of zygotic embryogenesis. For zygotic embryos and fruits of a number of woody perennials, a general state-network model for constructing response surfaces for metabolic phenotypes, based on nitrogen metabolism and plant growth regulators, has been proposed (Durzan, 1987b, d). This model, however, is currently more complex than can be applied for process control. The model also needs to be brought in line with current notions on metabolic control and its analysis (e.g. Sauro, Small and Fell, 1987).

Some of the dependent control variables cannot be measured directly or continuously. In such cases, other control variables (cell size, density, viscosity, conductivity, concentration, temperature, flow rate, etc.) that can be measured conveniently are selected. When these variables are grouped appropriately, they approximate the value of the fundamental control variables for cycles A and B. These variables are called *state variables*. Their values define the state of a system processing each step in cycles A and B.

From an engineering viewpoint, we must also balance mass, energy, nutrients, plant growth regulators and the momentum of the developmental process. Relationships are still needed to express thermodynamic equilibria, reaction rates and transport rates. Input effects introduced by a system commander (*Figure 8*) will not always be instantaneous. This time—internal or transportation lag, or distance-velocity lag, or pure delay—is called 'dead time'. Lag and hysteresis are problems common to all control systems.

Serious difficulties remain in our model for process control in somatic polyembryogenesis. These arise from poorly understood chemical, physical, biochemical and biological phenomena. Problems arise from inaccurate values of various system parameters and from the current size and complexity of the resulting model, i.e. we are still dealing with cyclic, branching, multicomponent reaction systems or with poorly known interactions among various components and imprecisely known kinetics. Moreover, from descriptions of physiological states and metabolic networks in zygotic embryogenesis, we have

observed metabolic characteristics that are non-linear and non-stationary. This situation should be simplified to vavour linear algorithms where possible.

The traditional notion of complex systems having an unique equilibrium or steady state has been superseded by the recognition that non-linear dynamic systems have multiple solutions. The nitrogen metabolism associated with zygotic polyembryogenesis, when described by equations, has multiple equilibria or steady states (Durzan 1987b, d). An example that deals with the biosynthesis of amino acids during development of the mesocarp of a cling peach is shown in *Figure 9*. Our controller function for embryogenesis must somehow adapt to similar parameter changes. We need an objective function that will guide the adaptation mechanism with its functions to the best adjustment of the controller parameters, i.e. we are currently faced with the basic notions of a model-reference adaptive control (MRAC).

## MODEL-REFERENCE CONTROLS

A reference model has been proposed, using arbitrarily selected aspects of the basal plans of conifer polyembryogenesis (*see* Dogra, 1978, 1984) to tell us how the controlled process output (growth and morphogenesis) ideally should respond to command signals (e.g. set points in *Figures 3* and *7*). The response of proembryonal somatic cells in suspension culture should mimic the zygotic system, i.e. the response becomes 'biomimetic'. The zygotic model output (e.g. *Figure 7*) is tracked and compared over time ($t$) to the actual process output wherever and whenever possible. The difference in error ($Em$) between the two outputs is used through the system commander (*Figure 8*) to adjust parameters of the controller to minimize the mean square error of measurements.

The model-reference adaptive control system is composed of two loops (*Figure 7*). The inner loop is an ordinary feedback control loop for somatic polyembryogenesis. The outer loop includes the adaptation mechanism to the zygotic model and it could also be a feedback loop. The zygotic model's initial output plays the role of the set point while the process output is the actual measurement of performance in somatic polyembryogenesis. There is a comparator, the output (error $EM$) of which is the input to the adjustment mechanism. A major problem remains to design the adaptation mechanism to provide a stable culture system, i.e. to bring $EM$ to zero. Initial experiences indicate that the foregoing is not a trivial problem.

Examination of jack pine seeds collected from a wide range of seed sources (Durzan and Chalupa, 1968) has shown that climate at the seed source correlates with the composition of the female gametophyte which nourishes the embryo, and the embryo itself. In pilot studies aimed at regenerating somatic embryos according to inferential or adaptive control, the boundary conditions represented by the range of climatic variables (temperature, light) at seed sources (provenances) should be imposed on the somatic production system. This will have several major effects that contribute to the ultimate field performance of recovered plants by establishing the level of nutrient reserves in cotyledons and the ability of plants to develop a functional root under field

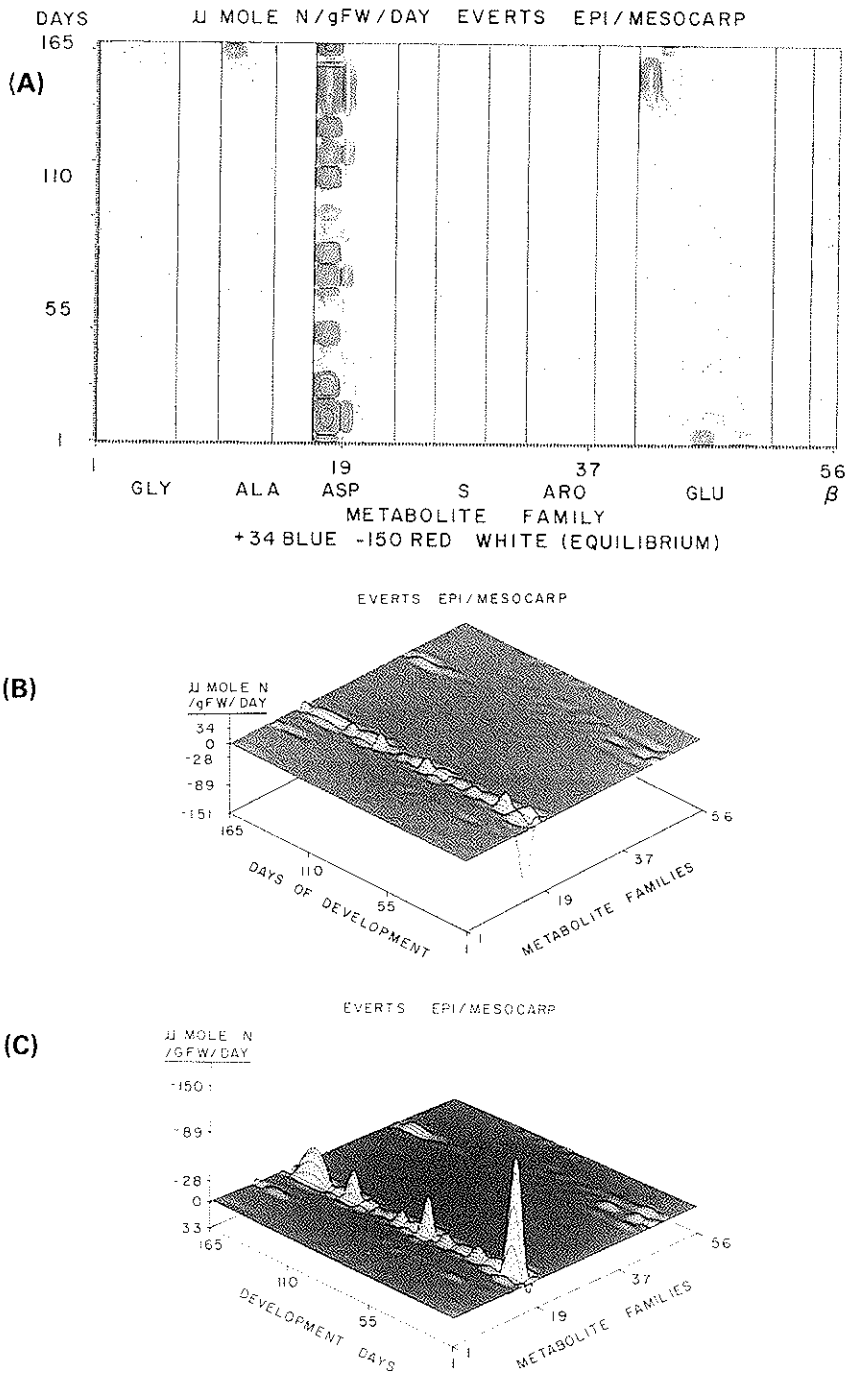


**Figure 9.** (A) An arbitrarily designed two-dimensional colour-coded state-network map

showing a signal surface for the biosynthesis of amino acids as families in peach tissue (*Prunus persica* cv. Everts). Contours represent maxima and minima in flux μmol N in each amino acid along a biosynthetic sequence over 165 days. The map represents a model-reference against which cells in culture must be able to adapt to reach the same discrete developmental outcome at day 165. The challenge for process control is to design a model-reference adaptive control system for such a situation. For somatic polyembryogenesis, a comparable map could serve as the zygotic reference model in *Figure 7*. The map is derived from graphic displays of the metabolic flux shown in more detail in *Figures 9(B)* and *9(C)*.

*Figure 9(B)* shows that the positive departures from equilibrium for the flux of N in each free amino acid in a hierarchy of biosynthesis are determined by a metabolite family. For example, glutamic acid is the first compound made in the glutamate family (GLU) and metabolites in this family are arranged in sequence along the corresponding axis. Metabolites include glutamine, ornithine, citrulline, arginine, proline, γ-aminobutyric acid, etc. (see Durzan 1987d).

*Figure 9(C)* is the underside of (B) to show the negative flux of N associated with each amino acid. When (B) and (C) are compressed into two dimensions as in *Figure (A)*, we see a mosaic contour of different intensities that represent the intensity of the positive or negative flux. This contour provides a physiological state or time-metabolic network signal surface as a model reference for sampled-data control systems and time stamping.

conditions. These variables precondition growth for precise environmental adaptation. Notions of physiological preconditioning (e.g. Rowe, 1964; Durzan and Chalupa, 1968) and metabolic phenotypes (Durzan, 1987b, d, 1988a) will have to be better understood to account for the variability of composition and performance in both somatic and zygotic embryos. This is indeed a major challenge that will take years of study, but one which will be very worthwhile in scientific returns and principles that will ensure superior products for certification and commercialization.

## RECOVERY OF COMPLETE PLANTS

In our experience, the efficiency of recovery of plantlets and transfer into soil, while low, has been steadily increasing. For loblolly pine, the *potential* for plantlet recovery, as judged by acetocarmine reactivity of cell suspensions, is over 1000 embryos per gram of packed cell volume. This number of plants has not been recovered from any batch of somatic embryos. Currently, and at best for Norway spruce (*Picea abies*), we have recovered 200–250 plantlets from a gram of packed cell volume (Durzan, 1987c). The efficient recovery of plants requires more experience and a better understanding of polyembryonic development. Somatic embryos should be developed in darkness for as long as possible before exposure to light (i.e. step 10 in *Figure 2*). Exposure is best delayed until germination or conversion is sought from mature and ripe embryos. Conversion is a term sometimes used to describe the production of plants from somatic embryos. In seeds the analogous process is germination.

The responsiveness and development of somatic embryos exposed to white light and especially to red and far-red light (Campbell and Durzan, 1979), can be easily assayed. For ripe 'zygotic embryos' in seeds, the phytochrome-response curve is estimated by use of an inexpensive helium–neon laser source

that is coupled to a camera shutter. The latter enables precise time exposures of embryos to light for the evaluation of germination. Atypical covalently bound metabolites derived from imbibition of tritiated water are useful in identifying metabolic blocks that occur during imbibition in darkness or light (Durzan, 1983).

The operational objectives for conversion and recovery of complete plantlets should involve: (1) discrete product specifications with defined genetic constitution involving true-to-type responses, and high conversion rates; (2) identification of batch operational constraints (e.g. underproduction, asynchrony, aberrations, cryopreservation, etc.), and (3) economic considerations. For the latter, conifers used in ornamental horticulture usually command greater prices than those used for forestry. However, in high demand and poor seed years, Douglas fir and some pine seeds can cost more than US $1000/lb (≈0·45 kg). Generally, however, seeds of genetically improved loblolly pine are available at $32/lb. Currently, artificial seeds or plantlets from somatic embryogenesis are economically justified only for research purposes or for unique situations involving endangered species.

From our first laboratory attempts at somatic polyembryogenesis, two species are now in soil (loblolly pine, and Douglas fir). Loblolly pine (*Pinus taeda*), which originated from Weyerhaeuser's tree improvement programme, is several feet tall and is normal in appearance (R. Timmis, personal communication). One Douglas fir (*Pseudotsuga menziesii*), now 1 foot (≈0·3 m) tall, also appears to be normal in development (Durzan and Gupta, 1988). Other species, sugar pine (*Pinus lambertiana*) and Norway spruce (*Picea abies*) were lost after several months through inexperience in establishing somatic embryos in various types of artificial soil substrates (Hiding, 1987).

## Somatic and gametophytic cell genetics

### CLONAL VARIABILITY BASED ON THE SPOROPHYTE

Mass vegetative propagation is employed in tree improvement to capture additive and non-additive genetic variation. Additive genetic variation is based on the independent effects of alleles, whereas the non-additive effects, often lost in sexual crossing, are based on the interfaction of alleles, either within or among loci. Mass propagation by reconstitution, restoration and reproductive regeneration is useful, provided that the genetic gains sought are stable and true-to-type. For reconstitution by somatic polyembryogenesis, the phenotypic variance ($V_p$) over time can be represented as:

$$V_p = V_a + V_d + V_r + V_e\,(V_{sp} + V_{cp} + V_R) + V_{a,d,r,exG} + V_{a,d,r,exGxe} + V_e$$

where

$V_a$ is the average effect of genes

$V_d$ is the genetic variance due to dominance
$V_r$ is the epistatic genetic variance
$V_c$ is the variation introduced by the clonal process
$V_{sp}$ is the variance due to simple or polyzygotic polyembryony (fraternal types)
$V_{cp}$ is the variance due to cleavage polyembryony
$V_R$ is the variance due to polyembryony derived from relic nuclei (rare) observed by Dogra (1983, 1984)
$V_{a,d,r,cxG}$ is the variance arising from the interaction of genes in the embryonal-suspensor mass (a,d,r,c) with the maternal female gametophyte (G) or its equivalent
$V_{a,d,r,cxGxe}$ is the variance arising from the interaction among genes in the embryonal–suspensor mass (a,d,r,c) with the female gametophyte (G) and environment (e)
$V_e$ is the variance due to different environments

Normally only one embryo per seed survives, but not so in cycles A and B of *Figure 2*. The complexity of the overall system reflects the opportunism and resilience of gymnosperm somatic polyembryogenesis.

Genetic instability in cellular populations is usually undesirable, but can lead to useful variants if the frequency can be controlled in derived plants.

This means that when plant regeneration occurs from callus or protodermal cells with apical initials having a variable genotype, we should expect some aberrations among the cloned populations, i.e. the genetic stability of cells initiating the proembryonal cell complexes, e.g. epidermal (tunica) or mesophyll (corpus) cells, contribute to subsequent aberrations in ontogeny and somatic cell genetics (Klekowski, 1987). 'Aberration' is used rather than 'variation', because the propagator is usually concerned more with aberrations from a true-to-type process in selection rather than its variations (Durzan, 1984a). The comparatively short cell cycle and the very long life of trees reinforces the view that for many aberrations, powerful repair or mutant cell displacement mechanisms exist to minimize deleterious effects.

The high levels of DNA per nucleus of gymnosperms (Price, Sparrow and Nauman, 1973) and the observation by Krimer and Van't Hoff (1983) that DNA is lost from chromosomes in elongating pea cells deserves attention. This displacement of DNA could reflect the removal of somatic mutations or of genetic information during cell differentiation. Do we have some metabolic control over mutant gene expression in these systems over and above the repair process? Mutant gene expression is complicated by the mobility of genetic elements, which can again contribute to variation in clonal performance of cells isolated as explants from the plant body. As yet, we do not have control systems in sight.

Clonal variability (genetic and epigenetic) leads to a dangerous situation in forest biotechnology where expectations are too high for the current knowledge base. We cannot pass on untested products to the consumer, to the poor, or to the uneducated. Just as we needed environmental technological

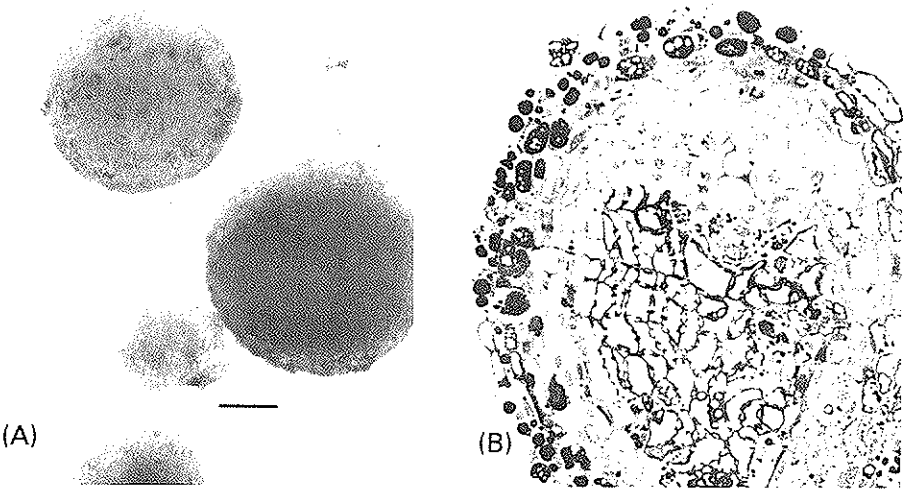


**Figure 10.** Sphaeroblast development in Douglas fir (*Pseudotsuga menziesii*) cell suspension cultures in diffuse white light. (A) Under the influence of N°-benzyladenine 1·1 mg/l and α-naphthalene acetic acid (0·1 mg/l) green globular sphaeroblasts develop after 3 weeks at 23°C (bar is 0·5 mm). (B) Cross-section sphaeroblast (*ca.* 0·8 mm diameter) to show precocious vascular development and cambial-like arrays of cells. Cell suspensions were derived from callus grown on cotyledons from a 1-week-old germinated seed.

impact studies in earlier years, we will need to assess the impact of somatic cell genetics in agriculture and forestry.

## CLONAL VARIABILITY BASED ON THE GAMETOPHYTE

The gametophytic generation provides unique opportunities for tree breeding and improvement (Winton and Stettler, 1974). As of now, we have little evidence yet to suggest that embryogenesis can be obtained from the haploid male gametophyte, although polyspermy and other interesting phenomena have been observed. The situation for the female gametophyte is different (Norstog, 1982). Haploid embryos have been reported among polyembryos of *Picea abies* (Illies, 1964). Dogra (1983) has reviewed situations where haploidy was observed in naturally and controlled pollinated *Pinus* and *Picea* sp.

Bonga, Von Anderkas and James (1988), Von Anderkas and Bonga (1988) and Von Anderkas, Bonga and Nagmani (1987) have described for *Larix decidua* the reproductive regeneration of haploid embryos bearing what appears to be a heavy load of lethal recessives. Nevertheless, haploid and some presumably spontaneously diploidized plantlets (J.M. Bonga, personal communication) were regenerated by somatic embryogenesis using the female gametophyte. With these cultures, we have a new source of élite cells and

protoplasts for the production of homozygous diploids. Mass selection used in reproductive regeneration enables the clean-up of heavy genetic lethal loads introduced by natural outbreeding and inbreeding. In conifers, indiscriminate inbreeding and pollen introgression brought on by wind currents often results in the abortion of embryos (Orr-Ewing, 1957; Owens and Blake, 1985). Polyembryony has been interpreted as a way of allowing considerable genetic load to be carried on in the population (Sorensen, 1982).

Haploid trees are useful for the following purposes: (1) to accelerate breeding strategies that employ homozygous lines produced by doubling the haploid genome either spontaneously or artificially with colchicine; (2) to remove by selection, cloned products with heavy lethal loads because all genes are expressed. These methods are usually a substitute for clonal propagation. In doubled haploids the recessive lethals are also expressed; (3) to serve as a source of morphogenic protoplasts (*see* Gupta and Durzan, 1987c) for the restoration and reconstruction of various ploidy types. We do not yet know if triploids formed by fusion of morphogenic protoplasts will yield seedless trees that will better utilize their biosynthetic potential for wood formation rather than for seed production. Novel conifer breeding strategies are possible, based on the recent progress using haploid barley plants (*see* Anderson and Reinbergs, 1985).

## PRECOCIOUS DEVELOPMENT: SPHAEROBLASTS AND CUTTINGS FROM THE MOTHER TREE

With callus derived from tissue explants from seeds, saplings, and mother trees, a variety of cell suspension cultures can be established. Often, cell suspensions originated from several tissues (epidermal, subepidermal mesophyll, etc.) depending on conditions selecting for cells at the callus formation stage (Durzan, 1984b).

Cells in suspension grow into nodules in diffuse light (*Figure 10*). Anatomical studies reveal that nodules may contain one or several meristemoids and that these tend to develop precociously with a secondary (cambial) meristem, rather than with the primary meristems associated with somatic embryos or restored organs. Sphaeroblasts are considered to be nodules of wood, separated from the central woody cylinder of trees and surrounded by bark. Globular stages can be confused with somatic embryos (Abo El-Nil, 1982). Stoutemeyer (1937) observed that adventitious shoots with juvenile characters could develop from sphaeroblasts of apple and pear trees. The significance of this observation was initially in the possible restoration of the juvenile phase of the mature tree (Wellensiek, 1952) and later as a source of tissue for reproductive regeneration. Sphaeroblasts have the ability to form roots and shoots in response to auxins and cytokinins (Durzan, 1982a).

If trees are cloned by restoration (cuttings) from saplings, i.e. from progressively more mature sources, traits associated with the maturity phase of development may be expressed (e.g. Libby, 1986). Explants taken from mature trees under field conditions exhibit positional, developmental and

microclimatic influences in their response to exogenously applied factors that can lead to rejuvenation and embryogenesis (Durzan, 1984b; Gupta and Durzan, 1987a). All of these phenomena must be related to the meristematic activity. Meristems can also become senile, (*see* Schaffalitzky de Muckadell, 1959).

## OPPORTUNITIES WITH PROTOPLASTS AND RECOMBINANT DNA

At least two decades of research, development, and pilot study were required to reach satisfactory cloning protocols for conifers, and a few fruit tree crops. For the latter, we now have several examples from temperate and subtropical tree species where micropropagules of organogenetic and embryogenic origin have been grown into trees and are currently being field-tested (*see* Bajaj, 1986; Bonga and Durzan, 1987). These morphogenic species provide opportunities for recombinant DNA applications.

Although the insertion and expression of foreign genes during plant development have been summarized elsewhere (Fraley, Rogers and Horsch, 1986; Schell, 1987; Sederoff *et al.*, 1987), it should be stated that the *luc* firefly gene has been introduced by electroporation and transiently expressed in Douglas fir and loblolly pine protoplasts (P.K. Gupta, A.M. Dandekar and D.J. Durzan, 1988). The recovery of somatic proembryos from protoplasts (Rao and Atkins, 1985; Gupta and Durzan, 1987c) enables the introduction of new genetic variation and overcomes some of the existing barriers to hybridization (Durzan, 1980). Tumours have been induced by *Agrobacterium* on micropropagated shoots from mature trees of Douglas fir and on seedlings grown *in vitro* (Dandekar *et al.*, 1987). Tumours were induced by two strains of *Agrobacterium tumefaciens* containing recombinant derivatives of the plasmid pTiA6. Transformed cells displayed autotrophic growth in culture, synthesis of octopine, presence of DNA sequences and expression of a chimeric, bacterial kanamycin-resistance gene as demonstrated by presence and expression of aminoglycoside phosphotransferase (APH(3′)II) activity. This is the first report of transfer and expression of a foreign gene, resulting in the genetic modification of Douglas fir, one of the most important conifer species in North America.

This work extends from earlier reports on gene transfer in loblolly pine (Sederoff *et al.*, 1986) and poplar (Parson *et al.*, 1986; Fillatti *et al.*, 1987). With such strategies it should now be possible to explore the additive and non-additive genetic gains present in populations, and introduce new genetic variability using *Agrobacterium tumefaciens*.

Currently, among the many genes now available, one of the most promising genetic modification of gymnosperm protoplasts appears to be the introduction of genes for the *Bacillus thuringiensis* toxin to provide resistance to lepidopteran insect attack (Herrnstadt *et al.*, 1986). Alternatively, the technology leading to the development of transgenic plants may be bypassed by recent production of more effective toxins (e.g. AGR, 1987); in other words, we must always ask whether a chemist or biochemist could do a better job.

Genes are also now available for altering phytohormone levels to affect

regenerative capacity of cells. Heat shock promotor genes can now be used to switch the synthesis of plant growth regulators on and off so that temperature becomes a control for the regeneration process. Moreover, the cloning of genes from the viral genome and their insertion into plants to confer resistance to virus may now be possible (Schell, 1987). The challenge remains to interface gene transfer methodologies with somatic embryogenesis and polyembryogenesis to enhance our knowledge of basic process in the long life cycle of trees.

The efficiency of integration, targeting and timing of gene expression represents a concern and major challenge. Criteria for gene expression form a sequence of events or vector with subsets of processes which have 'precedence' (Durzan, 1988a). Precedence of gene expression in clonal products relates to the priority in time, order, arrangement or significance of a series of events, direct and indirect. Seeds taken from a wide range of provenances, where insecticides have been used (e.g. fenitrothion), show the presence of new proteins (Pitel and Durzan, 1978a, b). The significance of variant phenotypes obtained under these conditions will remain hidden until we have a better understanding of somatic cell genetics and the responsiveness of cells to local environmental factors (Yablokov, 1987). For these and other reasons, the hope for successful applications of gene transfer methodologies to woody perennials will take us well into the next century.

We do not know how successful the above applications will be. For most woody perennials, we have yet to explore the potential of protoplast fusions for the rapid creation of new hybrids with more useful, naturally occurring traits.

## Artificial seeds and germplasm conveyance mechanisms

### ARTIFICIAL SEEDS

An artificial or synthetic seed is basically an encapsulated somatic embryo which forms a botanical analogue to a natural seed (Kitto and Janick, 1985; Redenbaugh *et al.*, 1986). When somatic embryos are encapsulated as artificial seeds, we have conceptually an ideal system to complement current seed and breeding orchard technology (*Figure 11*). The expectation is that élite material may be produced clonally year around and independent of damage and losses due to insects, climate, natural disasters, pollution or poor seed years. In non-woody species, encapsulated somatic embryos and seeds can be stored and handled by machine for planting in the field (Redenbaugh and Rusin, 1988). Artificial seeds, or at least encapsulated somatic embryos, have been produced for *Pinus, Picea*, and *Pseudotsuga* sp. (e.g. Gupta and Durzan 1986a, 1987b), and recently for *Eucalyptus* (T. Mascarhenas and P. Gupta, personal communication).

Alginate gels are often used to singulate, i.e. separate as an entity, and encapsulate somatic embryos. So far, with woody perennials, gels are only suitable for the experimental design and study of artificial seeds (Redenbaugh *et al.*, 1986). Although this technology provides many advantages (*Table 3*), several problems remain before this technology becomes a success story. We

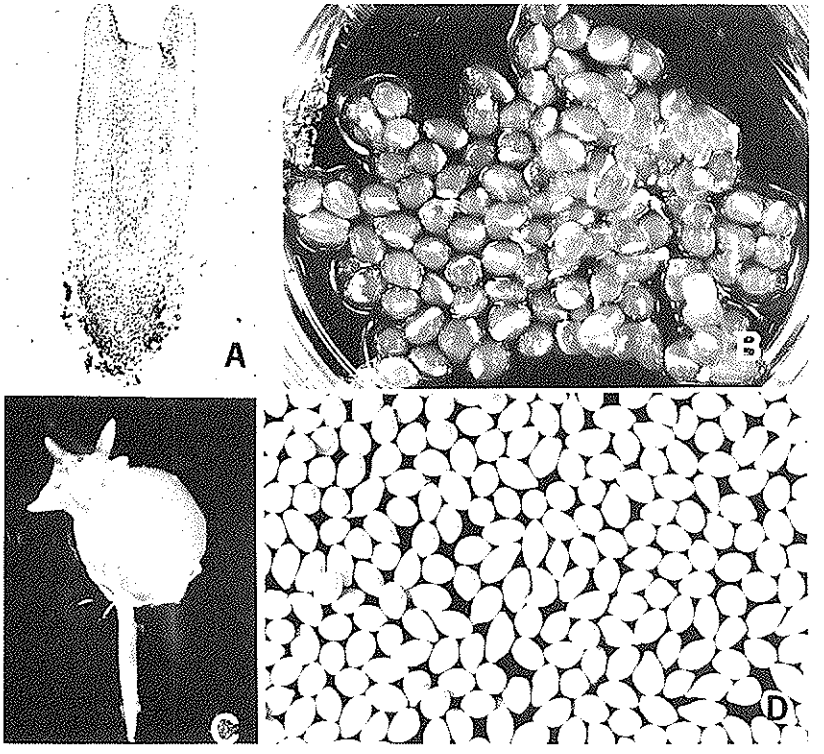


**Figure 11.** (A) Stained longitudinal section, stained with fastgreen and saffranine, of a somatic embryo of loblolly pine (*Pinus taeda*) at the time of encapsulation in alginate gel. The embryo is *c.* 3 mm long. (B) Somatic embryos of loblolly pine encapsulated in alginate gel (4). Each gel is *c.* 8–10 mm in diameter. (C) Emergence of a somatic embryo of Norway spruce (*Picea abies*) after encapsulation to show development of cotyledons and emergence of root. Currently, only a few individual embryos perform in this way because improvements are needed in the quality of encapsulating material and in understanding root growth and development. (D) Embryos encapsulated in gels can be further coated to protect gel and to add rigidity to the gel–embryo complex. Various types of coating materials are under investigation but greater reliability and cost-effectiveness are needed to ensure an acceptable product. Lack of root elongation and development in new environments as embryos emerge is a major problem to overcome.

need better synthetic endosperm or female gametophyte with osmotic nutritional controls, and a greater understanding of the local environment around the somatic embryos encapsulated with this material. More work is needed to evaluate the ability of somatic embryos with and without gels to survive in soils and to become associated with mycorrhizae. The machine-handling for the processing and field-planting of artificial seeds must not only be compatible with the plant material, but also be operationally stable under field conditions.

The production of artificial seeds assists the handling of clonal products with already well-established root primordia. While alginate encapsulation of

**Table 3.** Some potential advantages of using artificial seeds based on somatic embryogenesis (*cf.* Redenbaugh *et al.*, 1986)

| |
|---|
| Introduce agricultural chemicals, fungicides, micro-organisms |
| Precision planting by machine |
| Seed priming, yield uniformity, synchrony |
| Advantages mainly with high-valued germplasm |
| Efficient with conversion or germination rates |

somatic embryos enables their short-term storage at 4°C, several problems remain. Artificial seeds need to be nutritionally sound, preconditioned, dehydrated for storage and then hydrated to facilitate rapid establishment of roots in soil. More work is also required to evaluate the potential of natural or artificial soil substrates (Hiding, 1987).

Artificial seeds, somatic embryogenesis and polyembryogenesis offer great promise for clonal forestry, tree improvement (seed and breeding orchards) and for ornamental and environmental horticulture, where mature characteristics are sometimes expressed early.

## TRANSFER TO SOIL: GERMPLASM, STORAGE, CONVEYANCE AND FIELD TESTING

For root development in advance of transferring plants to soil, we have found that direct white light and excess water are inhibitory. While low levels of light are tolerated, the light must be highly diffused at the appropriate temperature in humid but not saturated atmospheres. High levels of culture nutrients also tend to be inhibitory. Media that promote conversion of somatic embryos to plantlets in soil usually have little or no sucrose (Redenbaugh *et al.*, 1986) and reduced levels of nutrients (<100 mosmols). Under these conditions, levels of other secondary inhibitory products are minimal. Inhibitors of root development can sometimes be removed mechanically by introducing fresh media of low osmolality and moderate pH. In this way, clonal products of the new generation can be established in the production nursery. Attempts are under way (Durzan, 1988a) to extend somatic embryogenesis to explants of rejuvenated branches from proven mature trees (e.g. Gupta and Durzan, 1987a).

In reality, current artificial seed technology is far from being representative of a true seed, because much of the biology is missing from the product. Hence, alternative and improved embryo conveyance mechanisms and devices to condition and transport regenerated plants are now the subject of interest of several laboratories.

Populations of clonal materials in the form of propagules or artificial seeds will require considerable field-testing. In some cases, testing could take at least 9 years, as in Douglas fir. As it would not be cost-effective to subculture source materials every 10 days for 9 years, we have explored the cryopreservation of embryonal cells in liquid nitrogen (−196°C). Thawed cells of Norway spruce and loblolly pine have regenerated visually normal plantlets (Gupta, Durzan and Finkle, 1987). Although cells have not yet been stored for a long time, their dense cytoplasmic and spore-like characteristics make them ideal material for

cryopreservation because cells are not as readily disrupted by the freezing and thawing process. We do not yet know the effect of long-term cryopreservation on embryo recovery and field performance.

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