DON J. DURZAN
Professor, Plant Sciences MS6
University of California
One Shields Ave. Old Davis Rd,
Davis, CA 95616 djdurzan@ucdavis.edu

MONOZYGOTIC CLEAVAGE POLYEMBRYOGENESIS AND CONIFER TREE IMPROVEMENT

The mass cloning of elite genotypes of commercially important conifers has led to the establishment of an industrial forest of two of the most important softwood species in the USA. Embryonal-suspensor masses, produced by monozygotic cleavage polyembryony (MCP), are rescued from controlled-pollinated seeds in tree breeding orchards. MCP is scaled up as cell suspensions and grown into mature somatic embryos. The embryos serve as a source for the production of various artificial and manufactured seeds used in replicated field trials to test genotype × environmental interactions. For the capture of genetic gains, early selections are based on correlations with known traits. This reduces the costs of years of tree improvement. Mass cloning and genotype cryopreservation enables field testing under a wider range of sites. Process-controlled bioreactors are proposed as artificial ovules to impose nutritional variables from the mother tree, and to simulate environmental factors that are known to affect the performance of the new generation. Comparisons among extant and modern conifer genotypes would provide new insights regarding their latent potentials for apomixis, the alternation of generations, and adaptive plasticity.

Introduction

It is an honor to contribute to Navashin's centenary volume. His discovery of double fertilization in angiosperms in 1898 continues as a landmark in all botanical textbooks today. My research deals with conifer embryology which indirectly relates to Navashin's interests in the embryology and karyology of higher plants. The recent knowledge gained in controlling the apomictic monozygotic cleave polyembryony (MCP) has contributed to the mass cloning and mechanized production of many elite genotypes for a wide range of genotype× ×environment trials for use in tree improvement and forestry. This has led to the development of prototype industrial forests.

Conifers comprise the largest and oldest living organisms that exist today (Sporne, 1965). Some are over 5000 years (*Pinus* sp.). Others are the tallest living organisms (*Sequoia, Fitzroya* sp.). In forests they often live on acidic soils, poor in nitrogen and some occupy tree lines where few other species survive. Conifers occur in most continents, occupy environmental extremes and high elevations. Some retain their needles for 20 to 30 years to insure a somewhat stable photosynthetic capacity that can carry a tree over several years of stress (Ferguson, 1968). They are economically important in providing shelter, building materials, biofuels, many secondary products including anticancer drugs, and can be pulped for paper and cardboard manufacture. Conifer populations are often self-pollinated and carry a 'genetic load', which together, controls their breeding system. Poor seed years and poor seed quality contribute to the problems in forest regeneration. Hence, the development of cloning methods based on MCP has great importance for tree breeding, improvement, and production programs.

In seeds, monozygotic cleavage polyembryony (MCP) is the production of multiple genetically embryos from a single zygote and its early embryo. Many angiosperms and gymnosperms have the potential to exhibit MCP. The potential for cleavage resides in the free nuclear stages of the zygote and in the 'embryo initials' of the early embryo. This potential is harnessed through the recovery or rescue of the embryonal suspensor mass (ESM) in the developing seeds. In normal seed development, only one embryo usually dominates. The remainder are found undeveloped at the start of the erosion cavity at the base of the seed.

'Breeding seed orchards' are needed to make

controlled crosses between selected parents. In this way, new and improved generations of high-quality genotypes can be collected as seeds. Even though the establishment of breeding seed orchards takes a long time, they have already contributed to the fourth generation of tree improvement in some countries. This review will show how the 'rescue' of the embryonal suspensor masses (ESMs) from single seeds, having the potential for MCP, has contributed to the massive clonal multiplication of many different genotypes. This type of cloning is very distinct from other methods in cell and tissue culture where 'explants' are cut from already differentiated tissues, e.g., cotyledons, shoot tips, leaves, etc., and «induced» to restore the missing apical and cambial meristems by 'organogenesis' or by 'adventitious' somatic embryogenesis. The criteria and guidelines for verifying the true-to-type development of clonal embryos are best modeled against the developmental processes in the seeds. As we will see, this is not always done.

MCP has enabled tree breeders in the forest industry to test a wide range of new genotypes for their adaptive plasticity, and insect and disease resistance, over a wide range of environments at many locations. Selection is highly standardized to rogue out 'somaclonal aberrations' and to provide a robust genetic mix of new well-adapted genotypes at any one new geographical location. This means that many years are needed for the evaluation of genotype × environment interactions, and for the subsequent establishment of new cycles of productive forests, based on the selected and clonal genotypes. The field performances of mixed genotypes from a wide range sources are compared and correlated with the performance of their parents. This takes into consideration the environments and cultural practices found at the sites of the genotype's geographical origin. Seed production orchards are re-established to bulk up and provide seeds for further testing and reforestation.

Elite genotypes, showing MCP and recovered as embryonal suspensor masses from seeds, are cryopreserved in liquid N until needed for breeding and tree improvement years later. When thawed and cultured, the early embryos continue to regenerate more clones by MCP. For research, elite clones also provide a source of 'totipotent' and morphogenic protoplasts for testing the recombining ability with other genotypes. It also provides tissues for genomic analyses that establish data bases for future research and development. The overall process represents the 'capturing genetic gains'.

Apomixis and polyembryony

Apomixis is the replacement of sexual reproduction by various types of asexual reproduction which does not result in the fusion of gametes. It can be described in one of three ways: budding, parthenogenesis, and polyembryony. The causes of apomixis and polyembryony are generally considered together (Mogie, 1992). Polyembryony is a recessive hereditary trait controlled by multiple genes, or by one or more recessive genes, which act together in suitable recombinations after hybridization. In biotechnology, apomixis is used to fix hybrid vigor, to save time, labor and money in establishing cultivated varieties.

Polyembryony was first reported by Leeuwenhoek in 1719 in Citrus seeds having more than two embryos. In 1878, Strassburger studied the adventitious origins of embryos, and referred to it as 'sporophytic polyembryony'. This term was not limited to any type of seed, nor to any type of embryo sac development (Webber, 1940). While the rescued ESM from seeds is initially a product of a single meiosis and fertilization reproduction, their cells retain the potential for producing multiple genetically identical embryos by MCP (Fig. 1). Lack of recognition of MCP has led to the misuse of the term 'induction' in the literature of somatic embryogenesis.

It is important to understand the differences between MCP and 'simple polyembryony'. The latter preferably could be called 'polyzygotic polyembryony'. In polyzygotic polyembryony, multiple embryos are each formed from more than one egg. The populations of embryos, being derived by meiosis and multiple different eggs, are therefore not genetically identical to one another. Polyzygotic polyembryony is found both in gymnosperms and angiosperms. In *Pinus* sp. a single ovule is reported to have the potential of producing 1 to 200 genetically different embryos by polyzygotic polyembrony.

Cellular totipotency and cell death

A newly fertilized egg (zygote) is considered 'totipotent'. Totipotency means that the zygote has the potential to differentiate into all tissues in a

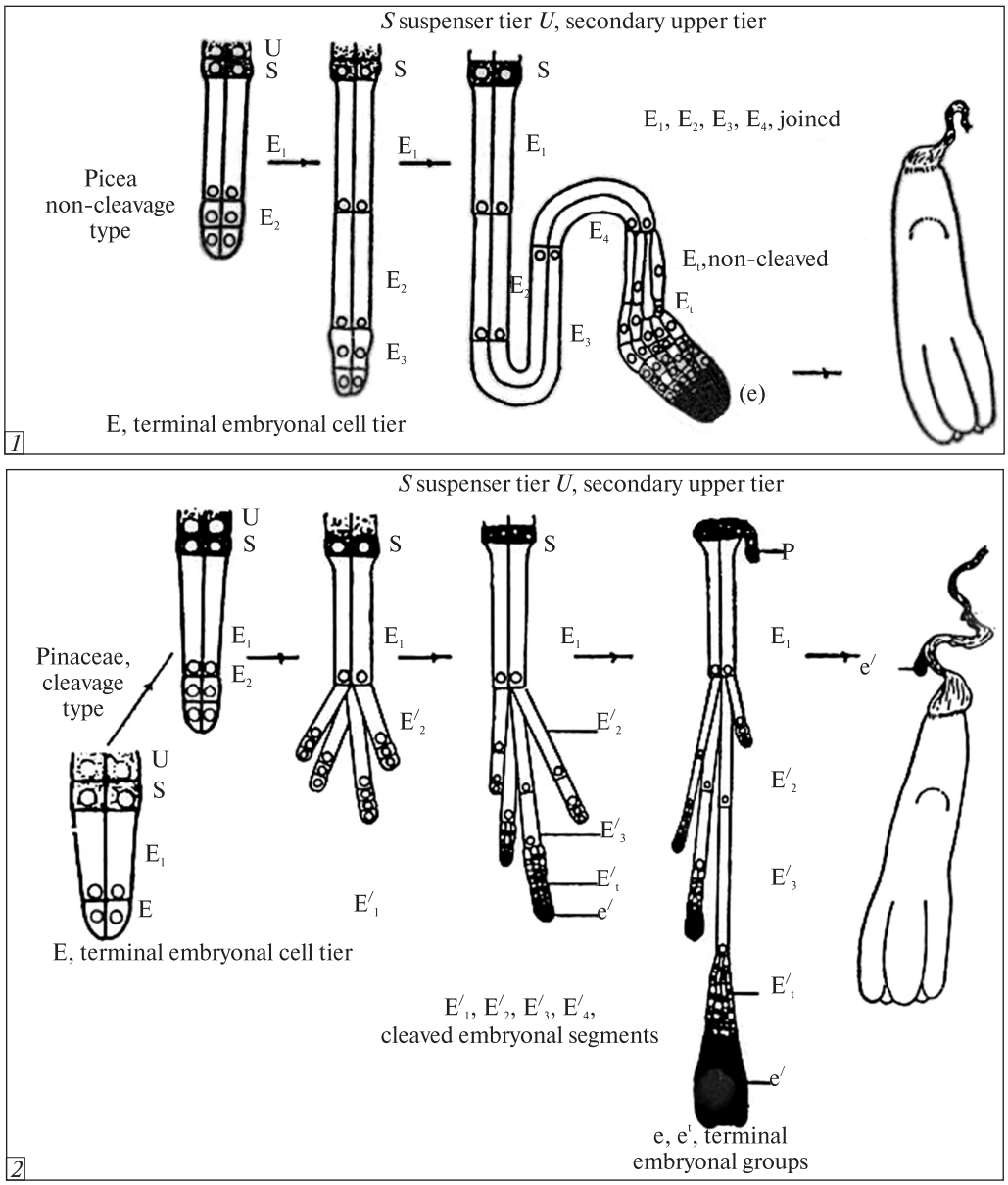


Fig. 1. Two USE basal plans for embryo formation in seeds based on Dogra (1978). The name 'tier' (U, S, E) is applied only to cells or nuclei (circles) which lie in a single horizontal plane. The pU tier is an open-celled tier which remains a single tier in most conifers. S represents the suspensor tier which will later elongate. Cells of the primary proembryonal group (pE) lie in different arrangements ranging from tiers to irregular storied arrangements. The suspensor system (S) develops from cleaved embryonal segments (E). E_t are proximal cells that do not contribute to the formation of tiers in the USE plan. In seeds not showing MCP (Fig. 1.*1.*), the tiers remain together as in *Picea*, and *Pseudotsuga* species. By contrast, seeds of the Pinaceae exhibit MCP (Fig. 1.*2.*). The last step in proembryo development is characterized by an internal division which affects all cells and duplicates the embryo. Secondary proembryos now arise separately by internal divisions. Rosette proembryos (p) produced by other tiers are not usually viable. Studies with rescued ESMs in suspension cultures show that while the basal plan is somewhat repeated, the addition of plant growth regulators stimulates the embryo initials in the proembryo to express MCP

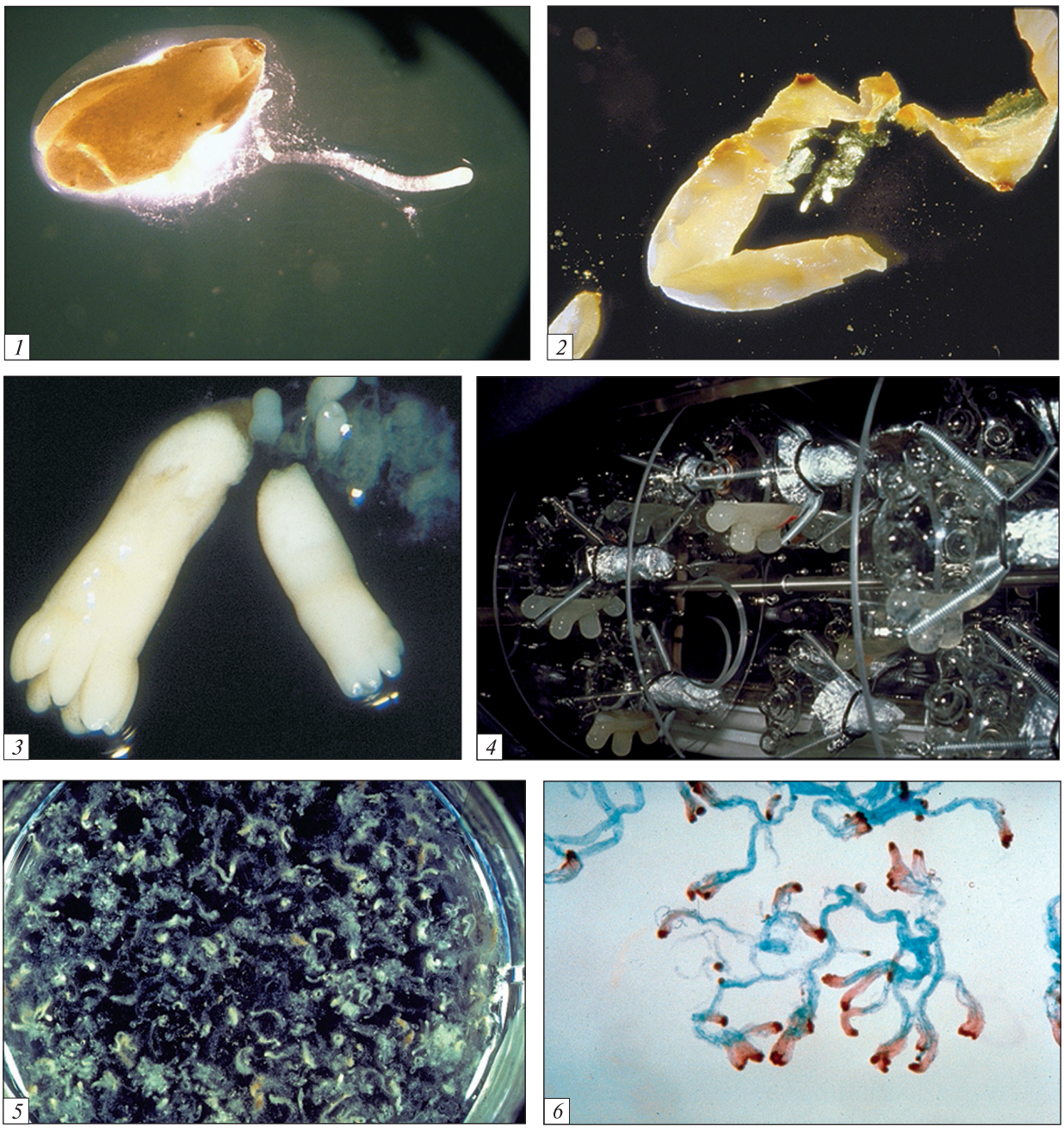


Fig. 2. Rescue of ESMs and MCP. 2.*1*. A non-cleaving early embryo with its 'embryo initials' escapes from a Douglas-fir seed on a semi-solid culture medium. It will multiply by MCP in suspension cultures. 2.*2*. Rescue of an ESM with multiple cleaving early embryos (MCP) from a Norway spruce seed. 2.*3*. Culture of a sugar pine ESM on an agar plate enables other embryos produced by MCP to develop. 2.*4*. Suspension cultures in one-liter nippled flasks, rotating at 1 rpm in darkness, enable the massive scale-up of ESMs and early embryos. Suspensions are sized according to their buoyant density to produce more uniform and synchronized cultures. 2.*5*. Suspensions in Petri dishes assist in assaying embryo development from different treatments. 2.*6*. A uniform population of ESMs stained with acetocarmine and Evan's blue. MCP is seen in proembryonal tier stained red with acetocarmine. The E_t cells react pink. The enucleated suspensors react with Evan's blue

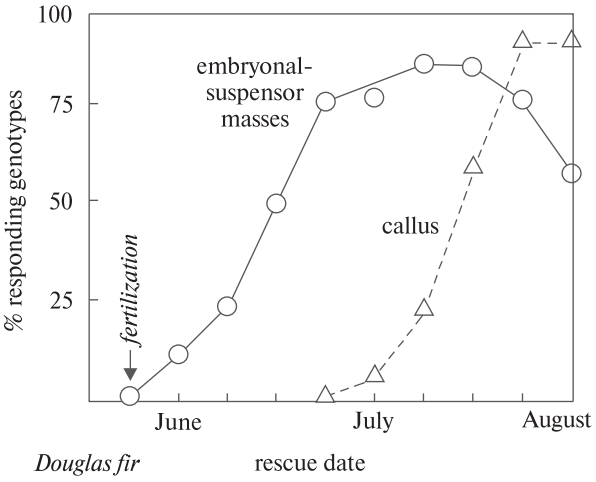
plant's life cycle. F.C. Steward at Cornell first demonstrated totipotency in cell suspensions from phloem explants of the carrot root. The plants grown from cells, and even those in the next generation retained their totipotency (Steward, 1968, 1975). He pointed out that zygotes in their ovules develop with a degree with infallibility, which cannot be easily emulated, even in the most favorable conditions by somatic cells. This conclusion was reached after years of study with growth regulating factors isolated from coconut milk and from extracts of plant tissues. Through differentiation, other cells become pluripotent, unipotent, or are developmentally programmed to die by apoptosis (programmed cell death) (Havel and Durzan, 1996a, b). Apoptosis is important in the formation of wood, in megasporogenesis, MCP, and in plant defenses that limit the attack by insects and diseases.

Totipotency also includes the apomictic potentials for development in a generalized life cycle showing the alternation of generations (Durzan and Steward, 1983). This cycle integrates with a nuclear-cytoplasmic cycle of cell determination at the molecular level by providing the inherited information, positional cellular organization, biochemical signals, and substrates that enable cells to function throughout the life cycle. Great advances in molecular biology have recently provided new methods, diagnostic tools, and insights into plant embryogenesis.

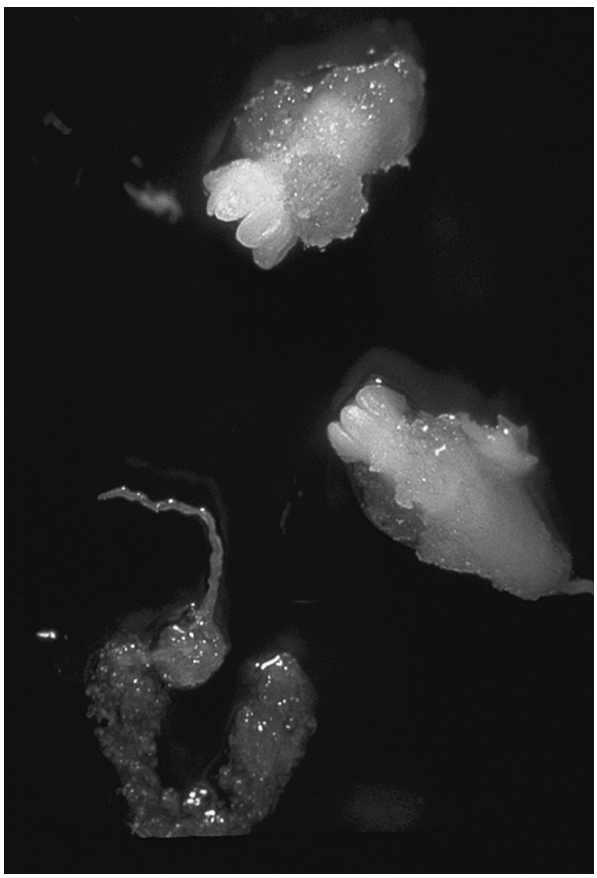
Basal developmental plans for MCP

Two different models have appeared for MCP in conifers. One is based on seed development and 'embryo initials' with or without MCP as formulated by Dogra (1979) in his USE basal plan (Fig. 1). This complex plan derives from the morphological development of the primary proembryo, which has lower primary embryonal cells (pE), and an upper open tier (pU) as first proposed by Doyle (1963). A free-nuclear zygote is formed after fertilization. The zygote produces a cellular 'primary proembryo' containing embryo initials. Doyle and Looby (1939) observed that every cell in a primary proembryo has the potential of being an embryo initial, and has the capacity for self-renewal. Embryo initials can give rise to independent embryos with their cell types, layers, and lineages, a fact that was long forgotten.

The *capacity* for the development of the early proembryo depends on the variable numbers of

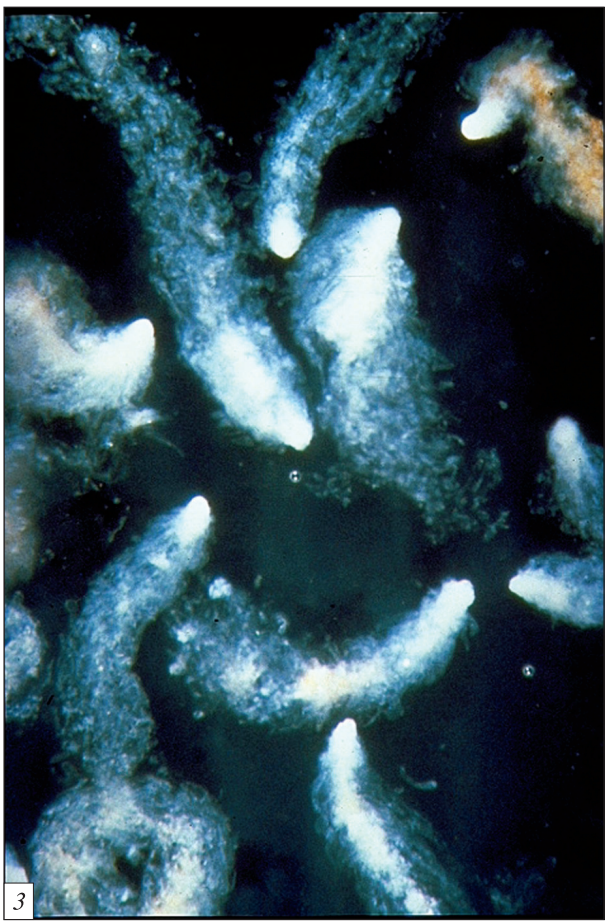


1



2

Fig. 3. ESM and callus initiation. 3.*1*. Percentage of Douglas-fir genotypes producing ESMs or callus (all or none) as a function of their rescue date from seeds (Hong et al. 1991). 3.*2*. The surfaces of excised embryos are induced by 2,4-D (5 to 50 ppm) to produce a mucilaginous 'callus' with the properties of an ESM



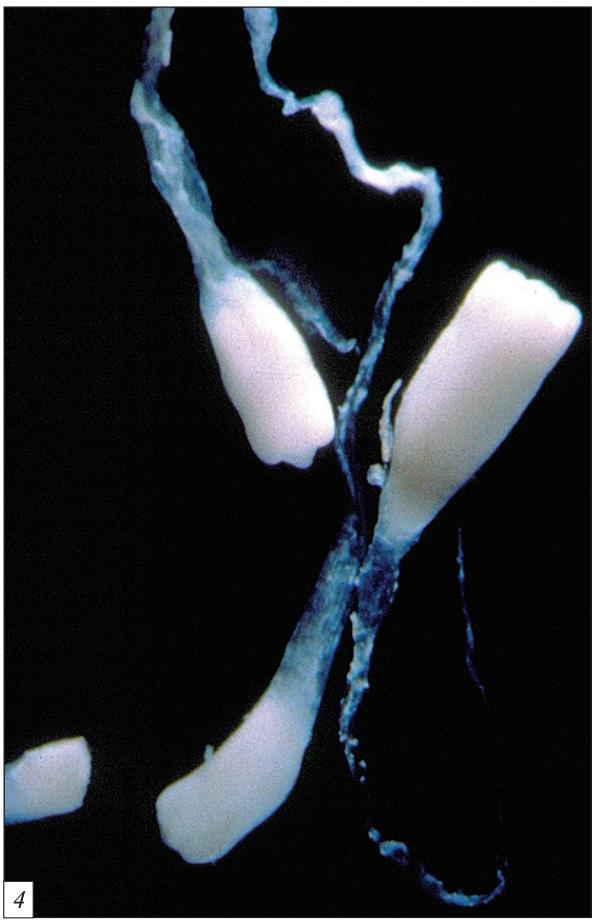


Fig. 3. ESM and callus initiation. 3.*3*. In suspension cultures individual Douglas-fir embryos will develop without cleavage as in seeds by controlling abscisic acid levels and osmotic pressures. 3.*4*. Early cotyledonary stages of individual embryos with elongated suspensors in a Petri dish

free-nuclear mitoses, the number of embryo initials, and on nutritional factors from the haploid female gametophyte in the developing seed. 'Proembryogeny' or 'proembryony' covers all stages before the elongation of the suspensor. All stages after the elongation of the suspensor, but before the establishment of the root meristem, are referred to as 'early embryogeny' (Singh, 1978).

In Dogra's USE model, embryo morphogenesis is partitioned into a terminal tier of embryonal cells (E), a suspensor tier (S) and cellular primary upper tier (U), which is not completely walled and remains open on the upper side to the cytoplasm of the zygote (Fig. 1). The suspensor tier (S) is derived from the U tier, and occurs after internal divisions in the primary proembryo. The developing embryo receives its nutrition from the egg cytoplasm, through the suspensor system. Later, the dominant embryo and other embryos, produced by MCP, are nourished by nutrition in the erosion zone of the haploid female gametophyte. The latter at maturity is often called an 'endosperm'. Late embryogeny refers to the establishment in the seed of the embryo with its cotyledons.

Dogra (1967, 1979, 1984) classified early embryo development into two types, based on dissections, and the X-ray analyses of seeds from Sweden and India. One type is represented by *Picea* sp., which does not show MCP (Fig. 1.*1*). In the other type MCP is initiated by the subdivision of a single zygote into a group of competing and genetically identical embryos (Fig. 1.*2*). These may or may not become independent from one another (Fig. 2 and 3). MCP greatly increases the chances that a seed has at least

one surviving embryo. In seeds, only one dominant embryo, which has competed successfully for a food supply, is commonly found. The remnants of the suppressed ESM are found at the basal end of the embryo.

Jäger (1899) suggested a way to reveal the capacity to form embryos of *Taxus baccata* which could be proven experimentally. It involved the provision of greater concentrations of food supplies. This postulate was supported nearly a century later when rescued embryos and ESMs were removed from the constraints of seed development. When this was done with cell cultures of Norway spruce, individual embryos in ESMs continued cleavage and freely regenerated embryos as long as cultural conditions were maintained (Durzan and Gupta, 1987; Gupta and Durzan, 1986 a, b, 1987 a, b).

The formation of enucleated suspensors along the axial tier involves apoptosis (Havel and Durzan, 1996 a, b). In this developmentally controlled process, the nuclei in distal tube cells (E_t) are fragmented with the release of nucleoli into the cytoplasm. Mucilaginous products are formed as the enucleated suspensor cells elongate. Mucilage was also released into the culture medium. During embryo growth, development, and differentiation, the turnover of cell regulatory proteins was regulated by the ubiquitination of amino acids in protein (Durzan, 1988 b, 1989 b, 1996 a; Durzan et al., 2006).

The ESMs from wide range of Norway spruce genotypes representing different geographical sites in Finland, when placed in the same culture dish, revealed different stages and patterns of ESM formation. This depended on their genomic capacity for MCP as established by differences in environments at the seed sources (Jokinen and Durzan, 1994). For jack pine (*Pinus banksiana*), the effects of local environment on seed size and biochemical composition are supported by the results of a seed collection study over wide range of seed sources across Canada and in the USA (Durzan and Chalupa, 1976). Because of the great genetic heterogeneity and geographic adaptation of some conifers, no one culture medium could be applied equally successfully to all genotypes.

In 1985 when Dogra visited my laboratory in Davis, he witnessed how ESMs from Douglas-fir, Norway spruce, and loblolly pine expressed MCP in cell suspension cultures (Fig. 2 and 3). This clearly demonstrated that both `non-cleavage` and `cleavage` species could express MCP when their ESMs and embryo initials were cultured in vitro. The degree of MCP was genotype dependent and controlled in culture media formulations with plant hormones, and especially abscisic acid.

An ESM-like 'callus' can also be 'induced' from the epidermal cells of embryos rescued from non-cleavage type seeds of Douglas-fir by adding auxins and cytokinins (Fig. 3.*2*, Hong et al., 1991). Somatic embryos induced by this process are 'adventitious' in origin. However, embryonal initial cells, which sloughed off in suspension cultures also continued to develop into somatic embryos (Durzan and Gupta, 1987).

A second model, based on immature Norway spruce embryos was initially developed in Sara von Arnold's laboratory, and later by several other investigators. 'Proliferating proembryogenic masses' (PEMs) containing 'meristematic' cells were initiated by auxins and cytokinins (Hakman et al., 1995). PEMs 'multiplied by unequal divisions' as a 'prevailing process'. This early model was expanded from a two-stage to a later three-stage PEM model for somatic embryogenesis. At stage PEM I, small cell aggregates are characterized by of a compact clump of densely cytoplasmic cells adjacent to a single enlarged vacuolated cell (Filonova et al., 2000 a, b; von Arnold et al., 2002). At PEM II, cell aggregates contain more than one vacuolated cell. Although the earliest stages of proembryogeny could not be characterized, the subsequent developmental processes correspond closely to what occurs in the course of early and late zygotic embryogeny. At PEM III, an enlarged clump of densely cytoplasmic cells appears 'loose rather than compact' and 'polarity is disturbed'. Withdrawal of plant growth regulators triggers embryo formation from PEM III. PEMs will not develop into somatic embryos unless stage PEM III has been achieved. Abscisic acid promotes the further development of somatic embryos through late embryogeny to mature forms. The passage through a series of three stages is required for the 'transdifferentiation' of cells into somatic embryos. Transdifferentiation is defined as 'the transition from one differentiated state of a cell to another'. An example of the transdifferentiation of cotyledon cells to PEMs in Norway spruce is shown in Fig. 4.*1* to 4.*4*.

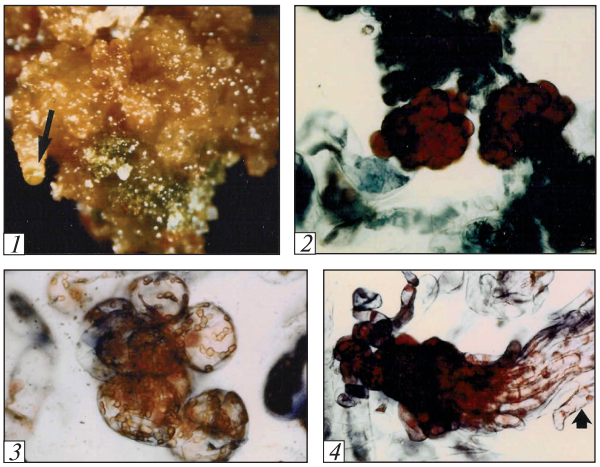


Fig. 4. A Norway spruce cotyledon is induced to form PEMs in an embryogenic callus on a semi-solid medium. The brown and white embryogenic callus is intermingled with nodular green tissues from the cotyledons. Fig. 4.*1*. Mucilage from MCP cultures was added to the culture medium to 'condition' somatic embryogenesis and callus formation. An early embryo emerging from the callus is shown at the arrow. Fig. 4.*2*. Fragmentation of the callus in a liquid medium and staining with acetocarmine reveals two red PEMs. Dead cells are stained with Evan's blue. Fig. 4.*3*. Acetocarmine-reactive cells, which are potentially embryonic, are also found in the callus. Note the red acetocarmine staining around the amyloplasts. Fig. 4.*4*. A somatic early embryo develops from PEMs. Several smaller PEMs are shown next to the tip of the proembryo. The arrow points to a binucleate and elongated cell

The three PEM stages emulate models for angiosperm embryogenesis (e.g., Komamine et al., 1992; Steward, 1968, 1975) even though angiosperms represent different phyllogenetic and evolutionary histories than the conifers, which are gymnosperms. The PEM model was later modified to include embryo cleavage. New molecular studies and encouraging field tests with conifers cloned by PEMs are underway in several countries (e.g., Dyachok et al., 2002; Stasolla et al., 2003, *cf*. Jain et al., 1995).

Others have shown that shoot apical meristems have special cells that initiate organ development much like '*stem cells*' in animals (Weigel and Järgens, 2002). Genes in stem cells are expressed only in the certain layers of the meristem and not in other apical zones or even in differentiating or transdifferentiating cells. Stem cells of shoot apical meristems have not yet been reported among the meristematic cells in PEMs. Stem cells are considered `pluripotent` and responsible for the formation of new organs rather than new embryos (Bhalla and Singh, 2006).It is also not yet known if and how meristematic PEM cells differ from the embryo initials in the USE model. The fact that different models account for conifer embryo development, and both express MCP, adds to the confusion and complexity in the tissue culture literature.

Somatic embryogenesis and polyembryogenesis in conifers

Somatic embryogenesis was first induced in immature embryos of Norway spruce (*Picea abies*) (Chalupa, 1985; Hakman et al., 1995). 'Somatic polyembryogenesis' in *Picea* and *Pinus* sp., referring to the cleavage process in rescued ESMs was first reported in cell suspensions (Durzan and Gupta, 1987; Gupta and Durzan, 1986 a, b, 1987 a, b). Abscisic acid levels were used to separate cleaving embryos and to recover individual mature embryo in cleavage and non-cleavage species (Boulay et al., 1988; Durzan and Gupta, 1988 a, b). The monitoring and control of abscisic acid-responsive gene expression has led to improved somatic embryo maturation in loblolly pine (Vales et al., 2007).

From 1990 to 1995, diploid and haploid somatic embryogenesis, and MCP, was reported for *Larix* sp. (Bonga et al., 1995). Most success was obtained with explants taken two weeks after fertilization in mid-June. Somatic embryogenesis was also induced on cotyledons with varying success. For Douglas-fir, the best time for the successful rescue of ESMs from developing seeds of the non-cleavage type was also just after fertilization (Hong et al., 1991) (Fig. 2 and 3). ESMs were rescued and cultured without the complications of callus formation. Clones from cell suspensions were planted in soil (Durzan and Gupta, 1987; Durzan, 1988a). This again demonstrated the 'latent potential' of 'embryo initials' in a species not known for MCP in seeds. Sugar pine seeds (cleavage type) with one fully developed embryo, and stored for five years, contained viable ESMs. When rescued, the ESM developed into embryos and plantlets (Gupta and Durzan, 1986 a).

The first planted pine produced by MCP in suspension cultures was transferred to Weyerhaeuser (Gupta and Durzan, 1991). The transfer of somatic polyembryogenesis technology to Weyerhaeuser was advanced by their recruitment of Pramod Gupta (see below).

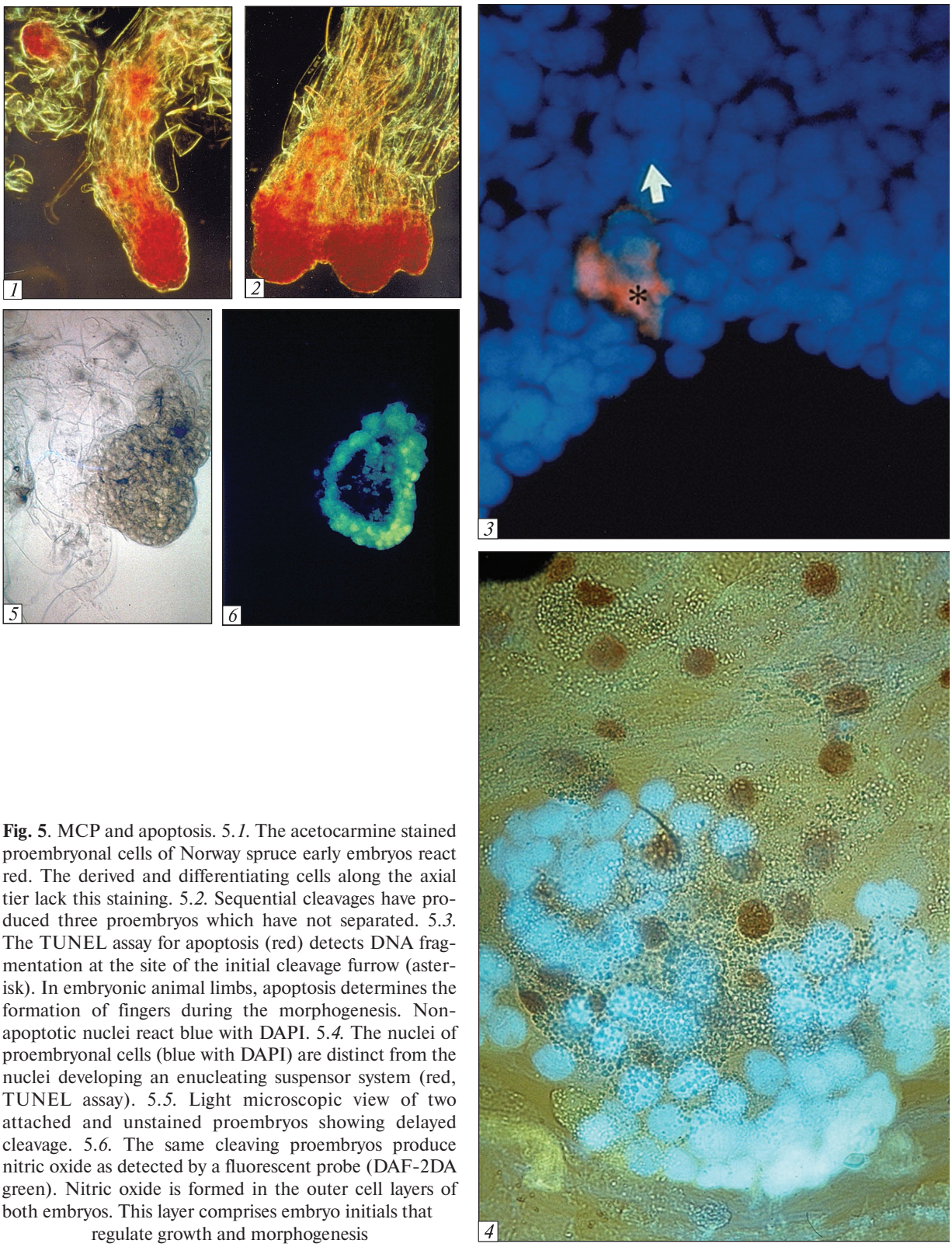


Fig. 5. MCP and apoptosis. 5.*1*. The acetocarmine stained proembryonal cells of Norway spruce early embryos react red. The derived and differentiating cells along the axial tier lack this staining. 5.*2*. Sequential cleavages have produced three proembryos which have not separated. 5.*3*. The TUNEL assay for apoptosis (red) detects DNA fragmentation at the site of the initial cleavage furrow (asterisk). In embryonic animal limbs, apoptosis determines the formation of fingers during the morphogenesis. Non-apoptotic nuclei react blue with DAPI. 5.*4*. The nuclei of proembryonal cells (blue with DAPI) are distinct from the nuclei developing an enucleating suspensor system (red, TUNEL assay). 5.*5*. Light microscopic view of two attached and unstained proembryos showing delayed cleavage. 5.*6*. The same cleaving proembryos produce nitric oxide as detected by a fluorescent probe (DAF-2DA green). Nitric oxide is formed in the outer cell layers of both embryos. This layer comprises embryo initials that regulate growth and morphogenesis

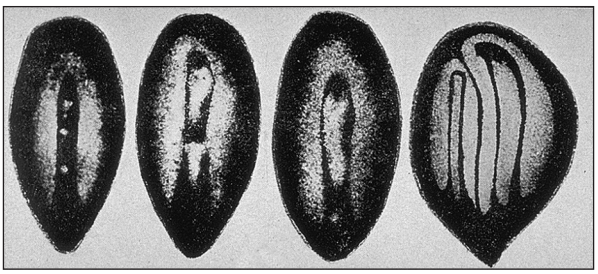
The above is a brief historical presentation. It is important to recognize that many other investigators have demonstrated MCP in a wide range coniferous species and hybrids using different culture methods. These investigators have made valuable contributions to our understanding of somatic embryogenesis in woody plants.

Diagnostic specificity and genotype cryopreservation

The fractionation of cell suspensions into size classes according to their buoyant density enabled the tracking of cell lineages, MCP, and embryo maturation. The re-inoculation of these cells increased culture uniformity and synchronization during scale-up production (Fig. 2 and 3, *cf.* Durzan and Chalupa, 1976). Double-staining with acetocarmine (Fig. 5.*1*) and Evan's blue (Fig. 2.*6*) distinguished between the proembryonal cells and the suspensors, and assessed the quality of the recovered ESMs after cryopreservation in liquid nitrogen (Gupta et al., 1987). Cryopreservation has now been repeated by others using the PEM model and its variations to store germplasm.

Specific antibodies to ubiquitin, PCNA (proliferating cell nuclear antigen), and the TUNEL assay were used to follow the turnover of cell regulatory proteins, DNA synthesis and cell cycling, and apoptosis (Fig. 5.*2* and 5.*3*), respectively (Durzan, 1996 a; Durzan et al., 2006; Havel and Durzan, 1999, 1996 a). With 'stains all', the distribution of proteins and nucleic acids in each cell type along the axial tier, and in the mucilage released into the culture medium, could be visually compared with the different types of proteins separated on polyacrylamide gels (Durzan, 1996 a).

Live and mechanically stressed conifer cells released rapid and transient nitric oxide (NO) bursts that preceded the release of ethylene (Magalhaes et al., 2000, Fig. 5.*4*). NO is a free radical that has beneficial and harmful effects (Durzan and Pedroso, 2002). At low levels, NO reduces the damage from reactive oxygen species. NO is also an integral part of cell signaling in plant defense. At high levels NO contributes to aging, DNA damage, and apoptosis (Magalhaes et al., 2000; Pedroso et al., 2000). It obtains its nitrogen from the amino acid arginine through the partial reactions of the urea cycle (Durzan and Steward, 1983; Durzan and Pedroso, 2002; Durzan et al., 2006). Arginine is also a precursor for monosubstituted guanidines, which are natural nitric oxide synthase inhibitors, for stress-related proline accumulation, and for urea, ornithine and agmatine biosynthesis. The latter polyamines are needed for the differentiation of the embryonic axial tier of early embryos (unpublished data).



A *B* *C* *D*

Fig. 6. X-Ray radiographs of *Pinus silvestris* seeds from Arctic regions (after Simak 1973). Left to Right. *A*. Three undevel 'oped point embryos' in the erosion cavity of the seed. *B*. Two equally differentiated embryos have formed by '*modificative polyembryony*'. There was no inhibitory effect of a dominant embryo. *C*. One normal and one 'point' embryo in the erosion cavity. The dominant embryo had an inhibitory effect on the point embryo. *D*. Rare example of a seed with two haploid female gametophytes, each having an embryo. This abnormality originates at the pre-fertilization stage when two of the three macrospores in pine do not form and develop their own embryo sacs. *Pinus silvestris* does not form tetrads. In rare cases, an archegonium can take a lateral position. When fertilization takes place, a seed with two erosion cavities cross each other in the gametophyte (not shown)

Filonova et al. (2002) have proposed that programmed cell death (PCD) is the major mechanism responsible for the competitive elimination of subordinate embryos in polyembryonic seeds. PCD is claimed to halt 'competition' among monozygotic embryos and ensure the survival of one embryo by a yet undefined 'autocatalytic self-destruction program'. This undefined program does not yet distinguish itself among various other forms of apoptosis, nor explain modificative polyembryony in *Pinus* sp., where 'elimination' does not occur (Simak, 1973, Fig. 6). Regarding 'competitive elimination' the roles of 'necrohormones', produced by dying cells (Haberlandt, 1922; Havel and Durzan, 1996 a; Durzan et al., 1994), and the degeneration of 'relic nuclei' extruded out of the proembryos (Dogra, 1984) may be due simply to wounding, developmental errors and somatic mutations. Apoptosis contributes to apomictic embryo formation in cell

suspensions in Norway spruce (Bell, 1994; Durzan et al., 1994). Apoptotic cells are 're-absorbed' during megaspore formation. Re-absorption has been considered a form of 'cannibalism' in conifer 'brood reduction' (Haig, 1992).

With angiosperms, molecular biology and laser-capture microdissection (LCM) has enabled the identification of genes in individual cells which are responsible for all stages of embryonic development (Goldberg, 2007). New information exists on the characteristics of gene families that are important in seed formation, seed size, and viability (e.g., Xiao et al., 2006 a, b). The LCM of cells, coupled to 'whole genome profiling' has identified many compartment-specific transcription factors in tissues, and shown how these factors are partitioned during embryo development. Gene-chip ESTs (expressed sequence tags) are short sub-sequences of transcribed nucleotide sequences that are now used in gene discovery, gene sequence determination, and in the identification of gene transcripts. Real-time quantitative polymerase chain reaction (qRT-PCR) studies can tell us if a gene of interest is found in a cell, if the gene changes due to an experimental condition, and to what degree does a treatment alter the level of gene expression.

For loblolly pine, a nine-stage system based on embryo morphology has been formulated to compare gene expression in somatic and zygotic embryos. The differential display reverse transcription (RT)-PCR method helped to find a gene transcript for an 'aquaglyceroporin', which was abundantly expressed in the suspensors of loblolly pine in zygotic and somatic embryos (Ciavatta et al., 2001). Its physiological function remains unclear. Improved phenotypic performance criteria for selected genotypic characteristics are still needed (Durzan, 1989 a).

Protoplasts and genetic engineering

After 15 weeks, an initial population of 10 protoplasts from a loblolly pine ESM developed over 100 somatic embryos (Gupta and Durzan, 1987 b). In a subsequent report, protoplasts from white spruce embryos produced plantlets (Attree et al., 1989).

Morphogenic coenocytic and non-coenocytic protoplasts from cell suspensions of Douglas-fir and loblolly pine ESMs were used to introduce an insect gene by electroporation (Gupta et al., 1988). Insertion of the luciferase (*luc*) gene from fireflies reduced the viability of protoplasts by half. The remaining viable protoplasts showed the transient expression of the insect gene 36 h after electroporation. The light flashes due to the expression of the insect gene were measured by a luminometer. Results were expressed as the number of light flashes produced by 1 pg of firefly tail enzyme in 1 min. Given the early successes with protoplasts, and the widespread introduction of other methods to insert foreign genes, the results have been disappointing in recovering transformed trees with a normal and stable genotype.

Polyembryony in seeds and environmental factors

Adverse climate conditions may result in late embryo mortality and empty seeds (Dogra, 2001).The severe climatic conditions in the Arctic regions of Scandinavia, and Russia contribute to poor seed development and to increased polyembryony (Simak, 1970, 1973, Fig. 5). Poor development correlates with insufficient temperature sums and a short growing season. Early frost correlates with the persistence of polyembryony in seeds. The later developmental stages are inhibited so that only one embryo usually dominates in the seed. The lack of dominance among multiple embryos was taken as resembling the behavior of the terminal and lateral shoots of a tree. If the terminal shoot looses it dominance, a competition arises among the nearest lateral shoots to form a new leader shoot.

In natural stands where self-pollination predominates, the resulting 'genetic load' with its lethal and semi-lethal genes may eliminate almost completely, the individuals originating from self-fertilization. Empty seeds or immature embryos are commonly found. Simple polyembryony counteracts the excessive losses due to self-fertilization. If one of the embryos in the ovule has originated through selfing and dies, a normal seed may still develop if the ovule contains additional embryos originating by cross-fertilization (Hagman, 1975).

Haploid embryos are sometimes found in polyembryonic seeds (Illies, 1964, Simak et al., 1968). Recessive lethal genes are directly expressed in haploids. The embryos containing these genes do not survive. The doubling of chromosomes in the remaining and viable haploids with or without colchicine would create double haploid embryos,

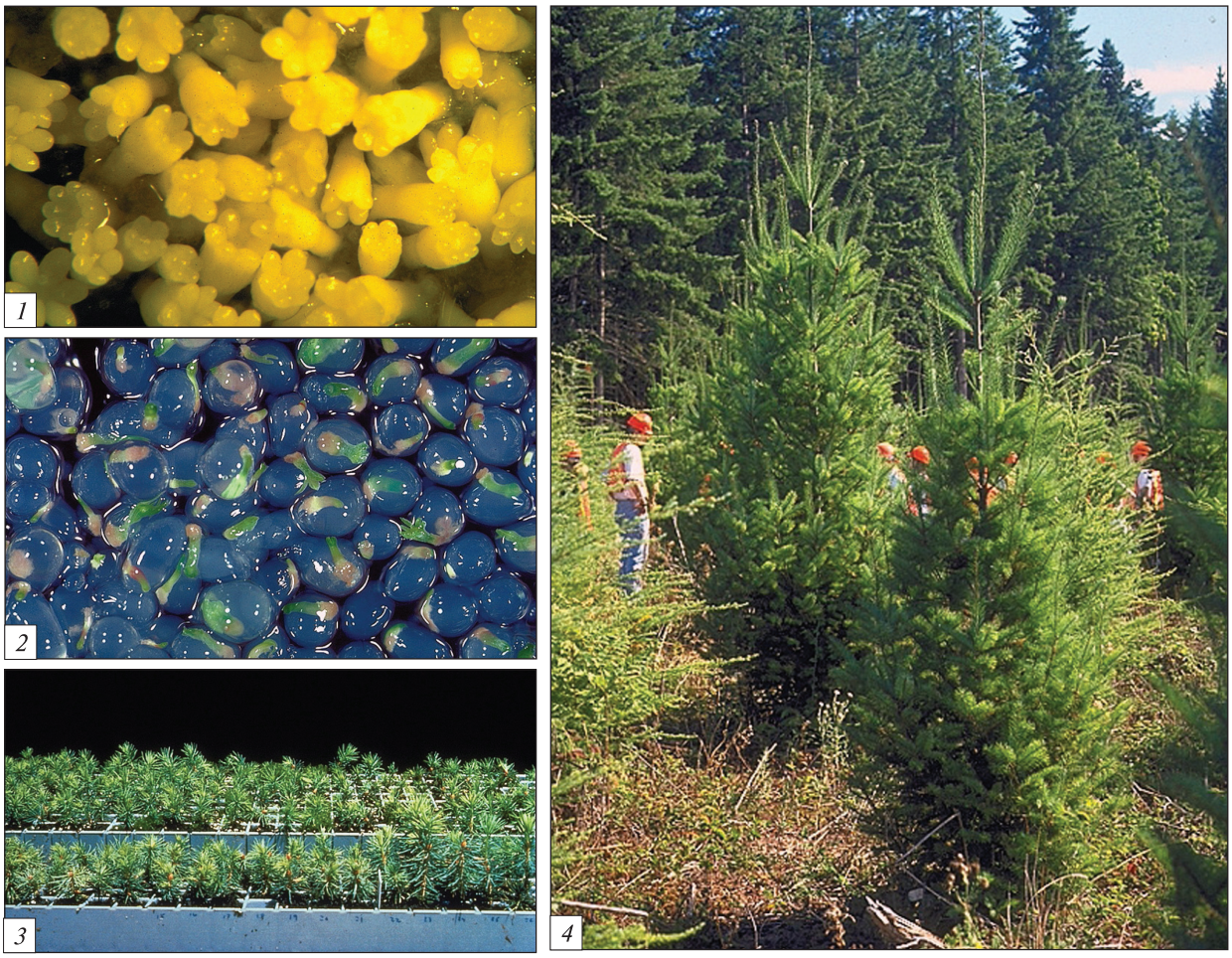


Fig. 7. Artificial seeds, clonal selection, and field testing. 7.*1*. A clone of Norway spruce embryos produced by MCP on a semi-solid support begins to develop cotyledons. 7.*2*. Spruce embryos 'germinate' in an alginate gel. These 'artificial seed's were intended to facilitate the mechanical handling of somatic embryos by a machine designed by Agricultural Engineering at Davis. The encapsulating material is not nutritive, nor equivalent to the food supply in the seed. 7.*3*. In capturing genetic gains, the clonal 'somatic seedlings' from a wide range of genotypes are assessed for specific markers at a juvenile stage before transfer to field trials. 7.*4*. A field plantation of clonal conifer genotypes is being evaluated for true-to-type development, clonal variations, and commercial potential (courtesy P.K. Gupta)

which are cleansed of the lethal factors. Genotypes also vary in their ability to release ethylene in cultures (Jokinen and Durzan, 1994). High ethylene producers can easily lead to the deterioration of other valuable genotypes if placed in the same closed culture dish unless the ethylene production is trapped and removed.

Protocols for Douglas-fir

Loblolly pine and Douglas-fir are two of the most important conifers in the USA for the timber and pulp and paper industry. Douglas-fir seeds do not exhibit MCP but do so when the early proembryos are rescued, cultured and cloned in cell suspensions (Durzan and Gupta, 1987). This process is protected as somatic polyembryogenesis by a US Patent (Durzan and Gupta, 1998) and licensed for use by Weyerhaeuser in the USA. Further improvements in 'somatic embryo production in liquid medium' and in the delivery of 'manufactured seeds' are protected by Weyerhaeuser patents to reduce their labor costs and increase the efficiency of mass clonal propagation (Carlson et al., 1997; Gupta et al., 1991, 1993).

At Weyerhaeuser, a semi-solid medium is still

used to induce ESM initiation (Gupta and Timmis 2005). 'Induction' and 'initiation' may obscure the fact that ESMs are initially 'rescued' from breeding orchards and not necessarily 'initiated' from seeds unless the 'ESM' is induced as a callus from the tissues of a single embryo (Fig. 3.*1*, 3.*2*, 4.*1*). With rescued ESMs, 'permissive conditions' may be more appropriate terminology rather than 'inductive conditions' since rescued ESMs already have embryo initials with the built-in capacity for MCP. Over 10,000 Douglas-fir somatic embryos have been produced in a single flask.

Weyerhaeuser uses a liquid medium in perfusion bioreactors with absorbent pads for the development and maturation of embryos (Timmis, 1998, e.g., Fig. 7.*1*). Over 700 genotypes have been cryopreserved in liquid N. Over 250,000 Douglas-fir somatic seedlings from a large number of genotypes have been produced for clonal field tests (Fig. 7.*3*, 7.*4*). Norway spruce embryos were encapsulated in alginate as artificial seeds to facilitate mechanical handling of the propagules (Fig.7.*2*).

Capturing genetic gains in clonal forestry

In tree breeding and improvement programs, vegetative propagation alone more than doubles the short-term genetic gain (G) compared to seed regeneration. Vegetative propagation offers monetary savings by avoiding the long life-cycles needed for seed production. Selection of an improved genotype is based on the principle that the *average* genetic value of selected individuals will be better than the *average* value of individuals in the population. 'G' is increased when genotypes are cloned and replicated many times. In the breeding seed orchard, the embryo(s) from a single cross can be rescued and 'bulked up' for full-scale field trials. The establishment of another seed production orchard for the new and tested genotypes can be reduced by 5 to 8 years. In the forest industry 'time is money'.

The formula for the capture of genetic gains comprises several complicating factors (Timmis et al., 1987, Fig. 8). Genotype × treatment interactions and clonal aberrations may introduce large variations in this process (Park et al., 1993). In 2005 alone, Weyerhaeuser field-planted 36,892 Douglas-fir, and 1250 Norway spruce produced by somatic embryogenesis (Fig. 7.*4*). With loblolly pine, 27,866 were field-planted, and 2,109 more

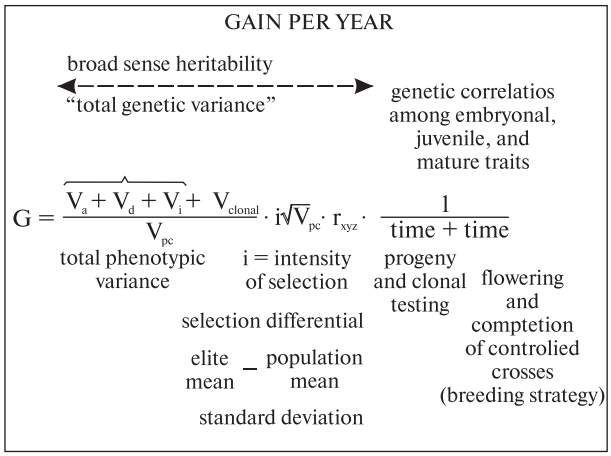


Fig. 8. Capturing genetic gains (G). *Heritability* is the proportion of variation in the population that is attributable to genetic differences among individuals in a particular environment. G requires a measure of the *total genetic variation*. It is partitioned into *additive* variations (V_a) due to differences among parents in general combining ability, and into *nonadditive* variances. V_a is also the 'breeding value' for the population. For regeneration by seeds, only V_a can be manipulated. Other components of the total genetic variance are specific combining effects, *viz.*, dominant (V_d), epistatic (V_i), clonal V_{clonal}), and the *total phenotypic variance for the clonal population* (V_{pc}). Maximum '*broad sense heritability*' is obtained when rejuvenation techniques are perfected. Heritability is a ratio between genetic and phenotypic variances. It is not a fixed value for a given characteristic of a given species. Volume growth for individual trees generally is an example of '*narrow-sense*' heritability, i.e., the ratio of additive genetic variance to the phenotypic variance (genetic variation + environmental variation). In genotypes with significant *nonadditive* variance, G is also captured through seed production, but this is only a small portion of the potential gain available compared to *vegetative propagation*. The total phenotypic variation determines the selection intensity '*i*'. It is the mean deviation of selected elite individuals from the population mean based on units of phenotypic standard deviation. It depends only on *p*, the proportion selected, assuming a normal distribution, or on *pj* for juvenile traits when selections are made in the field before crop maturity. The statistical correlation '*r*' enables time savings in field tests by using marker-aided selection over developmental stages to maturity (x, y, z). This increases the intensity of selection and saves labor and time. The last factor is the '*reciprocal generation interval*' dealing with the '*time costs*' for clonal propagation. It may include the time and costs for flowering and employing other breeding strategies. For in vitro cloning technologies, the selection differential, juvenile-mature correlations and the reciprocal of the generation interval are major factors in saving time and capturing genetic gains

elite clones were established in the greenhouse for field-testing.

In Europe, clones produced from PEMs have been field-tested for spruce, pine, and other conifers (e.g., Högberg et al., 2003). In North and South America, South-east Asia, and New Zealand, several industries and governments have scaled up the production of 'somatic seedlings' to commercial volumes. Long-term co-operative field trials have been started to evaluate the economic reality of forest clones (ArborGen USA, 2007).

G. Weyerhaeuser (2003) stated that the proof of concept testing for somatic embryogenesis, manufactured seed, and the automation required linking these technologies has been completed. The challenge is to lower costs and scale up commercial application.

Weyerhaeuser envisions scale production facilities capable of producing over 100 million seedlings of Douglas-fir and loblolly pine per year. These facilities will link somatic embryogenesis to manufactured seed via automation seamlessly into our existing nursery system. However, 'Clonal seedlings that cost significantly more than orchard seedlings will become an obstacle to the implementation of any cloning technology'.

Future challenges

Given the great technological advances towards commercialization of somatic embryogenesis, significant problems remain. These will challenge the economic reality for industrial forestry because the underlying biological processes are still not fully understood and controlled, nor are the benefits of mechanization and automation and plant-machine compatibility proven and acceptable in the market place.

Further research and development are needed for a wider range of species to: 1. Model the true-to-type process from embryo recovery and maturation using the rapidly-evolving new methods of molecular biology. 2. Develop reliable performance criteria for plant/machine compatibility and field testing. 3. Refine and improve feed-forward and feedback process controls in systems for MCP and true-to-type cloning based on cost-effectiveness. 4. Design more robust and flexible cloning systems to accommodate and physiologically precondition a wider range of genotypes. 5. Improve the current embryo-to-soil delivery systems to reduce the need for certain types of 'manufactured seeds'. 6. Develop a genomic and bioinformatics data base for silvicultural production cycles which feed into the unique properties of utilization cycles in the forest industries. 7. Learn more about the why some species are becoming endangered even if they are not yet ecologically or economically useful.

In the long run, the main benefit from studying MCP in extant and modern conifers will come from the new knowledge derived from future investigations of apomixis and it complexities. The discovery of latent diploid parthenogenesis coupled to MCP and apoptosis in Norway spruce is an example of unexpected hidden totipotency when new genotypes are challenged by the artificial environments under in vitro conditions (Bell, 1994; Durzan et al., 1994; Durzan and Durzan, 1991). Can we now control ploidy in the `alternation of the haploid gametophytic and diploid sporophytic generations` in cells undergoing reproductive development? Can totipotent cells exposed to simulated environmental conditions of the deep past provide clues as to how reproductive development may have evolved before the angiosperms appeared (Durzan, 1991, 1996 b)? With further progress in the basic analytical sciences, tree breeding, and genetics, we may be able to understand and predict how some genotypes might adapt and become selected by future genetic and climatic changes (UNESCO, 1992).

The author is grateful to the following colleagues who have contributed to the understanding and development of somatic embryogenesis in angiosperms and gymnosperms. These are F.C. Steward, P. Gupta, P.M. Dogra, V. Chalupa, L. Havel, K. Jokinen, M. Guerra, M. Boulay, A. Santerre, M. Simak, L. Stebbins, E.M. Gifford, G. Berlyn, N.W. Radforth, J.M. Bonga, L. Hong, among many others.

Don J. Durzan

MONOZYGOTIC CLEAVAGE POLYEMBRYOGENESIS AND CONIFER TREE IMPROVEMENT

Массовое клонирование элитных генотипов коммерчески важных хвойных деревьев привело к созданию промышленного леса двух наиболее важных хвойных видов в США. Эмбрионально-суспенсорные массы, которые образуются путем монозиготной ра сщепленной полиэмбрионии (МРС), вычленяют из семян, образовавшихся путем контролируемого опыления, в садах-питомниках. МРС растет как клеточная суспензия и формирует зрелые соматические эмбриоиды. Эти эмбриоиды служат источником для получения различных

искусственных семян, которые используются в повторяющихся полевых испытаниях для изучения взаимодействия генотипа и среды. Для достижения генетических целей отбор на ранних стадиях базируется на корреляциях с известными признаками. Это сокращает потерю лет при улучшении деревьев. Массовое клонирование и криосохранение генотипов дает возможность проводить полевые испытания по широкому кругу сайтов. Биореакторы с контролируемыми параметрами предлагаются как искусственные завязи для обеспечения питательными веществами материнского дерева и стимулирования факторов среды, которые влияют на новые поколения. Сравнение генотипов хвойных деревьев даст новые пути для понимания скрытых возможностей апомиксиса, чередования поколений и адаптивной пластичности.

Don J. Durzan

MONOZYGOTIC CLEAVAGE POLYEMBRYOGENESIS AND CONIFER TREE IMPROVEMENT

Масове клонування елітних генотипів комерційно важливих хвойних дерев привело до створення промислового лісу двох найбільш важливих хвойних видів в США. Ембріонально-суспенсорні маси, котрі утворюються шляхом монозиготної розщепленої поліембріонії (МРС), виділяють з насіння, яке утворилося шляхом контрольованого запилення в садах-розсадниках. МРС росте як клітинна суспензія і формує зрілі соматичні ембріоїди. Ці ембріоїди служать джерелом для отримання різного штучного насіння, яке використовується в повторних польових дослідах для вивчення взаємодії генотипа та середовища. Для досягнення генетичних цілей відбір на ранніх стадіях базується на кореляціях з відомими ознаками. Це скорочує втрату років при поліпшенні дерев. Масове клонування та кріозбереження генотипів дає можливість проводити польові досліди по широкому колу сайтів. Біореактори з контрольованими параметрами рекомендуються як штучні зав'язі для забезпечення поживними речовинами материнського дерева і стимулювання факторів середовища, які впливають на нові покоління. Порівняння генотипів хвойних дерев дасть нові шляхи для розуміння прихованих можливостей апоміксиса, чередування поколінь і адаптивної пластичності.

REFERENCES

ArborGen USA. 2007. Benefits of tree biotechnology. http://arborgen.com.

Attree S.M., Dunstan D., Fowke L.C. 1989. Plantlet regeneration from embryogenic protoplasts of white spruce (*Picea glauca*). Bio/Technology 7:1060–1061.

Bhalla P.L, Singh M.B. 2006. Molecular control of stem cell maintenance in shoot apical meristems. Plant Cell Rep. 25: 249–256.

Bell P.R. 1994. Apomictic features revealed in a conifer. Int. J. Plant Sci. 155: 621–622.

Bonga J.M., Durzan D.J. (eds). 1987. Cell and Tissue Culture in Forestry. Vol. 1. General Principles and Biotechnology (232 p.), Vol. 2. Specific Principles and Methods: Growth and Developments (447 pp), Vol. 3. Case Histories: Gymnosperms, Angiosperms and Palms (416 p.). Martinus Nijhoff, Dordrecht.

Bonga J.M., Klimaszewska K., Lelu M-A., von Aderkas P. 1995. Somatic embryogenesis in *Larix*. In: Somatic Embryogenesis in Woody Plants. Eds. Jain S., Gupta P.K., Newton R., Vol. 3. 315–339.

Boulay M.P., Gupta P.K, Krogstrup P., Durzan D.J. 1988. Development of somatic embryos from cell suspension culture of Norway spruce (*Picea abies* Karst.). Plant Cell Repts. 7:134–137.

Bozhkov P. 2006. Spruce embryogenesis – a model for developmental cell death in plants. In: Cell biology and instrumentation: UV radiation, nitric oxide, and cell death in plants. Blume, Y., *et al.* (eds), Proc. NATO Advanced Research Workshop, Yalta, Ukraine, Sept 8 to 11, 2004, IOS Press, Amsterdam. 276–280.

Ciavatta V., Morillon R., Pullman G.S., Chrispeels M.J., Cairney J. 2001. An aquagylceroporin is abundantly expressed early in the development of the suspensor and embryo proper of loblolly pine. Plant Physiol. 127: P. 1556–1567.

Bozhkov P., Filonova L., Suarez M., Helmersson A., Smertenko P., Zhivotovsky B., von Arnold S. 2003. VEIDase is a principal caspase-like activity involved in plant programmed cell death and essential for embryonic pattern formation**.** Cell Death and Differentiation 11: 175–182.

Carlson W.C., Hartle J. E., Salatas K., Harris A., Littke W.R. 1997. Manufactured seed with enhanced pregermination survivability. US Patent No. 5701699.

Chalupa V. 1985. Somatic embryogenesis and plantlet regeneration from cultured immature and mature embryos of *Picea abies* (L.) Karst. Commun. Inst. For. Cech. 14: 57–63.

Dogra P.D. 1967. Seed sterility and disturbances in embryogeny in conifers with particular reference to seed testing and tree breeding in Pinaceae. Stud. Forst. Suecica No. 45: 1–97.

Dogra P.D. 1979. Morphology, development and nomenclature of conifer embryo. Phytomorphology 28: P. 307–322.

Dogra P.D. 1984. The embryology, breeding systems and seed sterility in Cupressaceae. A monograph. In: P.K. Nair (ed.) Glimpses in Plant Research. Vices Press, New Delhi. Vol. VI, pp. 1–26.

Dogra P.D. 2001. Late embryo mortality – a prime cause of seed sterility in some exotic pine species adapted in Kumon Himalaya. Curr. Sci. 80: 335–336.

Doyle J. 1963. Proembryogeny in *Pinus* in relation to

that in other conifers – a survey. Proc. Roy. Irish. Acad. 62B: 181–216.
Doyle J., Looby W.J. 1939. Embryogeny in Saxegothaea and its relation to other podocarps. Sci. Proc. Roy. Dublin Soc. 22: 127–147.
Durzan D.J. 1988a. Somatic polyembryogenesis for the multiplication of tree crops. Biotech. Genetic Eng. Revs. 6: 341–378.
Durzan D.J. 1988b. Metabolic phenotypes in somatic embryogenesis and polyembryogenesis. In: Genetic Manipulation of Woody Plants. J. Hanover *et al.* (eds), Plenum Press, N.Y., 293–312.
Durzan D.J. 1989a. Performance criteria in response surfaces for metabolic phenotypes of clonally propagated woody perennials. In: Applications of Plant Biotechnology in Forestry and Agriculture. Ed. V. Dhawan, Plenum Press, New York, 181–203.
Durzan D.J. 1989b. Physiological aspects of somatic embryogenesis in suspension cultures of conifers. Ann. Sci. For. 46 Suppl.: 101s–107s.
Durzan D.J. 1991. Improving tree growth and quality through biotechnology. Kemia-Kemi (Finnish Chemistry) 18:192–199.
Durzan D.J. 1996a. Protein ubiquitination in diploid parthenotes of Norway spruce. Intl J. Plant Sci. 157:17–26.
Durzan D.J. 1996b. Asexual reproductive adaptation to simulated Cretaceous climatic variables by Norway spruce cells *in vitro*. Chemosphere 33: 1655–1673.
Durzan D.J. 2002. Stress-induced nitric oxide and adaptive plasticity in conifers. J. Forest Science 48: 281–291.
Durzan D.J. 2006. Nitric oxide, cell death and increased taxol recovery. In: Cell biology and instrumentation: UV radiation, nitric oxide, and cell death in plants. Blume, Y., *et al.*, (eds), NATO Advanced Research Workshop, Yalta, Ukraine, Sept 8 to 11, 2004, IOS Press, Amsterdam. 234–252.
Durzan D.J., Chalupa V. 1968. Free sugars, amino acids, and soluble proteins in the embryo and female gametophyte of jack pine as related to climate at the seed source. Can. J. Bot. 46:417–428.
Durzan D.J., Chalupa V. 1976. Growth and metabolism of cells and tissue of jack pine (*Pinus banksiana*). 3. Growth of cells in liquid suspension cultures in light and darkness. Can. J. Bot. 54:456–467.
Durzan D.J., Durzan P.E. 1991. Future technologies: Model-reference control systems for the scale-up of embryogenesis and polyembryogenesis in cell suspension cultures. In: Micropropagation. Debergh, P., Zimmerman, R.H., (eds.), Kluwer Academic Publ. 389–423.
Durzan D.J., Gupta P.K. 1987. Somatic embryogenesis and polyembryogenesis in Douglas-fir cell suspension cultures. Plant Sci. 52:229–235.
Durzan D.J., Gupta P.K. 1998a. Method for clonal propagation of gymnosperms by somatic polyembryogenesis. US Patent No. 5,821,126. Oct. 13, 1998.
Durzan D.J. Gupta P.K. 1988b. Somatic embryogenesis and polyembryogenesis in conifers. Advances in Biotechnological Processes. 9: 53–81.
Durzan D.J. Pedroso M.C. 2002. Nitric oxide and reactive nitrogen oxide species in plants. Biotechnology and Genetic Engineering Reviews. 19: 293–337.
Durzan D.J., Steward F.C. 1983. Nitrogen metabolism. In: Plant Physiology. A Treatise. VIII, Steward, F.C., Bidwell, R.G.S. (eds). Academic Press, NY. 55–265.
Durzan D.J., Jokinen K., Guerra M., Santerre A., Chalupa V., Havel L. 1994. Latent diploid parthenogenesis and parthenote cleavage in egg-equivalents in Norway spruce. Intl J. Plant Science 155: 677–688.
Durzan D.J., Santerre A., Havel L. 2006. Effects of chlorsulfuron on early embryo development in Norway spruce cell suspensions. In: Cell biology and instrumentation: UV radiation, nitric oxide, and cell death in plants. Blume, Y. *et al.* (eds), Advanced Research Workshop, Yalta, Ukraine, Sept 8 to 11, 2004, IOS Press, Amsterdam. 262–273.
Dyachok J.V., Wiweger M., Kenne L., von Arnold S. 2002. Endogenous Nod-factor-like signal molecules promote early embryo development in Norway spruce. Plant Physiol. 128: 523–533.
Ferguson C.W. 1968. Bristlecone pine: science and ethics. Science 159: 838–846.
Filonova L.H., Bozhkov P., von Arnold S. 2000a. Developmental pathways of somatic embryogenesis in Picea abies as revealed by time-lapse tracking. J. Exptl Bot. 51: 249–264.
Filonova L.H., Bozhkov P., Brukhin V.B., Daniel G., Zhivotovsky B., von Arnold S. 2000b. Two waves of programmed cell death occur during formation and development of somatic embryos in the gymnosperm, Norway spruce. J. Cell Science 113: 4399–4411.
Filonova L.H., von Arnold S., Daniel G., Bozhkov P.V. 2002. Programmed cell death eliminates all but one embryo in a polyembryonic plant seed. Cell Death & Differentiation. 9: 1057–1062.
Gupta P.K., Durzan D.J. 1986a. Somatic polyembryogenesis from callus of mature sugar pine embryos. Bio/Technology, 4:643–645.
Gupta P.K., Durzan D.J. 1986b. Plantlet regeneration via somatic embryogenesis from subcultured callus of mature embryos of *Picea abies* (Norway spruce). *In Vitro* Cellular and Development Biology 22:685–688.
Gupta P.K., Durzan D.J. 1987a. Biotechnology of somatic polyembryogenesis and plantlet regeneration in loblolly pine. Bio/Technology 5:147–151.
Gupta P.K., Durzan D.J. 1987b. Somatic embryos from protoplasts of loblolly pine proembryonal cells. Bio/Technology 5:710–712.

Gupta P.K., Durzan D.J. 1991. Loblolly pine (*Pinus taeda* L.). In: Biotechnology in Agriculture and Forestry. III. Trees. Y.S.P. Bajaj (ed.), Springer-Verlag, N.Y. 383–407.

Gupta P.K., Timmis R. 2005. Mass propagation of conifer trees in liquid cultures – progress towards commercialization. Plant Cell, Tissue and Organ Culture 2005: 81: 339–346.

Gupta P.K., Durzan D.J. Finkle B.J. 1987. Somatic polyembryogenesis in embryogenic cell masses of *Picea abies* (Norway spruce) and *Pinus taeda* (loblolly pine) after thawing from liquid nitrogen. Can. J. For. Res. 17:1130–1134.

Gupta P.K., Dandekar A.M., Durzan D.J. 1988. Somatic proembryo formation and transient expression of a luciferase gene in Douglas-fir and loblolly pine protoplasts. Plant Science 58: 85–92.

Gupta P.K., Pullman G., Timmis R., Kreitinger M., Carlson W.C., Grob J., Welty E. 1993. Forestry in the 21st century. Bio/Technology. 11: 454–259.

Gupta P.K., Shaw D., Durzan D.J. 1987. Loblolly pine: Micropropagation, somatic embryogenesis and encapsulation. In: Cell and Tissue Culture in Forestry. J.M. Bonga, D.J. Durzan (eds.), Martinus Nijhoff/Dr. W. Junk, Vol. 3, pp. 101–108.

Gupta P.K., Timmis R., Pullman G., Yancey M., Kreitinger M., Carlson W., Carpenter C. 1991. Development of an embryogenic system for automated propagation of forest trees. Cell Culture and Somatic Genetics of Plants. 8: 75–93.

Haberlandt G. 1922. Über Zellteilungshormone und ihre Beziehungen zur Wundheilung, Befruchtung, Parthenogenesis und Adventivembryonie. Biol. Zbl. 42: 145–172.

Hagman M. 1975. Incompatibility in forest trees. Proc. R. Soc. Lond. B 188: 313–326.

Haig D. 1992. Brood reduction in gymnosperms. In: Cannibalism. Ecology and evolution among diverse taxa. Elgar, M.A., Crespi, B.J. (eds). Oxford Univ. Press. 63–84.

Hakman I., Fowke L.C., von Arnold S., Eriksson T. 1985. The development of somatic embryos in tissue cultures initiated from immature embryos of *Picea abies* (Norway spruce). Plant Sci. 38: 53–59.

Havel L., Durzan D.J. 1996a. Apoptosis in plants. Botanica Acta 109: 268–277.

Havel L., Durzan D.J. 1996b. Apoptosis during diploid parthenogenesis and early somatic embryogenesis of Norway spruce. Intl J. Plant Sci. 157:8–16.

Havel L., Durzan D.J. 1999. Apoptosis during somatic embryogenesis in *Picea* sp. In: Somatic embryogenesis in woody plants. Jain, S.M., Gupta, P.K., Newton, R.J., Kluwer Academic, Dordrecht, 4: 125–147.

Högberg K.-A., Bozhkov P., von Arnold S. 2003. Height growth and within clone variation of Norway spruce clones propagated by somatic embryogenesis. Tree Physiol. 23:211–216.

Hong L., Boulay M., Gupta P.K., Durzan D.J. 1991. Variations in somatic polyembryogenesis: induction of adventitious embryonal-suspensor masses on developing Douglas-fir embryos. In: Woody Plant Biotechnology. M.R. Ahuja (ed.), Plenum Publ. Corp. N.Y. pp. 105–121.

Illies Z.M. 1964. Auftreten haploider Keimlinge bei *Picea abies*. Naturwiss. 51: 442.

Jäger L. 1899. Beiträge zur Kenntnis der Endospermbildung und zur Embryologie von *Taxus baccata*. Flora (Jena) 86: 241–288.

Jain S.M., Gupta P.K., Newton R.J. (eds) 1995. Somatic embryogenesis in woody plants. Vol. 3. Kluwer Academic Publ. Dordrecht.

Jokinen K., Durzan D.J. 1994. Properties of rescued embryonal suspensor masses of Norway spruce (*Picea abies* L. Karst.) determined by the genotype and the environment *in vitro*. Silva Fennica 28: 95–106.

Komamine A., Kawahara M., Matsumoto M., Sunabori S., Toya T., Fujiwara M., Smith J., Ito H. et al. 1992. Mechanisms of somatic embryogenesis in cell cultures: physiology, biochemistry, and molecular biology. In Vitro Cell. Dev. Biol. 28P: 11–14.

Lakshmanan K.K., Ambergaoker K.B. 1984. Polyembryony. In: Johri, B.M. (ed.), Embryology of angiosperms. Springer-Verlag, New York, 445–473.

Magalhaes J.R., Monte D.C., Durzan D.J. 2000. Nitric oxide and ethylene emission in *Arabidopsis thaliana*. Physiology and Plant Molecular Biology 6: 117–127.

Mogie M. 1992. Evolution of asexual reproduction in plants. Chapman & Hall, London.

Park V.S., Pond S.E., Bonga J.M. 1993. Initiation of somatic embryogenesis in white spruce (*Picea glauca*): genetic control, culture treatment effects, and implications for tree breeding. Theor. Appl. Genet. 86: 427–436.

Pedroso M.C., Magalhaes J.R., Durzan D.J. 2000. A nitric oxide burst precedes apoptosis in an angiosperm and a gymnosperm. J. Exptl Botany 51: 1027–1036.

Simak M. 1970. New uses of X-ray method for the analysis of forest seed. Inst. för Skogsföryngring, Skoghögskolan 23: 1–12.

Simak M. 1973. Polyembryonal seeds of *Pinus silvestris* in arctic regions. Institutionen för Skogsföryngring. Dept Reforestation. First All-Union Symp. Sexual Reproduction of Conifers, Novosibirsk, USSR. Research Notes 45: 1–14.

Simak M., Gustafsson A., Ching K. 1968. Occurrence of a mosaic aneuploid in polyembryonic Norway spruce seed. Stud. For. Suec. 67: 1–8.

Singh H. 1978. Embryology of Gymnosperms. Gebrüder, Borntraeger, Berlin.

Sporne K.R. 1965. The morphology of gymnosperms. Hutchinson Univ. Library, London.

Stasolla C. van Zyl L., Egertsdotter U., Craig D., Liu W. Sederoff R. 2003. The effects of polyethylene glycol on gene expression of developing white spruce embryos. Plant Physiol... 131: 49–60.

Steward F.C. 1968. Growth and Organization in Plants. Addison Wesley, Reading, Mass.

Steward F.C. 1975. Observations on growth and morphogenesis in cultured cells of carrot. Phil. Trans. Royal Soc. London, 273: 33–53.

Timmis R., Abo El-Nil M.M., Stonecypher R.W. 1987. Potential genetic gain through tissue culture. In: Bonga, J.M., Durzan, D.J. (eds.), Cell and Tissue Culture in Forestry. Genetic Principles and Biotechnology. Martinus Nijhoff, Dordrecht. Vol. 1, pp. 198–215.

Timmis R. 1998. Bioprocessing for tree production in the forest industry: Conifer somatic embryogenesis. Biotechnology Progress 14: 156–166.

UNESCO 1992. Contending with global change. Study No. 5. Human action to control global climate through designed ecosystems. Biodiversity and climate change workshop. Feb. 22–25. UNESCO/ROSTSEA, Jln. M. H. Thamrin 14, Jakarta, Indonesia. 26 p.

Vales T., Feng X., Ge L., Ku N., Cairney J., Pullman G., Peter G. 2007. Improved somatic embryo maturation in loblolly pine by monitoring ABA-responsive gene expression. Plant Cell Repts 26: 133–143.

Webber J.M. 1940. Polyembryony. The Botanical Review. 6: 575–598.

Weigel D., Jürgens G. 2002. Stem cells that make stems. Nature 415: 751–754.

Weyerhaeuser G.N. 2003. Biotechnology in forestry: The promise and the economic reality. Solutions for People, Progress and Paper, October, 2003. 28–30.

Xiao W., Custard K.D., Brown R.C., Lemmon B.E., Harada J.J., Goldberg R.B., Fischer R.L. 2006a. DNA methylation is critical for *Arabidopsis* embryogenesis and seed viability. Plant Cell 18: 805–814.

Xiao W., Brown R.C., Lemmon B.E., Harada J.J., Goldberg R.B., Fisher R.L. 2006b. Regulation of seed size of maternal and paternal genomes. Plant Physiol. 142: 1160–1168.

Received 30.09.07