# *Araucaria angustifolia* Biotechnology

**Neusa Steiner[1] • Claudete Santa-Catarina[2] • Júlia B. R. Andrade[3] • Tiago S. Balbuena[3] • Miguel P. Guerra[1] • Walter Handro[3] • Eny I. S. Floh[3] • Vanildo Silveira[4*]**

[1] Plant Developmental Physiology and Genetics Laboratory, Department of Plant Science, Federal University of Santa Catarina, Florianópolis-SC, Brazil
[2] Cellular and Tissue Research Laboratory (LBCT), CBB - State University of North Fluminense (UENF). Campos dos Goytacazes - RJ, Brazil
[3] Plant Cell Biology Laboratory, Department of Botany, IB-University of São Paulo. São Paulo-SP, Brazil
[4] Biotechnology Laboratory (LBT), CBB - State University of North Fluminense (UENF). Av. Alberto Lamego 2000. CEP: 28.013-602. Campos dos Goytacazes – Rio de Janeiro, Brazil

*Corresponding author*: * **vanildo@uenf.br**

## ABSTRACT

Biotechnological tools have a large application potential in the breeding programmes and biodiversity conservation of *Araucaria angustifolia* (Bert) O. Ktze, an endangered native coniferous species from the Brazilian Atlantic Rain Forest. An overview is presented of *A. angustifolia* seed and somatic embryo developmental biology, including physiological, biochemical studies and proteomic approaches. Significant advances in plant regeneration via somatic embryogenesis have been made over the last decade. Recent works on the induction, proliferation, development and morphogenesis of pro-embryogenic masses (PEMs) have been used to explain their role in somatic embryo development. In order to increase the efficiency of PEM development, biochemical and molecular events were studied. Among the former, the metabolism of polyamines and nitric oxide, besides the synthesis of specific proteins, such as late embryogenesis abundant and storage proteins, seem to be involved in the regulatory mechanisms of this complex process. Seed-development studies were performed to better understand the molecular and physiological basis of embryogenesis and system manipulation for *in vitro* multiplication via somatic embryogenesis. Genomics and proteomics are new tools for improving *A. angustifolia* biotechnology and providing more insight.

## CONTENTS

## INTRODUCTION

*Araucaria angustifolia* (Bert) O. Ktze is the only native conifer of economic importance in Brazil, representing the most exploited timber source until the 1970's (Astarita and Guerra 1998). This species is a valuable source of seeds, wood, fiber and resin. The seeds have high nutritional value, being consumed by both humans and wild fauna. The wood of adult trees is employed in the manufacture of furniture, structural timber, and almost all other kinds of wood application (Guerra *et al.* 2000).

Originally, the forests of *A. angustifolia* covered an area of 20 million hectares in Brazil. As a result of the clear-cutting form of exploitation, nowadays only relicts of the natural vegetation are found, these representing less than 2% of the original area (Guerra *et al.* 2002). Recently, this species was included in the official list of endangered Brazilian plants, under the "vulnerable" category (Guerra *et al.* 2000, 2002). Therefore, it is necessary to develop technologies for the conservation and genetic improvement of this subtropical conifer species.

Biotechnological tools have a large application potential in breeding programs and biodiversity conservation of *A. angustifolia*. Biotechnology is a significant affix to long-established traditional tree improvement practices, and is one that utilizes fundamental discoveries in the field of plant tissue culture for clone-forestry, gene transfer techniques, molecular biology, and genomics (Nehra *et al.* 2005). New discoveries in this field may provide an extended platform for the improvement of traits that were previously considered as unpractical via conventional breeding methods. Biotechnology provides exciting opportunities for further expanding our understanding of genome organization and the functioning of genes associated with complex value-added traits, and for transferring such genes into economically important tree species. This will lead to the development and deployment of trees in preparation for meeting the future demand of the world's ever-increasing population for timber and other forest products, while preserving natural forests for future generations (Merkle and Nairn 2005; Nehra *et al.* 2005).

## SOMATIC EMBRYOGENESIS IN CONIFERS

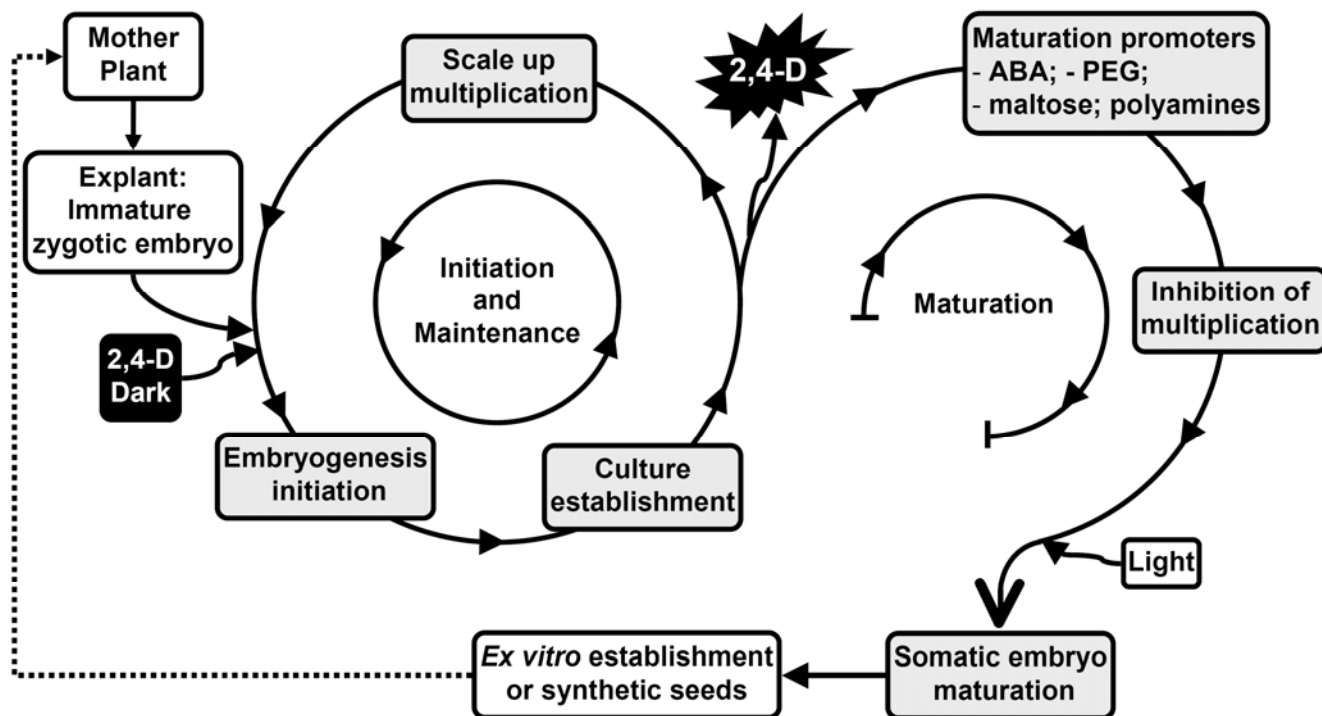Biotechnologically based techniques provide efficient methods for micropropagation, genetic improvement, and the

Invited Review

**Fig. 1 Conifer somatic embryogenesis modulation in two cycles**. Initiation, maintenance and maturation cycles.

germplasm conservation of tropical and subtropical trees (Litz *et al.* 1997). Among these, one micropropagation technique that has become widely utilized in forest biotechnology is somatic embryogenesis.

Somatic embryogenesis is a process analogous to zygotic embryogenesis, where a single cell or a small group of somatic cells are the precursors of somatic embryos (Tautorus *et al.* 1991). In conifers, somatic embryogenesis and plant regeneration were first reported in *Picea abies*, by using zygotic embryos as explants (Chalupa 1985; Hakman *et al.* 1985), and by the induction of somatic embryogenesis from megagametophytes in *Larix decidua* (Nagmani and Bonga 1985). The application of somatic embryogenesis in the genetic transformation of conifer species has been extensively reviewed recently (Malabadi and Nataraja 2007).

Conifer somatic embryogenesis is usually initiated from immature zygotic embryos by using auxins in the culture medium. Once induced embryogenic cultures can be maintained in periodical subcultures in a gelling or liquid culture medium. With the removal of the auxin and supplementation of maturation promoters into the culture medium, cultures are addressed for the formation of somatic embryos and subsequent *ex vitro* establishment (**Fig. 1**).

In conifers several factors affect the induction frequency of embryogenic cultures. These include the influence of the genotype, the effect of explant stage of development, and the induction potential of the culture medium (Radojevic *et al.* 1999), particularly, of sucrose, the nitrogen level and composition, mineral elements, agar, plant growth regulators and pH (Tautorus *et al.* 1991).

Maturation of conifer somatic embryos occurs in the presence of abscisic acid (ABA) and an osmotic agent (Attree and Fowke 1993; Stasolla *et al.* 2002; Stasolla and Yeung 2003). ABA inhibits cleavage polyembryony, thus allowing for embryo singulation, further development and maturation. Furthermore, ABA is involved in the accumulation of storage proteins (Dunstan *et al.* 1998), cellular expansion control (Gutmann *et al.* 1996) and triacylglycerol biosynthesis (Attree *et al.* 1992). The effectiveness of ABA treatment may be enhanced through an increase in the osmotic level of the culture medium by the addition of hexose sugars, sugar alcohols, or neutral polymers such as polyethylene glycol (Bonga *et al.* 1995).

## APPLICATIONS OF SOMATIC EMBRYOGENESIS

Somatic embryogenesis possesses great potential for clonal propagation in conifers. This technique allows additional genetic gain to be obtained, owing to possible capture of both additive and non-additive genetic variation (von Arnold *et al.* 1995). Somatic embryogenesis reveals several advantages when compared to other *in vitro* propagation systems, including its high multiplication rates, the cryopreservation of embryogenic callus, as well as the potential for scale-up in liquid suspension cultures, the use of bioreactors and somatic synthetic seed technologies and the fact that embryogenic cultures are suitable target tissues for gene transfer (Merkle and Dean 2000).

The applications of somatic embryogenesis include the provision of cell lines for genetic transformation, the *ex situ* conservation of rare and endangered species or populations, research to improve our understanding of conifer genetics, and the generation of high-value clone-forestry as a tool in tree-breeding programmes, the latter being the most promising application (Park *et al.* 1998).

High-value forestry is an intensive commercial activity, in which highly valuable genotypes or tree "varieties" are cloned for deployment at productive sites. The major advantages of clone-forestry are: (*i*) additional genetic gain achieved by capturing non-additive genetic variation; (*ii*) the speed in which such clones may be introduced to meet changing breeding programmes or market goals; (*iii*) the ability to introduce genetic variability into clone-forestry; and (*iv*) compensation for the shortage of improved seeds from orchards (Högberg *et al.* 1998; Park *et al.* 1998).

Somatic embryogenesis is a valuable tool in breeding programmes. The major advantage is to obtain a large number of cloned plants in a short amount of time (Högberg *et al.* 1998). Both somatic embryogenesis and cryopreservation permit the development of improved varieties through the re-growth of embryogenic cultures, followed by field performance (Park *et al.* 1998). The cryopreservation of embryogenic cultures efficiently links the breeding program to mass clone-propagation strategies. Thus, the inclusion of cryopreservation in the strategy of somatic embryogenesis allows for the use of superior clones in high-value forestry (Högberg *et al.* 1998). In addition, cryopreservation of em-
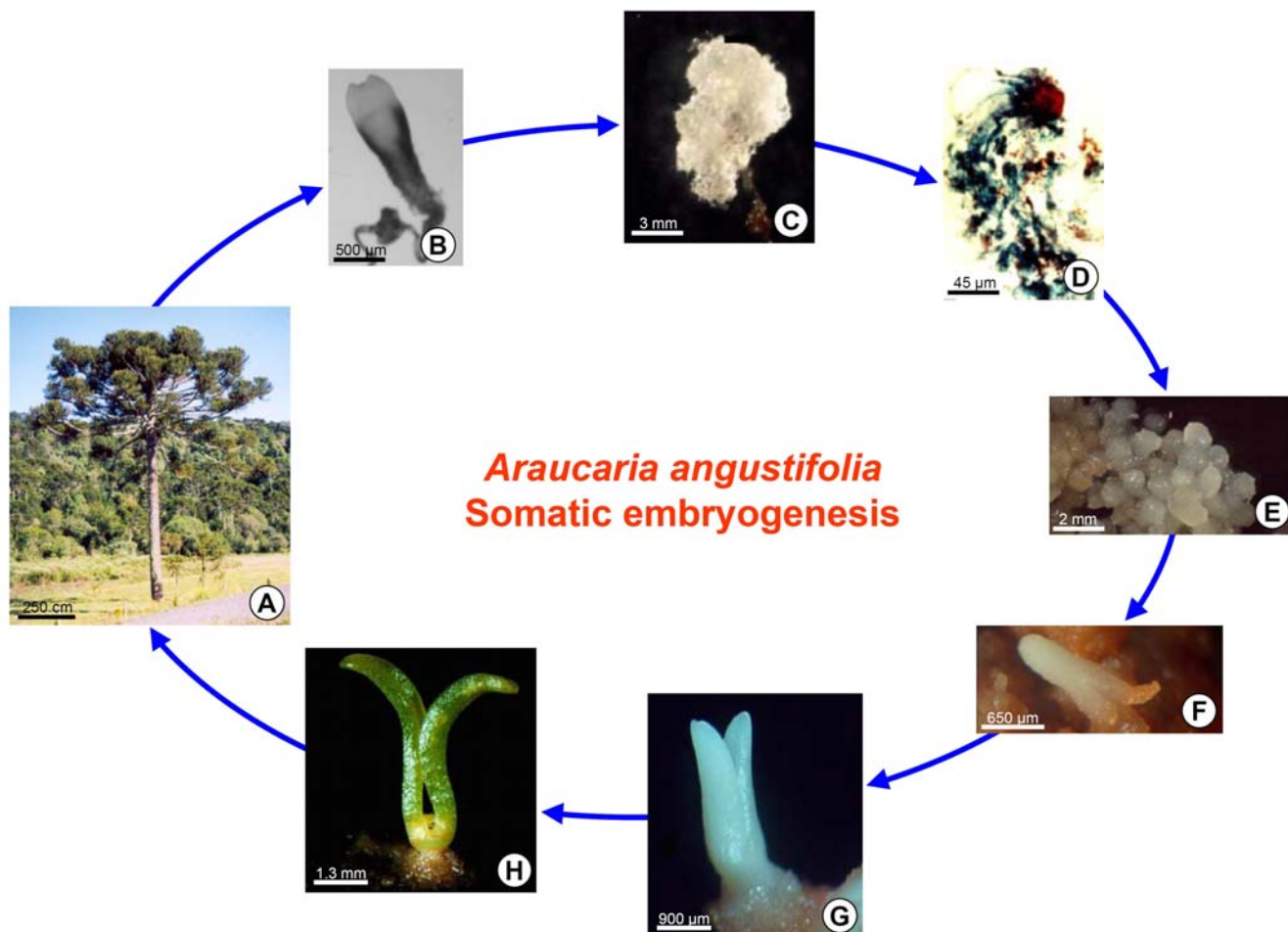
**Fig. 2 Somatic embryogenesis in *A. angustifolia*.** (**A**) Mother tree; (**B**) pre-cotyledonary zygotic embryo used as explant; (**C**) embryogenic culture induced in a basic medium (BM)(Gupta and Pullman 1991) supplemented with 5.0 μM 2,4-dichlorophenoxy acetic acid (2,4-D), 2.0 μM N6-benzylamino-purine (BA) and 2.0 μM kinetin (KIN); (**D**) somatic pro-embryo observed in the maintenance medium; (**E-H**) somatic embryo maturation in a BM medium supplemented with polyethyleneglycol (PEG 3350) (7%), maltose (9%) and abscisic acid (ABA) (150 μM).

bryogenic cultures permits stable maintenance of germ-plasm until clone-progeny have been evaluated under field conditions (Park *et al.* 1998).

Embryogenic cultures, obtained from culturing mature or immature zygotic embryos, constitute the most responsive tissue for plantlet regeneration in conifers. This tissue is the most adequate for studies on conifer transformation by using the biolistic bombardment approach, which thus enables the transfer of foreign DNA to virtually any cell types (Minocha and Minocha 1999).

## SOMATIC EMBRYOGENESIS IN *A. angustifolia*

The first study on somatic embryogenesis in *A. angustifolia* was undertaken by Guerra and Kemper (1992). The induction, establishment and multiplication of embryogenic cultures, as well as the effects of abscisic acid and osmotic agents on the maturation of these cultures, were reported by Astarita and Guerra (1998), Guerra *et al.* (2000) and Astarita and Guerra (2000). However, Guerra *et al.* (2000) did not manage to develop mature somatic embryos. It is important to consider that the *Araucariaceae* family, when compared to other conifer species, proves to be unique in early zygotic embryogenic features (Kaur and Bhatnagar 1983), through showing a high degree of specialization (Buchholz 1920). Furthermore, seeds of *A. angustifolia* are recalcitrant (Farrant *et al.* 1989), while most of conifers have orthodox seeds (Attree and Fowke 1993). These features should be taken into account in order to develop a specific somatic embryogenesis protocol for the mass clonal propagation of *A. angustifolia*. Studies with *A. angustifolia* somatic embryogenesis were held in the last decade (Asta-

rita and Guerra 1998, 2000; Guerra *et al.* 2000; dos Santos *et al.* 2002; Silveira *et al.* 2002; Steiner 2005; Steiner *et al.* 2005; Silveira *et al.* 2006; Steiner *et al.* 2007) (**Fig. 2**).

In the somatic embryogenesis of *A. angustifolia*, the developmental stage of zygotic embryos is a critical factor in the induction of embryogenesis. The highest induction rate of somatic embryogenesis in *A. angustifolia* is obtained by using pre-cotyledonary zygotic embryos (**Fig. 2B**) in basal medium (BM) (Gupta and Pullman 1991) supplemented with 5.0 μM 2,4-dichlorophenoxy acetic acid (2,4-D), 2.0 μM N6-benzylaminopurine (BA) and 2.0 μM kinetin (KIN) (dos Santos *et al.* 2002; Silveira *et al.* 2002; Steiner *et al.* 2005). Embryogenic induction in *A. angustifolia* is characterized by the proliferation of translucent to white, mucilaginous, embryogenic cultures (**Fig. 2C**) (dos Santos *et al.* 2002; Silveira *et al.* 2002; Steinet *et al.* 2005). Pre-cotyledonary embryos have been used as an explant source for many conifers species such as *Pinus taeda* (Becwar *et al.* 1991), *Pinus pinaster* (Lelu *et al.* 1999) and *Pinus strobus* (Klimaszewska and Smith 1997).

Somatic embryo maturation of *A. angustifolia* (**Fig. 2E-H**) is obtained in a BM culture medium supplemented with 7% polyethyleneglycol (PEG 3350), 9% maltose and 150 μM ABA (Steiner, N. unpublished data). In general, the maturation of conifer somatic embryos occurs in the presence of ABA and an osmotic agent (Attree and Fowke 1993). The presence of an osmotic agent permits the osmotic potential to be similar to the level observed during the early stages of zygotic embryo development. It is well documented that after complete embryonic formation, ABA concentrations rise while the embryo establishes dormancy and acquires storage reserves. ABA is involved in gene ex-

pression of storage proteins and LEA (late embryogenesis abundant) proteins that are synthesized in the seed after embryo differentiation (Bonetta and McCourt 2002; Wise and Tunnacliffe 2004). It was hypothesized that ABA acts through a standard signal transduction pathway, in which the binding of the hormone to a receptor elicits a transduction cascade (Bonetta and McCourt 2002).

## SCALE-UP MULTIPLICATION OF EMBRYOGENIC CULTURES

The use of suspension cultures is an important component in the scale-up and synchronization of conifer embryogenic cultures. Lulsdorf *et al.* (1992) showed that *Picea glauca-engelmannii* and *P. mariana* yielded 4000 and 6000 proembryos.l$^{-1}$ of suspension culture, respectively. Suspension cultures allow for a faster increase in culture fresh matter than does a culture on a semi-solid medium, thus reducing manipulation and the risk of contamination. In conifers, this system has been successfully employed for several species, among others *Picea abies* (von Arnorld *et al.* 1995), *Araucaria angustifolia* (Guerra *et al.* 2000; dos Santos *et al.* 2002; Steiner 2005; Silveira *et al.* 2006), *Picea glauca-engelmannii* and *Picea mariana* (Lulsdorf *et al.* 1992).

The growth dynamics of embryogenic suspension cultures is, in most cases, represented by a sigmoid curve showing a lag-phase, followed by exponential, linear and stationary phases. The lag-phase is considered as an adaptation period of cells to a new medium. If the subculture is started in the exponential or linear phases, the lag-phase can be limited to only a few days (1-3 days). In both the exponential and linear phases, the high rates of cell division result in growth of the suspension culture. The rate of cellular division is gradually reduced in the stationary phase (Szabados *et al.* 1993).

Suspension cultures also allow for the study of growth parameters such as sedimented-cell volume, packed-cell volume, mitotic index, fresh and dry weight (Guerra *et al.* 2000), cellular metabolism (Lulsdorf *et al.* 1992) and biochemical and physiological parameters (Silveira *et al.* 2004, 2006). Comprehension of these parameters is essential for elucidation of the mechanisms involved in somatic embryogenesis. However, available information on cellular metabolism of conifer embryogenic cultures is still limited (Nomura and Komamine 1995).

Silveira *et al.* (2002) observed that suspension cultures of *A. angustifolia*, growing in a liquid medium free from plant growth regulators (PGRs), resulted in an incremental rate of 5.6-fold in the initial sedimented-cell volume after 33 days of culture. Growth in a liquid medium supplemented with 5 μM of 2,4-D, 2 μM of BA and 2 μM of KIN showed a 7.8-fold increase in its initial volume after a culture period of 54 days (Silveira *et al.* 2002). This result possibly occurred as a response to the auxins and cytokinins present in the culture medium. Cytokinins act directly on the cell cycle where they are probably requested to regulate the synthesis of those proteins involved in the formation and operation of the mitotic spindle (Stals and Inzé 2001; Richard *et al.* 2002). Auxin acts in two ways during cellular growth: (i) it stimulates acidification of the cell wall, thus resulting in increased extensibility; and (ii) it induces the transcription of specific mRNAs that code for those proteins associated with cellular growth (Stals and Inzé 2001; Richard *et al.* 2002).

Both cytokinins and auxins are necessary for cell division in G1-S and G2-M transition in various cultured plant cells and *in planta* (Stals and Inzé 2001). In assays with tobacco leaf protoplasts, it was observed that the two hormones could synergistically influence transcription of the *cdc2a* kinase gene. It was concluded that hormone-induced *cdc2a* expression renders cells competent to divide, although additional factors are required to allow a cell to enter the division cycle (Coenen and Lomax 1997).

Embryogenic cell suspension growth in a culture medium free from PGRs, was probable mediated by biosynthesis of those plant hormones responsible for cell growth. Thus, this embryogenic culture could be considered habituated or autonomous. The acquisition of autonomy is based on the hypothesis that, when the cell cycle starts in the presence of plant growth regulators, the resulting cascade of transduction signals induces biosynthesis of plant hormones, thereby creating a positive feedback mechanism (George 1993).

Embryogenic cultures of conifers are generally induced and maintained in a culture medium containing both auxin and cytokinin (von Arnold *et al.* 1995). In *Pinus sylvestris*, *P. pinaster* (Lelu *et al.* 1999) and *A. angustifolia* (Guerra *et al.* 2000; dos Santos *et al.* 2002; Steiner *et al.* 2005), the induction and maintenance of embryogenic cultures were observed in the absence of exogenous PGRs. It has been suggested that the presence of auxins in the culture medium influences the formation of non-polar pro-embryos, thus affecting the further development of somatic embryos in the maturation phase (Korlach and Zoglauer 1995). Norway spruce embryo formation from PEMs was triggered by the withdrawal of PGRs. Furthermore, in this species a strong positive correlation has been shown between the frequency of somatic embryo formation and the percentage of cells with fragmented DNA, both of which stimulated by the withdrawal or partial depletion of PGRs in the culture medium (Filonova *et al.* 2000).

## BIOCHEMICAL ASPECTS OF EMBRYOGENIC CULTURES

In order to increase the efficiency of embryo development, an improved understanding of the biochemical and molecular events that occur during somatic embryo development is essential. Among biochemical events, polyamine (PA) and nitric oxide (NO) metabolisms, as well as amino acids, plant hormones and differential protein expression, seem to be involved in the regulatory mechanisms that play important roles in certain embryo-development processes.

Putrescine (Put), spermidine (Spd) and spermine (Spm) are the main PAs in plants, acting in cell division, somatic embryogenesis, root formation, floral initiation and development, fruit development, secondary metabolism, senescence and abiotic and biotic stress-response (Bais and Ravishankar 2002; Kuznetsov and Shevyakova 2007; Pang *et al.* 2007). PAs have been found to also regulate programmed cell death (PCD) and apoptosis (Kuehn and Phillips 2005). Some authors proposed that PAs and related compounds could both be considered as a type of growth regulator or secondary hormonal messenger, although they are found in plant cells at levels significantly higher than those of plant hormones (Bais and Ravishankar 2002).

Those small aliphatic amines that are positively charged in physiological pH have an essential role in cell proliferation and differentiation (Bouchereau *et al.* 1999). Put, Spd and Spm are the main PAs in plants, acting in essential stages of embryonic development. However, their action mechanisms have not yet been fully elucidated (Minocha *et al.* 1999). Silveira *et al.* (2006) observed that supplementation of PAs to the liquid culture medium resulted in a reduction of growth in embryogenic cultures. This inhibitory effect was more evident for 1 mM Spd and 1 mM Spm treatments (**Fig. 3**). Until now the role of exogenous PAs in the cellular growth of conifer embryogenic cultures, is not well-elucidated. According to Lainé *et al.* (1988), the use of Put (20 μM) and Spd (40 μM) stimulates cellular division of protoplast-derived cells of *Pinus oocarpa* and *Pinus patula*. During the growth of embryogenic suspension cultures of *Pinus taeda*, high levels of endogenous Put were associated with cell-growth reduction (Silveira *et al.* 2004). In *P. sylvestris*, Spd retarded cell proliferation and growth but enhanced somatic embryo maturation (Niemi *et al.* 2002).

Steiner *et al.* (2007) showed that the addition of PAs to the PGR-free BM medium, enhanced the growth of embryogenic cultures in *A. angustifolia*. PAs endogenous metabolism was significantly affected by exogenous PAs. PAs
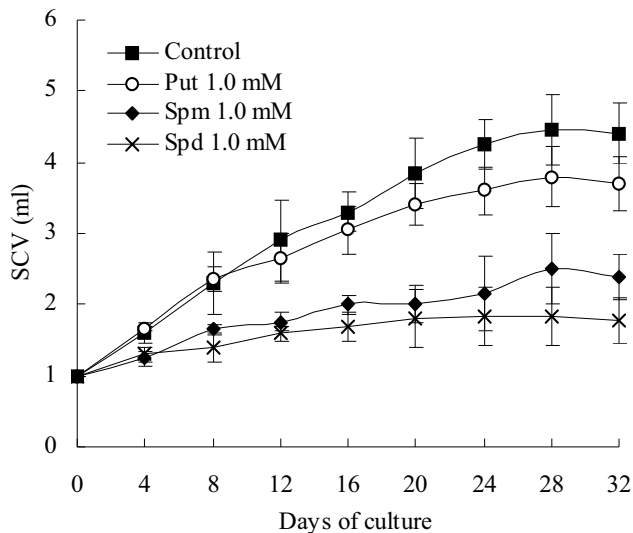
**Fig. 3 Growth of suspension cultures in *A. angustifolia*.** Cultures maintained in a culture medium supplemented with polyamines. SCV: sedimented cell volume, Put: putrescine, Spd: spermidine, Spm: spermine. (Reprinted from **Silveira *et al.*** (2006) *Plant Science* **171**, 91-98, ©2006, with kind permission from Elsevier).

supplemented to a PGR-free BM medium, mainly Put and Spm, increased the endogenous IAA and ABA levels, showing a direct relationship between PAs levels and ABA accumulation. These results direct new strategies to further improve *A. angustifolia* somatic embryogenesis, especially during the maturation phase, by culture medium manipulation using exogenous PAs.

NO, a highly diffusible gas-free radical, plays a key role as an intra- and inter-cellular messenger to induce various processes in plants, these including germination, induction of PCD and pathogen response, besides stomata and photosynthesis regulation (Yamasaki 2005). It is now a consensus among plant biologists that NO is an important gas molecule, comparable with the plant hormone ethylene (Yamasaki 2005). NO, in concert with auxin, plays an important role during the formation of embryogenic-type cells in *Medicago sativa* (Ötvös *et al.* 2005). The formation of embryogenic-type cells is enhanced by the use of the NO donor sodium nitroprusside at a low auxin concentration, although

this type of cell otherwise appears at higher auxin concentrations without SNP (Ötvös *et al.* 2005). These data indicate that NO, together with auxin, plays an important role during the embryogenesis process. Moreover, we recently found that PAs rapidly induce NO biosynthesis, this indicating PA-dependent NO biosynthesis as a potential link of NO and embryogenesis (Tun *et al.* 2006; Santa-Catarina *et al.* 2007).

Silveira *et al.* (2006) determined the effects of exogenous PAs supplemented to the liquid culture medium, on endogenous PA contents, NO release and the organization of PEMs, in embryogenic suspension cultures of *Araucaria angustifolia*. It was observed that PAs (Put, Spd and Spm) had a different effect on morphological evolution and NO biosynthesis in embryogenic cultures. Spd (1.0 mM) and Spm (1.0 mM) supplemented into the culture medium reduced cell-growth and allowed for morphogenetic evolution of aggregates from the PEM II to PEM III stages (**Fig. 4**). At PEM I, a cell aggregate is composed of a suspensor cell attached to an embryonal cell. PEM II presents similar cell aggregates that possess more than one suspensor cell. At the PEM III stage, an enlarged clump of embryonal and suspensor cells appears loose rather than compact, and with disturbed polarity.

Spd and Spm inhibitory effects in NO biosyntheses during suspension culture multiplication may be essential for PEM I and PEM II development, by reducing cell-growth and leading to the acquisition of PEM III structural characteristics. Additionally, Silveira *et al.* (2006) showed that Put addition to the culture medium increased NO release from the embryogenic culture, and Spd and Spm had an inhibitory effect on NO release when compared with the control treatment. These results suggest that Spd and Spm addition, and the consequent reduction in NO biosynthesis are correlated with the morphogenetic differentiation of PEMs, while Put is associated with PEM I and PEM II multiplication maybe by increasing endogenous NO in embryogenic cultures of *A. angustifolia* (**Fig. 5**). Whether NO metabolism is also associated with subsequent somatic embryo development remains a question to be answered. This opens a new perspective for work with species showing poor somatic embryogenetic responses, by the addition of NO donors to the culture medium, in an attempt to convert non-embryogenic cells to competent ones.

It was observed that embryonic cells of *A. angustifolia* accumulated more NO than suspensor cells suggesting that NO biosynthesis might be related to the maintenance of the
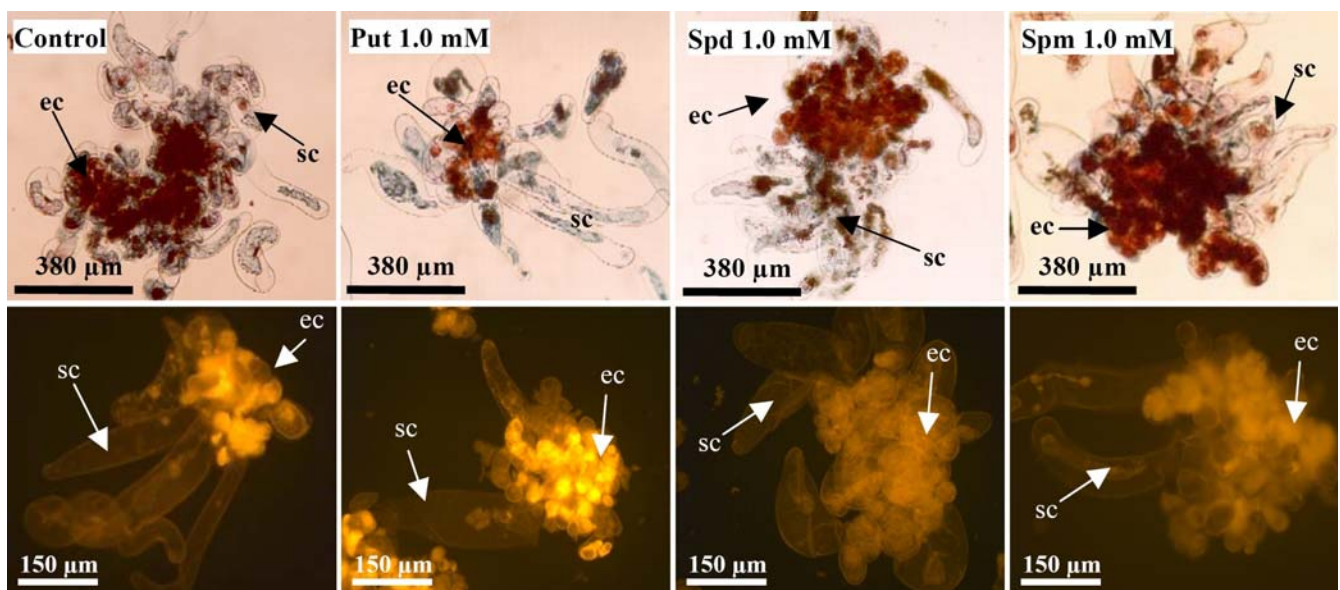


**Fig. 4 Morphological organization and NO-induced fluorescence in embryogenic masses from *A. angustifolia*.** Control treatment presenting aggregates in the pro-embryogenic mass (PEM) I stage. Put (1.0 mM) treatments presenting aggregates in the PEM II stage. Spd (1.0 mM) and Spm (1.0 mM) treatments presenting aggregates in the PEM III stage. ec: embryogenic cells; sc: suspensor cells. (Reprinted from **Silveira *et al.*** (2006) *Plant Science* **171**, 91-98, © 2006, with kind permission from Elsevier).
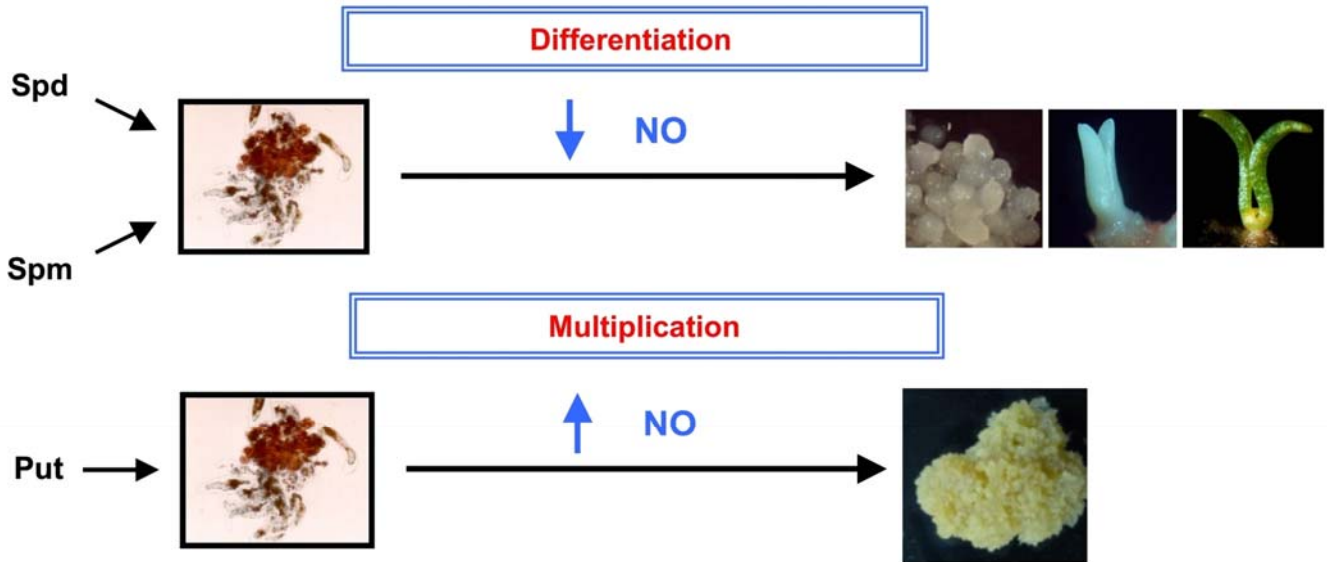
**Fig. 5 Possible correlations between polyamines and NO metabolisms on the growth and morphogenetic differentiation of embryogenic cultures in *A. angustifolia*.**

polarity (embryonic–suspensor cells) present in the PEM II stage (**Fig. 4**). This polarity could be a result of continuous cell division induced by NO, mainly in the embryonic region of PEM II aggregates. In *P. abies* embryogenic cultures in the PEM II stage high multiplication rates led to a pronounced increase in the size of the whole aggregate and to establishing a highly polarized morphology (Filonova *et al.* 2000). The key morphogenetic event in plant embryogenesis is bipolar pattern formation via establishment of the embryonic (apical) and suspensor (basal) regions (Bozhkov *et al.* 2005a). While embryonic cells will give rise to the plant body during a brief period, the suspensor functions as a conduit for growth factors to the developing embryo being subsequently eliminated by PCD (Filonova *et al.* 2000; Bozhkov *et al.* 2005a, 2005b).

The relationship between PA and NO biosynthesis has also been demonstrated in *Arabidopsis* seedlings (Tun *et al.* 2006) and *Ocotea catharinensis* (Santa-Catarina *et al.* 2007). PAs were described as mediators of embryogenesis (Gal-

ston *et al.* 1995; Shoeb *et al.* 2001; Bertoldi *et al.* 2004), and recently it was observed that in the presence of auxin, NO can stimulate the activation of cell division and embryogenic cell formation in leaf protoplasts derived from *Medicago sativa* (Ötvös *et al.* 2005).

Biosynthetic patterns of several macromolecules are similar in zygotic and somatic embryo development (Minocha *et al.* 1999), where an alternative approach could be to use analyses of zygotic tissues or growth environment to provide markers for somatic tissues (Pullman and Buchanan 2003).

Biochemical aspects during seed development have been disclosed for *A. angustifolia*. Some aspects, such as indole-3-acetic acid (Astarita *et al.* 2003a), ABA (Silveira *et al.* 2008), PA (Astarita *et al.* 2003b), amino acid (Astarita *et al.* 2003c) and protein (Silveira *et al.* 2008) contents, were studied during zygotic embryogenesis (**Fig. 6**). A better understanding of biochemical alterations during zygotic embryo development, besides providing basic information
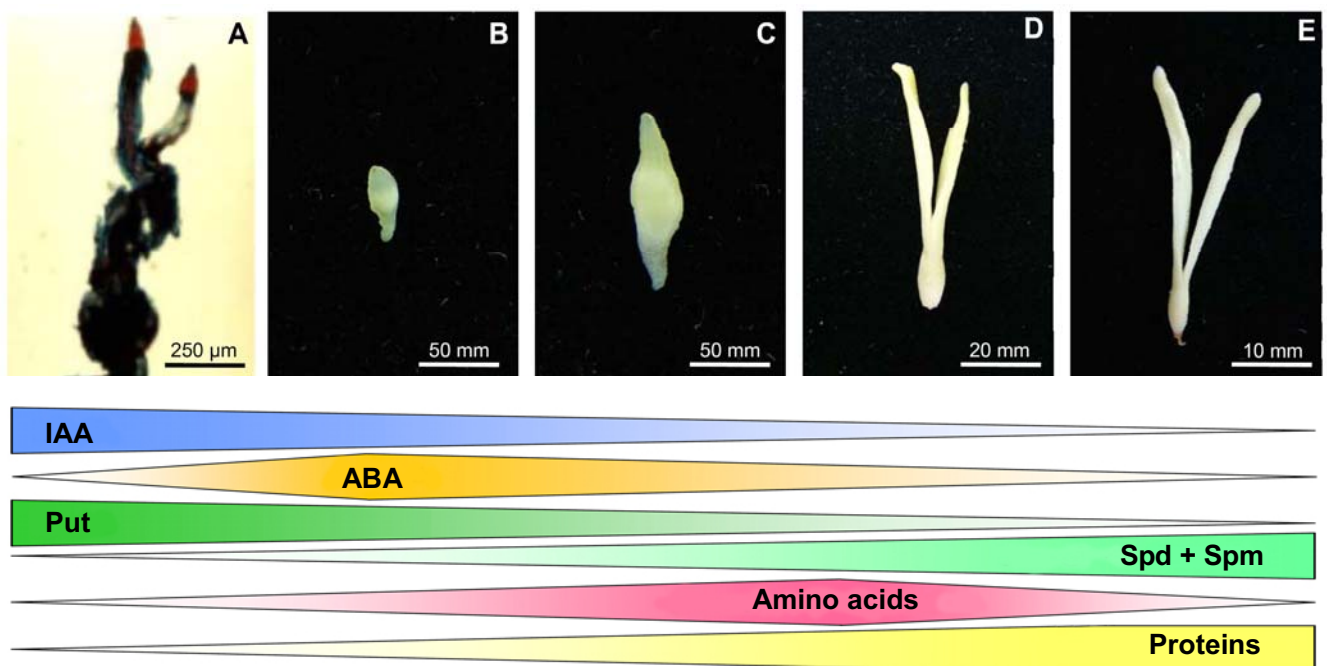


**Fig. 6 Biochemical changes during zygotic embryogenesis in *A. angustifolia*.** (**A**) pro-embryo stage; (**B**) torpedo stage; (**C**) pre-cotyledonary stage; (**D**) cotyledonary stage; (**E**) mature stage.
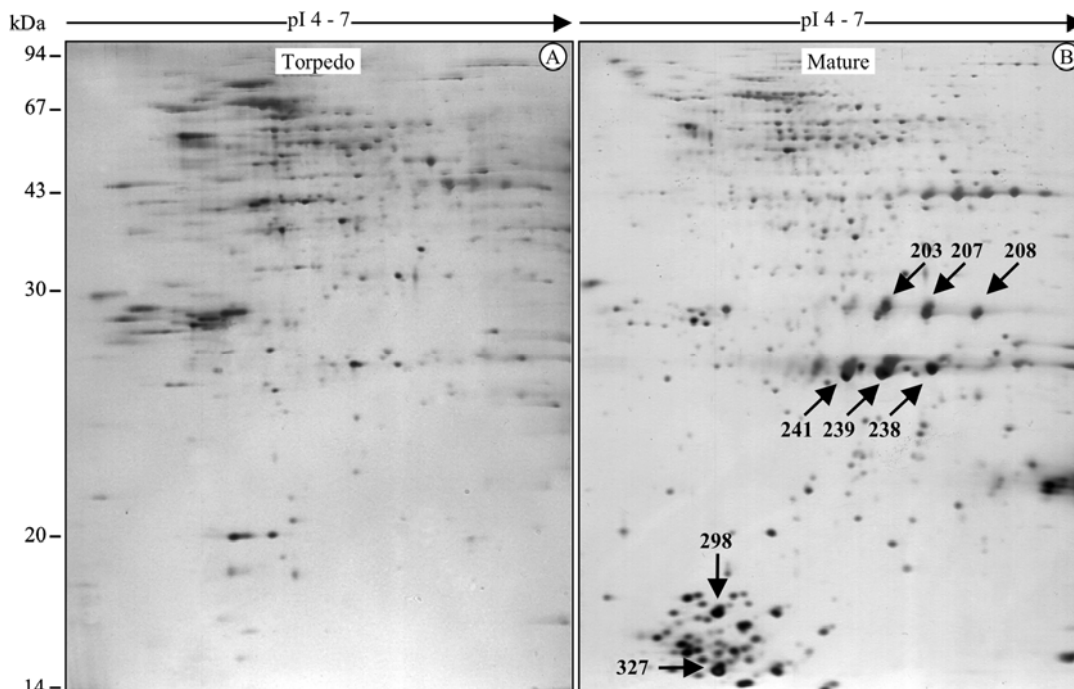
**Fig. 7 Proteome maps from *A. angustifolia* zygotic embryos. (A)** Torpedo stage; **(B)** Mature stage. Numbered spots correspond to polypeptides differentially expressed, selected for mass spectrometry and peptide mass fingerprinting (PMF) analyses. (Reprinted from **Silveira et al.** (2008) *Biologia Plantarum* **52**, 101-104, © 2008, with kind permission from Springer Science and Business Media).

on seed development, could be useful for further improvement in *A. angustifolia* somatic embryogenesis.

Silveira *et al.* (2008) describe a comparative proteome analysis and changes in endogenous abscisic acid (ABA), besides buffer soluble protein contents, during seed development in *A. angustifolia*. It was observed that ABA levels reach the maximum in the pre-cotyledonary stage, followed by a continuous decrease up to the mature stage. Protein contents in the embryonic axis increased until the cotyledonary stage, followed by stabilization in the mature seed. During zygotic embryogenesis, an increase in protein contents could be interpreted as a result of storage and LEA proteins synthesis of those that have a great affinity with water molecules, thus acting in seed dehydration protection (Wise and Tunnacliffe 2004). Storage proteins synthesis is one of the key processes of zygotic embryogenesis, providing compounds that will be used from the early stages of embryonic development until autotrophy after germination (Merkle *et al.* 1995).

To better understand protein variation during seed development, the protein pattern in two embryonic axis stages (torpedo and mature) were analyzed (Silveira *et al.* 2008). Silveira *et al.* (2008) showed that high molecular weight proteins, observed in the torpedo stage were of reduced expression in the mature stage, while new proteins of low molecular weight were specifically synthesized in mature embryos. In this work three polypeptides differentially expressed at mature stage were positively identified, by peptide mass fingerprinting, as AtSAC4, late embryogenesis abundant (LEA) and storage proteins, respectively (**Fig. 7**) (Silveira *et al.* 2008).

Seed development is a complex process, involving both physiological and biochemical factors, including ABA accumulation and synthesis of specific proteins such as LEAs and storage proteins. The cited works describe results related to the metabolism of developing seeds in *A. angustifolia*, and could be useful for optimizing somatic embryogenesis protocols for this species.

## CONCLUSIONS AND PROSPECTS

*A. angustifolia* is the only native coniferous species of economic importance in Brazil in which the natural reserves have been almost totally extirpated. The development of technologies for germplasm conservation and genetic improvement is a prerequisite for the establishment of reforestation and conservation programmes.

The application of somatic embryogenesis in conifer breeding allows for mass clone propagation of elite genotypes for use in the reforestation and conservation of endangered natural populations. Although our group has done much work on somatic embryogenesis in *A. angustifolia*, additional work is needed to support the conservation and mass propagation of this endangered conifer. The use of somatic embryogenesis for large-scale plant multiplication is very much dependent on the germination of somatic embryos. Seed-development studies have been performed to better understand the molecular and physiological basis of embryogenesis and system manipulation for *in vitro* multiplication via somatic embryogenesis.

The *A. angustifolia* genome has not yet been sequenced, this thus hindering protein identification from proteome maps. New strategies to improve *A. angustifolia* biotechnology results could be obtained using genomics and proteomics insights, by looking for markers associated with developmental stages and the optimization of somatic embryogenesis in this species.

## ACKNOWLEDGEMENTS

## REFERENCES

**Astarita LV, Guerra MP** (1998) Early somatic embryogenesis in *Araucaria angustifolia* – induction and maintenance of embryonal-suspensor mass culture. *Revista Brasileira de Fisiologia Vegetal* **10**, 113-118

**Astarita LV, Guerra MP** (2000) Conditioning of culture medium by suspension cells and formation of somatic proembryo in *Araucaria angustifolia* (Coniferae). *In Vitro Cellular and Developmental Biology – Plant* **36**, 194-200

**Astarita LV, Floh EIS, Handro W** (2003a) Changes in IAA, tryptophan and activity of soluble peroxidase associated with zygotic embryogenesis in *Araucaria angustifolia* (Brazilian pine). *Plant Growth Regulation* **39**, 113-118

**Astarita LV, Handro W, Floh EIS** (2003b) Changes in polyamines content associated with zygotic embryogenesis in the Brazilian pine, *Araucaria angustifolia* (Bert.) O Ktze. *Revista Brasileira de Botânica* **26**, 163-168

**Astarita LV, Floh EIS, Handro W** (2003c) Free amino acid, protein and water content changes associated with seed development in *Araucaria angustifolia*. *Biologia Plantarum* **47**, 53-59

**Attree SM, Pomeroy MK, Fowke, LC** (1992) Manipulation of conditions for the culture of somatic embryos of white spruce for improved triacylglycerol biosynthesis and desiccation tolerance. *Planta* **187**, 395-404

**Attree SM, Fowke LC** (1993) Embryogeny of gymnosperms: advances in synthetic seed technology of conifers. *Plant Cell, Tissue and Organ Culture* **35**, 1-35

**Bais HP, Ravishankar GA** (2002) Role of polyamines in the ontogeny of plants and their biotechnological applications. *Plant Cell, Tissue and Organ Culture* **69**, 1-34

**Becwar MR, Blush TD, Brown DW, Chesick EE** (1991) Multiple paternal genotypes in embryogenic tissue derived from individual immature loblolly-pine seeds. *Plant Cell, Tissue and Organ Culture* **26**, 37-44

**Bertoldi D, Tassoni A, Martinelli L, Bagni N** (2004) Polyamines and somatic embryogenesis in two *Vitis vinifera* cultivars. *Physiologia Plantarum* **120**, 657-666

**Bonetta D, McCourt P** (2002) Genetic analysis of ABA signal transduction pathways. *Trends in Plant Science* **3**, 231-235

**Bonga JM, Klimaszewska K, Lelu MA, von Aderkas P** (1995) Somatic embryogenesis in Larix. In: Jain S, Gupta P, Newton R (Eds) *Somatic Embryogenesis in Woody Plants* (Vol VI), Kluwer Academic Publishers, Dordrecht, Netherlands, pp 315-340

**Bouchereau A, Aziz A, Larher F, Martin-Tanguy J** (1999) Polyamines and environmental challenges: recent development. *Plant Science* **140**, 103-125

**Bozhkov PV, Filonova LH, Suarez MF** (2005a) Programmed cell death in plant embryogenesis. *Current Topics in Developmental Biology* **67**, 135-179

**Bozhkov PV, Suarez MF, Filonova LH, Daniel G, Zamyatnin AA, Rodriguez-Nieto S, Zhivotovsky B, Smertenko A** (2005b) Cysteine protease mcll-Pa executes programmed cell death during plant embryogenesis. *Proceedings of the National Academy of Sciences USA* **102**, 14463-14468

**Buchholz JT** (1920) Embryo development and polyembryony in relation to the phylogeny of conifers. *American Journal of Botany* **7**, 125-145

**Chalupa V** (1985) Somatic embryogenesis and plant regeneration from cultured immature and mature embryos of *Picea abies* (L.) Karst. *Communicationes Instituti Forestalis Cechosloveniae* **14**, 57-63

**Coenen C, Lomax TL** (1997) Auxin-cytokinin interactions in higher plants: old problems and new tools. *Trends in Plant Science* **2**, 351-356

**dos Santos ALW, Silveira V, Steiner N, Vidor M, Guerra MP** (2002) Somatic embryogenesis in Parana Pine (*Araucaria angustifolia* (Bert.) O. Kuntze). *Brazilian Archives of Biology and Technology* **45**, 97-106

**Dunstan DI, Dong JZ, Carrier DJ, Abrams SR** (1998) Events following ABA treatment of spruce somatic embryos. *In Vitro Cellular and Developmental Biology – Plant* **34**, 159-168

**Farrant JM, Pammenter NW, Berjak P** (1989) Germination associated events and the desiccation sensitivity of recalcitrant seeds: a study on three unrelated species. *Planta* **178**, 189-198

**Filonova L, Bozkov P, Von Arnold S** (2000) Developmental pathway of somatic embryogenesis in *Picea abies* as revealed by time-lapse tracking. *Journal of Experimental Botany* **51**, 343-249

**Galston AW, Kauw-Shawney R, Altabella T, Tiburcio AF** (1995) Plant polyamines in reproductive activity and response to abiotic stress. *Botanica Acta* **110**, 197-207

**George EF** (1993) *Plant Propagation by Tissue Culture - The Technology*, Exegetics, Edington, UK, 575 pp

**Guerra MP, Kemper E** (1992) Tecnologias Futuras: Aplicação da poliembriogênese somática para a propagação massal de plantas elite de *Araucaria angustifolia* (Bert) O Ktze. *Anais do Congresso Nacional Sobre Essências*, São Paulo, Brazil, pp 1233-1236

**Guerra MP, Silveira V, Santos ALW, Astarita LV, Nodari RO** (2000) Somatic embryogenesis in *Araucaria angustifolia* (Bert) O. Ktze. In: Jain S, Gupta P, Newton R (Eds) *Somatic Embryogenesis in Woody Plants* (Vol VI), Kluwer Academic Publishers, Dordrecht, Netherlands, pp 457-478

**Guerra MP, Silveira V, Reis MS, Schneider L** (2002) Exploração, manejo e conservação da araucária (*Araucaria angustifolia*). In: Simões LL, Lino CF (Eds) *Sustentável Mata Atlântica: A Exploração de Seus Recursos Florestais*, Editora SENAC, São Paulo, Brazil, pp 85-101

**Gupta PK, Pullman GS** (1991) Method for reproducing coniferous plants by somatic embryogenesis using abscisic acid and osmotic potential variation. US Patent 5,036,007

**Gutmann M, von Aderkas P, Label P, Lelu MA** (1996) Effects of abscisic acid on somatic embryo maturation of hybrid larch. *Journal of Experimental Botany* **47**, 1905-1917

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

**Högberg KA, Ekberg I, Norell L, von Arnold S** (1998) Integration of somatic embryogenesis in a tree breeding programe: a case study with *Picea abies*. *Canadian Journal of Forest Research* **28**, 1536-1545

**Kaur D, Bhatnagar SP** (1983) Studies in the family *Araucariaceae*. *Beiträge zur Biologie der Pflanzen* **58**, 369-381

**Klimaszewska K, Smith DR** (1997) Maturation of somatic embryos of *Pinus strobus* is promoted by a high concentration of gellan gum. *Physiologia Plantarum* **100**, 949-957

**Korlach J, Zoglauer K** (1995) Developmental patterns during direct somatic embryogenesis in protoplasts culture of european larch (*Larix decidua* Mill.). *Plant Cell Reports* **15**, 242-247

**Kuehn GD, Phillips GC** (2005) Role of polyamines in apoptosis and other recent advances in plant polyamines. *Critical Reviews in Plant Science* **24**, 123-130

**Kuznetsov VV, Shevyakova NI** (2007) Polyamines and stress tolerance of plants. *Plant Stress* **1**, 50-71

**Lainé E, Davide H, David A** (1988) Callus formation from cotyledon protoplasts of *Pinus oocarpa* and *Pinus patula*. *Physiologia Plantarum* **72**, 374-378

**Lelu MA, Bastien C, Drugeault A, Gouez ML, Klimaszewska K** (1999) Somatic embryogenesis and plantlet development in *Pinus sylvestris* and *Pinus pinaster* on medium with and without growth regulators. *Physiologia Plantarum* **105**, 719-728

**Litz RE, Chavez VM, Moon PA** (1997) Induction of embryogenic cultures from mature-phase tropical and subtropical trees and control of somatic embryo maturation and germination. In: Bruns S, Mantell S, Traegaardh C, Viana AM (Eds) *Proceedings of Recent Advances in Biotechnology for Tree Conservation and Management*, IFS, Stockholm, Sweden, pp 232-243

**Lulsdorf MM, Tautorus TE, Kikcio SI, Dunstan DI** (1992) Growth parameters of embryogenic suspension culture of Interior spruce (*Picea glauca – engelmanni* complex) and black spruce (*Picea mariana* Mill.). *Plant Science* **82**, 227-234

**Malabadi RB, Nataraja K** (2007) Genetic transformation of conifers: applications in and impacts on commercial forestry. *Transgenic Plant Journal* **1**, 289-313

**Merkle SA, Parrot WA, Flinn BS** (1995) Morphogenic aspects of somatic embryogenesis. In: Thorpe TA (Ed) *In Vitro Embryogenesis in Plants*, Kluwer Academic Publishers, Dordrecht, Netherlands, pp 155-203

**Merkle SA, Dean JFD** (2000) Forest biotechnology. *Current Opinion in Biotechnology* **11**, 298-302

**Merkle SA, Nairn CJ** (2005) Hardwood tree biotechnology. *In Vitro Cellular and Developmental Biology – Plant* **41**, 602-619

**Minocha SC, Minocha R** (1999) Genetic Transformation in Conifers. In: Jain S, Gupta P, Newton R (Eds) *Somatic Embryogenesis in Woody Plants* (Vol VI), Kluwer Academic Publishers, Dordrecht, Netherlands, pp 291-312

**Minocha R, Smith DR, Reeves C, Steele KD, Minocha SC** (1999) Polyamines levels during the development of zygotic and somatic embryos of *Pinus radiata*. *Physiologia Plantarum* **105**, 155-164

**Nagmani R, Bonga JM** (1985) Embryogenesis in subcultured callus of *Larix decidua*. *Canadian Journal of Forest Research* **15**, 1088-1091

**Nehra NS, Becwar MR, Rottmann WH, Pearson L, Chowdhury K, Chang S, Wilde HD, Kodrzycki, Zhang C, Gause KC, Parks DW, Hinchee MA** (2005) Forest biotechnoloy: innovative methods, emerging opportunities. *In Vitro Cellular and Developmental Biology – Plant* **41**, 701-717

**Niemi K, Sarjala T, Chen X, Häggman H** (2002) Spermidine and methylglyoxal bis(guanylhydrazone) affect maturation and endogenous polyamine content of Scots pine embryogenic cultures. *Journal of Plant Physiology* **159**, 1155-1158

**Nomura K, Komamine A** (1995) Physiological and biochemical aspects of somatic embryogenesis. In: Thorpe TA (Ed) *In Vitro Embryogenesis in Plants*, Kluwer Academic Publishers, Dordrecht, Netherlands, pp 249-266

**Ötvös K, Pasternak TP, Miskolczi P, Domoki M, Dorjgotov D, Szucs A, Bottka S, Dudits D, Feher A** (2005) Nitric oxide is required for, and promotes auxin-mediated activation of, cell division and embryogenic cell formation but does not influence cell cycle progression in alfalfa cell cultures. *The Plant Journal* **43**, 849-860

**Pang X-M, Zhang Z-Y, Wen X-P, Ban Y, Moriguchi T** (2007) Polyamines, all-purpose players in response to environment stresses in plants. *Plant Stress* **1**, 173-188

**Park YS, Barret DJ, Bonga JM** (1998) Application of somatic embryogenesis in high-value clonal forestry: deployment, genetic control, and stability of cryopreserved clones. *In Vitro Cellular and Development Biology – Plant* **34**, 231-239

**Pullman GS, Buchanan M** (2003) Loblolly pine (*Pinus taeda* L.): stage-specific elemental analyses of zygotic embryo and female gametophyte tissue. *Plant Science* **164**, 943-954

**Radojevic L, Älvarez C, Fraga MF, Rodríguez R** (1999) Somatic embryogenic tissue establishment from mature *Pinus nigra* Arn. ssp. *Salzmannii* embryos. *In Vitro Cellular and Developmental Biology – Plant* **35**, 206-209

**Richard C, Lescot M, Inze D,** (2002) Effect of auxin, cytokinin, and sucrose on cell cycle gene expression in *Arabidopsis thaliana* cell suspension cultures. *Plant Cell, Tissue and Organ Culture* **69**, 167-176

**Santa-Catarina C, Silveira V, Scherer GFE, Floh EIS** (2007) Polyamine and nitric oxide levels relate with morphogenetic evolution in somatic embryogenesis of *Ocotea catharinensis*. *Plant Cell, Tissue and Organ Culture* **90**, 93-101

**Shoeb F, Yadav JS, Bajaj S, Rajam MV** (2001) Polyamines as biomarkers for plant regeneration capacity: improvement of regeneration by modulation of polyamine metabolism in different genotypes of *indica* rice. *Plant Science* **160**, 1229-1235

**Silveira V, Steiner N, Santos ALW, Nodari RO, Guerra MP** (2002) Biotechnology tolls in *Araucaria angustifolia* conservation and improvement: inductive factors affecting somatic embryogenesis. *Crop Breeding and Applied Biotechnology* **2**, 463-470

**Silveira V, Floh EIS, Handro W, Guerra MP** (2004) Effect of plant growth

regulators on the cellular growth and levels of intracellular protein, starch and polyamines in embryogenic suspension cultures of *Pinus taeda*. *Plant Cell, Tissue and Organ Culture* **76**, 53-60

**Silveira V, Santa-Catarina C, Tun NN, Scherer GFE, Handro W, Guerra MP, Floh EIS** (2006) Polyamine effects on the endogenous polyamine contents, nitric oxide release, growth and differentiation of embryogenic suspension cultures of *Araucaria angustifolia* (Bert.) O. Ktze. *Plant Science* **171**, 91-98

**Silveira V, Santa-Catarina C, Balbuena TS, Moraes FMS, Ricart CAO, Sousa MV, Guerra MP, Handro W, Floh EIS** (2008) Endogenous abscisic acid and protein contents during seed development of *Araucaria angustifolia*. *Biologia Plantarum* **52**, 101-104

**Stals H, Inzé D** (2001) When plant cells decide to divide. *Trends in Plant Science* 6, 359-364

**Stasolla C, Yeung EC** (2003) Recent advances in conifer somatic embryogenesis: improving somatic embryo quality. *Plant Cell Tissue and Organ Culture* **74**, 15-35

**Stasolla C, Kong L, Yeung EC, Thorpe TA** (2002) Maturation of somatic embryos in conifers: morphogenesis, physiology, biochemistry and molecular biology. *In Vitro Cellular and Developmental Biology – Plant* **38**, 93-105

**Steiner N** (2005) Parâmetros fisiológicos e bioquímicos durante a embriogênese zigótica e somática de *Araucaria angustifolia* (Bert.) O. Kuntze. MSc Thesis, Universidade Federal de Santa Catarina, Florianópolis, 101 pp

**Steiner N, Vieira FN, Maldonado S, Guerra MP** (2005) Carbon source affects morphogenesis and histodifferentiation of *A. angustifolia* embryogenic cultures. *Brazilian Archives of Biology and Technology* **48**, 895-903

**Steiner N, Santa-Catarina C, Silveira V, Floh EIS, Guerra MP** (2007) Polyamine affects on growth and endogenous hormones levels in *Araucaria angustifolia* embryogenic cultures. *Plant Cell, Tissue and Organ Culture* **89**, 55-62

**Szabados L, Mroginski LA, Roca WM** (1993) Suspensiones celulares: descripción, manipulación y aplicaciones. In: Roca WM, Mroginski LA (Eds) *Cultivo de Tejidos en la Agricultura: Fundamentos y Aplicaciones*, CIAT, Cali, Colombia, pp 174-210

**Tautorus TE, Fowke LC, Dunstan DI** (1991) Somatic embryogenesis in conifers. *Canadian Journal of Botany* **69**, 1873-1899

**Tun NN, Santa-Catarina C, Begum T, Silveira V, Handro W, Floh EIS, Scherer GFE** (2006) Polyamines induce rapid biosynthesis of nitric oxide (NO) in *Arabidopsis thaliana* seedlings. *Plant and Cell Physiology* **47**, 346-354

**von Arnold S, Egertsdotter U, Ekberg I, Gupta P, Mo H, Nörgaard J** (1995) Somatic Embryogenesis in Norway spruce (*Picea abies*). In Jain S, Gupta P, Newton R (Eds) *Somatic Embryogenesis in Woody Plants* (Vol VI), Kluwer Academic Publishers, Dordrecht, Netherlands, pp 17-36

**Wise JM, Tunnacliffe A** (2004) POPP the question: what do LEA proteins do? *Trends Plant Science* **9**, 13-17

**Yamasaki H** (2005) The NO world for plants: achieving balance in an open system. *Plant, Cell and Environment* **28**, 78-84