

# Polyamine effects on the endogenous polyamine contents, nitric oxide release, growth and differentiation of embryogenic suspension cultures of *Araucaria angustifolia* (Bert.) O. Ktze.

Vanildo Silveira<sup>a,\*</sup>, Claudete Santa-Catarina<sup>a</sup>, Ni Ni Tun<sup>b</sup>, Günther F.E. Scherer<sup>b</sup>,  
Walter Handro<sup>a</sup>, Miguel P. Guerra<sup>c</sup>, Eny I.S. Floh<sup>a</sup>

<sup>a</sup> Plant Cell Biology Laboratory, Department of Botany, IB-University of São Paulo, CP 11461, 05422-970 São Paulo, Brazil

<sup>b</sup> Universität Hannover, Institut für Zierpflanzenbau, AG Spezielle Ertragsphysiologie, Herrenhäuser Str. 2, D-30419 Hannover, Germany

<sup>c</sup> Plant Developmental Physiology and Genetics Laboratory, Department of Plant Science, Federal University of Santa Catarina, Florianópolis, Brazil

Received 14 January 2006; received in revised form 23 February 2006; accepted 24 February 2006

Available online 22 March 2006

## Abstract

In the present work we determined the effects of exogenous polyamines (PAs), supplemented to the culture medium, on the endogenous PA contents, nitric oxide (NO) release, culture growth and organization of proembryogenic masses (PEMs), in embryogenic suspension cultures of *Araucaria angustifolia*. We observed that PAs (Putrescine (Put), spermidine (Spd) and spermine (Spm)) had a different effect on growth, morphological evolution and NO biosynthesis in embryogenic cultures. Spd (1.0 mM) and Spm (1.0 mM) supplemented into the culture medium reduced cellular growth and allowed a morphogenetic evolution of aggregates from PEM II to PEM III stages. Put increased NO release from the embryogenic culture, and Spd and Spm had an inhibitory effect on NO release when compared with the control treatment. In the control and Put (1.0 mM) treatments, it was observed that embryonic cells of *A. angustifolia* accumulated more NO than the suspensor cells, suggesting that NO biosynthesis might be related to the maintenance of the polarity (embryonic–suspensor cells) present at the PEM II stage.

© 2006 Published by Elsevier Ireland Ltd.

**Keywords:** Conifer; Nitric oxide; Somatic embryogenesis; Polyamine; Embryo polarity

## 1. Introduction

*Araucaria angustifolia* (Bert.) O. Ktze. is an endangered native conifer from Southern Brazil. Efforts have been made to use tissue cultures as an alternative in reforestation programs, but results are scarce. Despite the attempts to develop a protocol for inducing somatic embryogenesis in *A. angustifolia*, only somatic embryos in early developmental stages were obtained [1–3].

Micropropagation through somatic embryogenesis is an alternative pathway for the propagation of plants with high rates of multiplication [4]. Suspension cultures undergo an alter-

native system for embryogenic cultures multiplication that allows a faster increase in fresh matter than in a culture on a gelled medium. Suspension cultures also permit the study of growth parameters such as sedimented cell volume (SCV) [1,5] and cellular metabolism [6], besides biochemical and physiological parameters [5,7], during somatic embryogenesis development. Embryogenic suspension cultures of *A. angustifolia* have also been successfully employed to study the effect of plant growth regulators and carbon source in dynamic growth, extracellular protein release and glucose and fructose uptake [7,8]. The growth dynamics of embryogenic suspension cultures of *A. angustifolia* corresponds to a sigmoid curve showing a lag phase, followed by exponential, linear and stationary phases [8].

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 [9]. Among biochemical events, polyamine (PA) and nitric oxide (NO) metabolisms seem to be involved in the

**Abbreviations:** FM, fresh matter; HPLC, high-performance liquid chromatography; NO, nitric oxide; PA, polyamine; PEM, proembryogenic mass; Put, putrescine; SCV, sedimented cell volume; SNP, sodium nitroprusside; Spd, spermidine; Spm, spermine

\* Corresponding author. Tel.: +55 11 3091 7556; fax: +55 11 3091 7547.

E-mail address: [s\\_vanildo@yahoo.com](mailto:s_vanildo@yahoo.com) (V. Silveira).

regulatory mechanisms that play important roles in certain plant development processes [10–12].

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, abiotic stress response and biotic stress response (see review in ref. [10]). More recently, PAs have been found also to regulate the cell death program, apoptosis [13]. Some authors proposed that PAs and related compounds could 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 [10].

Nitric oxide, a highly diffusible gaseous free radical, plays a key role as an intra- and inter-cellular messenger to induce various processes in plants, these including germination, induction of cell death and pathogen response, besides stomata and photosynthesis regulation (see review in ref. [14]). It is now a consensus among plant biologists that NO is an important gas molecule, comparable with the plant hormone ethylene [14]. NO in concert with auxin plays an important role during the formation of embryogenic-type cells of *Medicago sativa* [12]. The formation of embryogenic-type cells is enhanced by the use of the NO donor sodium nitroprusside (SNP) at a low auxin concentration, although this type of cell otherwise appears at higher auxin concentrations without SNP [12]. These data indicate that NO in concert with auxin plays an important role during the embryogenesis process. Moreover, we recently found that PAs rapidly induce NO biosynthesis indicating PA-dependent NO biosynthesis as a potential link of NO and embryogenesis [15].

There are few reports comparing the NO and PAs metabolism between gymnosperm and angiosperm species. Pedrosa et al. [35] suggested that in both *Kalanchoe daigremontiana* (angiosperm) and *Taxus brevifolia* (gymnosperm) the NO is produced by a putative nitric oxide-synthase (NOS), common between these species. According to Fraga et al. [36] the morphogenetic competence in *Pinus radiata* (gymnosperm) is associated with decrease in the free PA content while in *Prunus persica* (angiosperm) this characteristic is related to the high levels of free PA. However, Franceschetti et al. [37] observed that mRNA sequences of *S*-adenosyl-*L*-methionine decarboxylase (AdoMetDC) are highly conserved between angiosperm and gymnosperm species. AdoMetDC is one of the key regulatory enzymes in the biosynthesis of PAs.

In the present work we determined the effects of exogenous PAs, supplemented to the culture medium, on the endogenous PAs contents, NO release, culture growth and organization of proembryogenic masses (PEMs) in the embryogenic suspension cultures of *A. angustifolia*. A correlation between NO release and polarity of forming cells in embryogenesis is indicated.

## 2. Materials and methods

### 2.1. Culture initiation

Embryogenic culture of *A. angustifolia* was initiated from immature seeds according to Santos et al. [2]. Seeds were

surface-sterilized in 70% ethanol (2 min), and 2% sodium hypochlorite (10 min), followed by rinsing three times in sterile water. Pre-cotyledonary zygotic embryos were excised from the seeds and inoculated on Petri dishes containing 20 ml BM medium [16] supplemented with plant growth regulators (2.0  $\mu$ M 2,4-dichlorophenoxyacetic acid; 0.5  $\mu$ M 6-benzylaminopurine and 0.5  $\mu$ M kinetin), 0.5 g/l casein enzymatic hydrolysate (from bovine milk, Sigma–Aldrich), 0.1 g/l myo-inositol, 1.0 g/l *L*-glutamine, 30.0 g/l sucrose and 2.0 g/l phytigel<sup>®</sup> (henceforth called as *basic medium*). The pH of the culture medium was adjusted to 5.7 before phytigel<sup>®</sup> was added. The culture medium was sterilized by autoclaving at 121 °C for 15 min. *L*-Glutamine was filter-sterilized and added to the culture medium after autoclaving. The cultures were incubated in the dark at 25  $\pm$  2 °C. Embryogenic cultures with a translucent to white and mucilaginous aspect, and with both embryonal and suspensor cell types, were subcultured every 21 days in the basic medium and maintained under the same culture conditions. After three subcultures, one part of these embryogenic cultures was maintained in the gelled (2.0 g/l phytigel) basic medium whereas 1.0 g fresh mass of each embryogenic culture was used to establish the suspension culture in Erlenmeyer flasks containing 40 ml of liquid basic medium. Suspension cultures were subcultured every 21 days by adding 5 ml of the old suspension culture in 35 ml of fresh liquid medium. Suspension cultures were kept on an orbital shaker (50 rpm) in the dark at 25  $\pm$  2 °C.

### 2.2. Experimental conditions

Putrescine, Spd and Spm treatments were carried out by addition of filter-sterilized solutions (pH 5.7) to give a final concentration of 1 mM. Basic medium was used as control treatment.

The experiments were performed with four-subcultured embryogenic suspension cultures, on the 15th day of growth. The suspensions were filtered (150  $\mu$ m), and the retained material was used to initiate the experiments. This was undertaken by inoculation of 0.5 g fresh mass (FM) into 40 ml of liquid medium using 12 normal Erlenmeyer flasks (125 ml) and 3 ones adapted for growth measurements [17]. The flasks were maintained on an orbital shaker (50 rpm) in the dark at 25  $\pm$  2 °C.

### 2.3. Cellular growth in liquid mediums

Cellular growth was measured via SCV by cell sedimentation in the side arm of the adapted flasks, and evaluated every four days until the 32nd day of culture. Data from each treatment was proportionally adjusted to an initial SCV of 1 ml. Each treatment was performed in triplicate.

### 2.4. PAs determination

Suspension cultures, maintained during 2, 12, 22 and 32 days in culture, were filtered, washed three times with Milli-Q<sup>®</sup> water and stored at –80 °C. The different periods correspond,

respectively, to the lag, exponential, linear and stationary growth phases, previously identified for the control treatment. Three suspension culture flasks of each treatment were used to obtain the samples in each collect point.

PAs were determined from three samples of 200 mg FM each. Samples were ground in 1.6 ml of a 5% (v/v) perchloric acid solution, and free PAs were extracted, dansylated and identified by reverse phase HPLC according to Silveira et al. [5]. The PA content was determined using a fluorescence detector at 340 nm (excitation) and 510 nm (emission). Peak areas and retention times were measured by comparison with standard PAs: Put, Spd and Spm.

### 2.5. Embryogenic cell masses organization

To evaluate the organizational stage of proembryogenic masses, suspensions at the 22nd day of growth were submitted to a double-staining procedure with acetocarmine (2%) and Evan's blue (0.1%), as described by Gupta and Durzan [18]. The stained embryogenic cultures were observed and identified by light microscope.

### 2.6. NO determinations

Samples (100 mg FM) of embryogenic cultures at 12th day of growth in the basic medium were used in short experiments for NO release quantification and NO visualization by fluorescence microscopy.

#### 2.6.1. NO release quantification

NO release to the medium was determined by binding to the cell-impermeant DAR-4M (Diaminorhodamine 4 M; Alexis) in a fluorometric assay [19,20]. Samples of embryogenic cultures (100 mg FM each) were transferred into 2 ml of water solution containing the PAs: Put, Spd and Spm, at different concentrations (0.5, 1.0 and 2.0 mM), and DAR-4M (2.5  $\mu$ M). Prior and after the experiment, viability was checked by the Trypan blue method [38]. Samples were incubated in the dark, on an orbital shaker (125 rpm). After 2 h of incubation, the supernatant (1 ml) without cells was taken, whereupon relative fluorescence was measured by emission at 560 nm and excitation at 575 nm in a LS-5 Luminescence spectrometer (Perkin-Elmer). All experiments were repeated at least twice, with similar results, albeit the data presented are from a single representative experiment. Samples incubated in the water solution with DAR-4M (2.5  $\mu$ M) were considered as the positive control, and those in the incubation solution without DAR-4M as the negative control.

#### 2.6.2. Intracellular NO observation by microscopy

NO was visualized under the fluorescence microscope by binding to the cell-permeable derivative DAR-4M AM (Alexis). Samples of embryogenic cultures (100 mg FM each) were transferred into 2 ml of water solution containing the PAs: Put, Spd and Spm (1.0 mM), and DAR-4M (5  $\mu$ M). Prior and after the experiment, viability was also checked by the Trypan blue method [38], thus indicating cells which were viable.

Samples were incubated in the dark, on an orbital shaker (125 rpm). After 2 h of incubation, plates with embryogenic cultures were prepared and observed under a fluorescence Axioskop2 Mot Plus microscope (Zeiss), Filter set no. 20 from Zeiss (excitation: BP 546/12; beam splitter: FT 560; emission: BP 575–640). Digital photos, which are compared, were taken at exactly the same camera settings of a digital camera and were not processed further. All experiments were repeated at least twice, with similar results, the photos presented being of a single representative experiment.

### 2.7. Data analysis

Experiments were performed in triplicate, whereupon the data presented were analyzed using means and standard errors.

## 3. Results and discussion

The first effect observed related to the supplementation of PAs to the culture medium was the reduction of growth in embryogenic cultures. This inhibitory effect was more evident for 1 mM Spd and 1 mM Spm treatments (Fig. 1). In our work we used the same combinations applied by Laukkanen and Sarjala [21]. These authors observed that callus of *Pinus sylvestris* cultured on the culture medium supplemented with 1 mM Put, Spd or Spm showed poor growth capacity [21].

Until now the role of exogenous PAs in the cellular growth of conifer embryogenic cultures is not well elucidated. According to Lainé et al. [22], the use of Put (20  $\mu$ M) and Spd (40  $\mu$ M) 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 [5]. In *P. sylvestris*, Spd retarded cell proliferation and growth but enhanced somatic embryo maturation [23].

When PAs were incorporated into the culture medium, PA levels in the embryogenic suspension cultures increased (Fig. 2). Free Put was the most abundant PA in the control

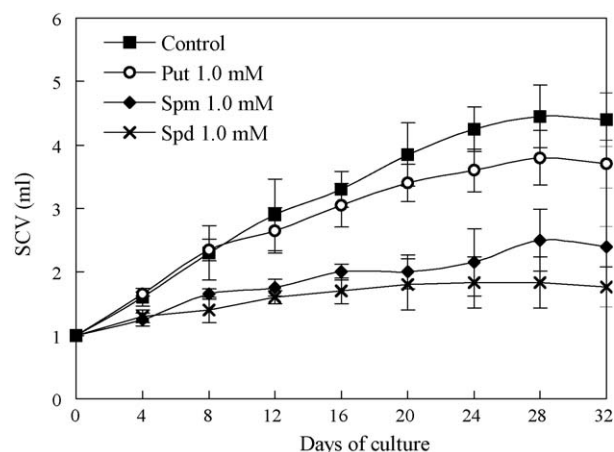


Fig. 1. Growth of suspension cultures of *A. angustifolia* maintained in culture medium supplemented with PAs. Data are presented as mean  $\pm$  standard error ( $n = 3$ ).

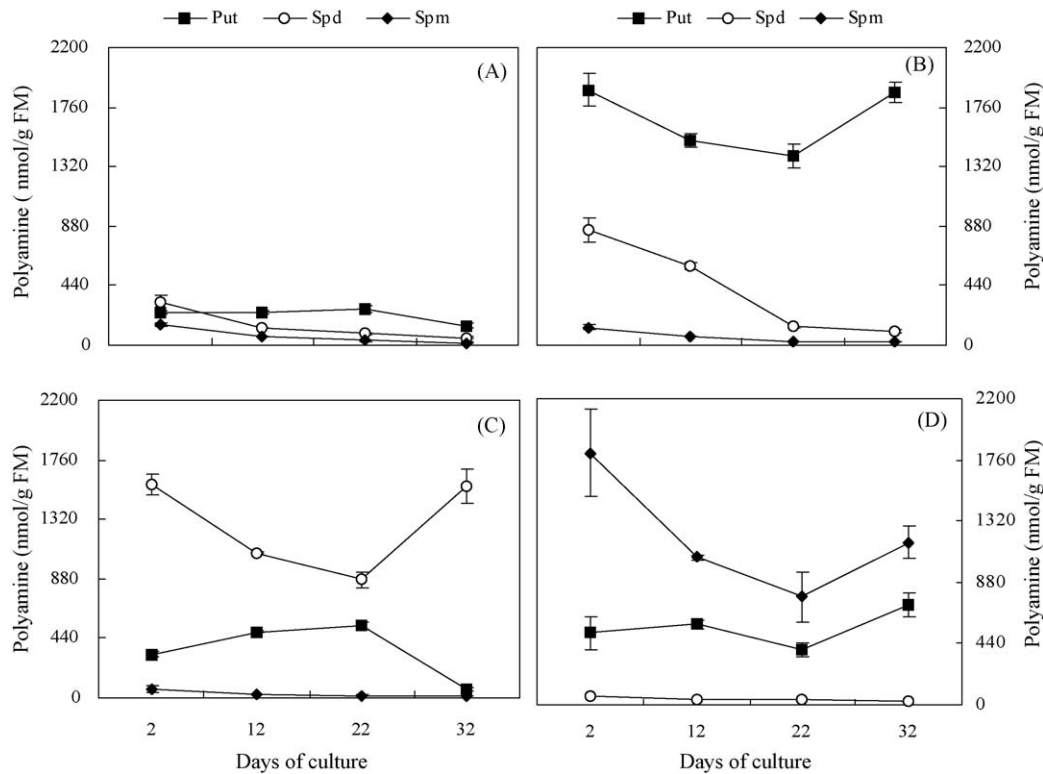


Fig. 2. PAs contents in suspension cultures of *A. angustifolia*. (A) Control—basic medium; (B) Put—basic medium supplemented with Put (1.0 mM); (C) Spm—basic medium supplemented with Spm (1.0 mM); (D) Spd—basic medium supplemented with Spd (1.0 mM). Data are presented as mean  $\pm$  standard error ( $n = 3$ ).

(Fig. 2A) and Put (1.0 mM) (Fig. 2B) treatments, while free Spm and Spd were the most abundant PAs in the Spd (1.0 mM) (Fig. 2C) and Spm (1.0 mM) (Fig. 2D) treatments, respectively (Fig. 2).

Free Spd and Spm levels decreased continuously during the culture, whereas free Put levels in the control treatment remained stable up to the 22nd day of culture, followed by a reduction until the 32nd day (Fig. 2A). Our results demonstrated cellular incorporation of PAs when they were added to the culture medium. Suspension cultures grown in the Put (1.0 mM) treatment showed a significant increase of endogenous Put levels. Put treatment also resulted in a slight increase of Spd levels in the second day, followed by a decrease until the end of culture (Fig. 2B). However, no significant variation of free Spm was observed in this treatment (Fig. 2B). The lack of the required aminopropyl groups might be the reason of the poor conversion from exogenously applied Put to endogenous free Spd and Spm. The first step in PA biosynthesis in higher plants is Put formation by decarboxylation of either ornithine or arginine [24]. The PAs Spd and Spm are synthesized from Put by subsequent addition of aminopropyl groups donated by decarboxylated *S*-adenosylmethionine (SAM) [10]. Polyamines and ethylene synthesis are linked through this common SAM precursor [10,24].

Spd treatment resulted in increase of free Spd and Put levels (Fig. 2C). Spm treatment induced an increase in the levels of free Spm and Put, and a reduction of Spd levels (Fig. 2D). These results suggested that the Spd (1 mM) and Spm (1 mM) supplementation into the culture medium, could have acted as a

stress factor for cells, resulting in Put accumulation. It has been demonstrated that Put is the main PA produced under stress conditions [10,24]. However, these conditions could be important and required for the PEM development. In conifers, generally the somatic embryo development occurs by abrupt changes in the culture medium. Alterations in the osmotic and environment conditions are important for allowing proper organization of embryonal heads of the developing embryos [39].

In all treatments two types of cells were observed: embryonal cells (rounded, with dense cytoplasm) and suspensor cells (highly vacuolated and elongated), these clustered into aggregates in different stages (Fig. 3). Aggregates present in conifer embryogenic cultures during the multiplication phase have been distinguished in three stages based on morphology, cellular organization, and cell number, as PEM I, PEM II and PEM III [25]. 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 stage PEM III, an enlarged clump of embryonal and suspensor cells appears loose rather than compact, with disturbed polarity.

Based on morphology and cell number, the aggregates observed in our experiment were classified according to Filonova et al. [25]. Embryogenic cultures grown in the control treatment presented aggregates at the PEM I and PEM II stages in equal proportions (Fig. 3A and B). Put treatment presented aggregates mainly at the PEM II stage (70%) (Fig. 3C). Spd and Spm treatments presented 85% of the aggregates at the PEM III

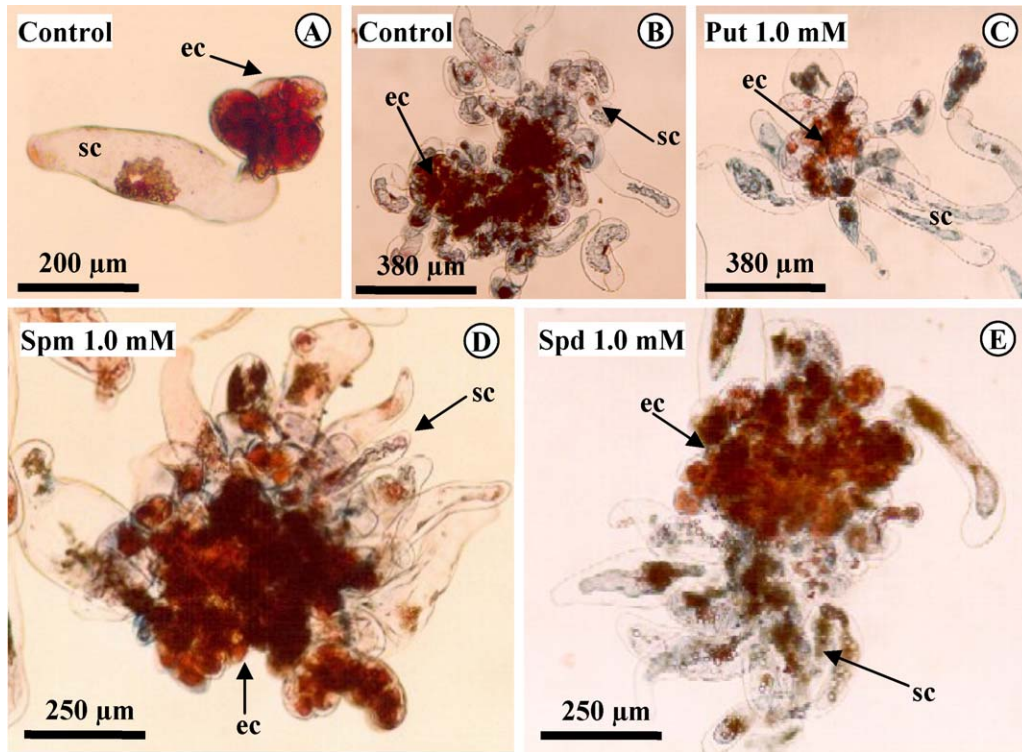


Fig. 3. Morphological organization of embryonic suspension cultures of *A. angustifolia*. (A) Control treatment presenting aggregates at the PEM I stage. (B and C) Control and Put (1.0 mM) treatments presenting aggregates at the PEM II stage. (D and E) Spm (1.0 mM) and Spd (1.0 mM) treatments presenting aggregates at the PEM III stage. ec: embryonic cells; sc: suspensor cells.

stage (Fig. 3D and E), these aggregates were larger with more number of cells when compared with the aggregates observed in the control and Put treatments. Although, it was observed aggregates at the stage PEM II (15%) in the Spd and Spm treatments, PEM III aggregates were not observed in the control and Put treatments, suggesting that Spd and Spm added to the culture medium promote conversion of the aggregates from the PEM I and PEM II (present in the control treatment) to PEM III, without forming new aggregates. According to Filonova et al. [25] aggregates at the PEM III stage are essential for somatic embryogenesis progression, since somatic embryos cannot develop directly from PEM I and PEM II aggregates.

The model for somatic embryogenesis in *P. abies* is divided into three phases: the successive growth of the PEMs, formation of somatic embryos from PEM III, and embryo maturation [25]. In this context, PEM III formation is essential for somatic embryogenesis development. Spd and Spm lead to PEM development, and from PEM II to PEM III stages, during multiplication in a suspension culture. This result can represent a new strategy in using these PAs in the multiplication phase to improve the number of mature embryos at the end of maturation. *A. angustifolia* is considered a recalcitrant species for somatic embryogenesis, as through the established protocols, only few pre-cotyledonary somatic embryos have been recovered [1–3]. In this experiment we did not submit the cultures throughout the maturation phase, but new essays using PAs during the multiplication and maturation phases have been performed with good preliminary results [26], and may represent a new insight in the studies of somatic embryogenesis in *A. angustifolia*. Kevers

et al. [27] showed that PAs are involved in the induction and regeneration of *Panax ginseng* somatic embryos and a five-fold increase in the number of embryonic structures has been recorded with Spd added to the culture medium.

In this study we also examined the regulation of NO biosynthesis by Put, Spd and Spm in suspension cultures of *A. angustifolia*. Using the fluorimetric method, employing the cell-impermeable NO-binding dye DAR-4M, we observed that Put increased NO release from the embryonic culture, whereas Spd and Spm had an inhibitory effect when compared with the control treatment (Fig. 4). These responses were

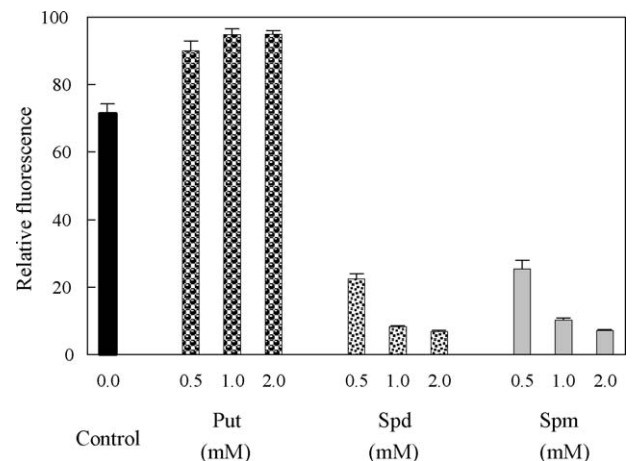


Fig. 4. NO release from embryonic suspension cultures of *A. angustifolia*. Data are presented as mean  $\pm$  standard error ( $n = 3$ ).

confirmed by fluorescence microscopy, using the cell-permeable NO-binding dye DAR-4M AM (Fig. 5). Put induced NO biosynthesis in embryonic cells of the PEMs, whereas suspensor cells presented no or little fluorescence (Fig. 5B). Spd and Spm inhibited the NO biosynthesis in both embryonic and suspensor cells (Fig. 5C and D).

The relationship between PAs and NO biosynthesis have been demonstrated in *Arabidopsis* seedlings, in which Spd and Spm stimulated NO release, while Put had little effect [15]. PAs probably affect differently in NO physiology depending on the species, but it remains unclear whether or not these PAs can regulate NR or other NOS-analogous plant enzymes directly, or by a PA-mediated process [15]. It may well be that in certain cases NO mediates the biological effects of primary signaling molecules, such as hormones [11]. For example, NO biosynthesis was rapidly induced by cytokinin in plant cell cultures of *Arabidopsis*, parsley and tobacco [28].

PAs were described as mediators of embryogenesis [29–31], 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 protoplast derived from *M. sativa* [12]. Our results showed that embryonic cells of *A. angustifolia* accumulated more NO than suspensor cells, mainly in the control and Put (1.0 mM) treatments (Fig. 5A and B), suggesting that PA-induced NO might have a physiological importance for *A. angustifolia* embryo development. NO biosynthesis in embryonic cells of PEMs might be related to the maintenance of polarity (embryonic–suspensor cells), present

at the PEM II stage. This polarity could be a result of continuous cell division induced by NO, mainly in the embryonic region of the PEM II aggregates. In *P. abies* embryogenic cultures at 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 [32]. The key morphogenetic event in plant embryogenesis is the bipolar pattern formation via establishment of the embryonic (apical) and suspensor (basal) regions [33]. 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 programmed cell death (PCD) [32–34].

Spd and Spm inhibit NO biosynthesis in PEM II aggregates (Fig. 5C and D). When Spd and Spm were applied to the culture medium, we observed an enhanced morphological development of embryogenic cultures up to the PEM III stage (Fig. 3). Spd and Spm inhibitory effects in NO biosyntheses during suspension culture multiplication may be essential for PEM I and PEM II development, reducing the cellular growth and leading to acquisition of PEM III structural characteristics. Recently, NO biosynthesis was correlated with the acquisition of embryogenic competence in leaf protoplast-derived cells of *M. sativa* [12]. Whether NO metabolism is also associated with the 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

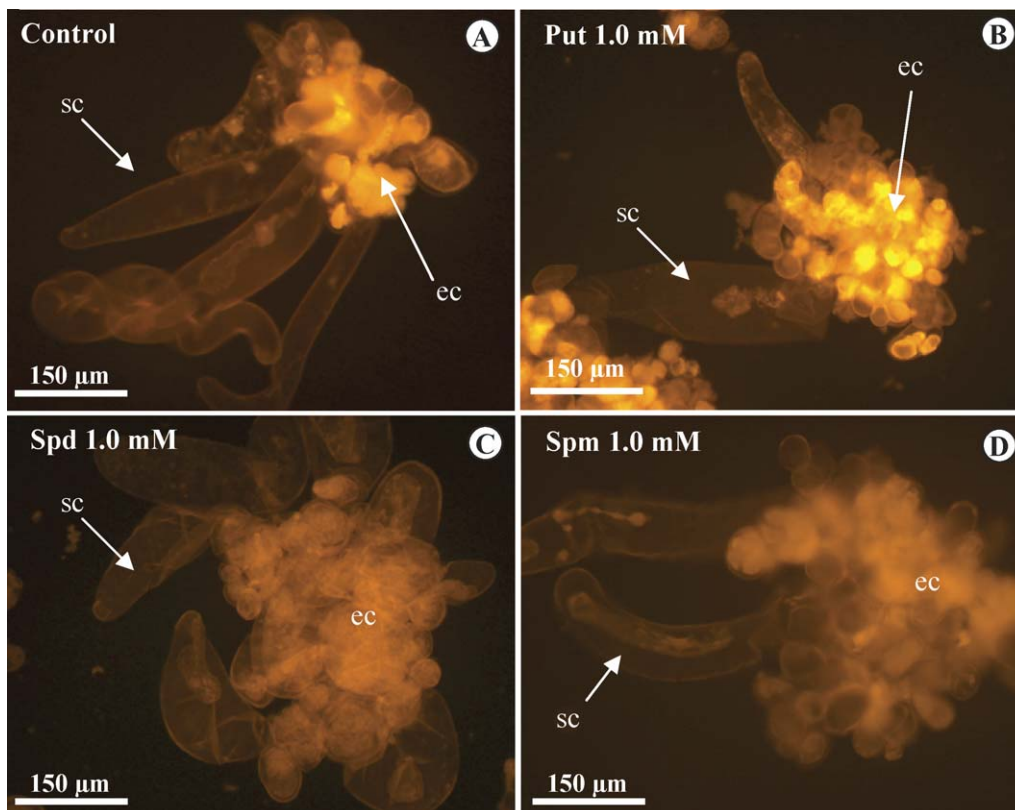


Fig. 5. NO-induced fluorescence observed by fluorescence microscopy in embryonic masses of *A. angustifolia*. (A) Control; (B) Put (1.0 mM); (C) Spd (1.0 mM); (D) Spm (1.0 mM). ec: embryonic cells; sc: suspensor cells.

addition of NO donors to the culture medium, in an attempt to convert non-embryogenic cells to competent ones.

In conclusion, we observed that PAs (Put, Spd and Spm) act in a distinct way on growth, morphological evolution and NO biosynthesis in embryogenic cultures of *A. angustifolia*. Spd and Spm supplemented into a culture medium reduced cell growth, and allowed a morphogenetic evolution of aggregates from the PEM II to PEM III stages. Put increased NO release from the embryogenic culture and Spd and Spm produced an inhibitory effect when compared to the control treatment. In the control and Put (1.0 mM) treatments, 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 polarity (embryonic–suspensor cells) present at the PEM II stage. Finally, our results support further insights in NO biosynthesis manipulation, using NO donors and NO scavengers, as well as Put, Spd and Spm supplementation to the culture medium and visualization of NO-dependent fluorescence might provide clues to study and eventually regulate development.

### Acknowledgements

This research was carried out with financial support from the State of São Paulo Research Foundation (FAPESP) and the National Council for Scientific and Technological Development (CNPq) to E.I.S.F. and a grant by the Deutsche Forschungsgemeinschaft (Sche 207/11-1) to G.E.F.S. This research was supported by a scholarship by the FAPESP to V.S., a travel grant by the DAAD (Germany) and FAPESP scholarship to C.S.C., and a scholarship by the Graduiertenförderung of the University of Hannover to N.N.T.

### References

- [1] M.P. Guerra, V. Silveira, A.L.W. Santos, L.V. Astarita, R.O. Nodari, Somatic embryogenesis in *Araucaria angustifolia* (Bert) O. Ktze., in: S.M. Jain, P.K. Gupta, R.J. Newton (Eds.), Somatic Embryogenesis In Woody Plants, 6, Kluwer Academic Publishers, Dordrecht, 2000 pp. 457–478.
- [2] A.L.W. dos Santos, V. Silveira, N. Steiner, M. Vidor, M.P. Guerra, Somatic embryogenesis in parana pine (*Araucaria angustifolia* (Bert.) O. Kuntze., Braz. Arch. Biol. Technol. 45 (2002) 97–106.
- [3] V. Silveira, N. Steiner, A.L.W. Santos, R.O. Nodari, M.P. Guerra, Biotechnology tolls in *Araucaria angustifolia* conservation and improvement: inductive factors affecting somatic embryogenesis, Crop Breed. Appl. Biotech. 2 (2002) 463–470.
- [4] C. Anjaneyulu, B. Shyamkumar, C.C. Giri, Somatic embryogenesis from callus cultures of *Terminalia chebula* Retz.: an important medicinal tree, Trees 18 (2004) 547–552.
- [5] V. Silveira, E.I.S. Floh, W. Handro, M.P. Guerra, 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 Org. Cult. 76 (2004) 53–60.
- [6] M.M. Lulsdorf, T.E. Taurus, S.I. Kikcio, D.I. Dunstan, Growth parameters of embryogenic suspension culture of interior spruce (*Picea glauca-engelmannii* complex) and black spruce (*Picea mariana* Mill.), Plant Sci. 82 (1992) 227–234.
- [7] L.V. Astarita, M.P. Guerra, Conditioning of culture medium by suspension cells and formation of somatic proembryo in *Araucaria angustifolia* (Coniferae), In Vitro Cell. Dev. Biol. Plant 36 (2000) 194–200.
- [8] L.V. Astarita, M.P. Guerra, Early somatic embryogenesis in *Araucaria angustifolia*: induction and maintenance of embryonal-suspensor mass cultures, Braz. J. Plant Physiol. 10 (1998) 13–118.
- [9] R. Minocha, S.C. Minocha, S. Long, Polyamines and their biosynthetic enzymes during somatic embryo development in red spruce (*Picea rubens* Sarg.), In Vitro Cell. Dev. Biol. Plant 40 (2004) 572–580.
- [10] H.P. Bais, G.A. Ravishankar, Role of polyamines in the ontogeny of plants and their biotechnological applications, Plant Cell Tissue Org. Cult. 69 (2002) 1–34.
- [11] S.J. Neill, R. Desikan, J. Hancock, Nitric oxide signalling in plants, New Phytol. 159 (2003) 11–35.
- [12] K. Ötvös, T.P. Pasternak, P. Miskolczi, M. Domoki, D. Dorjgotov, A. Szucs, S. Bottka, D. Dudits, A. Feher, 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, Plant J. 43 (2005) 849–860.
- [13] G.D. Kuehn, G.C. Phillips, Role of polyamines in apoptosis and other recent advances in plant polyamines, Crit. Rev. Plant Sci. 24 (2005) 123–130.
- [14] H. Yamasaki, The NO world for plants: achieving balance in an open system, Plant Cell Environ. 28 (2005) 78–84.
- [15] N.N. Tun, C. Santa-Catarina, T. Begum, V. Silveira, W. Handro, E.I.S. Floh, G.F.E. Scherer, Polyamines induce rapid biosynthesis of nitric oxide (NO) in *Arabidopsis thaliana* seedlings, Plant Cell Physiol. 47 (2006) 346–354.
- [16] P.K. Gupta, G.S. Pullman, Method for Reproducing Coniferous Plants by Somatic Embryogenesis Using Abscisic Acid and Osmotic Potential Variation, US patent 5,036,007 (1991).
- [17] P.J. King, Induction and maintenance of cell suspension cultures, in: I.K. Vasil (Ed.), Cell Culture and Somatic Cell Genetics in Plants, 1, Academic Press, New York, 1984, pp. 130–138.
- [18] P.K. Gupta, D.J. Durzan, Biotechnology of somatic polyembryogenesis and plantlet regeneration in loblolly pine, Biotechnology 5 (1987) 147–151.
- [19] H. Kojima, M. Hirotsu, Y. Urano, K. Kikuchi, T. Higuchi, T. Nagano, Fluorescence indicators for nitric oxide based on rhodamine chromophore, Tetrahedron Lett. 41 (2000) 69–72.
- [20] H. Kojima, M. Hirotsu, N. Nakatsubo, K. Kikuchi, Y. Urano, T. Higuchi, Y. Hirata, T. Nagano, Bioimaging of nitric oxide with fluorescent indicators based on the rhodamine chromophore, Anal. Chem. 73 (2001) 1967–1973.
- [21] H. Laukkanen, T. Sarjala, Effect of exogenous polyamines on Scots pine callus in vitro, J. Plant Physiol. 150 (1997) 167–172.
- [22] E. Lainé, H. Davide, A. David, Callus formation from cotyledon protoplasts of *Pinus oocarpa* and *Pinus patula*, Physiol. Plant 72 (1988) 374–378.
- [23] K. Niemi, T. Sarjala, X. Chen, H. Häggman, Spermidine and methylglyoxal bis(guanylhydrazone) affect maturation and endogenous polyamine content of Scots pine embryogenic cultures, J. Plant Physiol. 159 (2002) 1155–1158.
- [24] A. Bouchereau, A. Aziz, F. Larher, J. Martin-Tanguy, Polyamines and environmental challenges: recent developments, Plant Sci. 140 (1999) 103–125.
- [25] L.H. Filonova, P.V. Bozhkov, S. von Arnold, Developmental pathway of somatic embryogenesis in *Picea abies* as revealed by time-lapse tracking, J. Exp. Bot. 51 (2000) 249–264.
- [26] N. Steiner, M.P. Guerra, Parâmetros fisiológicos e bioquímicos durante a embriogênese zigótica e somática em *Araucaria angustifolia*, M.Sc. dissertation, Department of Plant Sciences, Federal University of Santa Catarina, Florianópolis, 2005, pp. 1–129.
- [27] C. Kevers, L.G. Nathalie, M. Monteiro, J. Dommes, T. Gaspar, Somatic embryogenesis of *Panax ginseng* in liquid cultures: a role for polyamines and their metabolic pathways, Plant Growth Regul. 31 (2000) 209–214.
- [28] N.N. Tun, A. Holk, G.F.E. Scherer, Rapid increase of NO release in plant cell cultures induced by cytokinin, FEBS Lett. 509 (2001) 174–176.
- [29] A.W. Galston, R. Kauw-Shawney, T. Altabella, A.F. Tiburcio, Plant polyamines in reproductive activity and response to abiotic stress, Bot. Acta 110 (1995) 197–207.

- [30] F. Shoeb, J.S. Yadav, S. Bajaj, M.V. Rajam, Polyamines as biomarkers for plant regeneration capacity: improvement of regeneration by modulation of polyamine metabolism in different genotypes of indica rice, *Plant Sci.* 160 (2001) 1229–1235.
- [31] D. Bertoldi, A. Tassoni, L. Martinelli, N. Bagni, Polyamines and somatic embryogenesis in two *Vitis vinifera* cultivars, *Physiol. Plant* 120 (2004) 657–666.
- [32] L.H. Filonova, P.V. Bozhkov, V.B. Brukhin, G. Daniel, B. Zhivotovsky, S. von Arnold, Two waves of programmed cell death occur during formation of somatic embryos in the gymnosperm, Norway spruce, *J. Cell Sci.* 113 (2000) 4399–4411.
- [33] P.V. Bozhkov, L.H. Filonova, M.F. Suarez, Programmed cell death in plant embryogenesis, *Curr. Top. Dev. Biol.* 67 (2005) 135–179.
- [34] P.V. Bozhkov, M.F. Suarez, L.H. Filonova, G. Daniel, A.A. Zamyatnin, S. Rodríguez-Nieto, B. Zhivotovsky, A. Smertenko, Cysteine protease mcll-Pa executes programmed cell death during plant embryogenesis, *PNAS* 102 (2005) 14463–14468.
- [35] M.C. Pedroso, J.R. Magalhaes, D. Durzan, A nitric oxide burst precedes apoptosis in angiosperm and gymnosperm callus cells and foliar tissues, *J. Exp. Bot.* 51 (2000) 1027–1036.
- [36] M.F. Fraga, M. Berdasco, L.B. Diego, R. Rodríguez, M.J. Cañal, Changes in polyamine concentration associated with aging in *Pinus radiata* and *Prunus persica*, *Tree Physiol.* 24 (2004) 1221–1226.
- [37] M. Franceschetti, C. Hanfrey, S. Scaramagli, P. Torrigiani, N. Bagni, D. Burtin, A.J. Michael, Characterization of monocot and dicot plant *S*-adenosyl-L-methionine decarboxylase gene families including identification in the mRNA of a highly conserved pair of upstream overlapping open reading frames, *Biochem. J.* 353 (2001) 403–409.
- [38] N. Steward, R. Martin, J.M. Engasser, J.L. Goergen, A new methodology for plant cell viability assessment using intracellular esterase activity, *Plant Cell Rep.* 19 (1999) 171–176.
- [39] C. Stasolla, E.C. Yeung, Recent advances in conifer somatic embryogenesis: improving somatic embryo quality, *Plant Cell Tissue Org. Cult.* 74 (2003) 15–35.