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## Research paper

# Proteomic analysis and polyamines, ethylene and reactive oxygen species levels of *Araucaria angustifolia* (Brazilian pine) embryogenic cultures with different embryogenic potential

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Somatic embryogenesis is an important biotechnological tool in the large-scale propagation of elite genotypes and ex situ conservation of conifer species. Protocols for the induction and proliferation of embryogenic cultures (ECs) of Brazilian pine (*Araucaria angustifolia* (Bert.) O. Ktze) are well established, although the proper formation of mature somatic embryos (SEs) is still problematic. Thus, the identification of molecular markers for the screening of ECs able to respond to maturation conditions (abscisic acid and osmotic agents) is highly desirable. To develop molecular markers for the early detection of ECs able to develop well-formed SEs under maturation conditions, we analyzed the proteins found during the proliferation phase of *A. angustifolia* cell lines with different embryogenic capabilities, with one cell line being responsive to maturation conditions (R cell line), and one cell line that presented blocked development of SEs (B cell line). In addition, based on the peptides identified, polyamine levels (free and conjugate), ethylene production and reactive oxygen species (ROS) emission were analyzed using both EC lines (R and B cell lines). A marked difference in the biochemistry of ECs between these two cell lines was observed. Eleven proteins that were differentially expressed in the cell lines were identified by the combination of two-dimensional electrophoresis (2-DE) and MALDI-TOF/TOF mass spectrometry. Among these, S-adenosylmethionine synthase, the enzyme associated with polyamines and ethylene biosynthesis, was observed exclusively in the R cell line, while a protein linked to the oxidative stress subunit F of NADH dehydrogenase was observed exclusively in the B cell lines. Additionally, B cell lines showed higher levels of diamine putrescine and lower levels of ethylene. Higher values of ethylene and ROS were observed for the cell line that showed normal development of SEs. Altogether, our results open new perspectives in the optimization of culture conditions for *A. angustifolia* somatic embryogenesis, as well as establishing biochemical markers for the early selection of ECs during maturation trials.

**Keywords:** biochemical markers, conifer biotechnology, somatic embryogenesis.

## Introduction

*Araucaria angustifolia* (Bert.) O. Ktze (Brazilian pine) is the only native conifer of economic importance in Brazil (Guerra et al. 2002). Due to intense wood exploitation and the expansion of agriculture in South Brazil, populations of *A. angustifolia* cover less than 2% of their original area (Seben et al. 2003, Wrege

et al. 2009). *Araucaria angustifolia* is a critically endangered species, according to the International Union of Conservation of Nature Red List of Threatened Species (2013). In spite of the studies on the genetic diversity and in situ conservation of *A. angustifolia* populations (de Sousa et al. 2005, Bittencourt and Sebbenn 2007, Stefenon et al. 2008), there is a need for the

development of complementary conservation strategies based on ex situ approaches (Seben et al. 2003).

Micropropagation tools, like somatic embryogenesis, have potential in clonal propagation and ex situ conservation of commercial and endangered plant species, especially conifers (Klimaszewska et al. 2011, Ma et al. 2012a, 2012b). Somatic embryogenesis comprises the coordinated execution of four steps (embryogenic culture (EC) induction, proliferation, maturation and plant regeneration) (von Arnold et al. 2002). As observed for other conifers, well-developed early somatic embryos (SEs) in the EC of *A. angustifolia* are essential for embryo maturation in media supplemented with abscisic acid (ABA) and osmotic agents (Steiner et al. 2005, Schlogl et al. 2012). In some genotypes even early SE do not assure embryo maturation (dos Santos et al. 2008, 2012). As SE morphology cannot be used as the only factor in EC selection, the development of molecular markers for the early detection of ECs responsive to maturation promoters (ABA and osmotic agents) is highly desirable in the SE protocol optimization for *A. angustifolia* (dos Santos et al. 2008, Schlogl et al. 2012, Steiner et al. 2012).

The induction and control of SE depend on the source of the explants, the genotype of the mother plant and the type and level of growth regulators in the culture medium (Guerra et al. 1999). To efficiently regulate the correct embryo development in vitro, an improved understanding of the physiological, biochemical and molecular events that are associated with embryogenic competence is essential (Zhang et al. 2010, Businge et al. 2012). The competence of embryogenic cells to respond adequately to exposure conditions during the different phases of SE is controlled by the molecular and biochemical components of embryogenic and suspensor cells (Stasolla et al. 2004, Abrahamsson et al. 2012).

Proteins directly affect the biochemistry of the cell, affording an analysis of the many biochemical events that occur during plant growth and development (Chen and Harmon 2006). Zygotic and SE development are closely associated with changes in protein expression (Balbuena et al. 2009, Rode et al. 2012). Thus, the characterization of proteins involved in embryogenesis is highly desirable, because perturbations in the expression of these proteins can indicate an aberrant developmental process (Lippert et al. 2005). Two-dimensional electrophoresis (2-DE) associated with protein identification via mass spectrometry (MS) is one of the most common approaches in plant proteomics (Balbuena et al. 2009, 2011). To date, the combination of 2-DE and MS has allowed the identification of several proteins linked to SE competence and development (Almeida et al. 2012, Correia et al. 2012, Rode et al. 2012, Sharifi et al. 2012).

To develop molecular markers for the early detection of *A. angustifolia* EC with high embryogenic potential, we carried out

a differential proteome analysis of ECs with different maturation capabilities. In addition, based on the peptides identified by MALDI-TOF/TOF MS, biochemical analyses that included the determination of polyamines (free and conjugate), ethylene production and reactive oxygen species (ROS) were performed in both EC lines (responsive and blocked cell lines). Our findings, besides providing a basis for the early selection of ECs with high embryogenic potential, may also become a useful tool in the improvement of a somatic embryogenesis protocol for *A. angustifolia*.

## Materials and methods

### Cone collection

Immature cones of *A. angustifolia* were collected during the month of January 2009, from three open-pollinated trees grown in the city of Canoas, Brazil ( $-26^{\circ}17'$ ,  $-50^{\circ}38'$ ). The immature seeds were detached from the cones and mixed to form a pool of seeds.

### EC initiation and proliferation

Embryogenic culture initiation was carried out according to dos Santos et al. (2008). For proliferation, ECs were subcultured every 21 days in MSG semi-solid medium and maintained in the dark at  $25 \pm 2^{\circ}\text{C}$ . To evaluate the degree of organization of proembryogenic masses (PEMs) among all embryogenic cell lines proliferated (each corresponding to a different genotype), 30 samples of 100 mg (fresh weight (FW)) were submitted to a double-staining procedure with acetocarmine (2%) and Evan's blue (0.1%) (Gupta and Durzan 1987). The stained ECs were observed and photographed using a SteREO Discovery V8 magnifier (Carl Zeiss, Jena, Germany) coupled to an ICC camera AxioCam (Carl Zeiss).

### EC maturation

Ten months after EC initiation, nine embryogenic cell lines were tested for their capacity to develop later-stage SEs. Maturation tests were performed according to Steiner et al. (2005) and dos Santos et al. (2008) with modifications. Embryonic cultures (100 mg, fresh weight) were transferred to filter paper disks (Whatman No. 1, 70 mm) in Petri dishes (100 × 15 cm) containing 25 ml of basic MSG, solidified with Gelrite® (Sigma-Aldrich) ( $3\text{ g l}^{-1}$ ), supplemented with L-glutamine ( $1.46\text{ g l}^{-1}$ ), sucrose ( $30\text{ g l}^{-1}$ ), activated charcoal ( $3\text{ g l}^{-1}$ ), maltose ( $70\text{ g l}^{-1}$ ) and PEG 4000 ( $90\text{ g l}^{-1}$ ) (maturation medium I) and kept in the dark at  $25 \pm 2^{\circ}\text{C}$ . Three repeats were performed, each consisting of a Petri dish inoculated with five ECs. After 30 days in maturation medium I, filter papers containing the ECs were transferred to maturation medium II (maturation medium I supplemented with  $120\text{ }\mu\text{M}$  ABA) and cultured for an additional 3 months (subcultured every 4 weeks in fresh maturation medium II).

During the whole maturation experiment (4 months), the morphology of SEs was observed and photographed. For each cell line, the number of ECs that showed the development of SEs was recorded. Cell lines showing the development of cotyledonary SE were classified as responsive to maturation conditions (R cell lines), whereas cell lines where SE did not develop were considered as cell lines with blocked development of SEs (B cell lines).

### *Protein extraction and quantification*

Protein extractions were carried out according to Balbuena et al. (2009). Proteome analysis was carried out in biological triplicates. Each biological replicate was prepared from an individual 21-day-old EC during the proliferation phase of R and B cell lines. Total protein was quantified using the 2-D Quant Kit (GE Healthcare, Freiburg, Germany) according to the manufacturer's instructions.

### *Two-dimensional electrophoresis and image analysis*

Two-dimensional electrophoresis was carried out according to Balbuena et al. (2009). Gels were stained in Colloidal Coomassie Brilliant Blue (Neuhoff et al. 1988) and digitalized in an Image Scanner (GE Healthcare) using the Labscan™ 5.0 software (GE Healthcare). After scanning, the gels were analyzed in the ImageMaster™ 2D Platinum software 6.0 (GE Healthcare). The parameters of spots automatic detection were optimized by analysis of different regions of the gels. When required, isolation, removal and addition of each spot were performed manually. To detect differential spots between 2-DE gels of R and B cell lines, all experimental gels were automatically matched based on 10 obviously common spots used as anchors, and again visually inspected for improper spot matches. Spots differentially expressed between the 2-DE maps of R and B cell lines were selected for further digestion and identification via MS.

### *Spot excision and protein digestion*

Digestion of peptides was performed in-gel, according to Shevechenko et al. (2006) using 15 ng l<sup>-1</sup> trypsin (Promega, Madison, WI, USA) for protein enzymatic digestion.

### *Protein identification using MALDI-TOF/TOF*

The solution containing the peptides was desalted on C18 reversed phase columns in ZipTip pipette tips (Merck Millipore, MA, USA). After desalting, a 1 µl sample dissolved in 50% (v/v) ACN acid and 1 µl α-cyano-4-hydroxycinnamic acid were applied to the MALDI plate. Mass spectra were obtained on platform AB SCIEX TOF/TOF 5800 (AB SCIEX, Framingham, MA, USA). The ranges of mass/charge (*m/z*) were adjusted to 800–4000 for MS spectra and 9–2500 for MS/MS. The spectra were calibrated externally using a mixture containing four peptides (des-arg1-bradykinin, angiotensin I, Glu1-Neurotensin

and Fibrinopeptide B). The five most abundant peaks in the MS spectra were used as selection parameters for MS/MS. The spectra of MS/MS were obtained after fragmentation of peptides via 'collision-induced dissociation'.

The database search parameters were similar to those described in Balbuena et al. (2011). The database used in searches was the NCBI 1.1., taxonomy Viridiplantae (green plants) using MASCOT v. 2.4 (Matrix Science, London, UK). Mass tolerance for precursor and fragmented ions were 100 ppm and 1.5 Da for precursor and fragmented peptides, respectively. Carbamidomethylation of cysteine was set as fixed modification, and oxidation of methionine and acetylation of N-terminal as variable modifications. Identification of proteins was considered confident if hits were produced by matching at least one MS/MS spectra with peptide ions scores above 50.

### *Free and conjugated polyamine extraction and quantification*

Extractions of free and conjugated polyamines were performed according to Silveira et al. (2006). Polyamine samples were prepared in biological triplicates. Each replicate was prepared from an individual 21-day-old EC during the proliferation phase of R and B cell lines. Polyamines were separated by high performance liquid chromatography (HPLC) on a reversed phase C18 column (Shim-pack CLC ODS, Shimadzu Corporation, Kyoto, Japan). The peak areas and retention times of each polyamine were evaluated by comparison with known concentrations of the main polyamines found in plants (putrescine (Put), spermidine (Spd) and spermine (Spm)).

### *Ethylene quantification*

Ethylene quantification was performed according to Thuler et al. (2003) and Kong and Yeung (1994) with modifications. Measurements were carried out using six biological replicates, with each replicate consisting of an individual 100 mg portion of 21-day-old ECs of R and B cell lines cultivated in 6 ml MSGO basal medium. Penicillin-type 60-ml flasks sealed with aluminum foil were used. Ethylene production was measured for two periods, 7 and 21 days after transfer to experimental flasks. Flasks with medium cultured without ECs were utilized as controls. Twenty-four hours before each measurement point, the aluminum foil was removed and the flasks were kept in a laminar air-flow cabinet for 2 h. Subsequently, rubber plugs tightened with metal cowls were used to seal the flasks, and ethylene production was quantified after 24 h.

For each measurement point, 1.0 ml of air from the experimental flasks was collected and analyzed in a gas chromatograph Shimadzu GC-14A (Shimadzu Corporation) with a PORAPAK-N 80/100—INOX column operated isothermally at 70 °C with nitrogen as the carrier gas and a flame ionization detector. Pure ethylene (White Martins, Rio de Janeiro, Brazil) was used as the standard.



### Endogenous ROS emission

For endogenous ROS quantification, suspension cultures of R and B cell lines were established according to [Silveira et al. \(2006\)](#). Reactive oxygen species quantification was carried out in biological triplicates. For each replicate, 14-day-old suspension cultures were filtered through 100 µm mesh sieves. Subsequently, 200 mg of cells were collected and incubated in 2 ml of fresh MSGO liquid medium. Then, fluorophore 2',7'-dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCFDA) was added to obtain a final concentration of 2.5 mM. Liquid MSGO medium containing H<sub>2</sub>DCFDA without cells was used as control. Cells were maintained under agitation in an orbital shaker (120 r.p.m.) and after 30 min of incubation in the dark, a 200 µl aliquot of each sample was transferred to a 96-multiwell opaque plate. The relative fluorescence emitted by the cells was monitored for 6 h (measurements every 10 min) in a fluorometer Victor 3TM (PerkinElmer®, Waltham, MA, USA), under emission of 522 nm and excitation of 498 nm.

### Data analysis

All means were compared using Student's *t*-test in the BIOESTAT® 5.0 software.

## Results and discussion

### *Araucaria angustifolia* SEs development

Forty days after the inoculation of immature zygotic embryos in MSGO culture medium, ECs of *A. angustifolia*, of all cell lines, were recorded and characterized as translucent, white or mucilaginous cell masses (Figure 1a). During the proliferation phase,

the Evans blue and acetocarmine double staining ([Gupta and Durzan 1987](#)) revealed the presence of cell aggregates, whose embryonic cells, which stained red, were surrounded by suspensor cells, which stained blue (Figure 1b). These structures, resembling PEMs III of *Picea abies* (L.) H. Karst. ECs ([von Arnold et al. 2002](#)), were observed in all EC lines induced and proliferated in the MSGO culture medium (nine cell lines).

Despite the presence of PEMs III in all EC lines established for *A. angustifolia*, a marked difference in the development of SEs during the maturation phase was observed. After 2 months, of the nine EC lines tested on maturation medium I, only two cell lines (MSGO1 and R1003) showed the development of globular SE (recorded in 10 SE for MSGO1 and 15 SE for R1003 of 15 embryogenic calli). These globular SE were characterized by spherical opaque embryonic heads and a degraded suspensor cell region (Figure 1c). After 4 months in maturation medium II (supplemented with ABA), the development of primordial cotyledon structures was observed (Figure 1d). The remaining EC lines (corresponding to seven cell lines) did not show the development of SE in maturation media I and II. Based on the results obtained during the maturation trials, two EC lines were classified as responsive cell lines (R), and seven EC lines were classified as blocked cell lines (B). To confirm this classification, the maturation trial was repeated 24 months after EC induction. No differences concerning the embryogenic potential were observed during the first (10 months after EC induction) or second maturation trials (24 months after EC induction).

In conifers, the maturation of SEs occurs when ECs are transferred to culture medium supplemented with ABA and

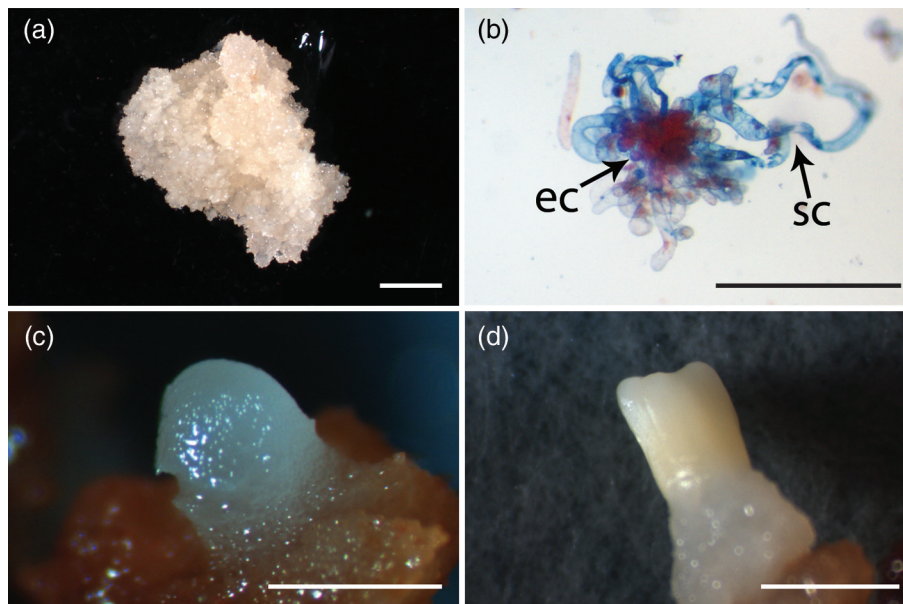


Figure 1. Morphology of *A. angustifolia* ECs and SEs during proliferation and maturation. (a) Twenty-one-day-old EC. (b) Evans blue and acetocarmine stained cellular aggregates at the PEM III stage. (c) Globular SE after 8 weeks of maturation. (d) Early cotyledonary SE after 16 weeks on maturation. ec, embryogenic cells; sc, suspensor cells. Bars (a and b): 2.5 mm; (c and d): 1.0 mm.

osmotic agents (Attree and Fowke 1993, Dunstan et al. 1998, von Arnold et al. 2002). In the present work, the development of SEs observed in the R cell lines indicates that a pretreatment with osmotic agents followed by ABA exposure can efficiently promote the development of pre-cotyledonary SE in *A. angustifolia*. However, the same pattern was not observed in B cell lines. In the presence of ABA and osmotic agents, the ECs of B cell lines did not show the development of SEs. This same precluded development of SEs was also observed in blocked cell lines of *P. abies* (Smertenko et al. 2003, Stasolla et al. 2004). This observation indicates that some cell lines are unable to respond properly to the stimulus imposed during the formation of SE, even under favorable conditions for the development of SEs.

The absence of differences in the overall morphology of R and B cell lines of *A. angustifolia* during the proliferation phase suggests that the differential SE maturation capabilities could be associated with differential endogenous components of these two cell lines. Cell lines of *Medicago truncatula* Gaertn. with different embryogenic capabilities showed differences in the level of transcripts linked to several biochemical pathways, such as hormone biosynthesis (Imin et al. 2008). Stasolla et al. (2004) observed that in *P. abies*, SE development is controlled by a precise pattern of gene expression. When compared with the blocked cell lines, the embryogenic-competent cell lines presented the differential expression of several genes that encode the proteins involved in detoxification processes, methionine synthesis and carbohydrate metabolism (Stasolla et al. 2004).

### Proteomic analysis

Based on the results obtained during maturation trials, two EC lines representative of the R (responsive cell lines) and B (blocked cell lines) were selected for proteomic analysis. The R cell line showed a higher abundance of proteins when compared with the B cell line (Table 1). According to Oropeza et al. (2001), differences in the levels of total protein between ECs and non-ECs may be due to higher metabolic activity in ECs, with higher levels of proteins, mRNA and other cytoplasmic components. Two-dimensional electrophoresis gel image analysis revealed a differential protein profile between gels of R and B lines. Two-dimensional electrophoresis gels of ECs of

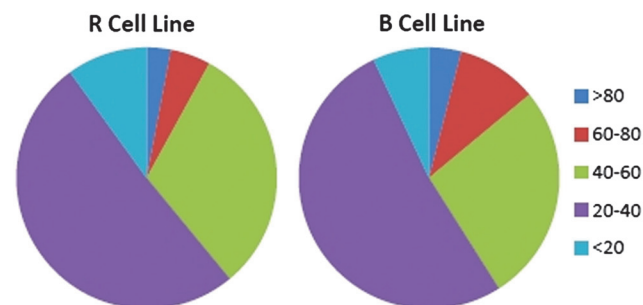


Figure 2. Percent of spots detected in the 2-DE maps *A. angustifolia* ECs of responsive (R) and blocked (B) cell lines, according to intervals of relative molecular weight (kDa).

the R cell line showed a higher abundance of spots compared with the B cell line (Table 1). Spot distribution on the 2-DE gels of both cell lines was similar. A higher number of 'spots' between 20–40 and 40–60 kDa occurs, regardless of embryogenic potential (Figure 2). A smaller proportion of spots with high (>80 kDa) and low (<20 kDa) relative molecular mass was observed in 2-DE gels of both cell lines (Figure 2).

The in silico alignment of 2-DE gels of both cell lines showed the presence of  $122 \pm 11$  spots exclusive to the R cell line, and  $65 \pm 10$  spots unique to the B cell line. Among the exclusive ones, the most distinct (20 spots from the R cell line 2-DE maps and 15 from the B cell line) were isolated and in-gel digested for MS analysis (Figure 3). A MASCOT stringent search resulted in the identification of 11 proteins. Among these, eight were exclusively found in the R cell line and three in the B cell line. Table 2 shows the list of the proteins identified, with their respective spot ID, protein name, accession number, MS score and number of unique peptides identified.

Among the proteins identified from the 2-DE gels of the R cell line, the enzyme *S*-adenosylmethionine (SAM) synthase (Spot 2) and mitochondrial ATPase beta subunit (Spot 1) were identified at relatively high scores (108 and 104, respectively). Besides the four hypothetical proteins in the NCBI database (Spots 5, 15, 17 and 19), a protein associated with protein synthesis (elongation factor II) (Spot 13) and a predicted protein from *Populus trichocarpa* Torr. & A.Gray (Spot 6) were also identified (Table 1). Mass spectrometry/MS spectra obtained from spots exclusively detected in 2-DE gels of the B cell line allowed the identification of two proteins, the F subunit of enzyme NADH dehydrogenase (Spot 23) and a porin protein (Spot 35) (Table 2).

Somatic embryogenesis is a morphogenetic process that requires high amounts of energy (Martin et al. 2000, Lyngved et al. 2008). In *Abies alba* Mill., an increase in the levels of adenosine triphosphate (ATP) is associated with the maturation of SEs (Petrussa et al. 2009). In addition, mitochondrial activities like ATP catabolism influence plant cell death, which is considered essential for correct SE maturation (Bozhkov et al. 2005, Petrussa et al. 2009). The proteomics analysis of

Table 1. Protein content (mg g<sup>-1</sup> FW) and number of spots detected in 2-DE maps of *A. angustifolia* ECs of responsive (R) and blocked (B) cell lines.

	Protein content (mg g <sup>-1</sup> FW)	Number of spots
R cell line	3.22 ± 0.47 <sup>a</sup>	245 ± 11 <sup>a</sup>
B cell line	2.43 ± 0.35 <sup>b</sup>	210 ± 2 <sup>b</sup>

For each column, means followed by different lowercase letters are significantly different according to Student's *t*-test ( $P < 0.05$ ) (mean ± SD;  $n = 3$ ).

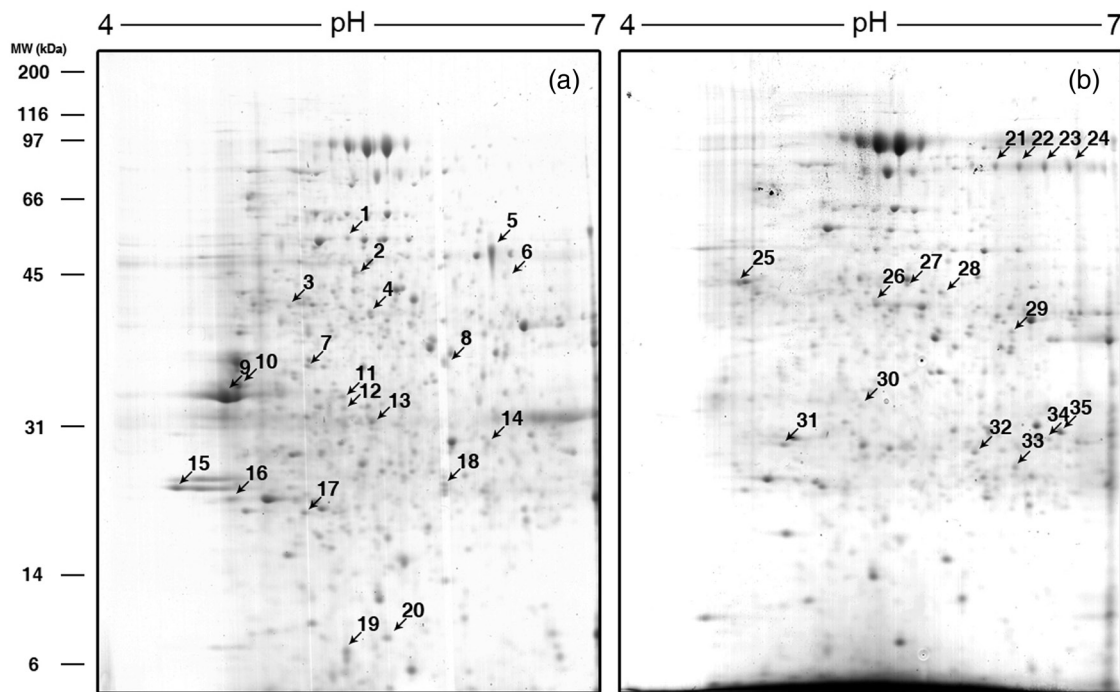


Figure 3. Two-dimensional electrophoresis map of proteins extracted from *A. angustifolia* ECs of responsive (a) and blocked (b) cell lines (pH 4–7 linear gradient). Arrows indicate specific spots of R and B cell lines 2-DE maps that were selected for protein identification. Proteins that were identified are numbered as specified in Table 2.

Table 2. List of identified proteins from spots isolated from 2-DE gels of *A. angustifolia* ECs of responsive (R) and blocked (B) cell lines.

Spot <sup>1</sup>	ID	Species	Accession <sup>2</sup>	Score <sup>3</sup>	Peptides <sup>4</sup>
1R	Mitochondrial ATPase beta subunit	<i>Nicotiana sylvestris</i>	AAD03392	104	3
2R	Full = S-adenosylmethionine synthase 3	<i>Hordeum vulgare</i>	Q4LB22	108	1
5R	Hypothetical protein OsJ_17535	<i>Oryza sativa</i>	EEE62732	76	1
6R	Predicted protein	<i>Populus trichocarpa</i>	XP_002301570	75	1
13R	Elongation factor 2	<i>Nicotiana tabacum</i>	CAC12818	55	2
15R	Hypothetical protein SELMODRAFT_404503	<i>Selaginella moellendorffii</i>	XP_002962607	53	1
17R	Hypothetical protein CHLNCDRAFT_27694	<i>Chlorella variabilis</i>	EFN51866	55	1
19R	Hypothetical protein VOLDACRAFT_84030	<i>Volvox carteri</i>	XP_002957246	54	1
23B	NADH dehydrogenase subunit F	<i>Anonidium sp.</i>	ABS45074	58	1
35B	Porin	<i>Prunus armeniaca</i>	AAD38145	66	1

<sup>1</sup>Spot numbers correspond to the numbers indicated in Figure 3.

<sup>2</sup>Accession number in NCBI protein database.

<sup>3</sup>Probability based MOWSE score of MASCOT software.

<sup>4</sup>Number of unique peptide sequences identified by MASCOT.

zygotic embryo development in *A. angustifolia* revealed a beta subunit of mitochondrial ATPase in mature SEs (Silveira et al. 2008). The same enzyme was detected in vitro in the R cell line during the proliferation phase. These results underline the importance of mitochondrial activity during SE development in conifers, and of the mitochondrial ATPase as the marker for selection of EC lines responsive to maturation treatments in *A. angustifolia*. The protein identified from Spot 13, SAM synthase, was detected in 2-DE gels of the R cell line (Table 2). SAM synthase converts the amino acid methionine into SAM, which is the donor of methyl groups to the DNA methylation system by SAM-dependent methyltransferase (Weretilnyk et al. 2001,

Moffatt et al. 2002). High levels of methylation during plant embryogenesis are associated with chromatin modeling, selective gene expression and growth of SEs (Stasolla et al. 2004). In addition, SAM synthase participates in the conversion of Put in spermidine, and in the production of ethylene (Baron and Stasolla 2008, Dias et al. 2010). In *Picea glauca* Hort. ex Beissn., SAM synthase has been characterized as a biochemical marker of early SE development (Lippert et al. 2005). In *P. abies*, cell lines that showed an arrested PEM III to SE transition contain lower levels of SAM synthase transcripts when compared with cell lines that showed a normal development of SEs (Stasolla et al. 2004). As observed for other conifers, the

presence of SAM synthase might be essential for the transition of PEM to SEs in *A. angustifolia*. Recently, this enzyme was also detected during proembryogeny, early embryogenesis and late embryogenesis of *A. angustifolia* zygotic embryos (Balbuena et al. 2009).

Spot 23, observed in the B cell line, has been identified as the subunit F of the enzyme NADH dehydrogenase (Table 2). NADH dehydrogenase (proton pumping complex I) is the first enzyme of the mitochondrial electron transport chain, catalyzing the oxidation of NADH and reduced coenzyme Q (Møller 2002). Under normal respiration conditions, complex I and complex III can act together, promoting the generation of ROS (Rhoads et al. 2006). During SE development, cell cultures undergo a rapid process of cell proliferation and active aerobic metabolism, provoking the accumulation of ROS in embryogenic cells (Stasolla et al. 2004, dos Santos et al. 2012). However, ROS overproduction can disturb the cell redox system, affecting embryogenic differentiation (Kairong et al. 2002). Mitochondria face excessive ROS production utilizing energy dissipating systems composed of enzymes like NADH dehydrogenase, alternative oxidase (AOX) and plant uncoupling mitochondrial protein (PUMP), as well as some antioxidant enzymes (Pastore et al. 2007, Valente et al. 2012).

The differences observed in *A. angustifolia* cell lines with different embryogenic potential in the proteome level suggest that these differences would also be observed in the levels of the compounds which these proteins are related to. To analyze this hypothesis, we compared the polyamines levels (free and conjugate), ethylene production and ROS emission of both EC lines (R and B cell lines).

### Polyamine quantification

Despite the morphological similarities between the PEM III of R and B cell lines, the levels of total polyamines (free and conjugated) were significantly different during the proliferation phase. R cell lines showed lower values of total polyamines, when compared with the B cell line (Table 3). This difference was observed mainly because of higher values recorded for Put (free and conjugated forms) in the B cell line, whereas for Spd and Spm similar levels were found in R and B cell lines (Table 3). For both cell lines, Put was the main polyamine accumulated, followed by Spd and Spm.

In gymnosperms and angiosperms, polyamines have been associated with various cell developmental processes, including the differentiation and morphogenetic evolution of SEs (Baron and Stasolla 2008). For conifer zygotic and SE development, a reduction in Put levels concomitant with an increase in Spd and Spm levels is necessary for correct embryo development (Kong et al. 1998, Minocha et al. 1999, Stasolla and Yeung 2003). Similar behavior was observed during zygotic embryo development in *A. angustifolia* (Astarita et al. 2003).

During the proliferation phase, B cell lines accumulate higher levels of Put, when compared with R cell lines. Similarly, in *Quercus ilex* Lour. and *Pinus nigra* Aiton, high levels of Put were also associated with the inability of cell cultures to develop SEs (Noceda et al. 2009, Mauri and Manzanera 2011). In *A. angustifolia*, the high accumulation of Put in the B cell line is not linked to a non-conversion of Put to Spd and Spm, since B and R cell lines showed similar levels of free and conjugated Spd and Spm. In addition, ornithine decarboxylase and arginine decarboxylase activities are similar in both B and R cell lines (L.F. Oliveira, A.L.W. dos Santos, A.F. Macedo and E.I.S. Floh, unpublished results). The reasons for the differential accumulation of Put in R and B cell lines in *A. angustifolia* remain to be elucidated.

Similar to the values of individual polyamines, the Put:Spd ratio is also used as an indicator of cell lines with high embryogenic competence (Shoeb et al. 2001, Li and Burritt 2003, Santa-Catarina et al. 2006). Shoeb et al. (2001) suggest that optimal ratios of Put:Spd are critical for the development of SE. For *A. angustifolia*, ECs of the R cell line showed lower Put:Spd ratios when compared with ECs of the B cell line. Similar profiles were observed in *Solanum melongena* L. (Yadav and Rajam 1998), *Medicago sativa* L. (Huang et al. 2001), *Oryza sativa* L. (Shoeb et al. 2001) and *Dactylis glomerata* L. (Li and Burritt 2003). Although the Put:Spd ratio has been reported as a biomarker of embryogenic competence in many angiosperm species, the observation of this relationship in gymnosperms has never been reported. According to Klimaszewska et al. (2009), reductions in Put levels and increases in Spd levels are associated with a more differentiated state of the ECs.

A close relationship between the Put:Spd ratio and embryogenic competence suggests that the regenerative potential of *A. angustifolia* ECs could be manipulated by optimization of endogenous polyamine levels. Silveira et al. (2006) observed that in ECs of *A. angustifolia*, supplementation of the medium with Spd

Table 3. Endogenous levels ( $\mu\text{g g}^{-1}$  FW) of free and conjugated forms of polyamines Put, Spd and Spm, total polyamine levels and total Put:Spd ratio of *A. angustifolia* ECs of responsive (R) and blocked (B) cell lines.

	Put		Spd		Spm		Total polyamines	Put:Spd
	Free	Conjugated	Free	Conjugated	Free	Conjugated		
R cell line	3.93 $\pm$ 0.89 <sup>b</sup>	4.36 $\pm$ 0.28 <sup>b</sup>	2.56 $\pm$ 0.38 <sup>a</sup>	2.74 $\pm$ 0.44 <sup>a</sup>	0.94 $\pm$ 0.36 <sup>a</sup>	1.15 $\pm$ 0.99 <sup>a</sup>	15.7 $\pm$ 2.22 <sup>b</sup>	1.57 $\pm$ 0.15 <sup>b</sup>
B cell line	34.92 $\pm$ 6.16 <sup>a</sup>	20.39 $\pm$ 5.58 <sup>a</sup>	2.99 $\pm$ 0.77 <sup>a</sup>	3.46 $\pm$ 0.62 <sup>a</sup>	0.67 $\pm$ 0.15 <sup>a</sup>	1.03 $\pm$ 1.25 <sup>a</sup>	63.5 $\pm$ 8.81 <sup>a</sup>	8.67 $\pm$ 1.17 <sup>a</sup>

For each column, means followed by different lowercase letters are significantly different according to Student's *t*-test ( $P < 0.05$ ) (mean  $\pm$  SD;  $n = 3$ ).



during the proliferation phase resulted in an increase of Spd levels, a decrease in nitric oxide production, reduction of cellular growth and a more differentiated state of PEMs. Our results suggest the involvement of Put levels in the embryogenic competence of *A. angustifolia*. The Put:Spd ratio could be used in *A. angustifolia* as a biomarker for selection during the proliferation phase of cell lines with a high embryogenic capability.

### Ethylene quantification

Similar to the results observed with polyamine accumulation, significant differences were observed in the concentrations of ethylene released by R and B cell lines. ECs of the R cell line showed much higher concentrations of ethylene after 7 and 21 days in proliferation medium MSGO (Table 4). However, in both cell lines a progressive accumulation of ethylene was recorded with EC growth. As observed for *A. angustifolia*, ethylene was positively correlated with the development of SEs of *Picea mariana* Britton, Sterns & Poggenb. (El Meskaoui and Tremblay 2001), *Pinus sylvestris* L. (Lu et al. 2011), *M. truncatula* (Mantiri et al. 2008) and *M. sativa* (Kepczynska and Zielinska 2011). In some cases ethylene production had a negative effect on the development of SEs. In *P. abies*, high ethylene concentrations decreased the quality of SEs as a result of formation of intercellular spaces in the shoot apex, resulting in low percentages of conversion into plantlets (Kong and Yeung 1994). In *Brassica napus*, the inhibition of ethylene production increased the frequency of SEs produced from microspores (Leroux et al. 2009). Taken together, the results of polyamine accumulation and ethylene production in R and B cell lines underline the importance of SAM synthase for SE maturation in *A. angustifolia*.

### Intracellular ROS analysis

A rapid increase in ROS emission was observed in the R cell line after 20 min of measurement (Figure 4). This increase stabilized only after 4 h of measurement. In contrast, the B cell line showed low levels of ROS emission, and no significant differences in ROS emission were detected throughout measurement (Figure 4). Reactive oxygen species are important signaling molecules that act in plant responses to biotic and abiotic stresses (Delledone et al. 2001), and in the development of SEs of conifers (Zhang et al. 2010). A precise balance between ROS and ROS scavengers results in the activation of pro-

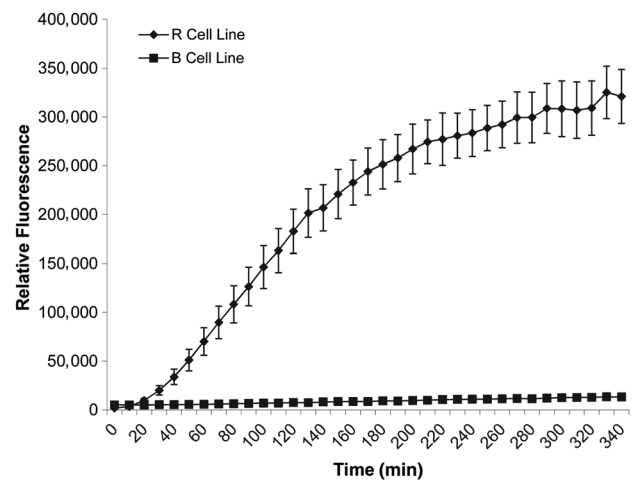


Figure 4. Intracellular ROS emission of *A. angustifolia* cell suspensions of responsive (R) and blocked (B) cell lines. (mean  $\pm$  SD;  $n = 3$ .)

grammed cell death (PCD) (Hoeberichts and Woltering 2002, Vianello et al. 2007, Parent et al. 2008), a crucial event for the trans-differentiation of PEMs to SEs in conifers (Filonova et al. 2000, Bozhkov et al. 2005). Similar results were also observed during SE development in Angiosperms. In *Mesembryanthemum crystallinum* L. and *Musa* spp., the generation of hydrogen peroxide and its regulation by the activation of antioxidant enzymes have been recorded at the beginning of SE formation (Lilik et al. 2005, Ma et al. 2012a, 2012b).

Apart from its function in the respiratory chain, some authors report the activity of an alternative complex of NADH dehydrogenase with the prevention of ROS generation and consequently inhibition of SE development (Møller 2002, Petrussa et al. 2009, O'Donnel et al. 2011). The presence of NADH dehydrogenase in the B cell line, and the observed levels of ROS suggest a differential antioxidant metabolism in this cell line. Recently, it was also demonstrated that PUMP proteins can also control ROS generation in ECs of *A. angustifolia* (Valente et al. 2012). However, no mention was made about the impact of this result on SE development. More studies are necessary to elucidate the importance of the cellular redox state during SE development in *A. angustifolia*.

A beta subunit of the protein ATPase was observed in the ECs of the R cell line (Spot 1) (Table 2). The presence of this protein suggests a high energetic metabolism in ECs of the cell line that showed the development of SEs. In plants (Vianello et al. 2007) and animals (Lemasters 1999) the energy status, particularly ATP levels, is crucial to the start of PCD. In animal cells, a decrease in ATP is recognized by the cell as a signal for PCD (Skulachev 2006). In cell suspensions of arabidopsis (Tiwari et al. 2002, Krause and Durner 2004) and tobacco BY-2 (Mlejnek et al. 2003), a rapid consumption of ATP was observed after the initiation of PCD. Thus, we can assume that the proper development of SEs in the R cell line could be linked

Table 4. Ethylene liberation (nmol ml<sup>-1</sup>) of 7- and 21-day-old ECs of *A. angustifolia* responsive (R) and blocked (B) cell lines.

	7-day-old	21-day-old
R cell line	2.77 $\pm$ 0.08 <sup>a</sup>	11.03 $\pm$ 1.96 <sup>a</sup>
B cell line	0 <sup>b</sup>	1.29 $\pm$ 2.24 <sup>b</sup>

For each column, means followed by different lowercase letters are significantly different according to Student's *t*-test ( $P < 0.05$ ) (mean  $\pm$  SD;  $n = 3$ ).



with an adequate PCD mechanism, which could be absent in the B cell line.

## Conclusions

We observed a marked difference between the biochemical composition in the embryogenic cells of the cell line responsive to maturation conditions and the cell line with blocked development of SEs. The observation of the enzyme SAM synthase in the cell line where SEs development was observed suggests the importance of this enzyme in *A. angustifolia* SE development. A contrasting difference in polyamine and ethylene levels between these two cell lines was recorded. We suggest the utilization of the differential biochemical parameters, particularly the Put:Spd ratio, as a biomarker for the early selection of *A. angustifolia* cell lines responsive to maturation conditions and able to develop SEs. The observation of a NADH dehydrogenase subunit, a possible ROS scavenger in the blocked cell line, suggests the importance of the cellular redox state in the formation of *A. angustifolia* SEs. Altogether, the data obtained in the present work open new perspectives on the optimization of culture conditions that improve responsiveness to SE maturation in *A. angustifolia* as well as other woody plants. Further studies including more cell lines whose genetic relationships are known should shed more light on the factors influencing embryogenic potential.

## Conflict of interest

None declared.

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