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BIOTECNOLOGIAS PARA O USO E CONSERVAÇÃO DE *Araucaria angustifolia* (Bertol.) Kuntze: CRIOPRESERVAÇÃO, ULTRAESTRUTURA E BIOQUÍMICA DE CULTURAS EMBRIOGÊNICAS

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À Leila do N. Vieira,
Por sua presença e apoio essenciais,
Ofereço.

À minha amada e saudosa mãe
Ademilde Pacheco (*in memoriam*),
Dedico.

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RESUMO

A conífera brasileira *Araucaria angustifolia* encontra-se dispersa no bioma Mata Atlântica e é conhecida popularmente como araucária ou pinheiro-brasileiro. Segundo a IUCN, a *A. angustifolia* encontra-se em perigo crítico de extinção, reforçando a necessidade de mais estudos que visem sua conservação e propagação. A cultura de tecidos vegetais engloba um conjunto de ferramentas biotecnológicas que possibilitam a conservação e melhoramento genético de plantas. A rota morfogenética *in vitro* de embriogênese somática (ES) pode ser empregada para a propagação massal clonal de genótipos de interesse, para a conservação de espécies ameaçadas de extinção ou para o estudo de processos fisiológicos, genéticos e bioquímicos envolvidos no desenvolvimento embrionário. O protocolo de ES para a *A. angustifolia* tem sido extensivamente estudado ao longo dos anos e encontra-se parcialmente desenvolvido, sendo seu principal limitante o processo de diferenciação e maturação das culturas embriogênicas (CE). Por outro lado, estudos envolvendo a criopreservação de CE desta espécie são ainda incipientes, apesar de toda sua importância estratégica na conservação de germoplasma *in vitro* de espécies recalcitrantes. Paralelamente, o cultivo *in vitro* e a suplementação do meio de cultura com reguladores de crescimento de plantas são conhecidos por afetar a metilação do DNA e os perfis de expressão de proteínas, modulando o fenótipo e/ou o potencial embriogênico. Neste contexto, o objetivo geral deste trabalho foi aprofundar os conhecimentos a respeito da ES e da criopreservação de CE de *A. angustifolia* visando à identificação e elucidação dos seus pontos de controle, por meio de estudos ultraestruturais e bioquímicos. Os resultados alcançados permitiram: a obtenção de um mapa de destino da ES de *A. angustifolia* através da técnica de *time-lapse cell tracking*, fornecendo informações relevantes a respeito da morfologia das CE durante as etapas de micropropagação e análises ultraestruturais dos tipos celulares que as compõem; a obtenção de um protocolo de criopreservação eficiente para as CE de *A. angustifolia*, aliado a análises morfológicas e ultraestruturais durante as etapas do protocolo; a análise dos níveis de metilação do DNA global das CE de *A. angustifolia* durante sucessivos ciclos de multiplicação em presença ou ausência de fitorreguladores, bem como a identificação de uma vasta gama de proteínas expressas diferencialmente nesses tratamentos através da técnica de proteômica *shotgun* revelou diferenças relevantes nos perfis de expressão protéica. Um maior detalhamento destes resultados e suas implicações encontra-se nos capítulos desta tese.

Palavras-chave: embriogênese somática, conífera brasileira, micropropagação, epigenética, conservação *in vitro*, proteômica *label-free*.

ABSTRACT

The Brazilian conifer *Araucaria angustifolia* naturally occurs in the Atlantic Forest Biome, and is popularly known as araucaria or Brazilian-pine. According to the IUCN, this species is critically endangered, reinforcing the need for further studies aimed at their preservation and propagation. Plant tissue culture encompasses a range of biotechnology tools that enable the conservation and plant breeding. The somatic embryogenesis (SE) morphogenetic route can be employed for mass clonal propagation of elite genotypes, conservation of endangered species, and for the study of physiological, genetic and biochemical processes involved in embryonic development. The SE protocol for *A. angustifolia* has been extensively studied over the past years and is partially developed. The differentiation process and embryogenic cultures (EC) maturation is this protocol limiting fator. Otherwise, studies involving the EC cryopreservation of this species are still incipient, in spite of its strategic importance in the *in vitro* germplasm conservation of recalcitrant species. In addition, *in vitro* tissue culture and culture médium supplementation with plant growth regulators are known to affect DNA methylation and protein expression profiles, modulating the phenotype and/or the embryogenic potential. In this context, the aim of this study was to deepen the knowledge about SE and EC cryopreservation of *A. angustifolia*, aiming at the identification and elucidation of its control points by means of ultrastructural and biochemical studies. The results achieved allow: obtaining a SE fate map of *A. angustifolia* by time-lapse cell tracking technique, providing relevant information about the EC morphology during the micropropagation stages and ultrastructural charaterization of cell types which constitute them; obtaining an efficient protocol for *A. angustifolia* EC cryopreservation, combined with morphological and ultrastructural analyses during steps of the protocol; analysis of global DNA methylation of *A. angustifolia* EC during successive multiplication cycles in the presence or absence of plant growth regulators as well as the identification of a wide range of proteins differentially expressed in these treatments by shotgun proteomics technique, revealing significant differences in protein expression profiles. More detailed results and their implicatins are presented in the specific section of this thesis.

Keywords: somatic embryogenesis, brazilian conifer, micropropagation, epigenetics, *in vitro* conservation, label-free proteomics.

LISTA DE FIGURAS

higher number and larger vacuoles, and thicker cell wall. D - Embryonal tube-like cell in detail showing large vacuole, Golgi bodies (GB), LB and mitochondrias. E – Suspensor cell, showing cytoplasmic degradation with large vacuoles throughout the cell space, with some isolated starch grains. F - Suspensor cell in detail showing thicker cell wall and starch grains. **Página 50**

Fig. 2 Ultrastructural features of different mitochondrial morphologies observed in embryogenic cells of *A. angustifolia*. A – General view of different morphologies found in the embryogenic cell, with sickle-like shape altered mitochondria (AM), and donut-shaped mitochondria (DM). B – Detail of different mitochondria morphology, with a typical cylindrical shape with well-packed and organized cristae (M), altered mitochondria appeared swollen, with cristae more prominent, and donut-shaped mitochondria. **Página 51**

Fig. 3 Overview of different cell fractions used in time-lapse cell tracking of *A. angustifolia*. A – 80-200 μm cell fraction showing numerous pro-embryogenic masses (PEM) I. B – 200-400 μm cell fraction showing mainly PEM I and II. C – 400-600 μm cell fraction showing PEM I, II, and III. >600 μm cell fraction showing mainly PEM III. **Página 53**

Fig. 4 Time–lapse tracking of different cell fractions of *A. angustifolia* embryogenic cultures during proliferation cycle. Cells aggregates found in fraction 200-400 μm started to proliferate from day 2 of culture, and with 12 days in culture, numerous PEM III could be observed. For the >600 μm fraction could be observed a large number of PEM III from the date of inoculation showing no proliferation after 12 days in culture, allied to a lower abundance of PEM I. **Página 54**

Fig. 5 Time–lapse tracking of *A. angustifolia* embryogenic cultures (EC) during maturation treatments (ABA-free and with ABA). ABA-free maturation was able to induce a polarization process in PEM III, with pro-embryos formation after 14 days of culture (arrows). The EC transfer directly to the maturation with ABA apparently started a process of cell death after 14 days in culture, becoming more translucent with no cell proliferation signal or morphological change after 28 days in culture. **Página 55**

Fig. 6 Histological analysis of the *A. angustifolia* somatic embryos development. A – Early globular (EG) somatic embryo with well-developed protoderm. B - Late globular-staged somatic embryo (LG) showing a layer of embryonal tube cells and suspensor-like cells. Note the presence of more vacuolated cells in the basal part of the embryo. C – Torpedo-staged somatic embryo showing meristematic cells in the apical part with embryonal tube cells in the middle part until the basal part consisting of suspensor cells. **Página 58**

CAPÍTULO 2

Fig. 1 Fresh mass increased ratio of *A. angustifolia* embryogenic cultures subjected to 0, 30, 60, 120 and 240 minutes of incubation in cryoprotectant solution after 2, 9, 16 and 23 days of culture in proliferation culture medium. Mean values followed by ± standard deviation (vertical bars). Uppercase letters represent differences between cryotreatment incubation times for each evaluated date, according to SNK test ($p < 0.05$). **Página 74**

Fig. 2 *A. angustifolia* embryogenic cultures features subjected to 0, 30, 60, 120 and 240 minutes of incubation in cryoprotectant solution after 2, 9, 16 and 23 days of culture in proliferation culture medium. Bar: 1 cm. **Página 75**

Fig. 3 Time–lapse cell tracking of *A. angustifolia* embryogenic cultures (EC) from two different cell lines (Cr01 and Cr02) subjected to 60 and 240 min cryoprotection incubation times, respectively, and then to cryopreservation procedures. The EC of both cell lines evaluated indicated the first cell regrowth signs after 30 days in culture (arrows), and after day 40 it was possible to observe a more pronounced cell proliferation. Note that the embryogenic cells were the major cell type that had proliferation in the initial culture phase after thawing. **Página 79**

Fig. 4 *A. angustifolia* embryogenic cultures (EC) features subjected to the cryopreservation process observed with double staining with acetic carmine and Evans blue in the different protocol steps. a) EC derived from proliferation cycles (Control); b) EC only subjected to 60 min cryotreatment; c) EC after 60 min cryotreatment and slow cooling in

Mr. Frosty, but not immersed in LN; d) cryotreated EC for 60 min subjected to slow cooling in Mr. Frosty, freezing in LN and then thawed. Three representative and independent samples were stained and evaluated per point of the cryopreservation protocol. **Página 81**

Fig. 5 *A. angustifolia* embryogenic cultures (EC) features subjected to the cryopreservation visualized with fluorescein diacetate vital staining in the different protocol steps. In the image are showed EC derived from proliferation cycles (Control); EC only subjected to 60 min cryotreatment (Cryotreated); EC after 60 min cryotreatment and slow cooling in Mr. Frosty, but not immersed in LN (After Mr. Frosty); cryotreated EC for 60 min subjected to slow cooling in Mr. Frosty, freezing in LN and then thawed (After thawing). Three representative and independent samples were stained and evaluated per point of the cryopreservation protocol. **Página 82**

Fig. 6 Ultrastructural analyses by transmission electron microscopy of *A. angustifolia* embryogenic cultures (EC) during different cryopreservation steps. a) and b) embryogenic cell from proliferation cycles showing meristematic cell features, with large and prominent nuclei, abundant presence of mitochondria and thin cell wall. c) and d) embryogenic cell from EC only subjected to 60 min cryotreatment in detail showing fewer small vacuoles, preserved nuclear envelope, amyloplast containing starch grain, and thickened and preserved cell wall. An improved presence of heterochromatin regions was also observed. e) embryogenic cell from EC that survived to cryopreservation recovery indicating preserved nuclear envelope with nuclei containing a huge amount of heterochromatin regions and thickened preserved cell wall. In this cell was also possible to observe a more irregular conformation of the plasma membrane, possibly due to severe dehydration caused by cryopreservation process. f) embryogenic cell that did not survived to the cryopreservation indicating cytoplasmic degradation and disintegration. V: vacuole; N: nuclei; Nu: nucleolus; M: mitochondria; A: amyloplast; GB: Golgi bodies; CW: cell wall; He: heterochromatin. **Página 83**

CAPÍTULO 3

Fig 1. Morphological features of plant material used for DNA methylation and proteomic analyses. A- Early globular-staged *A. angustifolia* zygotic embryos (explant used for somatic embryogenesis induction); B – Representative embryogenic culture (EC) obtained after 30 days culture in induction culture media; C - Representative EC derived from plant growth regulators-free treatment during multiplication step; D - Representative EC derived from plant growth regulators-supplemented treatment during multiplication step. **Página 99**

Fig 2. Global DNA methylation levels after somatic embryogenesis induction (cycle 1) and during successive subculture cycles of *A. angustifolia* embryogenic cultures subjected to plant growth regulators-free (A) and –supplemented treatments. Mean values followed by standard deviation (vertical bars). Means followed by lowercase letters are significantly different according to the SNK test ($p < 0.05$). **Página 104**

Fig 3. Volcano plot for expressed proteins of *A. angustifolia* embryogenic cultures from Cr01 cell line contrasting expression ratio between plant growth regulators (PGR)-supplemented/-free treatments. Green dots represent significant up-regulated proteins, red dots significant down-regulated proteins and blue dots unchanged expression level found between PGR-supplemented and –free treatments. **Página 105**

Fig 4. Functional classification of identified proteins in *A. angustifolia* embryogenic cultures from Cr01 cell line in both plant growth regulators treatments (supplemented or not) using Blast2GO software based on universal gene ontology (GO) annotation terms. The proteins were linked to at least one annotation term within the GO biological process (A), molecular function (B), and cellular component (C) categories. **Página 108**

LISTA DE TABELAS

SUMÁRIO

INTRODUÇÃO, JUSTIFICATIVA E ANTECEDENTES

As coníferas representam cerca de 650 espécies divididas em sete famílias (Araucariaceae, Cephalotaxaceae, Cupressaceae, Pinaceae, Podocarpaceae, Sciadopityaceae, Taxaceae). Algumas espécies desse grupo são os maiores e mais antigos organismos terrestres presentes no planeta (DOYLE, 1998; HENRY, 2005; AHUJA & NEALE, 2005). Além disso, as coníferas apresentam expressiva importância ecológica, pois dominam muitas paisagens terrestres e são os organismos terrestres com o maior potencial de fixação de carbono atmosférico. Elas estão presentes em um grande número de ecossistemas devido à aquisição de um eficiente sistema fisiológico de adaptação durante o processo de evolução (BECWAR et al., 1988).

Araucaria angustifolia (Bertol.) Kuntze é uma conífera dióica e perenifólia, que ocorre exclusivamente na América do Sul, na região da Floresta Ombrófila Mista, formação florestal que recebe seu nome (Floresta de Araucária) devido à abundância e ao grande porte da espécie, que imprime a fisionomia característica da floresta. Esta espécie é endêmica das regiões Sul e Sudeste do Brasil, com extensões em pequenas manchas no noroeste da Argentina e Paraguai, em áreas próximas às fronteiras brasileiras, onde encontra condições ideais para o desenvolvimento em altitudes entre 500 e 1800 m (CARVALHO, 1994; JUDD et al., 2009).

Do ponto de vista econômico e social, suas sementes servem de alimento com grande valor nutricional, sendo que a coleta e a venda destas sementes se constituem em relevante atividade econômica para um considerável número de famílias que vivem nas regiões de ocorrência (CARVALHO, 1994). Esta espécie também possui madeira de alta qualidade, motivo pelo qual, no início do século XX, foi altamente explorada (GUERRA et al., 2008), sendo empregada especialmente para construção civil, de móveis, e para a produção de celulose (CARVALHO, 1994).

Devido à drástica redução em sua área de ocorrência, em torno de 2% da sua extensão original, o pinheiro-brasileiro atualmente consta na lista internacional da IUCN de espécies ameaçadas como em perigo crítico de extinção (www.iucnredlist.org). Porém, mesmo após a inclusão da *A. angustifolia* na lista oficial de espécies brasileiras ameaçadas de extinção, remanescentes florestais com esta espécie continuam a ser explorados (STEFENON et al., 2009). Neste contexto, a

A. angustifolia é uma das espécies nativas do Brasil com grande potencial para estudos que auxiliem sua conservação genética, através do estabelecimento de coleções de germoplasma *in situ* e *ex situ* (GUERRA et al., 2008).

A biotecnologia oferece um conjunto de técnicas que oportunizam o estudo de células, tecidos, órgãos ou do organismo inteiro, por meio da indução e controle da morfogênese *in vitro* (RAO; RAVISHANKAR, 2002). Dentre as biotecnologias que visam à conservação e melhoramento genético de espécies vegetais destaca-se a micropropagação, ferramenta importante e de larga aplicação para a conservação da biodiversidade e programas de melhoramento. A micropropagação engloba um conjunto de técnicas que possibilitam a propagação massal de genótipos selecionados, permitindo a captura e fixação de ganhos genéticos (GUERRA; TORRES; TEIXEIRA, 1999), bem como a conservação de germoplasma. A aplicação desta técnica é dependente da indução e controle da morfogênese *in vitro* em suas duas rotas: a organogênese e a embriogênese somática.

A embriogênese somática (ES) é uma rota regenerativa *in vitro* pela qual células isoladas ou um pequeno grupo de células somáticas dão origem a embriões (TAUTORUS; FOWKE; DUNSTAN, 1991). Sendo uma expressão da totipotência, a ES envolve a desdiferenciação de uma ou mais células somáticas e subsequente rediferenciação (reprogramação), resultando na produção de células características de uma planta madura (ROSE et al., 2010).

Atualmente é bem aceito de que a chave do desenvolvimento que resulta na embriogênese somática é acionada em células temporariamente expostas a altos níveis de estresse e/ou altas concentrações não fisiológicas de reguladores de crescimento, entre outros fatores (FEHÉR, 2015). De acordo com este modelo, as condições indutoras resultam na desdiferenciação das células somáticas, seguidas, ou em paralelo, com a reaquisição da totipotência de desenvolvimento. Nesta fase totipotente, as células são competentes para perceber sinais de desenvolvimento apropriados que as "comprometerão" para uma rota embriogênica, que posteriormente prossegue de forma autónoma sob condições permissivas *in planta* ou *in vitro*, culminando na formação do embrião somático (Figura 1).

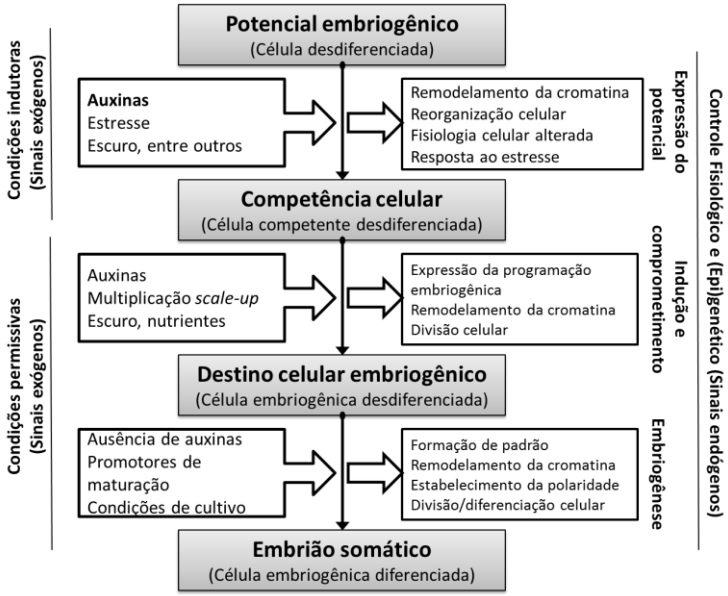

Figura 1. Sequência hipotética de eventos subjacentes à embriogênese somática (ES) em plantas. Vários sinais (endógenos e exógenos), incluindo auxinas, promotores de maturação, estresse, provocam uma resposta celular ampla, incluindo reorganizações em nível de estrutura celular, fisiologia, cromatina e expressão gênica. As principais etapas de modulação e controle da ES envolvem condições indutivas e permissivas, dirigindo células competentes a um destino celular embriogênico, um período que precede o início da embriogênese em si. Todos estes eventos são permeados por alterações (epi)genéticas e fisiológicas, que agem como moduladores da ES (Adaptado de FEHÉR, 2006).

A ES segue um padrão morfogenético análogo ao dos embriões zigóticos formados após a fertilização da célula ovo, apresentando estádios similares de desenvolvimento embrionário (ZIMMERMANN, 1993; GOLDBERG; DE PAIVA; YADEGARI, 1994). Não sendo limitada pela quantidade de tecido ou acessibilidade, como ocorre na embriogênese zigótica, a ES pode ser amplamente investigada por fornecer um sistema modelo para o estudo de aspectos morfológicos, fisiológicos, moleculares e bioquímicos relacionados ao processo de desenvolvimento da planta (ZHANG; LI; KONG, 2007; KARAMI; SAIDI, 2010).

A ES oferece várias vantagens quando comparada a outros sistemas de micropropagação, incluindo a sua alta taxa de multiplicação, a possibilidade de criopreservação de culturas embriogênicas, bem como o potencial para *scale-up* das culturas em suspensão. Permite ainda o uso de biorreatores e tecnologias de sementes sintéticas, além do fato de que as culturas embriogênicas são tecidos-alvo adequados para a transferência de genes específicos (CARNEROS et al., 2009; KARAMI; SAIDI, 2010).

Apesar do amplo campo de estudo que a ES proporciona seus mecanismos moleculares permanecem pouco compreendidos, sendo a maior parte dos estudos realizados focados principalmente em sua abordagem hormonal (ROSE et al., 2010). O paradigma clássico se configura na auxina inicialmente necessária para a indução da ES, enquanto que a sua retirada posterior, ou a redução da sua concentração, impulsiona o desenvolvimento de embriões (ROSE et al., 2010).

Na ES de coníferas são reconhecidas três fases principais: a) a fase pró-embrionária (estádios antes do alongamento do suspensor) que vai desde a fertilização até o rompimento do arquegônio pelo pró-embrião; b) embrionária inicial, compreendendo os estádios após o alongamento do suspensor e antes do estabelecimento dos meristemas; e c) embrionária tardia, na qual protoderme e procâmbio são originados e ocorre o estabelecimento dos meristemas apical e radicular (HAINES; PRAKASH, 1980).

A micropropagação de várias coníferas tem sido realizada com sucesso por meio da ES, principalmente as pináceas, como as espécies do gênero *Picea* (LI et al., 2008). Porém, as gimnospermas são, em geral, mais recalcitrantes à propagação *in vitro* que a maioria das angiospermas (VON ADERKAS; BONGA, 2000). Em decorrência disso, as culturas embriogênicas de gimnospermas têm sido obtidas em sua maioria a partir de embriões imaturos e jovens, os quais possuem tecidos que em geral respondem mais eficientemente ao processo de indução da desdiferenciação (STASOLLA et al., 2002).

Para a *A. angustifolia*, o processo básico de ES é caracterizado pela indução de culturas embriogênicas, originadas a partir do ápice do embrião zigótico imaturo. Neste processo são utilizados embriões zigóticos no estádio globular inicial, onde o conteúdo endógeno de auxinas é mais elevado, principalmente o ácido indolacético (ASTARITA et al., 2003). Dessa forma, o embrião é inoculado *in vitro* na presença ou não de auxinas e citocininas exógenas, resultando na formação de pró-embriões que caracterizam os estádios iniciais da

embriogênese (Figura 2). A formação de embriões somáticos é estimulada quando sinais químicos de ajuste osmótico (polietilenoglicol e maltose) e hormonal (ácido abscísico) são fornecidos aos pró-embriões durante a etapa de maturação (DOS SANTOS et al., 2002). Destaca-se que, as massas embriogênicas de coníferas são formadas por massas pró-embrionárias (PEMs). A partir das PEMs os processos de formação, maturação, dessecação e regeneração dos embriões somáticos são obtidos (VON ARNOLD et al., 2002).

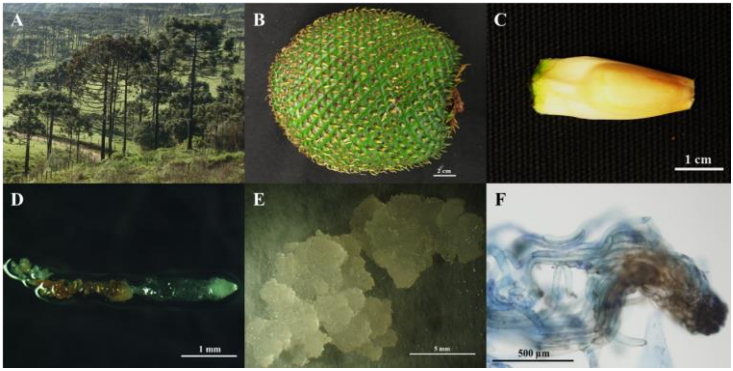

Figura 2. Etapas da indução da embriogênese somática (ES) em *A. angustifolia*. A: Indivíduos adultos de *A. angustifolia* em uma população natural em São Joaquim/SC; B: Cone feminino imaturo; C: Semente imatura destacada do cone feminino imaturo utilizada como fonte de explante para a indução da ES; D: Embrião zigótico em estádio globular excisado da sementes imatura; E: Cultura embriogênica obtida após o processo de indução da ES durante a fase de multiplicação; F: Embrião somático globular tratado com azul de Evans e carmim acético.

Sabe-se que o processo de ES é altamente dependente do estádio de desenvolvimento do explante utilizado para indução, do genótipo da planta mãe e das condições de cultivo (DOS SANTOS et al., 2002). Atualmente, em algumas linhagens celulares de *A. angustifolia* pode ser observada a formação de poucos embriões globulares, que raramente evoluem para estádios de desenvolvimento torpedo e pré-cotiledonar. Assim, embora uma série de avanços tenha sido gerada até o momento, a regeneração de plântulas por esta rota morfogenética ainda não foi obtida por completo. Apenas embriões em seu estádio de desenvolvimento inicial têm sido formados, sendo o processo de

maturação dos embriões o atual limitante da técnica (DOS SANTOS et al., 2008; FRAGA et al., 2015).

Uma vez estabelecidas, as culturas embriogênicas requerem subcultivos periódicos para a manutenção tanto de um elevado potencial proliferativo quanto a sua capacidade de se desenvolverem em pró-embriões somáticos (LAMBARDI; OZUDOGRU; BENELLI, 2008). Repicagens sucessivas não apenas consomem tempo e são trabalhosas, mas também podem culminar na perda das culturas devido à contaminação, erros humanos ou falhas técnicas. Além disso, a perda de potencial embriogênico e a ocorrência de alterações genéticas e/ou epigenéticas devido à repicagem em longo prazo tem sido frequentemente relatada (BHATTI et al., 1997; HARDING, 2004). Sendo assim, o desenvolvimento e a otimização de protocolos eficentes para a conservação de culturas embriogênicas com o objetivo de reduzir a frequência das manipulações e, consequentemente, os riscos de deterioração, perda ou alterações (epi)genéticas são de importância estratégica (LAMBARDI; OZUDOGRU; BENELLI, 2008).

Neste sentido, a criopreservação, que consiste no armazenamento de material biológico a temperaturas ultra-baixas, é uma das principais alternativas disponíveis para assegurar a conservação de germoplasma de forma segura e eficiente em longo prazo (ENGELMANN, 2004). Além das vantagens já apresentadas, a criopreservação é o único método capaz de armazenar recursos genéticos de plantas economicamente e ecologicamente importantes que produzem sementes recalcitrantes (sensíveis à dessecação) que, portanto, não podem ser desidratadas ao nível de umidade suficientemente baixa para permitir seu armazenamento a baixas temperaturas (HAZUBSKA-PRZYBYŁ et al., 2013), caso de inúmeras espécies nativas do Brasil, dentre elas a *A. angustifolia*.

Tanto o método de congelamento lento quanto os procedimentos de imersão direta em nitrogênio líquido têm sido testados com sucesso em culturas embriogênicas de várias espécies de plantas (PANIS; LAMBARDI, 2006; ENGELMANN, 2011). No entanto, o método tradicional de congelamento lento ainda é a abordagem mais comum para a criopreservação de culturas embriogênicas, e tem sido aplicada para diversas espécies de coníferas (LAMBARDI; OZUDOGRU; BENELLI, 2008). Recentemente, foi reportado um protocolo para a criopreservação de culturas embriogênicas de *A. angustifolia*, em que os autores avaliaram dezessete tratamentos crioprotetores, com base na combinação de diferentes soluções osmóticas, e dois métodos de

criopreservação (DEMARCHI et al., 2014). No entanto, maiores estudos focados na otimização desse processo se fazem necessários.

Sabe-se que a ES é acompanhada por dramáticas mudanças nos componentes celulares, tais como proteínas, fitormônios, poliaminas e polissacarídeos. Estas alterações requerem a expressão de vários genes, que são necessários para a síntese ou a mobilização destes compostos, e esta regulação gênica pode ser afetada por mecanismos epigenéticos, como o remodelamento da cromatina e a metilação do DNA. O crescimento e o desenvolvimento são regulados por hormônios vegetais específicos, sendo a modulação da metilação do DNA um dos mecanismos moleculares de ação hormonal na planta (VANYUSHIN et al., 2004).

O termo metilação do DNA refere-se à metilação pós-síntese de deoxicitosinas na posição 5' do anel de pirimidina da citosina para formar a metildeoxicitosina (FINNEGAN, 2010). Essa modificação pode virtualmente ocorrer em qualquer base, mas a transmissão dos padrões de metilação só ocorre em seqüências simétricas CpG, CpNpG, ou CpNpN, onde N pode ser qualquer deoxinucleotídeo. Nas plantas, a metilação do DNA é mais comum em ilhas CpNpG, característica de transposons, contribuindo ainda mais para o nível de metilação da citosina, especialmente devido à elevada presença destes elementos em genomas de plantas (VALLEDOR et al., 2007).

A quantificação da 5-metildeoxicitosina (5mdC) estabelece certos níveis de metilação global do DNA como marcadores para os processos de crescimento e de desenvolvimento (FRAGA et al., 2012). Além disso, do ponto de vista molecular, as variações de metilação global do DNA podem indicar que a estrutura da cromatina sofreu alterações durante determinados processos de desenvolvimento da planta (VALLEDOR et al., 2007).

Diversos trabalhos têm descrito que a proliferação de células desdiferenciadas, após o processo de indução da ES, durante longos períodos de subcultivo podem levar a uma perda gradual da estabilidade genética e epigenética dos regenerantes (ETIENNE; BERTRAND, 2003; SMULDERS; DE KLERK, 2011; NEELAKANDAN; WANG, 2012). De fato, a capacidade das culturas celulares em se rediferenciarem através da ES é gradualmente perdida ou alterada ao longo dos sucessivos subcultivos, sendo esses mecanismos associados à instabilidade não muito bem elucidados (RIVAL et al., 2013). O aumento no número de subcultivos e sua duração também pode causar o

aumento do surgimento de variantes somaclonais, especialmente em células em suspensão e cultura de calos (RIVAL et al., 2013). Neste sentido, é possível supor que alterações na metilação do DNA genômico, como um componente dos mecanismos epigenéticos que regulam a expressão gênica, estão envolvidas na modulação da expressão da competência embriogênica das culturas celulares obtidas através da indução da ES.

Células capazes de transitar do estado somático para o embriogênico são chamadas células competentes, ou seja, capazes de reagir a sinais específicos do desenvolvimento. Para entender os eventos associados ao processo de morfogênese *in vitro* dos embriões somáticos, alterações bioquímicas relacionadas devem ser elucidadas.

A identificação de mais proteínas e genes que atuam nas fases iniciais do desenvolvimento de plantas é um pré-requisito para uma melhor compreensão das redes regulatórias determinantes neste processo, sendo a análise das proteínas a abordagem mais direta para definir as funções dos respectivos genes (YIN et al., 2007). O desenvolvimento de diversas ferramentas computacionais e de bioinformática para a integração da proteômica com outras "ômicas" e o levantamento de dados fisiológicos tem possibilitado estudos de sinalização, regulação e redes metabólicas fundamentais para o fenótipo da planta (KITANO, 2002; Figura 3).

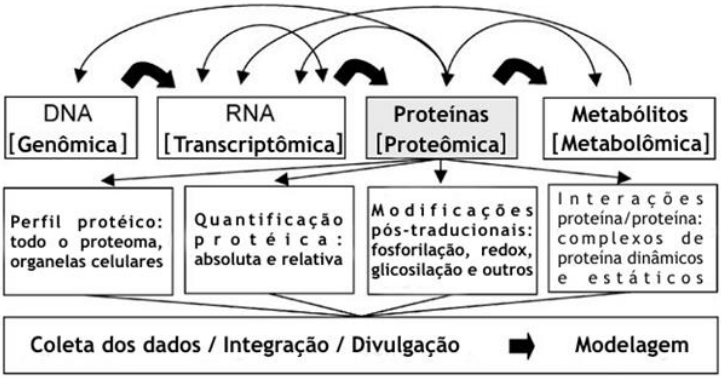

Figura 3. A proteômica tem um papel central nos sistemas biológicos porque complementa a análise do transcriptoma e do metaboloma (Adaptado de BAGINSKY, 2008).

O uso de proteínas como marcadores para a ES tem sido descrito com o objetivo de relacionar os estágios embriogênicos e suas alterações nos perfis protéicos com o desenvolvimento embrionário (MARSONI et al., 2008). Neste contexto, através de análises proteômicas têm sido possível a identificação de marcadores bioquímicos para os mais diversos processos associados a ES, como a diferenciação entre culturas embriogênicas e não-embriogênicas (MARSONI et al., 2008).

Estudos anteriores associados à embriogênese zigótica de *A. angustifolia* foram realizados visando analisar os padrões de acúmulo de quitinases e proteínas arabinogalactanas (DOS SANTOS et al., 2006), e também de proteômica comparativa dos estádios de desenvolvimento do embrião zigótico (BALBUENA et al., 2009). Além disso, esforços recentes têm sido realizados para descrever a embriogênese somática de *A. angustifolia* a nível molecular, com a utilização de abordagens proteômicas e transcriptômicas (JO et al., 2013; ELBL et al., 2015). No entanto, estudos proteômicos focados na resposta das culturas embriogênicas desta espécie em multiplicação mantidas ou não em meio de cultura suplementado com fitorreguladores ainda não foram realizados.

A tecnologia de identificação multidimensional de proteínas ("MudPIT") têm se tornado uma abordagem popular no contexto da proteômica *shotgun*, possibilitando uma separação ortogonal de alta resolução por cromatografia líquida de alta performance acoplada a espectrometria de massa em tandem (2D-nanoLC-MS/MS) (CHEN et al., 2006). Esta tecnologia tem permitido a identificação de proteínas em larga escala e também aquelas pouco abundantes, que em muitas vezes são perdidas nas técnicas que envolvem géis de eletroforese bidimensional (2-DE) (WASHBURN; WOLTERS; YATES, 2001). Neste sentido, a utilização de ferramentas proteômicas de alta resolução no estudo das alterações na expressão proteica de culturas embriogênicas de *A. angustifolia* pode auxiliar na elucidação de aspectos relacionados ao seu desenvolvimento embrionário, com a identificação de proteínas específicas ou expressas diferencialmente.

Dessa forma, no presente trabalho de tese, buscou-se avançar nos conhecimentos a respeito da ES nesta espécie, visando sua melhor caracterização e uso como estratégia de conservação e propagação *in vitro*, através do uso de diversas abordagens complementares. Sendo assim, a tese foi estruturada em três capítulos: no primeiro capítulo, análises morfológicas através da técnica de *cell tracking* e análises

ultraestruturais foram realizadas durante a ES de *A. angustifolia*, gerando novos *insights* a respeito desta rota morfogética e uma nova caracterização dos tipos celulares que compõem as culturas embriogênicas; no segundo capítulo, um protocolo otimizado de criopreservação das culturas embriogênicas foi descrito, aliado a caracterização morfológica e ultraestrutural das culturas ao longo do processo, permitindo a elucidação de aspectos relacionados ao comportamento dessas células durante cada etapa do protocolo de criopreservação; no terceiro e último capítulo, a análise da metilação global nas culturas embriogênicas durante a indução da ES e sucessivos ciclos de multiplicação em meio de cultura suplementado ou não com fitorreguladores permitiu avaliar a dinâmica deste processo ao longo de um ano de subcultivos. Aliado a isso, a identificação de uma vasta gama de proteínas expressas diferencialmente nesses tratamentos através da técnica de proteômica *shotgun* revelou diferenças relevantes nos perfis de expressão protéica. Ao final, as considerações finais e perspectivas futuras do trabalho são apresentadas.

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CAPÍTULO 1

Este manuscrito encontra-se publicado no periódico *Trees: Structure and Function*

Time-lapse cell tracking reveals morphohistological features in somatic embryogenesis of *Araucaria angustifolia* (Bert) O. Kuntze

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Abstract

Araucaria angustifolia has been extensively studied as a model system for somatic embryogenesis (SE). This protocol is characterized by the development of embryogenic cultures (EC), which are multiplied in pro-embryogenic masses (PEM). However, it hampers in the maturation of somatic embryos from PEM. The building of a SE fate map, allowing the analysis of individual stages of embryonic development, may help to identify the morpho-histological features that are causing failure in the maturation process. In this sense, the aim of this work was to characterize by means of time-lapse cell tracking the process of proliferation and maturation of *A. angustifolia* EC. We also performed transmission electron microscopy in EC and light microscopy analysis in early somatic embryos obtained. The TEM analysis showed a novel characterization of the different cell types that constitute the PEMs and the identification of intriguing mitochondrial structural morphology. In the cell tracking results, smaller cell aggregates showed to be more suitable for transfer to maturation step. Accordingly, the use of smaller cell aggregates together with a high osmotic potential culture medium during maturation phase I (ABA-free), and subsequently transfers to the maturation phase II (with ABA) proved to be more suitable for early somatic embryos obtainment. Moreover, the direct transfer of EC from proliferation to maturation with ABA seems to inhibit the further development of somatic embryos. Finally, the early somatic embryos characterized by light microscopy revealed the presence of intercellular spaces, which tended to develop poorly organized shoot apical meristems and abnormal embryos.

Keywords conifer, micropropagation, somatic embryo, altered mitochondria morphology.

Introduction

Araucaria angustifolia is a dioecious perennial conifer native to South America, occurring in South and Southeastern Brazil, and restricted areas of Northwestern Argentina and Paraguay (Guerra et al. 2000). Drastic population decline and habitat reduction culminated in the inclusion of *A. angustifolia* on the IUCN international list as "critically endangered" (IUCN 2014). However, the remaining forests of this species continue to be explored from commercial and scientific perspectives (Stefenon et al. 2009). In this context, *A. angustifolia* is a species with great potential for studies to support genetic conservation (Santos et al. 2010; Dutra et al. 2013). The application of tissue culture tools in this species, such as somatic embryogenesis (SE), is one of the most promising techniques for its conservation and mass propagation (Guerra et al. 2000).

Somatic embryogenesis is an ontogenetic process by which mitotically quiescent somatic cells can recover their embryogenic potential and produce new viable embryos, by reprogramming of gene expression (Marsoni et al. 2008). As an expression of totipotency, the SE involves dedifferentiation of a nonzygotic cell and subsequent redifferentiation (reprogramming), resulting in the production of all cells characteristic of the mature plant (Rose et al. 2010).

Somatic embryogenesis follows a similar morphogenetic pattern to zygotic embryos formed after fertilization of the egg cell, with similar stages of embryonic development (Zimmermann 1993; Goldberg et al. 1994). Unlike zygotic embryos, somatic embryos can be easily manipulated and growing conditions can be controlled. These features make the SE an efficient model system for the study of morphological, physiological, molecular and biochemical aspects that occur during the initiation and development in higher plants (Zhang et al 2007; Karami and Saidi 2010).

In general, SE in conifers includes five main steps: initiation of embryogenic cultures (EC) on a primary explant inoculated on culture medium generally supplemented with plant growth regulators (PGR), particularly auxins and cytokinins; multiplication of EC in liquid or on semi-solid medium culture, most often supplemented with auxins and cytokinins; maturation of somatic embryos in two different phases, maturation phase I and II. The maturation phase I consisting of PGR-free culture medium, in order to inhibit proliferation and promote cell differentiation, and the phase II of somatic embryos maturation in

culture medium supplemented with abscisic acid (ABA) and high osmotic potential; finally, plantlet development and conversion in PGR-free culture medium (von Arnold et al. 2002).

The early SE process in *A. angustifolia* is well characterized by the development of EC, which, in turn, are multiplied as pro-embryogenic masses (PEM) during the early stages of SE. This step is successfully achieved; however, further protocol optimization is required because an unknown factor hampers further maturation of somatic embryos from PEM (Vieira et al. 2012).

The maturation phase of *A. angustifolia* SE has been investigated over the past years by our research group, and different culture conditions have been employed, by testing osmotic agents, ABA, activated charcoal (AC), and other compounds. The first report of obtaining somatic pro-embryos in *A. angustifolia* after a maturation treatment used osmotic agents and ABA in the culture medium (Astarita and Guerra 1998; 2000). In the sequence, pro-embryos and globular-staged somatic embryos were obtained in response to the inclusion of osmotic agents in the culture medium, but independent on application of ABA (Santos et al. 2002; Silveira et al. 2002; Steiner et al. 2005; Santos et al. 2008; Vieira et al. 2012). Recently, it was reported in the maturation phase the use of osmotic agents, AC, and 120 µM ABA (Schlogl et al. 2012). Jo et al. (2013) proposed the inclusion of maturation phase I, consisting of culture medium with osmotic agents and AC for 30 days followed by maturation phase II, with AC and 120 µM ABA. However, to date, it has not been clearly demonstrated how the different EC cell aggregates morphologically respond when directly exposed to ABA (phase II), or when submitted to maturation phase I followed by phase II.

Considering that, the successive developmental stages leading to the formation of mature somatic embryos in conifers must be understood primarily for optimal management of somatic plant regenerative systems (Filonova et al. 2000a). Ideally, this task has to be accomplished by building a fate map of SE, including an adequate number of morphological and molecular markers specifying different stages in the whole process (Strehlow and Gilbert 1993; Filonova et al. 2000a). In addition, the construction of this map allows an analysis of individual stages of embryonic development. Specifically for *A. angustifolia*, the target is the identification of the morphological features

that may be causing failure in the proper maturation process of the initial somatic embryos obtained.

In this sense, the aim of the present work was to characterize by means of time-lapse cell tracking the processes of proliferation and maturation of *A. angustifolia* EC in order to identify cell types and aggregates present in the EC, and the way they respond to each step of the protocol. Along with that, we performed transmission electron microscopy analysis enabling the ultrastructural characterization of the cell types present in the EC. Additionally, light microscopy analysis was realized in globular-and torpedo-staged embryos.

Materials and methods

Somatic embryogenesis induction and proliferation

Embryogenic cultures were induced according to the methodology described by Santos et al. (2002). Immature female cones bearing globular-staged zygotic embryos were collected in December 2013, from an *A. angustifolia* natural population in Lages, Santa Catarina – Brazil (latitude 27° 49′ 0″, longitude 50° 19′ 35″, altitude 930 m). The seeds were submitted to disinfestation procedures with 70% ethanol for 5 minutes and 40% sodium hypochlorite for 15 minutes, followed by a triple-washed with autoclaved distilled water. Zygotic embryos were excised and inoculated in Petri dishes containing 25 ml of culture medium. The culture medium consisted of BM macro- and micro-salts (Gupta and Pullman 1991) supplemented with L-glutamine (1.0 g l^{-1}), myo-inositol (1.0 g l^{-1}), casein hydrolysate (0.5 g l^{-1}), Phytagel® (2 g l^{-1}) and sucrose (30 g l^{-1}). The pH of culture medium was adjusted to 5.8 and autoclaved at 121°C, 1.5 atm for 15 min. All the cultures were maintained in a growth room in the absence of light at temperature of 22 ± 2°C.

After 30 days, the EC were subcultured in Petri dishes containing 25 ml of the same culture medium composition described for EC induction. Subcultures were made every 21 days for 4 cycles in gelled culture medium for the EC scale-up. After that, EC were transferred for proliferation in liquid culture medium (cell suspension) with the same composition as described above without gelling agent. The cell suspension was established in 250 ml Erlenmeyer flasks containing 50 ml of liquid culture medium, kept in dark conditions with permanent agitation (90 rpm) in orbital shaker at temperature of 22 ± 2ºC.

Transmission electron microscopy

Aiming to evaluate the ultrastructural features of EC during cell proliferation, analysis of transmission electron microscopy (TEM) was performed. Representative samples of EC maintained in liquid proliferation culture medium were fixed with 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) plus 0.09 M sucrose overnight. The material was post-fixed with 1 % osmium tetroxide for 4 h, dehydrated in a graded ethanol series, and embedded in Spurr's resin, according to the manufacturer's instructions. Thin sections were contrasted with aqueous uranyl acetate, followed by lead citrate, according to Reynolds (1963). The samples were then examined under TEM JEM 1011 (JEOL Ltd., Tokyo, Japan, at 80 kV).

Fractionation of cell cultures and cell tracking

After the establishment of cell suspension, the cell fractionation was performed according to Filonova et al. (2000a) to assess the dynamics of cell aggregates proliferation. Briefly, 15-d-old cell suspensions were sieved through 80 μm mesh of tissue grinder homogenizer kit (Sigma-Aldrich), and the >80 μm fraction resuspended in proliferation medium. This procedure enabled dissociation of adhered cell aggregates. The resulting cell suspension greater than 80 μm was then passed through a series of meshes with successive 600, 400, 200, and 80 μm pore sizes. The single cells and few- and multi-celled aggregates obtained were 80-200, 200–400, 400-600, and >600, μm fractions.

These fractions were inoculated into either 0.5 ml aliquots of liquid proliferation medium held in 12 well plates (TPP, Switzerland), and subsequently immobilized by mixing with the same volume of 1.2 % (w/v) low melting point agarose (Conda Laboratories, Madrid, Spain) containing proliferation medium at 35°C. The cultures were incubated in the darkness at 22 ± 2°C. The plates were monitored at 0, 2, 4, 6, 8, 10, 12, 14 culture, with 4 wells per class of cell fraction, in inverted microscope (Olympus IX81), equipped with a computer-controlled digital camera (DP71, Olympus Center Valley, PA, USA).

Embryogenic cultures maturation

The EC maintained in proliferation cycles in cell suspension were used in two maturation treatments in order to evaluate how the different EC cell aggregates morphologically respond when submitted to maturation phase I or when directly exposed to maturation phase II. Thus, the process of EC polarization, pro-embryos formation, and establishment of the best culture conditions in the maturation processes were also monitored by cell tracking.

Embryogenic cultures were inoculated into either 0.5 ml aliquots of liquid maturation phase I culture medium held in 12 well plates, and subsequently immobilized by mixing with the same volume of 1.2 % (w/v) low melting point agarose containing maturation phase I culture medium at 35°C. This culture medium consisted of BM macro- and micro-salts supplemented with L-glutamine (1.0 g l^{-1}), myo-inositol (1.0 g l^{-1}), casein hydrolysate (0.5 g l^{-1}), 50 g l^{-1} maltose, 100 g l^{-1} polyethylene glycol 4000 (PEG). The cultures were incubated for 15 days with 16 h photoperiod, with a low light intensity of 5-10 µmol m^2 s^{-1} provided by cool-White OSRAM™ fluorescent lamps, at 22 ± 2°C. The plates were monitored at 0, 4, 6, 8, 10, 12, 14 culture, with 4 wells as replicates.

The same immobilization process described above was used for the EC maturation phase II treatment. Maturation phase II culture medium consisted of the same maturation phase I culture medium composition plus 100 µM abscisic acid (ABA). The ABA stock solution was filter sterilized and added to the culture medium after autoclaving. The cultures were incubated for 28 days with 16 h photoperiod, with a low light intensity of 5-10 µmol m^2 s^{-1}, at 22 ± 2°C. The plates were monitored at 0, 4, 7, 14, 21, 28 culture, with 4 wells as replicates. The cultures monitoring was carried out in inverted microscope (Olympus IX81), equipped with a computer-controlled digital camera (DP71, Olympus Center Valley, PA, USA).

In both treatments, maturation phase I and II, the total number of polarized and non-polarized PEMs were evaluated, as well as the number of cellular aggregates that survived or not at each step. The cell aggregates that remained stagnant in each treatment, i.e. that did not present cell proliferation or cell death, were not considered in the count. The percentages of PEMs polarization and survival for each treatment were compared by chi-square (χ^2) contingency test (99%).

Based on results obtained in the cell tracking monitoring, the EC were submitted to maturation processes in petri dishes. About 100–200 mg FW of EC suspended in 2 ml of proliferation culture medium was poured over a 70-mm diameter filter paper disks in a Büchner funnel. The drained and finely dispersed EC was then cultured over 90-mm diameter Petri dishes containing 25 ml of maturation phase I culture medium. This culture medium was the same described above plus 2 g l^{-1} activated charcoal and gelled with Phytagel® (3 g l^{-1}). Cultures were maintained in this step for 2 weeks in a low light intensity of 5-10 µmol m^2 s^{-1} and then transferred to the maturation phase II culture medium, consisting of the same composition as described above plus 2 g l^{-1} activated charcoal, 100 µM ABA and gelled with Phytagel® (3 g l^{-1}). Cultures were maintained for 12 wk in growth room at 22 ± 2°C and 16 h photoperiod, with a low light intensity of 5-10 µmol m^2 s^{-1}.

Light microscopy

Representative samples of somatic embryos obtained after maturation process were collected and fixed in paraformaldehyde (2.5 %) in sodium phosphate buffer 0.2 M (pH 7.2) for 24 h at 4°C. The samples were then washed three times in buffer without fixative and gradually dehydrated in graded ethanol series (30-100%). The samples were embedded in methacrylate resin (Leica Historesin®) according to manufacturer's instructions. Sections of 5-7 µm were obtained using a rotary microtome (Slee Technik®) were allowed to adhere to histological slides at 42 ± 2ºC, using few drops of water. After the water evaporation, sections were stained with 1 % toluidine blue in an aqueous solution of 1 % Borax, pH 9 (Kuo and O'Brien 1974), and relevant aspects have been identified and photographed using an inverted microscope, equipped with a computer-controlled digital camera.

Results and discussion

Transmission electron microscopy analysis

The EC ultrastructural features observed by TEM revealed three distinct cell types: embryogenic cells, intermediate cells (embryonal tube-like cells) and suspensor cells (Figure 1). The embryogenic cells morphology was characteristic of meristematic cells, with large and

prominent nuclei, high ratio nucleus: cytoplasm, numerous small vacuoles, high presence of mitochondria and thin cell wall (Figure 1A-B). In addition, these cells contained numerous amyloplasts with starch grains, some electron dense lipid bodies, and distinguishable regions of hetero-and euchromatin along the nuclei.

The embryonal tube-like cells differed from embryogenic cells by being more elongated, with thicker cell wall, and presenting higher number and larger vacuoles (Figure 1C-D). These characteristics are often related to low metabolic activity. However, these cells seemed to have an intense metabolic activity, characterized by the large presence of mitochondria, Golgi bodies, secretory vesicles and lipid bodies.

The third cell type, the suspensor cells, showed cytoplasmic degradation characteristics, large vacuoles, and some isolated starch grains which were possibly wrapped in an amyloplast before the cell collapse (Figure 1E-F). No organelle, besides the amyloplast, was observed in these cell types.

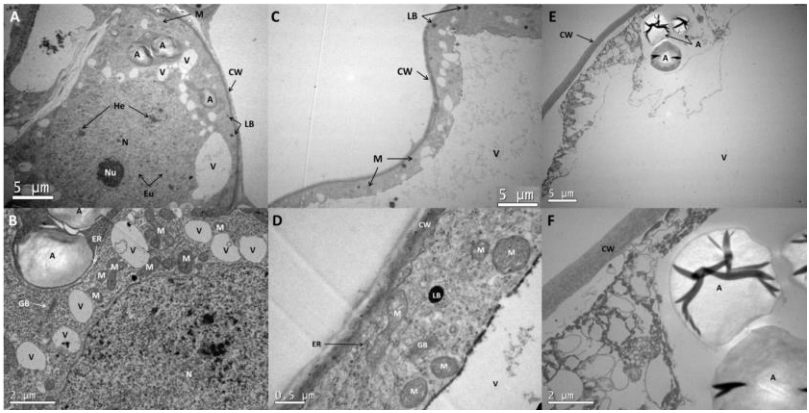

Fig. 1 Ultrastructural analyses of the *A. angustifolia* embryogenic cultures during proliferation step. A – Embryogenic cell containing numerous small vacuoles (V), large and prominent nuclei (N) and nucleolus (Nu), high presence of mitochondria (M), lipid bodies (LB), endoplasmic reticulum (ER), and thin cell wall (CW). B – Embryogenic cell in detail showing amyloplast (A) and starch grains, and distinguishable regions of hetero- (He) and euchromatin (Eu) along the nuclei. C – Embryonal tube-like cell, being more elongated, presenting higher number and larger vacuoles, and thicker cell wall. D - Embryonal tube-like cell in detail showing large vacuole, Golgi bodies (GB), LB and mitochondrias. E – Suspensor cell, showing cytoplasmic degradation with large vacuoles throughout the cell space, with some isolated starch grains. F - Suspensor cell in detail showing thicker cell wall and starch grains.

Filonova et al. (2000b; 2002) showed that the embryo suspensor is a terminally differentiated structure that is eliminated through programmed cell death (PCD) at the end of early embryogeny. These authors also described that the earliest morphological sign of PCD in the embryo suspensor was associated to an increase in the number and activity of Golgi bodies, which ultimately culminates in increased number of autolytic vacuoles and vesicles in the cytoplasm. During this process, one or more large vacuoles are formed and take up the majority of the cell volume, leading to its collapse and leaving a hollow-walled corpse (Smertenko et al. 2003).

In fact, among the embryonal tube-like cells analyzed in the present work, an increased secretory activity combined with a cell vacuolation process was observed, which apparently culminated with the development of suspensor cells. Therefore, it is possible to assume that there was a PCD process occurring in the intermediate cells of *A. angustifolia*, following a pattern quite similar to other conifers species, such as *Picea abies* (Smertenko et al. 2003).

The TEM analysis also revealed different types of mitochondria morphologies, mainly in the embryogenic cells (Figure 2A-B). Besides the standard mitochondria morphology, the typical cylindrically-shaped with well-packed and organized cristae, we observed swollen mitochondria, with more prominent cristae, "sickle-like mitochondria" and "donut-shaped mitochondria" (Figure 2). These structures were quite consistent and identified in almost all the embryogenic cells analyzed of *A. angustifolia*.

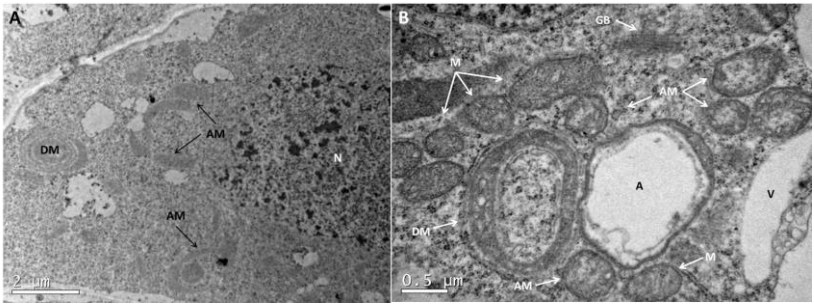

Fig. 2 Ultrastructural features of different mitochondrial morphologies observed in embryogenic cells of *A. angustifolia*. A – General view of different morphologies found in the embryogenic cell, with sickle-like shape altered

mitochondria (AM), and donut-shaped mitochondria (DM). B – Detail of different mitochondria morphology, with a typical cylindrical shape with well-packed and organized cristae (M), altered mitochondria appeared swollen, with cristae more prominent, and donut-shaped mitochondria.

Physiological conditions of mitochondrial metabolism are thought to evoke changes in mitochondrial dynamics, which control its structure (Liu and Hajnóczky 2011). In addition, it was suggested that the mitochondria may be the primary target of the many different stimuli that induce the stress response (Jendrach et al. 2008). Several structural changes in the mitochondria in response to stress have been reported, as in cells after heat-shock treatment (Welch and Suhan, 1985), oxidative stress (Jendrach et al. 2008), and cellular hypoxia and reoxygenation (Liu and Hajnóczky 2011). However, to date, there are no reports of such changes in mitochondrial structure of plant embryogenic cultures.

In vitro culture conditions represent an unusual combination of stress factors that plant cells encounter, e.g., oxidative stress, PGRs, low or high salt concentration, low or high light intensities (Zavattieri et al. 2010). Thus, the altered morphology of mitochondria found in this study suggested that these cells were extremely stressed, which can be due to many reasons. One possible explanation relates to the redox mechanism, by the oxidative stress. Jendrach et al. (2008) reported important structural changes in the mitochondria of animal cells after being exposed to specific dosages of hydrogen peroxide, a well-known and pivotal reactive oxygen species (ROS).

Another possible explanation relates to one of the structural conformations of mitochondria found in this study, the donut-shaped mitochondria. Liu and Hajnóczky (2011) systematically described its formation in animal cells and their possible role in the mitochondrial adaptation to hypoxia-reoxygenation-induced cellular stress. According to these authors, that mitochondrial conformation has some advantage over linear structures in tolerating a matrix volume increase, during hypoxia-reoxygenation events. Thus, reshaping of mitochondria to donuts might be a component of a protective mechanism that helps to preserve the organelles under conditions of metabolic stress.

Cell tracking of EC fractions during proliferation step

The cell tracking of *A. angustifolia* EC fractions revealed two cell types, i.e. highly vacuolated cells, which are more or less elongated (suspensor cells), and rounded densely cytoplasmic cells, the

embryogenic cells. Steiner et al (2005) and Farias-Soares et al. (2014) also reported the presence of these cell types in *A. angustifolia* EC evaluated by double-staining analysis with acetocarmine and Evans blue.

An overview of cell aggregates obtained with the different cell fractions analyzed is shown in Figure 3. Most of the cell aggregates with 80-200 µm started to proliferate at day 2 in culture. After 8 days in culture, the suspensor cells started to develop more noticeably, and with 14 days in culture, numerous PEM III could be observed. The 200-400 µm fraction showed a PEM multiplication pattern quite similar to 80-200 µm fraction, even though it presented a greater presence of suspensor cells (Figure 4). In addition, it was noticeable that the transition of PEM II to PEM III started mainly at day 10 in culture. At the end of 14 days in culture, an increased number of PEM III could be observed for this cell fraction. Furthermore, apparently, a better PEM III formation occurred in this cell fraction, with a better formed and individualized cell aggregates.

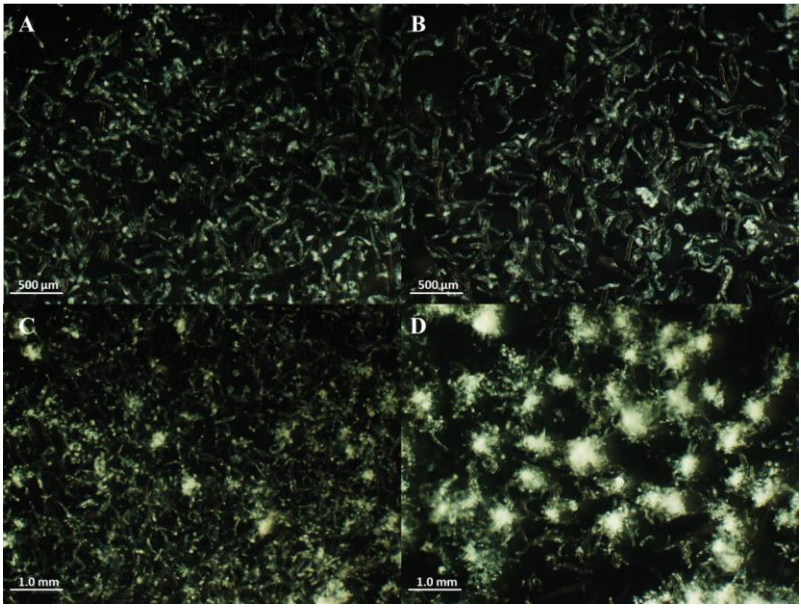

Fig. 3 Overview of different cell fractions used in time-lapse cell tracking of *A. angustifolia*. A – 80-200 µm cell fraction showing numerous pro-embryogenic masses (PEM) I. B – 200-400 µm cell fraction showing mainly PEM I and II. C

– 400-600 µm cell fraction showing PEM I, II, and III. >600 µm cell fraction showing mainly PEM III.

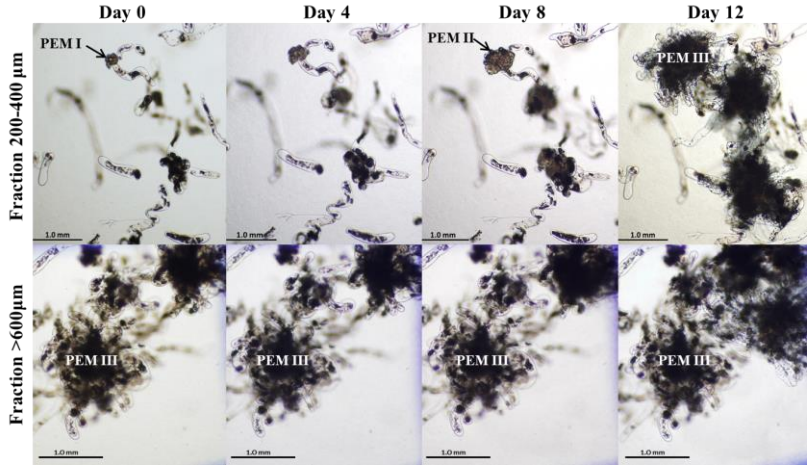

Fig. 4 Time–lapse tracking of different cell fractions of *A. angustifolia* embryogenic cultures during proliferation cycle. Cells aggregates found in fraction 200-400 µm started to proliferate from day 2 of culture, and with 12 days in culture, numerous PEM III could be observed. For the >600 µm fraction could be observed a large number of PEM III from the date of inoculation showing no proliferation after 12 days in culture, allied to a lower abundance of PEM I.

According to Smertenko et al. (2003), the asymmetric divisions of the most basally situated cells within the embryonal mass give rise to a layer of elongated embryonal tube cells which differentiate to form one layer of the suspensor cells. This pattern of cell division could be observed in *A. angustifolia* EC during cell tracking, particularly in the 200-400 µm fraction (Figure 4).

The 400-600 and >600 µm fractions were constituted by a higher number of PEM II and III at the inoculation date, and a lower abundance of PEM I. Over the days of culture, the PEM III started to fragment into smaller cell aggregates, and a greater proliferation of embryogenic cells in PEM II was observed. However, a large number of cell aggregates showed no proliferation over the evaluation dates (Figure 4).

PEM-somatic embryo transition is a critical developmental event which affects the final yield of somatic embryos produced in culture. Thus, a proper understanding of the factors affecting/regulating this transition would be of great value for the improvement of the overall

embryogenic process (Stasolla and Yeung 2003). Farias-Soares et al. (2014) proposed that a low numbers of early somatic embryos are ascribed to disturbances in the transition from PEM III to early somatic embryos of *A. angustifolia*. Based on our results, it can be assumed that smaller cell aggregates, as identified in the 80-200 μm and 200-400 μm cell fractions, may be more suitable for subsequent transfer to maturation step and obtaining somatic embryos in *A. angustifolia*. The EC derived from these cell fractions generated better formed PEM III and apparently more organized cell multiplication.

Cell tracking of EC during maturation treatments

Cell aggregates derived from proliferation cycles in cell suspension were also observed by cell tracking during two maturation treatments (with or without ABA), as shown in Figure 5. The ABA-free maturation treatment allowed the polarization process in PEM III, resulting in pro-embryos formation after 14 days in culture. This process started more markedly at day 10 in culture, with an organized proliferation of embryogenic and organization of suspensor cells. In addition, it is important to stress that cell tracking analysis during maturation in ABA-free treatment indicated the smaller cell aggregates as more responsive to induce the process of polarization, corroborating our results described in the cell proliferation.

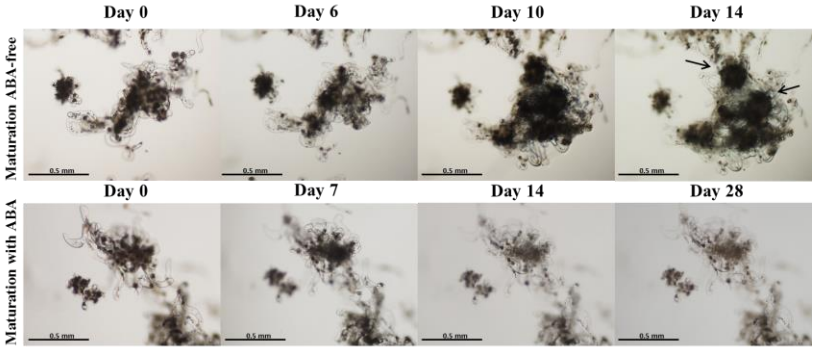

Fig. 5 Time–lapse tracking of *A. angustifolia* embryogenic cultures (EC) during maturation treatments (ABA-free and with ABA). ABA-free maturation was able to induce a polarization process in PEM III, with pro-embryos formation after 14 days of culture (arrows). The EC transfer directly to the maturation with ABA apparently started a process of cell death after 14 days in culture,

becoming more translucent with no cell proliferation signal or morphological change after 28 days in culture.

The transference of PEMs to a maturation PGR-free culture medium with a high osmotic potential has proven to promote PEMs transition to early somatic embryos in *A. angustifolia* EC (Santos et al. 2002; Vieira et al. 2012). However, to date it was not carried out an EC monitoring during this process by cell tracking.

EC transferred directly to the maturation medium containing ABA resulted in detrimental morpho-histological features (Figure 5). From 14 days in maturation culture medium, EC apparently started a process of cell death, becoming more translucent. In addition, there was no cell proliferation signal or morphological changes among the PEM analyzed. These results were also quite consistent, being observed in almost all EC analyzed.

The data obtained in the PEMs survival rate after maturation treatments (with or without ABA) also showed the same pattern (Table 1). A higher number of PEMs inoculated into ABA-free maturation treatment survived (80%), while in the maturation treatment with ABA the survival rate was only 20.8%.

Table 1. Survival and mortality rates of *A. angustifolia* pro-embryogenic masses as affected by maturation treatments.

Treatment	Survival (%)	Mortality (%)
0 µM ABA	78.95 A	21.05 B
100 µM ABA	20.88 B	79.11 A

Percentages followed by different letters in column differ significantly according to χ^2 2x2 contingency test (99% confidence), n = 152 and 158 cell aggregates for maturation ABA-free and maturation with ABA, respectively.

According to Stasolla and Yeung (2003), the endogenous ABA level in gymnosperms is normally low during the initial phases of embryonic development, increasing during the embryonic growth and then decreasing in the last stages of maturation, at the onset of the desiccation period. Based on this notion, the results of this study indicate that the EC seems to require an osmotic stress during the initial period of pro-embryo development, which in this case is mainly associated with high concentrations of PEG, but not by ABA.

PEM to embryo transition in conifers SE normally occurs after the withdrawal of auxin and cytokinin in the culture medium. The

inability of a cell line to form cotyledonary somatic embryos is to a large extent associated with disturbed or arrested PEM to embryo transition (von Arnold and Clapham 2008). These authors also stated that the pro-embryos should not be exposed to maturation treatments before they have reached the developmental stage corresponding to early embryogeny, corroborating our results, in which the direct transfer of EC from the proliferation step to maturation with ABA did not allow the further development of somatic embryos. The results presented in the Table 2 reinforce the findings of von Arnold and Clapham (2008). In our case, the polarization rate after the ABA-free maturation treatment were significantly higher (73.02%) than after maturation treatment with ABA (11.39%).

Table 2. Polarization and non-polarization rates of *A. angustifolia* pro-embryogenic masses (PEM) as affected by maturation treatments.

Treatment	Polarized PEM (%)	Non-polarized PEM (%)
0 µM ABA	73.02 A	26.98 B
100 µM ABA	11.39 B	88.61 A

Percentages followed by different letters in column differ significantly according to χ^2 2x2 contingency test (99% confidence), n = 152 and 158 cell aggregates for maturation ABA-free and maturation with ABA, respectively.

In Norway spruce SE, the insertion of maturation phase I (no PGR in the culture medium) between proliferation and maturation with ABA (phase II) improved the synchronization, enhanced the speed of somatic embryo development, and increased the number of developed embryos (Vágner et al. 2005). According to these authors, the beneficial effects could be ascribed to lower levels of auxin and cytokinin after this period, which combined with exogenous application of ABA stimulated the formation of new somatic embryos. In our study, no exogenous application of auxins and cytokinins was performed during the EC induction and proliferation steps; however, the osmotic stress caused by ABA-free maturation culture medium seemed to be essential to prepare the EC for subsequent maturation step (phase II).

Thus, the use of smaller cell aggregates of EC combined with a 14 days maturation phase I, and a high osmotic potential ABA-free before transfer to the maturation phase II should be performed for EC of

A. angustifolia in order to promote the further development of somatic embryos.

Light microscopy analysis of somatic embryos

Light microscopy technique was used to monitoring the development sequence of individually selected somatic embryos derived from cell aggregates maintained in maturation culture medium for 12 wk. In the somatic embryo clusters, different developmental stages were observed, revealing non-synchronized development (Figure 6A-B).

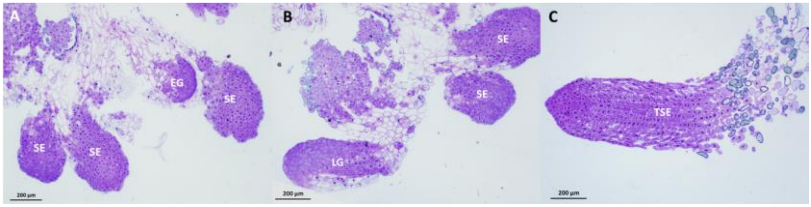

Fig. 6 Histological analysis of the *A. angustifolia* somatic embryos development. A – Early globular (EG) somatic embryo with well-developed protoderm. B - Late globular-staged somatic embryo (LG) showing a layer of embryonal tube cells and suspensor-like cells. Note the presence of more vacuolated cells in the basal part of the embryo. C – Torpedo-staged somatic embryo showing meristematic cells in the apical part with embryonal tube cells in the middle part until the basal part consisting of suspensor cells.

Globular somatic embryos at the onset of polarization showing a well-delimited protoderm were the first clearly distinguishable stages of somatic embryo development in culture (Figure 6A). A layer of embryonal tube cells and suspensor-like cells was also evident, which appeared to be more vacuolated as compared to cells of the embryonic head. Further development included their elongation, development of procambium and differentiation of the shoot meristem, reaching the late globular-staged somatic embryo (Figure 6B). Finally, an increased embryo elongation was observed with a subtle change in the shoot meristem conformation, which became sharper. In this last developmental stage, the embryo individualization from the cell aggregate was observed.

Histological analysis performed in distinct developmental stages of *Picea abies* somatic embryos showed similar pattern of ultrastructural organization (Filonova et al. 2000a). However, the later stages of torpedo-staged somatic embryos were not achieved in the present study,

unlike the work performed with *P. abies* somatic embryos, where the authors analyzed post-cotyledon-staged somatic embryos (Filonova et al. 2000a). Nevertheless, it is important to emphasize that the protocol used in this study for the early somatic embryos maturation of *A. angustifolia* allowed to obtain torpedo-staged somatic embryos (Figure 6C).

A relevant ultrastructural feature observed in the analyzed somatic embryos was the conspicuous presence of intercellular spaces, especially in torpedo-staged somatic embryo. Norway spruce somatic embryos cultured under control conditions (more reduced environment) showed poorly organized shoot apical meristems (SAM) with several abnormalities including the formation of intercellular spaces and large vacuoles within the sub-apical cells (Stasolla 2010). This author also pointed out the increased intercellular spaces associated with prolonged culture time, resulting in the deterioration of SAMs and the separation of the meristematic cells, compromising the overall post-embryonic performance of the embryo. Thus, an imposed oxidized environment can be tested aiming to improve the obtainment of normal somatic embryos of *A. angustifolia*.

Conclusion

In the present work, the novel characterization of the different cell types that constitute the *A. angustifolia* PEMs by TEM analysis allowed the identification of intriguing mitochondrial structural morphology. This was observed in embryogenic cells, which can be related to a protector mechanism to stress conditions. In addition, the embryonal tube-like cells were described for the first time for this species, showing unexpected features, with possible intense metabolic activity, characterized by the large presence of mitochondria, Golgi bodies, secretory vesicles and lipid bodies.

In the cell tracking results, smaller cell aggregates, as identified in the 80-200 μm and 200-400 μm cell fractions, were more suitable for transfer to maturation step after a cycle of 14 days in proliferation culture medium. The EC derived from these cell fractions generate well-developed PEM III and apparently more organized cell multiplication.

In the maturation treatment, the employ of smaller cell aggregates submitted to 14 days in maturation phase I (ABA-free) in culture medium with a high osmotic potential before transfer to the maturation

phase II (with ABA) proved to be more suitable for early somatic embryos development. Furthermore, the direct transfer of EC in proliferation to maturation culture medium containing ABA was shown to be highly detrimental.

Finally, the more advanced developmental stages of somatic embryos obtained were characterized by light microscopy at the end of the maturation process. Globular and torpedo-staged somatic embryos showed relevant ultrastructural features, as the presence of some intercellular spaces, especially in the torpedo-staged somatic embryo, which were associated to poorly organized SAMs and abnormal embryos.

Author Contribution Statement Conceived and designed the experiments: HPFF, LNV and MPG; Performed the experiments: HPFF, LNV and CCP; Performed the transmission electron microscopy analysis: HPFF, LNV and EMO; Contributed reagents/materials/analysis tools: MPG; Wrote the paper: HPFF, LNV and MPG.

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CAPÍTULO 2

Este manuscrito encontra-se publicado no periódico *Plant Cell, Tissue and Organ Culture*

High-efficiency cryopreservation of *Araucaria angustifolia* (Bertol.) Kuntze embryogenic cultures: ultrastructural characterization and morpho-physiological features

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Abstract

Araucaria angustifolia is a subtropical conifer belonging to the Araucariaceae family. The application of tissue culture tools, such as somatic embryogenesis (SE), is one of the most promising techniques for its conservation and mass propagation. The SE offers several advantages, including the potential for embryogenic cultures (EC) scale-up by suspension cultures and the application of cryopreservation techniques. The present study aimed at to evaluate and characterize the growth dynamics of the *A. angustifolia* EC submitted to different cryotreatments incubation times through morphological and time-lapse cell tracking analyzes. The EC regrowth rates submitted to cryopreservation protocol were also evaluated, and the ultrastructural characterization by transmission electron microscopy (TEM) analysis was performed. The results indicated that *A. angustifolia* EC support all the cryoprotection times evaluated, without cell proliferation inhibition, but with noticeable genotype-dependent response in cell lines tested. We obtained 100% cell regrowth in both cell lines, indicating that the proposed protocol can be successful applied to *A. angustifolia* EC cryopreservation. The cell tracking showed a survival and initial proliferation of embryogenic cells, with the first cell regrowth signs after 30 days in culture. The TEM analysis revealed a conspicuous cell wall thickening in embryogenic cells after cryotreatment and after thawing, which may be related to osmotic stress response caused by the cryopreservation process. An increased heterochromatin presence was also observed in cryotreated or after thawing cells, may possibly be acting as a cell defense mechanism, decreasing the DNA vulnerability to cleavage and preserving the cell integrity.

Keywords somatic embryogenesis, slow cooling, time-lapse cell tracking, cell regrowth, conifers

Introduction

Araucaria angustifolia is a dioecious perennial conifer native to South America, occurring in South and Southeastern Brazil, and restricted areas of Northwestern Argentina and Paraguay (Guerra et al. 2000). Drastic population decline and habitat reduction culminated in the inclusion of *A. angustifolia* on the IUCN international list as critically endangered status (IUCN 2014). However, the remaining forests of this species continue to be explored from commercial and scientific perspectives (Stefenon et al. 2009). In this context, *A. angustifolia* is a species with great potential for studies to support genetic conservation (Bittencourt and Sebbenn 2007). The application of tissue culture tools in this species, such as somatic embryogenesis (SE), is one of the most promising techniques for its conservation and mass propagation (Santos et al. 2008; Jo et al. 2013; Steiner et al. 2012; Schlögl et al. 2012; Vieira et al. 2012; Steiner et al. 2015; Fraga et al. 2015).

Somatic embryogenesis is the developmental restructuring of somatic cells toward the embryogenic pathway and forms the basis of cellular totipotency in higher plants (Karami et al. 2009). This morphogenetic route is associated with a variety of applications, such as obtaining a reference model for studies on basic physiology and biochemistry, large-scale propagation and genetic transformation, and the possibility of cryopreserving embryogenic cultures (EC), integrating it into breeding programs or *in vitro* germplasm conservation (Zoglauer et al. 2003; Guzmán-García et al. 2013).

Cryopreservation is the conservation of biological material at ultra-low temperatures, usually at -196ºC, the temperature of liquid nitrogen (LN) (Engelmann 2004). This process typically comprises many stages from culture, pregrowth (pretreatment), cryoprotection, freezing or vitrification, thawing, and recovery (regrowth) (Gonzalez-Arnao et al. 2008; Salaj et al. 2010). Specifically, EC cryopreservation can avoid the detrimental effects of long-term subculture such as contamination, somaclonal variation or loss of embryogenic competence (Lambardi et al. 2008). Moreover, this technique can prevent the loss of cell characteristics due to epigenetic instability such as structural changes in chromosome morphology, ploidy, cell morphology and the biosynthetic capacity of a cell line, mainly caused by prolonged subculturing (Mustafa et al. 2011). In this sense, the integrated use of SE

and cryopreservation enables the storage and efficient recovery of clones obtained from young reactive tissues, elite genotypes, cell lines with special attributes or derived from endangered species (Engelmann 2011).

Recent advances in the cryostorage of plant tissues and organs in LN opened the door to safe, low-cost, long-term maintenance of EC. Both the controlled rate cooling method (two-step freezing), and procedures allowing the direct immersion of specimens in LN (vitrification or one-step freezing) were successfully tested with EC lines from various plant species (Panis and Lambardi 2006; Engelmann 2011). The traditional controlled rate cooling method is still the most common approach for the cryopreservation of clumps from EC, and has been applied for several conifer species (Häggman et al. 1998; Ford et al. 2000; Marum et al. 2004; Salaj et al. 2007; Lambardi et al. 2008; Salaj et al. 2010; Álvarez et al. 2012; Krajňáková et al. 2013).

Recently, it was reported a protocol for EC cryopreservation of *A. angustifolia* in which the authors evaluated seventeen cryoprotective treatments, based on the combination of different osmotic solutions, and two cryopreservation methods, the slow cooling or the directly immersion in LN (Demarchi et al. 2014). The results indicated that the vitrification protocol using the PVS2 solution and 40 min of immersion in ethanol -20°C was promising for EC cryopreservation of *A. angustifolia*. However, further morphological and ultrastructural studies focusing on the behavior of these cells during the cryopreservation process are required.

In the present study we evaluated and characterized the growth dynamics of the *A. angustifolia* EC submitted to different cryotreatments incubation times through morphological and time-lapse cell tracking analyzes. The EC regrowth rates submitted to cryopreservation protocol by controlled rate cooling method were also evaluated, and further ultrastructural characterization by transmission electron microscopy analysis was performed.

Material and methods

Plant material

Immature female cones bearing globular-staged zygotic embryos were collected in December 2013, from an *A. angustifolia* open-pollinated natural population in Lages, Santa Catarina – Brazil (latitude

28° 02′ S, longitude 50° 17′ W, altitude 1030 m). Two independent cones were collected from different plants, and each cone generated a single cell line (Cr01 and Cr02).

Somatic embryogenesis induction and proliferation

Embryogenic cultures were induced according to the methodology described by Santos et al. (2002). The seeds were submitted to disinfestation procedures with 70% ethanol for 5 min and 40% sodium hypochlorite for 15 min, followed by a triple-washed with autoclaved distilled water. Zygotic embryos were excised and inoculated in Petri dishes containing 25 ml of culture medium. The culture medium consisted of BM macro-, micro-salts and vitamins (Gupta and Pullman, 1991) supplemented with L-glutamine (1.0 g L^{-1}), myo-inositol (1.0 g L^{-1}), casein hydrolysate (0.5 g L^{-1}), 2,4-dichlorophenoxyacetic acid (2,4-D; 4 µM), benzylaminopurine (BAP; 2 µM), kinetin (KIN; 2 µM), Phytagel® (2 g L^{-1}) and sucrose (30 g L^{-1}). The pH of culture medium was adjusted to 5.8 and autoclaved at 121°C, 1.5 atm for 15 min. All the cultures were maintained in a growth room in the absence of light at temperature of 22°C ± 2°C.

After 30 days, the EC were subcultured in Petri dishes containing 25 ml of the same culture medium composition described for EC induction, except the plant growth regulators (PGR) concentrations, which were reduced by half (BM_2 culture medium). Subcultures were made every 21 days for 4 cycles in gelled BM_2 culture medium for the EC scale-up. After that, EC were transferred for proliferation in BM_2 liquid culture medium (cell suspension), without gelling agent. The cell suspension was established in 250 ml Erlenmeyer flasks containing 50 ml of liquid culture medium, kept in dark conditions with permanent agitation (90 rpm) in orbital shaker with temperature of 22ºC (±2ºC).

Cryotreatment incubation times and cell growth dynamics

In this step, the focus was to evaluate the possible toxicity of cryoprotectants agents in EC of *A. angustifolia*. Thus, cultures were subjected to different cryotreatment incubation times without being subjected to freezing. For this experiment, we used EC of *A. angustifolia* at exponential growth phase (5 days after subculture) from

established cell suspension line (Cr01 cell line). The cryotreatment protocol used was based on Mustafa et al. (2011).

Briefly, EC were pre-incubated in an orbital shaker at 90 rpm for 2 days in the pretreatment solution, consisting of BM_2 culture medium plus mannitol (180 g L^{-1}). Subsequently, the pretreatment solution was replaced by the same volume of cryoprotectant solution composed by 2M sucrose, 1M glycerol, 1M dimethyl sulfoxide (DMSO) and 1% (wt/vol) l-proline (Mustafa et al. 2011) and different incubation times were tested (0, 30, 60, 120 and 240 min). The cryotreatment process was performed on an orbital shaker at 90 rpm at 4°C. Subsequently, aliquots of 1.8 mL containing the cells were removed from each treatment tested and transferred to 2 ml microtubes, and then centrifuged for 2 min at 100 xg. The upper liquid was removed with a pipette and the cells were transferred to Petri dishes containing 2 filter paper disks on BM_2 culture medium gelled with agarose (7.5 g L^{-1}). After 2 days incubation, the upper filter paper containing the EC were subcultured to a fresh BM_2 culture medium gelled with agarose and after 7 days incubation the EC were transferred to BM_2 culture medium gelled with Phytagel® (2 g L^{-1}) without filter paper. All the cultures were maintained in a growth room for 30 days in the dark at temperature of 22°C ± 2°C.

The EC were weighed at 2, 9, 16 and 23 days of culture and fresh weight increased ratio [final weight / initial weight] was calculated in different treatments. The experimental design was completely randomized with five replications, and the experimental unit consisted of a Petri dish containing a colony of EC. Data were submitted to analysis of variance (ANOVA), followed by Student-Newman-Keuls (SNK) mean separation test ($p<0.05$), using Statistica® 6.0 for Windows version 7.0.

Cryopreservation experiments

According to the results of different cryoprotetion incubation times evaluated in the experiment above, we performed the complete cryopreservation protocol (Mustafa et al. 2011). Two embryogenic cell lines of *A. angustifolia* were used in this experiment (Cr01 and Cr02). The procedures were the same as described above, with the EC pre-incubation in an orbital shaker at 90 rpm for 2 days in the pretreatment solution followed by incubation in a cryoprotectant solution for different times (30, 60, 120 and 240 min) at 90 rpm at 4°C. After this period, aliquots of 1.8 mL containing the EC were removed and transferred to

cryovials (2 mL), which was subsequently transferred to Mr. Frosty (Nalgene®) containers containing 250 mL isopropanol. The containers were placed in an ultra-freezer (-80°C) for controlled cooling, at a rate of approximately 1ºC min^{-1} for 4h. Then, the cryotubes were quickly removed and immediately immersed in LN for at least 24 h

After freezing in LN, cryopreserved EC were thawed by immersion of the cryovials in a 40ºC deionized water bath (≈ 2 min). Immediately after thawing, the liquid containing the EC was transferred to 2 mL microtubes, and then centrifuged for 2 min at 100 xg. The upper liquid was removed with a pipette and the cells were washed with 1 mL of pretreatment solution. The centrifugation process was repeated, the upper liquid removed and the content of each microtube was poured onto two sterile filter paper discs placed in a Petri dish containing 25 mL BM_2 culture medium gelled with agarose (7.5 g L^{-1}). After 2 days incubation, the upper filter paper containing the EC were subcultured to a fresh BM_2 culture medium gelled with agarose and after 7 days incubation the EC were transferred to BM_2 culture medium gelled with Phytagel® (2 g L^{-1}) without filter paper. All the cultures were maintained in a growth room for 60 days in the dark at temperature of 22°C ± 2°C.

The EC regrowth rate was evaluated at 30 and 60 days of culture in different cryotreatment incubation times and both cell lines (Cr01 and Cr02). The experimental design was completely randomized with six replications, and the experimental unit consisted of a Petri dish containing three colonies of EC. The percentages of regrowth for each treatment and cell line were compared by chi-square (χ^2) contingency test (95%), using Statistica® 6.0 for Windows version 7.0. Pairwise comparisons were carried out in 2x2 contingency tables in order to detect differences between two specific individual cryotreatment incubation times, for each evaluation date (30 and 60 days of culture).

Time-lapse cell tracking of cryopreserved embryogenic cultures

In order to monitor the new proliferation of the cultures after thawing, time-lapse cell tracking was performed to EC of *A. angustifolia* after being subjected to complete cryopreservation protocol in the different cryotreatment incubation times (30, 60, 120 and 240 min). The same two embryogenic cell lines of *A. angustifolia* were used in this experiment.

After thawing procedure, the EC were poured onto two sterile filter paper discs placed in a Petri dishes containing 25 ml BM_2 culture medium gelled with agarose (7.5 g L^{-1}). After 2 days incubation, the upper filter paper containing the EC were subcultured to a fresh BM_2 culture medium gelled with agarose. After 7 days incubation the EC were inoculated into 0.5 ml aliquots of liquid BM_2 culture medium (without gelling agent) held in 12 well plates (TPP, Switzerland), and subsequently immobilized by mixing with the same volume of BM_2 culture medium with 1.2% (w/v) low melting point agarose (Conda Laboratories, Madrid, Spain) containing BM_2 culture medium at 35°C (Filonova et al. 2000). The cultures were incubated in the darkness at 22±2°C for 50 days. The plates were monitored at 0, 10, 20, 30, 40, 50 days in culture, with 4 wells per different cryotreatment incubation times, in both cell lines, in inverted microscope (Olympus IX81), equipped with a computer-controlled digital camera (DP71, Olympus Center Valley, PA, USA).

Morphological analysis of cryopreserved embryogenic cultures

In order to assess the cell viability and morphology throughout the cryopreservation process, aliquots containing the EC from Cr01 cell line were collected at different points of the protocol: a) EC derived from proliferation cycles (control); b) EC only subjected to 60 min cryotreatment; c) EC after 60 min cryotreatment and slow cooling in Mr. Frosty, but not immersed in LN; d) cryotreated EC for 60 min subjected to slow cooling in Mr. Frosty, freezing in LN and then thawed.

Morphological assessments of EC were performed by double staining with acetic carmine (2%) and Evans blue (0.1%), according to the methodology described by Durzan (1988). In order to monitor the cell viability after cryopreservation steps, fluorescein diacetate (FDA) staining was also carried out. A stock solution of 1% (w/v) FDA in acetone was diluted with water up to a final concentration of 2% (v/v). Approximately 10 µl of this stain were added directly to the material and after 4 min of incubation, green fluorescence was observed under UV illumination using an inverted microscope (Olympus IX81), equipped with a computer-controlled digital camera (DP71, Olympus Center Valley, PA, USA). Three representative and independent samples were stained and evaluated per point of the cryopreservation protocol, in each morphological analysis performed.

Transmission electron microscopy analysis

Aiming to evaluate the ultrastructural features of EC during the cryopreservation protocol steps, analysis of transmission electron microscopy (TEM) was performed. Representative samples of EC from Cr01 cell line were collected at different points of the protocol: a) EC derived from proliferation cycles (control); b) EC only subjected to 60 min cryotreatment; c) cryotreated EC for 60 min subjected to slow cooling in Mr. Frosty, freezing in LN and then thawed.

The samples were fixed with 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) plus 0.09 M sucrose overnight. The material was post-fixed with 1 % osmium tetroxide for 4h, dehydrated in a graded ethanol series, and embedded in Spurr's resin, according to the manufacturer's instructions. Thin sections were contrasted with aqueous uranyl acetate, followed by lead citrate, according to Reynolds (1963). Three independent samples of each point of the cryopreservation protocol were then examined under TEM JEM 1011 (JEOL Ltd., Tokyo, Japan, at 80 kV) and the relevant aspects recorded.

Results and discussion

Cryotreatment incubation times and cell growth dynamics

The results of the cryoprotection time experiments indicated significant differences in the fresh weight increased ratio (FWIR) of EC subjected to cryotreatments (Figure 1). After 9 days of culture, EC not subject to cryotreatment (control) indicated the highest FWIR (1.94 mg mg^{-1} FM), followed by the EC submitted to 30 and 60 min of cryotreatment (1.56 and 1.41 mg mg^{-1} FM, respectively), which indicated no statistical difference between them. The EC submitted to the largest cryoprotection times (120 and 240 min) indicated the lowest FWIR (1.14 and 1.09 mg mg^{-1} FM, respectively).

The FWIR found in the other evaluation dates (16 and 23 days) showed no significant differences among the treatments (Figure 1). These results clearly demonstrate that the cryotreatment incubation time affects the initial FWIR, resulting in a retarding process to the beginning of the cell proliferation exponential phase. However, from day 16 in culture, mass increment rates were similar, indicating that the

cryoprotection process, in the evaluated incubation times, apparently did not affect the viability of *A. angustifolia* EC. In figure 2 it is possible to observe the EC morphological features of each cryotreatment incubation time throughout the days of culture.

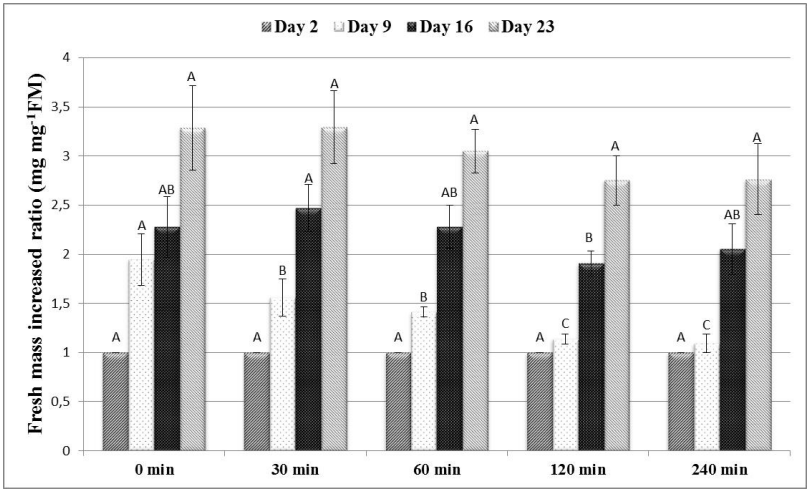

Fig. 1 Fresh mass increased ratio of *A. angustifolia* embryogenic cultures subjected to 0, 30, 60, 120 and 240 minutes of incubation in cryoprotectant solution after 2, 9, 16 and 23 days of culture in proliferation culture medium. Mean values followed by ± standard deviation (vertical bars). Uppercase letters represent differences between cryotreatment incubation times for each evaluated date, according to SNK test ($p < 0.05$)

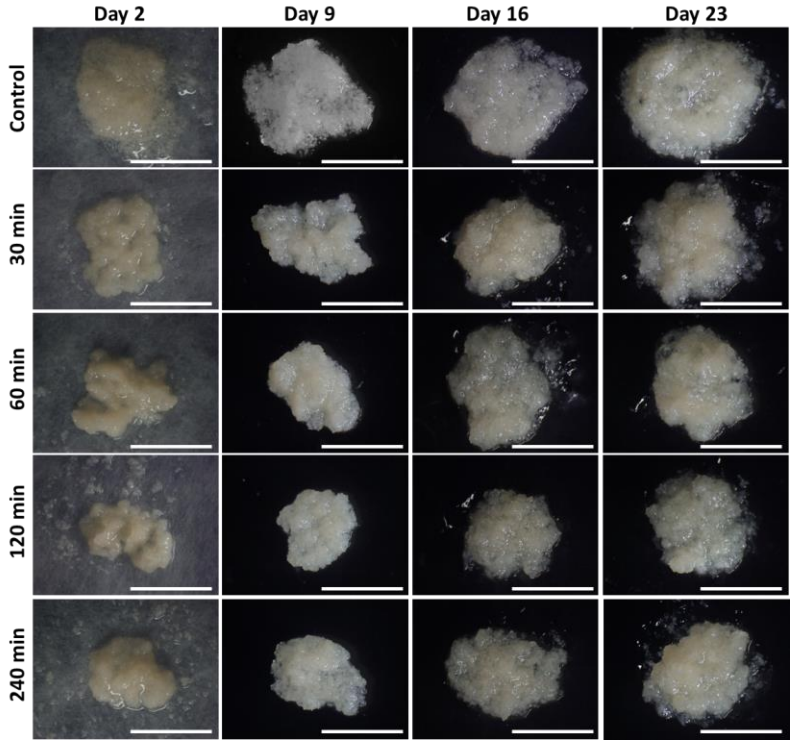

Fig. 2 *A. angustifolia* embryogenic cultures features subjected to 0, 30, 60, 120 and 240 minutes of incubation in cryoprotectant solution after 2, 9, 16 and 23 days of culture in proliferation culture medium. Bar: 1 cm

The growth stage of EC strongly affects survival after cryopreservation, and cells characterized by dense cytoplasm and few vacuoles have low water content, hence much better tolerating cryopreservation treatments (Lambardi et al. 2008). Normally, exponentially growing plant cells are more tolerant to freezing than are cells in the lag or stationary phase (Mustafa et al. 2011). In *Citrus* suspension cultures, EC at the early lag phase and at the stationary phase are large and highly vacuolated, thus having high water content. On the contrary, rapidly growing cells in the late lag and early to mid-exponential phase are small and contain dense cytoplasm, with fewer vacuoles and relatively low water content (Pérez 2000). Lainé et al. (1992) reported that, among the cryopreserved embryogenic cell

suspensions of *Pinus caribaea*, only rapidly growing cultures could be recovered successfully after cryostorage. In the present study, the use of EC during exponential growth phase may have positively influenced the FWIR after cryoprotection process, improving the FWIR of these cultures.

The cryoprotection process is one of the most important steps of the cryopreservation protocol, and the cryoprotectants can be divided into two categories: (i) permeating cryoprotectants, e.g. DMSO and glycerol, which can pass through cell membranes; and (ii) non-permeating cryoprotectants, e.g. polymers such as polyvinyl pyrrolidone and various sugars, which cannot enter cells (Panis and Lambardi 2006). The mechanism by which these agents protect cells against stresses encountered during cryopreservation is not known, but it is thought that permeating cryoprotectants reduce cell injury due to solution effects by reducing potentially harmful concentrations of electrolytes in the cell (Karlsson and Toner 1996). In our study, both permeating and non-permeating cryoprotectants were used, indicating no deleterious effects for further EC proliferation.

Evidence suggests that DMSO can lower the temperature at which freezing first occurs, alter the crystal habit of ice when it separates, and stabilize the plasma membrane by electrostatic interactions (Quatrano 1968; Karlsson and Toner 1996). In cultured cells, the optimum concentration of DMSO as a cryoprotectant ranges from 5-8% in many species (Klimaszewska et al. 1992). Although DMSO provides a cryoprotective effect, this has to be balanced with the phytotoxic effect. In white spruce EC, all the tested DMSO concentrations (5-20%) exerted the same level of toxicity prior to freezing when measured after 12 d of plating (Klimaszewska et al. 1992). Similar results were found for cryopreserved *Pinus nigra* EC, which almost all control tissues (pretreated with DMSO but not frozen) survived, suggesting limited negative effect of pretreatments on the embryogenic tissue behavior (Salaj et al. 2007; 2011). In our results, 1M DMSO showed non-inhibitory effects to *A. angustifolia* EC subjected to cryotreatment, independent of evaluated incubation time.

Due to possible toxic effects of the cryoprotection solution, which can negatively affect the embryogenic cell viability, the optimization of incubation time is the key to find a balance between appropriate dehydration and chemical toxicity (Panis and Lambardi 2006; Lambardi et al. 2008). The results found in the present study indicate that *A. angustifolia* EC support the longer cryoprotection time evaluated (240

min), without inhibition of cell proliferation (Figure 1 and 2). However, due to the possible deleterious effects of long exposure to cryoprotectant substances, such as DMSO, the use of shorter incubation time (30 or 60 min) should be applied for this cell line.

Embryogenic cultures regrowth after cryopreservation

The results of cell regrowth after cryopreservation procedures showed different responses to cryoprotection incubation time tested, according to the cell line analyzed (Table 1). For Cr01 cell line, it was observed 50% cell regrowth after 30 days culture for EC subjected to 60 min cryoprotection, without statistical difference when compared to cryoprotection treatments during 120 min (38.89%) and 240 min (44.45%). The EC derived from 30 min cryotreatment showed the lowest regrowth rate (16.67%), significantly lower than the 60 min cryotreatment. After 60 days in culture, EC derived from all cryoprotection incubation times indicated 100% cell regrowth (Table1).

For Cr02 cell line, different responses were found (Table 1). The EC derived from 30, 60 and 120 min cryotreatments indicated no cell regrowth after 30 days in culture. Otherwise, 240 min cryotreatment showed 88.89% cell regrowth, reaching 100% after 60 days of culture, significant higher than the others cryotreatment times evaluated, that resulted in regrowth rates lower than 30% in the same period. These results indicate a noticeable genotype-dependent response of the cell lines tested at different cryoprotection times evaluated.

Table 1 *A. angustifolia* embryogenic cultures regrowth rate after thawing subjected to different cryotreatment incubation times (30, 60, 120 and 240 min) after 30 and 60 days in culture

Incubation time		**Cell lines regrowth rate (%)**	
		Cr01	**Cr02**
30 min	Day 30	16.67 B	0 B
	Day 60	100 A	27.77 B
60 min	Day 30	50 A	0 B
	Day 60	100 A	5.56 B
120 min	Day 30	38.89 AB	0 B
	Day 60	100 A	11.12 B

240 min	Day 30	44.45 AB	88.89 A
	Day 60	100 A	100 A

Different letters for each cell line, within each evaluation date, indicate statistical difference between different incubation times, compared by chi-square (χ^2) contingency test (95%)

These different responses may be explained by different physiological conditions of tissues or genetic differences in cryotolerance (Salaj et al. 2007). For *Picea abies* EC it has been found that the regrowth capacity after cryopreservation, as determined by frequency of survival, length of lag-phase and growth rate, strongly depends on the genotype (Norgaard et al. 1993). *Pinus nigra* EC from five different cell lines subjected to cryoprotection with sucrose/DMSO and subsequent incubation in LN indicated variable recovery percentages, reaching values ranging from 62.5% to 100% (Salaj et al. 2007). Guzmán-García et al. (2013) also reported strong cell line-related differences in survival of cryopreserved avocado EC, with differential post-thawing survival and recovery values. Similar results were also reported by Uchendu and Reed (2008) testing different cryopreservation protocols in four varieties of *Mentha* spp.

Gale et al. (2007) evaluated five different genotypes of *Picea sitchensis* in cryopreservation experiments. The results indicated that all genotypes survived, although variable responses with respect to different cryoprotective and cryogenic treatments were observed, with specific cell lines being more tolerant compared to the others (Gale et al. 2007). Our results showed a similar response pattern, whereas the Cr02 cell line apparently required a longer exposure time to the cryoprotective solution that Cr01 to tolerate the freezing and recovery processes. Based on this, we can conclude that the Cr01 cell line is more tolerant to the applied cryopreservation protocol, and 30 or 60 min of cryoprotection should be used, since the EC have 100% cell regrowth in all the incubation times tested and a shorter exposure time to the cryoprotectant substances is preferable. For Cr02 cell line 240 min of cryoprotection should be used Cr02, where the best results were observed.

Demarchi et al. (2014) reported that *A. angustifolia* EC were able to proliferate after the cryopreservation process, indicating higher FWIR when cryotreated with PVS2. However, the authors did not present the percentage of cell regrowth, being impossible to compare with the results found in our study. Anyway, in the present study, we obtained 100% cell regrowth in both cell lines tested, indicating that the proposed

protocol can be successful applied to EC cryopreservation of this species.

Cell tracking of EC after thawing

The time-lapse cell tracking of EC from Cr01 and Cr02 cell lines subjected to 60 and 240 min cryoprotection incubation times, respectively, and then to cryopreservation procedures, is showed in figure 3. The EC of both cell lines evaluated indicated the first cell regrowth signs after 30 days in culture, and after day 40 it was possible to observe a more pronounced cell proliferation. After 50 days in culture a conspicuous cell growth was observed, corroborating the findings in cell regrowth analysis after cryopreservation process. The cell tracking observations showed that the embryogenic cells were the predominant cell type that had proliferation in the initial culture phase after thawing (Figure 3). Thereafter, the suspensor cells started to form, recovering the typical structuration of conifers EC.

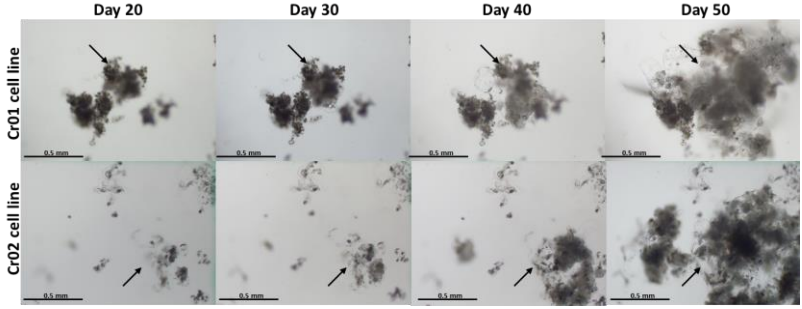

Fig. 3 Time–lapse cell tracking of *A. angustifolia* embryogenic cultures (EC) from two different cell lines (Cr01 and Cr02) subjected to 60 and 240 min cryoprotection incubation times, respectively, and then to cryopreservation procedures. The EC of both cell lines evaluated indicated the first cell regrowth signs after 30 days in culture (arrows), and after day 40 it was possible to observe a more pronounced cell proliferation. Note that the embryogenic cells were the major cell type that had proliferation in the initial culture phase after thawing

Conifer EC consist of small cells of the embryo proper attached to long highly vacuolated suspensor cells, and the cells surviving to cryopreservation are mainly the dense cells of the embryo proper, the

embryogenic cells (Häggman et al. 2000; Marum et al. 2004). Studies have reported that the recalcitrance of some cell lines to cryopreservation may reflect a high ratio of suspensors to embryogenic cells in the EC (Cyr et al. 1994; Salaj et al. 2011). This fact may be related to the embryogenic cells are relatively small, highly cytoplasmic with small vacuoles; these features improving their resistance capacity to the freezing process (Zoglauer et al. 2003). During cryopreservation of *Picea sitchensis* and *Abies* hybrids, respectively, long suspensor cells disrupted and newly formed somatic embryos developed from small meristematic cells in the embryonal end were reported (Kristensen et al. 1994; Salaj et al. 2010). The cell tracking results found in the present study corroborate with the previously report for both cell lines evaluated, which showed a massive survival and initial proliferation of embryogenic cells (Figure 3).

Time-lapse cell tracking offers the possibility to follow the developmental pathways in detail, for example, from single somatic cells to somatic embryos (Krens et al. 1998). This technique has been employed in the identification of morphological traits for the somatic embryogenesis induction in many plant cell cultures, such as barley and tobacco protoplasts, single suspension cells of carrot (*Daucus carota*), leaf explants from *Dactylis glomerata* and pro-embryogenic masses of Norway spruce (*Picea abies*) (Golds et al. 1992; Toonen et al. 1994; Somleva et al. 2000; Filonova et al. 2000). However, there are no reports of its use on the EC regrowth process after being subjected to cryopreservation procedures. Thus, to the best of our knowledge, this is the first study in which this process could be monitored by this technique, which proved to be reliable and applicable.

Cell viability and morphological features of cryopreserved EC

The results of morphological analysis by EC double staining indicated the maintenance of morphological EC integrity at all evaluated points throughout the cryopreservation process (Figure 4). Well defined pro-embryogenic masses were conspicuously observed, indicating intense reddish color of embryogenic cells, and the suspensor structure integrity, when compared to control (Figure 4A).

The FDA staining indicated the maintenance of cell viability in determined cell clusters (Figure 5). In the images we observed several regions with high fluorescence emission, as evidenced by comparing the same images to the bright field visualization. As demonstrated, these

cells presented a high viability before being subjected to cryopreservation procedures and also after the cryotreatment and thawing. It was also observed that regions with greater abundance of embryogenic cells showed higher viability, confirming the results found in the cell tracking analysis. These results reinforce the idea that these cells may be more suitable for survival after cryopreservation that the suspensor cells.

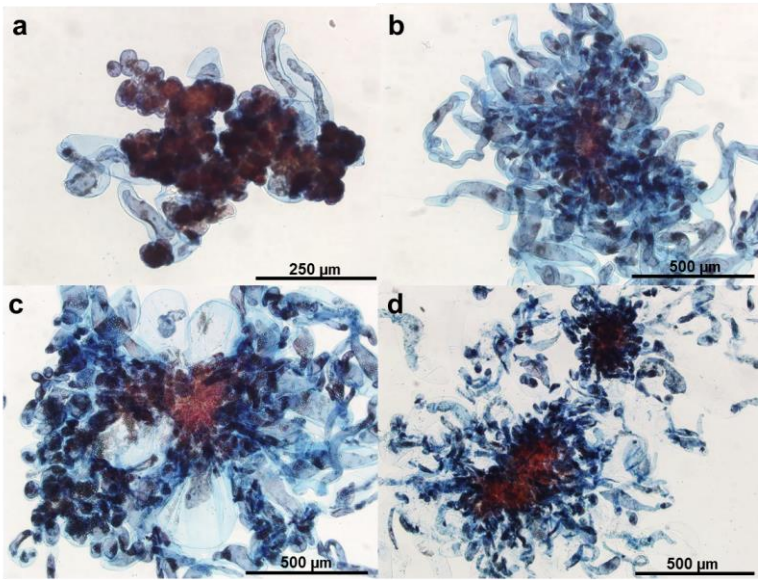

Fig. 4 *A. angustifolia* embryogenic cultures (EC) features subjected to the cryopreservation process observed with double staining with acetic carmine and Evans blue in the different protocol steps. a) EC derived from proliferation cycles (Control); b) EC only subjected to 60 min cryotreatment; c) EC after 60 min cryotreatment and slow cooling in Mr. Frosty, but not immersed in LN; d) cryotreated EC for 60 min subjected to slow cooling in Mr. Frosty, freezing in LN and then thawed. Three representative and independent samples were stained and evaluated per point of the cryopreservation protocol

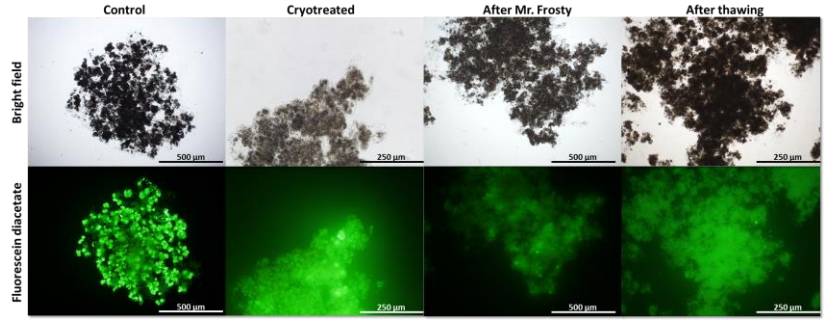

Fig. 5 *A. angustifolia* embryogenic cultures (EC) features subjected to the cryopreservation visualized with fluorescein diacetate vital staining in the different protocol steps. In the image are showed EC derived from proliferation cycles (Control); EC only subjected to 60 min cryotreatment (Cryotreated); EC after 60 min cryotreatment and slow cooling in Mr. Frosty, but not immersed in LN (After Mr. Frosty); cryotreated EC for 60 min subjected to slow cooling in Mr. Frosty, freezing in LN and then thawed (After thawing). Three representative and independent samples were stained and evaluated per point of the cryopreservation protocol

FDA test has been repeatedly used in cryopreservation studies as an indicator of cell viability (Ford et al. 2000; Salaj et al. 2007; Guzmán-García et al. 2013). This assay is based on the fact that only cells with intact plasma membranes can accumulate fluorescein (Malpique et al. 2007). After entering into a living cell, FDA is transformed by cytoplasmic esterases into fluorescein, which is brightly fluorescent under blue light (Jones and Senft 1985). In our study, FDA test confirmed a differential survival between embryogenic cells and suspensor cells, besides indicating the viability maintenance of a large number of cells after each main cryopreservation steps. This differential cryotolerance observed for the embryogenic and suspensor cells was also reported by Ford et al (2000), which observed the fluorescence in the intact embryonal head and no suspensor tissue visible when irradiated with UV light.

Transmission electron microscopy analysis

The ultrastructural features of the EC observed by TEM are shown in Figure 6. Embryogenic cells from EC of control showed characteristic morphology of meristematic cells, with thin cell wall, large and prominent nuclei, high nucleus/cytoplasm ratio, numerous

small vacuoles, and abundant presence of mitochondria (Figure 6A-B). Abundant amiloplasts containing starch grains, and some Golgi bodies were also noted. The nucleus was well preserved and indicated few regions of heterochromatin. These observations are in accordance with the findings of Fraga et al. (2015), which reported the same ultrastructural features for this cell type (embryogenic cells) for EC of *A. angustifolia* during proliferation cycles.

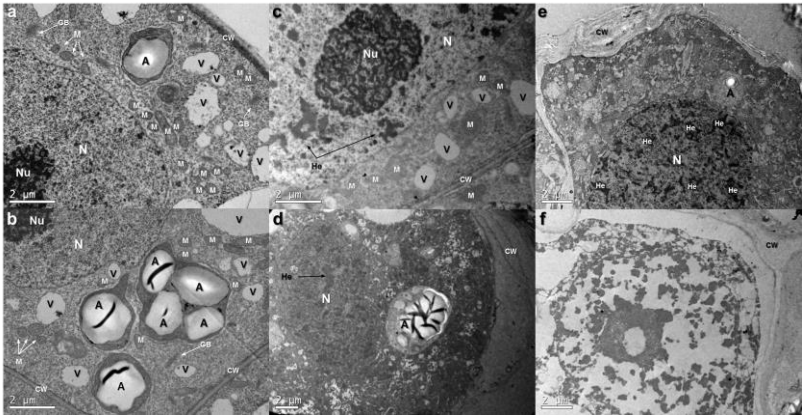

Fig. 6 Ultrastructural analyses by transmission electron microscopy of *A. angustifolia* embryogenic cultures (EC) during different cryopreservation steps. a) and b) embryogenic cell from proliferation cycles showing meristematic cell features, with large and prominent nuclei, abundant presence of mitochondria and thin cell wall. c) and d) embryogenic cell from EC only subjected to 60 min cryotreatment in detail showing fewer small vacuoles, preserved nuclear envelope, amyloplast containing starch grain, and thickened and preserved cell wall. An improved presence of heterochromatin regions was also observed. e) embryogenic cell from EC that survived to cryopreservation recovery indicating preserved nuclear envelope with nuclei containing a huge amount of heterochromatin regions and thickened preserved cell wall. In this cell was also possible to observe a more irregular conformation of the plasma membrane, possibly due to severe dehydration caused by cryopreservation process. f) embryogenic cell that did not survived to the cryopreservation indicating cytoplasmic degradation and disintegration. V: vacuole; N: nuclei; Nu: nucleolus; M: mitochondria; A: amyloplast; GB: Golgi bodies; CW: cell wall; He: heterochromatin

The embryogenic cells from EC only subjected to 60 min cryotreatment indicated a similar pattern of organelle organization.

However, some differences could be noted, as a substantial thickening of the cell wall and an important increase of cytoplasm electrondensity, possibly due to the cellular contents concentration with the dehydration caused by the cryotreatment (Figure 6C-D). A subtle increase in heterochromatin regions in the nucleus was also observed.

For the embryogenic cells from EC that survived to the entire cryopreservation protocol, it was observed the maintenance of nuclear envelope integrity, as well as the plasma membrane, which showed no retraction in relation to the cell wall. The cell wall also showed a substantial thickening, plenty of material deposited, with no apparent cell wall disruption (Figure 6E). A large number of condensed chromatin regions was also observed in these cells, when compared to embryogenic cells of control. Otherwise, the portion of embryogenic cells that did not survive to the cryopreservation indicated protoplast degradation and disintegration (Figure 6F).

Tissue desiccation and chemical permeation induced by cryoprotectant solutions affect cellular freezing properties, dehydrating the tissues and changing the behavior of water remaining (Volk and Walters 2006; Volk and Kaspersen, 2007). On one hand, smaller cells with only small vacuoles and electrondense cytoplasm, such as embryogenic cells, show the least plasmolysis during osmotic dehydration treatments. On the other hand, large and vacuolated cells, such as the suspensor cells, exhibit high levels of plasmolysis with cryoprotectant treatment (Volk and Kaspersen, 2007). The amount of water lost from the protoplasm of these larger cells appeared to be lethal since these cell types did not generally survive the imposed osmotic stresses (Volk and Kaspersen, 2007). In the present study, the ultrastructural features observed in embryogenic cells agree with the reported by these authors, being possible to observe the maintenance of its cellular integrity, unlike the suspensor cells (data not shown).

Another important aspect observed in the survived cells, the cell wall thickening, has also been associated with the increased resistance to low temperature exposure (Tahtamouni and Shibli 1999). It was showed that the accumulation of unsaturated lipids on cell membrane (which is induced by cold hardening) and consequent cell wall thickening would improve the cell cryotolerance, and may also be linked with the protective effect of DMSO (Engelmann et al. 1995). Our results indicated a conspicuous cell wall thickening in embryogenic cells after cryotreatment and thawing, especially in the surviving cells, which may

be related to osmotic stress response caused by cryopreservation process.

Epigenetic variation in chromatin and DNA methylation of gene sequences has been found in plants after cryopreservation, suggesting altered patterns of gene expression (Harding 2006). Chromatin conformation and nucleosomal structures have been implicated as cellular processes that undergo modulation during changes in gene expression, as chromatin relaxes (euchromatin) DNA no longer becomes associated with histones in these structures, being vulnerable to enzymic digestion (Harding 2006). Several studies have reported an increased proportion of heterochromatin to euchromatin in embryogenic cells after subjected to cryopreservation processes (Wen et al. 2012; Heringer et al. 2013a, 2013b). This change in heterochromatin/euchromatin ratio can possibly act as a cell defense mechanism, decreasing the DNA vulnerability to cleavage and preserving the cell integrity. Our results showed an increased heterochromatin presence in the embryogenic cells cryotreated or after thawing, suggesting that this protection process may possibly be occurring. However, further analysis should be carried out to prove that this increase is significant.

Taken together, the results found in the present study indicate that *A. angustifolia* EC support all the cryoprotection times evaluated, without expressive cell damage and cell proliferation inhibition. The use of 1M DMSO also showed non-inhibitory effects to EC subjected to cryotreatment, independent of evaluated incubation time. It was also found a noticeable genotype-dependent response of the cell lines tested at different cryoprotection times evaluated. For the cell regrowth results, we obtained 100% cell regrowth in both cell lines tested, indicating that the proposed protocol can be successful applied to EC cryopreservation of this species.

The cell tracking results showed an expressive survival and initial proliferation of embryogenic cell type, with the first cell regrowth signs after 30 days in culture. The morphological analysis by EC double staining showed the maintenance of morphological EC integrity at all evaluated points throughout the cryopreservation process. The FDA test confirmed a differential survival between embryogenic cells and suspensor cells, besides indicating the viability maintenance of a large number of cells after each main cryopreservation steps. Finally, TEM analysis indicated a conspicuous cell wall thickening in embryogenic cells after cryotreatment and thawing, especially in the survived cells,

which may be related to osmotic stress response caused by cryopreservation process. In addition, an increased heterochromatin presence in the embryogenic cells cryotreated or after thawing was also observed; this feature possibly acting as a cell defense mechanism, thus decreasing the DNA vulnerability to cleavage and preserving the cell integrity.

Author Contribution Statement Conceived and designed the experiments: HPFF, LNV and MPG; Performed the experiments: HPFF, LNV, CCP, JMS, KGA; Performed the transmission electron microscopy analysis: HPFF, LNV and EMO; Contributed reagents/materials/analysis tools: MPG; Wrote the paper: HPFF, LNV and MPG.

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CAPÍTULO 3

Este manuscrito segue as normas de formatação do períodico *Plant Cell, Tissue and Organ Culture* e foi submetido em 12/09/2015

DNA methylation and proteome profiles of *Araucaria angustifolia* (Bertol.) Kuntze embryogenic cultures as affected by plant growth regulators supplementation

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Abstract

Araucaria angustifolia is a critically endangered conifer native to South America, and somatic embryogenesis (SE) is one of the most promising biotechnological tools for its conservation and mass propagation. *In vitro* tissue culture and chemical compounds supplemented to culture medium, especially plant growth regulators (PGRs), are known to affect DNA methylation and protein expression profiles, modulating the phenotype and/or the embryogenic potential. Here, we evaluated the global DNA methylation (GDM) levels of *A. angustifolia* embryogenic cultures (EC) during SE induction and multiplication steps for one year subcultures, and identified a wide range of differentially expressed proteins in PGR-free or –supplemented treatments. During long-term subcultures, PGR-supplementation proved to gradually increase the GDM, which may compromise genomic stability and evoke gene expression modifications. Label-free proteomics enabled a robust protein identification and quantification in *A. angustifolia* EC. Exclusively expression of PIN-like protein in PGR-supplemented treatment indicated a possible differential response of the EC to polar auxin transport, which can generate implications in its morphogenetic response to maturation step. Up-regulation of stress-related proteins in EC from PGR-supplemented treatment suggests its more stressful environment, triggering notable responses to hormonal, osmotic and oxidative stresses. Improved expression of proteins involved with protein folding and stabilization processes in PGR-free treatment could play a protective function in response to stress conditions caused by *in vitro* culture, and may provide an adaptive advantage to these EC. The expression of several proteins associated to terpenoid biosynthesis suggests that EC from both treatments and cell lines are possibly producing these compounds.

Keywords: label-free proteomics; somatic embryogenesis; epigenetics; stress-related proteins; terpenoid biosynthesis.

Introduction

Araucaria angustifolia is a dioecious perennial conifer native to South America, occurring in South and Southeastern Brazil, and restricted areas of Northwestern Argentina and Paraguay (Guerra et al. 2008). Drastic population decline and habitat reduction culminated in the inclusion of *A. angustifolia* on the IUCN international list as "critically endangered" (www.iucnredlist.org). Many efforts have been carried out in order to propagate and conserve this species; however, conventional strategies are hampered mostly due to the recalcitrant nature of the seeds (Salmen-Espindola et al. 1994; Panza et al. 2002). Micropropagation techniques, such as somatic embryogenesis (SE), have potential for clonal propagation and *ex situ* conservation of commercial and endangered plant species, especially conifers (Guerra et al. 2000; Klimaszewska et al. 2011; Jo et al. 2013).

Somatic embryogenesis is the developmental restructuring of somatic cells toward the embryogenic pathway, and forms the basis of cellular totipotency in higher plants (Karami and Saidi 2010). Unlike zygotic embryos, somatic embryos can be easily manipulated and growing conditions can be controlled. These features make the SE an efficient model system for the study of morphological, physiological, molecular and biochemical aspects that occur during the initiation and development in higher plants (Zhang et al. 2007; Karami and Saidi 2010).

The early SE process in *A. angustifolia* is well characterized by the development of embryogenic cultures (EC), which, in turn, are multiplied as pro-embryogenic masses (PEM) during the early stages of SE. This step is successfully achieved; however, unknown factors hamper further somatic embryos maturation from PEM (Santos et al. 2008, 2010; Vieira et al. 2012). Recently, promising results were achieved with EC induced and proliferated in plant growth regulators-free (PGR-free) culture medium, generating somatic embryos in advanced maturation stages with reproducibility (Fraga et al. 2015). Although these recent advances, there are still several bottlenecks that require a better understanding of the underlying causes.

Ontogenetic development in trees is characterized by successive phase changes which are accompanied by drastic morphological, biochemical and physiological modifications (Poethig 1990; Teyssier et al. 2014). Cell differentiation and development are controlled by means

temporal and spatial activation and silencing of specific genes (Noceda et al. 2009). Among the mechanisms involved in the regulation of plant development, DNA methylation, a pivotal epigenetic mechanism, is one of the key factors controlling gene/protein expression (Feng et al. 2010; Miguel and Marum 2011). In cell and tissue culture systems, differentiation and dedifferentiation processes, as well as cell division, are followed by methylation and demethylation events in genomic DNA tissue-specific (Msogoya et al. 2011).

In vitro tissue culture techniques and chemical compounds supplemented to culture medium, especially PGRs, are known to induce modifications in global DNA methylation (GDM) levels, which may ultimately affect the phenotype and/or the embryogenic potential (LoSchiavo et al. 1989; Valledor et al. 2007; Rodríguez Lopéz et al. 2010). Specific changes in GDM in plants were proved to be related to the developmental phase (Fraga et al. 2002), being also possible to relate the specific methylation status to the further *in vitro* morphogenic ability of EC (Valledor et al. 2007). Several recent reports on SE of different conifer species have suggested causal relationships between GDM levels and *in vitro* morphogenetic competence (Klimaszewska et al. 2009; Noceda et al. 2009; Leljak-Levanic et al. 2009; Teyssier et al. 2014).

Proteomics is a global analysis strategy that provides information of a multitude of processes in complex events, such as SE (Balbuena et al. 2011). Proteins directly influence cellular biochemistry, providing more accurate analysis of cellular changes during growth and development (Chen and Harmon 2006). Changes in the proteome profile during SE of woody species have been reported (Marsoni et al. 2008; Cangahuala-Inocente et al. 2009; Pan et al. 2009, 2010; Sghaier-Hammami et al. 2010). In addition, recently efforts have been made to describe *A. angustifolia* somatic and zygotic embryogenesis at the molecular level, using proteomic and transcriptomic approaches (Balbuena et al. 2009, 2011; Jo et al. 2013; Elbl et al. 2015).

The multidimensional protein identification technology "MudPIT" has become a popular approach for performing shotgun proteomics, with high resolution orthogonal separation coupled to tandem mass spectrometry (2D-nanoLC-MS/MS) (Chen et al. 2006; Heringer et al. 2015). High-definition $HDMS^E$ (data independent acquisition with ion mobility) (Lalli et al. 2013), with increased selectivity and specificity, are required for shotgun proteomics and complex samples due to their resolving power for overlapping chimeric

peptides (Geromanos et al. 2009). This technology has enabled the identification of low-abundant proteins, which are often missed when using two-dimensional electrophoresis (Washburn et al. 2001). Here, we describe for the first time for *A. angustifolia* a 2D-nanoLC-MS/MS strategy for proteome investigation.

A remarkable aspect in the induction and maintenance process of *A. angustifolia* EC is the possibility of establishing cell lines both in the presence or absence of PGR supplementation to culture medium. This striking ability allows the establishment of a reliable model system to investigate the possible effects of these PGRs in both GDM levels and proteome profile of EC, with minimal biological variation. In this sense, the aim of the present work was to investigate the involvement of individual proteins in response to PGR supplementation during EC proliferation and to identify the wide range of proteins that are differentially regulated in these different treatments (PGR-free or – supplemented). We used the HDMS^E technique, a gel- and label-free approach which allows the qualitative and quantitative analysis of large number of protein in complex samples. In addition, we quantified the GDM levels of EC induced and maintained in PGR-free or – supplemented treatments for one year subculture.

Materials and methods

Plant material

Immature female cones bearing early globular-staged zygotic embryos were collected in December 2012, from an *A. angustifolia* open-pollinated natural population in Lages, Santa Catarina – Brazil (latitude 28° 02′ S, longitude 50° 17′ W, altitude 1030 m). Three different female individuals were chosen as donor plants (Cr01, Cr02, and Cr03). Two independent cones were collected from each donor plant, wherein one cone was used to establish an individual cell line (named Cr01, Cr02, and Cr03 according to the respective donor plant), and the other one for global DNA methylation analysis.

Somatic embryogenesis induction and EC proliferation

Embryogenic cultures were induced according to Santos et al. (2002). The seeds were submitted to disinfestation procedures with 70%

ethanol for 5 min and 1.5% sodium hypochlorite for 15 min, followed by a triple-washed with autoclaved distilled water. Zygotic embryos were excised and inoculated in Petri dishes containing 25 mL of induction culture medium. The basic induction culture medium consisted of BM macro-, micro-salts and vitamins (Gupta and Pullman 1991) containing L-glutamine (1.0 g L^{-1}), myo-inositol (1.0 g L^{-1}), casein hydrolysate (0.5 g L^{-1}), and sucrose (30 g L^{-1}). Two different induction treatments were established: PGR-free induction culture medium (BM_0), and PGR-supplemented induction culture medium (4 µM 2,4-D, 2 µM BAP, 2 µM KIN - BM_4). All culture media were gelled with Phytagel® (2 g L^{-1}), the pH adjusted to 5.8 and autoclaved at 121°C, 1.5 atm for 15 min. All the cultures were maintained in a growth room in the absence of light at temperature of 22°C ± 2°C.

After 30 days induction, the EC obtained were subcultured in Petri dishes containing 25 mL of BM_0 or BM_2 (2 µM 2,4-D, 0.5 µM BAP, 0.5 µM KIN), according to the respective induction culture media composition (with or without PGR supplementation). Subcultures were made every 21 days in gelled BM_0 or BM_2 culture medium. All the cultures were maintained in a growth room in the absence of light at temperature of 22°C ± 2°C.

Global DNA methylation analysis

Samples for GDM analysis were collected from the three different *A. angustifolia* lines (Cr01, Cr02, and Cr03), wherein each line were considered as a biological replicate. These samples consisted of: a) early globular-staged zygotic embryos (explant used for SE induction); b) EC after 30 days induction in culture media BM_0 or BM_4; c) EC derived from multiplication in BM_0 or BM_2 culture media, every two multiplication cycles, from cycle 3 to cycle 17, totaling a period of one year collections (Fig. 1). The aim of such sampling was to follow up the dynamics of global DNA methylation in EC induced and long-term maintained in PGR presence or absence.

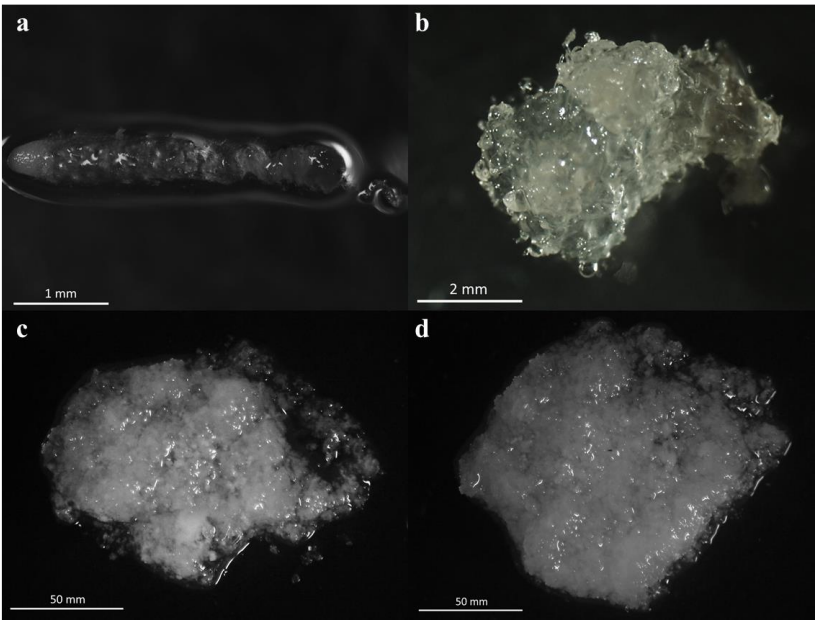

Fig 1. Morphological features of plant material used for DNA methylation and proteomic analyses. A- Early globular-staged *A. angustifolia* zygotic embryos (explant used for somatic embryogenesis induction); B – Representative embryogenic culture (EC) obtained after 30 days culture in induction culture media; C - Representative EC derived from plant growth regulators-free treatment during multiplication step; D - Representative EC derived from plant growth regulators-supplemented treatment during multiplication step.

DNA extraction was performed in samples consisting of three different biological replicates (Cr01, Cr02 and Cr03 cell lines), for each collection time, according to Doyle (1987). Nucleic acids digestion procedures were based on the method described by Johnston et al. (2005) and Fraga et al. (2012). HPLC analysis was performed according to Johnston et al. (2005). A HyperClone™ 5 µm ODS (C18) 120 Å, LC Column 250 x 4.6 mm (Phenomenex®, Torrance, USA), guard column (4.0 × 3.0 mm) (Phenomenex®), and UV detector (280 nm) were used. The gradient program consisted of 3 min with 100% buffer A (0.5% v/v methanol in 10 mM KH_2PO_4 adjusted to pH 3.7 with phosphoric acid, 0.22 µm filtered), followed by a linear gradient from 3 to 20 min to 100% of buffer B (10% v/v methanol in 10 mM KH_2PO_4 adjusted to pH

3.7 with phosphoric acid, 0.22 µm filtered), followed by 20–25 min with 100% of buffer B. A flow rate of 1 mL min^{-1} and 20 µL of sample injection volume were applied.

The dNTPs (Fermentas®, Hanover, MD, USA) used as standards (dA, dT, dC and dG) and 5mdC were digested for 2h with alkaline phosphatase (10 U mL^{-1}) and Tris-HCl (0.5 M, pH 8.3) at 37°C to obtain the nucleosides. The standard nucleosides (5-50 mM) were prepared in deionized H_2O and stored at -20°C. 5mdC quantification (%) was performed according to 5mdC concentration divided by 5mdC concentration plus dC concentration multiplied by 100. The obtained peak area was analyzed by LC Solution software (Shimadzu®, Kyoto, Japan). Data were analyzed by Statistica® (Statsoft Inc., Tulsa, USA) for Windows® version 7.0 and submitted to ANOVA. Treatments were compared by Student-Newman-Keuls (SNK) post hoc test ($p < 0.05$).

Proteomic analyses

Total protein extraction

Embryogenic cultures from Cr01 and Cr02 cell lines established in PGR-free and –supplemented culture media were collected after 7 cycles in multiplication step and subjected to total protein extraction (Fig. 1C and 1D). Protein extracts were prepared in biological triplicate (500 mg FM) for each treatment (PGR-free or –supplemented) in each cell line evaluated. Protein extraction protocol used was based on that described by Balbuena et al. (2009). Briefly, the samples were ground and transferred to clear 2-mL microtubes containing 1.5 mL of extraction buffer (7 M urea, 2 M thiourea, 1% DTT, 2% Triton X-100, 0.5% Pharmalyte, 1 mM PMSF, all reagents purchased from Sigma-Aldrich). The extracts were briefly vortexed and kept in extraction buffer on ice for 30 min followed by centrifugation at 12,000 g for 5 min at 4°C. The supernatants were collected, and protein concentration was measured using a 2-D Quant Kit (GE Healthcare, Piscataway, NJ, USA). The five protein extracts resulting from each treatment were pooled, totaling 100 µg of protein (Luge et al. 2014). The samples were stored at −20°C until proteomic analysis.

Protein digestion

The protein samples (100 μg) were purified using a methanol–chloroform method according to Komatsu et al. (2013). Briefly, 600 μL methanol and 150 μL chloroform was added and the sample was mixed by vortexing. Then, 450 μL water was added to the sample to induce phase separation, mixed by vortexing, and centrifuged at 20,000 ×g for 10 min at room temperature. The upper phase was carefully discarded, and 450 μL methanol was then added slowly to the remaining organic phase. The samples were centrifuged again with the same conditions, the supernatant was removed, and the pellet was dried in air for 10 min. The dried pellet was resuspended in 50 μL 50 mM NH_4CO_3.

For trypsin digestion, a 2 μg μL^{-1} solution of 50 μL of the previous sample plus 25 μL of 0.2% v/v *Rapi*GEST (Waters, USA) (Yu et al. 2003) was added to a 1.5-mL microfuge tube, vortexed and heated in an Eppendorf Thermomixer Comfort device at 80°C for 15 min. Then, 2.5 μL of 100 mM DTT was added and placed in the thermomixer at 60°C for 30 min. The tubes were placed on ice (30 sec), and 2.5 μL of 300 mM iodoacetamide (IAA) was added, followed by vortexing and incubation in the dark for 30 min at room temperature. Afterwards, 20 μL of trypsin (50 ng μL^{-1}) solution that was prepared with 50 mM NH_4HCO_3 (pH 8.5) was added and the tubes placed in the thermomixer at 37°C overnight. After that, 10 μL of trifluoroacetic acid (TFA) 5% v/v was added to precipitate the surfactant *Rapi*GEST, the tubes were vortexed and incubated at 37°C for 90 min (without shaking), and centrifuged at 4,000 × g for 30 min at 4°C. Then, 100 μL of the supernatant was collected and transferred to Total Recovery Vial (Waters, USA) for further shotgun mobility-DIA proteomics analysis.

Label-free protein quantification by MS

Qualitative and quantitative nano-ultra-high pressure chromatography (nanoUPLC) tandem nanoESI-HDMS^E (multiplexed DIA—data-independent acquisition) experiments were conducted using both a 1h reverse-phase gradient from 7% to 40% (v/v) acetonitrile (0.1% v/v formic acid) and a 350 nL min^{-1} nanoACQUITY UPLC 2D Technology system. A nanoACQUITY UPLC HSS T3 1.8 μm, 75 μm × 150 mm column (pH 3) was used in conjunction with a reversed-phase (RP) XBridge BEH130 C_{18} 5 μm, 300 μm × 50 mm nanoflow column (pH 10).

For every measurement, the mass spectrometer was operated in resolution mode with a typical *m/z* resolving power of at least 35000 FWHM and an ion mobility cell that was filled with nitrogen gas and a cross-section resolving power at least 40 $\Omega/\Delta\Omega$. The effective resolution with the conjoined ion mobility was 1,800,000 FWHM. Analyses were performed using nano-electrospray ionization in positive ion mode nanoESI (+) and a NanoLockSpray (Waters, Manchester, UK) ionization source. The lock mass channel was sampled every 30 sec. The mass spectrometer was calibrated with an MS/MS spectrum of [Glu1]-Fibrinopeptide B human (Glu-Fib) solution (100 fmol μL^{-1}) that was delivered through the reference sprayer of the NanoLockSpray source. $[M + 2H]^{2+}$ = 785.8426) was used for initial single-point calibration, and MS/MS fragment ions of Glu-Fib were used to obtain the final instrument calibration.

DIA scanning with added specificity and selectivity of a non-linear 'T-wave' ion mobility ($HDMS^E$) device (Giles et al. 2011) was performed with a SYNAPT G2-Si HDMS mass spectrometer (Waters), which was automatically planned to switch between standard MS (3 eV) and elevated collision energies $HDMS^E$ (19–45 eV) applied to the transfer 'T-wave' CID (collision-induced dissociation) cell with argon gas; the trap collision cell was adjusted to 1 eV, using a millisecond scan time that was previously adjusted based on the linear velocity of the chromatographic peak that was delivered through nanoACQUITY UPLC to generate a minimum of 20 scan points for each single peak, both in low-energy and high-energy transmission at an orthogonal acceleration time-of-flight (oa-TOF) and a mass range from *m/z* 50 to 2000. The RF offset (MS profile) was adjusted such that the nanoESI-$HDMS^E$ data were effectively acquired from *m/z* 400 to 2000, ensuring that any masses that were observed in the high-energy spectra with less than m/z 400 arose from dissociations in the collision cell. The samples and conditions were injected with the same amount on the column. Stoichiometric measurements based on scouting runs of the integrated total ion account (TIC) prior to analysis were performed to ensure standardized molar values across all conditions.

Database searching and quantification

Progenesis QI for Proteomics Software V.2.0 (Nonlinear Dynamics, Newcastle, UK) was used to process the MS^E data. A protein

databank from Pinidae subclass (Gymnosperm) was used, obtained from UniProt database (http://www.uniprot.org/taxonomy/3313).

The search conditions were based on taxonomy (Pinidae); maximum missed cleavages by trypsin allowed up to 1, variable modifications by carbamidomethyl (C) and acetyl N-terminal and oxidation (M); and a default maximum false discovery rate (FDR) value of 4%. The obtained proteins were organized by software algorithm into a statistically significant list corresponding to increased and decreased regulation ratios between the different groups. Normalizations were performed automatically by Expression^{E} software, which was included inside PLGS informatics, using the recommended default parameters. Co-expressed proteins were filtered based on a fold change of $\log_2$ 1.2, as determined by the overall coefficient of variance for all quantified proteins across all replicates, and classified as up-regulated when $\log_2$ was 1.2 or greater and as down regulated when $\log_2$ was -1.2 or less.

For a functional context of the EC proteomics results, the identified proteins were further analyzed using Blast2GO, a bioinformatics tools for gene ontology (GO) based DNA or protein sequence annotation, to classify the biological process, cellular component, and molecular functional of the identified proteins (Götz et al. 2008).

Results

Global DNA methylation levels during long-term EC proliferation

Embryogenic cultures induced and maintained in PGR-free culture medium showed a heterogeneous pattern of global DNA methylation (GDM) between successive multiplication cycles. From the first to the third subculture cycle the GDM remained constant (15.38%), as compared to the levels found in zygotic embryos (15.98%), with a decrease in the cycle 7, and then showing an increase. This unstable behavior persisted until the cycle 17 multiplication (Figure 2A). EC maintained in the PGR-supplemented culture medium showed a more regular pattern, with an initial significant reduction of the GDM in the cycle 3 (10.62%), then remaining stable until the cycle 7 (Figure 2B). From the ninth cycle (12.96%) there was a gradual and steady increase in GDM, resulting in 14.43% at the cycle 17. The range of GDM

variation in PGR-free treatment was almost twice that observed in PGR-supplemented treatment.

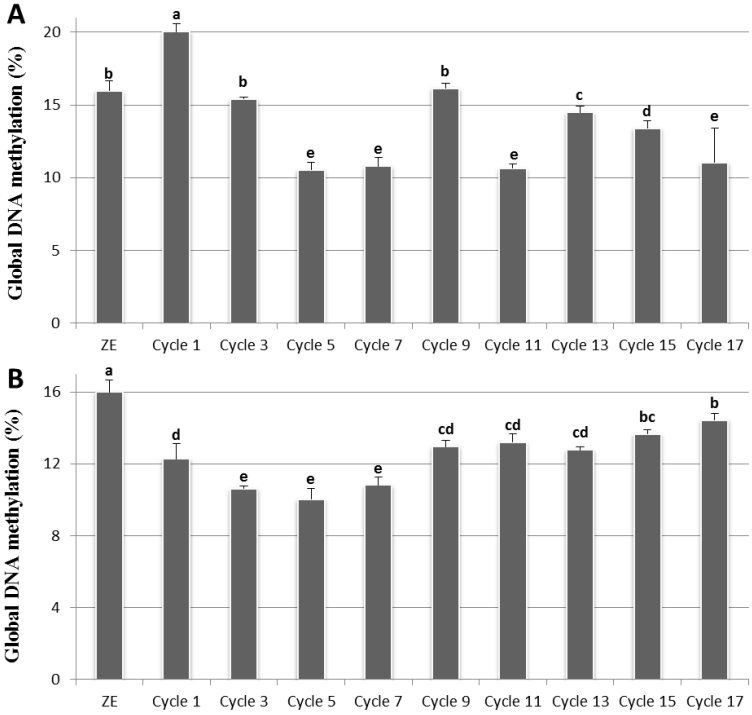

Fig 2. Global DNA methylation levels after somatic embryogenesis induction (cycle 1) and during successive subculture cycles of *A. angustifolia* embryogenic cultures subjected to plant growth regulators-free (A) and –supplemented treatments. Mean values followed by standard deviation (vertical bars). Means followed by lowercase letters are significantly different according to the SNK test ($p < 0.05$).

Protein identification between PGR-free and –supplemented treatments

High-throughput proteomic screening identified 993 and 969 proteins for Cr01 and Cr02 cell lines, respectively, which were concomitantly expressed in both treatments evaluated (PGR-free or –supplemented). Among these, 293 and 283 (Cr01 and Cr02 cell lines, respectively) could be cross-referenced and annotated with the corresponding homolog protein in the accessed database. For Cr01cell

line, 51 were differentially expressed (up- or down-regulated) between the two conditions (Figure 3), and 41 proteins indicated differential expression for Cr02 cell line (Supplementary figure 1). For EC subjected to PGR-supplemented treatment, 5 and 3 unique proteins, from Cr01 and Cr02 cell lines, respectively, were identified and for EC from PGR-free treatment 2 unique proteins were found, from each cell line evaluated (Table 1).

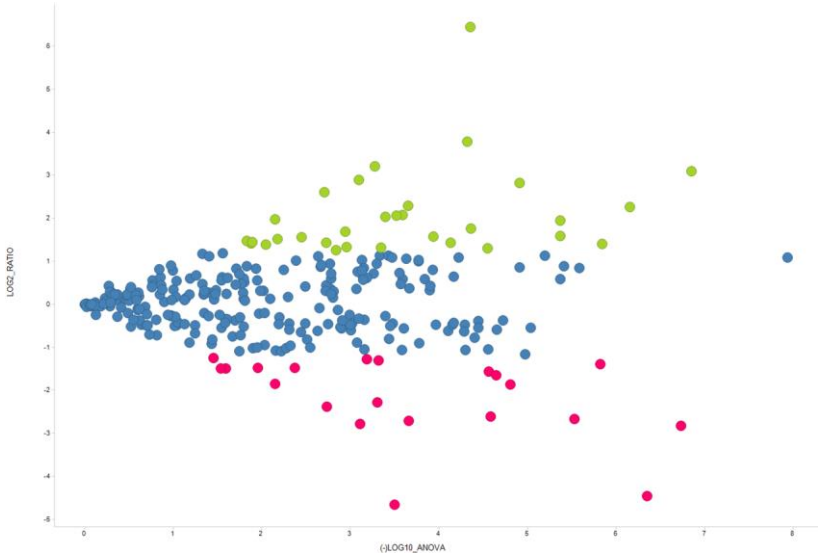

Fig 3. Volcano plot for expressed proteins of *A. angustifolia* embryogenic cultures from Cr01 cell line contrasting expression ratio between plant growth regulators (PGR)-supplemented/-free treatments. Green dots represent significant up-regulated proteins, red dots significant down-regulated proteins and blue dots unchanged expression level found between PGR-supplemented and –free treatments.

Table 1. Unique proteins identified in *A. angustifolia* embryogenic cultures from both cell lines (Cr01 and Cr02) subjected to plant growth regulators (PGR)-free or –supplemented treatments.

Accession number	Description	Peptide count	Peptides used for quantitation	Confidence score

Unique proteins in PGR-supplemented treatment (Cr01)				
D0PPL9_9SPER	ATP synthase subunit alpha (Fragment)	16	1	168.55
C0PRH2_PICSI	Phosphoglycerate kinase	13	1	105.83
G8HSH5_9SPER	ATP synthase subunit alpha, chloroplastic	4	1	24.12
A9NZJ5_PICSI	Histone H2B	2	1	20.98
B8RIJ0_9SPER	Putative epoxide hydrolase (Fragment)	2	1	10.86
Unique proteins in PGR-free treatment (Cr01)				
B2KZJ2_PICAB	Putative senescence-associated protein (Fragment)	2	1	20.24
D5A831_PICSI	T-complex protein 1 subunit gamma	2	1	11.46
Unique proteins in PGR-supplemented treatment (Cr02)				
A0A075IEQ1_9SPER	PIN-like protein (Fragment)	4	1	22.28
A0A075E5K9_9SPER	DNA-directed RNA polymerase subunit alpha	3	1	18.02
C1IXS9_PINGE	DNA-directed RNA polymerase subunit beta	1	1	5.20
Unique proteins in PGR-free treatment (Cr02)				
B2KZJ2_PICAB	Putative senescence-associated	2	1	20.24

	protein (Fragment)			
Q9BAN2_9SPER	Ribulose-1,5-bisphosphate carboxylase large subunit (Fragment)	9	1	58.35

Confidence scores were calculated by ProteinLynx Global Server (PLGS).

Although the genome of *A. angustifolia* has not been sequenced, 27% and 26% of the differentially expressed proteins, from Cr01 and Cr02 cell lines, respectively, could be identified and annotated using sequence data from Pinidae subclass.

Protein functional classification

Based on predicted biological process, most of the identified proteins in both cell lines and evaluated treatments were likely to be involved in metabolic, cellular and single-organism processes (Figure 4A; Supplementary figure 2A). Regarding the molecular function, the three top GO processes were catalytic activity, binding and structure molecule activity, also in both cell lines and evaluated treatments (Figure 4B; Supplementary figure 2B). For cellular component GO category, most of proteins were located in cell, organelle and macromolecular complex (Figure 4C; Supplementary figure 2C).

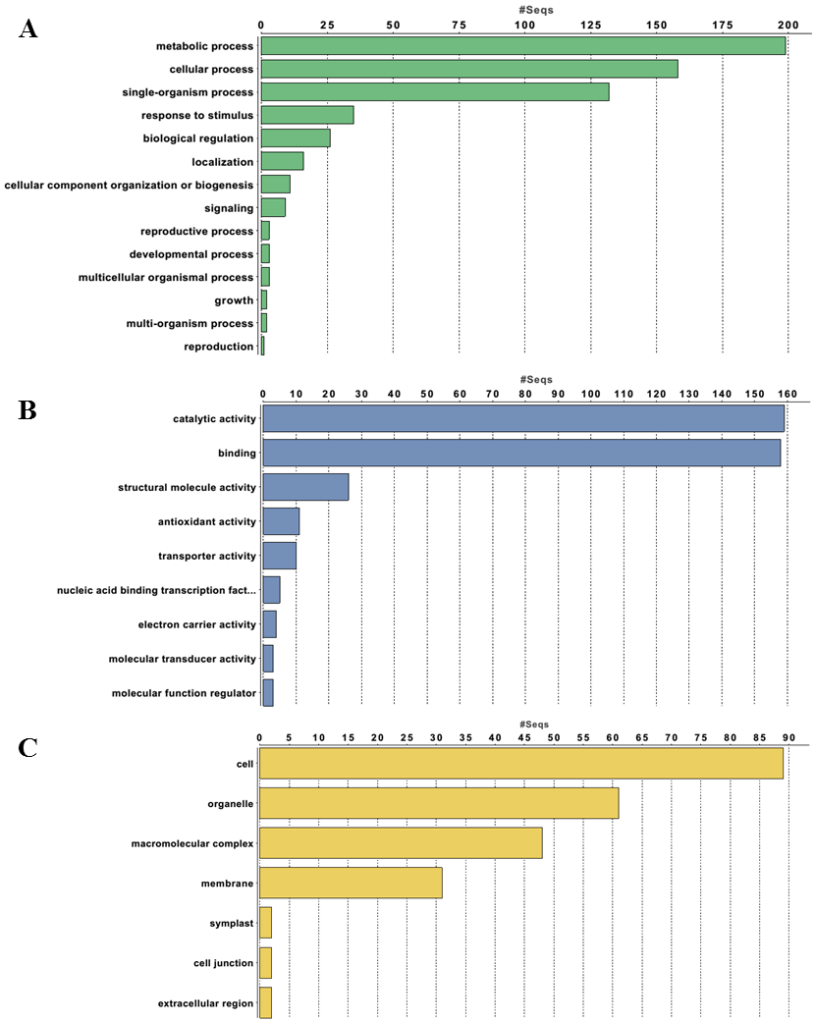

Fig 4. Functional classification of identified proteins in *A. angustifolia* embryogenic cultures from Cr01 cell line in both plant growth regulators treatments (supplemented or not) using Blast2GO software based on universal gene ontology (GO) annotation terms. The proteins were linked to at least one annotation term within the GO biological process (A), molecular function (B), and cellular component (C) categories.

With respect to the differentially expressed proteins for Cr01 cell line, the most up-regulated protein in PGR-supplemented treatment was

E,E-α-farnesene synthase, associated to terpene synthase activity, followed by Tau class glutathione S-transferase, related to oxidation-reduction process (Table 2). Many other proteins associated to the redox metabolism were up-regulated in this treatment (cinnamyl alcohol dehydrogenase, isoflavone reductase homolog 2, isocitrate dehydrogenase, among others), being the highest assigned biological process. Between down-regulated proteins in PGR-supplemented treatment, emphasis on heat shock protein (70 kDa) and thaumatin-like protein, proteins involved in protein folding and stabilization. Again, several oxidative stress-related proteins were identified.

Differentially expressed proteins for Cr02 cell line indicated slight differences (Table 2). The most up-regulated protein in PGR-supplemented treatment was histone H3, related to protein heterodimerization activity, followed by DNA-directed RNA polymerase and Elongation factor 1-alpha. Regarding to down-regulated proteins, we highlight two proteins associated to methyltransferase activity (mRNA cap guanine-N7 methyltransferase and cathechol O-methyltransferase) which were not differentially expressed in Cr01 EC and that may indicate a different pattern of response to PGR-supplementation between evaluated cell lines.

Table 2. Differentially expressed proteins identified in *A. angustifolia* embryogenic cultures from both cell lines (Cr01 and Cr02) subjected to plant growth regulators (PGR)-free or –supplemented treatments.

Accession number	Description of homologous protein	Peptide count[a]	Ratio PGR-supplemented/-free[b]	Function[c]	Sub-cellular localization[d]
Up-regulated proteins in PGR-supplemented treatment (Cr01 cell line)					
Q675K8_PICAB	E,E-α-farnesene synthase	1	86.95	Terpene synthase activity	Not assigned
L7S0Y4_PINTB	Tau class glutathione S-transferase	3	13.76	Glutathione transferase activity	Not assigned
B8LMY9_PICSI	Ubiquinone biosynthesis	1	9.25	Ubiquinone biosyntheti	Mitochondria

	protein COQ4 homolog			c process	
Q0Q418_PINTA	Class III HD-Zip protein HDZ34 (Fragment)	1	8.50	Lipid binding	Not assigned
HSP02_PSEMZ	Putative heat shock protein 2 (Fragment)	1	7.44	Response to heat	Cytoplasm
I6Q476_9SPER	4-coumarate: coenzyme A ligase (Fragment)	4	7.04	Catalytic activity	Not assigned
R9QW62_9SPER	Cinnamyl alcohol dehydrogenase (Fragment)	5	6.07	Oxidation-reduction process	Not assigned
B2KZF6_PICAB	Zinc finger protein-related protein (Fragment)	2	4.89	Protein ubiquitination	Not assigned
Q9MR99_TSUCA	ATP synthase subunit beta (Fragment)	10	4.77	ATP-binding	Plastid
G8J144_9SPER	30S ribosomal protein S2	1	4.22	Structural constituent of ribosome	Plastid
G4LAW1_TAICR	30S ribosomal protein S3	2	4.16	Structural constituent of ribosome	Plastid
E6Y3C3_PINMO	WRKY transcription factor PmWRKY91	1	4.09	Sequence-specific DNA binding	Not assigned

	(Fragment)				
Q9AVU0_PICAB	40S ribosomal protein S2	5	3.94	Structural constituent of ribosome	Ribosome
A9NLS7_PICSI	60S ribosomal protein L6	3	3.84	Structural constituent of ribosome	Ribosome
Q9FSD2_PINSY	Phytochrome (Fragment)	2	3.39	Photoreceptor activity	Not assigned
TCTP_PSEMZ	Translationally-controlled tumor protein homolog	5	3.00	Not assigned	Not assigned
Q6J0T7_PICGL	Type II CPD DNA photolyase (Fragment)	2	2.97	DNA photolyase activity	Not assigned
Q5TIN4_PINTA	Alpha-1,6-xylosyltransferase	1	2.94	Transferase activity	Golgi, Plasma membrane
Q29VH9_TAXWM	Taxadiene synthase	6	2.86	Terpene synthase activity	Not assigned
Q5EKC1_PINTA	Phenylalanine ammonia-lyase 1 (Fragment)	1	2.78	Catalytic activity	Not assigned
B5T3M6_JUNVI	RNA polymerase subunit (Fragment)	1	2.72	DNA-directed RNA polymerase activity	Plastid
V5W951_9SPER	Gamma class glutathione S-transferase	10	2.70	Transferase activity	Not assigned
R4L9Z6_CUN	Cytochrome	1	2.70	Cytochrom	Plastid

LA	c biogenesis protein CcsA			e complex assembly	
IFRH2_PSEMZ	Isoflavone reductase homolog 2 (Fragments)	1	2.67	Oxidation-reduction process	Not assigned
A9NVG2_PICSI	Isocitrate dehydrogenase [NAD] subunit	11	2.64	Oxidation-reduction process	Mitochondria
L7RY37_9SPER	Rab GTPase (Fragment)	2	2.61	Protein transport	Intracellular
V5L1A8_9SPER	UDP-glucose 6-dehydrogenase	22	2.53	Oxidation-reduction process	Not assigned
A9NXV4_PICSI	Thioredoxin reductase	3	2.48	Removal of superoxide radicals	Cytoplasm
A9NNS9_PICSI	Ribosomal protein	6	2.45	Structural constituent of ribosome	Ribosome
B8LNN0_PICSI	40S ribosomal protein S12	2	2.38	Structural constituent of ribosome	Ribosome
Down-regulated proteins in PGR-supplemented treatment (Cr01 cell line)					
C6FCI5_PSEMZ	Heat shock protein 70 kDa (Fragment)	11	0.42	Not assigned	Not assigned
E0ZBJ0_PICSI	SKP1-interacting-like protein (Fragment)	2	0.41	Not assigned	Not assigned
Q5DWG0_CRYJA	Thaumatin-like protein (Fragment)	1	0.40	Not assigned	Not assigned
A9NVT9_PICSI	Malate dehydrogena	15	0.38	Oxidation-reduction	Not assigne

	se			process	d
G3PC_PINSY	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	16	0.36	Oxidation-reduction process	Not assigned
D5A7Y5_PICSI	Auxin-responsive protein	1	0.36	Auxin-activated signaling pathway	Nucleus
A9NN85_PICSI	Small ubiquitin-related modifier	1	0.36	Not assigned	Nucleus
Q2L638_TAXDI	Light-independent protochlorophyllide oxidoreductase subunitN (Fragment)	1	0.36	Oxidation-reduction process	Plastid
X2D433_JUNMO	Apocytochrome f	2	0.34	Oxidation-reduction process	Plastid
D5AAB1_PICSI	Histone H2A	3	0.32	Protein heterodimerization activity	Nucleosome
Q944N7_TAXWC	Paclitaxel/taxanoid biosynthesis susceptibility protein TS1 (Fragment)	1	0.28	Endonuclease activity	Not assigned
CADH7_PICAB	Probable cinnamyl alcohol dehydrogenase 7/8	6	0.27	Oxidation-reduction process	Intracellular

Q711T3_PINPS	Histone H2A	4	0.21	Protein heterodimerization activity	Nucleosome
G8IVK5_PINPU	ATP synthase subunit c, chloroplastic	1	0.19	Ion transport	Plastid
Q9M3Y3_PINHA	Phosphoenolpyruvate carboxylase (Fragment)	3	0.16	Catalytic activity	Not assigned
D2CNK0_PINBU	Maturase R (Fragment)	1	0.16	Not assigned	Not assigned
A0A023W6Y7_PINPA	Trans-cinnamate 4-hydroxylase 2 (Fragment)	3	0.15	Oxidation-reduction process	Intracellular
A9NUH0_PICSI	Endoglucanase	1	0.15	Polysaccharide catabolic process	Not assigned
O65863_PINEL	Reverse transcriptase (Fragment)	3	0.14	RNA-directed DNA polymerase activity	Not assigned
G3C8Z9_PINPS	Phenylcoumaran benzylic ether reductase	5	0.05	Oxidation-reduction process	Not assigned
Q8GUU4_PINPS	Chalcone synthase	4	0.04	Transferase activity, transferring acyl groups	Not assigned
Up-regulated proteins in PGR-supplemented treatment (Cr02 cell line)					
K7NYB2_LARDC	Histone H3 (Fragment)	6	54.56	Protein heterodime	Nucleosome

				rization activity	
G8IUJ9_PINRI	DNA-directed RNA polymerase	7	34.06	DNA-directed RNA polymerase activity	Plastid
R4IKK9_9SPER	Elongation factor 1-alpha	22	20.72	Translational elongation	Cytoplasm
Q9AVU0_PICAB	40S ribosomal protein S2	5	11.09	Structural constituent of ribosome	Ribosome
A9NVN6_PICSI	Serine/threonine-protein phosphatase	1	8.66	Phosphatase activity	Not assigned
E0ZA86_PICSI	Leucine rich repeat-like protein (Fragment)	2	7.21	Not assigned	Not assigned
Q9XEW8_PICGL	14-3-3 protein	23	4.55	Protein domain specific binding	Not assigned
BAS1_PINST	Putative 2-Cys peroxiredoxin BAS1 (Fragments)	3	4.12	Oxidation-reduction process	Plastid
R9QW62_9SPER	Cinnamyl alcohol dehydrogenase (Fragment)	5	4.04	Oxidation-reduction process	Not assigned
A9NZJ5_PICSI	Histone H2B	2	4.04	Protein heterodimerization activity	Nucleosome
B2KZF6_PIC	Zinc finger	2	3.85	Protein	Not

AB	protein-related protein (Fragment)			ubiquitination	assigned
Q9FSD2_PINSY	Phytochrome (Fragment)	2	2.68	Photoreceptor activity	Not assigned
LIAS_PICSI	Lipoyl synthase	2	2.63	Protein lipoylation	Mitochondria
I3VMC1_PINMU	ABA and WDS induced 1 protein (Fragment)	1	2.49	Response to stress	Not assigned
E0ZCV6_PICSI	Basic endochitinase-like protein (Fragment)	1	2.41	Cell wall catabolic process	Not assigned
C6FGA1_9SPER	Putative 60S ribosomal protein L10A (Fragment)	3	2.37	Translational process	Ribosome
Down-regulated proteins in PGR-supplemented treatment (Cr02 cell line)					
O24271_9SPER	Actin 4 (Fragment)	3	0.43	Not assigned	Not assigned
A0A076U5K7_PINBN	CYP720B1v2	1	0.43	Oxidation-reduction process	Not assigned
B9VLC7_9SPER	AtpA	1	0.42	Ion transport	Mitochondria
Q9M520_TSUHE	Pinoresinol-lariciresinol reductase TH2	1	0.42	Not assigned	Not assigned
C4XUQ2_CRYJA	30S ribosomal protein S11	1	0.41	Translational process	Plastid

D5A8F9_PICSI	Succinyl-CoA ligase subunit beta	9	0.41	Catalytic activity	Not assigned
G8IU03_9SPER	Cytochrome b6-f complex subunit 6	1	0.41	Oxidation-reduction process	Plastid
A0A097ZMV6_CRYJA	Putative PHYN2	1	0.40	Photoreceptor activity	Intracellular
B6V6Q0_9SPER	Putative carbonic anhydrase (Fragment)	3	0.37	Carbonate dehydratase activity	Not assigned
C6F655_PSEMZ	Luminal binding protein (Fragment)	1	0.36	Not assigned	Not assigned
A0A068PCB8_9SPER	UDP-glucose pyrophosphorylase	5	0.35	Uridylyltransferase activity	Not assigned
E0ZBJ0_PICSI	SKP1-interacting-like protein (Fragment)	2	0.34	Not assigned	Not assigned
O24270_9SPER	Actin 2 (Fragment)	2	0.34	Not assigned	Not assigned
B1PID6_9SPER	Putative chaperonin family protein	2	0.32	UTP-binding	Not assigned
C0PS33_PICSI	mRNA cap guanine-N7 methyltransferase	1	0.32	Methyltransferase activity	Nucleus
CADH7_PICAB	Probable cinnamyl alcohol dehydrogenase 7/8	1	0.25	Oxidation-reduction process	Intracellular

B0ZR82_PINRA	Catechol O-methyltransferase (Fragment)	1	0.25	Methyltransferase activity	Nucleus
A9NUH1_PICSI	N-acetyl-gamma-glutamyl-phosphate reductase	1	0.20	Oxidation-reduction process	Cytoplasm
A9NUH0_PICSI	Endoglucanase	1	0.19	Polysaccharide catabolic process	Not assigned
C3VYB7_9SPER	DNA-directed RNA polymerase subunit alpha	3	0.18	DNA-directed RNA polymerase activity	Plastid
Q1XG58_CRYJA	Putative elongation factor	1	0.13	Translational process	Cytoplasm
A9NMW7_PICSI	Thioredoxin	1	0.12	Oxidation-reduction process	Intracellular
Q5DWG0_CRYJA	Thaumatin-like protein (Fragment)	1	0.10	Not assigned	Not assigned
A9NZ28_PICSI	Ribosomal protein L15	1	0.09	Structural constituent of ribosome	Ribosome
Q944N7_TAXWC	Paclitaxel/taxanoid biosynthesis susceptibility protein TS1 (Fragment)	1	0.08	Endonuclease activity	Not assigned

[a]"Peptide count" indicates number of peptides identified in this analysis. [b]"Ratio PGR-supplemented/-free" indicates the differential protein expression ratio between embryogenic cultures subjected to plant growth regulators (PGR)–supplemented compared to -free treatment. [c]"Function" indicates protein

function categorized using Blast2GO results. [d]"Sub-cellular localization" indicates protein localization predicted by Blast2GO results.

Between five unique proteins expressed in PGR-supplemented treatment (Cr01 cell line), two were assigned for ATP synthesis, one for glycolytic process, one for hydrolase activity and the last one for DNA-binding (Table 1). Unique proteins for Cr02 cell line in PGR-supplemented treatment were different, with two DNA-directed RNA polymerase, associated in the transcription process, and the interesting PIN-like protein, known for its involvement in polar auxin transport. Unique proteins found in PGR-free treatment showed one protein in common for both cell lines, the putative senescence-associated protein, involved in the translation process, and a uniquely expressed protein in each cell line, which were related to protein folding (Cr01) and carbon fixation (Cr02).

Discussion

Global DNA methylation is affected by PGRs supplementation during successive EC subcultures

In the present study, results of GDM for EC induced and maintained in PGR-free or –supplemented treatments indicated, as expected, diverse behaviors. During one year subcultures, GDM could be monitored and contrasted with the initial explant levels.

Results indicated a differentiated initial response (cycle 1) of EC from PGR-free treatment, showing a significant increase in GDM levels, the opposite situation observed in PGR-supplemented treatment (Fig. 2). Dedifferentiation process occurred during SE induction is a key-step to obtaining EC. This process is thought to be achieved through the erasing of some, if not all, of the pre-existing epigenetic marks (and among them, DNA methylation) across the genome (Feng et al. 2010; Jacob and Martienssen 2011; Rival et al. 2013). Our results showed that, in fact, GDM differed from the initial status (zygotic embryo); however, diverse responses were observed for EC from PGR-free or –supplemented treatments.

Several studies have suggested the existence of a progressive DNA methylation along with plant development, in a similar way to that described throughout the mammals embryonic development (Goto and

Monk 1998; Hsieh 2000; Ruiz-García et al. 2005). Teyssier et al. (2014) suggested that global DNA hypermethylation in EC is associated with somatic embryos differentiation in hybrid larch (*Larix* × *eurolepis*). In our results, increased GDM in the induced EC on PGR-free culture medium may indicate that, despite the occurrence of the dedifferentiation process in most cells, a portion of these cells may have committed to the beginning of embryonic development process, causing an increase in GDM levels. On the other extreme, the significant decrease in GDM after EC induction on PGR-supplemented treatment may suggest a more uniform pattern in cell dedifferentiation and maintenance of the cells in an undifferentiated state.

Subsequent subculture cycles of EC in PGR-free treatment maintained an oscillating GDM pattern, but without achieving the highest values found after the first subculture. For EC maintained in PGR-supplemented culture medium GDM gradually increased, until cycle 17 of subcultures. Although no other studies comparing the GDM of EC maintained in PGR presence or absence, the consequences of long-term *in vitro* propagation on GDM levels of cultured plant cells have been the subject of several recent investigations.

In *Taxus media* cell cultures propagated over 5 years, GDM quantitation by HPLC showed a time-dependent increase (from 17 to 25.7%, on average), whereas MSAP (methylation sensitive amplification polymorphism) analyses revealed a number of locus-specific methylation gains and losses throughout the period (Fu et al. 2012). In oil palm (*Elaeis guineensis*) cell suspensions, four out of five clonal lines studied showed time-dependent increased GDM, and at the end of 12-months proliferation period, the GDM average of the three ‘‘hypermethylating’’ lines have increased by approximately 10% (Rival et al. 2013). The same authors stated that GDM could be modulated by the environmental conditions imposed on *in vitro* cultivated cells, with a long series of subcultures inducing a sizeable increase in GDM. Our results for EC derived from PGR-supplemented treatment corroborate this idea, with increased GDM during prolonged subcultures; however, the same is not true for EC maintained on PGR-free treatment, highlighting the possible PGR role in this process.

Correlations between GDM levels and morphogenetic response have previously been reported for several plant species (Lambé et al. 1997; Fraga et al. 2002; Noceda et al. 2009; Klimaszewska et al. 2009; Fraga et al. 2012; Maury et al. 2012; Rival et al. 2013; Teyssier et al. 2014). Many angiosperm species show variations in GDM during SE,

with embryogenic cell lines generally exhibiting lower GDM levels than non-embryogenic lines (LoSchiavo et al. 1989; Miguel and Marum 2011; Maury et al. 2012). In gymnosperms, Noceda et al. (2009) also reported a clear negative correlation between GDM level and embryogenic competence in *Pinus nigra* EC.

In the present study, we do not present results associated with morphogenetic response of EC from PGR-free or –supplemented treatments subjected to the maturation step; however, a recent study published by our research group reported promising results with EC from the same cell line evaluated in this study (Cr01) induced and maintained in PGR-free culture medium (Fraga et al. 2015). In that study, EC were subsequently inoculated in maturation culture medium and torpedo-staged somatic embryos could be successfully achieved. In addition, preliminary assays performed in our laboratory with EC derived from PGR-supplemented treatment demonstrated poor responsiveness to maturation treatment (data not shown).

The use of synthetic auxin analogues, such as 2,4-D, used in PGR-supplemented treatment in the present study, have been frequently linked to compromise genomic stability through the promotion of DNA methylation deregulation coupled with gene expression modifications (LoSchiavo et al. 1989; Bairu et al. 2006; Krogan and Long, 2009). A recent report by Zhang et al. (2012) provides evidence of a functional interplay between environmentally induced epigenetic modifications, response to PGRs and phenotypic plasticity. Our results showed that PGR-supplementation affects GDM in a long-term maintained EC; nonetheless, more evidences are necessary to demonstrate a possible deregulatory effect on embryogenic competence of those EC.

To date, the interplay between GDM and embryogenic competence has yet been clearly established (Klimaszewska et al. 2009); however, apparently, genome hypo-methylation appears to be a characteristic of EC that are able to regenerate whole plants (Noceda et al. 2009; Teyssier et al. 2014). Based on this notion, it can be assumed that the induction and maintenance of *A. angustifolia* EC in PGR-free culture medium may be recommended, thus avoiding possible epigenetic deleterious effects during their subsequent morphogenetic process.

Label-free proteomic analysis

Proteomic studies have been shown to be powerful tools for monitoring the physiological status of plant organs under specific developmental conditions (Rose et al. 2004). Unlike model biological systems, the full potential of proteomics is far from being fully exploited in plant biology research (Abril et al. 2011). Thus, only a low number of plant species have been investigated at the proteomics level and, mainly, by using strategies based on 2-DE coupled to MS, resulting in low proteome coverage (Carpentier et al. 2008). *A. angustifolia* is a non-model conifer, for which bioinformatic resources are scarce or even absent; consequently, protein identification mostly relies on limited sequence coverage. In addition, little information about non-Pinaceae conifer species is available in the database. Nevertheless, some recent efforts using 2-DE coupled to MS approach have attempted to characterize at a proteomic level the zygotic (Balbuena et al. 2009, 2011) and somatic embryogenesis (Jo et al. 2013) of this species.

Here, we describe for the first time in this species a label-free proteomic approach, using 2D-nanoESI-HDMS^E technology, which proved to be a reliable alternative for large-scale protein identification in *A. angustifolia* EC. Shotgun proteomics allowed the identification of 293 and 283 proteins of EC from Cr01 and Cr02 cell lines, respectively, with 51 proteins differentially expressed (up- or down-regulated) between two conditions (PGR-free or –supplemented) for Cr01, and 41 differentially expressed for Cr02.

Regarding protein functional classification, most of the identified proteins in both cell lines and evaluated treatments were likely to be involved in metabolic, cellular and single-organism processes, for biological process GO category; catalytic activity, binding and structure molecule activity, for molecular function; and located in cell, organelle and macromolecular complex, for cellular component GO category. A recent study performed a functional annotation of the embryogenesis reference transcriptome for this species (Elbl et al. 2015). In that study, the most highly represented categories, which comprised at least 4,000 genes, were almost the same found in our results for all three GO categories, indicating consistency in proteomic data obtained in our study.

PIN-like protein is exclusively expressed in PGR-supplemented treatment

Here, we describe for the first time in a proteomic study the PIN-like protein expression during somatic embryogenesis (Table 1). This protein was found exclusively expressed in EC from Cr02 cell line subjected to PGR-supplemented treatment.

Auxin is a central regulator in many processes during plant growth and development. An important aspect of auxin action is its directional transport through the plant (Friml 2003). This polar auxin transport (PAT) is important for many auxin-regulated processes and requires the activity of polarly localized efflux regulators, represented by members of the PIN-FORMED family (Weijers et al. 2005). The PIN-FORMED (PIN) proteins are a plant-specific family of transmembrane proteins that transport the plant signal molecule (phytohormone) auxin as their substrate (Křeček et al. 2009). In land plants, the PIN proteins act as key regulators in multiple developmental events ranging from embryogenesis through morphogenesis and organogenesis to growth responses to environmental stimuli (Křeček et al. 2009).

Treatments of embryos with various PAT inhibitors hampers the establishment of bilateral symmetry, i.e., cotyledon and shoot apical meristem development, resulting in reduced PAT and several morphological abnormalities, often including deformed or fused cotyledons (Palovaara et al. 2010). Investigating *Picea abies* SE, Palovaara et al. (2010) suggested that auxin efflux proteins of the PIN family are also involved in modulating (creating local) auxin maxima/gradients and directing auxin flow in distinct regions of developing conifer somatic embryos.

Auxin itself upregulates the transcription of PINs, and other phytohormones and PGRs also influence the activity of the PIN promoters to various degrees; however, the effects are organ- or even cell-type-specific and strongly depend on the particular part of the plant examined and PGR used (Křeček et al. 2009). Not surprisingly, PIN-like protein was found as unique expressed protein in PGR-supplemented treatment in our results. Nevertheless, the specific effect of this protein expression in EC subjected to PGR-supplementation and its consequences in the maturation step remain unclear.

Recently, Elbl et al. (2015), based on transcriptome data of *A. angustifolia* SE, suggested that early somatic embryos fails to establish the correct auxin distribution due to *WUSCHEL* over-expression, which may be culminates in altered polar auxin flux. In that study, the authors

used another culture medium composition, plant genotype and PGR-free treatment, culture conditions quite distinct from those used in this study.

Differentially expressed proteins in PGR-free and –supplemented treatments are related to terpenoid biosynthesis

E,E-α-farnesene synthase were the most up-regulated protein detected in EC induced and maintained in PGR-supplemented treatment, from Cr01 cell line (Table 2). This enzyme converts farnesyl diphosphate (FDP) to (E,E)-α-farnesene, a sesquiterpene hydrocarbon produced by many plant species in a range of tissues, in response to pathogens (Huang et al. 2003), or on wounding by herbivores (Vuorinen et al. 2004). To date, there are no reports on up-regulation of this protein in plant SE. However, studies have shown that prevention of scald in pome fruits, a physiological storage disorder that affects certain cultivars of apples and pears, by 1-methylcyclopropene treatment is correlated with both strong inhibition of ethylene production and marked reduction in α-farnesene synthesis (Apolo Arquiza et al. 2005; Lurie et al. 2005). In addition, there is ample evidence that expression of the apple gene encoding α-farnesene synthase is induced by ethylene during the initial weeks of storage (Gapper et al. 2006).

Jo et al. (2013) reported improved ethylene levels in *A. angustifolia* EC responsive to maturation from PGR-free culture medium during proliferation. However, these results are in disagreement with that reported by Leroux et al. (2009), which observed that the inhibition of ethylene production increased the frequency of somatic embryos formation from microspores of *Brassica napus*. Similarly, ethylene was found to negatively affect *Picea glauca* somatic embryo development (Kong and Yeung, 1994). Furthermore, higher levels of this hormone have been found in non-embryogenic than in EC of white spruce and carrot (Kumar et al. 1990; Feirer and Simon 1991). In another report involving modulation of ethylene levels, an increased synthesis was observed in response to application of high amounts of 2,4-D, which impaired embryo development (Minocha and Minocha 1995). In our results, the up-regulation of E,E-α-farnesene synthase may be related to ethylene synthesis and, consequently, PGR supplementation.

Surprisingly, one of the up-regulated proteins found in PGR-supplemented treatment was taxadiene synthase. Taxadiene synthase is a key enzyme catalyzing the first committed step of the biosynthetic

pathway of Taxol™ (paclitaxel), a diterpenoid that can be found in the bark and needles of different species of *Taxus* (Khan et al. 2009). The ability to produce Taxol in plant cell culture has been viewed as a promising alternative production system for this chemotherapeutic drug, as well as a useful model to delineate and manipulate the complex biosynthetic pathway (Brincat et al. 2002). Suspension cultures of *Taxus baccata* can produce and release significant amounts of Taxol and other taxanes, achieving levels of up to 13 mg L^{-1} extracellular (Hirasuna et al. 1996).

Kim et al. (2006) reported enhanced Taxol production in *Taxus* suspension cultures in response to phytosulfokine-α plant peptide supplementation, along with increased levels of mRNA of taxadiene synthase, in comparison to control cultures. Obviously, taxadiene synthase up-regulation found in our results does not ensure that *A. angustifolia* EC from PGR-supplemented treatment are producing significant levels of this compound. However, this finding is an evidence that this synthesis may be occurring in these EC, may be a first report for a species that does not belong to *Taxus* genus.

Other strong evidence of our results that there might be terpenoid synthesis, such as Taxol, in these *A. angustifolia* EC is the phenylalanine ammonia-lyase 1 (PAL) up-regulation (Table 2). PAL is not directly involved in Taxol biosynthesis, although its substrate, phenylalanine, is an important precursor for the side chain and the C2 benzoyl group (Brincat et al. 2002). These authors investigated the use of cinnamic acid and other PAL inhibitors to inhibit phenolic production in *Taxus canadensis*, with the prediction that greater availability of phenylalanine might result in enhanced Taxol production. Once again, up-regulation of this protein may be related to the Taxol production in these EC; however, further studies quantifying the Taxol levels can confirm whether or not this hypothesis.

Intriguingly, another protein predicted to be involved in Taxol biosynthesis, paclitaxel/taxanoid biosynthesis susceptibility protein TS1, were found to be up-regulated in EC from PGR-free treatment, in both evaluated cell lines (Table 2). Hu et al. (2004) provided evidences at transcriptional level and successfully isolated a clone (TS1) with specific expression in the Taxol synthesis phase from *Taxus chinensis* cells; nevertheless, the accurate relationship between TS1 and Taxol production needs to be further elucidated. The interesting here is that, apparently, *A. angustifolia* cell lines from both PGR-free and -

supplemented treatments showed involvement in the terpenoid biosynthesis process.

Stress-related proteins are differentially expressed between PGR-free and –supplemented treatments

Another interesting up-regulated protein detected in PGR-supplemented EC was Tau class glutathione S-transferase. Glutathione-S-transferases (GSTs) are the enzymes that catalyze the conjugation of the tripeptide glutathione (GSH) to a wide variety of hydrophobic, electrophilic, and cytotoxic substrates. Many GSTs also act as GSH-dependent peroxidases by catalyzing the reduction of organic hydroperoxide to the less toxic monohydroxy alcohols (Gong et al. 2005). Plant GSTs have been intensively studied for their ability to detoxify herbicides (Flocco et al., 2004). Members of the GST gene family are upregulated during auxin-induced SE, and GST induction is auxin regulated (Marsoni et al. 2008). This enzyme catalyzes GSH conjugation to herbicide molecules, to form glutathione-S-conjugates, which are then imported to vacuoles, thus protecting the plants from damage by herbicides (Gong et al. 2005). So, in general, plants with higher GST levels are more tolerant to herbicide exposure. Apparently, the high expression level of this enzyme on EC subjected to PGR-supplemented treatment is related to improved capability of herbicide detoxification via GSH conjugation by activation of GST activity.

Other stress-related proteins were also up-regulated in PGR-supplemented treatment (Table 2). Oxidative stress caused by increased levels of radical oxygen species (ROS) has been reported to enhance SE in many plant species (Caliskan et al. 2004). ROS has been implicated also as a second messenger during auxin and stress-induced embryogenesis, and maybe act as signaling molecules playing an important role during auxin induced SE (Marsoni et al. 2008). Thus, the improved expression of these proteins in EC subjected to PGR-supplemented treatment maybe also associated to auxin stress responses.

Between stress-related proteins up-regulated in PGR-supplemented treatment, special attention should be paid to ABA and WDS induced 1 protein (Table 2). The ABA and water-deficit stress (WDS)-induced proteins represent a family of plant proteins induced by WDS or ABA stress and ripening (Sang et al. 2012). The role of these proteins at the molecular level is unclear, but they have been observed to

be up-regulated in a number of plant species as a consequence of WDS (Padmanabhan et al. 1997).

The up-regulation of ABA and WDS induced 1 protein found in the present study may also indicate that these EC can contain high endogenous ABA levels, compared to EC maintained in PGR-free culture medium. According to Stasolla and Yeung (2003), the endogenous ABA levels in gymnosperms are normally low during the initial phases of embryonic development, and higher levels were associated with developmental failure. Farias-Soares et al. (2014) reported that the decrease in ABA levels was coincident with an increase in the frequency of pro-embryos formation in *A. angustifolia*. Based on this notion, PGR supplementation may be causing WDS on *A. angustifolia* EC, possibly related to undesirable improved ABA levels.

A pathogenesis-related thaumatin-like protein was up-regulated in PGR-free treatment, in both cell lines evaluated. This protein accumulates in response to stress, such as wounding, infection by a virus or fungus, and osmotic stress (Yasuda et al. 2001). Their synthesis during *in vitro* conditions could be related with the adaptation of the plant cells to new environmental conditions, which, in a first phase, may be perceived by the cells as unfavorable, hence resulting in the activation of defense mechanisms probably not directly related with a specific morphogenetic pathway, such as SE (Correia et al. 2012). The same authors hypothesized that, alternatively, the stress response of plant cells may activate signaling pathways, triggering cellular events leading to the formation of embryonary structures. Our evidences that EC derived from PGR-free treatment have improved responses to maturation treatment may be in accordance to this hypothesis.

Proteins involved in protein folding and stabilization appear to be enhanced in PGR-free treatment

The most down-regulated protein in PGR-supplemented treatment for Cr01 cell line was assigned to the family of 70 kDa heat shock proteins (Hsp70s). The Hsp70s act as molecular chaperones and play essential roles in protein biogenesis, transport and degradation (Morano et al. 2012). Their function may be of increased importance under stress conditions, where misfolding of polypeptides occurs more commonly (Zhang et al. 2009). In addition, Hsp70s have been associated to normal development during embryogenesis and seed

germination (Waters et al. 2008). Furthermore, these proteins play a role in translocation of proteins across membranes by maintaining proteins in an extended translocation-competent conformation that also affects reserve mobilization (Latijnhouwers et al. 2010).

Members of the heat shock protein family have been reported to be highly expressed during initiation of SE from somatic cells, microspores and developing pollen in alfalfa and tobacco and from hypocotyls in carrots (Györgyey et al. 1991; Zarsky et al. 1995; Kitamya et al. 2000). Marsoni et al. (2008) also found Hsp70s up-regulated during *Vitis vinifera* SE. Fraga et al. (2013) reported the presence of these proteins in the normal somatic plantlets and its absence in the off-types plantlets derived from somatic embryos of *Acca sellowiana*.

In the same way, luminal binding protein, also a member of the Hsp70s, was up-regulated in PGR-free treatment for Cr02 cell line (Table 2). In Douglas fir, this protein was shown to be regulated during seed development and early seedling growth (Forward and Misra, 2000). On the contrary, Teyssier et al. (2014) reported that Hsps were present in higher levels at the late embryogenesis stage than at the early stage, probably in order to prepare the embryos for desiccation. Thus, the up-regulation of Hsp70s in PGR-free treatment could also play a protective function in response to the stress conditions that characterize *in vitro* growth, and provide an adaptive advantage to these EC.

Putative chaperonin family protein was also up-regulated in PGR-free treatment (Cr02 cell line). Chaperones are overexpressed under several types of stress in *in vitro* conditions (Correia et al. 2012). These proteins are also known for their roles in the maturation of protein complexes and in facilitating the folding process of newly synthesized proteins, similarly to above described proteins from Hsp70s family.

Proteins with methyltransferase activity are up-regulated in PGR-free treatment

Among up-regulated proteins in PGR-free treatment is the mRNA cap guanine-N7 methyltransferase (Table 2). Eukaryotic mRNAs are modified at the 5'-end by the addition of a m7GpppN cap, catalyzed by the sequential action of three enzymatic activities, RNA 5'-triphosphatase, guanylyltransferase, and guanine-N7 methyltransferase (Mandal et al. 2004). The cap with its unique structural characteristics has important, positive impacts on multiple downstream steps in gene

expression, including RNA stability, splicing, transport, and translation initiation (Maniatis and Reed 2002).

Guanine-N7 methyltransferase catalyzes methyl transfer from S-adenosylmethionine (AdoMet) to GpppRNA to form m7GpppRNA, which directs pre-mRNA to the processing and transport pathways in the cell nucleus and regulates both mRNA turnover and the initiation of translation (Bujnicki and Rychlewski 2002). Although there are no reports in the literature on the impact of this protein expression in plants, mutations in guanine-N7 methyltransferase of the yeast capping apparatus that inhibit its activity are lethal *in vivo* (Bujnicki and Rychlewski 2002). Thus, the up-regulation of this protein in the PGR-free treatment possibly affects the translation process, altering the EC proteome profile. However, the methyltransferase activity of this protein cannot be directly linked to GDM levels evaluated in this study.

The other protein with methyltransferase activity found up-regulated in PGR-free treatment was catechol O-methyltransferase. This protein plays an important role in the central nervous system of animals, metabolizing catecholamine neurotransmitters, such as dopamine and epinephrine (Joshi and Chiang 1998). In plants, the enzyme caffeoyl CoA O-methyltransferase from alfalfa showed a high level of similarity in the core of the enzyme, despite poor sequence similarity to catechol O-methyltransferase (Ferrer et al. 2005). In a general way, many O-methyltransferases are involved in the synthesis of secondary products such as lignin, flavonoids and phytoalexins (Joshi and Chiang 1998). However, due to poor characterization of catechol O-methyltransferase in plants, their specific role remains unknown.

Conclusion

Our approach to investigate the effect of PGR-free and -supplemented treatments in *A. angustifolia* EC allowed the identification of different scenarios between them in an unprecedented way.

GDM differed from the initial status (zygotic embryo) in both treatments, and diverse responses were observed between EC from PGR-free and –supplemented treatments. Increased GDM in EC induced on PGR-free culture medium may indicate that, despite the occurrence of the dedifferentiation process in most cells, a portion of these cells may have committed to the beginning of embryonic development process, causing an increase in GDM levels. Otherwise, the significant

decrease in GDM after EC induction on PGR-supplemented treatment may indicate a more uniform pattern in cell dedifferentiation and maintenance of the cells in an undifferentiated state. During long-term subcultures, PGR-supplementation proved to gradually increase the GDM levels, which has frequently been linked to compromise genomic stability and evoke gene expression modifications.

Label-free proteomics proved to be a reliable method for large-scale protein identification in *A. angustifolia* EC, enabling a robust protein identification and quantification. Exclusively expression of PIN-like protein in PGR-supplemented treatment indicated a possible differential response of the EC to polar auxin transport, which can generate implications in its morphogenetic response to maturation step. Regarding to differentially expressed proteins, up-regulation of stress-related proteins in EC from PGR-supplemented treatment, such as GSTs, suggests a more stressful environment, triggering notable responses to hormonal, osmotic and oxidative stresses. In the same way, improved expression of proteins involved with protein folding and stabilization processes in PGR-free treatment, such as Hsps, could play a protective function in response to the stress conditions caused by *in vitro* culture, and may provide an adaptive advantage to these EC. The expression of several proteins associated to terpenoid biosynthesis pathways suggests that EC from both evaluated treatments and cell lines possibly are producing these compounds, may be a first report for a conifer species that does not belong to *Taxus* genus.

Author Contribution Statement Conceived and designed the experiments: HPFF, LNV and MPG; Performed the *in vitro* culture experiments and DNA methylation analysis: HPFF, LNV and CCP; Performed the label-free proteomics and data analysis: HPFF, ASH and VS; Contributed reagents/materials/analysis tools: VS and MPG; Wrote the paper: HPFF, LNV and MPG.

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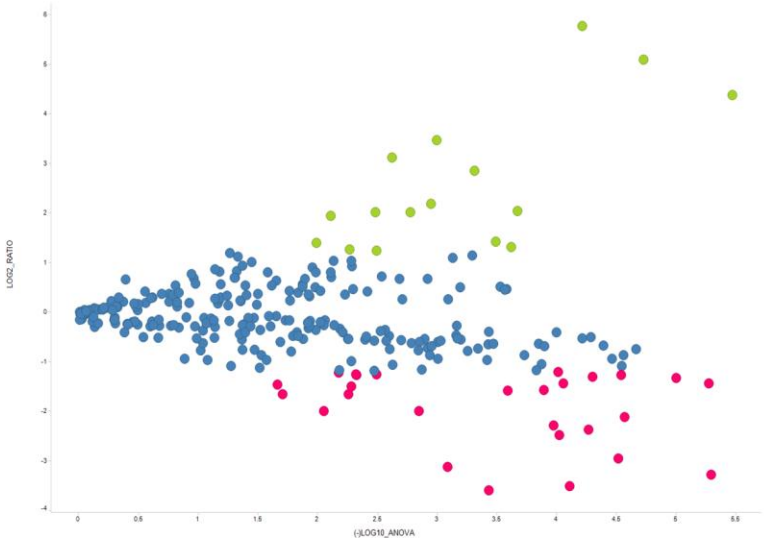
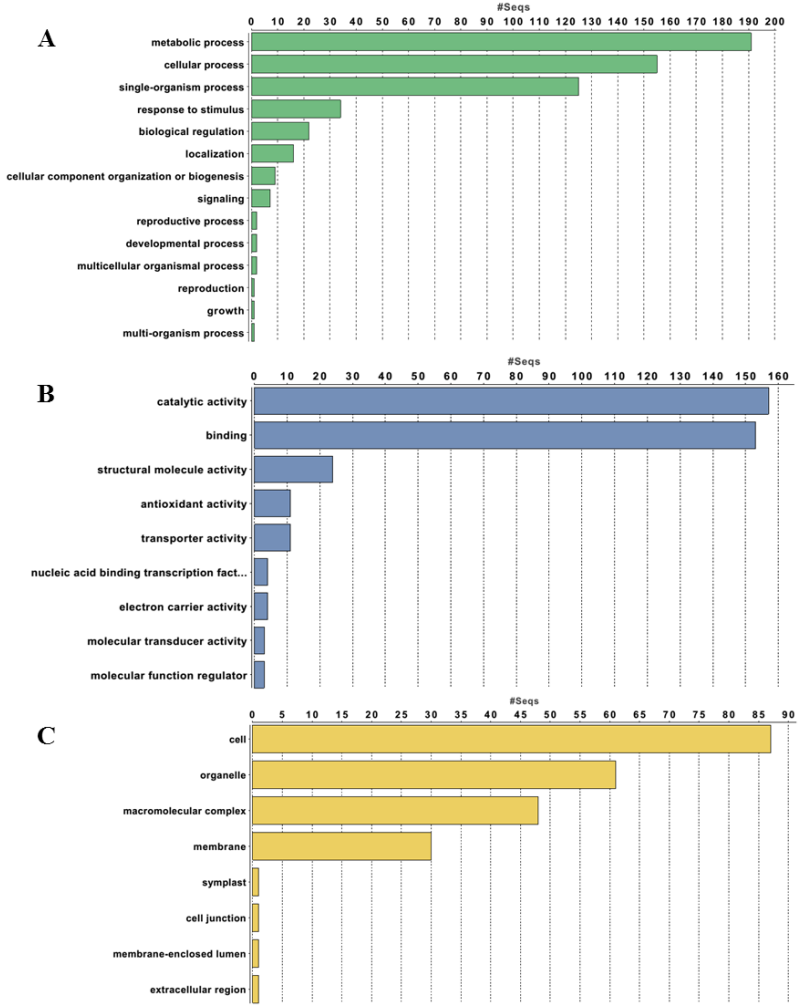
Supplementary material



Supplementary figure 1. Volcano plot for expressed proteins of *A. angustifolia* embryogenic cultures from Cr02 cell line contrasting expression ratio between plant growth regulators (PGR)-supplemented/-free treatments. Green dots represent significant up-regulated proteins, red dots significant down-regulated proteins and blue dots unchanged expression level found between PGR-supplemented and –free treatments.



Supplementary figure 2. Functional classification of identified proteins in *A. angustifolia* embryogenic cultures from Cr02 cell line in both plant growth regulators treatments (supplemented or not) using Blast2GO software based on universal gene ontology (GO) annotation terms. The proteins were linked to at least one annotation term within the GO biological process (A), molecular function (B), and cellular component (C) categories.

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CONSIDERAÇÕES FINAIS E PERSPECTIVAS FUTURAS

Os avanços alcançados no presente trabalho permitiram a elucidação de aspectos morfo-estruturais durante a embriogênese somática da *A. angustifolia* até então pouco compreendidos, como demonstram os resultados do capítulo 1. Baseando-se nestes resultados, comprovou-se que os aglomerados celulares são compostos por três diferentes tipos celulares (as células embrionárias, as células intermediárias e as células do suspensor) que possuem características ultraestruturais bastante distintas entre elas. Somado a isso, observou-se de forma inédita em culturas embriogênicas vegetais diferentes conformações mitocondriais, que puderam ser correlacionadas com as condições de estresse que o ambiente *in vitro* impõe a estas culturas. Adicionalmente aprofundou-se o entendimento da etapa de maturação das culturas, que aparentemente necessitam ser mantidas em meio de cultura com alto potencial osmótico e isento de ABA antes de serem transferidas para o meio de cultura contendo este fitorregulador. Por fim, as análises de microscopia de luz indicaram um processo bastante assincrônico de obtenção dos embriões somáticos, que quando em estádios de desenvolvimento mais avançados apresentaram diversos espaços intercelulares que podem gerar consequências para o seu posterior desenvolvimento.

Os estudos de criopreservação, apresentados no capítulo 2, permitiram um avanço substancial como uma estratégia de conservação desta espécie, que até então não possuía um protocolo de criopreservação eficiente mesmo para outros propágulos, indicando uma alta taxa de sucesso para diferentes linhagens celulares. Os resultados apresentados indicaram que as culturas suportam bem a exposição à solução crioprotetora, mantendo sua morfologia e viabilidade. No entanto, expressivas alterações ultraestruturais foram observadas no período posterior ao criotratamento e de recuperação das culturas do nitrogênio líquido. Respostas genótipo-dependentes foram também observadas; no entanto, ambas as linhagens avaliadas demonstraram altas taxas de sobrevivência. Sendo assim, este estudo abre perspectivas para futuros estudos básicos e aplicados, ampliando os fundamentos para o desenvolvimento desta técnica para outras espécies, incluindo espécies bastante correlacionadas, como o *Podocarpus lambertii*. Além disso, o protocolo proposto pode ser uma ferramenta útil para manutenção das culturas recém-induzidas, reduzindo drasticamente os efeitos das repicagens em longo prazo.

Por fim, os resultados apresentados no capítulo 3 indicaram que a suplementação com fitorreguladores durante o período de indução e manutenção em longo prazo das culturas embriogênicas de *A. angustifolia* de fato afetam diferencialmente os níveis de metilação global dessas culturas. No entanto, futuros estudos focados no processo de metilação em genes específicos ou mesmo a avaliação da expressão gênica em genes relacionados com esse processo, como as metiltransferases, podem complementar estes resultados. Os resultados das análises de proteômica *shotgun* indicaram inúmeras diferenças nos perfis de expressão de proteínas entre os tratamentos isentos ou suplementados com fitorreguladores. A identificação sem precedentes da expressão exclusiva da proteína PIN-like nas culturas embriogênicas submetidas ao tratamento suplementando com fitorreguladores forneceu indícios de que a presença dos fitorreguladores pode, de fato, interferir no processo de transporte polar de auxinas; no entanto, estudos mais específicos, como de imunolocalização das proteínas PIN, podem trazer informações mais precisas sobre este processo. Os resultados das proteínas diferencialmente expressas indicaram inúmeras proteínas relacionadas a estresse superexpressas no tratamento com suplementação de fitorreguladores, sendo um indicativo de que essa suplementação pode estar contribuindo para um ambiente ainda mais estressante para as culturas. Além disso, puderam ser identificadas diversas proteínas superexpressas no tratamento isento de fitorreguladores que estão envolvidas com a estabilização e conformação de outras proteínas, o que pode auxiliar na resposta dessas culturas ao estresse do ambiente *in vitro*. Por fim, a identificação de diversas proteínas envolvidas direta ou indiretamente com a síntese de terpenóides, entre eles o Taxol, gerou fortes indícios de que as culturas embriogênicas de *A. angustifolia* podem estar sintetizando estes compostos. Sendo assim, futuros estudos visando à quantificação destes poderão comprovar estes resultados e evidenciar se essa síntese é relevante do ponto de vista bioquímico.