UNIVERSIDADE DE SÃO PAULO
INSTITUTO DE BIOCIÊNCIAS

LEANDRO FRANCISCO DE OLIVEIRA

Metabolismo de poliaminas na embriogênese zigótica e somática de *Araucaria angustifolia* (Bertol.) Kuntze

Polyamine metabolism in zygotic and somatic embryogenesis of *Araucaria angustifolia* (Bertol.) Kuntze

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Orientadora: Profa. Dra. Eny Iochevet Segal Floh

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COMISSÃO JULGADORA

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Aos meus pais e família, José e Lúcia,

os quais foram e são a base de tudo o que sou,

Dedico.

“*Alegria é aceitar a vida com tudo que ela oferece*

e poder escolher com o coração, para

aprender o que o espírito realmente deseja!”

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"Quem caminha sozinho pode até chegar mais rápido,

mas aquele que vai acompanhado,

com certeza vai mais longe."

Clarice Lispector

RESUMO

DE OLIVEIRA, Leandro Francisco. **Metabolismo de poliaminas na embriogênese zigótica e somática de *Araucaria angustifolia* (Bertol.) Kuntze.** 2017. 180 p. Tese (Doutorado em Ciências, área de Botânica) – Instituto de Biociências, Universidade de São Paulo, São Paulo, 2017.

A *Araucaria angustifolia* é uma conífera nativa do Brasil. Em função da sua intensa exploração florestal, a espécie ocupa apenas 2% de sua vegetação natural. Neste sistema, a aplicação de técnicas biotecnológicas, como a embriogênese somática, podem ser integradas a programas de melhoramento genético e conservação. A similaridade entre a embriogênese somática e zigótica, tem sido utilizada para o estabelecimento de estudos visando o aperfeiçoamento do cultivo *in vitro* dos embriões somáticos, bem como para um maior conhecimento dos aspectos moleculares e fisiológicos que regulam a embriogênese. O metabolismo de poliaminas (PAs), mais especificamente putrescina, espermidina e espermina, tem se mostrado como fundamental para a compreensão e evolução da embriogênese zigótica e somática. Entretanto, a biossíntese das PAs e seu envolvimento nos vários processos biológicos que regulam a embriogênese, são pouco conhecidas em coníferas. Inserido nessa perspectiva, o presente trabalho teve como objetivo o estudo do metabolismo de PAs durante três estádios de desenvolvimento da semente (contendo as fases da embriogênese inicial até a tardia) e na proliferação de linhagens embriogênicas com diferentes potenciais embriogênicos de *A. angustifolia*. Foram investigados: a) os perfis de PAs (livres e conjugadas) e aminoácidos; b) determinação da via preferencial da biossíntese de putrescina, através da atividade enzimática da arginina descarboxilase (ADC) e ornitina descarboxilase (ODC); c) identificação e caracterização do padrão de expressão dos genes envolvidos no metabolismo de PAs; e d) a identificação das relações entre os perfis de PAs e aminoácidos presentes nas sementes das matrizes, e sua potencial influência nas fases de indução, proliferação e maturação dos embriões somáticos. Durante a embriogênese zigótica, a expressão dos genes *AaADC* (arginina descarboxilase) e *AaSAMDC* (S-adenosilmetionina descarboxilase) aumentaram no estádio cotiledonar, juntamente com o aumento de PAs. A biossíntese da putrescina é realizada preferencialmente via ADC, enquanto que a citrulina foi o principal aminoácido presente nas sementes. Em relação ao metabolismo de PAs nas culturas embriogênicas, os dados obtidos demonstraram que a arginina e ornitina parecem ter diferentes funções em cada linhagem testada. Na linhagem com alto potencial embriogênico, a arginina parece estar associada com a ativação dos genes relacionados ao catabolismo de PAs (*AaPAO2*, *AaCuAO* e *AaALDH*), enquanto que esse efeito não foi observado na linhagem bloqueada. A ODC tem uma maior atividade na linhagem responsiva, enquanto que na linhagem bloqueada, as atividades da ADC e ODC são similares. Dependendo da matriz foram observados diferentes perfis de PAs e aminoácidos, sendo estes perfis relacionados com as taxas de indução, proliferação e desenvolvimento dos embriões somáticos. Putrescina total, ornitina e asparagina foram os

metabólitos diferencialmente identificados entre as matrizes, os quais podem ser propostos como marcadores bioquímicos para a seleção de matrizes com alto potencial para a embriogênese somática. Os resultados obtidos fornecem informações relevantes e inéditas sobre o metabolismo de PAs e aminoácidos na embriogênese zigótica e somática de *A. angustifolia*, bem como fornece novos subsídios para o aprimoramento das condições artificiais utilizadas para o desenvolvimento dos embriões somáticos.

Palavras-chave: Arginina descarboxilase. *Araucaria angustifolia*. Embriogênese somática. Embriogênese zigótica. Ornitina descarboxilase. Poliaminas.

ABSTRACT

DE OLIVEIRA, Leandro Francisco. **Polyamine metabolism in zygotic and somatic embryogenesis of *Araucaria angustifolia* (Bertol.) Kuntze.** 2017. 180 p. Tese (Doutorado em Ciências, área de Botânica) – Instituto de Biociências, Universidade de São Paulo, São Paulo, 2017.

The *Araucaria angustifolia* is a native conifer species of Brazil. Due to its intense exploitation, the species cover only 2% of its original forest area. In this system, biotechnological tools, like somatic embryogenesis, may be integrated into breeding and conservation programs. The similarity between zygotic and somatic embryogenesis have been used to establishment of studies in order to optimization of somatic embryos in vitro culture, as well as for a better understanding of physiologic and molecular aspects that modulates the embryogenesis. The metabolism of polyamines (PAs), specifically putrescine, spermidine and spermine, has been demonstrated as fundamental for the comprehension and evolution of zygotic and somatic embryogenesis. However, the biosynthetic pathways of PAs and their involvement in various biological process that regulate the embryogenesis are little known in conifers. Inserted in this perspective, the aim of the current work was to study the metabolism of PAs during three seeds development stages (containing the early till late embryogenesis phases) and in proliferation of cell lines with different embryogenic potential of *A. angustifolia*. Were investigated: a) PAs (free and conjugated) and amino acids profiles; b) determination of preferential pathway for putrescine biosynthesis, through enzymatic activity of arginine decarboxylase (ADC) and ornithine decarboxylase (ODC); c) identification and characterization of gene expression profile of genes related to metabolism of PAs; and d) identification of the relationship between PAs and amino acids profiles in seeds of mother plants, and their potential influence in initiation, proliferation and maturation phases of somatic embryos. During the zygotic embryogenesis, *AaADC* (arginine decarboxylase) and *AaSAMDC* (S-adenosylmethionine decarboxylase) genes were up-regulated at cotyledonary stage along with the increasing of PAs. The biosynthesis of putrescine is performed preferentially by ADC pathway, while citrulline was the main amino acid recorded during the seed development. Regarding the metabolism of PAs in embryogenic cultures, the data demonstrated that arginine and ornithine seem to have different functions in each cell line tested. In cell line with high embryogenic potential, arginine seems to be associated to activation of genes related to PAs catabolism (*AaPAO2*, *AaCuAO* e *AaALDH*), while in blocked cell line this effect was not observed. ODC has a higher enzymatic activity in responsive cell line, while in blocked cell line, both ADC and ODC activities are similar. Depending of mother plant, were observed different PAs and amino acids profiles, being these profiles related with the rate of initiation, proliferation and maturation of somatic embryos. Total putrescine, ornithine and asparagine were the differentially metabolites identified between the mother plants, which can be proposed as biochemical marker to select mother plant with high potential to somatic embryogenesis. The results obtained provide relevant and inedited information about the metabolism of PAs and amino acids in zygotic and somatic embryogenesis of *A. angustifolia*, as well as provide news subsidies for optimization of *in vitro* conditions for somatic embryos development.

Keywords: Arginine decarboxylase. *Araucaria angustifolia*. Somatic embryogenesis. Zygotic embryogenesis. Ornithine decarboxylase. Polyamines.

SUMÁRIO

Introdução Geral

INTRODUÇÃO GERAL

1. O processo de embriogênese zigótica

A embriogênese zigótica é um processo biológico complexo e altamente organizado e representa o papel central no ciclo de vida das plantas superiores (Cairney *et al.*, 1999; von Arnold *et al.*, 2002; Santa-Catarina *et al.*, 2006). Nos vegetais o termo embriogênese corresponde as fases de desenvolvimento compreendidas entre a fertilização até o início da dormência do embrião zigótico (Harada *et al.*, 2010). A embriogênese é considerada como um modelo para estudos de desenvolvimento e diferenciação em plantas, pois é através dela que é estabelecida a arquitetura do indivíduo, pela geração da forma (morfogênese), associada com a organização de estruturas (organogênese) e diferenciação das células que resultam nos diversos tecidos vegetais (histogênese) (Floh *et al.,* 2015).

Nas angiospermas, o processo de embriogênese tem início com a dupla fecundação dando origem ao zigoto (2n) e o endosperma (3n). Dentro do saco embrionário ocorre a primeira divisão celular do zigoto (divisão assimétrica), resultando na formação de uma estrutura polarizada formada por um polo superior voltado para o endosperma (embrião) e pelo polo inferior voltado para a micrópila (suspensor) (Floh *et al.*, 2015). A primeira divisão do zigoto é acompanhada pela formação da parede celular, pela determinação dos três eixos embrionários (longitudinal, lateral e radial), e por uma sequência de alterações na morfologia do embrião passando, no caso de dicotiledôneas, pelos estádios globular, cordiforme, torpedo e cotiledonar (Floh *et al.,* 2015).

Por outro lado, a embriogênese em gimnospermas é caracterizada, após a fusão dos gametas masculino e feminino, por uma etapa de núcleos livres na qual inicialmente não é possível observar a formação da parede celular (Hakman e Oliviusson, 2002). Em gimnospermas são reconhecidas três fases distintas durante o desenvolvimento embrionário (Figura 1): a) fase proembrionária que vai desde a fertilização até o rompimento da arquegônia pelo proembrião (estádios anteriores ao alongamento do suspensor primário); b) fase embrionária inicial, que compreende aos estádios após o alongamento do suspensor secundário e antes do estabelecimento dos meristemas e c) fase embrionária tardia, na qual a protoderme e o procâmbio são diferenciados e os meristemas apical e radicular são estabelecidos (Singh, 1978; Haines e Prakash, 1980; von Arnold *et al.*, 2002).

Para a maioria das gimnospermas ocorre a formação de múltiplos embriões durante a fase embrionária inicial (Figura 1) cuja formação pode ocorrer via dois processos: a

poliembrionia simples ou por clivagem (Singh, 1978; Gifford e Foster, 1989; Floh *et al.*, 2015). Na poliembrionia simples ocorre a formação de mais de um embrião a partir da fertilização de mais de um arquegônio. Já na poliembrionia por clivagem, poliembriões podem ser formados a partir da bipartição por clivagem das células proembrionárias resultando na formação de até 24 proembriões. Independente do processo de poliembriogênese (simples ou por clivagem), apenas o embrião zigótico que atinge a cavidade de corrosão é mantido na semente, sendo que os demais (embriões subordinados) são eliminados por morte celular programada (Gifford e Foster, 1989; Sharma e Thorpe, 1995; Floh *et al.*, 2015).

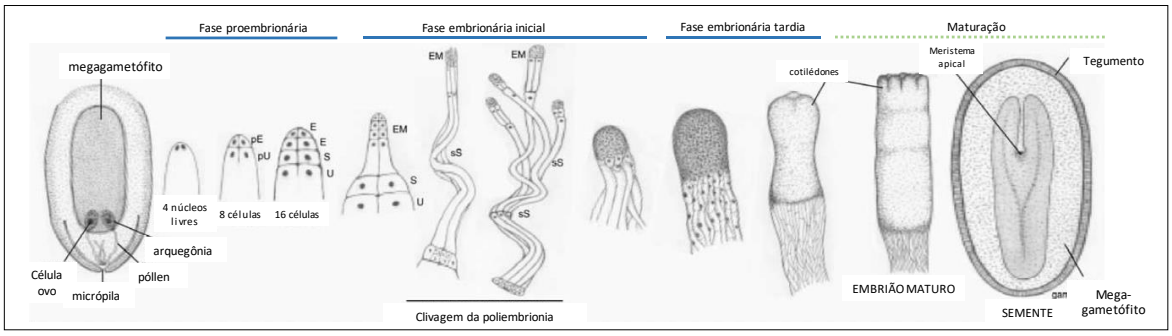

Figura 1. Modelo de desenvolvimento do embrião em gimnospermas (*Pinus sp*). E – camada embrionária; EM – massa embrionária; pE – camada embrionária primária; pU – camada superior primária; S – camada do suspensor; sS – suspensor secundário; U – camada superior. Fonte: adaptado de von Arnold *et al.* (2002).

Nas fases tardias do desenvolvimento, o embrião reduz drasticamente os processos de divisão celular dando início a fase de maturação (Ikeda *et al.*, 2006). Tanto para angiospermas como em gimnospermas, a maturação é considerada como a etapa final e fundamental do desenvolvimento do embrião (Perán-Quesada *et al.*, 2004). Durante maturação, ocorre o desenvolvimento dos cotilédones e acúmulo de substâncias de reserva (lipídeos, carboidratos e proteínas), as quais são consideradas fundamentais para o período pós-germinação (desenvolvimento inicial da plântula) até a autotrofia (Bewley e Black, 1994; Merkle *et al.*, 1995). Dependendo do desenvolvimento fisiológico da semente, a fase de maturação pode ser ainda caracterizada pela diminuição da atividade metabólica e aquisição da tolerância a dessecação mediada ou não pelo ácido absíscico (sementes ortodoxas) (Bewley e Black, 1994; von Arnold *et al.*, 2002; Walters *et al.*, 2008), ou então

pelo aumento da atividade metabólica e acúmulo de água (sementes recalcitrantes) (dos Santos *et al.,* 2006).

O desenvolvimento embrionário vegetal é caracterizado por um controle espacial e temporal na expressão de centenas de genes que desencadeiam processo de transcrição, biossíntese e transporte de hormônios vegetais (principalmente auxinas) ao longo do eixo embrionário (Floh *et al.,* 2015). Grande parte do nosso conhecimento sobre os aspectos moleculares do desenvolvimento embrionário são decorrentes do uso de sistemas modelos como *Arabidopsis thaliana* ou *Medicago truncatula* (Karami *et al.,* 2010; Elhiti *et al.,* 2013; Radoeva e Weijers, 2014; Fehér, 2015). Contudo, de acordo com Ballester *et al.,* (2016), o desenvolvimento embrionário de espécies arbóreas com longo ciclo de vida, sobretudo nas coníferas, apresenta uma série de particularidades que não são encontradas nos sistemas modelos. Os recentes avanços obtidos com uso dos sequenciadores de última geração, e a rápida expansão da base de dados de sequências genéticas vem permitindo o aprofundamento dos estudos moleculares em sistemas não-modelo, como por exemplo, as arbóreas com longo ciclo de vida (Elbl *et al.,* 2015a; Ballester *et al.,* 2016). O desenvolvimento de estudos envolvendo aspectos moleculares em arbóreas é considerado atualmente como fundamental para a ampliação do conhecimento básico relacionado ao desenvolvimento embrionário, bem como para a sua potencial aplicação via ferramentas biotecnológicas (Ballester *et al.,* 2016).

2. Embriogênese somática em gimnospermas

A embriogênese somática é um processo análogo à embriogênese zigótica, no qual uma célula ou um grupo de células vegetativas são precursoras de embriões somáticos (Tautorus *et al.*, 1991; Zimmerman, 1993). Esse processo envolve uma série de estádios de desenvolvimento, que se aproximam da sequência de eventos observados na embriogênese zigótica. As similaridades entre os dois processos envolvem aspectos morfológicos, bioquímicos e moleculares (Hakman, 1993; Thibaud-Nissen *et al.,* 2003; Schmidt *et al.,* 2006; Tereso *et al.*, 2007**;** Sghaier *et al.,* 2008; Floh *et al.*, 2015). Em virtude dessas similaridades, vários estudos têm buscado marcadores comuns dos diferentes estádios do desenvolvimento da embriogênese somática e zigótica (Klimaszewska *et al.,* 2004; Domoki *et al.*, 2006; Namasivayam, 2007; Sghaier *et al.*, 2008). Abordagens integrativas da embriogênese zigótica e somática são fundamentais para o a compreensão destes processos,

estabelecimento de protocolos eficientes, e que mimetizem as condições necessárias para o correto desenvolvimento embrionário (Garcia-Mendiguren *et al.*, 2015).

O emprego da embriogênese somática pode ter diferentes objetivos, que vão desde a obtenção de um modelo de referência para estudos básicos em biologia celular e molecular, fisiologia e bioquímica, até a propagação clonal, visando à conservação e o melhoramento genético das espécies (Floh *et al.*,2015). Como modelo para estudo de um processo morfogenético, a embriogênese somática é ideal para investigar o processo de diferenciação em plantas, bem como os mecanismos de totipotencialidade da célula vegetal. Incluem-se aqui abordagens diferenciais da competência celular que é definida como o potencial de reprogramação de uma célula em resposta a sinais específicos, por meio de processos de desdiferenciação e rediferenciação (Floh *et al.*, 2007). Adicionalmente, a ES permite eliminar as dificuldades encontradas em se analisar os estádios iniciais da embriogênese zigótica, durante o desenvolvimento do embrião no interior do tecido materno (Smertenko e Bozhkov, 2014). Como exemplo, estão as fases pró-embrionária e embrionária inicial, caracterizadas como de difícil ou impossíveis manipulações nas condições *in vivo*.

Sistemas de embriogênese somática e micropropagação têm sido utilizados, com relativo sucesso, para diferentes sistemas vegetais, objetivando a multiplicação e conservação de espécies lenhosas, em especial gimnospermas (Jain e Ishii, 1998; Klimaszewska *et al.*, 2016; Ballester *et al.*, 2016), sejam aquelas de interesse comercial ou ameaçadas de extinção, para futuros programas de reflorestamento clonal (Guerra *et al.*, 2000; Neale e Kremer, 2011; Santa-Catarina *et al.*, 2013). A utilização da cultura de tecidos, mais especificamente da embriogênese somática, como uma técnica biotecnológica, possibilita que sejam transpostas barreiras e dificuldades encontradas na propagação convencional de espécies nativas recalcitrantes (Santa-Catarina *et al.*, 2013). Comparativamente às demais técnicas de micropropagação, a embriogênese somática apresenta as seguintes vantagens sobre outros sistemas de propagação *in vitro*: a) permite a obtenção de uma grande quantidade de propágulos (embriões somáticos); b) o sistema permite um alto grau de automatização (biorreatores), permitindo baixar os custos por unidade produzida; c) os embriões somáticos podem ser produzidos de forma sincronizada, com alto grau de uniformização e qualidade genética; d) pode ser utilizada como uma ferramenta integrada a programas de melhoramento vegetal; e) e como ferramenta para pesquisa de genômica funcional em coníferas, por meio da transformação genética (Högberg *et al.*, 1998; Guerra *et al.*, 1999; Santa-Catarina *et al.*, 2013; Klimaszewska *et al.*, 2016).

A modulação da embriogênese somática pode ser dividida em quatro etapas distintas (Figura 2):

a) Etapa de indução **–** tem início após a inoculação dos explantes em meio de cultura suplementado ou não com reguladores de crescimento, especialmente auxinas e citocininas (von Arnold *et al.*, 2002), ou ainda com a aplicação de condições estressantes como baixas temperaturas e exposição a luz, estressores osmóticos, aumento ou diminuição na concentração salina ou então do pH do meio de cultura (Lee *et al.*, 2001). Frequentemente, para o estabelecimento das culturas embriogênicas (CEs) em espécies arbóreas são utilizados embriões imaturos como explantes iniciais (Stasolla e Yeung, 2003; Klimaszewska *et al.*, 2007; Klimaszewska *et al.*, 2016). A presença, na superfície do explante, de um calo branco-translúcido indica o início da formação das massas proembriogênicas (MPEs) (von Arnold *et al.*, 2002; Stasolla e Yeung, 2003);

b) Etapa de multiplicação ou proliferação **–** quando alteradas as concentrações de auxinas e citocininas, suplementadas ao meio de cultura, as CEs podem ser proliferadas e/ou multiplicadas. Frequentemente os ciclos de subcultivos, que dão origem às novas MPEs, ocorrem de duas a três semanas em meio semi-sólido ou líquido (von Arnold *et al.*, 2002; Stasolla e Yeung, 2003). Com base na morfologia, organização celular e número de células, podem ser identificados diferentes padrões de MPEs, descritas como: a) **MPE I**, composta por uma célula do suspensor vacuolizada adjacente as células embrionárias com citoplasma denso; b) **MPE II**, composta por agregados celulares similares a MPE I, entretanto com mais de uma célula do suspensor; c) **MPE III** caracterizada por um agregado com um número maior de células, constituído por células do suspensor e embrionárias, as quais formam agregados sem qualquer padrão de organização (von Arnold *et al.*, 2002);

c) Etapa de maturação – a partir da formação das MPE III e a retirada dos reguladores de crescimento ocorre a formação do ES inicial (Filonova *et al.*, 2000; von Arnold *et al.*, 2002). A adição de promotores da maturação, como o ABA, poliaminas e agentes osmóticos, promovem a inibição da proliferação e a evolução dos embriões somáticos (Attre e Fowke, 1993; Stasolla *et al.*, 2002; von Arnold *et al.*, 2002; Stasolla e Yeung, 2003). Durante a maturação são observadas as várias etapas da embriogênese tardia, incluindo os estádios globular e cotiledonar. Nesta evolução podem ser identificados diferentes eventos morfológicos (histodiferenciação da protoderme, crescimento axial e radial do embrião, desenvolvimento e expansão dos cotilédones, degradação do suspensor e desenvolvimento dos meristemas) e bioquímicos (acúmulo de substâncias de reserva,

redução da atividade metabólica e aquisição da tolerância à dessecação) (Stasolla e Yeung, 2003; von Arnold *et al.*, 2002);

d) Etapa de germinação e estabelecimento da plântula – após sua completa formação, a transferência dos ES para meios isentos de reguladores de crescimento permite a germinação e subsequente desenvolvimento da plântula (Attre e Fowke, 1993; Stasolla e Yeung, 2003; von Arnold *et al.*, 2002). O posterior desenvolvimento do sistema radicular permite a transferência destas plântulas para uma condição *ex vitro* (Högberg *et al.*, 2003).

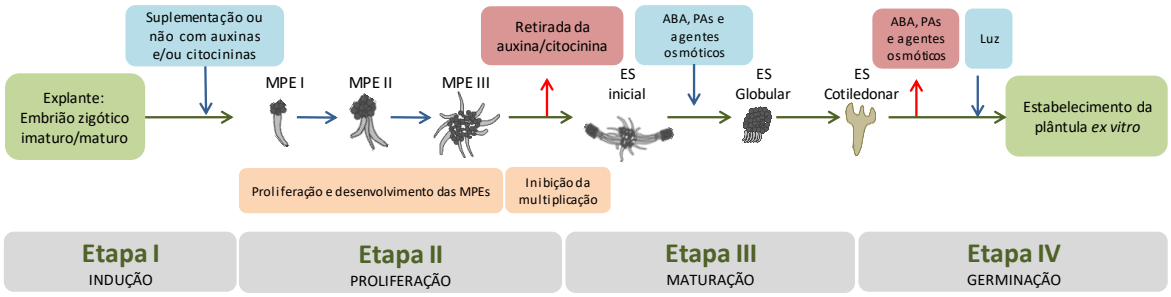

Figura 2. Esquema do desenvolvimento da embriogênese somática em coníferas. ABA, ácido abscísico; ES, embrião somático; MPEs, massas proembriogênicas; PAs, poliaminas. Fonte: Adaptado de Stasolla e Yeung (2003), von Arnold *et al.* (2002), Steiner *et al.* (2008) e Jo (2012).

O primeiro relato da obtenção de embriões somáticos em gimnospermas foi realizado por Norstog e Rhamstine (1967) utilizando embriões zigóticos de *Zamia* spp. Para coníferas o processo foi inicialmente descrito para *Picea abies* (Chalupa, 1985; Hakman e von Arnold, 1985; Hakman *et al.*, 1985) e *Larix decidua* (Nagmani e Bonga, 1985), e atualmente está descrito para 30 espécies da família Pinaceae e para espécies das famílias Cupressaceae (quatro), Taxaceae (uma), Podocarpaceae (uma), Cephalotaxaceae (uma) e Araucariaceae (uma) (Guerra *et al.*, 2016; Klimaszewska *et al.*, 2016; Fraga *et al.*, 2016).

O uso da ES em coníferas tem proporcionado a produção de embriões em larga escala, como por exemplo em *Picea glauca*, onde é possível a obtenção de aproximadamente 300 embriões somáticos a partir de 50 mg (peso fresco) de células em proliferação (Klimaszewska *et al.*, 2016). Além disso, a criopreservação de CEs tem sido obtida com sucesso em pelo menos 26 espécies, incluindo aquelas pertencentes ao gênero *Abies*, *Larix*, *Picea*, *Pinus* e *Pseudotsuga* (Cyr e Klimaszewska, 2002; Klimaszewska *et al.*, 2016). Entretanto, a genótipo-dependência, a rápida perda do potencial embriogênico após poucos

meses de subcultivo e a baixa taxa de embriões somáticos formados durante a maturação, devido as diversas condições impostas nessa etapa, tem impedido o emprego da ES na regeneração de plantas de algumas coníferas (Santa-Catarina *et al.*, 2013). Apesar de inúmeros protocolos de ES já terem sido estabelecidos para espécies de coníferas, grande parte do sucesso obtido ainda se restringe aos membros da família *Pinaceae* (Lelu-Walter *et al.*, 2013). Para as demais famílias de coníferas ainda existem uma série de limitações que impossibilitam a regeneração de plantas via ES (Sutton, 2002).

Grande parte destas dificuldades estão associadas às condições de cultivo empregadas (estímulos físicos e químicos) ainda ineficazes para o correto desenvolvimento embrionário *in vitro* (Santa-Catarina *et al.*, 2013). Com isso, estudos comparativos envolvendo abordagens moleculares, incluindo proteômica e transcriptômica, têm levado a identificação molecular e caracterização funcional dos vários genes envolvidos na indução e desenvolvimento do embrião somático (Vestman *et al.*, 2011; Champagne *et al.*, 2013; Martin *et al.*, 2013; Elbl *et al.*, 2015a; dos Santos *et al.*, 2016).

Estudos dos diferentes aspectos fisiológicos, bioquímicos e moleculares nestas várias fases são fundamentais para a compreensão dos processos básicos da diferenciação celular, bem como para o estabelecimento de protocolos mais eficientes de propagação através da embriogênese somática. Destaca-se que a indução e controle da embriogênese somática são dependentes da fonte de explante, do genótipo da planta matriz, e do tipo e concentração dos reguladores de crescimento adicionados ao meio de cultura (Guerra *et al.*, 1999; Steiner *et al.*, 2008; Santa-Catarina *et al.*, 2013).

3. Poliaminas

As poliaminas (PAs) são substâncias de ampla distribuição nos seres vivos tendo sido identificadas em bactérias, fungos, animais e plantas (Vuosku *et al.*, 2006; Majumdar *et al.*, 2013; Lasanajak *et al.*, 2014). A presença em cristais de sêmen humano foi descrita por Antonie van Leeuwenhoek, em 1678, embora a sua constituição bioquímica tenha sido determinada em 1924, e a sua síntese realizada dois anos mais tarde (Wallace *et al.*, 2003).

A natureza policatiônica, associada às interações eletrostáticas observada junto a diversas macromoléculas essenciais (DNA, RNA, fosfolipídeos e proteínas), fazem com que as PAs apresentem funções essenciais para a sobrevivência da célula. Estudos destacam a sua interação com a cromatina (afetando as modificações epigenéticas do DNA), processamento do RNA, tradução e ativação de proteínas (Childs *et al.*, 2003; Vuosku *et al.*,

2006; Kusano *et al.*, 2007; Baron e Stasolla, 2008; Vuosku *et al.*, 2012; Minocha *et al.*, 2014). Acredita-se também que as PAs podem agir tanto como um sequestrador de radicais livres, protegendo o DNA de espécies reativas de oxigênio, bem como gerador dos mesmos, através de seu catabolismo, liberando peróxido de hidrogênio (Aloisi *et al.*, 2016).

Em plantas, as PAs têm sido consideradas como uma nova classe de reguladores de crescimento (Kakkar *et al.*, 2000; Bais e Ravinshanlar, 2002; Steiner *et al.*, 2007; Steiner *et al.*, 2008; Lasanajak *et al.*, 2014). Geralmente estão envolvidas nas respostas ao estresse biótico e abiótico (Baron e Stasolla, 2008; Alcázar *et al.*, 2010). Entretanto, estudos também indicam seu envolvimento na morfogênese, agindo na divisão e diferenciação celular, morte celular programada, embriogênese, formação das raízes, iniciação e desenvolvimento floral, crescimento do tubo polínico, desenvolvimento e amadurecimento de frutos, metabolismo secundário, senescência, sinalização e balanço de N:C (Bouchereau *et al.*, 1999; Wallace *et al.*, 2003; Silveira *et al.*, 2006; Kuznetsov e Shevyakova, 2007; Kusano *et al.*, 2008; Steiner *et al.*, 2008; Gemperlová *et al.*, 2009; Alcázar *et al.*, 2010; Kovács *et al.*, 2010; Moschou *et al.*, 2012; Tavladoraki *et al.*, 2012; Majumdar *et al.*, 2013; Minocha *et al.*, 2014; Lasanajak *et al.*, 2014; Majumdar *et al.*, 2016).

As PAs mais comuns nos vegetais são: putrescina (Put), espermidina (Spd) e espermina (Spm) (Silveira *et al.*, 2006; Vieira *et al.*, 2012; Jo *et al.*, 2014; de Oliveira *et al.*, 2017) (Figura 2). Em alguns casos, a termoespermina (tSpm), pode ocorrer em conjunto ou substituindo a Spm (Minocha *et al.*, 2014; Pál *et al.*, 2015). As PAs podem ser encontradas nas formas de aminas livres ou conjugadas (Minocha *et al.*, 2014; Pál *et al.*, 2015). Na sua forma conjugada, elas geralmente ocorrem associadas com pequenas moléculas, tais como os ácidos fenólicos, ou ligadas a macromoléculas (Pál *et al.*, 2015). Em células vegetais, as PAs são frequentemente armazenadas no vacúolo e na parede celular, entretanto, a Spm também está presente no núcleo celular (Belda-Palazon *et al.*, 2012; Aloisi *et al.*, 2016).

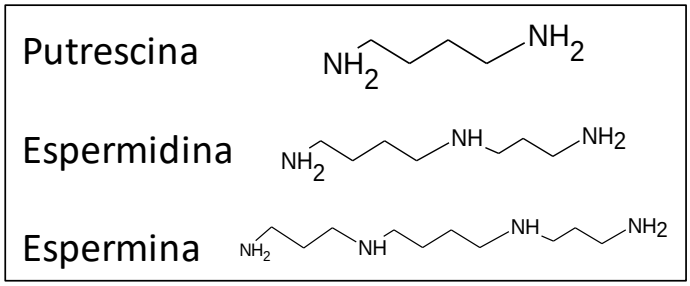

Figura 3. Estrutura molecular das poliaminas putrescina, espermidina e espermina. Fonte: de Oliveira (2017).

3.1. Metabolismo de poliaminas em plantas

Os níveis endógenos de PAs nas células são afetados por sua biossíntese, degradação, influxo e efluxo celular, compartimentalização e mecanismos de conjugação (Tiburcio *et al.*, 1997; Kusano *et al.*, 2007; Carbonell e Blázquez, 2009; Jiménez-Bremont *et al.*, 2014). Embora existam relatos de trabalhos sobre o estudo do metabolismo de PAs em gimnospermas desde a década de 90, os mecanismos moleculares da ação das PAs ainda não foram totalmente elucidados, e pouco se sabe sobre a sua função a nível celular em coníferas (Vuosku *et al.*, 2006; Jiménez-Bremont *et al.*, 2014).

Revisões abordando e descrevendo a biossíntese e catabolismo das PAs foram recentemente publicadas (Liu *et al.*, 2015; Jancewicz *et al.*, 2016; Majumdar *et al.*, 2016). Em plantas, a biossíntese de Put, pode ocorrer através de duas rotas independentes, reguladas por duas diferentes enzimas (Figura 4) (Jiménez-Bremont *et al.*, 2014; Majumdar *et al.*, 2016) ou seja: a primeira rota se inicia com a descarboxilação do aminoácido arginina, pela ação da enzima arginina descarboxilase (ADC; EC 4.1.1.19), localizada principalmente no cloroplasto em células fotossintéticas e direcionada ao núcleo em células não-fotossintéticas (Bortolotti *et al.*, 2004). O produto desta reação é a agmatina, a qual é subsequentemente metabolizada a *N*-carbamoilputrescina e em Put, pela ação das enzimas agmatina deiminase (AIH; EC 3.5.3.12) e *N*-carbamoilputrescina amidohidrolase (CPA; EC 3.5.1.53), respectivamente (Alcázar *et al.*, 2010; Vuosku *et al.*, 2006); a segunda rota inicia com o aminoácido ornitina, o qual é convertido em Put, em uma única reação catalisada pela enzima ornitina descarboxilase (ODC; EC 4.1.1.17), encontrada principalmente no citosol de células vegetais (Jiménez-Bremont *et al.*, 2014). A partir da Put pode ocorrer a síntese da Spd e Spm, onde a S-adenosilmetionina descarboxilada (dcSAM) atua como doador de grupos aminopropil, gerados pela ação da enzima S-adenosilmetionina descarboxilase (SAMDC; EC 4.1.1.50) (Figura 3). A síntese da Spd e Spm é controlada por uma combinação da SAMDC e mais duas aminopropiltransferases, chamadas espermidina sintase (SPDS; EC 2.5.1.16) e espermina sintase (SPMS; EC 2.5.1.22) (Shao *et al.*, 2014; Majumdar *et al.*, 2016). A biossíntese de PAs e etileno são ligadas através da SAM, o qual é um precursor comum entre esses dois metabolismos (Bouchereau *et al*., 1999; Bais e Ravishankar, 2002; Silveira *et al*., 2006; Jo *et al*., 2014).

A biossíntese de PAs está relacionada com o fluxo metabólico de outros aminoácidos, como o glutamato, ornitina, arginina, prolina, citrulina e metionina (Majumdar *et al.*, 2016). A biossíntese de arginina ocorre via citrulina, a partir da ornitina (Figura 3), o

qual envolve a ação de três enzimas: ornitina carbamoiltransferase (OTC; EC 2.1.3.3), argininosuccinato sintase (ASS; EC 6.3.4.5) e argininosuccinato liase (ASL; EC 4.3.2.1). Por outro lado, a arginina pode ser convertida em ornitina em uma via simples, pela ação da arginase (EC 3.5.3.1), participando do ciclo da ureia. A arginina também pode ser convertida em citrulina e óxido nítrico (ON), como produtos pela ação da enzima óxido nítrico sintase (ainda não caracterizada em plantas) (Page *et al.*, 2012; Minocha *et al.*, 2014; Corpas e Barroso, 2014; Majumdar *et al.*, 2016; Santolini *et al.*, 2016). Juntos, o metabolismo das PAs e do glutamato conferem uma das maiores interações de vias na assimilação e particionamento do carbono e nitrogênio, apresentando uma ampla variedade de funções fisiológicas em plantas, produzindo outros aminoácidos, moléculas sinalizadoras, como o óxido nítrico, e GABA, que desempenham funções críticas no desenvolvimento vegetal e respostas ao estresse (Page *et al.*, 2012; Minocha *et al.*, 2014; Guo *et al.*, 2014; Majumdar *et al.*, 2016).

O catabolismo de PAs tem a ação de duas enzimas específicas: a poliamina oxidase (PAO; EC 1.5.3.3), que degrada tanto a Spm em Spd, como a Spd em Put, e a diamino oxidase (DAO; EC 1.4.3.6), ou amina oxidase contendo cobre (CuAO), que degrada a putrescina em Δ^{-1}-pirrolina, posteriormente em GABA e então direcionado para o ciclo do ácido tricarboxílico (Bhatnagar *et al.*, 2002; Minocha *et al.*, 2014; Majumdar *et al.*, 2016). Essas enzimas se localizam na parede celular, e o catabolismo das PAs acontece no apoplasto. Pesquisas tem demonstrado a importância do catabolismo das PAs no padrão da parede celular, através da liberação de H_2O_2 (produto do catabolismo) e influenciando os processos de lignificação e rigidez da parede (Moschou *et al.*, 2012; Saha *et al.*, 2015; Aloisi *et al.*, 2016).

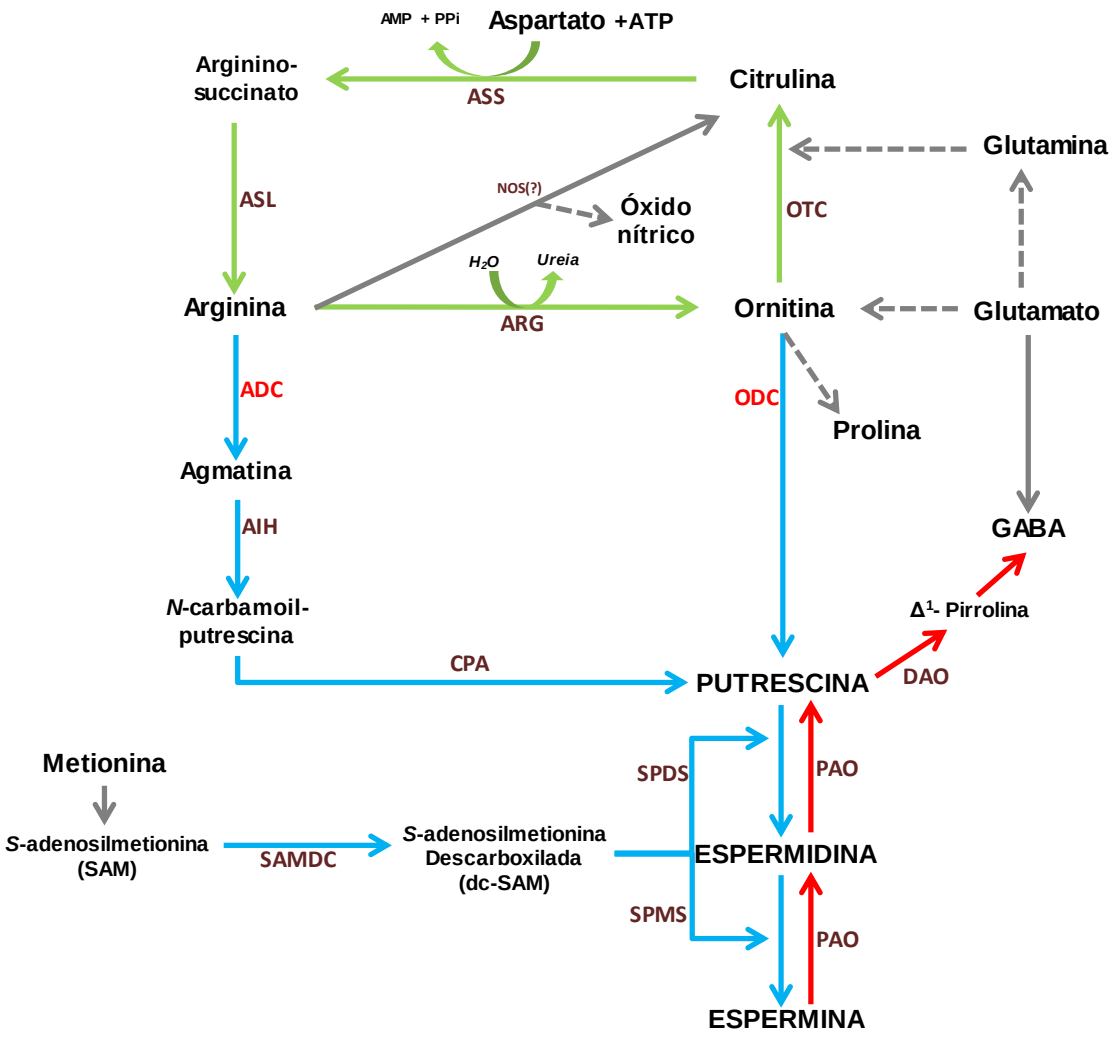

Figura 4. Vias de biossíntese da arginina/ornitina e poliaminas. AIH, agmatina deiminase; ARG, arginase; ADC, arginina descarboxilase; ASL, argininosuccinato liase; ASS, argininosuccinato sintase; CPA, N-carbamoilputrescina amidase; DAO, diamino oxidase; NOS, óxido nítrico sintase (putativa); ODC, ornitina descarboxilase; OTC, ornitina carbamoiltransferase; PAO, poliamina oxidase; SAMDC, S-adenosilmetionina descarboxilase; SPDS, espermidina sintase; SPMS, espermina sintase. Fonte: de Oliveira (2017).

3.2. Importância das poliaminas na embriogênese

A importância das PAs no processo de embriogênese zigótica tem sido amplamente estudada no sistema *Arabidopsis thaliana*. Grande parte deste conhecimento está relacionado com a utilização de mutantes das principais enzimas das vias de biossíntese como ADC, ODC, SPDS, SPMS e SAMDC (Imai *et al.*, 2004; Urano *et al.*, 2005; Ge *et al.*, 2006).

Diferentes funções podem ser associadas a cada rota biossintética (ADC ou ODC), as quais podem ser tecido-específica e terem uma regulação distinta, dependendo das condições fisiológicas do material (Kumar *et al.*, 1997; Page *et al.*, 2012; Majumdar *et al.*, 2013; Minocha *et al.*, 2014). Ressalta-se que a via da ODC é geralmente identificada como mais ativa em células e tecidos com intensa atividade de divisão celular, como por exemplo, nos meristemas (Hummel *et al.*, 2004; Vuosku *et al.*, 2006), enquanto que a atividade da ADC tem sido encontrada em células em processo de alongamento, células embrionárias e células expostas a vários tipos de estresse (Tiburcio *et al.*, 1997; Vuosku *et al.*, 2006). Diferenças também foram observadas nos vários sistemas vegetais. Durante a embriogênese zigótica de *Pinus sylvestris* foi identificada a existência das duas rotas de biossíntese de PAs com o predomínio da ADC para a produção da Put. Entretanto, em *Arabidopsis thaliana*, bem como em outras espécies da família Bracicaceae, a ODC é ausente e a biossíntese só ocorre via a ADC (Hanfrey *et al.*, 2001).

Alterações nos perfis e conteúdo de Put, Spd e Spm ocorrem durante as diferentes fases de desenvolvimento *in vivo* dos embriões zigóticos, sugerindo a sua utilização como marcador bioquímico da diferenciação celular (Minocha *et al.*, 1999, Astarita *et al.*, 2003c, Silveira *et al.*, 2004). Em *Pinus radiata* (Minocha *et al.*, 1999), *P. taeda* (Silveira *et al.*, 2004), *P. silvestrys* (Vuosku *et al.,* 2006) e *P. abies* (Malá *et al.*, 2009) o conteúdo de Put é alto nos estádios iniciais do desenvolvimento embrionário, em comparação a Spd e Spm. Contudo, durante as fases de estabelecimento dos meristemas apical e radicular, alongamento dos cotilédones e acúmulo de substâncias de reserva, a Spd passa a ser a PA predominante em relação à Put e Spm (Minocha *et al.,* 1999; Vuosku *et al.,* 2006).

Embora as PAs demonstrem grande importância durante o desenvolvimento dos embriões zigóticos (Minocha *et al.*, 1999; Astarita *et al.*, 2003b, 2003c; Silveira *et al.*, 2004; Vuosku *et al.*, 2006; Kusano *et al.*, 2008; Gemperlová *et al.*, 2009), ainda é desconhecido a relação da expressão gênica e as mudanças associadas com a biossíntese de PAs e arginina/ornitina durante o desenvolvimento de sementes. Em coníferas, como ainda não existe a disponibilidade de mutantes, o estudo do metabolismo de PAs tem sido baseado no uso de precursores marcados, quantificação por HPLC, ensaios enzimáticos, e estudos de expressão gênica (Gemperlová *et al.*, 2009; Vuosku *et al.*, 2006; Vuosku *et al.*, 2012).

A participação das PAs no desenvolvimento do embrião somático foi primeiramente descrita por Montague *et al.* (1978) em *Daucus carota*. Desde então, diversos trabalhos têm confirmado a importância das PAs para o desenvolvimento embrionário *in*

vitro em diferentes sistemas (Astarita *et al.*, 2003a; Silveira *et al.*, 2006; Steiner *et al.*, 2007; Vieira *et al.*, 2012; Santa-Catarina *et al.*, 2013; Cheng *et al.*, 2015; Salo *et al.*, 2016). Em *Allium cepa*, a adição de Put associada com a Spd promove a indução de embriões somáticos, enquanto que a presença apenas da Spd estimula a maturação e a conversão de embriões em plantas (Martinez *et al.*, 2000). Em *Daucus carota* a Spm estimula a formação de embriões somáticos (Takeda *et al.*, 2002). Baixas concentrações de ABA e de Put e níveis elevados de Spd foram associadas às altas taxas de conversões de embriões somáticos em *Quercus petraea* (Cvikrová *et al.*, 1998).

Os maiores níveis de Put estão associadas à intensa atividade de divisão celular, que ocorre durante a fase de proliferação celular da embriogênese somática (Feirer, 1995; Minocha *et al.*, 2004). Durante a fase de maturação a qual está associada à diferenciação celular são observadas maiores concentrações de Spd e Spm (Minocha *et al.*, 1999; Stasolla *et al.*, 2002; Minocha *et al.*, 2004; Gemperlová *et al.*, 2009). A Spm é considerada biologicamente mais ativa que as demais PAs, atuando em diferentes processos como: estabilização das membranas celulares pela redução do transporte transmembrana dos fosfolipídios, interagindo com proteínas nucleares que determinam a configuração espacial do DNA, estimulando a atividade da síntese de pectinas e hemicelulose, além de exercer ação antioxidante (Bouchereau *et al.*, 1999).

A adição de Spd e Spm ao meio de cultura pode induzir a formação de ON em culturas embriogênicas de *Ocotea catharinensis* (Santa-Catarina *et al.,* 2007) e *A. angustifolia* (Silveira *et al.*, 2006) e em plântulas de *Arabidopsis thaliana* (Tun *et al.*, 2006). Estes trabalhos foram pioneiros e constituem as primeiras evidencias da relação em plantas de PAs e ON, bem como da importância deste processo de sinalização na competência e formação de embriões somáticos. A associação entre poliaminas e ON vem proporcionando novas perspectivas para o estudo da biossíntese e catabolismo de PAs e produção de espécies reativas de oxigênio (EROs) em gimnospermas. Esses aspectos indicam que as PAs podem conferir maior tolerância a variados tipos de estresse atuando na remoção de espécies reativas de oxigênio e na estabilização das membranas celulares (Larher *et al.*, 2003; Groppa & Benavides, 2008).

Em espécies arbóreas, estudos indicam que as PAs estão envolvidas no estabelecimento da competência dos tecidos em produzir uma resposta à indução embriogênica e, portanto, serem utilizadas como marcadores para a identificação precoce de linhagens com diferentes capacidades embriogênicas (Niemi *et al.*, 2002; Silveira *et al.*,

2006; Nakagawa *et al.*, 2011; Santa-Catarina *et al.*, 2013). Segundo Shoeb *et al.* (2001), os níveis celulares de PAs e a relação Put/Spd constituem importantes biomarcadores da capacidade regenerativa em um sistema vegetal. Uma menor razão Put/Spd foi associada a intensa diferenciação de embriões somáticos globulares em culturas de *P. sylvestris* (Niemi *et al.*, 2002).

4. A *Araucaria angustifolia* como modelo de estudo

4.1. A escolha do sistema

O gênero *Araucaria* é composto por 19 espécies, constituindo o mais abundante dentro da família Araucariaceae, presente em parte da Oceania, sudeste Asiático e América do Sul (Liu *et al.*, 2009). A *A. angustifolia* (Bertol.) Kuntze é uma espécie arbórea nativa do Brasil, dominante nas formações de Floresta Ombrófila Mista (Guerra *et al.*, 2002). Existente há mais de 300 milhões de anos, a espécie pertence a um dos grupos mais basais dentre as coníferas ainda vivas (Ordem Pinales) (Liu *et al.*, 2009). Apesar da sua ampla distribuição no período mesozóico, as mudanças edafoclimáticas sofridas após a separação dos continentes (Haworth *et al.*, 2011) e o surgimento das angiospermas, fizeram com que a *A. angustifolia* ficasse restrita a regiões de altitude no sul da América do Sul (Zonnenveld, 2012), com ocorrência natural nos estados do Paraná, Santa Catarina, Rio Grande do Sul, podendo também ser encontrada e em áreas esparsas de São Paulo, Minas Gerais, Rio de Janeiro, Argentina e Paraguai (Auler *et al.*, 2002; Guerra *et al.*, 2002; Liu *et al.*, 2009).

A *A. angustifolia* possui um papel fundamental na estrutura e funcionamento dos ecossistemas nos quais está inserida, sendo fonte de recursos também para os seres humanos. Sua área original era de aproximadamente 200 mil Km² (Guerra *et al.*, 2000; Auler *et al.*, 2002; Duarte *et al.*, 2002; Guerra *et al.*, 2002). Entretanto, em função da alta qualidade da sua madeira, a espécie foi intensivamente explorada ao longo de sua história, de modo que atualmente restam somente 2% da área original por ela ocupada (Figura 4) (Guerra *et al.*, 2000; Duarte *et al.*, 2002). Adicionalmente, a redução da Floresta Ombrófila Mista resultou na degradação das populações de Araucária, o que dificulta ainda mais sua regeneração natural (Shimizu *et al.*, 2000). Como consequência, a espécie foi classificada como "criticamente em perigo de extinção" pela Lista Vermelha de Espécies ameaçadas da IUCN (The World Conservation Union) (disponível em: http://www.iucnredlist.org/details/32975/0, acesso em dezembro de 2016), e "em perigo" no Livro Vermelho da Flora do Brasil (Martinelli e Moraes, 2013), dados que foram

insuficientes, entretanto, para o recuo na exploração dos remanescentes florestais por ela ocupados (Guerra *et al.*, 2002; Mantovani *et al.*,2006). Somado a isso, o aquecimento global é outro fator que poderá afetar diretamente a Araucária, uma vez que a espécie necessita de baixas temperaturas e elevada umidade relativa para se desenvolver adequadamente (Souza e Aguiar, 2012).

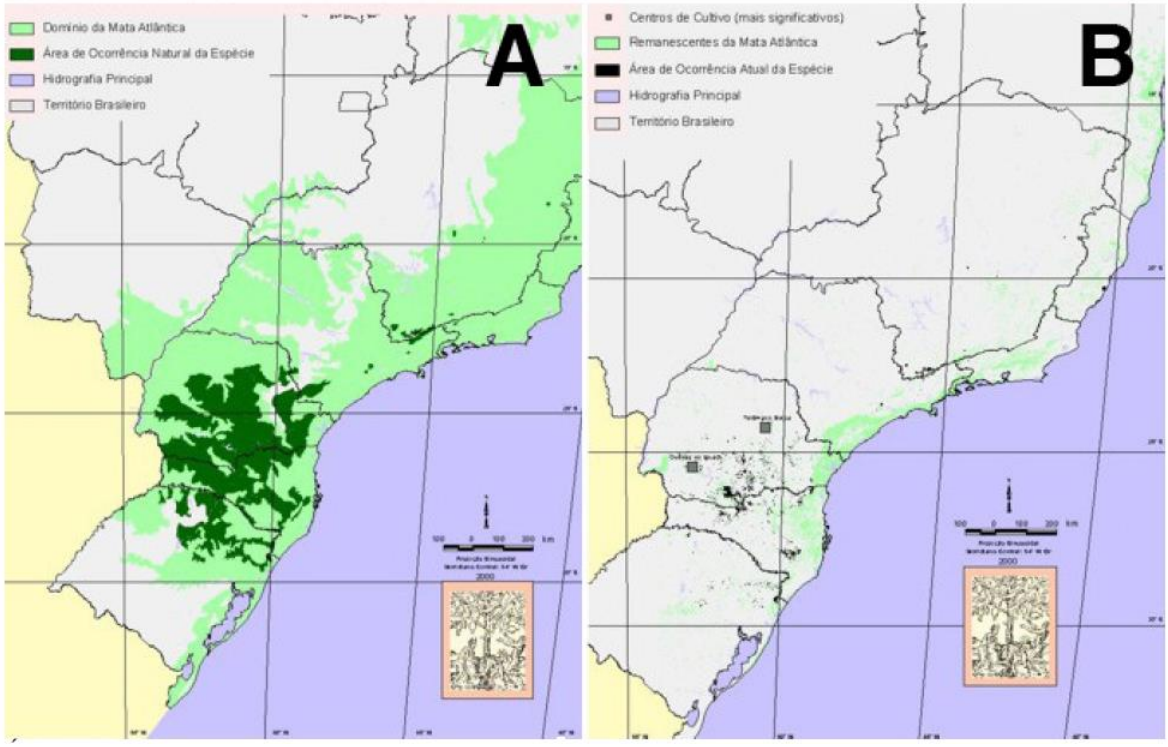

Figura 4. Distribuição geográfica da espécie *Araucaria angustifolia* no Brasil. Área de ocorrência destacada em verde escuro. Distribuição no início do século XX (**A**), e a área de cobertura atual da espécie (**B**). Fonte: Conselho Nacional da Reserva da Biosfera da Mata Atlântica (2012).

Além da exploração exacerbada da espécie, a dificuldade da recuperação natural de sua população também está ligada ao seu ciclo de vida longo, que leva em torno de 15 anos até atingir a maturidade reprodutiva (Garcia, 2002). A polinização inicia-se em setembro, e estende-se até dezembro, com o amadurecimento das sementes no período de março a julho. Sua fenologia é irregular, e seu ciclo reprodutivo leva, em geral, em torno de dois a quatro anos, considerando-se o início do desenvolvimento do cone feminino até a dispersão das sementes (Shimoya, 1962; Sousa e Hattemer, 2003a, 2003b; Mantovani *et al.*, 2004).

Outro ponto que dificulta sua conservação é o fato de suas sementes apresentarem características recalcitrantes, e consequentemente a sua reduzida longevidade e viabilidade dificultam o armazenamento e conservação, resultando em um curto período de vida pós-colheita (Balbuena *et al.*, 2009; Pieruzzi *et al.*, 2011; Berjak e Pammenter, 2013; Araldi *et al.*, 2016). De acordo com Santos (2000), a conservação de pólen ou o cultivo *in vitro* de suas estruturas vegetativas, são alternativas de maior viabilidade quando comparadas a conservação utilizando sementes. A macropropagação, e os métodos convencionais de propagação dessa espécie, são dificultados pelo estádio de maturação dos propágulos, seja via estaquia ou enxertia, em função do plagiotropismo e crescimento lateral dos explantes (Kageyama e Ferreira, 1975; Wendling, 2011).

Assim, outras estratégias como é o caso de técnicas biotecnológicas, devem ser adotadas para assegurar a sobrevivência da espécie. Salienta-se que estas metodologias apresentam um grande potencial de aplicação nos programas de melhoramento genético e conservação de germoplasma de *A. angustifolia* (Steiner *et al.*, 2008; Pieruzzi *et al.*, 2011). Dentre as técnicas biotecnológicas, a micropropagação, através da embriogênese somática, é considerada como a mais promissora em plantas, para a clonagem massal de genótipos superiores (Steiner *et al.*, 2008) bem como para conservação *ex situ* de germoplasma de espécies em risco de extinção (Johnson *et al.*, 2012; Ma *et al.*, 2012).

Associado a estas abordagens, estudos utilizando a *A. angustifolia* como modelo ou sistema referência, para estudos básicos dos processos de diferenciação, totipotencialidade e desenvolvimento embrionário são muito escassos. Em partes, a dificuldade em se estudar os processos de embriogênese desta espécie se deve à natureza recalcitrante das sementes, dificuldade em isolar os estádios iniciais do desenvolvimento dos embriões, referente a proembrionia, além do fato de seu ciclo reprodutivo ser longo.

4.2. Embriogênese zigótica

O desenvolvimento proembrionário na família Araucariaceae é considerado como um dos mais basais, e divergentes em relação às demais Coniferophytas (Johansen, 1950; Durzan, 2012). Diferentemente ao descrito para as demais famílias de coníferas, onde ocorre a formação de até quatro núcleos livres, em espécies de Araucariaceae há a formação de 32 a 64 núcleos livres, formadas logo após a fusão dos gametas masculinos e femininos, e que precede a formação das paredes celulares (Guerra *et al.*, 2008). Esta estrutura não fica posicionada nas extremidades do arquegônio, mas sim em uma posição central onde os

proembriões se formam (Raghavan e Sharma, 1995; Durzan, 2012; Floh *et al.*, 2015). A polaridade dos proembriões ocorre pela organização dos núcleos livres, constituindo três tipos celulares bem definidos: as células do suspensor, da capa e do grupo embrionário (Guerra *et al*., 2008). A presença de uma camada de células denominada de capa, e o desenvolvimento de um proeminente sistema de suspensor secundário são consideradas características exclusivas da família Araucariaceae (Durzan, 2012; Floh *et al.*, 2015).

Nos estádios iniciais do desenvolvimento embrionário, o suspensor constitui a principal via de transporte de nutrientes para o proembrião o qual degenera após o final do estádio proembrionário (com o rompimento da arquegônia) (Astarita e Guerra, 2000). Neste estádio foi demonstrado a ocorrência de um aumento nos níveis de AIA (Astarita *et al.*, 2003a). Destaca-se que este fitormônio está diretamente relacionado com o estabelecimento da simetria e polaridade (eixo ápice-base) do embrião. A formação dos cotilédones é caracterizada por um aumento no número e tamanho das células, devido à presença de proteínas e um grande número de grãos de amido (Guerra *et al*., 2008; Rogge-Renner *et al*., 2013). A seguir tem início a fase de maturação da semente com um aumento da concentração do ácido abscísico (ABA) durante a fase pré-cotiledonar dos embriões zigóticos, seguido de uma diminuição nos níveis durante as fases da embriogênese tardia (estádio cotiledonar e maturo) (Silveira *et al.*, 2008). O ABA desempenha um papel fundamental no processo de maturação dos embriões zigóticos sendo a sua presença associada ao aumento na concentração de lipídeos e acúmulo de proteínas de reserva. Adicionalmente, a diminuição do ABA na embriogênese tardia, pode estar associada ao comportamento recalcitrante das sementes de *A. angustifolia*, ou seja, elas mantêm altos teores de água, são sensíveis à dessecação e continuam metabolicamente ativas até a germinação (dos Santos *et al*., 2006; Pieruzzi *et al*., 2011).

A associação entre PAs e os processos de embriogênese zigótica em *A. angustifolia,* tem sido reportado em diversos trabalhos (Astarita *et al*., 2003*c*; Silveira *et al*., 2004; Silveira *et al*., 2006; Steiner *et al*., 2007; Jo *et al*., 2014; de Oliveira *et al*., 2017). Neste sistema vegetal, o perfil de PAs é caracterizado por uma redução brusca dos níveis de Put, e aumento da Spm durante as fases da embriogênese tardia (estabelecimento dos meristemas e formação dos cotilédones) (Astarita *et al.,* 2003a, 2013b; Pieruzzi *et al.*, 2011). Conteúdos elevados de Spd e Spm são essenciais ao final do desenvolvimento do embrião, quando o crescimento é decorrente, principalmente, do alongamento celular (Silveira *et al.,* 2006). Adicionalmente, as PAs também estão relacionadas com os eventos pós-embriogênicos, se apresentando

também como marcadores do término da germinação das sementes de *A. angustifolia* (Pieruzzi *et al.*, 2011).

A presença de proteínas estádio-específicas, sugeridas como marcadores bioquímicos foram identificadas ao longo do processo de embriogênese zigótica de *A. angustifolia* (Silveira *et al.*, 2008; Balbuena *et al.*, 2011). Destaca-se que nas etapas iniciais do desenvolvimento embrionário ocorre maior expressão do metabolismo do estresse oxidativo, enquanto que nas fases tardias da embriogênese ocorre o acúmulo de proteínas de reserva (Balbuena *et al.*, 2009, 2011). A expressão diferencial de oito genes (Argonaute (*AaAGO*), Cup-shaped cotyledon1 (*AaCUC*), wushel-related WOX (*AaWOX*), S-locus lectin protein kinase (*AaLecK*), Scarecrow-like (*AaSCR*), Vicilin 7S (*AaVIC*), Leafy Cotyledon 1 (*AaLEC*), e Reversible glycosylated polypeptide (*AaRGP*)) durante o desenvolvimento das sementes de *A. angustifolia*, foi relatada por Schlogl *et al*. (2012a, b). Neste trabalho, os autores verificaram que genes relacionados a morfogênese (*AaAGO, AaCUC, AaWOX, AaLeckin, AaLEC, AaRPG-like*) e ao acúmulo de reservas na semente (*AaVIC*) foram mais expressos durante as fases globular e cotiledonar dos embriões zigóticos, enquanto que na fase de maturação foi possível observar uma diminuição no padrão de expressão.

A construção de uma plataforma de transcriptoma da *A. angustifolia* tem fornecido relevantes informações sobre os aspectos moleculares do desenvolvimento embrionário desta espécie, tanto na embriogênese zigótica como na somática (Elbl *et al.*, 2015a). Durante o desenvolvimento das sementes, os autores relataram que 13 genes foram diferencialmente expressos entre o estádio globular e cotiledonar. No estádio globular, foi identificada a expressão de genes envolvidos no armazenamento e biossíntese de carboidratos, enquanto que no estádio cotiledonar genes associados com a produção de metabólitos secundários e fatores responsivos ao etileno foram mais expressos. A comparação entre os estádios cotiledonar e maturo evidenciou 78 genes diferencialmente expressos. No estádio cotiledonar, foram identificados transcritos associados às categorias de processo do desenvolvimento e divisão celular, enquanto que no estádio maturo, foi observado maior abundância de transcritos relacionados com os fotossistemas e metabolismo da clorofila (Elbl *et al*., 2015a). A análise do transcriptoma evidenciou que a maioria dos unigenes identificados em *A. angustifolia* representam funções basais, comuns em Viridiplantae, mas também há genes compartilhados com espécies de Monocotiledôneas e Eudicotiledôneas, que podem representar funções que surgiram entre as espécies basais de Viridiplantae e gimnospermas (Elbl *et al*., 2015a).

4.3. Embriogênese somática em *Araucaria angustifolia*

Os trabalhos com a embriogênese somática em *A. angustifolia* tiveram início com Guerra e Kemper (1992). Desde então, diversos estudos foram desenvolvidos visando o aperfeiçoamento do protocolo para obtenção de embriões somáticos nesta espécie (Astarita e Guerra 1998, 2000; Guerra *et al.*, 2000; dos Santos *et al.*, 2002; Silveira *et al.*, 2002; Steiner *et al.*, 2005; Silveira *et al.*, 2006; Steiner *et al.*, 2007; dos Santos *et al.*, 2008, 2010; Schlogl *et al.*, 2012a; Vieira *et al.*, 2012, Jo *et al.*, 2014).

Especificamente para *A. angustifolia*, as MPEs são originadas a partir do ápice do embrião zigótico imaturo em estádio globular (dos Santos *et al*., 2002), e podem ser induzidas e proliferadas em meio de cultura, na ausência de reguladores de crescimento (Figura 5). Este fato, apesar de ser considerado como uma vantagem, por amenizar os problemas associados à variação somaclonal, impossibilita a formação de poliembriões somáticos pela simples retirada das auxinas e citocininas do meio de cultura (dos Santos *et al.*, 2008). Como resultado, as culturas embriogênicas são formadas apenas por agregados celulares contendo células embrionárias e de suspensor com diferentes tamanhos, e por um baixo número de embriões somáticos globulares, os quais raramente evoluem para o estádio cotiledonar (dos Santos *et al*., 2008; de Oliveira *et al.*, 2015; Jo *et al*.,2014; Elbl *et al.*,2015a; dos Santos *et al*., 2016).

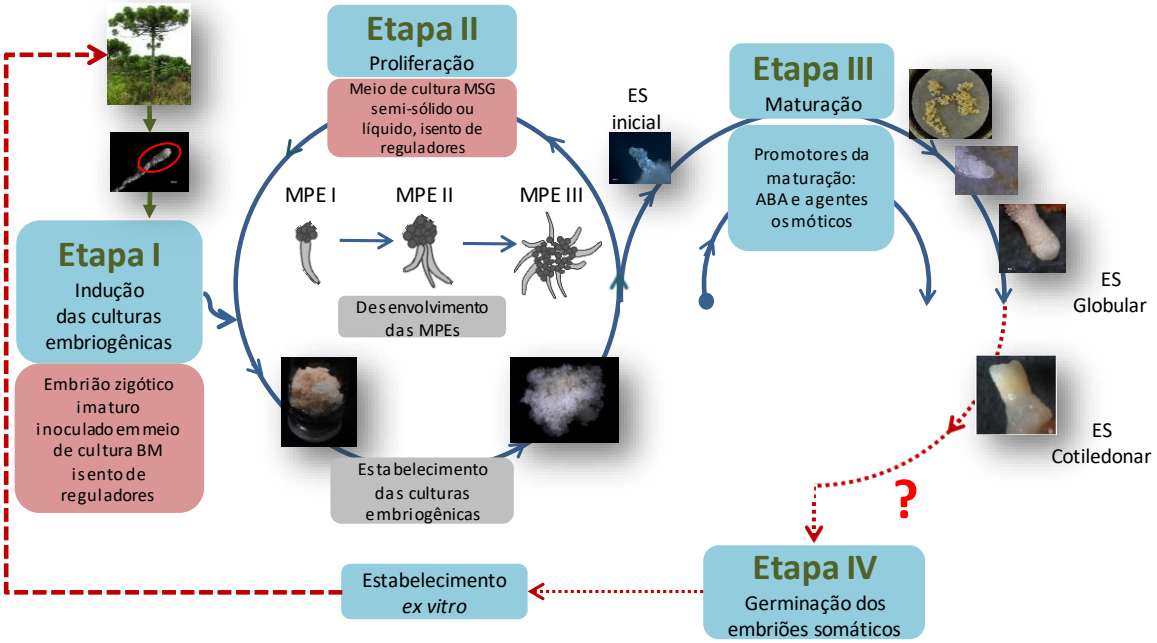

Figura 5. Esquema da modulação da embriogênese somática em *A. angustifolia*. MPE, massas proembriogênicas; ES, embrião somático; ABA, ácido abscísico; BM, BM (Gupta e Pullman, 1991); meio de cultura MSG básico (Becwar *et al.*, 1989). Caixas vermelhas indicam particularidades para este sistema. Caixas azuis indicam processos comuns aos protocolos para gimnospermas em geral. Linhas vermelhas tracejadas indicam as etapas nas quais o protocolo ainda não está completamente estabelecido. Fonte: Adaptado de Steiner *et al.* (2008) e Jo *et al.* (2014).

A ausência de embriões somáticos e a genótipo-dependência da resposta das culturas ao ABA tem dificultado a fase de maturação dos embriões de *A. angustifolia*, onde o ABA provoca a oxidação das estruturas embrionárias menos evoluídas morfologicamente (dos Santos *et al.*, 2008). Esta incapacidade de alguns genótipos apresentarem o desenvolvimento de embriões somáticos é geralmente identificada apenas nas etapas finais do processo de embriogênese, resultando na formação de embriões maduros com anormalidades, ou então com baixas taxas de conversão em plântulas (Stasolla *et al.*, 2002; Stasolla e Yeung, 2003; dos Santos *et al.*, 2006; Ochatt *et al.*, 2016). Adicionalmente, diversos estudos têm mostrado a influência da planta mãe na eficiência em várias etapas da embriogênese somática, como relatado em *Pinus sylvestris* (Lelu-Walter *et al.*, 2008; Aronen *et al.*, 2009; Vuosku *et al.*, 2009) e *A. angustifolia* (dos Santos *et al.*, 2002). Estes trabalhos demonstraram que a utilização de diferentes matrizes como doadores de explantes, tem revelado diferentes

respostas no estabelecimento de culturas embriogênicas e maturação dos embriões somáticos (Niskanen *et al.*, 2004; Montalbán *et al.*, 2012). Além disso, o efeito materno tem sido reportado como mais influente na indução e maturação dos embriões somáticos (Niskanen *et al.*, 2004). Neste sentido, a identificação de marcadores bioquímicos e moleculares para uma seleção precoce de genótipos aptos e competentes para a formação e evolução de embriões somáticos viáveis, é considerada importante para o aperfeiçoamento dos protocolos de embriogênese somática (Dowlatabadi *et al*., 2009; Klimaszewska *et al*., 2004; Lipert *et al*., 2005; Silveira *et al*., 2008; Jo *et al*., 2014; Elbl *et al*., 2015a; dos Santos *et al.*, 2016).

As altas concentrações endógenas de auxinas no embrião zigótico (Astarita *et al*., 2003a), fonte inicial das culturas, podem suprimir sua suplementação no meio de indução das culturas embriogênicas. Contudo, a possível manutenção dos altos níveis de auxinas endógenas (provenientes dos explantes utilizados na fase de indução) nas culturas embriogênicas, durante a fase de proliferação, possivelmente dificultam a transdiferenciação das massas embrionárias em embriões somáticos, e possivelmente afetam a morfologia dos estádios tardios do embrião somático. Esse fato foi observado ao avaliar-se o banco de transcritos de *A. angustifolia* (Elbl *et al*., 2015a), em que vinte e um genes relacionados à via de auxina e outras vias auxina-independentes foram identificados como possíveis promotores da inadequada formação e evolução dos embriões somáticos durante a fase de maturação (Elbl *et al*., 2016, dados não publicados).

Considerando-se as similaridades morfológicas, fisiológicas, bioquímicas e moleculares, estudos com abordagens integrativas dos processos de embriogênese somática e zigótica têm sido estudados em *A. angustifolia,* em especial pelo grupo do BIOCEL e associados (Silveira *et al*., 2006; Silveira *et al*., 2008; Balbuena *et al*., 2009, 2011; Steiner *et al*., 2007; Durzan *et al*., 2012; Steiner *et al*., 2012; Schlogl *et al*., 2012a, 2012b; Vieira *et al*., 2012; Elbl *et al.*, 2015a, 2015b; dos Santos *et al*., 2016). Adicionalmente, a manipulação do meio de cultura com relação ao metabolismo de aminoácidos (dos Santos *et al.* 2010), óxido nítrico (Silveira *et al.* 2006; Vieira *et al.* 2012) e poliaminas (Silveira *et al.*, 2006; Steiner *et al.*, 2007; Jo *et al.*, 2014) tem desencadeado mudanças morfológicas adequadas para a formação dos poliembriões somáticos. Este tipo de abordagem tem contribuído para o desenvolvimento de condições artificiais de cultivo semelhantes ao ambiente natural do desenvolvimento embrionário em coníferas, diminuindo o tempo para o estabelecimento e aumentando a eficiência dos protocolos de ES (Pullman *et al.* 2015).

Nesse aspecto, foram identificados marcadores relacionados aos perfis de expressão gênica e protéicos (Silveira *et al*., 2008; Jo *et al*. 2014; Elbl *et al*., 2015a; dos Santos *et al*., 2016; de Oliveira *et al*., 2017). Jo *et al.* (2014), utilizando linhagens responsiva (SE1) e bloqueada (SE6) para o tratamento de maturação, identificaram possíveis marcadores bioquímicos e moleculares associados à competência para embriogênese somática em *A. angustifolia.* Os resultados obtidos foram essenciais para a identificação de proteínas estádio-específicas de culturas celulares com alto potencial embriogênico, além de confirmarem a importância das vias de sinalização (poliaminas, aminoácidos, etileno, carboidratos, óxido nítrico, espécies reativas de oxigênio e auxina) estudadas pelo grupo de pesquisa na temática (Silveira *et al*., 2006; Steiner *et al*., 2007; Silveira *et al.*, 2008; Balbuena *et al.*, 2009, 2011; Durzan *et al*., 2012; Steiner *et al*., 2012; Vieira *et al.*, 2012; Schlogl *et al*., 2012a, 2012b; Jo *et al*., 2014; Elbl *et al.*, 2015a, 2015b; dos Santos *et al*., 2016; de Oliveira *et al.*, 2017).

A ausência de um genoma de referência para a espécie constituiu um fator limitante para uma abordagem mais aprofundada neste sistema. Para contornar este problema, foi realizada uma plataforma de transcriptoma (Elbl *et al*., 2015a), que a partir daí tem sido referência para todos os trabalhos em desenvolvimento (Elbl *et al*, 2015 a, b; dos Santos *et al*., 2016; de Oliveira *et al.*, 2017). A montagem *de novo* do transcriptoma foi realizada utilizando-se três diferentes estádios de desenvolvimento da semente e duas linhagens celulares estabelecidas (SE1 e SE6) (Jo *et al*., 2014), tendo permitido: i) a identificação de unigenes, ii) obtenção dos perfis de expressão diferencial entre os diferentes estádios e linhagens celulares, e iii) a correlação destes perfis com os processos biológicos capazes de modular a embriogênese *in vitro* e *in vitro* no sistema *A. angustifolia* (Elbl *et al*., 2015a). Adicionalmente, foi construída uma base de dados comparativa entre os estádios globular da embriogênese somática e da zigótica, a qual permitiu a identificação dos possíveis genes relacionados com as anormalidades morfológicas. Além disso, foi elaborado um banco de dados de sequencias de proteínas o que permitiu pela primeira vez a identificação em larga escala de proteínas presentes em linhagens celulares embriogênicas de *A. angustifolia* (dos Santos *et al.*, 2016).

As atuais possibilidades de integração dos conhecimentos, obtidos ao longo dos anos, e os estudos de genômica funcional no sistema, tem acenado para resultados promissores e poderá ser fundamental para modular de forma correta o desenvolvimento embrionário *in vitro.* Além disso, um maior conhecimento dos aspectos moleculares envolvidos durante a

embriogênese poderá fornecer uma base de referência para o aperfeiçoamento das condições artificiais utilizadas durante o cultivo *in vitro* de *A. angustifolia*.

5. Contextualização da tese

Ao longo da última década, o Laboratório de Biologia Celular de Plantas (BIOCEL) do IBUSP vem estudando parâmetros morfofisiológicos, bioquímicos e moleculares, em torno do processo da embriogênese zigótica e somática em diferentes sistemas vegetais, com ênfase na *A. angustifolia*. As abordagens incluem o estudo e compreensão dos aspectos básicos (fisiológicos, bioquímicos e moleculares) na competência, determinação e diferenciação celular em uma arbórea nativa. Do ponto de vista biotecnológico, numa analogia com a embriogênese zigótica, os estudos visam o aperfeiçoamento das condições artificiais de cultivo *in vitro* de embriões somáticos. Neste contexto, insere-se a possibilidade de um aumento na quantidade, qualidade e evolução morfogenética, para *A. angustifolia*, com perspectivas de uso em programas de melhoramento genético que visam a propagação massal clonal de genótipos superiores e na conservação de material genético, via estabelecimento de bancos de germoplasma. Dentro desta perspectiva, o grupo de pesquisa vem utilizando metodologias inovadoras, que permitem um maior aprofundamento dos aspectos moleculares, e que incluem o estudo do transcriptoma (*high throughtput* (RNAseq) e de proteômica quantitativa (GeLC-MS/MS) (Elbl *et al.*, 2015a, b; dos Santos *et al*., 2016), aspectos bioquímicos e fisiológicos (Jo *et al.*, 2014; de Oliveira *et al*., 2017) responsáveis pela regulação do desenvolvimento embrionário do pinheiro brasileiro. Dentre as principais vias de sinalização celular estudadas, as PAs foram identificadas como extremamente relevantes ao longo do desenvolvimento da semente, e em linhagens celulares com alto potencial embriogênico da *A. angustifolia* (Astarita *et al*., 2003c; Jo *et al*., 2014; Elbl *et al.*, 2015a; Santos *et al*., 2016; de Oliveira *et al*., 2017).

Pesquisas desenvolvidas pelo grupo demonstraram que embriões zigóticos de *A. angustifolia* apresentam um perfil de aminoácidos diferente de outras coníferas, tais como as da família Pinaceae. Enquanto que, em sementes de *Pinus taeda* e de outras coníferas, o principal aminoácido encontrado é a arginina, precursor das PAs, em *A. angustifolia* o ácido aspártico e glutâmico são os aminoácidos mais abundantes (Astarita *et al.*, 2003c). Esta observação levanta uma importante questão sobre sua principal via de biossíntese das PAs, e seu reflexo na embriogênese. O uso de aminoácidos marcados e a expressão gênica das enzimas de biossíntese podem ser ferramentas importantes para elucidação do metabolismo

das PAs, e para a compreensão da evolução da embriogênese *in vitro* e *in vivo.* Através da plataforma de transcriptoma da *A. angustifolia* foram identificados genes envolvidos tanto no metabolismo de PAs como na biossíntese de arginina e ornitina (de Oliveira *et al.*, 2015, 2017). A análise *in silico* tem revelado que essa é uma via conservada e a *AaADC* e *AaSAMDC* foram identificados como diferencialmente expressos em diferentes linhagens celulares, demonstrando a importância do estudo dos genes envolvidos na regulação do metabolismo de PAs (de Oliveira *et al.*, 2015). Adicionalmente, trabalhos anteriores desenvolvidos pelo grupo descrevem as PAs como potencial marcador bioquímico para a capacidade embriogênica, sendo que diferentes perfis de PAs foram associados a linhagens responsivas e bloqueadas aos agentes da maturação (Jo *et al.*, 2014). Esses dados sugerem a necessidade de um estudo mais aprofundado sobre a relação dos perfis de PAs, desde a semente até o estabelecimento de culturas embriogênicas.

Inserido nestas pesquisas a presente tese tem como temática o estudo do metabolismo das poliaminas durante a embriogênese zigótica e em culturas embriogênicas de *A. angustifolia*. Para tanto foram investigados: a) os perfis de aminoácidos e poliaminas (livres e conjugadas) em diferentes estádios de desenvolvimento de embriões zigóticos e em culturas embriogênicas com diferentes potenciais embriogênicos; b) determinação da via preferencial de biossíntese da putrescina, através da análise da atividade enzimática da ADC e ODC em embriões zigóticos e culturas embriogênicas com diferentes potencias embriogênicos; c) identificação, através do banco de dados de transcritos da embriogênese de Araucária (Elbl *et al.*, 2015a), dos principais genes envolvidos na via de PAs; d) caracterização do padrão de expressão dos genes envolvidos no metabolismo de PAs em diferentes estádios de desenvolvimento de embriões zigóticos e em culturas embriogênicas; e) identificação das relações entre os perfis de PAs e aminoácidos presentes nas sementes das matrizes, e sua potencial influência nas fases de indução, proliferação e maturação dos embriões somáticos.

O fluxograma de trabalho para o desenvolvimento da tese é apresentado na figura 6.

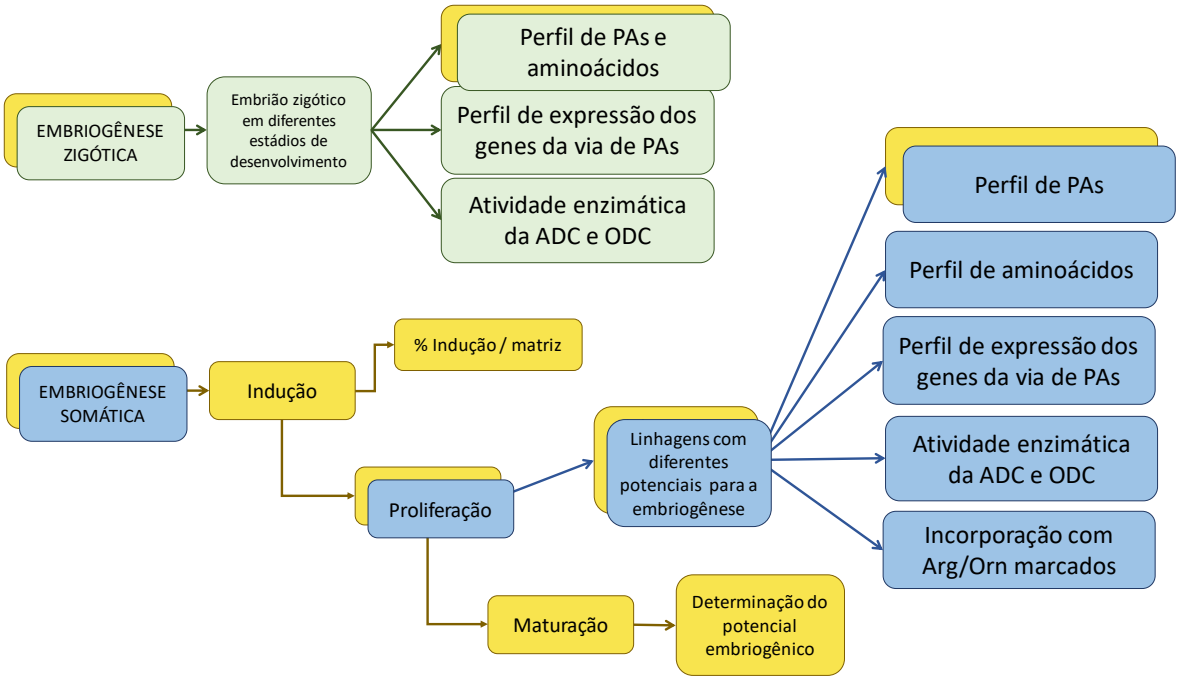

Figura 6. Fluxograma para o desenvolvimento do trabalho. ▢ - Capítulo I; ▢ - Capítulo II; ▢ - Capítulo III. Fonte: de Oliveira (2017).

Neste contexto o presente trabalho foi estruturado nos seguintes capítulos:

Capítulo I. Elucidação da biossíntese de poliaminas durante o desenvolvimento de sementes do pinheiro brasileiro (*Araucaria angustifolia*)

Capítulo II. Biossíntese de poliaminas em culturas embriogênicas de *Araucaria angustifolia*

Capítulo III. Poliaminas e aminoácidos em diferentes matrizes de *Araucaria angustifolia* e sua associação com o potencial embriogênico

6. Referências bibliográficas

Alcázar, R., Altabella, T., Marco, F., Bortolotti, C., Reymond, M., Koncz, C., Carrasco, P., Tiburcio, A. F. 2010. Polyamines: molecules with regulatory functions in plant abiotic stress tolerance. Planta 231:1237-1249.

Aloisi, I., Cai, G., Serafini-Fracassini, D., Del Duca, S. 2016. Polyamines in Pollen: From Microsporogenesis to Fertilization. Front Plant Sci. 7, doi: 10.3389/fpls.2016.00155.

Araldi, C. G., Coelho, C. M. M., Gaziola, S. A., Azevedo, R. A. 2016. Storage elicits a fast antioxidant enzyme activity in *Araucaria angustifolia* embryos. Acta Physiol Plant 38:201, doi: 10.1007/s11738-016-2219-2.

Aronen, T., Pehkonen, T., Ryynänen, L. 2009. Enhancement of somatic embryogenesis from immature zygotic embryos of *Pinus sylvestris*. Scandinavian Journal of Forest Research 24:372-83.

Astarita, L. V., Floh, E. I. S., Handro, W. 2003a. Changes in IAA, tryptophan and activity of soluble peroxidase associated with zygotic embryogenesis in *Araucaria angustifolia* (Brazilian pine). Plant Growth Regul 39:113–118.

Astarita, L. V., Floh, E. I. S., Handro, W. 2003b. Free amino acid, protein and water content changes associated with seed development in *Araucaria angustifolia*. Biol Plant 47:53–59.

Astarita, L. V., Guerra, M. P. 1998. Early somatic embryogenesis in *Araucaria angustifolia*– induction and maintenance of embryonal-suspensor mass culture. Rev. Bras. Fisiol. Veg 10:113-118.

Astarita, L. V., Guerra, M. P. 2000. Conditioning of culture medium by suspension cells and formation of somatic proembryo in *Araucaria angustifolia* (Coniferae). In Vitro Cell Dev Biol Plant. 36:194-200.

Astarita, L. V., Handro, W., Floh, E. I. S. 2003c. Changes in polyamines content associated with zygotic embryogenesis in the Brazilian pine, *Araucaria angustifolia* (Bert.) O Ktze. Rev Bras Bot 26:163–168.

Attre S. M., Fowke L. C. 1993. Embryogeny of gymnosperms: advances in synthetic seed technology of conifers. Plant Cell Tissue and Organ Culture 35:1-35.

Auler, N. M. F., Reis, M. S., Guerra, M. P., Nodari, R. O. 2002. The genetics and conservation of *Araucaria angustifolia*: I. Genetic structure and diversity of natural populations by means of non-adaptive variation in the state of Santa Catarina, Brazil. Genetics and Molecular Biology 25:329-338.

Bais, H. P., Ravinshankar, G. A. 2002. Role of polyamines in the ontogeny of plants and their biotechnological applications. Plant Cell Tissue Organ Cult 69:1-34.

Balbuena, T. S., Jo, L., Pieruzzi, F. P., Dias, L. L. C., Silveira, V., Santa-Catarina, C., Junqueira, M., Thelen, J. J., Shevchenko, A., Floh, E. I. S. 2011. Differential proteome analysis of mature and germinated embryos of *Araucaria angustifolia*. Phytoch 72:302-311.

Balbuena, T. S., Silveira, V., Junqueira, M., Dias, L. L. C., Santa-Catarina, C., Shevchenko, A., Floh, E. I. S. 2009. Changes in the 2-DE protein profile during zygotic embryogenesis in the Brazilian Pine (*Araucaria angustifolia*). Journal of Proteomics 72:337-352.

Balbuena, T. S., Silveira, V., Junqueira, M., Dias, L. L. C., Santa-Catarina, C., Shevchenko, A., Floh, E. I. S. 2009. Changes in 2-DE proteins protein profiles during zygotic embryogenesis in *A. angustifolia*. J Proteomics 72:337-352.

Ballester, A., Corredoira, E., Vieitez, A. M. 2016. Limitations of somatic embryogenesis in hardwood trees: Vegetative propagation of forest trees. Park, Y. S., Bonga, J. M., Moon, H. K. (eds) National Institute of Forest Science, Seoul, Korea, pp. 56–74.

Baron, K., Stasolla, C. 2008. The role of polyamines during *in vivo* and *in vitro* development. In Vitro Celll Dev Biol Plant. 44:384-95.

Becwar, M. R., Noland, T. L., Wyckoff, J. L. 1989. Maturation, germination, and conversion of Norway spruce (*Picea abies* L.) somatic embryos to plants. In Vitro Cell Dev Biol Plant 26: 575-580.

Belda-Palazon, B., Ruiz, L., Marti, E., Tarraga, S., Tiburcio, A. F., Culianez, F., Farràs, R., Carrasco, P., Ferrando, A. 2012. Aminopropyltransferases involved in polyamine biosynthesis localize preferentially in the nucleus of plant cells. PLoS ONE 7:e46907. doi: 10.1371/journal.pone.0046907.

Berjak, P., Pammenter, N. W. 2013. Implications of the lack of desiccation tolerance in recalcitrant seeds. Front Plant Sci 5:123.

Bewley, J. D., Black, M. 1994. Seeds: Physiology of development and germination. Plenum Press, New York, pp. 341.

Bhatnagar, P., Minocha, R., Minocha, S. C. 2002. Genetic manipulation of the metabolism of polyamines in poplar cells. The regulation of putrescine catabolism. Plant Physiol. 128:1455–1469.

Bortolotti, C., Cordeiro, A., Alcazar, R., Borrell, A., Culiañez-Macià, F. A., Tiburcio, A. F., Altabella, T. 2004. Localization of arginine decarboxylase in tobacco plants. Physiol Plant 120:84–92.

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

Cairney, J., Xu, N., Pullmann, G. S., Ciavatta, V. T., Johns, B. 1999. Natural and somatic embryo development in Loblolly pine. Appl Bioch Biotech 77-79:5-17.

Carbonell, J., Blázquez, M. A. 2009. Regulatory Mechanisms of polyamine biosynthesis in plants. Genes & Genomics 31:107-118.

Chalupa, V. 1985. Somatic embryogenesis and plant regeneration from cultured immature and mature embryos of *Picea abies* (L.) Karst. Comm Inst For Cecholosveniae 14:57-63.

Champagne, A., Boutry, M. 2013. Proteomics of nonmodel plant species. Proteomics 13:663-673.

Childs, A. C., Mehta, D. J., Gerner, E. W. 2003. Polyamine-dependent gene expression. Cell Mol Life Sci. 60:1394-13406.

Conselho Nacional Reserva da Biosfera da Mata Atlântica. Disponível em: <http://www.rbma.org.br/anuario/mata_03_anosdedesttuicao_dest_araucaria.asp>. (Acesso em 01 out 2016).

Corpas, F. J., Barroso, J. B. 2014. Peroxisomal plant nitric oxide synthase (NOS) protein is imported by peroxisomal targeting signal type 2 (PTS2) in a process that depends on the cytosolic receptor PEX7 and calmodulin. FEBS Letters 588:2049–2054.

Cvikrová, M., Malá, J., Eder, J., Hrubcová, M., Vágner, M. 1998. Abscisic acid, polyamines and phenolic acids in sessile oak somatic embryos in relation to their conversion potential. Plant Physiol. Biochem. 36:247-255.

Cyr, D. R., Klimaszewska, K. 2002. Conifer somatic embryogenesis: II. Applications. Dendrobiology 48:41–49.

de Oliveira, L. F., Elbl, P., Navarro, B. V., Macedo, A. F., Dos Santos, A. L. W., Floh, E. I. S. 2017. Elucidation of the polyamine biosynthesis pathway during Brazilian pine (*Araucaria angustifolia*) seed development. Tree Physiol. 37(1):116-130.

de Oliveira, L.F., Macedo, A.F., dos Santos, A.L.W. and Floh, E.I.S. 2015. Polyamine levels, arginine and ornithine decarboxylase activity in embryogenic cultures of *Araucaria angustifolia* (Bert.) O. Kuntze. Acta Hortic. 1083, 419-425 DOI: 10.17660/ActaHortic.2015.1083.54.

Domoki, M., Györgyey, J., Biró, J., Pasternak, T. P., Zvara, A., Bottka, S., Puskás, L. G., Dudits, D., Fehér, A. 2006. Identification and characterization of genes associated with the induction of embryogenic competence in leaf-protoplast-derived alfalfa cells. Biochim Biophys Acta 1759:543–551.

dos Santos, A. L. W., Elbl, P., Navarro, B. V., de Oliveira, L. F., Salvato, F., Balbuena, T. S., Floh, E. I. S. 2016. Quantitative proteomic analysis of *Araucaria angustifolia* (Bertol.) Kuntze cell lines with contrasting embryogenic potential. J Proteomics 130:180–189.

dos Santos, A. L. W., Gueddarri, N. E., Wiedholteher, N., Moerschbacher, B. M. 2006. Seed proteins of *Araucaria angustifolia*: Temporal expression of class IV chitinase and arabinogalactan proteins. Physiol Plant 127:138-148.

dos Santos, A. L. W., Silveira, V., Steiner, N., Maraschin, M., Guerra, M. P. 2010. Biochemical and morphological parametres during the growth of suspension cultures of *Araucaria angustifolia*. Brazi Archi Bio/Tech. 53:497-504.

dos Santos, A. L. W., Silveira, V., Steiner, N., Vidor, M., Guerra, M. P. 2002. Somatic embryogenesis in Parana Pine (*Araucaria angustifollia* (Bert.) O. Kuntze). Braz. Arch. Biol. Technol. 45:97-106.

dos Santos, A. L. W., Steiner, N., Guerra, M. P., Zoglauer, K., Moerschbacher, B. M. 2008. Somatic embryogenesis in *Araucaria angustifolia*. Biol Plant 52:195-199.

Dowlatabadi, R., A.M. Weljie, T.A. Thorpe, E.C. Yeung and H.J. Vogel. 2009. Metabolic foot printing study of white spruce somatic embryogenesis using NMR spectroscopy. Plant Physiol. Biochem. 47:343–350.

Duarte, L. S., Dillenburg, L. R., Rosa, L. M. G. 2002. Assessing the role of light availability in the regeneration of *Araucaria angustifolia* (Araucariaceae). Aust J Bot. 50:741-751.

Durzan, D. J. 2012. Female parthenogenetic apomixis and androsporogenetic parthenogenesis in embryonal cells of *Araucaria angustifolia*: interpolation of progenesis and asexual heterospory in an artificial sporangium. Sex Plant Reprod. 25:227-246.

Elbl, P., Campos, R. A., Lira, B. S., Andrade, S. C. S., Jo, L., dos Santos, A. L. W., Coutinho, L. L., Floh, E. I. S., Rossi, M. 2015a. Comparative transcriptome analysis of early somatic embryo formation and seed development in Brazilian pine, *Araucaria angustifolia* (Bertol.) Kuntze. Plant Cell Tiss Organ Cult 120:903–915.

Elhiti, M., Stasolla, C., Wang, A. 2013. Molecular regulation of plant somatic embryogenesis. In Vitro Cell.Dev.Biol.-Plant 49:631–642.

Fehér, A. 2015. Somatic embryogenesis — Stress-induced remodeling of plant cell fate. Biochim Biophys Acta 1849:385-402.

Feirer, R. P. 1995. The biochemistry of conifer embryo development: amino acids, polyamines and storage proteins. In: Jain, S. M., Gupta, P. K., Newton, R. J. (eds) Somatic embryogenesis in woody plants, vol. 1. Dordrecht: Kluwer Academic Publishers; pp. 317-336.

Filonova, L. H., Bozhkov, P. V., Brukhin, V. B., Daniel, G., Zhivotovky, B., von Arnold, S. (2000) Two waves of programmed cell death occur during formation and development of somatic embryos in the gymnosperm Norway spruce. J Cell Sci 113:4399–4411.

Floh, E. I. S. dos Santos, A. L. W., Demarco, D. 2015. Embriogênese vegetal: abordagens básicas e biotecnológicas. In: Resende, R. R., Soccol, C. R. (eds.). 1ed. São Paulo: Editora Edgard Blücher Ltda, v. 1, pp. 01-15.

Floh, I. E. S. 2007. Marcadores bioquímicos e moleculares para estudo da morfogênese *in vitro*. Revista Brasileira de Horticultura Ornamental 13:1992-2001.

Garcia, R. J. F. 2002. Araucareaceae. In: Wanderley, M. G. L., Shepherd, G. J., Giulietti, A. M. (eds) Flora Fanerogâmica do Estado de São Paulo. São Paulo: Hucitec, v.2. Araucareaceae, p.1-4.

Garcia-Mendiguren, O., Montalbán, I. A., Stewart, D., Moncaleán, P., Klimaszewska, K., Rutledg, R. G. 2015. PLoSOne, Jun 3;10(6):e0128679.

Ge, C., Cui, X., Wang, Y., Hu, Y., Fu, Z., Zhang, D., Cheng, Z., Li, J. 2006. *BUD2*, encoding an S-adenosylmethionine decarboxylase, is required for Arabidopsis growth and development. Cell Res. 16:446-456.

Gemperlová, L., Fischerová, L., Cviková, M., Malá, J., Vondráková, Z., Martincová, O., Vágner, M. 2009. Polyamine profiles and biosynthesis in somatic embryo development and comparison of germinating somatic and zygotic embryos of Norway spruce. Tree Physiol 29:1287–1298.

Gifford, E. M., Foster, A. S. 1989. Morphology and evolution of vascular plants. New York: W. H. Freeman and Company.

Groppa, M. D., Benavides, M. P. 2008. Polyamines and abiotic stress: recent advances. Amino Acids 34:35-45.

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

Guerra, M. P., Silveira, V., Dos Reis, M. S., Schneider, L. 2002. Exploração, manejo e conservação da Araucária (*Araucaria angustifolia*). In: Simões, L. L., Lino, C. F. (eds) Sustentável Mata Atlântica: a exploração de seus recursos florestais. São Paulo: Editora Senac. Exploração, manejo e conservação da araucária (*Araucaria angustifolia*), p.85-101.

Guerra, M. P., Silveira, V., Santos, A. L. W., Astarita, L.V., Nodari, R.O. 2000. Somatic embryogenesis in *Araucaria angustifolia* (Bert) O. Ktze. In: Jain, S., Gupta, P., Newton, R. (eds) Somatic embryogenesis in woody plants. Kluwer Academic Publishers, Amsterda 457-478.

Guerra, M. P., Steiner, N., Farias-Soares, F. L., Vieira, L. do N., Fraga, H. P., Rogge-Renner, G. D., Maldonado, S. B. 2016. Somatic Embryogenesis in *Araucaria angustifolia* (Bertol.) Kuntze (Araucariaceae). In: Germana, M. A., Lambardi, M. (eds) In Vitro Embryogenesis in Higher Plants, 1359:439-50

Guerra, M. P., Steiner, N., Mantovani, A., Nodari, R. O., Dos Reis, M. S., dos Santos, K. L. 2008. Araucária. In: Barbieri, R. L., Stumpf, E. R. T. (eds). Origem e Evolução de Plantas Cultivadas. Brasília: Embrapa Informação Tecnológica, 2008. Araucária, p.149-184.

Guerra, M. P., Torres, A. C., Teixeira, J. B. 1999. Embriogênese somática e semente sintética. In: Torres, A. C., Caldas, L. S., Buso, J. A. (eds.) Cultura de Tecidos e Transformação de Plantas. Embrapa-SPI/CNPH vol.2, pp. 533-568.

Guerra, M. P., Torres, A. C., Teixeira, J. B. 1999. Embriogênese somática e semente sintética. In: Torres, A. C., Caldas, L. S., Buso, J. A. (eds.) Cultura de Tecidos e Transformação de Plantas. Embrapa-SPI/CNPH vol.2, pp. 533-568.

Guo, Z., Tan, J., Zhuo, C., Wang, C., Xiang, B., Wang, Z. 2014. Abscisic acid, H_2O_2 and nitric oxide interactions mediated cold-induced S-adenosylmethionine synthetase in *Medicago sativa* subsp. *falcata* that confers cold tolerance through up-regulating polyamine oxidation. Plant Biotechnol. J. Epub 12:601-612.

Gupta, P.K., Pullman, G.S. 1991. Method for reproducing coniferous plants by somatic embryogenesis using abscisic acid and osmotic potential variation. *US patent* 5,036,007.

Haines, R. J., Prakasha, N. 1980. Proembryo development and suspensor elongation in *Araucaria* Juss. Aust. J. Bot. 28:511-522.

Hakman, I. 1993. Embryology in Norway spruce (*Picea abies*): An analysis of the composition of seed storage proteins and deposition of storage reserves during seed development and somatic embryogenesis. Physiol. Plant. 87: 148-159.

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

Hakman, I., Oliviusson, P. 2002. High expression of putative aquaporin genes in cells with transporting and nutritive functions during seed development in Norway spruce (*Picea abies*). Journal of Experimental Botany 53: 639-649.

Hakman, I., von Arnold, S. 1985. Plantlet regeneration through somatic embryogenesis in Picea abies (Norway spruce). J. Plant Physiol. 121:149-158.

Hanfrey, C., Sommer, S., Mayer, M. J., Burtin, D., Michael, A. J. 2001. Arabidopsis polyamine biosynthesis: absence of ornithine decarboxylase and the mechanism of arginine decarboxylase activity. Plant J 27:551–560.

Harada, J. J., Belmonte, M. F., Kwong, R. W. 2010. Plant Embryogenesis (Zygotic and Somatic). eLS. DOI: 10.1002/9780470015902.a0002042.pub2.

Haworth, M., Elliot-Kingston, C., McElwain, J. C. 2011. The stomatal CO_2 proxy does not saturate at high atmospheric CO_2 concentrations: evidence from stomatal index responses of Araucariaceae conifers. Oecologia 167: 11-19.

Högberg, K. A., Bozhkov, P. V., von Arnold, S. 2003. Early selection improves clonal performance and reduces intraclonal variation of Norway spruce plants propagated by somatic embryogenesis. Tree Physiol. 23:211-6.

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

Hummel, I., Bourdais, G., Gouesbet, G., Couée, I., Malmber, R. L., Amrani, A. E. 2004 Differential gene expression of *ARGININE DECARBOXYLASE ADC1* and *ADC2* in Arabidopsis thaliana: characterization of transcriptional regulation during seed germination and seedling development. New Phytol 163:519-531.

Ikeda, M., Umehara, M., Kamada, H. 2006. Embryogenesis-related genes; its expression and roles during somatic and zygotic embryogenesis in carrot and Arabidopsis. Plant Biotech 23:153–161.

Imai, A., Matsuyama, T., Hanzawa, Y., Akiyama, T., Tamaoki, M., Saji, H., Shirano, Y., Kato, T., Hayashi, H., Shibata, D. *et al.* 2004. Spermidine synthase genes are essential for survival of Arabidopsis. Plant Physiol 135:1565-1573.

Jain, S. M., Ishii, K. 1998. Recent advances in somatic embryogenesis in forest trees. In: Mantell, S. H., Bruns, S., Tragardh, C., Viana, A. M. (eds) Recent advances in biotechnology for conservation and management. International Foundation for Science, Stockholm, p.214-231.

Jancewicz, A. L., Gibbs, N. M., Masson, P. H. 2016. Cadaverine's Functional Role in Plant Development and Environmental Response. Front Plant Sci. 7. doi: 10.3389/fpls.2016.00870.

Jiménez-Bremont, J. F., Marina, M., Guerrero-González, M. L., Rossi, F. R., Sánchez-Rangel, D., Rodríguez-Kessler, M., Ruiz, O. A., Gárriz, A. 2014. Physiological and molecular implications of plant polyamine metabolism during biotic interactions. Front Plant Sci. 5, doi: 10.3389/fpls.2014.00095.

Jo, L. 2012. Estabelecimento de marcadores bioquímicos para embriogênese somática em *Araucaria angustifolia*. Dissertação de Mestrado. Universidade de São Paulo, 116p.

Jo, L., dos Santos, A. L. W., Bueno, C. A., Barbosa, H. R., Floh, E. I. S. 2014. Proteomic analysis and polyamines, ethylene and reactive oxygen species levels of *Araucaria angustifolia* (Brazilian pine) embryogenic cultures with different embryogenic potential. Tree Physiol 34: 94–104.

Johansen, D. A. 1950. Chronica Botanica Company. Waltham, Massachusettes, pp.305.

Johnson, T., Cruse-Sanders, J. M., Pullman, G. S. 2012. Micropropagation and seed cryopreservation of the critically endangered species Tennessee yellow-eye grass, *Xyris tennesseensis* Kral. In Vitro Celll Dev Bio Plant. 48:369-376.

Kageyama, P. Y., Ferreira, M. 1975. Propagação vegetativa por enxertia com *Araucaria angustifolia* (Bert.) O. Ktze. IPEF 11:95-102.

Kakkar, R. K., Nagar, P. K., Ahuja, P. S., Rai, V. K. 2000. Polyamines and plant morphogenesis. Biologia Plantarum 43:1-11.

Karami, O., Saidi, A. 2010. The molecular basis for stress-induced acquisition of somatic embryogenesis. Mol. Biol. Rep. 37:2493-2507.

Klimaszewska, K., Hargreaves, C., Lelu-Walter, M., Trontin, J. 2016. Advances in Conifer Somatic Embryogenesis since year 2000. In: Germana, M. A., Lambardi, M. (eds) In Vitro Embryogenesis in Higher Plants, 1359, pp 131-166.

Klimaszewska, K., Morency, F., Jones-Overtona, C., Cooke, J. 2004. Accumulation pattern and identification of seed storage proteins in zygotic embryos of *Pinus strobus* and in

somatic embryos from different maturation treatments. Physiologia Plantarum 121:682–690.

Klimaszewska, K., Trontin, J. F., Becwar, M. R., Devillard, C., Park, Y. S., Lelu-Walter, M. A. 2007. Recent Progress in Somatic Embryogenesis of Four *Pinus* spp. Tree and Forestry Science and Biotechnology 1:11-25.

Kovács, Z., Simon-Sarkadi, L., Szücs, A., Kocsy, G. 2010. Differential effects of cold, osmotic stress and abscisic acid on polyamine accumulation in wheat. Amino Acids 38:623-631.

Kumar, A., Altabella, T., Taylor, M. A., Tiburcio, A. F. 1997. Recent advances in polyamine research. Trends in Plant Science 2:124-130.

Kusano, T., Berberich, T., Tateda, C., Takahashi, Y. 2008. Polyamines: essential factors for growth and survival. Planta 228:367–381.

Kusano, T., Yamaguchi, K., Berberich, T., Takahashi, Y. 2007. Advances in polyamine research in 2007. J Plant Res. 120:345-350.

Kuznetsov, V. L., Shevyakova, N. I. 2007. Polyamines and stress tolerance of plants. Plant Stress 1:50–71.

Larher, F. R., Aziz, A., Gibon, Y., Trotel-Aziz, P., Sulpice, R., Bouchereau, A. 2003. An assessment of the physiological properties of the so-called compatible solutes using in vitro experiments with leaf discs. Plant Physiol Bioch. 41:657-666.

Lasanajak, Y., Minocha, R., Minocha, S. C., Goyal, R., Fatima, T., Handa, A. K., Mattoo, A. K. 2014. Enhanced flux of substrates into polyamine biosynthesis but not ethylene in tomato fruit engineered with yeast S-adenosylmethionine decarboxylase gene. Amino Acids 46:729–742.

Lee, E. K., Cho, D. Y., Soh, W. Y. 2001. Enhanced production and germination of somatic embryos by temporary starvation in tissue cultures of *Daucus carota*. Plant Cell Rep 20:408–415.

Lelu-Walter, M. A., Bernier-Cardou, M., Klimaszewska, K. 2008. Clonal plant production from self- and cross-pollinated seed families of *Pinus sylvestris* (L.) through somatic embryogenesis. Plant Cell Tissue Org. Cult. 92:31–45.

Lelu-Walter, M.A., Thompson, D., Harvengt, L., Sanchez, L., Toribio, M., Pâques, L.E. 2013. Somatic embryogenesis in forestry with a focus on Europe: stateof-the-art, benefits, challenges and future direction. Tree Genet Genome 9:883–899.

Lipert, D., J. Zhuang, S. Ralph, D.E. Ellis, M. Gilbert, R. Olafson, K. Ritland, C.J. Douglas, J. Bohlmann. 2005. Proteome analysis of early somatic embryogenesis in Picea glauca. Proteomics 5:461–473.

Liu, J. H., Wang, W., Wu, H., Gong, X., Moriguchi, T. 2015. Polyamines function in stress tolerance: from synthesis to regulation. Front Plant Sci. 6, doi: 10.3389/fpls.2015.00827.

Liu, Q., Chen, Y. Q. 2009. Insights into the mechanism of plant development: Interactions of miRNAs pathway with phytohormone response. Biochem. Biophys. Res. Comm, 384:1–5.

Ma, X., Bucalo, K., Determann, R. O., Cruse-Sanders, J. M., Pullman, G. S. 2012. Somatic embryogenesis, plant regeneration and cryopreservation for *Torreya taxifolia*, a highly endangered coniferous species. In Vitro Cell Dev Biol Plant. 48:324-334.

Majumdar, R., Barchi, B., Turlapati, S. A., Gagne, M., Minocha, R., Long, S., Minocha, S. C. 2016. Glutamate, ornithine, arginine, proline, and polyamine metabolic interactions: the pathway is regulated at the posttranscriptional level. Front Plant Sci 7:78.

Majumdar, R., Shao, L., Minocha, R., Long, S., Minocha, S. C. 2013. Ornithine: the overlooked molecule in the regulation of polyamine metabolism. Plant Cell Physiol 54:990–1004.

Malá, J., Cvikrová, M., Máchová, P., Martincová, O. 2009 Polyamines during somatic embryo development in Norway spruce (*Picea abies* [L.]). Journal of Forest Science 55:75–80.

Mantovani, A., Morellato, L. P. C., dos Reis, M. S. (2004) Fenologia reprodutiva e produção de sementes em *Araucaria angustifolia* (Bert.) O. Kuntze. Rev Brasil Bot 27:787-796.

Mantovani, L. A., Morellato, P. C., dos Reis, M. S. 2006. Internal Genetic Structure and Outcrossing Rate in a Natural Population of *Araucaria angustifolia* (Bert.) O. Kuntze. J Hered. 97:466–472.

Martin, L. B. B., Fei, Z., Giovanni, J. J., Rose, J. K. C. 2013. Catalyzing plant science research with RNA-seq. Front. Plant Sci. 4, doi.org/10.3389/fpls.2013.00066.

Martinelli, G., Moraes, M.A. 2013. Livro Vermelho da flora do Brasil. Rio de Janeiro: Andrea Jakobsson, Instituto de Pesquisas Jardim Botânico do Rio de Janeiro. 1100 p.

Martínez, L. E., Agüero, C. B., López, M. E., and Galmarini, C. R. 2000. Improvement of *in vitro* gynogenesis induction in onion (*Allium cepa* L.) using polyamines. Plant Sci. 156:221–226.

Merkle, S. A., Parrott, W. A., Flinn, B. S. 1995. Morphogenic aspects of somatic embryogenesis. In: Thorpe, T. A. (eds) In Vitro Embryogenesis in Plants. Dordrecht, Kluwer Academic Publishers, p. 155-203.

Minocha, R., Majumdar, R., Minocha, S. C. 2014. Polyamines and abiotic stress in plants: a complex relationship. Front. Plant Sci. 5, doi.org/10.3389/fpls.2014.00175.

Minocha, R., Minocha, S. C., Long, S. 2004. Polyamines and their biosynthetic enzymes during somatic embryo development in red spruce (*Picea rubens* Sarg.). In Vitro Cell. Dev. Biol.-Plant 40:572-580.

Minocha, R., Smith, D. R., Reeves, C., Steele, K. D., Minocha, S. C. 1999. Polyamine levels during the development of zygotic and somatic embryos of *Pinus radiata*. Physiol Plant 105:155–164.

Minocha, S. C., Minocha, R. 1995. Role of polyamines in somatic embryogenesis. In: Bajaj, Y. P. S. (ed) Biotechnology in agriculture and forestry, somatic embryogenesis and synthetic seed I. Springer-Verlag, Heidelberg, pp 55–72.

Montague, M., Koppenbrink, J., Jaworski, E. 1978. Polyamine metabolism in embryogenic cells of *Daucus carota*. I. Changes in intracellular content and rates of synthesis. Plant Physiol. 62:430-3.

Montalbán, I. A., De Diego, N., Moncalean, P. 2012. Enhancing initiation and proliferation in radiata pine (*Pinus radiata* D. Don) somatic embryogenesis through seed family screening, zygotic embryo staging and media adjustments. Acta Physiologiae Plantarum 34: 451–460.

Moschou, P. N., Wu, J., Cona, A., Tavladoraki, P., Angelini, R., Roubelakis-Angelakis, K. A. 2012. The polyamines and their catabolic products are significant players in the turnover of nitrogenous molecules in plants. Journal of Experimental Botany 63:5003-5015.

Nagmani, R., Bonga, J. M. 1985. Embryogenesis in subcultured callus of *Larix decidua*. Canadian Journal of Forest Research 15:1088-1091.

Nakagawa, R., Kurushima, M., Matsui, M., Nakamura, R., Kubo, T. and Funada, R. 2011. Polyamines promote the development of embryonal-suspensor masses and the formation of somatic embryos in *Picea glehnii*. In Vitro Cell. Dev. Biol. Pl. 47:480-487.

Namasivayam, P. 2007. Acquisition of embryogenic competence during somatic embryogenesis. Plant Cell Tiss Organ Cult 90:1–8.

Neale, D. B., Kremer, A. 2011. Forest tree genomics: growing resources and applications. Nat Rev Genet 12:111-122.

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

Niskanen, A.-M., Lu, J., Seitz, S., Keinonen, K., von Weissenberg, K., Pappinen, A. 2004. Effect of parent genotype on somatic embryogenesis in Scots pine (*Pinus sylvestris*). Tree Physiology 24:1259–1265.

Norstog, K., Rhamstine, E. 1967. Isolation and culture of haploid and diploid cycad tissue. Phytomorphology 17:374-381.

Ochatt, S. J., Revilla, M. A. 2016. From Stress to Embryos: Some of the Problems for Induction and Maturation of Somatic Embryos. In vitro embryogenesis in higher plants 1359:523-536.

Page, A. F., Minocha, R., Minocha, S. C. 2012. Living with high putrescine: expression of ornithine and arginine biosynthetic pathway genes in high and low putrescine producing poplar cells. Amino Acids 42:295–308.

Pál, M., Szalai, G., Janda, T. 2015. Speculation: Polyamines are important in abiotic stress signaling. Plant Sci. 237:16-23.

Perán-Quesada, R., Sánchez-Romero, C., Barceló-Muñoz, A., Pliego-Alfaro, F. 2004. Factors affecting maturation of avocado somatic embryos. Scientia Horticulturae 102:61-73.

Pieruzzi, F. P., Dias, L. L. C., Balbuena, T. S., Santa-Catarina, C., Santos, A. L. W., Floh, E. I. S. 2011. Polyamines, IAA and ABA during germination in two recalcitrant seeds: *Araucaria angustifolia* (Gymnosperm) and *Ocotea odorifera* (Angiosperm). Annals of botany (Print) 108:337-345.

Pullman, G. S., Xiaoyan, Z., Copeland-Kamp, B., Crockett, J., Lucrezi, J., May, S. W., Bucalo, K. 2015. Conifer somatic embryogenesis: improvements by supplementation of medium with oxidation-reduction agents. Tree Physiol 35:209-224.

Radoeva, T., Weijers, D. 2014. A roadmap to embryo identity in plants. Trend Plant Sci. 19:709-716.

Raghavan, V., Sharma, K. K. 1995. Zygotic embryogenesis in gymnosperms and angiosperms. In: Thorpe, T. A. (ed) In vitro embryogenesis in plants, Dordrecht, The Netherlands: Kluwer Academic Publishers, 73-115.

Rogge-Renner, G. D., Steiner, N., Schmidt, E. C., Bouzon, Z. L., Farias, F. L., Guerra, M. P. 2013. Structural and component characterization of meristem cells in *Araucaria angustifolia* (Bert.) O. Kuntze zygotic embryo. Protoplasma 250:731-739.

Saha, J., Brauer, E. K., Sengupta, A., Popescu, S. C., Gupta, K., Gupta, B. 2015 Polyamines as redox homeostasis regulators during salt stress in plants. Front. Environ. Sci. 3, doi.org/10.3389/fenvs.2015.00021.

Salo, H. M., Sarjala, T., Jokela, A., Häggman, H., Vuosku, J. 2016. Moderate stress responses and specific changes in polyamine metabolism characterize Scots pine somatic embryogenesis. Tree Physiol 36:392–402.

Santa-Catarina, C., Silveira, V., Balbuena, T. S., Viana, A. M., Estelita, M. E. M., Handro, W., Floh, E. I. S. 2006. IAA, ABA, polyamines and free amino acids associated with zygotic embryo development of *Ocotea catharinensis*. Plant Growth Regul 49:237-247.

Santa-Catarina, C., Silveira, V., Guerra, M. P., Steiner, N., Macedo, A. F., Floh, E. I. S., dos Santos, A. L. W. 2013. The use of somatic embryogenesis for mass clonal propagation and biochemical and physiological studies in woody plants. Curr. Opin. Plant Biol. 13:103-119.

Santa-Catarina, C., Silveira, V., Scherer, G. F. E., Floh, E. I. S. 2007. Polyamine and nitric oxide levels relate with morphogenetic evolution in somatic embryogenesis of *Ocotea catharinensis*. Plant Cell Tiss Organ Cut 90:93-101.

Santolini, J., André, F., Jeandroz, S., Wendehenne, D. 2016. Nitric oxide synthase in plants: Where do we stand? Nitric Oxide, In Press, doi: 10.1016/j.niox.2016.09.005.

Santos, I. R. I. 2000. Criopreservação: Potencial e perspectivas para a conservação de germoplasma vegetal. R. Bras. Fisiol. Veg. 12:70-84.

Schlogl, P. S., dos Santos, A. L. W., Vieira, L. N., Floh, E. I. S., Guerra, M. P. 2012a. Gene expression during early somatic embryogenesis in Brazilian pine (*Araucaria angustifolia* (Bert) O. Ktze). Plant Cell Tiss Org 108: 173-180.

Schlogl, P. S., dos Santos, A. L. W., Vieira, L. N., Floh, E. I. S., Guerra, M. P. 2012b. Cloning and expression of embryogenesis-regulating genes in *Araucaria angustifolia* (Bert.) O. Kuntze (Brazilian Pine). Genet Mol Biol 35:172-181.

Schmidt, T., Ewald, A., Seyring, M., Hohe, A. 2006. Comparative analysis of cell cycle events in zygotic and somatic embryos of *Cyclamen persicum* indicates strong resemblance of somatic embryos to recalcitrant seeds. Plant Cell Rep 25:643–650.

Sghaier, B., Bahloul, M., Bouzid, R. G., Drira, N. 2008. Development of zygotic and somatic embryos of *Phoenix dactylifera* L. cv. Deglet Nour: Comparative study. Scientia Horticulturae 116:169–175.

Shao, L., Bhatnagar, P., Majumdar, R., Minocha, R., Minocha, S. C. 2014. Putrescine over production does not affect the catabolism of spermidine and spermine in poplar and Arabidopsis. Amino Acids 46:743–757. doi:10.1007/s00726-013-1581-2.

Sharma, K. K., Thorpe, T. A. 1995. Asexual embryogenesis in vascular plants in nature. In: In vitro embryogenesis in plants. Thorpe, T. A. (eds) Dordrecht: Kluwer Academic Publisher. p. 17-72.

Shimizu, J. Y., Jaeger, P., Sopchaki, S. A. 2000. Genetic variability in a remnant population of araucaria in the Iguaçu National Park, Brazil. Bol Pesq Florestais 41:18-36.

Shimoya, C. 1962. Contribuição ao estudo do ciclo biológico de *Araucaria angustifolia* (Bertolini) O. Ktze. Experientie 02:519-540.

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

Silveira, V., Floh, E. I. S., Handro, W., Guerra, M. P. 2004. Effect of plant growth regulators on the cellular growth and levels of intracellular protein, starch and polyamines in embryogenic suspension cultures of *Pinus taeda*. Plant Cell, Tissue and Organ Culture 76:53-60.

Silveira, V., Santa-Catarina, C., Balbuena, T. S., Moraes, F. M. S., Ricart, C. A. O., Sousa, M. V., Guerra, M. P., Handro, W., Floh, E. I. S. (2008) Endogenous abscisic acid levels and comparative proteome during seed development of *Araucaria angustifolia* (Bert.) O. Ktze. Biol Plantarum 52:101-104.

Silveira, V., Santa-Catarina, C., Tun, N. N., Scherer, G. F. E., Handro, W., Guerra, M. P., Floh, E. I. S. 2006. Polyamine effects on the endogenous polyamine contents, nitric oxide release, growth and differentiation of embryogenic suspension cultures of *Araucaria angustifolia* (Bert.) O. Ktze. Plant Science 171:91-98.

Singh H. Embryology of Gimnosperms. Encyclopedia of plant anatomy. Berlin:Gebruder Borntraeger; 1978.

Smertenko, A., Bozhkov, P. V. 2014. Somatic embryogenesis: life and death processes during apical–basal patterning. Journal of Experimental Botany 65:1343-1360.

Sousa, V. A., Hattemer, H. H. 2003a. Fenologia Reprodutiva da *Araucaria angustifolia* no Brasil. Bol. Pesq. Fl. 47:19-32.

Sousa, V. A., Hattemer, H. H. 2003b. Pollen dispersal and gene flow by pollen in *Araucaria angustifolia*. Aust J Bot 51:309-317.

Souza, V. A., Aguiar, A. V. 2012. Programa de melhoramento genético de Araucária da Embrapa Florestas: situação atual e perspectivas. Embrapa Florestas, v1, 38 p.

Stasolla, C., Kong, L., Yeung, E. C., Thorpe, T. A. 2002. Maturation of somatic embryos in conifers: morphogenesis, physiology, biochemistry, and molecular biology. In Vitro Cell Dev Biol Plant 38:93-105.

Stasolla, C., Yeung, E. C. 2003. Recent advances in conifer somatic embryogenesis: improving somatic embryo quality. Plant Cell Tissue Organ Cult. 74:15-35.

Steiner, N., Santa-Catarina, C., Andrade, J. B. R., Balbuena, T. S., Guerra, M. P., Handro, W., Floh, E. I. S., Silveira, V. 2008. *Araucaria angustifolia* Biotechnology. Functional Plant Science and Biotechnology 2:20-28.

Steiner, N., Santa-Catarina, C., Guerra, M. P., Cutri, L., Dornelas, M. C., Floh, E. I. S. 2012. A gymnosperm homologo of SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE-1 (*SERK1*) is expressed during somatic embryogenesis. Plant Cell Tiss Organ Cul 109:41-50.

Steiner, N., Santa-Catarina, C., Silveira, V., Floh, E. I. S., Guerra, M. P. 2007. Polyamine effects on growth and endogenous hormones levels in *Araucaria angustifolia* embryogenic cultures. Plant Cell Tiss Organ Cult 89:55-62.

Sutton, B. 2002. Commercial delivery of genetic improvement to conifer plantations using somatic embryogenesis. Ann For Sci. 59:657-661.

Takeda, T., Hayakawa, F., Oe, K., Matsuoka, H. 2002. Effects of exogenous polyamine on embryogenic carrot cells. Biochem Eng J 12: 21–28.

Tautorus, T. E., Fowke, L. C., Dunstan, D. I. 1991. Somatic embryogenesis in conifers. Canadian Journal of Botany 69:1873-1899.

Tavladoraki, P., Cona, A., Federico, R., Tempera, G., Viceconte, N., Saccoccio, S., Battaglia, V., Toninello, A., Agostinelli, E. 2012. Polyamine catabolism: target for antiproliferative therapies in animals and stress tolerance strategies in plants. Amino Acids 42:411-426.

Tereso, S., Zoglauer, K., Milhinhos, A., Miguel, C., Oliveira, M. M. 2007. Zygotic and somatic embryo morphogenesis in *Pinus pinaster*: comparative histological and histochemical study. Tree Physiology 27:661–669.

Thibaud-Nissen, F. O., Shealy, R. T., Khanna, A., Vodkin, L. O. 2003. Clustering of microarray data reveals transcript patterns associated with somatic embryogenesis in soybean. Plant Physiol 132:118–136.

Tiburcio, A. F., Altabella, T., Borrell, A., Masgrau, C. 1997. Polyamine metabolism and its regulation. Physiol Plantarum 100:664-674.

Tun, N. N., Santa-Catarina, C., Begum, T., Silveira, V., Handro, W., Floh, E. I. S., Scherer, G. F. E. 2006. Polyamines induce rapid biosynthesis of nitric oxide (NO) in *Arabidopsis thaliana* seedlings. Plant and Cell Physiology 47:346-354.

Urano, K., Hobo, T., Shinozaki, K. 2005. *Arabidopsis ADC* genes involved in polyamine biosynthesis are essential for seed development. FEBS Lett 579:1557–1564.

Vestman, D., Larsson, E., Uddenberg, D., Cairney, J., Claphan, D., Sundberg, E., von Arnold, S. 2011. Important process during differentiation and early development of somatic embryos of Norway spruce as revealed by changes in global gene expression. Tree Genetics & Genomes 7: 347-362.

Vieira, L. N., Santa-Catarina, C., Fraga, H. P. F., dos Santos, A. L. W., Steinmacher, D. A., Schlogl, P. S., Silveira, V., Steiner, N., Floh, E. I. S., Guerra, M. P. 2012. Glutathione improves early somatic embryogenesis in *Araucaria angustifolia* (Bert) O. Kuntze by alteration in nitric oxide emission. Plant Science 195:80-87.

von Arnold, S., Sabala, I., Bozhkov, P., Dyachok, J., Filanova, L. 2002. Developmental pathways of somatic embryogenesis. Plant Cell Tissue Organ Cult 69:233-49.

Vuosku, J., Jokela, A., Läärä, E., Sääskilahti, M., Muilu, R., Sutela, S., Altabella, T., Sarjala, T., Häggman, H. 2006. Consistency of polyamine profiles and expression of arginine decarboxylase in mitosis during zygotic embryogenesis of Scots pine. Plant Physiology 142:1027-1038.

Vuosku, J., Suorsa, M., Ruottinen, M., Sutela, S., Muilu-Mäkelä, R., Julkunen-Tiitto, R., Sarjala, T., Neubauer, P., Häggman, H. 2012. Polyamine metabolismo during exponential growth transition in Scots pine embryogenic cell culture. Tree Physiol 32:1274–1287.

Vuosku, J., Sutela, S., Tillman-Sutela, E., Kauppi, A., Jokela, A., Sarjala, T., Häggman, H. 2009. Pine embryogenesis many licences to kill for a new life. Plant Signaling & Behavior 4:10, 928-932.

Wallace, H. M., Fraser, A. V., Hughes, A. 2003. A perspective of polyamine metabolism. Biochem. J. 376:1–14.

Walters, C., Wesley-Smith, J., Crane, J., Hill, L. M., Chmielarz, P., Pammenter N. W., Berjak, P. 2008. Cryopreservation of recalcitrant (i.e. desiccation-sensitive) seeds. pp. 465-484. In: Reed, B. M. (ed.) Plant Cryopreservation: A practical guide. Springer Publishing, New York, NY.

Wendling, I. 2011. Enxertia e florescimento precoce em *Araucaria angustifolia*. Comunicado Técnico 272:1-7.

Zimmerman, J. L. 1993. Somatic Embryogenesis: A Model for Early Development in Higher Plants. Plant Cell. 5:1411-1423.

Zonneveld, B. J. M. 2012. Genome sizes of all 19 Araucaria species are correlated with their geographical distribution. Plant Syst Evol 298:1249–1255.

Capítulo I

Elucidação da biossíntese de poliaminas durante o desenvolvimento de sementes do pinheiro brasileiro (*Araucaria angustifolia*)

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Research paper

Elucidation of the polyamine biosynthesis pathway during Brazilian pine (*Araucaria angustifolia*) seed development

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Polyamines (PAs), such as spermidine and spermine, as well as amino acids that are substrates for their biosynthesis, are known to be essential for plant development. However, little is known about the gene expression and metabolic switches associated with the ornithine/arginine and PA biosynthetic pathway during seed development in conifers. To understand these metabolic switches, the enzyme activity of arginine decarboxylase and ornithine decarboxylase, as well as the contents of PAs and amino acids were evaluated in three *Araucaria angustifolia* (Bertol. Kuntze) seed developmental stages in combination with expression profile analyses of genes associated with the ornithine/arginine and PA biosynthetic pathway. Twelve genes were selected for further analysis and it was shown that the expression profiles of *AaADC* and *AaSAMDC* were up-regulated during zygotic embryo development. Polyamines and amino acids were found to accumulate differently in embryos and megagametophytes, and the transition from the globular to the cotyledonary stage was marked by an increase in free and conjugated spermidine and spermine contents. Putrescine is made from arginine, which was present at low content at the late embryogenesis stage, when high content of citrulline was observed. Differences in amino acids, PAs and gene expression profiles of biosynthetic genes at specific seed stages and at each seed transition stage were investigated, providing insights into molecular and physiological aspects of conifer embryogenesis for use in future both basic and applied studies.

Keywords: amino acids, arginine decarboxylase, conifer, embryogenesis, ornithine decarboxylase, polyamine biosynthesis.

Introduction

The Brazilian pine, *Araucaria angustifolia* (Bertol. Kuntze), is a native conifer in the southern part of Brazil. The high value of its timber and the uncontrolled exploitation of wood resources at the end of the 19th century have led to this species being classified as critically endangered by the International Union of Conservation of Nature Red List of Threatened Species (http://www.iucnredlist.org/details/32975/0). Currently, populations of *A. angustifolia* cover less than 2% of its original forest area (Koch and Corrêa 2002, Jo et al. 2014). Unlike those of most conifers, *A. angustifolia* seeds maintain a high content of water and active metabolic rates at maturity, resulting in a rapid loss of viability (Steiner et al. 2008). There is therefore considerable interest in developing biotechnological tools for *A. angustifolia* germplasm conservation and genetic improvement in order to support reforestation and conservation programs (Steiner et al. 2008, Schlögl et al. 2012*a*, Elbl et al. 2015*a*). Somatic embryogenesis is an alternative to ex situ clonal propagation of commercially important and endangered plant species, especially conifers (Klimaszewska et al. 2011, Jo et al. 2014, Rupps et al. 2016). However, the establishment of a protocol for efficient *A. angustifolia* somatic embryogenesis has been limited by a poor understanding of the underlying genetic programs and biochemical pathways that regulate zygotic embryogenesis (Astarita et al. 2003*a*, 2003*b*, 2003*c*, Silveira et al. 2008). Indeed, somatic embryogenesis has been defined as being

analogous to zygotic embryogenesis, and a number of morphological, physiological, biochemical and molecular similarities between the two have been identified (Silveira et al. 2006, 2008, Steiner et al. 2007, Lara-Chavez et al. 2012, Schlögl et al. 2012*a*, 2012*b*, Elbl et al. 2015*a*, dos Santos et al. 2016). Studies of the molecular processes and biochemical activities during *A. angustifolia* zygotic embryogenesis have included a comparative transcriptome analysis (Elbl et al. 2015*a*) and profiling of proteins (Silveira et al. 2008), abscisic acid (Silveira et al. 2008), indole-3-acetic acid (Astarita et al. 2003*a*), amino acids (Astarita et al. 2003*b*) and polyamines (PAs) (Astarita et al. 2003*c*).

Polyamines are multifunctional aliphatic nitrogen polycationic compounds that are present in plants, animals and microorganisms (Minguet et al. 2008, Alcázar et al. 2010). They can exist as free molecules, as forms that are conjugated to small molecules such as coumaroyl, ferulic or hydrocinnamic acids, or bound to larger molecules such as lipids, nucleic acids and proteins (Grimes et al. 1986, Tiburcio et al. 1993, Kong et al. 1998). Putrescine (Put), spermidine (Spd) and spermine (Spm) are three common plants PAs that are known to play key roles in developmental processes that include embryogenesis, morphogenesis, fruit development and ripening, programmed cell death and responses to biotic and abiotic stresses (Kusano et al. 2008, Gemperlová et al. 2009, Alcázar et al. 2010, Vuosku et al. 2012, Akhtar 2013). Putrescine is produced directly from the amino acid ornithine (Orn), by ornithine decarboxylase (ODC; EC 4.1.1.17), or indirectly from arginine (Arg) in a reaction catalyzed by three enzymes: arginine decarboxylase (ADC; EC 4.1.1.19), agmatine deiminase (AIH; EC 3.5.3.12) and *N*-carbamoylputrescine amidase (CPA; EC 3.5.1.53) (Bais and Ravishankar 2002). The co-expression of the ADC and ODC enzymes in some species may reflect differences in their tissue specificity or in their contribution to stress responses and development. Putrescine is the immediate precursor of the tri- and tetra-amines, Spd and Spm, which are made by combined actions of S-adenosylmethionine decarboxylase (SAMDC; EC 4.1.1.50) and two different aminopropyltransferases, Spd synthase (SPDS; EC 2.5.1.16) and Spm synthase (SPMS; EC 2.5.1.22) (Bais and Ravishankar 2002, Lasanajak et al. 2014).

Polyamines function as regulators during seed development in both angiosperms and gymnosperms (Stasolla and Yeung 2003), and a number of studies using *Arabidopsis thaliana* mutants with defects in PA metabolism have established the importance of PAs in embryogenesis (Imai et al. 2004). Changes in either the profile or ratio of PAs correlate with defined stages of zygotic embryogenesis, suggesting that PAs are potential developmental markers of this process (Minocha et al. 1999, Astarita et al. 2003*c*, Silveira et al. 2004). Although there have been reports of an association between content of PAs and zygotic and somatic embryogenesis in conifers, the exact mechanisms by which they exert an effect is not clear (Minocha et al. 1999, Astarita et al. 2003*c*, Silveira et al. 2004, 2006, Steiner et al. 2007, Gemperlová et al. 2009, Jo et al. 2014). During conifer seed development, PA content increases at the early stages and then decreases at the later stages of embryogenesis (Vuosku et al. 2006). Putrescine abundance was reported to remain stable during *Pinus sylvestris* embryo development, while Spd content was shown to be higher than that of Put in mature *Picea abies* zygotic embryos, accompanied by an increase in the activity of PA biosynthetic enzymes (Vuosku et al. 2006, Gemperlová et al. 2009). Similar profiles were observed during *A. angustifolia* zygotic embryo development (Astarita et al. 2003*c*).

The PA biosynthetic pathway is known to be regulated by the contents of amino acids that are required for their biosynthesis (Majumdar et al. 2013, 2016). Moreover, Orn, even though present in small quantities, may function as a regulator of not only PA biosynthesis, but also pathways involved in the conversion of glutamate to Arg, and proline biosynthesis (Page et al. 2007, 2012, Majumdar et al. 2013).

Although PAs are important for plant development (Minocha et al. 1999, Astarita et al. 2003*b*, 2003*c*, Silveira et al. 2004, Vuosku et al. 2006, Kusano et al. 2008, Gemperlová et al. 2009), relatively little is known about the relationship between gene expression and metabolic switches associated with Orn/Arg and PA biosynthesis during seed development (Fait et al. 2006). The aim of the current study was to investigate *A. angustifolia* PA biosynthesis during zygotic embryogenesis. To this end we measured the activities of ADC and ODC, as well as PA and amino acid abundance, in combination with quantitative real-time polymerase chain reaction (qRT-PCR) analyses of gene expression of genes involved in the Orn/Arg and PA biosynthetic pathways. These analyses were performed for three different seed developmental stages involving zygotic embryos and megagametophytes.

Materials and methods

Plant materials

The following seed developmental stages were analyzed: (i) megagametophytes containing globular embryos (GZE) (due to their small size, zygotic embryos and megagametophytes at the globular stage were analyzed together); (ii) isolated cotyledonary embryos (CZE); (iii) isolated mature embryos (MZE); (iv) megagametophytes at the cotyledonary stage (CZE MG); and (v) megagametophytes at the mature stage (MZE MG). Immature and mature *A. angustifolia* seeds were harvested from five trees located in the Parque Estadual de Campos do Jordão (22°41.792′ S; 045° 29.393′ W, 1.529 m above sea level) (authorization by Secretaria do Meio Ambiente, Instituto Florestal, in accordance with CARTA COTEC no 066/2014 D139/2013 AP), Campos do Jordão, São Paulo, Brazil. For each zygotic embryo developmental stage, we collected two or three seed cones per mother tree, mixed the seeds and divided them in three subsamples containing 70 seeds. Each subsample was considered a biological replicate. Plant material was

homogenized in liquid nitrogen with a pestle and mortar, stored at −80 °C and used for analyses of biochemical composition, enzymatic activity and gene expression.

Database searches, phylogenetic analyses and functional annotation

Protein sequences of *A. thaliana* ADC (arginine decarboxylase), AIH (agmatine deiminase), ARG (arginase), ASL (argininosuccinate lyase), ASS (arginosuccinate synthase), CPA (*N*-carbamoylputrescine amidase), OTC (ornithine carbamoyltransferase), SAMDC (*S*-adenosylmethionine decarboxylase), SPDS (spermidine synthase) and SPMS (spermine synthase), as well as *Prunus persica* ODC (ornithine decarboxylase), were used in tBLASTn (e-value > e^{-10}) (Altschul et al. 1990) searches of an *A. angustifolia* transcriptome database (Elbl et al. 2015*a*).

Phylogenetic analyses were performed using other plant sequences that are homologous to the protein sequences described above, obtained by searching the Phytozome (http://www.phytozome.net), NCBI (http://www.ncbi.nlm.nih.gov) and SustainPineDB (http://www.scbi.uma.es/sustainpinedb/home_page) databases. The sequences were aligned using the MUSCLE/CLUSTAW program with default parameters (MEGA software, version 6.0, Tamura et al. 2013). The alignment was analyzed using the neighborjoining method and the distances were calculated using the JTT model. The tree topology was evaluated with 1500 bootstrap replications.

In order to assess the predicted localization, molecular function and involvement in biological processes of each of these proteins, gene ontology analysis was performed using Blast2GO software (Conesa et al. 2005).

Quantitative RT-PCR analysis

RNA extraction, DNAse treatment, cDNA synthesis, primer design and qRT-PCR analysis was performed as in Elbl et al. (2015*b*). Gene specific primers (see Table S1 available as Supplementary Data at *Tree Physiology* Online) used in the qRT-PCR assay were designed using the OligoAnalyzer 3.1 (https://www.idtdna.com/calc/analyzer) according to Minimum Information for Publication of qRT-PCR Experiments guidelines (Bustin et al. 2009). The quantification cycle values from two technical replicates and the primer efficiency were calculated using LinRegPCR software (Ruijter et al. 2009). The expression values of the target genes were normalized against the geometric average of the *AaEIF4B-L* (translational initiation factor 4B) and *AaPP2A* (protein phosphatase 2A) reference genes (Elbl et al. 2015*b*). The relative expression of all the genes tested was calculated based on the average expression levels in the GZE sample and presented as Log_2 fold changes.

Determination of free amino acids

The amino acid content was determined as previously described (Santa-Catarina et al. 2006). Amino acids were derivatized with *o*-phthalaldehyde and separated by high-performance liquid chromatography (HPLC, Shimadzu, Japan) on a C_{18} reverse-phase column (5 μm × 4.6 mm × 250 mm—Shim-pack CLC ODS, Shimadzu, Japan). The gradient was developed by mixing proportions of 65% methanol with a buffer solution (50 mM sodium acetate, 50 mM sodium phosphate, 20 ml l^{-1} methanol, 20 ml l^{-1} tetrahydrofuran and adjusted to pH 8.1 with acetic acid). The 65% methanol gradient was programmed to 20% over the first 32 min, from 20% to 100% between 32 and 71 min, and 100% between 71 and 80 min, with a flow rate of 1 ml min^{-1}, at 40 °C. A fluorescence detector (Shimadzu, RF-20A), set at 250 nm excitation and 480 nm emission wavelengths, was used for detection and quantification.

PA profiles and content

Free and soluble conjugated PAs were extracted with 5% (v/v) perchloric acid and perchloric acid-insoluble PAs were extracted by acidic hydrolysis in 12 N HCl, as previously described by Jo et al. (2014) and Shevyakova et al. (2006), respectively. Polyamine derivatization was performed using a benzoylation method (Naka et al. 2010) with modifications: 1 ml of 2 N NaOH, 10 μl of benzoyl chloride and 40 μl of 0.05 mM diaminoheptane (used an as internal standard), were added to 60 μl of plant extract. Each sample was vortexed, incubated at room temperature for 40 min in the dark, and then 2 ml saturated sodium chloride solution was added to stop the reaction. After the addition of 2 ml diethyl ether, the samples were vortexed, centrifuged (13,000*g* for 5 min at 4 °C), and the upper organic phase was collected and evaporated with gaseous nitrogen (40 °C). The benzoylated PAs were solubilized in 175 μl of acetonitrile and analyzed by HPLC using a C_{18} reverse-phase column (as described above), operating at a flow rate of 1.0 ml. The gradient was developed by mixing 42% acetonitrile in water and 100% acetonitrile. The elution consisted of 42% acetonitrile over the first 20 min, from 42% to 100% between 20 and 38 min and then a column cleaning/regeneration cycle up to 56 min. PAs were detected and quantified at 229 nm (Shimadzu, Japan, UV-VIS Detector SPD-10).

ADC and ODC assays

Enzymatic activities were determined as previously described (Vuosku et al. 2006), with modifications. Three samples (200 mg fresh weight) were homogenized in an ice-cold mortar with liquid nitrogen and transferred to 200 μl of extraction buffer (50 mM Tris-HCl, pH 8.5, 0.5 mM pyridoxal-5-phosphate, 0.1 mM EDTA and 5 mM dithiothreitol). The solution was vortexed, centrifuged (13,000*g* for 20 min at 4 °C) and the supernatant was used for ADC and ODC enzyme assays. For the assays, a reaction mixture containing 200 μl of protein extract, 8.3 μl of extraction buffer, 12 mM unlabeled L-Arg or L-Orn and 25 nCi of either L-[^{14}C(U)]-Arg (specific activity 274.0 mCi.$mmol^{-1}$, PerkinElmer, USA) or L-[1-^{14}C]-Orn (specific activity 57.1 mCi $mmol^{-1}$, PerkinElmer, USA) was used. Blank samples contained 200 μl of extraction

buffer only. The reaction mixtures were incubated in glass tubes fitted with a rubber stopper and a filter paper disc soaked in 2 N KOH. The material was maintained at 37 °C, at 120 rpm (orbital shaker) for 90 min, and the reaction was stopped by addition of 200 µl of 5 % (v/v) perchloric acid followed by a further incubation for 15 min. The filter paper, containing $^{14}CO_2$, was immersed in 1 ml of scintillation fluid (PerkinElmer) and the radioactivity was measured using a scintillation counter (Tri-Carb2910TR, PerkinElmer). The activities were expressed as nmol $^{14}CO_2$ mg protein^{-1} h^{-1}. Protein content was measured using the Bradford method (Bradford 1976) with bovine serum albumin as a standard. The specific activities were measured for three biological replicates.

Statistical and correlation analysis

Data were analyzed by analysis of variance (ANOVA) followed by a Tukey's test ($P < 0.01$) and log transformed when appropriate. Pairwise comparisons between stages of development and between tissues were analyzed by Student's *t*-test (Benjamini-Hochberg method used for *P*-value adjustment) and the most abundant metabolites (3-fold change and $P < 0.01$ as the threshold cutoff) were visualized using Volcano plots. Pearson's ($P < 0.01$, $r > 0.90$) was performed for correlation analysis. For metabolic profiles separation between the samples, principal component analysis (PCA) and linear discriminant analysis (LDA) were performed, using MASS and ggplot2 package. These analyses were performed using R version 3.2.2 (R Development Core Team 2015). Heat map graphical and Hierarchical cluster by Euclidean distance were performed using Gene-E software (https://software.broadinstitute.org/GENE-E/).

Results

Identification of genes predicted to encode enzymes in the Orn/Arg and PA biosynthetic pathways

Twelve putative genes associated with the Orn/Arg and PA biosynthetic pathways were identified in the *A. angustifolia* transcriptome database. A protein alignment revealed high similarity to *A. thaliana* amino acids sequences. For *AaODC*, a fragment with 161 amino acids was obtained, showing high similarity to *P. persica* ODC (*PpODC*) (Table 1).

A phylogenetic analysis was performed to identify the *A. angustifolia* homologs of the *ADC*, *AIH*, *ARG*, *ASL*, *ASS*, *CPA*, *ODC*, *OTC* and *SAMDC* genes from Viridiplantae and *SPDS* and *SPMS* genes from Eukarya and Archaea species, in order to evaluate the diversity of Orn/Arg and PA biosynthetic enzymes. Most of the trees obtained had a topology that was congruent with the established phylogenetic relationships of the constituent species (see Figure S1 available as Supplementary Data at *Tree Physiology* Online). One copy of each gene was identified, except for the aminopropyltranferase, where three putative genes (*SPDS*, *SPDS3* and *SPMS*) were identified in distinct positions in the phylogenetic tree, being orthologous to *A. thaliana* (Figure 1). Gene ontology functional analysis of the *SPDS*, *SPDS3* and *SPMS* genes revealed a total of 518 terms, which were associated with

Table 1. Comparison of *A. angustifolia* putative genes related to the Orn/Arg and PA biosynthetic pathways with *A. thaliana* and *P. persica* sequences in the NCBI database (http://www.ncbi.nlm.nih.gov) using Blastp analysis.

Gene	EC number	*A. angustifolia* protein length (aa)	Blastp	E-value	Identities (%)	Positives (%)
AaAIH	3.5.3.12	370	*A. thaliana* AT5G08170 Agmatine deiminase	0	259/371(70)	309/371(83)
AaARG	3.5.3.1	343	*A. thaliana* AT4G08900.1 Arginase	0	273/342(80)	301/342(88)
AaADC	4.1.1.19	723	*A. thaliana* AT2G16500 Arginine decarboxylase 1	0	385/670(57)	500/670(74)
AaASL	4.3.2.1	519	*A. thaliana* AT5G10920 Argininosuccinate lyase	0	342/459(75)	398/459(86)
AaASS	6.3.4.5	551	*A. thaliana* AT4G24830 Argininosuccinate synthase	0	355/464(77)	396/464(85)
AaCPA	3.5.1.53	291	*A. thaliana* AT2G27450 *N*-carbamoylputrescine amidase	0	247/318(78)	271/318(85)
AaODC	4.1.1.17	161	*P. persica* BAD97830.1 Ornithine decarboxylase	$2e^{-83}$	116/161(72)	135/161(83)
AaOTC	2.1.3.3	380	*A. thaliana* AT1G75330 Ornithine carbamoyltransferase	$5e^{-177}$	232/309(75)	269/309(87)
AaSAMDC	4.1.1.50	300	*A. thaliana* AT3G02470.1 *S*-adenosylmethionine decarboxylase	$7e^{-136}$	188/291(65)	225/291(77)
AaSPDS	2.5.1.16	341	*A. thaliana* AT1G23820.1 Spermidine synthase 1	0	249/341(73)	284/341(83)
AaSPDS3	2.5.1.16	322	*A.thaliana* AT5G53120 Spermidine/spermine synthase 3	$3e^{-147}$	225/352(64)	267/352(75)
AaSPMS	2.5.1.22	346	*A. thaliana* AT5G19530 Spermine synthase	$4e^{-165}$	212/346(61)	268/346(77)

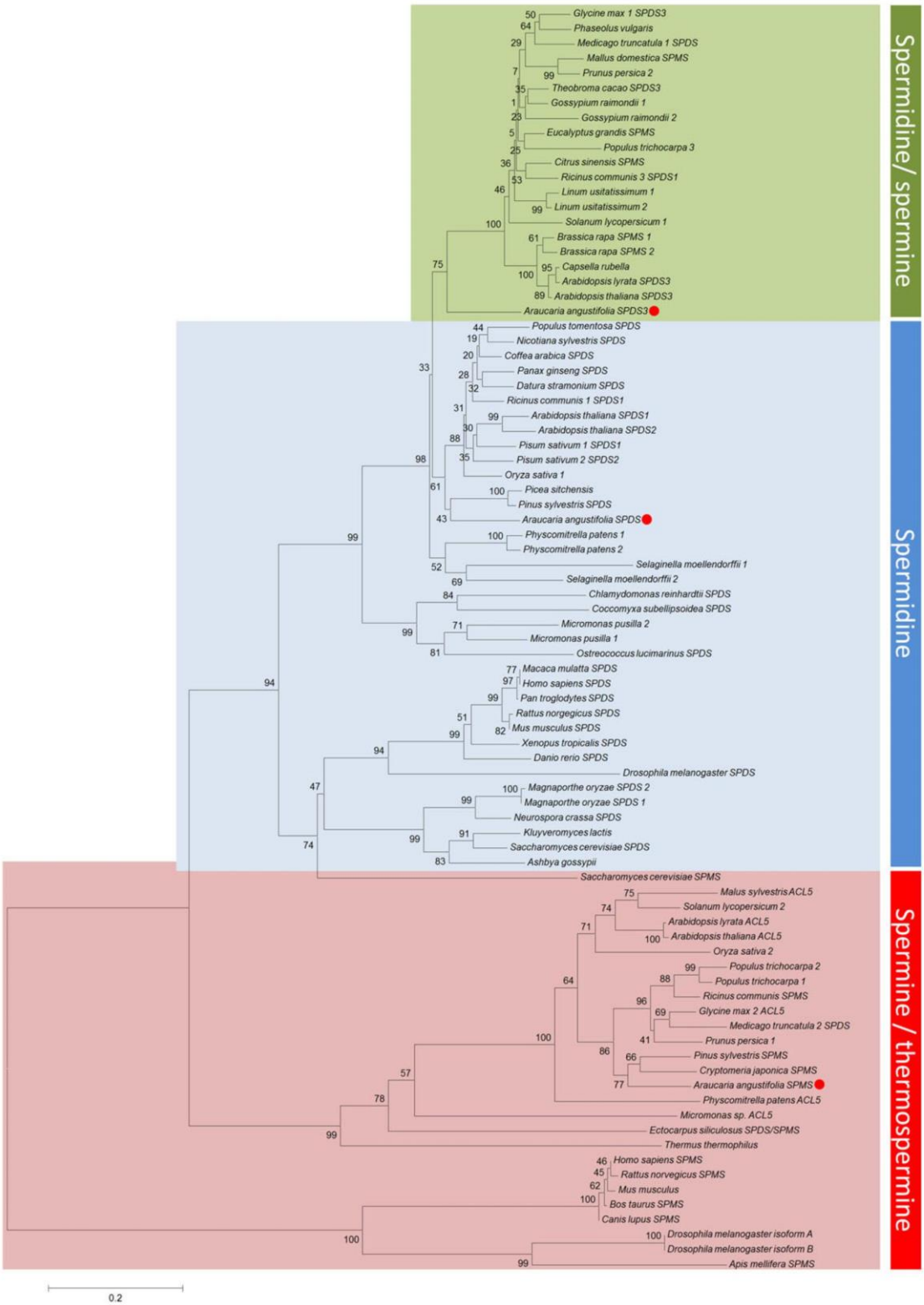

Figure 1. Phylogenetic tree constructed from sequences with homology to *A. angustifolia* aminopropyltransferases. About 85 spermidine synthase (SPDS), spermidine synthase 3 (SPDS3) and spermine synthase (SPMS) sequences from 52 different species were analyzed. *AaSPDS*, *AaSPDS3* and *AaSPMS* sequences from *A. angustifolia* are indicated with red points. Boxes highlighted in blue, green and red indicate the products of each enzyme: spermidine, spermidine/spermine and spermine/thermospermine, respectively. Genetic distances were inferred using the neighbor-joining method and 1500 replicates bootstrap calculations. Database and accession numbers are listed in Table S2, available as Supplementary Data at *Tree Physiology* Online.

the 'biological process' (419), 'molecular function' (62) and 'cellular component' (37) categories (see Tables S3 and S4 available as Supplementary Data at *Tree Physiology* Online).

Transcriptome and metabolic profiles

A hierarchical cluster analysis (HCA) was performed to identify associations between transcripts and metabolite profiles related to the Orn/Arg and PA biosynthetic pathways during seed development. For this purpose, the GZE stage was considered to be the starting point of development and the rest of the data were normalized to this stage.

Twelve genes (*AaADC*, *AaAIH*, *AaARG*, *AaASL*, *AaASS*, *AaCPA*, *AaODC*, *AaOTC*, *AaSAMDC*, *AaSPDS*, *AaSPDS3* and *AaSPMS*) related to Orn/Arg and PA biosynthesis were identified and characterized using RT-qPCR. We noted that even though the *AaODC* gene was identified in the *A. angustifolia* transcriptome database (Elbl et al. 2015*a*), its mRNA levels were very low and the associated fluorescence signal was only detected after 40 cycles, which we considered to be below the cutoff threshold of detection. Other than *AaODC*, all tested genes, amino acids and PAs were detected in all the seed stages analyzed (see Figure S2, Tables S5 and S6 available as Supplementary Data at *Tree Physiology* Online). HCA of gene expression and metabolite profiles was performed comparing GZE with isolated embryos (CZE and MZE) or megagametophytes (CZE MG and MZE MG) (Figure 2), and the clusters (C) shown represent different groups of genes or metabolites with similar profiles over the course of seed development. When comparing the different embryo stages (Figure 2a), cluster CI had two genes (*AaADC* and *AaSAMDC*) with increasing expression during the GZE–CZE transition, while *AaCPA* showed a constant increase during seed development. The remaining genes were placed in cluster CII and were characterized by similar or lower expression values compared with GZE. In the megagametophytes (Figure 2b), the expression of *AaARG*, *AaSPDS* and *AaSPMS* decreased during seed development (CI), while the genes in CII, with the exception of *AaASL*, displayed similar or lower expression values compared with GZE. *AaCPA* was most abundantly expressed at the mature stage, and was placed alone outside clusters CI and CII.

During the zygotic embryo development, among the metabolites tested, citrulline, Spd and Spm showed the highest contents of accumulation (Figure 2c, CI). In cluster CII, Orn and agmatine contents were slightly higher in CZE and MZE than in GZE, while

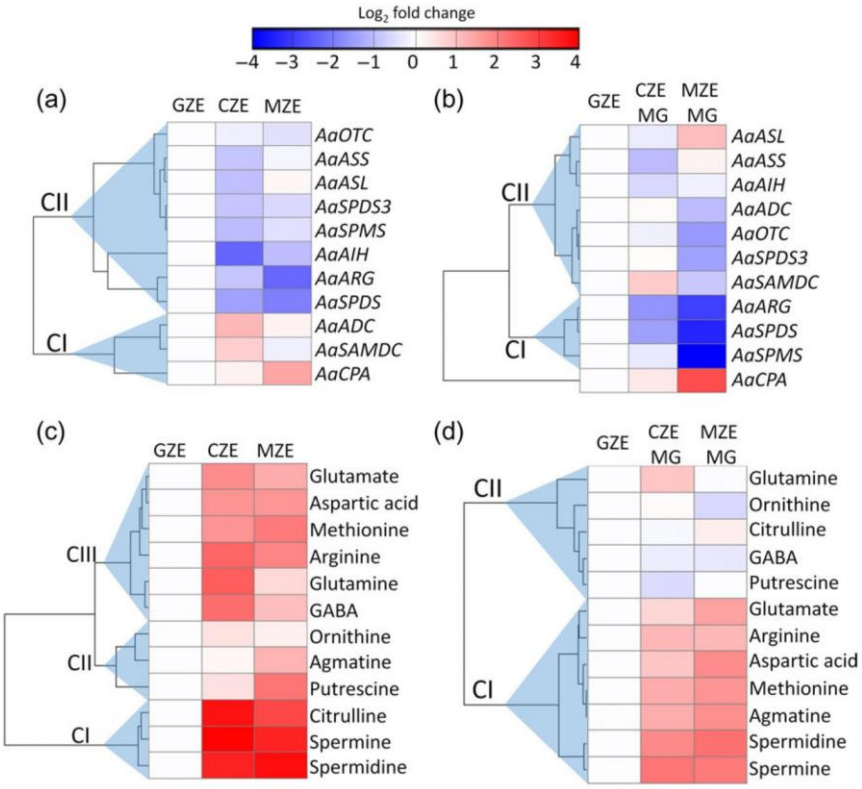

Figure 2. Hierarchical cluster analysis of transcript and metabolic profiles related to the Orn/Arg and PA biosynthetic pathway in three different seed developmental stages of *A. angustifolia* using Euclidean distance. Metabolites and gene expression are indicated using a color scale with high (red) or low (blue) values ($\log_2$ fold change). Data from isolated embryos at cotyledonary (CZE) and mature (MZE) stages (a, b), or megagametophytes at the cotyledonary (CZE MG) and mature (MZE MG) (c, d) stages were compared with the megagametophytes containing globular embryos (GZE) sample. All data (metabolites and gene expression values) are listed in Tables S5 and S6, available as Supplementary Data at *Tree Physiology* Online.

Put showed the highest value in MZE. For CIII, six amino acids [glutamate, aspartic acid, methionine, Arg, glutamine and γ-aminobutyric acid (GABA)] showed greater values in CZE and MZE than in GZE.

In the megagametophytes, the metabolites were grouped in two clusters (Figure 2d). In cluster CI, four amino acids (glutamate, Arg, aspartic acid and methionine), as well as agmatine and the two PAs, Spd and Spm, showed increasing accumulation during seed development. In contrast, in cluster CII, Put and four amino acids (glutamine, Orn, citrulline and GABA) showed little such variation. The expression of most of the genes related to the amino acid and PA biosynthetic pathways showed few changes between embryos and megagametophytes. However, at the end of seed development, some metabolite profiles (GABA, citrulline and Put) showed a different pattern in the mature zygotic embryo and its corresponding megagametophyte. During seed development, zygotic embryos accumulated up to eight times greater amounts of citrulline, twice the amount of GABA and five times the amount of Put. Few changes were observed for these metabolites during seed development in the respective megagametophytes (see Table S5 available as Supplementary Data at *Tree Physiology* Online).

ADC and ODC enzymatic activities

In both embryos and megagametophytes, during all stages of seed development, ADC activity was higher than ODC activity, indicating that ADC likely acts in the predominant Put synthesis pathway (Figure 3). Both activities increased sharply from GZE to CZE, and declined at the MZE stage (Figure 3a). In the megagametophytes, a constant increase in ADC specific activity during the seed development was observed, whereas ODC activity remained constant (Figure 3b). The total protein content increased from the GZE to the CZE stage and remained constant until the MZE stage in isolated embryos, while the content declined in mature megagametophytes (Figure 3).

PCA, LDA and pairwise comparison analysis

Amino acid and PA changes in abundance were analyzed by PCA and considered together with LDA of 28 identified metabolites, including free and conjugated PAs, agmatine and free amino acids (see Table S5 available as Supplementary Data at *Tree Physiology* Online). Metabolites that showed the same vector were not considered.

Principal components (PCs) 1 and 2 together explained 88.7% of the total variance among the samples (Figure 4a); linear discriminants (LDs) 1 and 2 explained 98.2% (Figure 4b). It was possible to distinguish the GZE, CZE MG and MZE MG tissues from the CZE and MZE stages based on PC1 (explaining 67.1%) and PC2 (explaining 21.6%) (Figure 4a). Free Spd, free Spm and serine were major compounds contributing to the separation of the MZE stage from the other tissues, while free Put, citrulline, Orn and GABA contributed to the separation of CZE stage. Agmatine was the main compound that separated the MZE MG tissue. GZE was isolated of all other tissues by PC1. Linear discriminant 1 (84.8%) indicated the existence of isolated embryos (CZE and MZE) group that differed from the megagametophytes (CZE MG and MZE MG) group, while GZE was a distinct group, based on LD2 (13.4%) (Figure 4b). Thus, distinct metabolites with the highest impact on the separation of each seed stage development were identified.

The more abundant (≥3-fold difference with a $P < 0.01$) metabolites between GZE and the others developmental stages, were identified through pairwise comparisons in a volcano plot analysis. This revealed that the greatest differences in abundance occurred

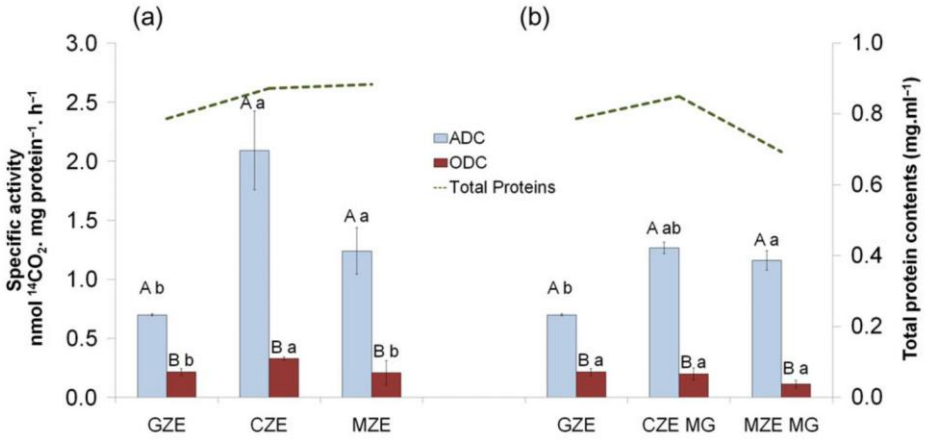

Figure 3. Specific enzymatic activity of ADC and ODC in *A. angustifolia* seeds tissues. Data from megagametophytes containing globular embryos (GZE) and isolated embryos at cotyledonary (CZE) and mature (MZE) stages (a), or GZE and isolated megagametophytes at the cotyledonary (CZE MG) and mature (MZE MG) (b) stages. Enzymatic activities were analyzed by measuring $[^{14}]CO_2$ released by decarboxylated radiolabeled L-arginine and L-ornithine. Vertical bars indicate standard error of the mean values derived from three biological replicates. Means followed by uppercase letters are significantly different between ADC and ODC, according to the Tukey's test ($P < 0.05$). Means followed by lowercase letters are significantly different for developmental stages according to the Tukey's test ($P < 0.05$).

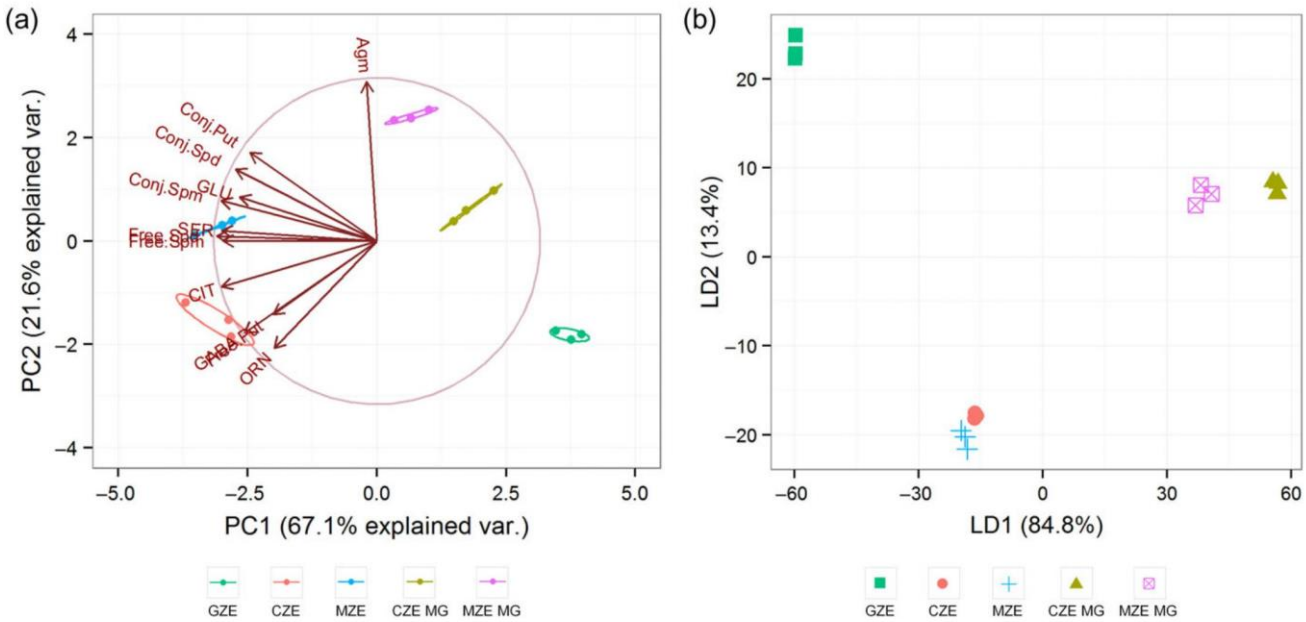

Figure 4. (a) PCA and (b) LDA on metabolite correlations of different *A. angustifolia* seed tissues. Principal component analysis and LDA were performed with the 28 metabolites identified, including PAs (free and conjugated forms), agmatine and amino acids. Red arrows indicate the loading plots showing the contribution of the compounds to the PC1 or -2. Agm, agmatine; CIT, citrulline; GLU, glutamic acid; ORN, ornithine; SER, serine; GZE, megagametophytes containing globular embryos; CZE, isolated cotyledonary embryos; MZE, isolated mature embryos; CZE MG, megagametophytes at the cotyledonary stage; MZE MG, megagametophytes at the mature stage.

in the CZE stage (free and conjugated Spm), CZE MG (conjugated Spd) and in MZE (conjugated Put and Spm) (Figure 5a–c, respectively). A comparison (Figure 5d) between GZE and MZE MG did not reveal metabolites with fold change (≥3-fold).

Correlations among metabolites, gene expression and seed development stage

Given the role of Arg, Orn and methionine in PA biosynthesis, we performed a linear correlation analysis of these amino acids, PAs and Put/Spd/Spm biosynthesis-related genes (see Table S8 available as Supplementary Data at *Tree Physiology* Online). Arginine and PA were correlated in all samples analyzed, with positive correlations in the globular and cotyledonary stages (Figure 6a–c), while a negative correlation was observed at the mature stage (Figure 6d and e). A correlation involving Orn was not detected in the first seed developmental stage, although a positive correlation was detected in the zygotic embryo with Put and Spd in the cotyledonary stage (CZE). At the mature stage, a negative correlation between Orn and Put for both embryo (MZE) and megagametophyte (MZE MG) was observed. No correlations were observed for methionine at the GZE and CZE MG stages, while positive correlations were found with Spm in the cotyledonary stage (CZE) and mature embryos (MZE). In the mature stage, methionine correlated positively with Put in the embryo and negatively in the megagametophyte.

The correlation between gene expression and PA content highlighted *AaSAMDC* with PAs when methionine was present. Positive correlations were found between *AaSAMDC* and Spd and Put, at the CZE and MZE stages, respectively. In contrast, negative correlations were observed between *AaSAMDC* and Spm in the mature embryo, and between *AaSAMDC* and both Spd and Spm in the corresponding megagametophyte. Positive correlations between *AaSPDS* and Spm were also observed until the cotyledonary stages, but a negative correlation at the mature stage, while we noted a negative correlation between *AaSPDS3* and Spd in the GZE and CZE stages.

Discussion

Previous studies of *A. angustifolia* seed development studies have aimed to develop a better understanding of the molecular and physiological basis of embryogenesis, in order to enhance in vitro multiplication via somatic embryogenesis (Steiner et al. 2008). The globular stage of *A. angustifolia* seed development is marked by intensive cell division and differentiation, expression of genes involved in carbohydrate biosynthesis and oxidative stress metabolism (dos Santos et al. 2006, Balbuena et al. 2009, Elbl et al. 2015*a*). Subsequently, the transition from the globular to the cotyledonary stage has been associated with a range of physiological, biochemical and molecular changes (Astarita et al. 2003*b*, Elbl et al. 2015*a*), involving the switch from embryogenesis (cell division) to seed filling (reserve deposition) (dos Santos et al. 2006, Balbuena et al. 2009). There is an absence of morphological transitions in zygotic embryos from the cotyledonary to the mature stage (Astarita et al. 2003*b*, dos Santos et al. 2006, Balbuena et al. 2009). Compared with most Pinaceae family species, the seeds of

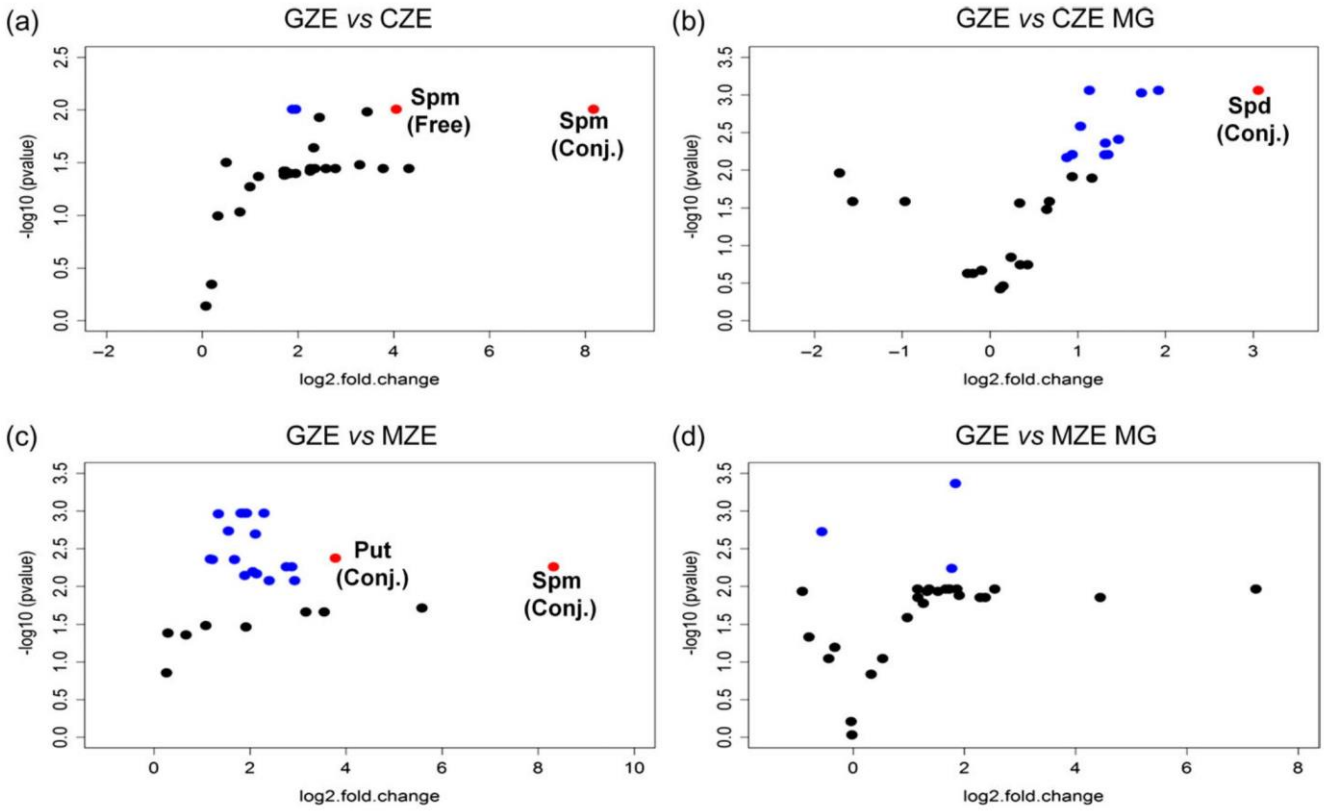

Figure 5. Volcano plot of pairwise comparisons between the globular and other stages. Comparisons of 28 metabolites including PAs (free and conjugated forms), agmatine and free amino acids. The plot displays the metabolites that are differentially abundant in pairwise comparisons of *A. angustifolia* seed tissues. Metabolites that showed a significant difference in abundance ($P < 0.01$) are highlighted by blue dots. Metabolites identified and represented by red dots have the most significantly altered abundance, using 3-fold change and $P < 0.01$ as the threshold cutoff. Put, putrescine; Spd, spermidine; Spm, spermine; GZE, megagametophytes containing globular embryos; CZE, isolated cotyledonary embryos; MZE, isolated mature embryos; CZE MG, megagametophytes at the cotyledonary stage; MZE MG, megagametophytes at the mature stage.

A. angustifolia are recalcitrant (Steiner et al. 2008), meaning they have a higher water content, are sensitive to desiccation and are metabolically active until germination (dos Santos et al. 2006).

Notwithstanding previous studies with respect to PA profiles in *A. angustifolia* (Astarita et al. 2003c, Silveira et al. 2006, Jo et al. 2014), there are no studies about the relationship between gene expression and metabolic switches associated with Orn/Arg and PA biosynthesis pathway. In this study, we identified genes and metabolites that participate on the Orn/Arg and PAs biosynthetic pathway, providing an opportunity to unravel the complexity of coordinated switches during embryo development.

In silico analyses of genes related to the Orn/Arg and PA biosynthetic pathway

A search for genes related to the Orn/Arg and PA biosynthetic pathways revealed 12 genes with ≥57% amino acid sequence identity to their homologs in *A. thaliana*, or *P. persica* in the case of the *ODC* gene (Liu et al. 2006) (Table 1). Of all genes identified in the present study, only those encoding aminopropyltransferases were found to have more than one copy in *A. angustifolia* (*AaSPDS*, *AaSPDS3* and *AaSPMS*). This appears to differ from the situation in *P. sylvestris* (Vuosku et al. 2012) and *Pinus pinaster* (http://www.scbi.uma.es/sustainpinedb/home_page), since a search of their respective transcriptome databases revealed only one copy of *SPDS* and *SPMS*. It is likely that *AaSPDS* is responsible for the biosynthesis of Spd, and *AaSPMS* catalyzes the conversion of Spd into Spm. We further propose that *AaSPDS3* is involved in the biosynthesis of both Spd and Spm, as has been reported for its *A. thaliana* homolog (Hanzawa et al. 2002). Although *AaSPDS3* homologs were found in other species (Figure 1), as far as we are aware, only one study has mentioned this gene, and its enzymatic activity remains to be determined (Hanzawa et al. 2002).

Only a partial sequence of *AaODC* (corresponding to a polypeptide of 161 amino acids) was retrieved and to date no complete *ODC* sequence has been reported in any conifer. While the size of ODC in some angiosperms is approximately 433 amino acids (Alabadí and Carbonell 1998, Delis et al. 2005, Yoda et al. 2009), only a 391 amino acids fragment has been

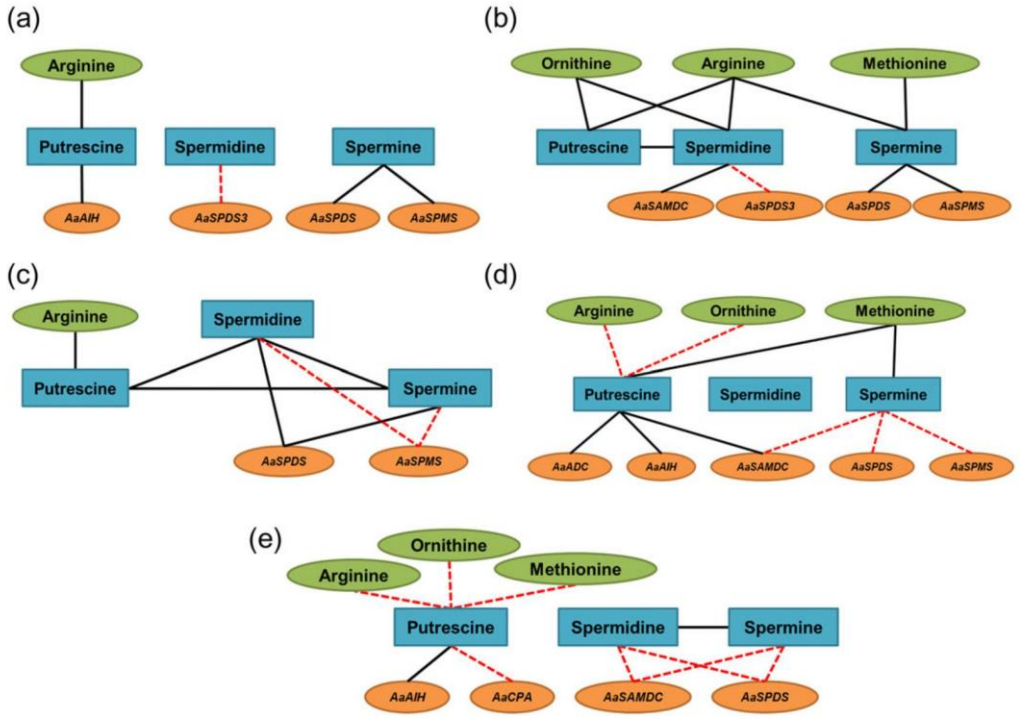

Figure 6. Correlations between PAs and arginine, ornithine, methionine and biosynthetic-related genes in three different seed developmental stages of *A. angustifolia*. Metabolites are represented by green circles and genes are represented by orange circles. Lines connecting two nodes represent significant Pearson's correlations ($P < 0.01$; $r > 0.90$): black lines represent a positive correlation and dashed red lines represent a negative correlation. (a) GZE, megagametophytes containing globular embryos; (b) CZE, isolated cotyledonary embryos; (c) CZE MG, megagametophytes at the cotyledonary stage; (d) MZE, isolated mature embryos; (e) MZE MG, megagametophytes at the mature stage.

identified in *P. sylvestris* (Vuosku et al. 2012, accession number ADQ37307.1) and a 351 amino acids fragment in *P. pinaster* (http://www.scbi.uma.es/sustainpinedb/home_page).

Polyamine, amino acid and gene expression profiles during seed development

To identify switches associated with specific seed stages and transition stage, complementary statistical tools for unsupervised (PCA) and supervised (LDA) data analysis were implemented to look at the global metabolic changes. Based on PAs and amino acid profiles, PCA and LDA separated the globular stage from the cotyledonary and mature stages. This led to the identification of two groups of tissues with distinct metabolic profiles: one composed of the isolated embryos and another of the megagametophytes. These two distinct profiles were characterized by higher contents of amino acids and PAs in the zygotic embryo than in the megagametophyte. For the second approach, Euclidean distance and pairwise comparison analyses between the globular and the other developmental stages revealed that most changes occurred at the transitions, as described below.

The globular stage

In this study, the globular stage was considered the developmental starting point, to which all other stages were compared. Early embryogenesis is marked by the lowest PA content and a high Put/(Spd + Spm) ratio, due to the high content of Put compared with Spd and Spm. This result is in accordance with reports of early embryogenesis in *Pinus radiata* (Minocha et al. 1999), *Pinus taeda* (Silveira et al. 2004) and *P. sylvestris* (Vuosku et al. 2006). This ratio is considered a biochemical marker of developmental stage and it also corresponds to cell division and elongation (Astarita et al. 2003c, Silveira et al. 2004). In plants, Put promotes the cell cycle and mitotic division (Astarita et al. 2003c), and has been reported to be the most abundant PA at the globular seed stage of *P. taeda* (Silveira et al. 2004) and *P. sylvestris* (Vuosku et al. 2006). Furthermore, Put can modulate the expression of peroxidases and other related proteins, in addition to promoting Spd synthesis and reducing oxidative stress induced by an excessive production of reactive oxygen species (ROS) during early *A. angustifolia* embryogenesis (Balbuena et al. 2009, Reis et al. 2016).

Aspartic acid, glutamic acid and glutamine were the main amino acids presents at the *A. angustifolia* globular stage. Although these amino acids are part of the Orn/Arg pathway, Arg content was lower than that of Orn, which differs from the profile observed in the seeds of *P. taeda* (Silveira et al. 2004). Arginine plays an important role as a substrate for Put biosynthesis and as an intermediate in nitrogen metabolism (Page et al. 2012,

Majumdar et al. 2013). In plants, the biosynthesis of PAs is initiated with the formation of the Put by two alternative enzymes (ADC and/or ODC) (Alcázar et al. 2010). In the present study, the positive correlations observed at the globular stage between Arg, Put and *AaAlH*, as well as the higher activity of ADC than ODC, suggest that the Arg pathway is more important for Put biosynthesis during early embryogenesis. The use of Arg for Put biosynthesis via the ADC pathway has also been reported to occur in the zygotic embryogenesis of *P. sylvestris* (Vuosku et al. 2006) and *P. abies* (Santanen and Simola 1999, Gemperlová et al. 2009), suggesting that this pathway is common to early conifer embryo development.

Ornithine decarboxylase activity was barely detected, and was approximately four times lower than that of ADC. The co-existence of the ADC and ODC pathways in some species may relate to their differential contribution to stress responses, development processes and tissue specificity. In support of this idea, expression of the *ADC* gene and protein have been detected in the mitotic cells of developing zygotic embryos and shown to be activated in response to stress (Tiburcio et al. 1997, Vuosku et al. 2006).

The cotyledonary stage

The transition from a globular to a cotyledonary stage in *A. angustifolia* seeds is marked by biochemical, transcriptional and morphological processes that support the differentiation of meristems, the formation of the embryo body and the early development of cotyledons in zygotic embryos, while the accumulation of reserves occurs in the megagametophytes (dos Santos et al. 2006, Balbuena et al. 2009, Elbl et al. 2015*a*). During the cotyledonary stage, the pool of free amino acids and PAs, the expression profiles of genes related to the Orn/Arg and PA biosynthetic pathways, and both ADC and ODC enzymatic activities all reached the maximal levels. In the zygotic embryo, Put, GABA, citrulline and Orn contents increased during development, while in the megagametophyte contents tended to show only a minor change (Figure 2). This suggests an important role of these compounds in embryo development, consistent with the idea of a possible flux from the megagametophyte and embryonic axis to the cotyledons (Astarita et al. 2003*b*).

The highest ADC activity and gene expression of *AaADC* were measured at the cotyledonary stage. A predominance of PA pathway has also been observed during seed development in *P. abies* (Gemperlová et al. 2009) and *P. sylvestris* (Vuosku et al. 2006, 2012). In contrast, the ODC activity was lower and *AaODC* transcripts were below the cutoff threshold of detection.

In the megagametophyte, the constant *AaADC* expression was accompanied by minimal changes in Put content. In contrast, an increase in ADC activity was observed between the megagametophyte containing early zygotic embryos (GZE MG) and the mature stage megagametophyte (MZE MG). This suggests a different regulation of the PAs biosynthesis in megagametophyte than observed into embryos; however, the exact molecular mechanism that regulates PAs genes translation has not been determined.

Through a pairwise comparison between globular and zygotic embryos or the megagametophytes at the cotyledonary stage we observed that Spd and Spm, in both the free and conjugated forms, were more abundant at the cotyledonary stage. In plants, PAs can conjugate with hydroxycinnamic acid to produce acylated PAs (Luo et al. 2009), or conjugate with macromolecules (insoluble conjugated PAs) (Shevyakova et al. 2006). These conjugated PAs serve as nitrogen reserves to support germination (Luo et al. 2009) and may participate simultaneously in the scavenging of oxygen radicals (O_2^- and OH.), thereby providing protection against oxidative stress and in H_2O_2 generation via PA oxidation (Shevyakova et al. 2006). These conjugated PAs may represent one of the ROS scavenger mechanisms necessary to control and maintain the oxidative stress metabolism in *A. angustifolia*, where high respiratory levels are present in the recalcitrant seeds (Leprince et al. 1999, Balbuena et al. 2009).

Citrulline has the highest content among amino acids detected, increasing approximately 14-fold during the transition from the globular to the cotyledonary stage. In plants, citrulline is synthesized from arginine and it has been reported to be an efficient hydroxyl radical scavenger and a strong antioxidant (Kusvuran et al. 2013). Furthermore, citrulline has a high nitrogen content (three atoms per molecule) and has been associated with drought tolerance in *Cucumis melo* (Akashi et al. 2001, Slocum 2005, Kusvuran et al. 2013). The role of citrulline in embryogenesis is not well understood; however, a possible role in *A. angustifolia* seeds may be as an antioxidant, again due the high respiratory levels at the late stages (Balbuena et al. 2009), associated with PAs as demonstrated in the Euclidean distance analysis (Figure 2c).

Different GABA patterns were observed, being more abundant in the embryo than megagametophyte. GABA is an amino acid that is not used for protein synthesis, and it is synthesized through glutamic acid decarboxylation, or as a product of PA catabolism (Satya-Naraian and Nair 1990, Bouchereau et al. 1999, Santa-Catarina et al. 2006). It is associated with several processes and in plants has some similar functions to those of proline, such as its contribution to the C:N balance, regulation of pH, protection against oxidative stress, osmoregulation, response to biotic and abiotic stress, protection against heat shock and as a signaling molecule (Bouché and Fromm 2004, Dowlatabadi et al. 2009, Winkelmann et al. 2015). During seed development GABA is present in *P. taeda* (Silveira et al. 2004), *Cedrela fissilis* (Aragão et al. 2015) as well as in recalcitrant seeds of *Ocotea catharinensis* (Santa-Catarina et al. 2006). The biphasic nature of GABA concentration may in part be interpreted in the context of a requirement for PA biosynthesis during embryo development (Minocha and Minocha 1995); however, while GABA is thought to play an important role during embryo

development (Aragão et al. 2015), its detailed modes of action in conifer embryogenesis remain poorly understood.

The mature stage

The transition from the cotyledonary to the mature stage in particular is known to be marked by intensive reserve (proteins, starch and lipids) and water accumulation (Balbuena et al. 2009). Spermidine and Spm contents were higher than that of Put in mature seeds, and were more similar to contents reported in the conifers, *P. radiata* and *P. sylvestris* (Minocha et al. 1999, Vuosku et al. 2006). The high Spd and Spm contents were also reflected in a reduction in the Put/(Spd + Spm) ratio (see Table S5 available as Supplementary Data at *Tree Physiology* Online), which has been demonstrated to be important for seed filling (Astarita et al. 2003*c*, Santa-Catarina et al. 2006). Through studies of gene knockout mutants of SPDS and SAMDC in *A. thaliana*, Spd is known to be essential for embryo development (Imai et al. 2004, Ge et al. 2006). In contrast, Spm seems not to be required for embryogenesis because loss-of-function mutants of the *A. thaliana* SPMS genes show normal embryo development (Imai et al. 2004).

The highest Spd and Spm contents in the cotyledonary and mature stages were accompanied by an increase in *AaSAMDC* expression and a decrease in the expression of *AaSPDS*, *AaSPDS3* and *AaSPMS*. The positive correlation of *AaSAMDC* with Put, and negative correlation with Spd and Spm, at the mature zygotic embryo stage suggest a regulatory mechanism involving *AaSAMDC* to control PA content. However, such a putative regulatory mechanism seems to be species- and tissue-specific, since leaves of *Brassica juncea* show up-regulation of *SAMDC* expression following exogenous Put treatments (Hu et al. 2005). Additionally, accumulation of Put in hybrid poplar (*Populus nigra* × *maximowiczii*) cells may inhibit the expression of some members of the *SAMDC* family, leading to decreased SAMDC activity (Hu et al. 2005, Page et al. 2007). It should be noted that the SPDS enzyme is very stable and that its activity does not correlate with mRNA levels (Page et al. 2007, Salo et al. 2016). Putrescine, which can be used to provide more substrate for Spd/Spm biosynthesis, was more abundant at the mature stage than in the globular stage (Figure 2). Putrescine has also been reported to enhance drought resistance and freezing stress in *A. thaliana* mutants defective in Put biosynthesis (Cuevas et al. 2008, Alcázar et al. 2010, Bitrián et al. 2012) and it may have a similar protective role mechanism in *A. angustifolia* seeds during late embryogenesis.

Seven amino acids had higher contents in zygotic embryos at the mature stage than in the globular stage. During zygotic embryo development, high contents of glutamic acid, aspartic acid and glutamine coincided with an increase in the abundance of Arg and PAs, which are involved in the Orn/Arg pathway, providing substrates for the biosynthesis of Put (Page et al. 2012, Majumdar et al. 2013). Ornithine content only changed slightly and the fact that ODC activity was lower than that of ADC activity suggests Orn can be used for Arg biosynthesis. Methionine content increased proportionately to PA profiles, indicating a direct relationship between high content of PA precursors and high PA accumulation (Silveira et al. 2004). Similarly, previous studies have reported that the megagametophyte has less PAs and amino acids than the zygotic embryos (Astarita et al. 2003*b*, Balbuena et al. 2009).

Interestingly, Arg was not the main amino acid observed in late embryogenesis. Arg is the amino acid with the highest nitrogen content (four atoms per molecule) and constitutes a large proportion of the amino acid pool of seed reserve proteins, particularly in conifers (King and Gifford 1997, Cantón et al. 2005). However, we found that Arg represented only 1–2% of the total amino acids present during the late embryogenesis in *A. angustifolia* seeds (see Table S5 available as Supplementary Data at *Tree Physiology* Online). This contrasts with profiles from other conifers, where it can represent more than 23% of seed reserves (King and Gifford 1997, Cantón et al. 2005). The amino acid composition of vicilin-like proteins in *A. angustifolia* seeds indicates that more than 10% of its sequence is composed of glutamic acid, while among the major amino acid residues of seed storage proteins in *P. taeda* and *P. pinaster* is Arg (Allona et al. 1992, Rodríguez et al. 2006, Balbuena et al. 2009). The low expression of *AaARG* and small changes in Orn content in mature seeds suggest that the low Arg content may be due to its use in Put biosynthesis being in accordance with the increase in PA content. Further support for this hypothesis was the negative correlation between Arg and PAs observed here. Another piece of evidence was the fact that Arg can be used in nitric oxide (NO) biosynthesis, thereby releasing citrulline, which was present at high content in the mature stage (Figure 2, see Table S5 available as Supplementary Data at *Tree Physiology* Online).

Conclusions

In summary, this study describes the changes in the Orn/Arg and PA biosynthetic pathways associated with *A. angustifolia* seed development. Out data suggest two genes that may encode key enzymes in the PA biosynthetic pathway: *AaADC* for the regulation of the ratio of Put biosynthesis, and *AaSAMDC* for Spd/Spm synthesis. Based on PA and amino acid profiles, it was possible to distinguish the different tissues and development stages of the seeds. Specific sets of metabolites accumulate differentially in the embryo and megagametophyte during late embryogenesis. Arginine, in contrast to in other conifers, is not the main amino acid during *A. angustifolia* embryogenesis and its function may be in NO or PA biosynthesis, rather than incorporation into seed reserve proteins. In addition, citrulline was the main amino acid recorded during seed development. The results described here will help to guide future testing of in vitro embryogenic cell

lines, to identify the optimal in vitro conditions for development of somatic embryos.

Supplementary Data

Supplementary data for this article are available at *Tree Physiology* Online.

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Conflict of interest

None declared.

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References

Akashi K, Miyake C, Yokota A (2001) Citrulline, a novel compatible solute in drought-tolerant wild watermelon leaves, is an efficient hydroxyl radical scavenger. FEBS Lett 508:438–442.

Akhtar N (2013) Endogenous polyamines: a temporal cellular modulator of somatic embryogenesis in guava (*Psidium guajava* L.) cv. Allahabad Safeda. Res Plant Sci 1:4–14.

Alabadí D, Carbonell J (1998) Expression of ornithine decarboxylase is transiently increased by pollination, 2,4-dichlorophenoxyacetic acid, and gibberellic acid in tomato ovaries. Plant Physiol 118:323–328.

Alcázar R, Altabella T, Marco F, Bortolotti C, Reymond M, Koncz C, Carrasco P, Tiburcio AF (2010) Polyamines: molecules with regulatory functions in plant abiotic stress tolerance. Planta 231:1237–1249.

Allona I, Casado R, Aragoncillo C (1992) Seed storage proteins from *Pinus pinaster* Ait.: homology of major components with 11S proteins from angiosperms. Plant Sci 87:9–18.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410.

Aragão VPM, Navarro BV, Passamani LZ, Macedo AF, Floh EIS, Silveira V, Santa-Catarina C (2015) Free amino acids, polyamines, soluble sugars and proteins during seed germination and early seedling growth of *Cedrela fissilis* Vellozo (Meliaceae), an endangered hardwood species from the Atlantic Forest in Brazil. Theor Exp Plant Physiol 27:157–169.

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

Astarita LV, Floh EIS, Handro W (2003*b*) Free amino acid, protein and water content changes associated with seed development in *Araucaria angustifolia*. Biol Plant 47:53–59.

Astarita LV, Handro W, Floh EIS (2003*c*) Changes in polyamines content associated with zygotic embryogenesis in the Brazilian pine, *Araucaria angustifolia* (Bert.) O Ktze. Rev Bras Bot 26:163–168.

Bais HP, Ravishankar GA (2002) Role of polyamines in the ontogeny of plants and their biotechnological applications. Plant Cell Tiss Organ Cult 29:1–34.

Balbuena TS, Silveira V, Junqueira M, Dias LLC, Santa-Catarina C, Shevchenko A, Floh EIS (2009) Changes in the 2-DE protein profile during zygotic embryogenesis in the Brazilian pine (*Araucaria angustifolia*). J Proteomics 72:337–352.

Bitrián M, Zarza X, Altabella T, Tibrucio AF, Alcázar R (2012) Polyamines under abiotic stress: metabolic crossroads and hormonal crosstalks in plants. Metabolites 2:516–528.

Bouché N, Fromm H (2004) GABA in plants: just a metabolite? Trends Plant Sci 9:110–115.

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

Bradford M (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254.

Bustin SA, Benes V, Garson JA et al. (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55:611–622.

Cantón FR, Suárez MF, Cánovas FM (2005) Molecular aspects of nitrogen mobilization and recycling in trees. Photosynth Res 83: 265–278.

Conesa A, Götz S, Garcia-Gomez JM, Terol J, Talon M, Robles M (2005) Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics 21:3674–3676.

Cuevas JC, Lopez-Cobollo R, Alcázar R, Zarza X, Koncz C, Altabella T, Salinas J, Tiburcio AF, Ferrando A (2008) Putrescine is involved in Arabidopsis freezing tolerance and cold acclimation by regulating abscisic acid levels in response to low temperature. Plant Physiol 148: 1094–1105.

Delis C, Dimou M, Efrose RC, Flemetakis E, Aivalakis G, Katinakis P (2005) Ornithine decarboxylase and arginine decarboxylase gene transcripts are co-localized in developing tissues of *Glycine max* etiolated seedlings. Plant Physiol Bioch 43:19–25.

dos Santos ALW, Wiethölter N, Gueddari NE, Moerschbacher BM (2006) Protein expression during seed development in *Araucaria angustifolia*: transient accumulation of class IV chitinases and arabinogalactan proteins. Physiol Plant 127:138–148.

dos Santos ALW, Elbl P, Navarro BV, de Oliveira LF, Salvato F, Balbuena TS, Floh EIS (2016) Quantitative proteomic analysis of *Araucaria angustifolia* (Bertol.) Kuntze cell lines with contrasting embryogenic potential. J Proteomics 130:180–189.

Dowlatabadi R, Weljie AM, Thorpe TA, Yeung EC, Vogel HJ (2009) Metabolic foot printing study of white spruce somatic embryogenesis using NMR spectroscopy. Plant Physiol Bioch 47:343–350.

Elbl P, Campos RA, Lira BS, Andrade SCS, Jo L, dos Santos ALW, Coutinho LL, Floh EIS, Rossi M (2015*a*) Comparative transcriptome analysis of early somatic embryo formation and seed development in Brazilian pine, *Araucaria angustifolia* (Bertol.) Kuntze. Plant Cell Tiss Organ Cult 120:903–915.

Elbl P, Navarro BV, de Oliveira LF, Almeida J, Mosini AC, dos Santos ALW, Rossi M, Floh EIS (2015*b*) Identification and evaluation of reference genes for quantitative analysis of Brazilian pine (*Araucaria angustifolia* Bertol. Kuntze) gene expression. PLoS One 10:1–15.

Fait A, Angelovici R, Less H, Ohad I, Urbanczyk-Wochniak E, Fernie AR, Galili G (2006) Arabidopsis seed development and germination is associated with temporally distinct metabolic switches. Plant Physiol 142:839–854.

Ge C, Cui X, Wang Y, Hu Y, Fu Z, Zhang D, Cheng Z, Li J (2006) *BUD2*, encoding an S-adenosylmethionine decarboxylase, is required for *Arabidopsis* growth and development. Cell Res 16:446–456.

Gemperlová L, Fischerová L, Cviková M, Malá J, Vondráková Z, Martincová O, Vágner M (2009) Polyamine profiles and biosynthesis in somatic

embryo development and comparison of germinating somatic and zygotic embryos of Norway spruce. Tree Physiol 29:1287–1298.

Grimes HD, Slocum RD, Boss WF (1986) α-Difluoromethylarginine treatment inhibits protoplast fusion in fusogenic wild-carrot protoplasts. Biochim Biophys Acta 886:130–134.

Hanzawa Y, Akihiro I, Michael AJ, Yoshibumi K, Takahashi T (2002) Characterization of the spermidine synthase-related gene family in *Arabidopsis thaliana*. FEBS Lett 527:176–180.

Hu W, Gong H, Pua E (2005) Molecular cloning and characterization of S-adenosylmethionine decarboxylase genes from mustard (*Brassica juncea*). Physiol Plant 124:25–40.

Imai A, Matsuyama T, Hanzawa Y et al. (2004) Spermidine synthase genes are essential for survival of Arabidopsis. Plant Physiol 135: 1565–1573.

Jo L, dos Santos ALW, Bueno CA, Barbosa HR, Floh EIS (2014) Proteomic analysis and polyamines, ethylene and reactive oxygen species levels of *Araucaria angustifolia* (Brazilian pine) embryogenic cultures with different embryogenic potential. Tree Physiol 34: 94–104.

King JE, Gifford DJ (1997) Amino acid utilization in seeds of Loblolly pine during germination and early seedling growth. Plant Physiol 113: 1125–1135.

Klimaszewska K, Overton C, Stewart D, Rutledge RG (2011) Initiation of somatic embryos and regeneration of plants from primordial shoots of 10-year-old somatic white spruce and expression profiles of 11 genes followed during the tissue culture process. Planta 233: 635–647.

Koch Z, Corrêa MC (eds) (2002) Araucária: a Floresta do Brasil Meridional. Olhar Brasileiro, Curitiba, Brasil.

Kong L, Attree SM, Fowke LC (1998) Effects of polyethylene glycol and methylglyoxal bis(guanylhydrazone) on endogenous polyamine levels and somatic embryo maturation in white spruce (*Picea glauca*). Plant Sci 133:211–220.

Kusano T, Berberich T, Tateda C, Takahashi Y (2008) Polyamines: essential factors for growth and survival. Planta 228:367–381.

Kusvuran S, Dasgan HY, Abak K (2013) Citrulline is an important biochemical indicator in tolerance to saline and drought stresses in melon. Scientific World J 2013:1–8.

Lara-Chavez A, Egertsdotter U, Flinn BS (2012) Comparison of gene expression markers during zygotic and somatic embryogenesis in pine. In Vitro Cell Dev Biol Plant 48:341–354.

Lasanajak Y, Minocha R, Minocha SC, Goyal R, Fatima T, Handa AK, Mattoo AK (2014) Enhanced flux of substrates into polyamine biosynthesis but not ethylene in tomato fruit engineered with yeast *S*-adenosylmethionine decarboxylase gene. Amino Acids 46:729–742.

Leprince O, Buitink J, Hoekstra FA (1999) Axes and cotyledons of recalcitrant seeds of *Castanea sativa* Mill. exhibit contrasting responses of respiration to drying in relation to desiccation sensitivity. J Exp Bot 50: 1515–1524.

Liu J, Nada K, Pang X, Honda C, Kitashia H, Moriguchi T (2006) Role of polyamines in peach fruit development and storage. Tree Physiol 26: 791–798.

Luo J, Fuell C, Parr A, Hill L, Bailey P, Elliot K, Fairhurst SA, Martin C, Michael AJ (2009) A novel polyamine acyltransferase responsible for the accumulation of spermidine conjugates in *Arabidopsis* seed. Plant Cell 21:318–333.

Majumdar R, Shao L, Minocha R, Long S, Minocha SC (2013) Ornithine: the overlooked molecule in the regulation of polyamine metabolism. Plant Cell Physiol 54:990–1004.

Majumdar R, Barchi B, Turlapati SA, Gagne M, Minocha R, Long S, Minocha SC (2016) Glutamate, ornithine, arginine, proline, and polyamine metabolic interactions: the pathway is regulated at the post-transcriptional level. Front Plant Sci 7:78.

Minguet EG, Vera-Sirera F, Marina A, Carbonell J, Blázquez MA (2008) Evolutionary diversification in polyamine biosynthesis. Mol Biol Evol 25:2119–2128.

Minocha R, Smith DR, Reeves C, Steele KD, Minocha SC (1999) Polyamine levels during the development of zygotic and somatic embryos of *Pinus radiata*. Physiol Plant 105:155–164.

Minocha SC, Minocha R (1995) Role of polyamines in somatic embryogenesis. In: Bajaj YPS (ed) Biotechnology in agriculture and forestry, somatic embryogenesis and synthetic seed I. Springer-Verlag, Heidelberg, pp 55–72.

Naka Y, Watanabe K, Sagor GHM, Niitsu M, Pillai MA, Kusano T, Takahashi Y (2010) Quantitative analysis of plant polyamines including thermospermine during growth and salinity stress. Plant Physiol Biochem 48:527–533.

Page AF, Mohapatra S, Minocha R, Minocha SC (2007) The effects of genetic manipulation of putrescine biosynthesis on transcription and activities of the other polyamine biosynthetic enzymes. Physiol Plant 129:707–724.

Page AF, Minocha R, Minocha SC (2012) Living with high putrescine: expression of ornithine and arginine biosynthetic pathway genes in high and low putrescine producing poplar cells. Amino Acids 42:295–308.

R Development Core Team (2015) R: A language and environment for statistical computing: reference index version 2.8.0. Vienna foundation for statistical computing. http://www.r-project.org (10 January 2016, date last accessed).

Reis RS, Vale EM, Heringer SA, Santa-Catarina C, Silveira V (2016) Putrescine induces somatic embryo development and proteomic changes in embryogenic callus of sugarcane. J Proteomics 130:170–179.

Rodríguez MJP, Suárez MF, Heredia R et al. (2006) Expression patterns of two glutamine synthetase genes in zygotic and somatic pine embryos support specific roles in nitrogen metabolism during embryogenesis. New Phytol 169:35–44.

Ruijter JM, Ramakers C, Hoogaars WMH, Karlen Y, Bakker O, Van den Hoff MJB, Moorman AFM (2009) Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. Nucleic Acids Res 37:e45. doi:10.1093/nar/gkp045

Rupps A, Raschke J, Rümmler M, Linke B, Zoglauer K (2016) Identification of putative homologs of *Larix decidua* to BABYBOOM (BBM), LEAFY COTYLEDON1 (LEC1), WUSCHEL-related HOMEOBOX2 (WOX2) and SOMATIC EMBRYOGENESIS RECEPTOR-like KINASE (SERK) during somatic embryogenesis. Planta 243:473–488.

Salo HM, Sarjala T, Jokela A, Häggman H, Vuosku J (2016) Moderate stress responses and specific changes in polyamine metabolism characterize Scots pine somatic embryogenesis. Tree Physiol 36:392–402.

Santa-Catarina C, Vanildo S, Balbuena TS, Viana AM, Estelita MEM, Handro W, Floh EIS (2006) IAA, ABA, polyamines and free amino acids associated with zygotic embryo development of *Ocotea catharinensis*. Plant Growth Regul 49:237–247.

Santanen A, Simola LK (1999) Metabolism of L[U-^{14}C]-arginine and L[U-^{14}C]-ornithine in maturing and vernalised embryos and megagametophytes of *Picea abies*. Physiol Plant 107:433–440.

Satya-Naraian V, Nair PM (1990) Metabolism, enzymology and possible roles of 4-aminobutyrate in higher plants. Phytochemistry 29:367–375.

Schlögl PS, dos Santos ALW, Vieira LN, Floh EIS, Guerra MP (2012*a*) Cloning and expression of embryogenesis-regulating genes in *Araucaria angustifolia* (Bert.) O. Kuntze (Brazilian pine). Genet Mol Biol 35:172–181.

Schlögl PS, dos Santos ALW, Vieira LN, Floh EIS, Guerra MP (2012*b*) Gene expression during early somatic embryogenesis in Brazilian pine (*Araucaria angustifolia* (Bert) O. Ktze). Plant Cell Tiss Organ Cult 108:173–180.

Shevyakova NI, Rakitin VY, Stetsenko LA, Aronova EE, Kuznetsov VV (2006) Oxidative stress and fluctuations of free and conjugated polyamines in the halophyte *Mesembryanthemum crystallinum* L. under NaCl salinity. Plant Growth Regul 50:69–78.

Silveira V, Balbuena TS, Santa-Catarina C, Floh EIS, Guerra MP, Handro W (2004) Biochemical changes during seed development in *Pinus taeda* L. Plant Growth Regul 44:147–156.

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

Silveira V, Santa-Catarina C, Balbuena TS, Moraes FMS, Ricart CAO, Souza MV, Guerra MP, Handro W, Floh EIS (2008) Endogenous abscisic acid levels and comparative proteome during seed development of *Araucaria angustifolia* (Bert.) O. Ktze. Biol Plant 52:101–104.

Slocum RD (2005) Genes, enzymes and regulation of arginine biosynthesis in plants. Plant Physiol Bioch 43:729–745.

Stasolla C, Yeung EC (2003) Recent advances in conifer somatic embryogenesis: improving somatic embryo quality. Plant Cell Tiss Organ Cult 74:15–35.

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

Steiner N, Santa-Catarina C, Andrade JBR, Balbuena TS, Guerra MP, Handro W, Floh EIS, Silveira V (2008) *Araucaria angustifolia* biotechnology. Funct Plant Sci Biot 2:20–28.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30: 2725–2729.

Tiburcio AF, Kaur-Sawhney R, Galston AW (1993) Spermidine biosynthesis as affected by osmotic stress in oat leaves. Plant Growth Regul 13:103–109.

Tiburcio AF, Altabella T, Borrell A, Masgrau C (1997) Polyamine metabolism and its regulation. Physiol Plant 100:664–674.

Vuosku J, Jokela A, Läärä E, Sääskilahti M, Muilu R, Sutela S, Altabella T, Sarjala T, Häggman H (2006) Consistency of polyamine profiles and expression of arginine decarboxylase in mitosis during zygotic embryogenesis of scots pine. Plant Physiol 142:1027–1038.

Vuosku J, Suorsa M, Ruottinen M, Sutela S, Muilu-Mäkelä R, Julkunen-Tiitto R, Sarjala T, Neubauer P, Häggman H (2012) Polyamine metabolism during exponential growth transition in Scots pine embryogenic cell culture. Tree Physiol 32:1274–1287.

Winkelmann T, Ratjens S, Bartsch M, Rode C, Niehaus K, Bednarz H (2015) Metabolite profiling of somatic embryos of *Cyclamen persicum* in comparison to zygotic embryos, endosperm, and testa. Front Plant Sci 6:597. doi:10.3389/fpls.2015.00597

Yoda H, Fujimura K, Takahashi H, Munemura I, Uchimiya H, Sano H (2009) Polyamines as a common source of hydrogen peroxide in host- and nonhost hypersensitive response during pathogen infection. Plant Mol Biol 70:103–112.

Capítulo II

Biossíntese de poliaminas em culturas embriogênicas de *Araucaria angustifolia*

Manuscrito a ser submetido para publicação (Anexo II).

Polyamine biosynthesis in embryogenic cell lines of *Araucaria angustifolia* (Bertol. Kuntze) (Brazilian pine)

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Abstract

In embryogenic cultures of *Araucaria angustifolia*, an endangered conifer from Brazil, polyamines (PAs) are associated to embryogenic potential, with exogenous PAs showing better development of pro-embryogenic masses. Even so, to date a completely somatic embryo has never been developed. A better understanding of PA biosynthetic pathway regulation could help in modulating optimal somatic embryogenesis conditions. In this context, arginine (Arg) and ornithine (Orn), substrates for putrescine (Put) biosynthesis, have played important roles in PA regulation. Until now, the mechanisms that control PA metabolism in cell lines with different embryogenic potential have not been completely elucidated. Herein, investigation focused on the role of Arg and Orn in the regulation of PA metabolism in two cell-lines of *A. angustifolia* with different embryogenic potential. Cellular PAs and amino acid content and incorporation of labeled precursors were analyzed, as were arginine decarboxylase (ADC) and ornithine decarboxylase (ODC) enzymatic activity. This was also the case of the expression profile of genes involved in biosynthesis and catabolism, with the supplementation or not of either 5 mM Arg or Orn into the culture medium. Although both changed PA levels, Arg was more efficiently incorporated labeled precursors and changed gene expression related to biosynthesis and catabolism. With Arg supplementation, responsive cells maintain PA gene catabolism activity, whereas the blocked cells seem to simply accumulate PAs. Exogenous Arg and Orn not only changed PAs levels, but also effectively altered the entire pool of amino acids, with Citrulline being outstanding. This knowledge should facilitate experimental manipulation of the culture medium or growth conditions, to so induce biochemical and molecular changes that normally occur in the developing embryos, thereby improving the process of somatic embryogenesis.

Key words: Amino acids, arginine decarboxylase, conifer embryogenesis, ornithine decarboxylase, polyamine biosynthesis.

1. INTRODUCTION

Through the similarity with zygotic embryogenesis, somatic embryogenesis (SE) constitutes an efficient model for studying factors that affect embryo development (von Arnold *et al.,* 2002; Salo *et al.,* 2016). Even though protocols for SE have been successfully described for many conifer species (Klimaszewska *et al.,* 2016), this is not the case of *A. angustifolia*, an endangered conifer that occurs in south Brazil. The lack of knowledge on the underlying genetic programs and biochemical pathways that regulate embryogenesis in this species, restricts *in vitro* development to only a few somatic embryos (Jo *et al.,* 2014; Elbl *et al.,* 2015a; dos Santos *et al.,* 2016). Nonetheless, studies of molecular processes and biochemical activities, using comparative transcriptome analysis, are under way in somatic and zygotic embryogenesis (Elbl *et al.,,* 2015a), and profiling proteins (Silveira *et al.,* 2008; Jo *et al.,* 2014; dos Santos *et al.,* 2016), abscisic acid (Silveira *et al.,* 2008), indole-3-acetic acid (Astarita *et al.,* 2003a), amino acids (Astaria *et al.,* 2003b; de Oliveira *et al.,*2017), and polyamines (Astarita *et al.,* 2003; Jo *et al.,* 2014; de Oliveira *et al.,* 2017).

Polyamines (PAs) are small and positively charged aliphatic amines present in all living organisms (Minguet *et al.,* 2008; Silveira *et al.,* 2013; Minocha *et al.,* 2014). Their charged structure is responsible for electrostatic interaction with several macromolecules, such as DNA, RNA, phospholipids and proteins that can influence various development processes in plants (Baron and Stasolla, 2008; Minocha *et al.,* 2014). In this sense, PAs play a role in cell division, the regulation of plant development processes, as flowering and fructification, programmed cell-death, senescence, rooting, response to biotic and abiotic stress, and embryogenesis (Bais and Ravishankar 2002; Kuehn and Phillips 2005; Kuznetsov and Shevyakova 2007; Floh *et al.,* 2007; Gemperlová *et al.,* 2009).

PA metabolism has been connected to both zygotic and somatic embryogenesis in many plants species (Minocha *et al.,* 1999; Astarita *et al.,* 2003c; Silveira *et al.,* 2004; Vuosku *et al.,* 2006; Steiner *et al.,* 2008; Gemperlová *et al.,* 2009; Vuosku *et al.,* 2012; Jo *et al.,* 2014; Salo *et al.,* 2016; de Oliveira *et al.,* 2017). Subtle changes in PA levels and amino acid content related to PA biosynthesis are associated to development stages in somatic embryogenesis, influence ranging from the induction of embryogenic cultures to embryo germination (Minocha *et al.,* 1999; Astarita *et al.,* 2003a; Astarita *et al.,* 2003b; Silveira *et al.,* 2004; Pieruzzi *et al.,* 2011). Furthermore, the accumulation of PAs may play a role in the protection of cells against reactive oxygen species (ROS) damage (Salo *et al.,*

2016). These multifaceted functions ensure crucial PA homeostasis through the regulation of biosynthesis, catabolism and transport (Kusano *et al.,* 2008).

Putrescine (Put), spermidine (Spd) and spermine (Spm) are three common plant PAs. Put is synthesized directly by ornithine decarboxylase (ODC; EC 4.1.1.17), or, during intermediate steps, by arginine decarboxylase (ADC; EC 4.1.1.19), agmatine iminohydrolase (AIH; EC 3.5.3.12) and *N*-carbamoylputrescine amidohydrolase (CPA; EC 3.5.1.53) (Bais and Havishankar, 2002). The co-existence of ADC and ODC enzymes in some species may be related to their differential contribution to stress, development and tissue specificity (Vuosku *et al.,* 2006). Spd and Spm are synthesized by the sequential addition of aminopropyl groups to Put, through reactions involving *S*-adenosylmethionine decarboxylase (SAMDC; EC 4.1.1.50), spermidine (SPDS; EC 2.5.1.16) and spermine synthases (SPMS; EC 2.5.1.22) (Tiburcio *et al.,* 1997; Vuosku *et al.,* 2012).

The PA biosynthetic pathway in plants is regulated by enzymatic activities, and the availability of substrates, such as arginine and ornithine (Page *et al.,* 2007; Page *et al.,* 2012; Majumdar *et al.,* 2013; Majumdar *et al.,* 2016). In poplar cell-lines, up-regulation of a single step in this pathway, such as that from ornithine to putrescine, can alter expression in a broad spectrum of genes, as well as in more than half of the 200 detected metabolites (Page *et al.,* 2016). Furthermore, possibly increased metabolic conversion of amino acids into Put may considerably affect the pool of other amino acids in the cell (Majumdar *et al.,* 2016). Current results presuppose that PA biosynthesis is under complex regulation, possibly even post-translational (Fortes *et al.,* 2011). The mechanisms that control PA metabolism in cell-lines with different embryogenic potential, are not yet clearly understood. Moreover, high Put concentration has been associated with the incapacity to induce somatic embryo production in other species, such as *P. nigra* and *P. sylvestris* (Noceda *et al.,* 2009; Salo *et al.,* 2016), thereby leading to queries regarding the regulation of PA metabolism in various cell-lines.

PA profiles from embryogenic cultures of *A. angustifolia* have already been correlated with distinct embryogenic cell-lines (Jo *et al.,* 2014; de Oliveira *et al.,* 2015). However, although PA supplementation in the culture medium apparently enhanced the morphology of pro-embryogenic masses (Silveira *et al.,* 2006), evolution to somatic-embryo maturation did not occur. Furthermore, cell-lines producing somatic embryos were associated with lower PA levels than the unproductive cell-lines, which presented higher ones. Hence, a better understanding of the mechanisms that regulate PA metabolism in embryogenic cell-lines with distinct embryogenic capacity, is required, to so improve

artificial conditions for obtaining an efficient protocol for *A. angustifolia* somatic embryogenesis.

The roles of Arg and Orn in the regulation of PA metabolism in two distinct embryogenic cell-lines of *A. angustifolia* were investigated. Accordingly, ADC and ODC activities were measured, Arg and Orn flux quantified using labeled precursors, and PA and amino acid abundance analyzed, all together with quantitative real-time polymerase chain reaction (qRT-PCR) analysis of gene expression in the main genes involved in the Orn/Arg/PA biosynthetic pathway. The results showed that, besides changing the pool of PAs and amino acids through biosynthesis and catabolism, Arg and Orn can play distinct roles in PA metabolism regulation during the proliferation phase of both cell-lines. This should facilitate experimental manipulation of tissue culture media and growth conditions, to so induce biochemical and molecular changes that normally occur in the developing embryos, thereby improving the process.

2. MATERIALS AND METHODS

2.1. Plant material and experimental conditions

Two embryogenic cell (EC) lines of *A. angustifolia,* previously established according to dos Santos *et al.* (2008), were used in the assay. According to Jo *et al.* (2014), selection was based on the various responses in an MSG medium (Becwar *et al.*, 1989) supplemented with 7% (w/v) maltose, 3% (w/v) sucrose, 1.46 $g.l^{-1}$ L-glutamine, 0.3% (w/v) activated charcoal, 1% (w/v) Gelrite®, and 120 µM abscisic acid. Identification was focused on: a) Responsive: cells capable of producing pre-cotyledonary embryos under the maturation conditions (about 45 ± 5 [Mean ± S.D.] somatic embryos per 100 mg FW of embryonal mass); and b) Blocked: cells incapable of developing somatic embryos in identical circumstances.

The cell-lines remained for 14 days in a semi-solid MSG medium (Becwar *et al.*, 1989), containing 1.46 $g.l^{-1}$ L-glutamine and 3% (w/v) sucrose. Prior to autoclaving, the pH of the medium was adjusted to 5.8. One hundred mg (fresh weight) of each cell-line were then dissected into small pieces and transferred to six-multiwell plates (Techno Plastic Products, Switzerland), containing 5 ml of liquid MSG medium per well (as described before, except for Gelrite®), with or without 5 mM Arg or Orn (Sigma-Aldrich, USA). The experiment took place during the proliferation phase of embryogenic cultures.

For incorporation of labeled precursors, 0.25 µCi of either L-[^{l4}C(U)]-Arg (specific activity 274.0 mCi.mmol^{-1}, PerkinElmer), or L-[l-^{14}C]-Orn (specific activity 57.1 mCi.mmol^{-1}, PerkinElmer), plus 5 mM (final concentration) of cold Arg or Orn, were added into each well. However, in the control treatment, only L-^{14}C-Arg or L-^{14}C-Orn were added.

The suspension cultures were grown in the dark, at 25 ± 1 ºC, on a gyratory shaker at 110 r.p.m. These were sequentially collected into 15 ml tubes, at 6, 24, 48, 72, 168 and 336 h intervals. Centrifugation was 11,000*g* for 5 minutes at room temperature, to so pellet the cells. The supernatant was discarded, and the pellets washed three times with 2 mM cold Arg or Orn, followed by three washes with distilled water, with additional centrifugation after each wash. The pellets were weighed, frozen and stored at -80ºC for biochemical analysis, as described below.

2.2. Determination of free amino acids

The amino acid content was determined according to Santa-Catarina *et al.* (2006). One hundred milligrams (fresh weight) of cells were homogenized in an ice-cold mortar with liquid nitrogen, mixed in 3 ml 80% (v/v) ethanol, and concentrated in a Speed-Vac. The samples were then re-suspended in 1 ml Milli'Q water, and centrifuged at 11,000*g* for 10 minutes, whereupon the supernatant was filtered through a 20 µm membrane (Sartorius Stedim Biotech, Germany). Amino acid derivation was with *o*-phthalaldehyde, and separation by high-performance liquid chromatography (HPLC, Shimadzu, Japan) on a C_{18} reverse-phase column (5 µm × 4.6 mm × 250 mm - Supelcosil LC-18, Sigma-Aldrich, USA). The gradient was developed by mixing proportions of 65% methanol with a buffer solution (50 mM sodium acetate, 50 mM sodium phosphate, 20 ml l^{-1} methanol, 20 ml l^{-1} tetrahydrofuran and adjusted to pH 8.1 with acetic acid). The 65% methanol gradient was programmed to 20% during the first 32 minutes, from 20% to 100% between 32 and 71 minutes, and 100% between 71 and 80 minutes, with a flow rate of 1 ml min^{-1} at 40 ºC. Detection and quantification was with a fluorescence detector (RF-20A, Shimadzu, Japan), set at 250 nm excitation and 480 nm emission wavelengths.

2.3. Analysis of Free Polyamines

Free PA extraction was according to Bhatnagar *et al.* (2001). Samples were mixed with cold 5% (v/v) perchloric acid (PCA) at a ratio of 1:4 (w/v; 100 mg fresh-weight-tissue in 400 µl PCA), followed by three cycles of freezing (-20°C) and thawing (room

temperature), prior to centrifugation at 11,000*g* for 10 minutes, and subsequent supernatant collection. The final step was storage at -20ºC until PA analysis.

Free PA derivation was according to Silveira *et al.* (2004). Forty µl of the sample were added to 100 µl of dansyl chloride (5 mg ml^{-1} in acetone), 20 µl of 0.05 mM diaminoheptane (internal standard), and 50 µl of saturated sodium carbonate. After 50 minutes incubation in the dark at 70 °C, the excess of dansyl chloride was converted to dansylalanine by adding 25 µl of alanine (100 mg ml^{-1}). Subsequent incubation was for 30 minutes at room temperature. Polyamines were extracted with 200 µl of toluene, and the supernatant collected and dried in Speed-Vac at 45 °C. Dansyl-PAs were dissolved in 200 µl of acetonitrile.

Polyamines were separated by HPLC on a reversed-phase C_{18} column. The gradient was developed by mixing increasing proportions of absolute acetonitrile to 10% acetonitrile in water (pH 3.5). The gradient of absolute acetonitrile was programmed 0 to 65% for the first 10 minutes, 65 to 100% from 10 to 13, and at 100% from 13 to the final 21, in a flow of 1 ml min^{-1} at 40 °C. Polyamines were detected at 340 nm (excitation) and 510 nm (emission) wavelengths with an RF-20A fluorescence detector (Shimadzu, Japan).

2.4. Analysis of labeled precursor incorporation

After extraction and dansylation of free polyamines, a 10 µl aliquot of PCA extract and 10 µl of an aqueous fraction (post-dansylation, and containing amino acids and other charged by-products), were counted separately for radioactivity.

After dissolution in 10 µl of acetonitrile, the dansyl-Pas were then spotted onto 20 x 20 cm thin-layer-chromatograph (TLC) plates (silica gel 60, Merck KGaA). Plate development was in a solvent mix of chloroform:triethylamine (3:1 [v/v]) in a chromatograph chamber. When the solvent front had shifted 15 cm from origin, the plates were air-dried and the respective PA bands marked under UV light and scraped for radioactivity counting.

L-[^{l4}C(U)]-Arg and L-[l-^{14}C]-Orn incorporation into other amino acids related to the PA biosynthesis pathway was assayed by disposal of 20 µl of amino acid extract onto TLC plates, and resolution in a solvent mix of *n*-butanol:acetic acid:water (4:1:1 [v/v]). On the solvent front shifting 15 cm from the origin, the plates were air-dried and the spots corresponding to ornithine, arginine, citrulline, proline and GABA visualized by spraying

with 1% (w/v) ninhydrin in a 100 ml acetone solution, followed by heating at 90 °C for 5-7 minutes to ensure plateau intensity of the colored complex.

PA and amino acid bands were scraped and immersed in 1 ml of scintillation fluid (PerkinElmer). Radioactivity counting was with a Tri-Carb2910TR - PerkinElmer scintillation counter, and expression as counts per minute (CPM) g^{-1} FW. The percentage of incorporation in each PA, i.e. Put, Spd and Spm, was considered as a fraction of the sum of radioactivity present in all the three (100%). Analysis was with three biological replicates.

2.5. ADC and ODC enzyme assay

Enzyme activities were defined according to de Oliveira *et al.* (2017). Samples were homogenized in an ice-cold mortar with liquid nitrogen, whereupon an extraction buffer (50 mM Tris-HCl, pH 8.5, 0.5 mM pyridoxal-5-phosphate, 0.1 mM EDTA and 5 mM dithiothreitol) was added at the rate of 100 µl to 100 mg (fresh weight) of tissue. The solution was vortexed and centrifuged (13,000*g* for 20 min at 4 ºC), and the supernatant used for ADC and ODC enzyme assay. A reaction mixture containing 50 µL of protein extract, 8.3 µL of extraction buffer, 12 mM of unlabeled L-Arg or L-Orn, and 25 nCi of either L-[^{l4}C(U)]-Arg (specific activity 274.0 mCi.mmol^{-1}, PerkinElmer, USA), or L-[1-^{14}C]-Orn (specific activity 57.1 mCi.mmol^{-1}, PerkinElmer, USA) was used for this. Blank samples contained only 50 µL of extraction buffer. Reaction mixtures were incubated in glass tubes fitted with rubber stoppers and filter-paper discs soaked in 2 N KOH. The material was maintained at 37 ºC and 120 rpm (orbital shaker) for 90 minutes. The reaction ended by adding 200 µL of perchloric acid, followed by further incubation for another 15 minutes under the same conditions. Filter paper containing $^{14}CO_2$ was immersed in 1 mL of scintillation fluid (PerkinElmer). Radioactivity was then measured using a scintillation counter (Tri-Carb2910TR, PerkinElmer). The activities were expressed as nmol $^{14}CO_2$ mg protein^{-1} h^{-1}. Protein content was measured by the Bradford method (Bradford, 1976) with bovine serum albumin as the standard. Specific activities were measured for three biological replicates.

2.6. Quantitative RT-PCR analysis

The ReliaPrepTM RNA Cell Miniprep System kit (Promega, USA) was employed for RNA extraction. cDNA synthesis, primer design and qRT-PCR analysis were according to Elbl *et al.* (2015b). Gene specific primers used in qRT-PCR assay were designed using

OligoAnalyzer 3.1 software (http://www.idtdna.com/calc/analyzer) according to Minimum Information for Publication of qRT-PCR Experiments (MIQE) guidelines (Bustin *et al.*, 2009). Quantification cycle (Cq) values from two technical replicates and primer efficiency were calculated using LinRegPCR software (Ruijter *et al.*, 2009). Target-gene expression values were normalized against geometric averages of *AaEF-1α* (elongation factor 1α) and *AaPP2A* (protein phosphatase 2A) reference genes (Elbl *et al.*, 2015*b*). Calculations of gene relative expression were based on average expression levels in control samples of the responsive-cell line, and presented as Log_2 fold changes.

2.7. Statistical analysis

Data analysis was by Student *t*-test or variance analysis (ANOVA), followed by Tukey test for comparison and control, as indicated in figure legends. Data were log-transformed when appropriate. R version 3.2.2 (R Core Team, 2015) and BioEstat (Version 5.0) software were employed for analysis.

3. RESULTS

3.1. Uptake of L-[U-^{14}C]Arg and L-[1-^{14}C]Orn

The choice of the two cell-lines was based on their capacity to produce somatic embryos adequate for analysing L-[U-^{14}C]Arg and L-[1-^{14}C]Orn uptake in 5 mM treated and untreated cells, by counting the amounts of radioactivity in PCA-soluble fraction. There was a linear increase in [^{14}C] between 6 and 48 hours, in both treatments and cell-lines, followed by a reduction in uptake at 72 hours (Figure 1). Although L-[U-^{14}C]Arg uptake was higher than L-[1-^{14}C]Orn in both cell-lines and treatments, it was lower with 5 mM cold Arg supplementation in Responsive cells during the interval 6-24h (Figure 1A) and Blocked, 6-72h (Figure 1C). On the other hand, L-[1-^{14}C]Orn uptake was similar between untreated and 5 mM Orn treated cells, except in the Blocked at 336 hours (Figure 1B and D).

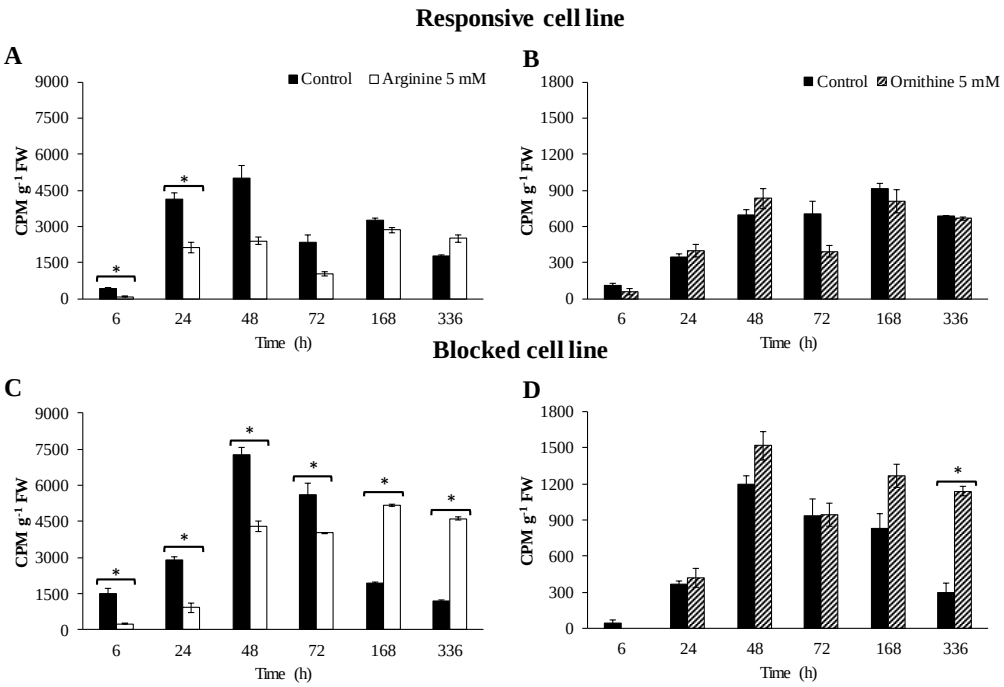

Figure 1. Amount of radioactivity in a ^{14}C-PCA extract showing the uptake of L-[U-^{14}C]Arg and L-[1-^{14}C]Orn in Responsive (**A, B**) and Blocked (**C, D)** cells of *Araucaria angustifolia* at different times of incubation in a liquid MSG medium, supplemented with, or without, 5 mM cold Arg or Orn. Data are the averages ± SD of three replicates. Asterisks indicate that 5 mM cold Arg or Orn values are significantly differently (Student *t*-test; $P < 0.01$) from control at a given time.

Furthermore, following dansylation of the PCA-soluble fraction, and partitioning of dansyl-PAs into toluene, radioactivity in the remaining aqueous fraction was also counted. This fraction represents mostly unincorporated L-[U-^{14}C]Arg or L-[1-^{14}C]Orn taken up by

either cells derived from ^{14}C-labeled precursors, or polar catabolic products of Put. The total [^{14}C] present in the aqueous fraction was higher in cells labeled with L-[U-^{14}C]Arg than with L-[1-^{14}C]Orn, in both cell-lines (Figure 2). This profile was quite compatible with that observed in PCA-radioactivity profiles.

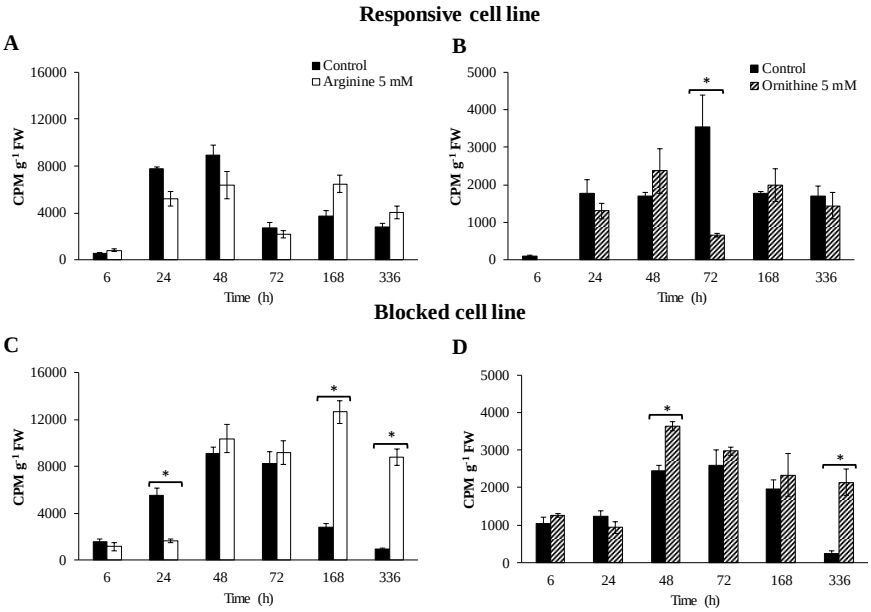

Figure 2. Radioactivity from L-[U-^{14}C]Arg and L-[1-^{14}C]Orn present in aqueous fractions after PCA dansylation in Responsive (**A, B**) and Blocked (**C, D**) cells of *Araucaria angustifolia* at different times of incubation in a liquid MSG medium supplemented with, or without, 5 mM cold Arg or Orn. Data are the averages ± SD of three replicates. Asterisks indicate that 5 mM cold Arg or Orn values are significantly differently (Student *t*-test; $P < 0.01$) from control at a given time.

3.1.1. Labelling with L-[U-^{14}C]Arg

Apart from uptake analysis of amino acids, the source of the Arg and Orn substrates involved in Put, Spd and Spm biosynthesis was also defined. Accordingly, measurements were taken of the incorporation of radioactivity from ^{14}C-labeled precursors of Put (i.e., L-[U-^{14}C]Arg or L-[1-^{14}C]Orn), for varying lengths of time, with or without the supply of cold 5 mM Arg or Orn. Dansyl-PAs were separated by TLC (Supplementary Fig. S1), and the spots of the three PAs were counted for radioactivity. In general, radioactivity counts were higher in untreated-cell lines than in the 5 mM Arg-treated (Figures 3 and 4).

In the Responsive cell-line, ^{14}C-Put recovered from L-[U-^{14}C]Arg without adding 5 mM Arg, was higher at 24h (70%) and 72h (85%) (Figure 3A and Supplementary Figure S2A). [^{14}C] incorporation into ^{14}C-Spd and ^{14}C-Spm until 48h had reached 39% and 23% respectively, followed by a decrease until 72h (down to 8% and 5%, respectively) (Figures

3B-C and Supplementary Figure S2A). [^{14}C] incorporation into cells supplied with 5 mM Arg was lower, with alterations in percentage in relation to control. Whereas until 48h of culture, radioactivity was the highest in Spd (37-56%), followed by Put and Spm (Supplementary Figure S2B), after 72h, the highest rate of incorporation was in Put (Figure 3A) followed by Spd and Spm (Supplementary Figure S2B).

In the Blocked cell-line, the highest amount of radioactivity until 48h of culture was in Put (49 to 74%) (Figures 3D-F and Supplementary Figure S2C), followed by Spd (21 to 43%) and Spm (4 to 9%). By 72h, the flow of L-[U-^{14}C]Arg was higher into Spd (46%) and Spm (16%) (Supplementary Figure S2C). In cells supplied with 5 mM Arg, until 24h L-[U-^{14}C]Arg flow into Spm was higher (48%) (Supplementary Figure S2D). Towards the end of incubation, incorporation in both treatments was the highest in Put, followed by Spd and Spm.

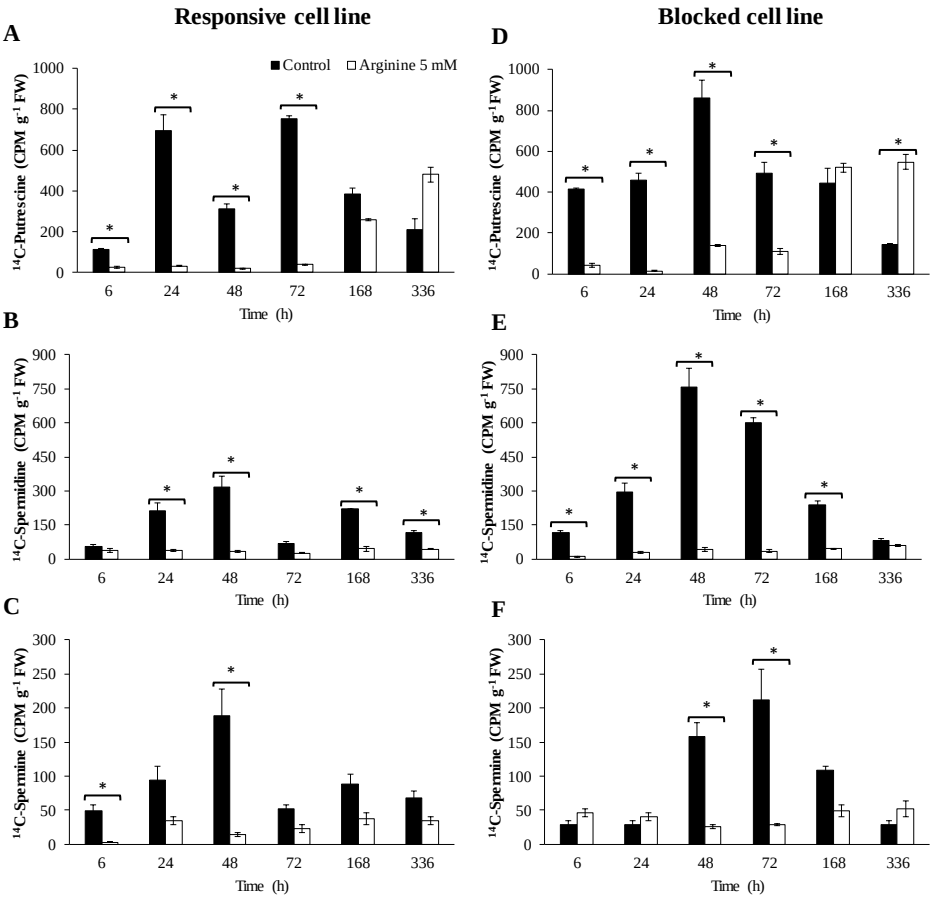

Figure 3. The amount of radioactivity incorporated from L-[U-^{14}C]Arg into free putrescine, spermidine and spermine, in Responsive (**A, B, C**) and Blocked (**D, E, F**) cells of *Araucaria angustifolia* at different times of incubation in a liquid MSG medium supplemented with, or without, 5 mM cold Arg. Data are the averages ± SD of three replicates. Asterisks indicate that 5 mM cold Arg values are significantly differently (Student *t*-test; $P < 0.01$) from control at a given time.

Radioactivity incorporation into Arg, Om, Cit, Pro and GABA was assessed, in order to check whether conversion of ^{14}C-precursors into other amino acids that are also products of the pathway, occurred. Overall, radioactivity from L-[^{14}C]-Arg was the highest in Arg, in both cell lines (Supplementary Tables S1 and S2).

As regards L-[^{14}C]-Arg incorporation, this into [^{14}C]-Orn was higher than in any other amino acid, thereby indicating the possible conversion of Arg into Orn through the action of arginase. In the Responsive cell-line, high rates of incorporation were found at 24h and 48h (Supplementary Table S1). Occurrence in cells supplied with 5 mM Arg was lower in relation to control. Even so the highest rate was found at 24h. In Blocked cells, incorporation was the highest at 48h and 72h, in both untreated cells and those treated with 5 mM Arg (Supplementary Table S2). On the other hand, L-[^{14}C]-Arg incorporation into Cit, Pro and GABA was much lower in both cell-lines.

3.1.2. Labelling with L-[1-^{14}C]Orn

The pattern of changes in the incorporation of L-[1-^{14}C]Orn into Put, Spd and Spm was different from that noted for L-[U-^{14}C]Arg, and lower when into Pas (Figure 4 and Supplementary Figure S3). In Responsive cell lines, incorporation of L-[1-^{14}C]Orn into Put and Spd was similar between untreated and 5 mM Orn treated cells in most periods (Figure 4A). In these cells, an increase in ^{14}C-Spm incorporation rate occurred between 6 and 24 h, followed by a decrease|drop after 48h (Supplementary Figure S3B). On the other hand, incorporation of ^{14}C from Spd into Spm occurred after the 6-72h period, when the amount of radioactivity in Spd decreased and in Spm, increased (Figure 4B and C).

Blocked cells containing L-[1-^{14}C]Orn showed that incorporation of ^{14}C into PAs was more pronounced at around 6h (Figure 4D, E and F), with a significant drop after 24h. Considering the lower incorporation of L-[1-^{14}C]Orn into PAs, compared to L-[U-^{14}C]Arg, the addition of 5 mM Orn had little effect on the rate, when compared to untreated cells. The further transfer of ^{14}C from Put into Spd and Spm did not follow the same trend as that observed in cells with L-[U-^{14}C]Arg (Figure 4F).

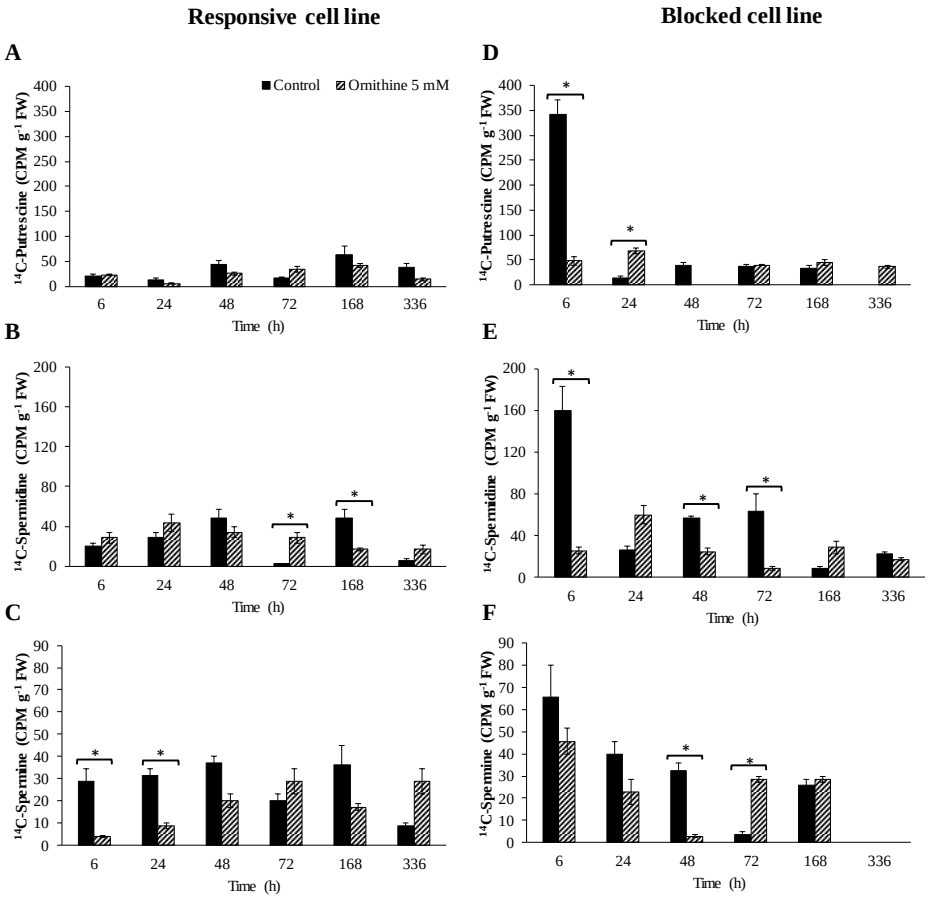

Figure 4. The amount of radioactivity incorporated from L-[1-^{14}C]Orn into free putrescine, spermidine and spermine, in Responsive (**A, B, C**) and Blocked (**D, E, F**) cells of *Araucaria angustifolia* at different times of incubation in a liquid MSG medium supplemented with, or without, 5 mM cold Arg or Orn. Data are the averages ± SD of three replicates. Asterisks indicate that 5 mM cold Orn values are significantly differently (Student *t*-test; $P < 0.01$) from control at a given time.

Although L-[1-^{14}C]Orn uptake was lower than L-[U-^{14}C]Arg, incorporation into other amino acids, notably Arg, was higher (Supplementary Tables S1 and S2). In Responsive cells, incorporation from L-[1-^{14}C]Orn into ^{14}C-Arg increased during the 6h-48h period, followed by a reduction until 72h in both treated and untreated 5 mM Orn-cells (Supplementary Table S1). In Blocked cells, this increase occurred until 48h in untreated cells, and 72h in the 5 mM Orn-treated (Supplementary Table S2). As regards the other amino acids, incorporation was the highest in Pro, followed by Cit and GABA.

3.2. The effect of exogenous Arg and Orn supplementation on polyamine content

The main PAs found were, in order, Put, Spd and Spm in both cell-lines analyzed (Figure 5). Overall, compared to untreated cells, exogenous Arg or Orn supplementation

significantly increased endogenous Put content in both cell-lines, mainly at 48h, 168h and 336h of incubation (Figure 5A and D). Comparatively, Blocked cells showed higher levels of PA than Responsive.

Compared to untreated cells, in Responsive 5 mM Arg treated, the effect on Spd and Spm levels was less, this dropping further still until 72h (Figure 5B and C). Counterwise, in Blocked cells, an early increase in Spd and Spm had occurred by 48h (Figure 5E and F).

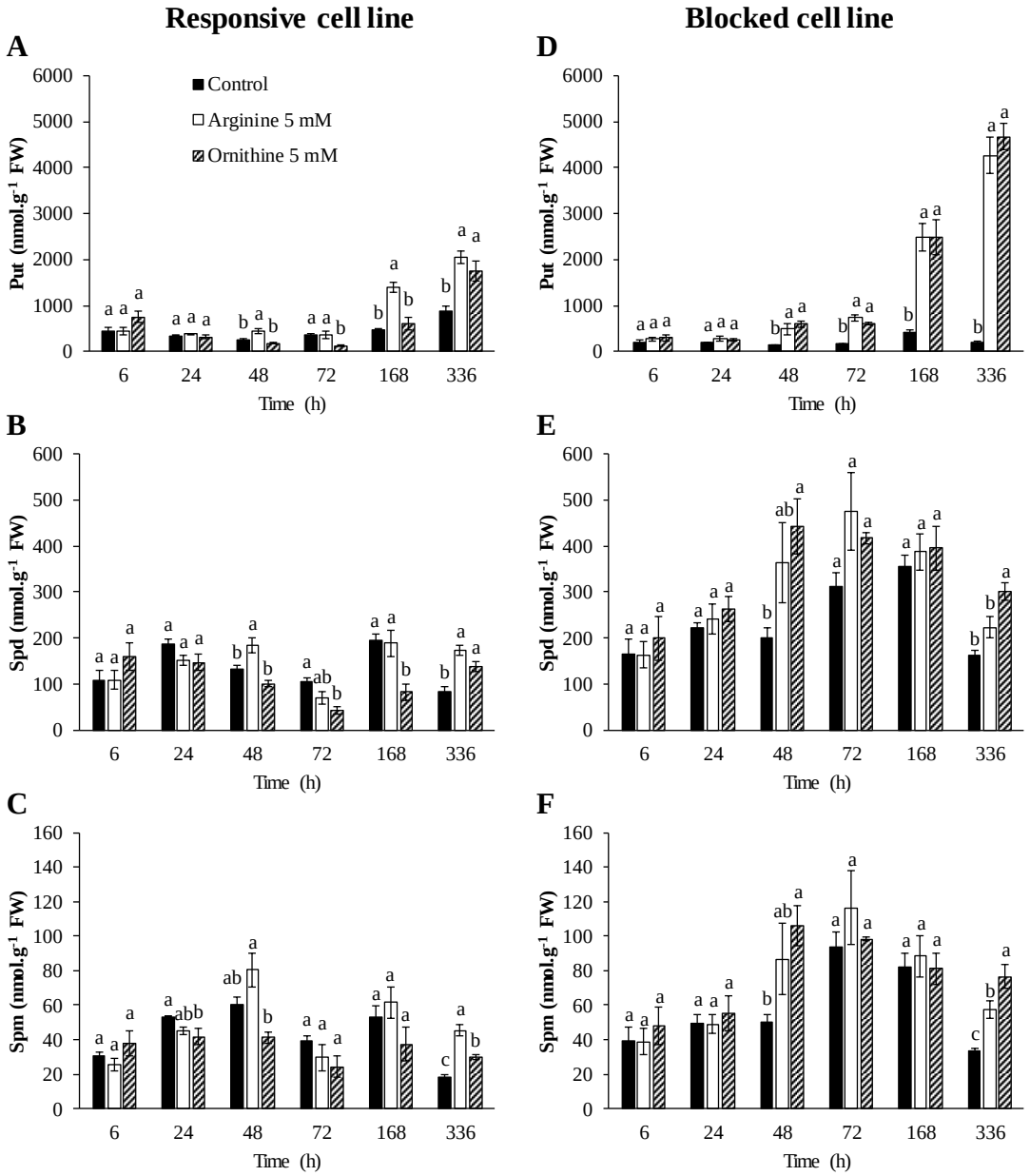

Figure 5. Cell contents of free polyamines (putrescine, spermidine and spermine) in Responsive (**A, B, C**) and Blocked (**D, E, F**) cells of *Araucaria angustifolia* at different times of incubation in a liquid MSG medium supplemented with, or without, 5 mM cold Arg or Orn. Data are the averages ± SD of three replicates. Statistically significant differences (Tukey test; $p<0.01$) among treatments are indicated by different letters at a given time.

3.3. Effects of exogenous Arg or Orn on ADC and ODC enzymatic activities

On checking whether ADCs or ODCs, the enzymes involved on Put biosynthesis, are affected by adding Arg or Orn, the rate of decarboxylation of ^{14}C-Arg (ADC activity) and ^{14}C-Orn (ODC activity) were measured in Responsive and Blocked cells, following 48h incubation, with or without amino acid supplementation. The choice of time was based on the incorporation rate of labeled precursors throughout amino acid profiles. In most cases, the amino acids proved to have already been metabolized until 48h after Arg or Orn supplementation (Figures 1-4, 8; Supplementary Tables S3 and S4).

ADC activity was lower in Responsive cells with 5 mM Orn, while, ODC was 2.5-fold less in cells with both 5 mM Arg and Orn (Figure 6A). On comparing the activities of both, ODC was higher than ADC, in control and cells with Orn, in Responsive cells. The profiles of the two enzymes remained alike in all the conditions tested in Blocked cells (Figure 6B).

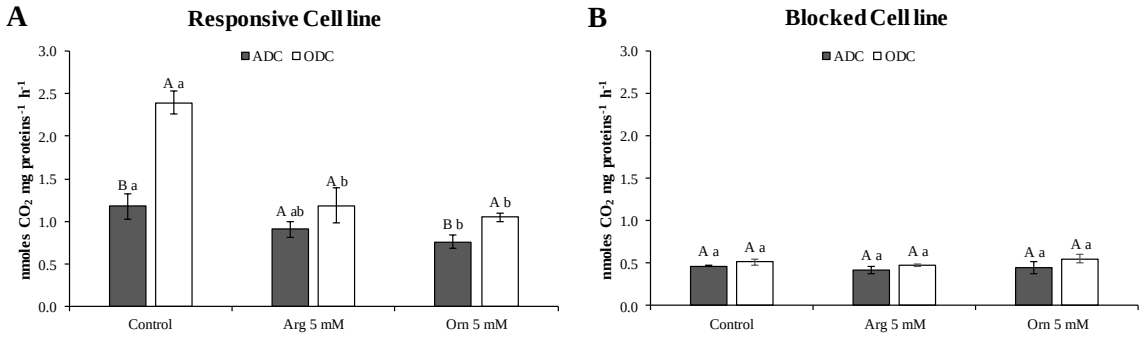

Figure 6. Specific ADC and ODC enzymatic activities in Responsive (**A**) and Blocked (**B**) cells of *Araucaria angustifolia* after 48 hours of incubation in a liquid MSG medium supplemented with, or without, 5 mM cold Arg or Orn. Activities were analyzed by measuring [^{14}]CO_2 released by decarboxylated radiolabeled Arg and Orn. Vertical bars indicate standard deviation of the average values derived from three biological replicates. Averages followed by uppercase letters are significantly different between ADC and ODC, according to the Student *t*-test ($P < 0.01$). Averages followed by lowercase letters are significantly different by treatment according to the Tukey test ($P < 0.01$).

3.4. Expression of Arg|Orn-Polyamine metabolism related genes under the effects of exogenous Arg and Orn supplementation

Expression analysis of the genes involved in Arg, Orn and PA biosynthesis (*AaADC*, *AaODC*, *AaARGINASE* and *AaOTC*) and in PA catabolism (*AaPAO2*, *AaCuAO* and *AaADLH*) was to understand transcriptional regulation in response to exogenous Arg and Orn supplementation, and was simultaneous with the analysis of ADC and ODC activities.

Expression levels were adjusted to control conditions in Responsive cells, which are known to promote the formation of globular somatic embryos under maturation conditions. Individual gene-profile expression between the two cell-lines was predominantly the opposite (Figure 7).

Compared with untreated cells, *AaADC* expression was higher among Responsive cells and lower among Blocked supplemented with 5 mM Arg (Figure 7A). However, 5 mM Orn supplementation had no effect on *AaADC* expression in either cell-line. *AaARGINASE*, involved in the conversion of Arg into Orn showed the highest expression in Blocked cells supplied with 5 mM Orn, whereas in other samples there were no changes (Figure 7B). Five mM Orn suplementation caused a decrease in *AaOTC* expression in Responsive cells, thereby indicating down-regulation under these circumstances. There were no changes among Blocked cells (Figure 7C).

Of the more than four genes that participate in PA catabolism in *A. angustifolia* embryogenic cells three were successfully amplified. The highest *AaPAO2* expression was found among Responsive cells supplemented with 5 mM Arg, followed by 5 mM Orn (Figure 7D). Among the Blocked cells, expression of those supplied with Arg was lower compared to untreated. Supplementation by both amino acids had no effect on *AaCuAO* expression in the two cell-lines (Figure 7E), whereas *AaALDH* expression levels were higher among Responsive cells supplemented with both Arg and Orn, and among Blocked with Orn, there was a decrease (Figure 7F).

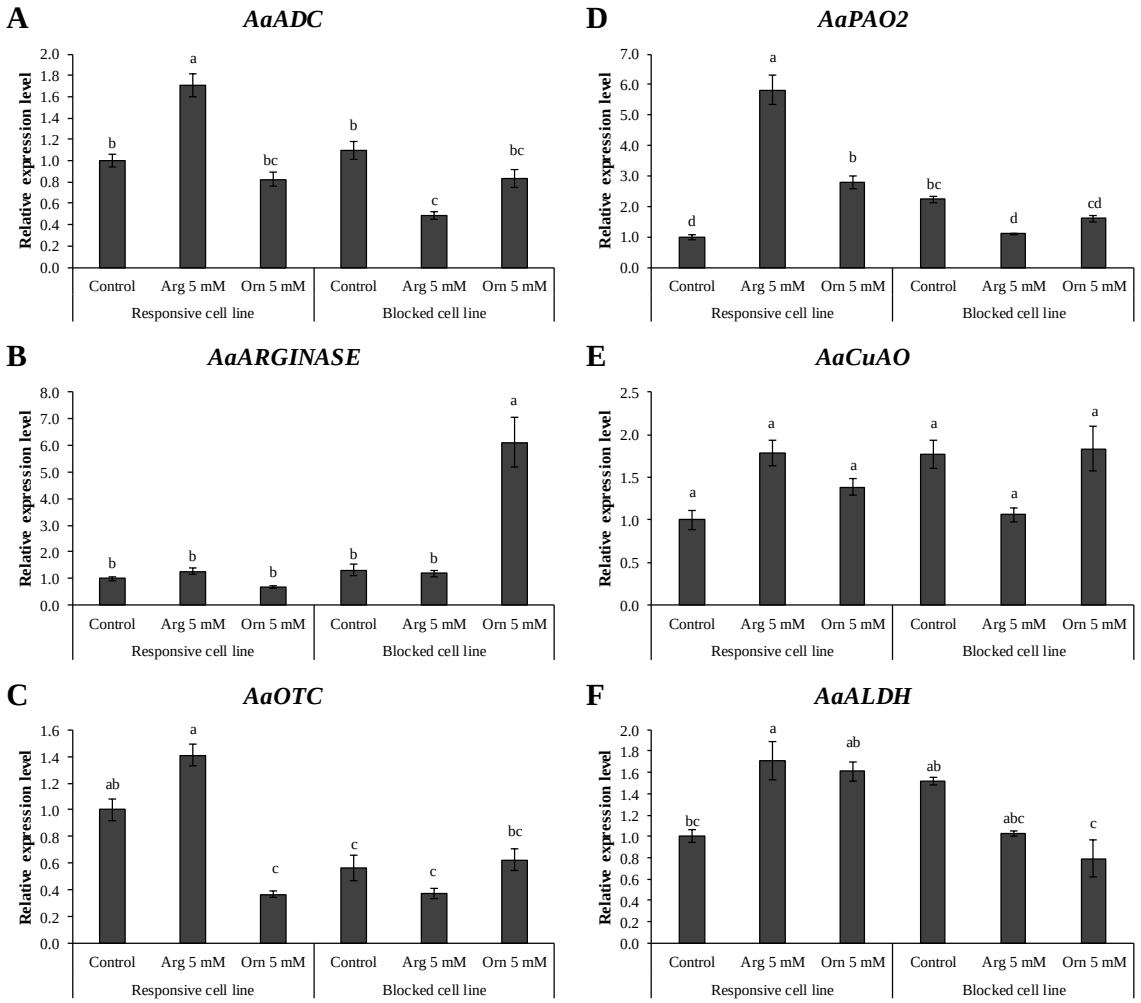

Figure 7. Relative gene expression in Orn-Arg-PA biosynthetic (**A, B, C**) and catabolism (**D, E, F**) pathway associated genes in Responsive and Blocked cells of *Araucaria angustifolia* after 48 hours of incubation in a liquid MSG medium supplemented with, or without, 5 mM cold Arg or Orn. Vertical bars indicate standard error of the average values derived from three biological replicates. Statistically significant differences ($P < 0.05$) between samples are indicated with different letters according to the Tukey test.

3.5. Changes in amino acid content by the exogenous supply of Arg or Orn

Differences in polyamine content in both cell-lines, together with Arg and Orn for Put substrate status, indicate the latter as also regulating other amino acids derived from the PA biosynthetic pathway. Thus, apart from PA analysis, focus was placed on the occurrence of free soluble amino acids in the presence or absence of exogenous Arg and Orn.

In both cell lines, exogenous 5 mM Arg or Orn supplementation increased cellular Put along with own accumulation (Arg and Orn) absorbed from the medium (Supplementary Tables S3 and S4). In the case of these two amino acids, bar graphs were plotted showing

only the effects of 5 mM Arg supplementation on Orn content, and of 5 mM Orn on Arg (Figures 8A-D).

The 5 mM Orn supplementation increased Arg content in both cell lines (Figures 8A, B). In the Responsive, the highest increase was reached at 48h (Figure 8A), and in the Blocked, there was a growing increase until 168h (Figure 8B). The effects of 5 mM Arg supplementation on Orn levels in both cell lines were only slight (Figures 8C and 8D).

Citrulline (Cit) is an intermediate product from Arg and NO biosynthesis. Endogenous Cit contents were small (<1%) in relation to the pool of amino acids in both cell-lines (Suppementary Tables S3 and S4). However, 5 mM Arg|Orn supplementation caused an increase in the two (Figures 8E and 8F), the highest in the Responsive cells, where exogenous Arg caused a 165-fold rise until 48h (Figure 8E). In the Blocked cells this reached about 67-fold by 168h (Figure 8F), when compared to control. Worthy of note, the increase of Cit in Responsive cells through 5 mM Orn supplementation, was followed by an increase in Arg contents (Figure 8A and E), thereby revealing a certain similarity in profiles. On the other hand, in Blocked cells, Arg and Cit profiles were different throughout the period of incubation, thereby implying the conversion of Arg into Cit during the period analyzed (Figure 8B and F).

γ-aminobutyric acid (GABA) is a possible product of Put catabolism. Contents of this amino acid were modified by 5 mM Arg and Orn supplementation. The increase, mainly caused by 5 mM Arg in Responsive cells (48h-168h) (Figure 8G) indicates Put catabolic activity. This was reduced in the Blocked (Figure 8H).

Changes in the cell contents of other amino acids, indirect products or that do not participate in the Arg-Orn-PAs biosynthetic pathway, are shown in Supplementary Tables S1 and S2. Intense changes in amino acid contents involving control, and 5 mM Arg and Orn treated cell-lines, were observed until 48h. In the Responsive cells, there was an increase in most cases (Supplementary Table S3). As to the Blocked cells, there was a general reduction until 48h, with a subsequent increase until 336h (Supplementary Table S4).

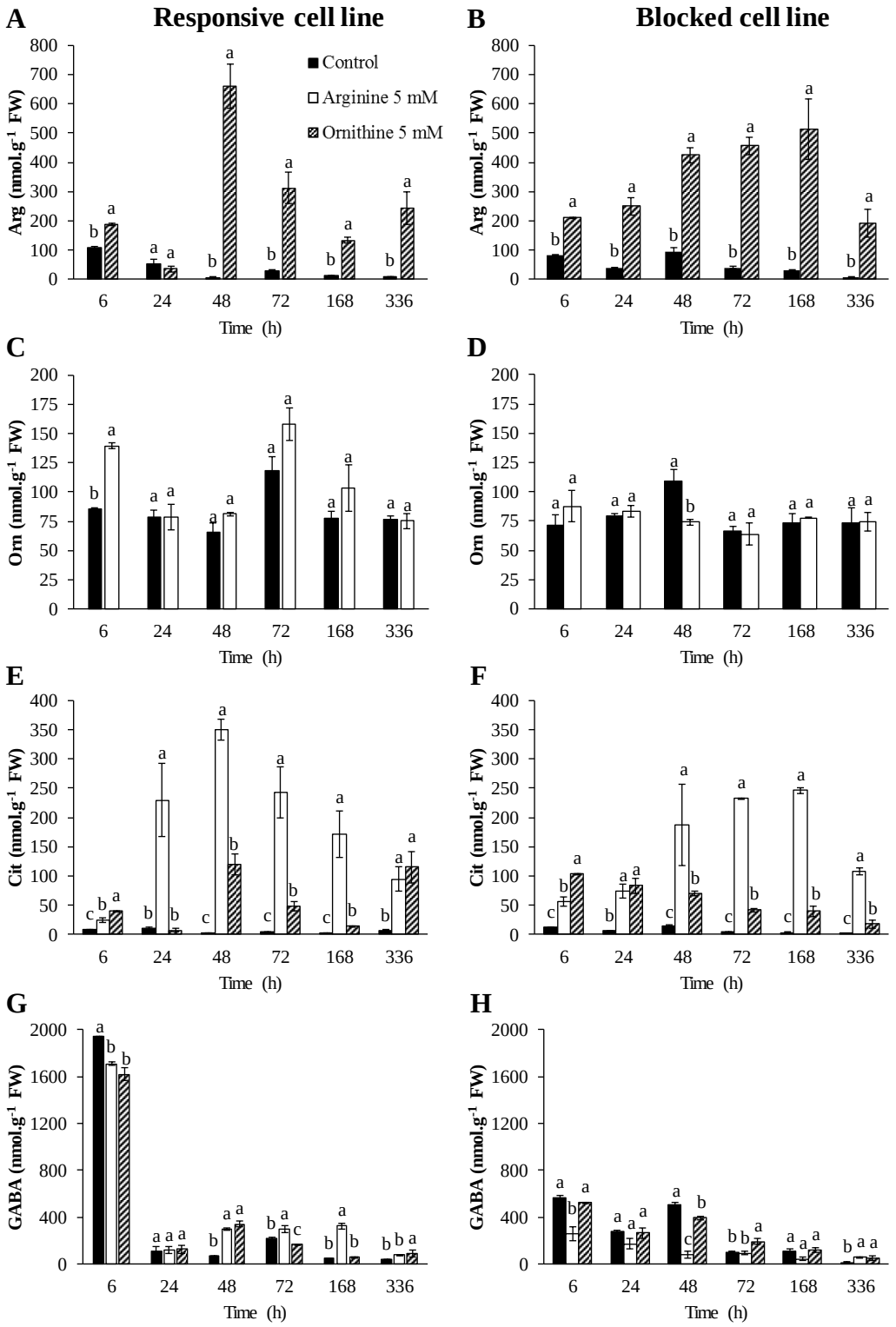

Figure 8. Cell content of arginine, ornithine, citrulline and γ-aminobutyric acid in Responsive (**A, C, E** and **G**) and Blocked (**B, D, F** and **H**) cells of *Araucaria angustifolia* at different times of incubation in a liquid MSG medium supplemented with, or without, 5 mM cold Arg or Orn. Data are the averages ± SD of three replicates. Statistically significant differences (Tukey test; $P < 0.01$) among treatments are indicated by different letters at a given time.

4. DISCUSSION

Elucidation of the regulation of PA and amino acid metabolism in plants is of major interest, due to the fundamental role of PAs in somatic embryogenesis, as reported in studies of conifers, such as *Pinus radiata, P. sylvestris, P. pinaster, P. nigra, Picea abies, P. rubens* and *P. glauca* (Minocha *et al.,* 1996; Kong *et al.,* 1998; Minocha *et al.,* 1999; Minocha *et al.,* 2004; Silveira *et al.,* 2004; Gemperlová *et al.,* 2009; Klimaszewska *et al.,* 2009; Noceda *et al.,* 2009; Vuosku *et al.,* 2012; Muilu-Mäkela *et al.,* 2015; Salo *et al.,* 2016). The amount of free Put present in plant tissue is the net result of its complex metabolism, as coordinated by biosynthesis, conjugation transport to other tissues, and degradation during a given period (Bhatnagar *et al.,* 2002).

In *A. angustifolia*, PAs have an effect, not only on growth and morphological evolution in embryogenic cultures, but also on interaction with other metabolic pathways, such as NO and ethylene, both related to embryogenic capacity (Silveira *et al.,* 2006; Jo *et al.,* 2014). Treatments that modify PA levels, such as the exogenous application of PAs and amino acids, are interesting ways for improving morphogenesis (Kevers *et al.,* 2000). In the present study, a comparison was made of PA metabolism and its precursors, Arg and Orn, in two cell-lines with different embryogenic potential, through the characterization of labeled precursors incorporated into PA, enzyme activities, and the identification of genes and metabolites that are changed by exogenous-amino-acid supplementation. Thus, a more precise indication of their importance was revealed, thereby providing an opportunity for unraveling the complexity of PA metabolism-regulation and of the amino acids associated in different cell-lines.

4.1. Polyamine biosynthesis from Arg versus Orn in embryogenic cultures of *A. angustifolia*

Polyamine contents can vary in embryogenic cells during the various stages of the SE process, as well as in different, potentially embryogenic cell-lines (Gemperlová *et al.,* 2009; Vuosku *et al.,* 2012; Jo *et al.,* 2014; Salo *et al.,* 2016). Herein, *A. angustifolia* cell-lines were characterized by distinct PA profiles. The Responsive presented lower Put levels than the Blocked, thereby reflecting their capacity to produce somatic embryos (Jo *et al.,* 2014). In order to understand Put metabolism in cell-lines with variable embryogenic capacity, use was made of supplementing the culture medium with the PA biosynthesis precursors,

arginine (Arg) and ornithine (Orn). A preliminary study (data unpublished) showed their capacity for changing Put, Spd and Spm levels (de Oliveira *et al.*, 2014).

As Arg and Orn can be alternative precursors for PAs in *A. angustifolia* (de Oliveira *et al.*, 2015), the focus was on the source of Put biosynthesis. The use of labeled precursors is opportune for studying appropriate precursors that facilitate inferring substrate pathway flux, in biosynthesis and catabolism (Santanen *et al.*, 1999; Bhatnagar *et al.*, 2001; Bhatnagar *et al.*, 2002; Majumdar *et al.*, 2013). Although the effect of exogenous Orn was similar to Arg in Put biosynthesis, its incorporation into PAs from ^{14}C-Orn was lower than from ^{14}C-Arg. The rapid absorption of exogenous Arg, proved its accessibility as a substrate in cell-lines (Andersen *et al.*, 1998; Majumdar *et al.*, 2013).

Labeled precursor-incorporation analysis clearly indicated changes in coordenation between the moment of amino acid absorption and incorporation into PAs. The incorporation rate of labeled precursors implies that, in most cases, amino acids are metabolized until 48h after supplementation. From then on and until 72 hours of incubation, there was a decrease in amino-acid uptake in both cell lines, probably due to the accumulation of endogenous with exogenous Arg and Orn. Absorption was resumed around 168h. Amino-acid metabolism in *A. angustifolia* seems to be slower than observed in poplar cell-lines expressing a mouse ODC (*mODC*), where Orn was metabolized into Put within 2 hours of supplementation (Bhatnagar *et al.*, 2002).

The addition of either Arg or Orn into the medium induces increased Put accumulation in both cell-lines, showing that these precursors may be limiting in the cells (Bhatnagar *et al.*, 2001; Bhatnagar *et al.*, 2002; Majumdar *et al.*, 2013). In the Blocked cell-line, the increase in Put levels was produced by both precursors, whereas in the Responsive cell-line the effect of Arg was higher than of Orn. A higher accumulation of Put was observed at the end of incubation (168h and 336h), which was to be expected, since high Put concentration is a characteristic of the exponential phase in *A. angustifolia* (Silveira *et al.*, 2006).

All the Put converted into Spd or Spm constituted only a small fraction of the total cell Put contents. In embryogenic cultures of *A. angustifolia*, Put has been reported as the main PA, followed by Spd and Spm (Jo *et al.*, 2014). Although the correlations between the incorporation and biochemical profiles described here are not clear, a consistent ^{14}C-Arg flux through Put, Spd and Spm was noted. In Responsive cells, a decreasing of incorporation of ^{14}C into Put was at 48h, followed by increase in Spd and Spm. However, was identified

at 72h a higher level of ^{14}C-Put, which was not observed by HPLC analysis. This event, although apparently derived from a decrease in Spd and Spm biosynthesis, could be Spd reconversion into Put (Vuosku *et al.*, 2012). In Blocked cells, the flow from ^{14}C-Arg into Put and Spd occurred simultaneously, and into Spm, later (72h). Clearly, metabolism was redirected to Put biosynthesis towards the end of incubation, thereby implying an important role in Put cell proliferation (Silveira *et al.*, 2006; Vuosku *et al.*, 2012).

4.2. Effect of Arg and Orn on ADC/ODC enzymatic activity and gene expression in PA metabolism

Analysis of ADC and ODC activities and gene expression were at 48h of incubation with 5 mM Arg or Orn. As explained above, this is when most changes in labeled precursor incorporation occur and amino acids are metabolized. However, other periods (6h and 336h) require analysis in order, to understand the process of PA cell metabolism during the subculture proliferation cycle.

There was no increase in ADC enzymatic activity in Responsive cells by adding 5 mM Arg, the contrary to that observed in Put levels (Figure 5A). There are two possibilities: a) inhibition of ADC feedback by the increase in Put levels, a somewhat different situation to that reported in poplar cells (Bhatnagar *et al.*, 2002) and in embryogenic cultures of *P. sylvestris* (Vuosku *et al.*, 2012); b) the increase in Put levels was caused by Spd catabolism, through an increase in *AaPAO2* gene expression (Figure 7D). This could also be partially explained by an increase in *AaADC* expression levels, the preferential pathway for Put synthesis in zygotic embryos (de Oliveira *et al.*, 2017). *ADC* genes have also been identified as predominant in Put biosynthesis at the embryogenic callus stage and early embryo differentiation in *Gossypium hirsutum* (Cheng *et al.*, 2015).

ADC and ODC activities in Blocked cells were not affected by Arg or Orn supplementation. Even though ODC activities in Responsive cells seem to be more than ADC, Arg|Orn supplementation seemed to reduce its activity. Although ODC activities seem to persist, *AaODC* transcripts were below the cutoff detection threshold, as was seen in zygotic embryos of *A. angustifolia* (de Oliveira *et al.*, 2017), thereby precluding expression-pattern analysis in this study. Furthermore, *AaODC*, retrieved early as a partial sequence, corresponded to a polypeptide of 161 amino acids, whereas approximately 433 have been observed in certain species of angiosperms (de Oliveira *et al.*, 2017). Studies indicate the importance of ADC and ODC enzymes in embryogenesis. Overall, ADC is the prime

regulatory enzyme in Put biosynthesis in zygotic and somatic embryogenesis in *P. sylvestris* (Vuosku *et al.*, 2006, 2012). In *P. abies*, whereas ADC was evidently the preferential Put pathway in zygotic embryo development, during somatic embryogenesis Put synthesis is by the ODC pathway (Gemperlová *et al.*, 2009). While in various plant tissues, the latter is particularly active in cell proliferation, the ADC is involved in embryo and organ differentiation and stress response (Kevers *et al.*, 2000; Vuosku *et al.*, 2006). Nevertheless, in some plant species the ODC gene is absent, as occurs with several members of the *Brassicaseae* family, including *Arabidopsis thaliana* (Jiménez-Bremont *et al.*, 2014).

Arg|Orn supplementation into the culture medium changed the expression profile of PA catabolism-related genes. The relative importance of the two components of Put loss (i.e. conversion into Spd and catabolic breakdown) can be inferred from the data presented herein. 5 mM Arg supplementation, while affecting the rate of ^{14}C-Arg incorporation, directed mainly to Put, and less to Spd and Spm, also implemented Spd and Spm starting at 48h. Thus, the establishment of steady-state equilibrium between PA biosynthesis and degradation can be inferred, the former being largely responsible for regulating the amounts of PAs at a given time (Bhatnagar *et al.*, 2002). PA catabolism seems to be more active in Responsive cells than in Blocked. Oxidation by CuAOs and PAOs contributes to the regulation of PA homeostasis, thereby generating catabolic products which have been linked to biological functions attributed to PAs (Cona *et al.*, 2006; Angelini *et al.*, 2010; Moschou *et al.*, 2012). Hydrogen peroxide (H_2O_2), a product of PA catabolism (Moschou *et al.*, 2012), is, in *A. angustifolia*, an important signaling molecule from oxidative metabolism, and associated to embryogenesis (Jo *et al.*, 2014).

GABA can be a product of Put catabolism, under the action of CuAO and ALDH (Majumdar *et al.*, 2016; Page *et al.*, 2016). The regulation of GABA metabolism in plants is complex, since various enzymes associated with this are spatially compartmentalized in the cell (Shelp *et al.*, 2012b). Whether its biosynthesis and catabolism are regulated at the transcription level is not well known (Majumdar *et al.*, 2016). Herein, it was shown that Put catabolism into GABA is differentially regulated in each cell line. In Responsive cells, the increase in GABA levels was accompanied by an increase in *AaCuAO* and *AaALDH* expression (Figure 7E, F), which came about by Arg or Orn supplementation. On the other hand, in the Blocked, Arg decreased not only GABA levels (Figure 8H), but also *AaCuAO* and *AaALDH* expression (Figures 7E, F). The importance of GABA in plants has been amply reported. In general, it is associated with several processes and functions similar to those of

proline, such as its contribution to C:N balance, pH regulation, protection against oxidative stress, osmoregulation, response to biotic and abiotic stress, and as a signaling molecule (Bouché and Fromm, 2004; Dowlatabadi *et al.*, 2009; Winkelmann *et al.*, 2015). However, although GABA is thought to play an important role during embryo development (Aragão *et al.*, 2015; de Oliveira *et al.*, 2017), details on its action in conifer embryogenesis remain poorly understood, and the relative contributions of its pathway in maintaining PA homeostasis in plants, remain obscure (Majumdar *et al.*, 2016).

4.3. Changes in the Arg-Orn pathways by exogenous Arg and Orn supplementation

Here, it was shown that after 48 hours, most total amino acids can be affected by exogenous Arg or Orn supplementation into the culture medium. This led to investigating Arg/Orn biosynthetic pathways by expression-genes, amino acid content and labeled incorporation, mainly to determine the conversion of Arg into Orn, and Orn into Arg. In higher plants, arginase converts arginine into ornithine, which can be applied by ODC in Put synthesis, or metabolized back to arginine through the ornithine cycle (Bais and Ravishankar, 2002). In the present study, even though arginase enzymatic activity was not assayed, it was concluded that Arg conversion into Orn by this can be low, as indicated by low *AaARGINASE* expression (Figure 7B), and the low incorporation rate from ^{14}C-Arg into Orn (Supplementary Tables S1 and S2). In young *Arabidopsis* seedlings, the primary source of Orn was Glu, and not Arg, which was produced by N assimilation from the medium. Page *et al.* (2012) reported that genes involved in the Glu-Orn-Arg pathway are constitutively coordinated by an increase in flow, whence the assumption of greater involvement of biochemical regulation than changes in gene expression.

As observed in zygotic embryos in embryogenic cultures of *A. angustifolia*, Orn levels are higher than Arg (de Oliveira *et al.*, 2017). Furthermore, conversion of the former into the latter is easy. Ornithine, even though present in small quantities in some species, may be not only the key regulator of PA biosynthesis, but also regulate the entire subset of pathways of glutamate to arginine and proline (Page *et al.*, 2007; Page *et al.*, 2012; Majumdar *et al.*, 2013; Majumdar *et al.*, 2016). On the other hand, through catabolism, arginine is an essential metabolite in nitrogen distribution, whereby it has been clearly shown that arginase, ADC and NO synthesis, can metabolize arginine to other amino acids, as well as PAs (Silveira *et al.*, 2006; Tun *et al.*, 2006; Flores *et al*, 2008; Brauc *et al.*, 2012; Shi *et al.*, 2013; Winter *et al.*, 2015). As is the case of the morphology of embryo development, the

amino acid and PA profiles of *A. angustifolia* during zygotic embryogenesis also seem to differ from those of species of the *Pinaceae* family. In *A. angustifolia*, in contrast to other conifers, low arginine levels are accumulated during embryo development. Thus, its role seems to be more PA biosynthetic than seed reserve proteins. Congruously, glutamic acid, glutamine, aspartic acid and citrulline are the main amino acids accumulated in seeds (Astarita *et al.*, 2003; de Oliveira *et al.*, 2017).

Cit contents were increased by exogenous Arg and Orn supplementation in both cell lines. The former induced a higher increase than the latter. This revealed the accumulated trend for Arg convertion into Cit/NO or PAs. As an extension of early studies on the role of Orn and Cit in regulating Arg-Orn pathways (Ramos *et al.*, 1970), and in the Glu-Orn flux (Majumdar *et al.*, 2016), our argument was in favor of the importance of not only Orn, but also Arg, in regulating the entire Orn/Arg/Cit biosynthetic pathway. The increase of Cit by Arg supplementation is particularly interesting, since in zygotic embryos of *A. angustifolia*, there was an expressive increase in Cit levels during embryogenesis (de Oliveira *et al.*, 2017), thus mimicking actual *in vivo* conditions. Furthermore, NO can be produced likewise (Flores *et al.*, 2008). PAs are biochemically related to NO through arginine, a common precursor in their biosynthetic routes, thereby implying that alteration in NO homeostasis can affect PA bioavailability and vice-versa, through a still uncharacterized mechanism (Tun *et al.*, 2006; Silveira *et al.*, 2006; Filippou *et al.*, 2013; Tanou *et al.*, 2014). The overlapping roles between PAs and NO raises the question of how both molecules act together during plant development (Tun *et al.*, 2006, Silveira *et al.*, 2006). Furthermore, along with PAs, NO has a physiological importance for embryo development in *A. angustifolia*, associated to the maintenance of polarity (embryonic-suspensor cells) in pro-embryogenic masses (Silveira *et al.*, 2006). Based in our findings, the results open a new perspective for work with species showing poor somatic embryogenesis response, by regulating NO biosynthesis mediated by Arg or Cit, in an attempt to optimize *in vitro* conditions for correct somatic-embryo development.

5. CONCLUSIONS

Taken these results together, our findings provide a new viewpoint about Put metabolism in two *A. angustifolia* cell-lines with different embryogenic capacity. Although exogenous Arg and Orn supplementation induced changes in PA levels, Arg had a greater effect on labeled precursor incorporation and gene expression related to biosynthesis and catabolism. Cell-lines producing somatic embryos responded at the gene expression level to Arg treatment. This was different with nonproductive ones. In Responsive cells, the genes of PA catabolism became active following Arg supplementation, whereas, in Blocked cells PA accumulation occurred. Further studies of enzymatic activities are required to confirm the possible roles of PA catabolism in embryogenic cultures. Arg and Orn supplementation not only changed PA levels, but also altered the entire pool of amino acids. Citrulline was the main one recorded. As its increase was mainly linked to Arg supplementation in Responsive cells, this was possibly due to higher NO metabolism. Ornithine was converted to arginine, however, conversion from Arg to Orn was low, in both cell lines. The information obtained in the present study will not only improve understanding of PA metabolism regulation, but also help as a guide in future *in vitro* testing to identify the best conditions for a somatic embryogenesis protocol of *A. angustifolia*.

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Conflict of interest

None declared.

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6. References

Andersen, S. E., Bastola, D. R., Minocha, S. C. 1998. Metabolism of Polyamines in Transgenic Cells of Carrot Expressing a Mouse Ornithine Decarboxylase cDNA. Plant Physiol. 116: 299–307.

Angelini, R., Cona, A., Federico, R., Fincato, P., Tavladoraki, P., Tisi, A. 2010. Plant amine oxidases 'on the move': an update. Plant Physiology and Biochemistry 48:560–564.

Aragão, V. P. M., Navarro, B. V., Passamani, L. Z., Macedo, A. F., Floh, E. I. S., Silveira, V., Santa-Catarina, C. 2015. Free amino acids, polyamines, soluble sugars and proteins during seed germination and early seedling growth of *Cedrela fissilis* Vellozo (Meliaceae), an endangered hardwood species from the Atlantic Forest in Brazil. Theor Exp Plant Physiol 27:157–169.

Astarita, L. V., Floh, E. I. S., Handro, W. 2003a. Changes in IAA, tryptophan and activity of soluble peroxidase associated with zygotic embryogenesis in *Araucaria angustifolia* (Brazilian pine). Plant Growth Regul 39:113–118.

Astarita, L. V., Floh, E. I. S., Handro, W. 2003b. Free amino acid, protein and water content changes associated with seed development in *Araucaria angustifolia*. Biol Plant 47:53–59.

Astarita, L. V., Handro, W., Floh, E. I. S. 2003c. Changes in polyamines content associated with zygotic embryogenesis in the Brazilian pine, *Araucaria angustifolia* (Bert.) O Ktze. Rev Bras Bot 26:163–168.

Bais, H. P., Ravinshankar, G. A. 2002. Role of polyamines in the ontogeny of plants and their biotechnological applications. Plant Cell Tissue Organ Cult. 69:1-34.

Baron, K., Stasolla, C. 2008. The role of polyamines during *in vivo* and *in vitro* development. In Vitro Celll Dev Biol Plant. 44:384-95.

Becwar, M. R., Noland, T. L., Wyckoff, J. L. 1989. Maturation, germination, and conversion of Norway spruce (*Picea abies* L.) somatic embryos to plants. In Vitro Cell Dev Biol Plant 26: 575-580.

Bhatnagar, P., Glasheen, B. M., Bains, S. K., Long, S. L., Minocha, R., Walter, C., Minocha, S. C. 2001. Transgenic Manipulation of the Metabolism of Polyamines in Poplar Cells. Plant Physiology 125:2139-2153.

Bhatnagar, P., Minocha, R., Minocha, S. C. 2002. Genetic manipulation of the metabolism of polyamines in poplar cells. The regulation of putrescine catabolism. Plant Physiol. 128:1455–1469.

Bouché, N., Fromm, H. 2004. GABA in plants: just a metabolite? Trends Plant Sci. 9:110–115.

Bradford, M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254.

Brauc, S., De Vooght, E., Claeys, M., Geuns, J. M. C., Höfte, M., Angenon, G. 2012. Overexpression of arginase in Arabidopsis thaliana influences defence responses against *Botrytis cinerea*. Plant Biology 14:39–45.

Bustin, S. A., Benes, V., Garson, J. A. *et al.* 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55:611–622.

Cheng, W. H., Wang, F. L., Cheng, Z. Q., Zhu, Q. H., Sun, Y. Q., Zhu, H. G., Sun, J. 2015. Polyamine and its metabolite H_2O_2 play a key role in the conversion of embryogenic callus into somatic embryos in upland cotton (*Gossypium hirsutum* L.). Frontiers in Plant Science 6, doi: 10.3389/fpls.2015.01063.

Cona, A., Rea, G., Angelini, R., Federico, R., Tavladoraki, P. 2006. Functions of amine oxidases in plant development and defence. Trends in Plant Science 11:80–88.

de Oliveira, L. F., Elbl, P. M., Navarro, B. V., dos Santos, A. L. W., Floh, E. I. S. 2015a. *In silico* identification and characterization of genes related to polyamines biosynthesis in embryogenic cultures of Brazilian pine. In Proceedings of the IUFRO Tree Biotechnology 2015 Conference: "Forests: the importance to the planet and society". Eds: Vettori C, Vendramin G.G., Paffetti D., Travaglini D. - doi: 10.13140/RG.2.1.4603.6882; ID: S3.P76.

de Oliveira, L. F., Elbl, P., Navarro, B. V., Macedo, A. F., Dos Santos, A. L. W., Floh, E. I. S. 2017. Elucidation of the polyamine biosynthesis pathway during Brazilian pine (*Araucaria angustifolia*) seed development. Tree Physiol 37(1):116-130, doi:10.1093/treephys/tpw107.

de Oliveira, L.F., Macedo, A.F., dos Santos, A.L.W., Floh, E.I.S. 2015b. Polyamine levels, arginine and ornithine decarboxylase activity in embryogenic cultures of *Araucaria angustifolia* (Bert.) O. Kuntze. Acta Hortic. 1083:419-425, Doi:10.17660/ActaHortic.2015.1083.54.

dos Santos, A. L. W., Elbl, P., Navarro, B. V., de Oliveira, L. F., Salvato, F., Balbuena, T. S., Floh, E. I. S. 2016. Quantitative proteomic analysis of *Araucaria angustifolia* (Bertol.) Kuntze cell lines with contrasting embryogenic potential. J Proteomics 130:180–189.

dos Santos, A. L. W., Steiner, N., Guerra, M. P., Zoglauer, K., Moerschbacher, B. M. 2008. Somatic embryogenesis in *Araucaria angustifolia*. BiolPlant, 52:195-199.

Dowlatabadi, R., A.M. Weljie, T.A. Thorpe, E.C. Yeung and H.J. Vogel. 2009. Metabolic foot printing study of white spruce somatic embryogenesis using NMR spectroscopy. Plant Physiol. Biochem. 47:343–350.

Elbl, P., Campos, R. A., Lira, B. S., Andrade, S. C. S., Jo, L., dos Santos, A. L. W, Coutinho, L. L., Floh, E. I. S., Rossi, M. 2015a. Comparative transcriptome analysis of early somatic embryo formation and seed development in Brazilian pine, *Araucaria angustifolia* (Bertol.) Kuntze. Plant Cell Tiss Organ Cult 120:903–915.

Elbl, P., Navarro, B. V., de Oliveira, L. F., Almeida, J., Mosini, A. C., dos Santos, A. L. W., Rossi, M., Floh, E. I. S. 2015b. Identification and evaluation of reference genes for quantitative analysis of Brazilian pine (*Araucaria angustifolia* Bertol. Kuntze) gene expression. PLoS One 10:1–15.

Filippou, P., Antoniou, C., and Fotopoulos, V. 2013. The nitric oxide donor sodium nitroprusside regulates polyamine and proline metabolism in leaves of *Medicago truncatula* plants. Free Radic. Biol. Med. 56:172–183. doi:10.1016/j.freeradbiomed.2012.09.037.

Floh, E. I. S., Santa-Catarina, C., Silveira, V. 2007. Marcadores bioquímicos e moleculares para estudos da morfogênese *in vitro*. Revista Brasileira de Horticultura Ornamental 13:1992-2001.

Flores, T., Todd, C. D., Tovar-Mendez, A., Dhanoa, P. K., Correa-Aragunde, N., Hoyos, M. E., Brownfield, D. M., Mullen, R. T., Lamanttina, L., Polacco, J. C. 2008. Arginase-negative mutants of *Arabidopsis* exhibit increased nitric oxide signaling in root development. Plant Physiology 147:1936–1946.

Fortes, A. M., Costa, J., Santos, F., Seguí-Simarro, J. M., Palme, K., Altabella, T., Tiburcio, A. F., Pais, M. S. 2011. Arginine decarboxylase expression, polyamines biosynthesis and reactive oxygen species during organogenic nodule formation in hop. Plant Signaling & Behavior 6:258-269.

Gemperlová, L., Fischerová, L., Cviková, M., Malá, J., Vondráková, Z., Martincová, O., Vágner, M. 2009. Polyamine profiles and biosynthesis in somatic embryo development

and comparison of germinating somatic and zygotic embryos of Norway spruce. Tree Physiol 29:1287–1298.

Jiménez-Bremont, J. F., Marina, M., Guerrero-González, M. L., Rossi, F. R., Sánchez-Rangel, D., Rodríguez-Kessler, M., Ruiz, O. A., Gárriz, A. 2014. Physiological and molecular implications of plant polyamine metabolism during biotic interactions. Front Plant Sci. 5, doi: 10.3389/fpls.2014.00095.

Jo, L., dos Santos, A. L. W., Bueno, C. A., Barbosa, H. R., Floh, E. I. S. 2014. Proteomic analysis and polyamines, ethylene and reactive oxygen species levels of *Araucaria angustifolia* (Brazilian pine) embryogenic cultures with different embryogenic potential. Tree Physiol 34:94–104.

Kevers, C., Le Gal, N., Monteiro, M., Dommes, J., Gaspar, T. 2000. Somatic embryogenesis of *Panax ginseng* in liquid cultures: a role for polyamines and their metabolic pathways. Plant Growth Regulation 31:209–214.

Klimaszewska, K., Hargreaves, C., Lelu-Walter, M., Trontin, J. 2016. Advances in Conifer Somatic Embryogenesis since year 2000. In: In Vitro Embryogenesis in Higher Plants, 1359, pp 131-166.

Klimaszewska, K., Noceda, C., Pelletier, G., Label, P., Rodriguez, R., Lelu-Walter, M. A. 2009. Biological characterization of young and aged embryogenic cultures of *Pinus pinaster* (Ait.). In Vitro Cell.Dev.Biol.-Plant 45:20–33.

Kong, L., Attree, S. M., Fowke, L. C. 1998. Effects of polyethylene glycol and methylglyoxal bis(guanylhydrazone) on endogenous polyamine levels and somatic embryo maturation in white spruce (*Picea glauca*). Plant Science 133:211–220.

Kuehn, G. D., Phillips, G. C. 2005. Roles of polyamines in apoptosis and other recent advances in plant polyamines. Critical Reviews in Plant Sciences 24:123–130.

Kusano, T., Berberich, T., Tateda, C., Takahashi, Y. 2008. Polyamines: essential factors for growth and survival. Planta 228:367–381.

Kuznetsov, V. L., and Shevyakova, N. I. 2007. Polyamines and stress tolerance of plants. *Plant Stress*. 1:50–71.

Majumdar, R., Barchi, B., Turlapati, S. A., Gagne, M., Minocha, R., Long, S., Minocha, S. C. 2016. Glutamate, ornithine, arginine, proline, and polyamine metabolic interactions: the pathway is regulated at the posttranscriptional level. Front Plant Sci 7:78.

Majumdar, R., Shao, L., Minocha, R., Long, S., Minocha, S. C. 2013. Ornithine: the overlooked molecule in the regulation of polyamine metabolism. Plant Cell Physiol 54:990–1004.

Minguet, E. G., Vera-Sirera, F., Marina, A., Carbonell, J., Blázquez, M. A. 2008. Evolutionary diversification in polyamine biosynthesis. Mol Biol Evol 25:2119–2128.

Minocha, R., Majumdar, R., Minocha, S. C. 2014. Polyamines and abiotic stress in plants: a complex relationship. Front. Plant Sci. 5, doi.org/10.3389/fpls.2014.00175.

Minocha, R., Minocha, S. C., Long, S. 2004. Polyamines and their biosynthetic enzymes during somatic embryo development in red spruce (*Picea rubens* Sarg.). In Vitro Cell. Dev. Biol.-Plant 40:572-580.

Minocha, R., Shortle, W. C., Coughlin, D. J. J., Minocha, S. C. 1996. Effects of aluminum on growth, polyamine metabolism, and inorganic ions in suspension cultures of red spruce (*Picea rubens*). Can. J. For. Res. 26:550-559.

Minocha, R., Smith, D. R., Reeves, C., Steele, K. D., Minocha, S. C. 1999. Polyamine levels during the development of zygotic and somatic embryos of *Pinus radiata*. Physiol Plant 105:155–164.

Moschou, P. N., Wu, J., Cona, A., Tavladoraki, P., Angelini, R., Roubelakis-Angelakis, K. A. 2012. The polyamines and their catabolic products are significant players in the turnover of nitrogenous molecules in plants. Journal of Experimental Botany 63:5003-5015.

Muilu-Mäkela, R., Vuosku, J., Hamberg, L., Latva-Mäenpää, H., Häggman, H., Sarjala, T. 2015. Osmotic stress affects polyamine homeostasis and phenolic content in proembryogenic liquid cell cultures of Scots pine. Plant Cell Tiss Organ Cult 122: 709-726.

Noceda, C., Salaj, T., Pérez, M., Viejo, M., Cañal, M. J., Salaj, J., Rodriguez, R. 2009. DNA demethylation and decrease on free polyamines is associated with the embryogenic capacity of *Pinus nigra* Arn. cell culture. Trees 23:1285–1293.

Page, A. F., Cseke, L. J., Minocha, R., Turlapati, S. A., Podila, G. K., Ulanov, A., Li, Z., Minocha, S. C. 2016. Genetic manipulation of putrescine biosynthesis reprograms the cellular transcriptome and the metabolome. BMC Plant Biology 16:113, doi:10.1186/s12870-016-0796-2.

Page, A. F., Minocha, R., Minocha, S. C. 2012. Living with high putrescine: expression of ornithine and arginine biosynthetic pathway genes in high and low putrescine producing poplar cells. Amino Acids 42:295–308.

Page, A. F., Mohapatra, S., Minocha, R., Minocha, S. C. 2007. The effects of genetic manipulation of putrescine biosynthesis on transcription and activities of the other polyamine biosynthetic enzymes. Physiologia Plantarum 129: 707–724.

Pieruzzi, F. P., Dias, L. L. C., Balbuena, T. S., Santa-Catarina, C., Santos, A. L. W., Floh, E. I. S. 2011. Polyamines, IAA and ABA during germination in two recalcitrant seeds: *Araucaria angustifolia* (Gymnosperm) and *Ocotea odorifera* (Angiosperm). Annals of botany (Print), v.108:337-345.

R Development Core Team. 2015. R: A language and environment for statistical computing: reference index version 2.8.0. Vienna foundation for statistical computing. Disponível em: http://www.r-project.org (15 October 2016, date last accessed).

Ramos, F., Thuriaux, P., Wiame, J. M., Bechet, J. 1970. The participation of ornithine and citrulline in the regulation of arginine metabolism in *Saccharomyces cerevisiae*. Eur. J. Biochem. 12:40–47. doi:10.1111/j.1432-1033.1970.tb00818.x.

Ruijter, J.M., Ramakers, C., Hoogaars, W. M. H., Karlen, Y., Bakker, O., Van den Hoff, M. J. B., Moorman, A. F. M. 2009. Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. Nucleic Acids Res 37:e45. doi:10.1093/nar/gkp045.

Salo, H. M., Sarjala, T., Jokela, A., Häggman, H., Vuosku, J. 2016. Moderate stress responses and specific changes in polyamine metabolism characterize Scots pine somatic embryogenesis. Tree Physiol 36:392–402.

Santa-Catarina, C., Silveira, V., Balbuena, T. S., Viana, A. M., Estelita, M. E. M., Handro, W., Floh, E. I. S. 2006. IAA, ABA, polyamines and free amino acids associated with zygotic embryo development of *Ocotea catharinensis*. Plant Growth Regul 49:237-247.

Santanen, A., Simola, L. K. 1999. Metabolism of L[U-^{14}C]-arginine and L[U-^{14}C]-ornithine in maturing and vernalised embryos and megagametophytes of *Picea abies*. Physiol Plant 107:433–440.

Shelp, B. J., Mullen, R. T., Waller, J. C. 2012. Compartmentation of GABA metabolism raises intriguing questions. Trends in Plant Science 17:57–59.

Shi, H., Ye, T., Chen, F., Cheng, Z., Wang, Y., Yang, P., Zhang, Y., Chan, Z. 2013. Manipulation of arginase expression modulates abiotic stress tolerance in *Arabidopsis*: effect on arginine metabolism and ROS accumulation. J Exp Bot 64:1367-1379.

Silveira, V., Floh, E. I. S., Handro, W., Guerra, M. P. 2004. Effect of plant growth regulators on the cellular growth and levels of intracellular protein, starch and polyamines in embryogenic suspension cultures of *Pinus taeda*. Plant Cell, Tissue and Organ Culture 76:53-60.

Silveira, V., Santa-Catarina, C., Balbuena, T. S., Morais, F. M. S., Ricart, C. A. O., Sousa, M. V., Guerra, M. P., Handro, W., Floh, E. I. S. 2008. Endogenous abscisic acid levels and comparative proteome during seed development of *Araucaria angustifolia* (Bert.) O. Ktze. Biol Plantarum 52:101-104.

Silveira, V., Santa-Catarina, C., Tun, N. N., Scherer, G. F. E., Handro, W., Guerra, M. P., Floh, E. I. S. 2006. Polyamine effects on the endogenous polyamine contents, nitric oxide release, growth and differentiation of embryogenic suspension cultures of *Araucaria angustifolia* (Bert.) O. Ktze. Plant Science 171:91-98.

Silveira, V., Vita, A. M., Macedo, A. F., Dias, M. F. R., Floh, E. I. S., Santa-Catarina, C. 2013. Morphological and polyamine content changes in embryogenic and non-embryogenic callus of sugarcane. Plant Cell Tiss Organ Cult 114:351–364.

Steiner, N., Santa-Catarina, C., Andrade, J. B. R., Balbuena, T. S., Guerra, M. P., Handro, W., Floh, E. I. S., Silveira, V. 2008. *Araucaria angustifolia* Biotechnology. Functional Plant Science and Biotechnology 2:20-28.

Tanou, G., Ziogas, V., Belghazi, M., Christou, A., Filippou, P., Job, D., Fotopoulus, J. D., Molassiotis, A. 2014. Polyamines reprogram oxidative and nitrosative status and the proteome of citrus plants exposed to salinity stress. Plant Cell Environ. 37:864–885. doi: 10.1111/pce.12204.

Tiburcio, A. F., Altabella, T., Borrell, A., Masgrau, C. 1997. Polyamine metabolism and its regulation. Physiol Plant 100:664–674.

Tun, N. N., Santa-Catarina, C., Begum, T., Silveira, V., Handro, W., Floh, E. I. S., Scherer, G. F. E. 2006. Polyamines induce rapid biosynthesis of nitric oxide (NO) in *Arabidopsis thaliana* seedlings. Plant and Cell Physiology, 47:346-354.

von Arnold, S., Sabala, I., Bozhkov, P., Dyachok, J., Filanova, L. 2002. Developmental pathways of somatic embryogenesis. Plant Cell Tissue Organ Cult 69:233-49.

Vuosku, J., Jokela, A., Läärä, E., Sääskilahti, M., Muilu, R., Sutela, S., Altabella, T., Sarjala, T., Häggman, H. 2006. Consistency of polyamine profiles and expression of arginine decarboxylase in mitosis during zygotic embryogenesis of Scots pine. Plant Physiology 142:1027-1038.

Vuosku, J., Suorsa, M., Ruottinen, M., Sutela, S., Muilu-Mäkelä, R., Julkunen-Tiitto, R., Sarjala, T., Neubauer, P., Häggman, H. 2012. Polyamine metabolismo during exponential growth transition in Scots pine embryogenic cell culture. Tree Physiol 32:1274–1287.

Winkelmann, T., Ratjens, S., Bartsch, M., Rode, C., Niehaus, K., Bednarz, H. 2015. Metabolite profiling of somatic embryos of *Cyclamen persicum* in comparison to zygotic embryos, endosperm, and testa. Front Plant Sci 6:597. doi:10.3389/fpls.2015.00597.

Winter, G., Todd, C. D., Trovato, M., Forlani, G., Funck, D. 2015. Physiological implications of arginine metabolism in plants. Front. Plant Sci. 6, doi.org/10.3389/fpls.2015.00534.

Capítulo III

Poliaminas e aminoácidos em diferentes matrizes de *Araucaria angustifolia* e sua associação com o potencial embriogênico

(Anexo III).

1. Introdução

O sucesso da embriogênese somática é afetado por fatores intra- e extracelulares (Cairney e Pulllman, 2007; Pullman e Bucalo, 2014) sendo a eficiência da embriogênese somática dependente da planta mãe (Niskanen *et al.*, 2004; Lelu-Walter *et al.*, 2008; Montalbán *et al.*, 2012). O período de coleta de sementes, estádio de desenvolvimento do embrião zigótico e as condições de cultivo são outros fatores que podem afetar a indução e eficiência dos processos de embriogênese somática (Niskanen *et al.*, 2004).

Em *Araucaria angustifolia*, a embriogênese somática é iniciada a partir do ápice de embriões zigóticos imaturos (dos Santos *et al.*, 2002). A indução de culturas embriogênicas nesta espécie é genótipo-dependente, geralmente identificada apenas nas etapas finais do processo de embriogênese somática, mais especificamente durante a maturação dos embriões somáticos. Neste estádio é possível identificar linhagens embriogênicas que evoluem no processo até o estádio pré-cotiledonar, quando submetidas a substâncias promotoras da maturação, como o ABA e agentes osmóticos (dos Santos *et al.*, 2008; Jo *et al.*, 2014). Ressalta-se que os protocolos testados e estabelecidos até o momento, para esta espécie, permitem apenas a evolução do embrião até o estádio pré-cotiledonar (dos Santos *et al*., 2002; Steiner *et al*., 2005; Jo *et al.*, 2014).

Grande parte dos esforços no desenvolvimento e otimização do protocolo de embriogênese somática para *A. angustifolia* envolvem o aperfeiçoamento da composição dos meios de cultura (Astarita e Guerra 1998; Steiner *et al.*, 2005; Silveira *et al.*, 2006; Steiner *et al.*, 2007; Jo *et al.*, 2014; Vieira *et al.*, 2012; Guerra *et al.*, 2016). Entretanto, para algumas etapas não foram totalmente estabelecidas, como por exemplo a etapa de maturação completa dos embriões somáticos (Jo *et al.*, 2014). Ressalta-se ainda, a importância da seleção precoce de genótipos aptos e competentes para a formação e evolução de embriões somáticos viáveis (Klimaszewska *et al*., 2004; Lipert *et al*., 2005; Silveira *et al*., 2008; Dowlatabadi *et al*., 2009; Jo *et al*., 2014; Elbl *et al*., 2015a; dos Santos *et al.*, 2016). Em coníferas, as taxas de indução e maturação podem ser incrementadas a partir da escolha de uma matriz, como fonte de explante, com maior potencial para embriogênese somática, como observado em *Pinus sylvestris* (Lelu *et al.*, 1999; Niskanen *et al.*, 2004; Park *et al.*, 2006; Lelu-Walter *et al.*, 2008; Aronen *et al.*, 2009). Além disso, Niskanen *et al.* (2004) destacam que o genótipo materno é considerado como o fator de maior efeito durante as etapas de indução e maturação dos embriões somáticos. Em espécies de *P. glaucia*, a fase

de indução tem forte influência do efeito genético (em torno de 69%), embora esta tenda a diminuir nas etapas de proliferação, maturação e germinação (Park *et al.*, 1998; Park, 2002).

Considerando-se as similaridades morfológicas, fisiológicas, bioquímicas e moleculares, entre a embriogênese zigótica e somática, estudos que visem compreender os perfis bioquímicos, fisiológicos e físicos nas sementes podem fornecer não somente informações para mimetizar as condições *in vitro* (Silveira *et al.*, 2008; Balbuena *et al.*, 2009, 2011; Schlögl *et al.*, 2012a, 2012b; Lara-Chavez *et al.*, 2012), mas também permitem o estabelecimento de marcadores bioquímicos para a identificação de genótipos com alta competência embriogênica (Jo *et al.*, 2014).

Neste contexto, as poliaminas (PAs) tem se apresentado como fundamental e possíveis marcadores da embriogênese zigótica e somática em diversas espécies de coníferas (Minocha *et al.*, 1999; Silveira *et al.*, 2004; Vuosku *et al*., 2006; Jo *et al*., 2014; de Oliveira *et al.*, 2017). Alterações no metabolismo das PAs podem afetar o desenvolvimento de embriões zigóticos e somáticos, especialmente quando associados às mudanças nos conteúdos entre putrescina (Put), espermidina (Spd) e espermina (Spm). Assim, é proposto a utilização das PAs como marcadores bioquímicos dos vários estádios de desenvolvimento embrionário (Minocha *et al.*, 1999; Silveira *et al*., 2006; Steiner *et al*., 2007; Santa-Catarina *et al*., 2007; Jo *et al*., 2014). Conforme proposto, diferentes potenciais embriogênicos poder ser identificadas e selecionados durante a etapa de proliferação, baseado nos perfis de PAs (Noceda *et al.*, 2009; Mauri e Manzanera, 2011; Jo *et al.*, 2014). Adicionalmente, o padrão e conteúdo de aminoácidos também reflete as alterações ao longo da embriogênese (Santa-Catarina *et al.*, 2006; de Oliveira *et al.*, 2017) e podem ser considerados com potenciais candidatos a marcadores moleculares. Assim, o presente trabalho tem como objetivo estudar a relação entre os níveis de PAs e aminoácidos, presentes nas sementes provenientes de diferentes matrizes de *A. angustifolia*, com a caracterização do potencial embriogênico. Os resultados permitirão uma melhor compreensão do metabolismo de PAs e aminoácidos, e sua possível utilização como marcadores da embriogênese somática em *A. angustifolia.* Os resultados obtidos poderão fornecer informações essenciais para a seleção de matrizes com alto potencial para culturas embriogênicas, bem como auxiliar na otimização dos protocolos de embriogênese somática para esta espécie.

2. Materiais e métodos

2.1. Material vegetal

A partir de quatro plantas matrizes foram obtidas as sementes imaturas (três cones/matriz) de *A. angustifolia*. O material foi coletado nos meses de novembro e dezembro de 2013 e 2014 e são provenientes de populações naturais dos municípios de Campos do Jordão, SP (22°44’20”S 45°35’27”W) e de Rio Negrinho, SC (26º15’16”S 49º31’06”W). Os materiais foram codificados da seguinte maneira: Matriz A (proveniente de Campos do Jordão), Matriz B, Matriz C e Matriz D (provenientes de Rio Negrinho).

2.2. Cultivo *in vitro*

2.2.1. Indução e proliferação das culturas embriogênicas

A indução das culturas embriogênicas foi realizada de acordo com Jo *et al.* (2014), com modificações. As sementes foram esterilizadas com 70% (v/v) etanol por 5 min e posteriormente em solução de 50% (v/v) de hipoclorito de sódio (2% [p/v] cloro ativo) por 25 min, seguido de três lavagens em água destilada autoclavada. Os embriões zigóticos imaturos (150/matriz) foram isolados e inoculados (um embrião/tubo de ensaio) em de meio básico BM (Gupta e Pullman, 1991) (10 ml/tubo), suplementado com 3% (p/v) de sacarose e 0,3% (p/v) Gelrite® (Sigma-Aldrich). O pH foi ajustado para 5.8 antes de adicionar o Gelrite®. As culturas foram mantidas por 45 dias no escuro, à temperatura de 25 ± 2ºC. Após esse período, os explantes que apresentaram proliferação celular constituída por massa celular branco translúcida (Figura 1), foram considerados como induzidos.

Figura 1. Aspecto morfológico de linhagem embriogênica induzida a partir de embrião zigótico imaturo de *A. angustifolia*, após 45 dias de cultivo no escuro, em meio BM (Gupta e Pullman, 1991), suplementado com 3% (p/v) de sacarose e 0,3% (p/v) Gelrite®. *Barra*: 1 cm.

As respostas foram avaliadas considerando-se a porcentagem de indução de culturas embriogênicas (CEs) e o número de embriões que formaram CEs/por matriz. Cada linhagem celular foi codificada de acordo com sua matriz de origem (A, B, C ou D) e ano de coleta (I ou II).

A proliferação das CEs foi realizada em meio de cultura MSG (Becwar *et al.*, 1989), de acordo com o Jo *et al.* (2014). Nessa etapa foram avaliados o número e a porcentagem de CEs provenientes de cada matriz que responderam ao meio de proliferação.

2.2.2. Maturação das culturas embriogênicas

Um ano após a indução, dezessete CEs oriundas de explantes inoculados em 2013, e vinte em 2014, foram submetidas à maturação. Nas superfícies das CEs foram identificadas as massas proembriogênicas (MPEs) constituídas por agregados celulares contendo células embrionárias e células do suspensor, segundo classificação de von Arnold *et al.* (2002). As MPEs com 14 dias de cultivo em meio de proliferação, foram coletadas e dissociadas em meio de cultura líquido MSG. A suspensão celular obtida foi filtrada em um funil, contendo papel filtro Whatman® nº1 (70 mm), acoplado a uma bomba de vácuo. As MPEs retidas no papel filtro foram coletadas, pesadas (100 mg de massa fresca [MF]) e diluídas em meio de cultura MSG líquido, a uma proporção de 1:10 (p/v), obtendo uma concentração final de 1 g (MF).10 ml^{-1} de meio de cultura MSG.

Posteriormente, foram inoculadas 1 ml dessa suspensão em placas de Petri (100 × 1,5 cm) contendo papel de filtro, sobreposto em 25 ml do meio de cultura de maturação. Este meio básico MSG foi suplementado com 1,46 g l^{-1} de L-glutamina, 3% (p/v) de sacarose, 0,3% (p/v) de carvão ativado, 7% (p/v) de maltose e 1% (p/v) de Gelrite®. O pH foi ajustado para 5.8 antes da adição do Gelrite®. A L-glutamina foi filtro-esterilizada e adicionada após autoclavar os meios de cultura. Após 30 dias de cultivo, os papéis filtros contendo as CEs foram transferidas para o meio de cultura fresco, de mesma constituição. Foram utilizadas quatro placas de Petri para cada linhagem celular. As culturas foram mantidas no escuro, à temperatura de 25 ± 2ºC. Após oito semanas de cultivo, as superfícies das CEs foram avaliadas morfologicamente e fotografadas. O número de embriões somáticos (ESs) por 100 mg de MPEs iniciais, bem como a frequência de ESs, foram contabilizados em cada uma das linhagens testadas. Para esta avaliação, foram contabilizados ESs em estádio II de desenvolvimento (Figura 2), seguindo a descrição morfológica estabelecida por von Arnold *et al.* (2002). As linhagens celulares apresentando embrião somático estádio II foram

classificadas como responsivas às condições de maturação, enquanto que linhagens celulares onde os embriões não se desenvolveram, foram consideradas como bloqueadas às condições de maturação, de acordo com Jo *et al.* (2014).

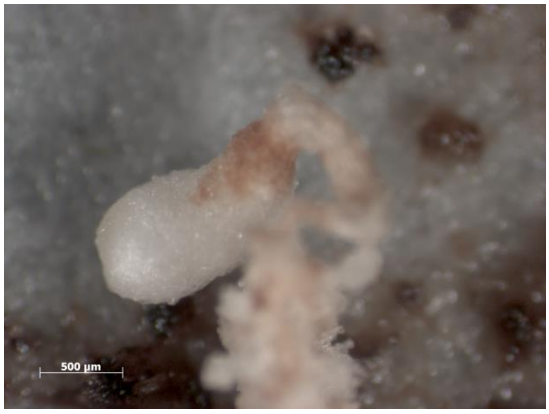


Figura 2. Desenvolvimento de um embrião somático no estádio II de *Araucaria angustifolia,* após dois meses de cultivo nas condições de maturação, em meio MSG (Becwar *et al.*, 1989) suplementado com 1,46 g l^{-1} de L-glutamina, 3% (p/v) de sacarose, 0,3% (p/v) de carvão ativado, 7% (p/v) de maltose e 1% (p/v) de Gelrite®.

2.3. Determinações bioquímicas nas sementes

Para as análises bioquímicas, os megagametófitos contendo embriões zigóticos imaturos foram pulverizados em nitrogênio líquido e armazenados a -80°C até sua utilização.

2.3.1. Perfil e conteúdo de aminoácidos

O conteúdo de aminoácidos presente no megagametófito foi determinado de acordo com a metodologia descrita por Santa-Catarina *et al.* (2006). Os megagametófitos (200 mg MF) pulverizados em nitrogênio líquido, foram homogeneizados em 6 ml de etanol 80% (v/v), e a seguir concentrados até a evaporação do etanol. O volume das amostras foi ajustado para 2 ml com água Milli-Q e centrifugadas a 20.000*g* por 10 min a 45°C. O sobrenadante foi filtrado em membrana de 0.20 µm (Sartorius®) e utilizado para determinação dos aminoácidos.

Os aminoácidos foram derivatizados com oftalaldeído-borato e identificados por cromatografia líquida de alta eficiência (CLAE) em coluna de fase reversa C_{18} (5 µm × 4.6 mm × 250 mm – Supelcosil LC-18, Sigma-Aldrich), de acordo com a metodologia descrita por Santa-Catarina *et al.* (2006). Foram utilizados como solventes: metanol 65% e uma solução de acetato de sódio 50 mM, fosfato de sódio 50 mM, metanol (20 $ml.l^{-1}$) e

tetrahidrofurano (20 $ml.l^{-1}$), com o pH de 8,6 ajustado com ácido acético glacial. O gradiente de 65% de metanol foi programado para 20% durante os primeiros 4 min, de 20 a 44% entre 4 e 11 min, 44% até 15 min, de 44 a 60% entre 15 e 43 min e 100% até 100 min, com fluxo de 1 $m.min^{-1}$, a 40°C. O detector de fluorescência (Shimadzu, RF-20A) foi ajustado para excitação em 250 nm e emissão em 480 nm.

As áreas dos picos e os tempos de retenção foram mensuradas pela comparação com quantidades conhecidas de padrões de aminoácidos: ácido aspártico (Asp), ácido glutâmico (Glu), asparagina (Asn), serina (Ser), glutamina (Gln), histidina (His), glicina (Gly), arginina (Arg), treonina (Tre), alanina (Ala), ácido γ-aminobutírico (GABA), tirosina (Tir), metionina (Met), triptofano (Trp), valina (Val), fenilalanina (Phe), isoleucina (Ile), leucina (Leu), ornitina (Orn), lisina (Lis) e citrulina (Cit). As análises foram realizadas em triplicatas biológicas.

2.3.2. Perfil e conteúdo de PAs

Para a determinação do perfil de PAs em tecidos do megagametófito foram avaliadas as PAs solúveis (livres e conjugadas) e as insolúveis em PCA. As extrações dessas substâncias foram realizadas de acordo com Silveira *et al.* (2006) e Shevyakova *et al.* (2006). Alíquotas de 200 mg MF, pulverizadas em nitrogênio líquido, foram homogeneizadas em 1,6 ml de ácido perclórico 5% (v/v), mantidas no gelo por 1h, e posteriormente centrifugadas a 14.000*g* por 20 min a 4°C. O sobrenadante, contendo as PAs livres foi coletado. O pellet e uma alíquota de 200 µl do sobrenadante obtido foram hidrolizados com HCl (12 N) por 19h a 100°C, para a obtenção das PAs conjugadas insolúveis e solúveis, respectivamente. Posteriormente, as frações contendo as PAs conjugadas solúveis e insolúveis foram secas a 40°C sob jato de nitrogênio e solubilizadas em ácido perclórico 5% (v/v) (200 µl para conjugadas solúveis e 300 µl para as insolúveis).

A derivatização das PAs foi realizada de acordo com Silveira *et al.* (2006). Alíquotas de 40 µl de amostra contendo as PAs (livres ou conjugadas) foram homogeneizadas com 100 µl de cloreto de dansil (5 $mg.ml^{-1}$ em solução de acetona), 50 µl de solução saturada de carbonato de sódio e 20 µl de 1,7-diaminoheptano (0.05 mM) (como padrão interno). As amostras foram incubadas, no escuro, por 50 min a temperatura de 70°C. Posteriormente, 25 µl de prolina (100 $mg.ml^{-1}$) foi adicionado às amostras para a retirada do excesso de dansil. Após 30 min de incubação no escuro, em temperatura ambiente, as PAs

dansiladas foram extraídas em 200 µl de tolueno. A fase do tolueno, contendo as PAs, foi coletada, secada em nitrogênio gasoso e ressuspendida em 175 µl de acetonitrila.

Os perfis de PAs foram obtidos em CLAE, em coluna de fase reversa C_{18} (5 µm × 4.6 mm × 250 mm – Supelcosil LC-18, Sigma-Aldrich). A fase móvel foi constituída de acetonitrila absoluta e acetonitrila 10% em água, com pH 3,5 ajustado com HCL 1 N. O gradiente de acetonitrila foi programado para 65%, durante os primeiros 6 min, de 65 a 100% entre 6 e 13 min, e 100% até 23 min com fluxo de 1 $ml.min^{-1}$, a 40°C. O conteúdo de PAs foi determinado usando um detector de fluorescência a 340 nm (excitação) e 510 nm (emissão). Picos de áreas e tempo de retenção foram mensurados pela comparação com concentrações conhecidas de Put, Spd e Spm. As análises foram realizadas em triplicatas biológicas.

2.4. Determinações bioquímicas nas culturas embriogênicas

2.4.1. Perfil e conteúdo de PAs

Para a determinação do perfil de PAs nas CEs, durante a fase de proliferação, foram analisadas as frações de PAs solúveis (livres e conjugadas). As extrações desse material foram realizadas de acordo com Minocha *et al.* (1994). Alíquotas, de 100 mg MF das CEs, foram homogeneizadas com 400 µl de ácido perclórico 5% (v/v) e submetidas a três ciclos de congelamento (a -20°C durante 1h30) e descongelamento (em temperatura ambiente, em torno de 3h). Posteriormente, as amostras foram centrifugadas a 14.000*g*, por 20 min a 4°C. O sobrenadante contendo as PAs livres foi coletado. As PAs conjugadas foram extraídas por hidrólise a partir deste sobrenadante, conforme descrito no item 2.3.2. A derivatização das PAs livres e conjugadas foram realizadas de acordo com Silveira *et al.* (2006). As análises foram realizadas em triplicatas biológicas.

2.5. Avaliação dos resultados

2.5.1. Caracterização morfo-histológica das culturas embriogênicas

Durante a etapa de proliferação, o grau de organização das MPEs (Figura 3) presentes nas linhagens embriogênicas foi avaliado utilizando a metodologia de dupla coloração (Gupta e Durzan, 1987), com 1% (p/v) acetocarmine e 0,05% (p/v) azul de Evans. Os resultados foram avaliados utilizando-se uma lupa SteREO Discovery. V8 (Carl Zeiss®, Göttingen, Alemanha) acoplada a uma máquina fotográfica AxioCam ICC 1 (Carl Zeiss®, Göttingen, Alemanha).

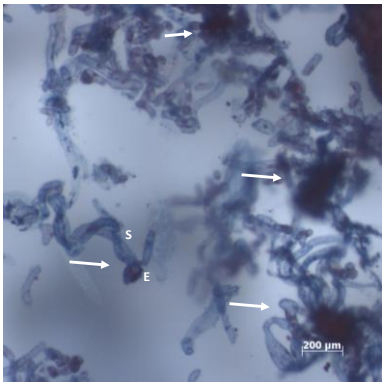


Figura 3. Aspecto morfológico de massas proembriogênicas de *A. angustifolia*, durante a etapa de proliferação, após 14 dias de cultivo em meio MSG (Becwar *et al.*, 1989) suplementado com 1,46 g l^{-1} de L-glutamina, 3% (p/v) de sacarose e 0,3% (p/v) de Gelrite®. Setas indicam massas proembriogênicas, E – cabeça embrionária, S – células do suspensor.

2.5.2. Análises estatísticas

Os dados obtidos foram analisados por ANOVA, seguido pelo teste de comparação de média (Tukey) a $P < 0.05$. Os dados foram transformados por log quando necessário. Comparações entre duas amostras foram realizadas pelo Teste *t* de Student a $P < 0.01$. Para a separação dos perfis metabólicos entre as amostras, foram utilizados os métodos de análise de componentes principais (PCA) e análise de discriminantes lineares (LDA), usando os pacotes MASS, ggplot2 e vegan. Testes de comparação de médias e análises de correlações foram feitas utilizando o software R versão 3.2.2 (R Core Team, 2015).

3. Resultados e discussão

3.1. Efeito de diferentes matrizes no estabelecimento de culturas embriogênicas

Estudos preliminares (dados não publicados) têm demonstrado que diferentes períodos de coleta das sementes, bem como a utilização de diferentes matrizes como fonte de explantes, tem proporcionado a obtenção de diferentes taxas de indução de CEs de *A. angustifolia* (dos Santos *et al.*, 2002; Steiner *et al.*, 2005). Com o objetivo de identificar marcadores entre as matrizes, associadas ao estabelecimento das CEs, neste trabalho foi realizada uma análise do perfil de PAs e aminoácidos presentes nos megagametófitos de sementes imaturas de *A. angustifolia*. A associação entre os perfis das PAs e aminoácidos e os processos de embriogênese, tem sido estudada em diversos trabalhos para o sistema *A. angustifolia* (Astarita *et al.*, 2003c; Silveira *et al.*, 2004; Silveira *et al.*, 2006; Steiner *et al.*, 2007; Jo *et al.*, 2014; de Oliveira *et al.*, 2017). As alterações nos conteúdos de Put, Spd e Spm, que ocorrem durante as diferentes fases de desenvolvimento *in vivo* dos embriões zigóticos, sugerem a possibilidade de sua utilização como marcadores bioquímicos da diferenciação celular (Astarita *et al.*, 2003c; Silveira *et al.*, 2004). Nos estádios iniciais da embriogênese zigótica de coníferas, geralmente é marcada com maiores níveis de Put, enquanto que nos estádios tardios de desenvolvimento, os níveis de Spd e Spm são maiores em relação aos níveis de Put (Minocha *et al.*, 1999; Astarita *et al.*, 2003c; Silveira *et al.*, 2004; de Oliveira *et al.*, 2017). Adicionalmente, em culturas embriogênicas de *A. angustifolia*, os níveis de PAs foram descritos com uma possível relação ao potencial embriogênico (Jo *et al.*, 2014). Uma menor razão Put/(Spd+Spm) está associada ao desenvolvimento dos embriões somáticos em linhagens com alto potencial embriogênico, comparado à linhagens bloqueadas, que apresentam uma maior razão (Jo *et al.*, 2014).

No presente trabalho, os perfis de PAs e aminoácidos foram selecionados como parâmetros bioquímicos a serem avaliados nos megagametófitos imaturos. Levando-se em consideração a abundância de dados gerados dos perfis bioquímicos das sementes provenientes das coletas de 2013 e 2014 (Tabelas suplementares S1 e S2), optou-se por utilizar análises multidimensionais. Para esta avaliação, foram utilizados os métodos de análise de componentes principais (PCA) e análise de discriminantes lineares (LDA), que permitem sumarizar e identificar particularidades existentes em cada matriz (Businge, 2014), baseado nos perfis de PAs e aminoácidos presentes no megagametófito das sementes imaturas.

Levando-se em conta os eixos com maior captura da variação (PC1 e LD1), foi possível classificar as matrizes em quadrantes distintos. Nas sementes coletadas em dezembro de 2013, o componente principal (PC) 1, explicando 56,4% da variação (Figura 4A), e o discriminante linear (LD) 1, explicando 95,8% (Figura 4B), mostram claramente a distinção da matriz A (Campos do Jordão, SP) das matrizes B, C e D (Rio Negrinho, SC). Em sementes de 2014, os perfis de PAs e aminoácidos presentes nas sementes novamente apresentaram uma distinção entre as matrizes analisadas, baseado nas análises de PCA e LDA, os quais os eixos PC1 e PC2, em conjunto, explicaram 71,7% da variação, e o eixo LD1 explicou 61,4% da variação (Figura 4C e 4D). A matriz C não apresentou formação de sementes com embriões zigóticos imaturos em 2014 e, portanto, não foi incluída nessas análises.

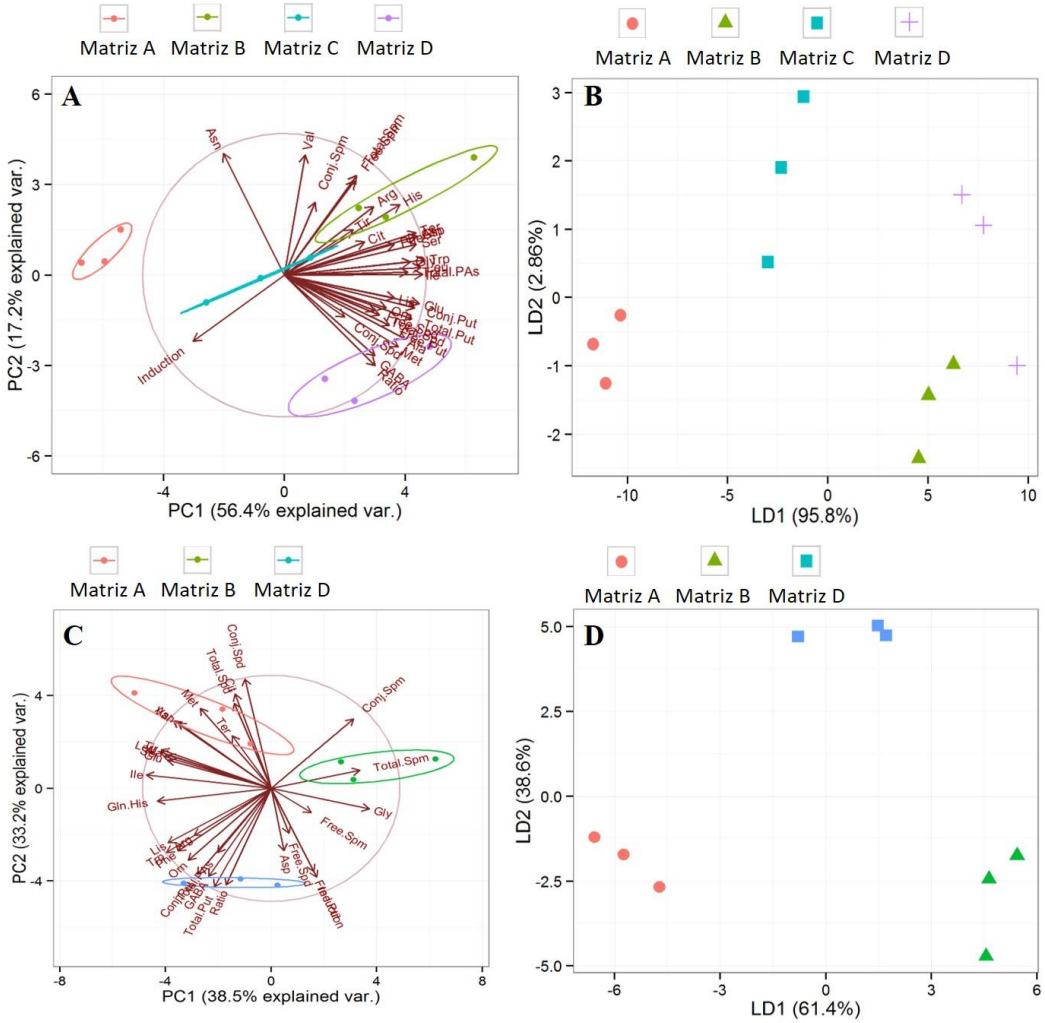


Figura 4. Análise de componentes principais (**A, C**) e discriminante linear (**B, D**) dos perfis de PAs e aminoácidos em sementes imaturas de *A. angustifolia*, coletadas em dezembro de 2013 (**A, B**) e 2014 (**C, D**).

A comparação entre os dados dos dois anos de coleta, identificou-se que os metabólitos que mais contribuíram para a separação das matrizes em 2013 (arginina, histidina, GABA, metionina, PAs totais, Spm total, Put livre e Put/(Spd+Spm) foram diferentes daqueles observados em 2014 (asparagina, aspartato, citrulina, metionina, valina, Spd e Spm totais, Put conjugada e Put/(Spd+Spm). Esses dados demonstram as variações dos perfis de PAs e aminoácidos associados a cada matriz, ocorrentes em cada ano. No presente estudo as sementes foram coletadas em populações naturais de *A. angustifolia*, portanto, essa variação pode ser decorrente das alterações no clima ou solo, uma vez que as PAs são moléculas que respondem à diversos tipos de estresse biótico e abiótico (Minocha *et al.*, 2014; Jiménez-Bremont *et al.*, 2014). Para uma melhor compreensão da influência destes fatores, um estudo mais aprofundado deve ser realizado.

Os metabólitos indicados pelos vetores na análise de PCA foram submetidos à análise de variância, e comparados entre matrizes. Para esta análise, duas matrizes foram selecionadas (matriz A e D), as quais apresentaram indução, proliferação e maturação nos dois anos de coleta e com diferentes respostas na caracterização do potencial embriogênico. Essa análise revelou que a Put total (livres e conjugadas), ornitina e asparagina apresentaram diferenças significativas ($P < 0.01$) entre as duas matrizes analisadas (Figura 5).

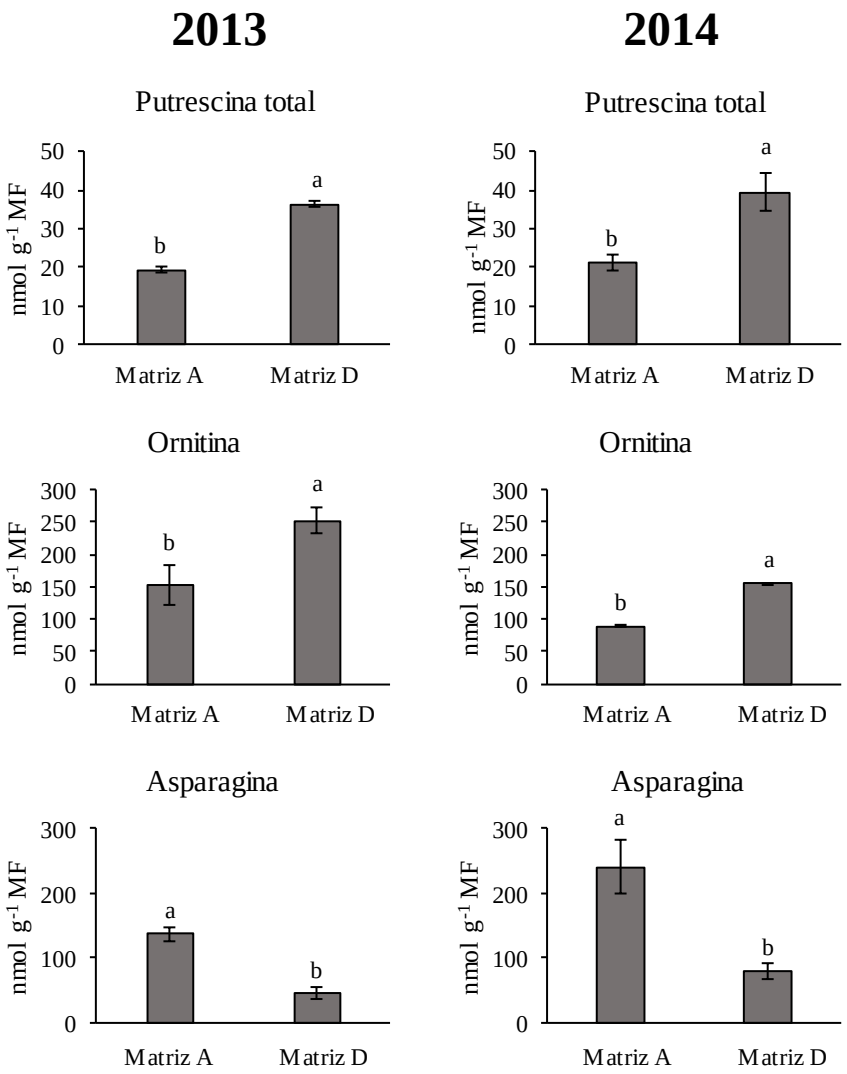


Figura 5. Conteúdos de putrescina total (livre e conjugada), ornitina e asparagina, presentes em megagametófitos de sementes imaturas de duas matrizes de *A. angustifolia*. Os metabólitos foram selecionados previamente com base na análise de PCA/LDA (figura 1) e que fossem diferencialmente expressos nos dois anos de coleta das sementes. Para esta análise foram selecionadas as matrizes A e D, pois foram as únicas que apresentaram linhagens induzidas, proliferadas e testadas na maturação nos dois anos de coleta. Letras diferentes indicam diferenças significativas de acordo com o teste "*t*" de Student ($P < 0.01$).

Em megagametófitos da matriz A, o conteúdo de Put total foi menor em relação a matriz D (Figura 5). Esta PA tem sido reportada como a mais abundante poliamina em embriões zigóticos imaturos de coníferas como *P. taeda* (Silveira *et al.*, 2004), *P. sylvestris* (Vuosku *et al.*, 2006) e *A. angustifolia* (Astarita *et al.*, 2003c; de Oliveira *et al.*, 2017). Em plantas, a Put está associada ao ciclo celular e a divisão mitótica, modula a expressão de enzimas, como as peroxidases e outras proteínas relacionadas, além de ser substrato para a

biossíntese de Spd e Spm (de Oliveira *et al.*, 2017; Reis *et al.*, 2016). Adicionalmente, a Put tem se mostrado como um importante marcador molecular para a identificação de linhagens celulares com capacidade embriogênica (Jo *et al.*, 2014) onde altos conteúdos desta substância foram associados com a incapacidade das células em desenvolver embriões somáticos (Noceda *et al.*, 2009; Mauri e Manzanera, 2011; Jo *et al.*, 2014).

Da mesma forma, para a ornitina foram observados menores conteúdos na matriz A (Figura 5). A ornitina é um aminoácido que não é incorporado às proteínas, sintetizado a partir da via do glutamato, consistindo o ponto de maior entrada de nitrogênio inorgânico em plantas (Slocum, 2005; Minocha *et al.*, 2014). Esse aminoácido é um metabólito intermediário na via de biossíntese da arginina, de onde são derivados outros compostos tais como a prolina, citrulina e PAs, conhecidos como osmoprotetores (Kalamaki *et al.*, 2009; Page *et al.*, 2012; Majumdar *et al.*, 2013; de Oliveira *et al.*, 2017). A ornitina também pode ser convertida em Put através da ação da enzima ornitina descarboxilase (ODC) (Bais e Ravishankar, 2002). Entretanto, em sementes de *A. angustifolia*, a baixa atividade da ODC indica que esta pode ser utilizada em sua maior parte na biossíntese de arginina (de Oliveira *et al.*, 2017). Em *Cryptomeria japonica*, a ornitina foi descrita como um fator chave na regulação do desenvolvimento das MPEs em embriões somáticos (Nakagawa *et al.*, 2006). Entretanto, estudos associando os perfis de ornitina nas sementes à capacidade embriogênica não foram desenvolvidos.

A asparagina foi mais presente na matriz A em relação a matriz D (Figura 5). Em plantas, a asparagina é normalmente associada com o transporte de nitrogênio e seus conteúdos podem ser regulados pela luz (Buchanan *et al.*, 2000; Cangahuala-Inocente *et al.*, 2014). A asparagina também acumula sob condições de estresse e contribui para a manutenção da pressão osmótica (Lea *et al.*, 2007). Embora na literatura não tenham sido encontrados estudos sobre a associação entre os conteúdos de asparagina em sementes com os processos de embriogênese somática, a aplicação exógena deste aminoácido ao meio de cultura incrementou o desenvolvimento dos embriões somáticos de algumas espécies, como *Triticum aestivum*, onde atuou como um doador de nitrogênio orgânico (Sarker *et al.*, 2007).

Desta forma, os resultados apresentados podem propor a Put total, ornitina e a asparagina como possíveis marcadores bioquímicos para a seleção de matrizes com maior potencial para a embriogênese. A importância da composição do megagametófito no processo de embriogênese somática em *A. angustifolia*, foi avaliada verificando-se as taxas de indução, proliferação e maturação *in vitro*, provenientes das quatro matrizes analisadas.

Verificou-se que a matriz A apresentou a maior taxa de indução (14%) em 2013, enquanto que em 2014 as matrizes B e D apresentaram taxa acima de 44% de indução (Tabela 1). Para o ano de 2014, não foi possível isolar o embrião zigótico imaturo dentro do megagametófito de sementes da matriz C.

Tabela 1. Efeito da matriz e ano de coleta na taxa de indução de culturas embriogênicas provenientes de embriões zigóticos imaturos de *Araucaria angustifolia*.

Matriz	**Taxa de indução de culturas embriogênicas**	
	2013	**2014**
A	14% a	11% b
B	7% b	48% a
C	7% b	-
D	11% ab	44% a

Letras diferentes indicam diferenças significativas de acordo com teste de Tukey ($P \leq 0,05$). (Média ± desvio padrão, n=150).

A indução da embriogênese somática em coníferas é fortemente influenciada pelo genótipo da planta matriz (Klimaszewska *et al.*, 2007), e esse fator tem sido demonstrado nas espécies de coníferas *Picea glauca*, *Pinus pinaster*, *P. sylvestris*, *P. pinea*, *P. strobus* e *P. taeda* (Park *et al.*, 1993; Bercetche e Pâques, 1995; Garin *et al.*, 1998; Lelu *et al.*, 1999; Klimaszewska *et al.*, 2001; Miguel *et al.*, 2004; Niskanen *et al.*, 2004; Lelu-Water *et al.*, 2006; MacKay *et al.*, 2006; Carneros *et al.*, 2009; Klimaszewska *et al.*, 2016). Em muitos programas de melhoramento, cruzamentos controlados são realizados para obtenção de explantes favoráveis para iniciar a embriogênese somática (Klimaszewska *et al.*, 2001; Klimaszewska *et al.*, 2007). A seleção de uma planta matriz eficiente permite um aumento de 1,5 a 9 vezes na frequência de indução de culturas embriogênicas (Häggman *et al.*, 1999; Klimaszewska *et al.*, 2016).

A utilização de diferentes matrizes como fonte de embriões zigóticos imaturos permitiu obter uma taxa de indução das CEs de até 48% (matriz B, em 2014). Esse efeito também foi observado em estudos anteriores com *A. angustifolia*, nos quais foram obtidas taxas de indução variando entre 9% a 55%, utilizando diferentes matrizes (dos Santos *et al.*, 2002; Silveira *et al.*, 2002).

O ano de coleta das sementes influenciou as taxas de indução de CEs em *A. angustifolia*, variando de 7% a 11% em 2013, e de 11% a 48% em 2014, entre as diferentes

matrizes testadas (Tabela 1). Em *P. pinaster* e *P. pinea*, a frequência de indução das CEs também foi dependente do ano de coleta das sementes (Bercetche e Pâques, 1995; Carneros *et al.*, 2009). Por outro lado, em *Larix* × *eurolepis* o ano de coleta dos embriões não teve qualquer influência na taxa de indução das CEs (Lelu-Walter *et al.*, 2009).

Para as CEs de 2013, durante a etapa de proliferação, a matriz A apresentou maior porcentagem de linhagens (52,4%) que proliferaram em meio de cultura MSG, seguido das matrizes D (25%) e C (20%) (Tabela 2). Linhagens proveniente da matriz B necrosaram em meio de proliferação. Para as CEs induzidas em 2014, as matrizes B (74%) e D (73%) apresentaram as maiores porcentagens de linhagens que proliferaram em meio MSG, seguido das linhagens provenientes da matriz A (62%).

Tabela 2. Efeito da matriz e do ano de coleta das sementes na proliferação de culturas embriogênicas de *A. angustifolia* em meio de cultura líquido.

Matriz	**Frequência de linhagens celulares que proliferam em meio MSG**	
	2013	**2014**
A	52.4%	62.0%
B	0	74.0%
C	20.0%	0
D	25.0%	73.0%

Após a etapa de proliferação, as linhagens celulares foram testadas nas condições de maturação para a avaliação do potencial embriogênico. Dentre as linhagens testadas do ano de 2013, aquelas provenientes da matriz A apresentou maior frequência (72%) de embriões somáticos estádio II, seguido das matrizes D (50%) e C (50%) (Tabela 3). Para o ano de coleta de 2014, a matriz B apresentou a maior porcentagem (75%) de linhagens que formaram embriões somáticos, seguido pelas matrizes D e A (Tabela 3).

Tabela 3. Efeito da matriz e do ano de coleta das sementes na maturação das linhagens celulares de *A. angustifolia*, após 60 dias de cultivo em meio de maturação MSG.

Matriz	Frequência de linhagens celulares[1] que apresentaram a formação de embriões somáticos no estádio II	
	2013	**2014**
A	72%	75%
B	-	88%
C	50%	-
D	50%	50%

[1]Número de linhagens testadas em: 2013 - 11 (matriz A), 2 (matriz C) e 4 (matriz D); 2014 – 4 (matriz A), 8 (matriz B) e 8 matriz (D).

Nesta etapa, observou-se uma maior influência da matriz e menor do ano de coleta das sementes na identificação de linhagens responsivas. Uma vez que o genótipo materno tem sido considerado a de maior consequência para a etapa de indução de culturas de coníferas, como é o caso de *P. sylvestris* (Niskanen *et al.*, 2004), a utilização de diferentes matrizes pode aumentar a porcentagem de indução de culturas embriogênicas em *A. angustifolia* (dos Santos *et al.*, 2002). Embora esse efeito seja reduzido durante as etapas de proliferação, maturação e germinação, como observado em *P. glaucia* (Park *et al.*, 1998; Park, 2002), esse fator ainda constitui uma vantagem devido ao alto número de linhagens induzidas a serem obtidas para futuros testes na etapa de maturação.

3.2. Correlação entre as matrizes, perfis de PAs e o potencial embriogênico das linhagens estabelecidas

Dentre as 37 linhagens testadas no meio de maturação, foi possível observar diferentes respostas na formação de embriões somáticos estádio II (Figura 6). As linhagens foram classificadas de acordo com Jo *et al.* (2014), em: responsivas aos agentes de maturação: aquelas que apresentaram a formação de embriões somáticos estádio II (Figura 6A); não-responsivas aos agentes de maturação: aquelas que não apresentaram a formação de embriões somáticos e/ou necrosaram quando submetidas à maturação (Figura 6B).

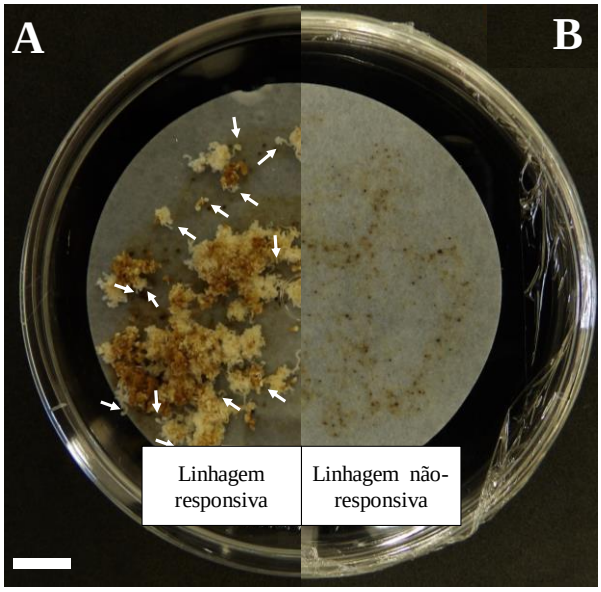


Figura 6. Aspectos de duas linhagens celulares de *A. angustifolia*, com diferentes potenciais embriogênicos, após dois meses em meio de maturação: (**A**) Linhagem AI.02, responsiva aos agentes da maturação, e (**B**) Linhagem DI.01, não-responsiva aos agentes de maturação. Setas indicam regiões onde há a formação de embriões somáticos estádio II. *Barra:* 1 cm.

A frequência na formação dos embriões somáticos (estádio II) de *A. angustifolia* foi dependente da linhagem celular (Tabela 4), um fenômeno comum observado para coníferas (Högberg *et al.*, 1998; Park, 2002; Lelu-Walter *et al.*, 2006; 2008; Jo *et al.*, 2014). De um total de 37 linhagens testadas no meio de maturação, 11 linhagens de 2013 e 14 linhagens de 2014 apresentaram a formação de embriões somáticos estádio II (Tabela 4), após oito semanas de cultivo no meio de maturação. Dentre as linhagens induzidas em 2013, a linhagem AI.02 (Figura 6A) apresentou a maior média com 45,5 embriões somáticos (por 100 mg de MPE) no estádio II de desenvolvimento. Em contrapartida, a linhagem DI.01 (Figura 6B) não apresentou o desenvolvimento de embriões somáticos, seguido de necrose total das células (Tabela 4). Resultados similares foram observados em CEs provenientes de genótipos com o desenvolvimento bloqueado de *P. abies* (Smertenko *et al.*, 2003; Stasolla *et al.*, 2004) e de *A. angustifolia* (Jo *et al.*, 2014). Para os genótipos induzido em 2014, seis genótipos apresentaram número médio de embriões somáticos globulares acima de 20, sendo a linhagem BII.075 com a maior média de embriões somáticos globulares formados (48,3) no meio de maturação (Tabela 4).

Tabela 4. Efeito do genótipo das linhagens celulares na frequência de formação de embriões somáticos globulares de *A. angustifolia* em meio de maturação.

	2013			2014		
	Linhagem celular	**NES[1].100 mg MPE[2]**	**Frequência de ESs[3] estádio II**	**Linhagem celular**	**NES[1].100 mg MPE[2]**	**Frequência de ESs[3] estádio II**
Matriz A	AI.02	**45,5**	**100%**	AII.130	24,5	100%
	AI.04	0	0	AII.064	4,0	100%
	AI.05	0,5	25%	AII.077	14,0	100%
	AI.06	8,8	100%	AII.091	0	0
	AI.07	0,5	50%	*	*	*
	AI.08	8,5	100%	*	*	*
	AI.12	0	0	*	*	*
	AI.14	10,3	100%	*	*	*
	AI.15	0,3	25%	*	*	*
	AI.16	0,3	25%	*	*	*
	AI.17	0	0	*	*	*
Matriz B	*	*	*	BII.123	4,3	100%
	*	*	*	BII.144	0	0
	*	*	*	BII.150	26,7	100%
	*	*	*	BII.020	4,5	75%
	*	*	*	BII.029	4,0	100%
	*	*	*	BII.047	1,8	100%
	*	*	*	BII.075	**48,3**	**100%**
	*	*	*	BII.078	28,7	100%
Matriz C	CI.03	0,8	75%	*	*	*
	CI.05	0	0	*	*	*
Matriz D	DI.01	0	0	DII.100	0	0
	DI.03	0,5	25%	DII.012	0	0
	DI.04	15,0	100%	DII.125	29,8	100%
	DI.09	0	0	DII.140	0	0
	*	*	*	DII.144	22,3	100%
	*	*	*	DII.033	0	0
	*	*	*	DII.037	2,3	100%
	*	*	*	DII.097	0,3	25%

[1]NES: número médio de embriões somáticos por 100 mg de MPE[2]: massas proembriogênicas; [3]ESs: embriões somáticos; *Amostra não efetivada.

Após a caracterização do potencial embriogênico, as respostas observadas durante a etapa de maturação (número de embriões somáticos estádio II por 100 mg de MPE) foram correlacionados com os perfis de PAs obtidos durante a etapa de proliferação. Para isso, os conteúdos de Put, Spd e Spm das linhagens celulares foram determinados após 14 dias de cultivo em meio de proliferação MSG. Para essa análise multivariada, os dados obtidos

(Tabelas suplementares S3 e S4) foram submetidos a análise LDA e agrupamento hierárquico, através da construção de um dendograma.

Os resultados apresentam três grupos de linhagens celulares com características distintas, baseado no perfil de PAs e respostas ao meio de maturação (Figuras 7 e 8). Esses grupos de linhagens foram classificados como: Classe I – linhagens altamente responsivas (> cinco embriões por 100 mg de MPE), Classe II – linhagens com baixa resposta (≤ cinco embriões por 100 mg de MPE) e, Classe III – linhagens que não respondem às condições de maturação.

Para as culturas embriogênicas induzidas em 2013, as linhagens que foram classificadas como Classe I foram separadas das Classes II e III, baseado no LD1 (que explica 94,2% da variação) (Figura 7A). Dentre as linhagens da Classe I, 80% (4) delas eram provenientes de sementes da matriz A, e 20% (1) da matriz D (Figura 7B).

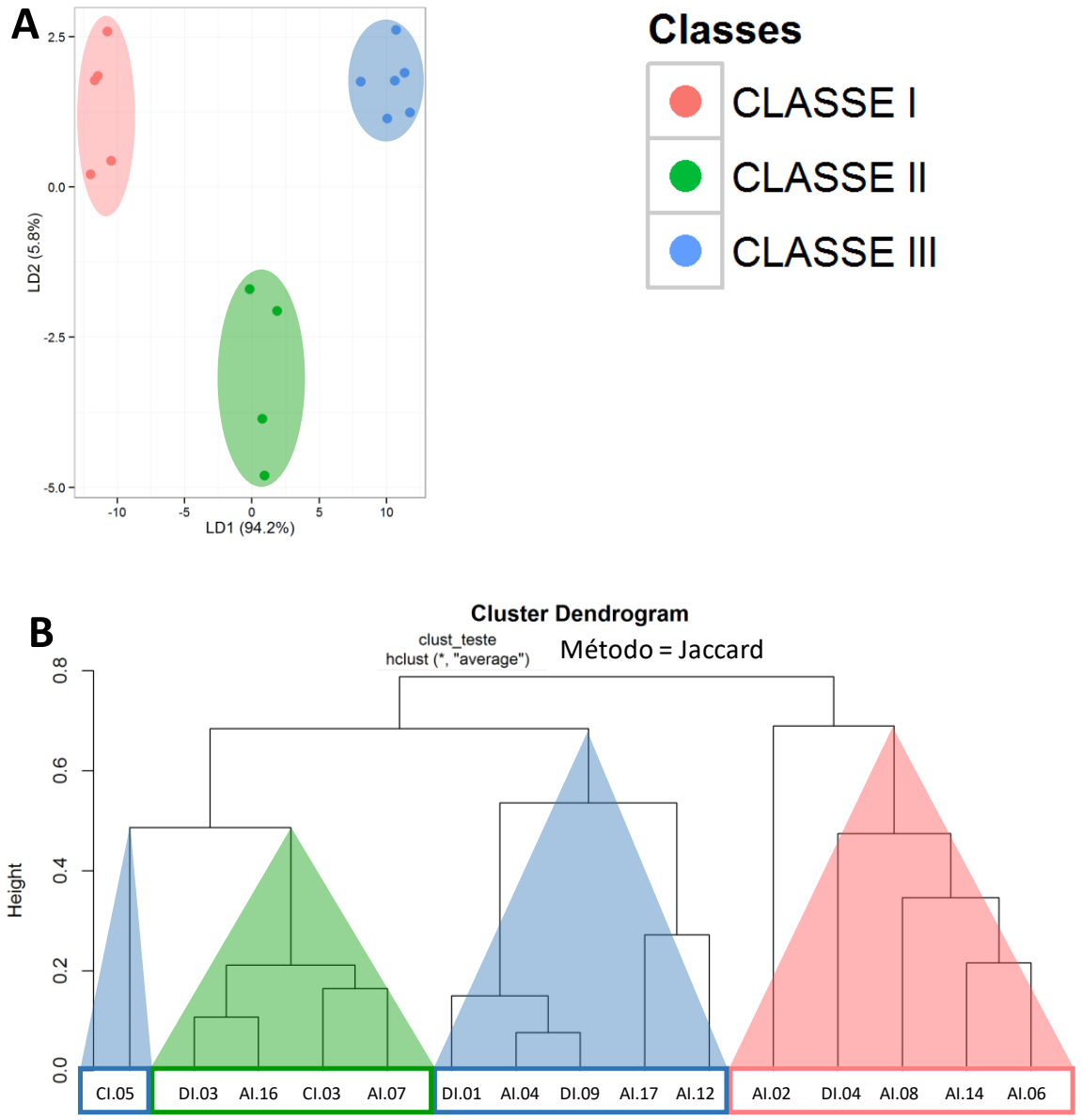


Figura 7. Análise multivariada do perfil de PAs (livres e conjugadas) e sua relação com o potencial embriogênico em linhagens embriogênicas de *A. angustifolia*, induzidas em dezembro de 2013. **A** – Análise de discriminante linear, **B** – Dendograma por clusterização. As linhagens embriogênicas foram classificadas de acordo com sua resposta aos agentes da maturação (formação de embriões somáticos globular): **Classe I** – altamente responsiva, **Classe II** – baixa resposta, **Classe III** – não respondem.

Em linhagens celulares provenientes das sementes coletadas em dezembro de 2014, também foi possível observar uma clara separação entre as linhagens de Classe I, II e III, baseado no LD1 explicando 69,1% da variação (Figura 8A). Entre as linhagens presentes na Classe I, 42,8% (3) eram provenientes da matriz B, 28,6% (2) da matriz D, e outros 28,6%

(2) da matriz A (Figura 8B). Nas linhagens da Classe III, as quais não formaram embriões somáticos em meio de maturação, cinco linhagens eram provenientes da matriz D.

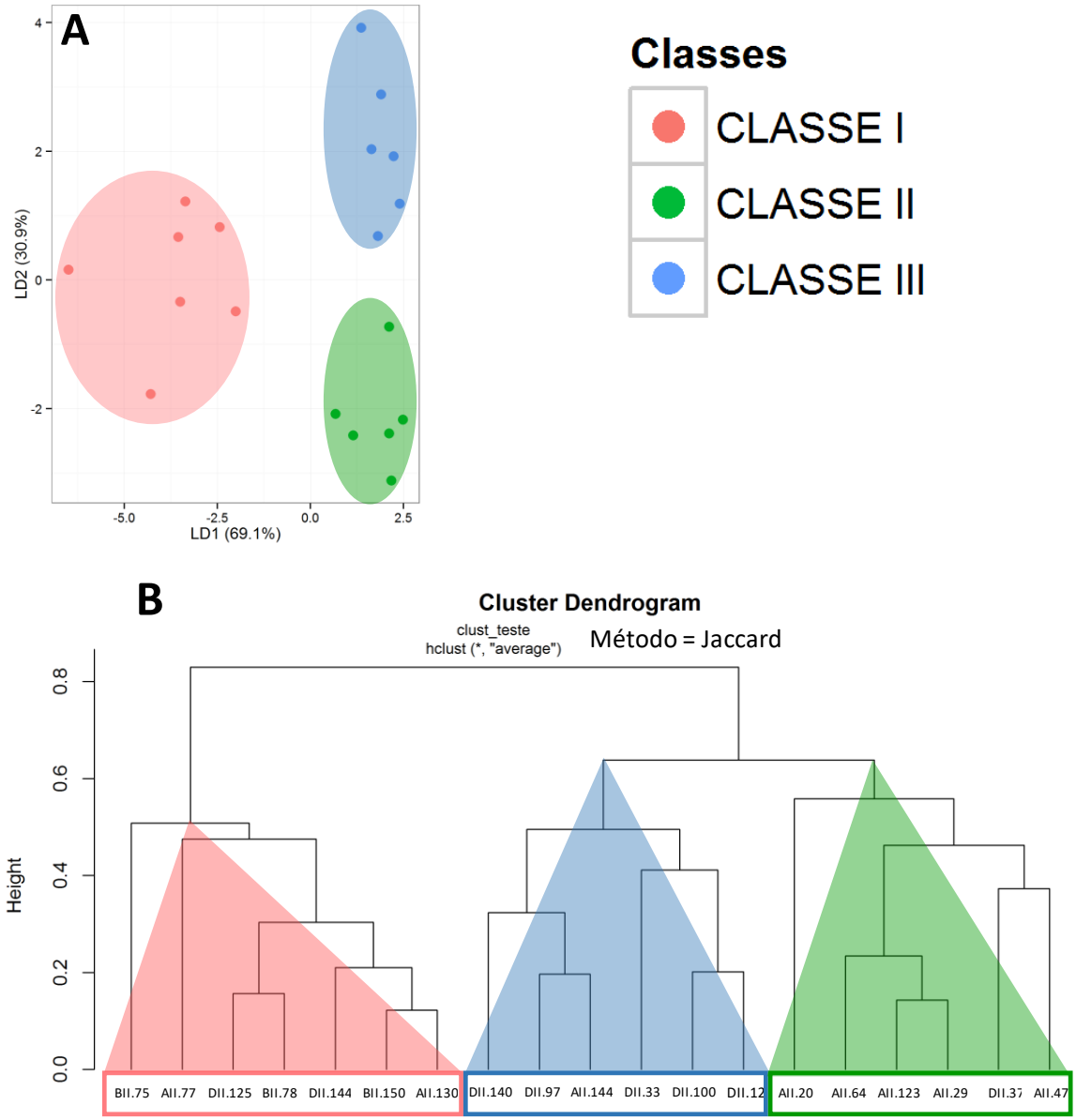


Figura 8. Análise multivariada do perfil de PAs (livres e conjugadas) e sua relação com o potencial embriogênico em linhagens embriogênicas de *A. angustifolia*, induzidas em dezembro de 2014. **A** – análise de discriminante linear, **B** – Dendograma por clusterização. As linhagens embriogênicas foram classificadas de acordo com sua responsividade aos agentes da maturação (formação de embriões somáticos globular): **Classe I** – altamente responsiva, **Classe II** – baixa resposta, **Classe III** – não respondem.

A partir desta análise, foi possível observar o efeito das matrizes juntamente com o perfil de PAs das CEs que apresentam diferentes potenciais embriogênicos. Os resultados

obtidos reforçam a importância dos fatores genéticos e bioquímicos na competência para a embriogênese somática (Cairney e Bucalo, 2007). A escolha de diferentes matrizes, como observado, pode aumentar as taxas de indução e maturação, como observado em *Pinus sylvestris* (Lelu *et al.*, 1999, Niskanen *et al.*, 2004, Park *et al.*, 2006, Lelu-Walter *et al.*, 2008, Aronen *et al.*, 2009). Além disso, o perfil de PAs presente nas linhagens celulares também contribuíram para uma variação nas respostas ao meio de maturação. De acordo com Jo *et al.* (2014), menores níveis de Put em relação à Spd e Spm em CEs de *A. angustifolia* são possíveis indicadores de um maior potencial embriogênico. Esse fato pode ser explicado devido as PAs estarem associadas ao desenvolvimento dos embriões em plantas onde a Put está relacionada à divisão celular, enquanto que a Spd e Spm estão associados à diferenciação celular e a formação de embriões somáticos (Niemi *et al.*, 2006; Silveira *et al.*, 2006; Santa-Catarina *et al.*, 2007).

4. Conclusões

No presente estudo, foi avaliado o perfil bioquímico do megagametófito das sementes imaturas de diferentes matrizes de *A. angustifolia* e sua associação com o estabelecimento de CEs. A análise dos perfis de PAs e aminoácidos dos megagametófitos revelaram que as matrizes são diferentes entre si, e essas diferenças foram refletidas durante as etapas de indução, proliferação e maturação das CEs. Dentre as CEs estabelecidas, as linhagens com maior potencial embriogênico são provenientes de matrizes que também apresentaram maior porcentagem de indução e proliferação e maturação, sugerindo haver uma correlação entre a escolha da matriz e a porcentagem de linhagens que formam embriões somáticos no meio de maturação. Os perfis de três metabólitos (Put total, ornitina e asparagina) do megagametófito foram associados à diferentes taxas de maturação nas matrizes A e D, indicando que estes compostos podem ser utilizados como marcadores bioquímicos para a seleção de matrizes com potencial embriogênico. Entretanto, futuras análises deverão ser realizadas a fim de estabelecer uma correlação entre essas duas variáveis. As abordagens utilizadas neste trabalho, ou seja, a análise dos perfis de PAs e aminoácidos das matrizes e sua associação com as respostas observadas *in vitro*, são fundamentais para a validação e o estabelecimento de potenciais marcadores bioquímicos durante a seleção de matrizes com alto potencial embriogênico no sistema *A. angustifolia*.

5. Referências bibliográficas

Aronen, T., Pehkonen, T., Ryynänen, L. 2009. Enhancement of somatic embryogenesis from immature zygotic embryos of *Pinus sylvestris*. Scandinavian Journal of Forest Research 24:372-83.

Astarita, L. V., Floh, E. I. S., Handro, W. 2003b. Free amino acid, protein and water content changes associated with seed development in *Araucaria angustifolia*. Biol Plant 47:53–59.

Astarita, L. V., Guerra, M. P. 1998. Early somatic embryogenesis in *Araucaria angustifolia*– induction and maintenance of embryonal-suspensor mass culture. Rev. Bras. Fisiol. Veg 10:113-118.

Astarita, L. V., Handro, W., Floh, E. I. S. 2003c. Changes in polyamines content associated with zygotic embryogenesis in the Brazilian pine, *Araucaria angustifolia* (Bert.) O Ktze. Rev Bras Bot 26:163–168.

Bais, H. P., Ravinshankar, G. A. 2002. Role of polyamines in the ontogeny of plants and their biotechnological applications. Plant Cell Tissue Organ Cult. 69:1-34.

Balbuena, T. S., Silveira, V., Junqueira, M., Dias, L. L. C., Santa-Catarina, C., Shevchenko, A., Floh, E. I. S. 2009. Changes in the 2-DE protein profile during zygotic embryogenesis in the Brazilian Pine (*Araucaria angustifolia*). Journal of Proteomics 72:337-352.

Becwar, M. R., Noland, T. L., Wyckoff, J. L. 1989. Maturation, germination, and conversion of Norway spruce (*Picea abies* L.) somatic embryos to plants. In Vitro Cell Dev Biol Plant 26: 575-580.

Bercetche, J., Pâques, M. 1995. Somatic embryogenesis in maritime pine (*Pinus pinaster*). In: Jain, S. M., Gupta, P. K., Newton, R. J. (eds) Somatic embryogenesis in woody plants. Gymnosperms, vol 3. Kluwer Academic Publishers, Dordrecht, Netherlands, pp 221–242.

Buchanan, B., Groissem, W., Jones, R. 2000. Biochemistry and Biology of Plants. In: Coruzzi, G., Last, R. (eds) Amino Acids. American Society of Plant Physiologists. pp. 358-410.

Businge, E. 2014. Regulation of metabolic events during embryo development in Norway Spruce (*Picea abies* L. Karst). Tese de doutorado. Faculty of Forest Sciences, Swedish University of Agricultural Sciences, Umeå, Sweden, 72p.

Cairney, J., Pullman, G. S. 2007. The cellular and molecular biology of conifer embryogenesis. New Phytologist 176:511–536.

Cangahuala-Inocente, G. C., Silveira, V., Caprestano, C. A., Floh, E. I. S., Guerra, M. P. 2014. Dynamics of physiological and biochemical changes during somatic embryogenesis of *Acca sellowiana*. In Vitro Cell.Dev.Biol.—Plant 50:166–175.

Carneros, E., Celestino, C., Klimaszewska, K., Park, Y. S., Toribio, M., Bonga, J. M. 2009. Plant regeneration in Stone pine (*Pinus pinea* L.) by somatic embryogenesis. Plant Cell Tiss Organ Cult 98:165–178.

de Oliveira, L. F., Elbl, P., Navarro, B. V., Macedo, A. F., Dos Santos, A. L. W., Floh, E. I. S. 2017. Elucidation of the polyamine biosynthesis pathway during Brazilian pine (*Araucaria angustifolia*) seed development. Tree Physiol 37(1):116-130, doi:10.1093/treephys/tpw107.

dos Santos, A. L. W., Silveira, V., Steiner, N., Vidor, M., Guerra, M. P. 2002. Somatic embryogenesis in Parana Pine (*Araucaria angustifollia* (Bert.) O. Kuntze). Braz. Arch. Biol. Technol. 45:97-106.

dos Santos, A. L. W., Steiner, N., Guerra, M. P., Zoglauer, K., Moerschbacher, B. M. 2008. Somatic embryogenesis in *Araucaria angustifolia*. Biol. Plant. 52:195-199.

Garin, E., Isabel, N., Plourde, A. 1998. Screening of large numbers of seed families of *Pinus strobus* L. for somatic embryogenesis from immature and mature zygotic embryos. Plant Cell Rep 18:37–43.

Guerra, M. P., Steiner, N., Farias-Soares, F. L., Vieira L do N., Fraga, H. P., Rogge-Renner, G. D., Maldonado, S. B. 2016. Somatic Embryogenesis in *Araucaria angustifolia* (Bertol.) Kuntze (Araucariaceae). In: Germana, M. A., Lambardi, M. (eds) In Vitro Embryogenesis in Higher Plants, 1359:439-50.

Gupta, P. K., Durzan, D. J. 1987. Biotechnology of somatic polyembryogenesis and plantlet regeneration in loblolly pine. Nat Biotechnol 5:147–151.

Gupta, P. K., Pullman, G. S. 1991. Method for reproducing coniferous plants by somatic embryogenesis using abscisic acid and osmotic potential variation. *US patent* 5,036,007.

Häggman, H., Jokela, A., Krajnakova, J., Hauppi, A., Niemi, K., Aronen, T. 1999. Somatic embryogenesis of Scots pine: cold treatment and characteristics of expiants affecting induction. Journal of Experimental Botany 50:1769-1778.

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

Jiménez-Bremont, J. F., Marina, M., Guerrero-González, M. L., Rossi, F. R., Sánchez-Rangel, D., Rodríguez-Kessler, M., Ruiz, O. A., Gárriz, A. (2014) Physiological and molecular implications of plant polyamine metabolism during biotic interactions. Front Plant Sci.; 5, doi: 10.3389/fpls.2014.00095.

Jo, L., dos Santos, A. L. W., Bueno, C. A., Barbosa, H. R., Floh, E. I. S. 2014. Proteomic analysis and polyamines, ethylene and reactive oxygen species levels of *Araucaria angustifolia* (Brazilian pine) embryogenic cultures with different embryogenic potential. Tree Physiol 34:94–104.

Kalamki, M. S., Merkouropoulos, G., Kanellis, A. K. 2009. Can ornithine accumulation modulate abiotic stress tolerance in Arabidopsis? Plant Signaling & Behavior 4:11, 1099-1101.

Klimaszewska, K., Hargreaves, C., Lelu-Walter, M., Trontin, J. 2016. Advances in Conifer Somatic Embryogenesis since year 2000. In: Germana, M. A., Lambardi, M. (eds) In Vitro Embryogenesis in Higher Plants, 1359, pp 131-166.

Klimaszewska, K., Park, Y. S., Overton, C., MacEacheron, I., and Bonga, J. M. 2001. Optimized somatic embryogenesis in *Pinus strobus* L. In Vitro Cell Dev Biol – Plant 37: 392–399.

Klimaszewska, K., Trontin, J. F., Becwar, M. R., Devillard, C., Park, Y. S., Lelu-Walter, M. A. 2007. Recent Progress in Somatic Embryogenesis of Four *Pinus* spp. Tree and Forestry Science and Biotechnology 1:11-25.

Lara-Chavez, A., Egertsdotter, U., Flinn, B. S. 2012. Comparison of gene expression markers during zygotic and somatic embryogenesis in pine. In Vitro Cell Dev Biol Plant 48:341–354.

Lea, P. J., Sodek, L., Parry, M. A. J., Shewry, P. R., Halford, N. G. 2007. Asparagine in plants. Ann Appl Biol 150: 1–26.

Lelu-Walter, M. A., Batien, C., Drugeault, A., Gouez, M. L., Klimaszewska, K. 1999. Somatic embryogenesis and plantlet development in *Pinus sylvestris* and *Pinus pinaster* on medium with and without growth regulators. Physiologia Plantarum 105: 719–728.

Lelu-Walter, M. A., Bernier-Cardou, M., Klimaszewska, K. 2008. Clonal plant production from self- and cross-pollinated seed families of *Pinus sylvestris* (L.) through somatic embryogenesis. Plant Cell Tissue Org. Cult. 92: 31–45.

Lelu-Walter, M. A., Pâques, L. E. 2009. Simplified and improved somatic embryogenesis of hybrid larches (*Larix* × *eurolepis* and *Larix* × *marschlinsii*). Perspectives for breeding. Ann. For. Sci. 66:104, doi: 10.1051/forest/2008079.

Lelu-Walter, M.A., Bernier-Cardou, M., and Klimaszewska, K. 2006 Simplified and improved somatic embryogenesis for clonal propagation of *Pinus pinaster* (Ait.). Plant Cell Rep 25:767–776.

MacKay, J. J., Becwar, M. R., Park, Y. S., Corderro, J. P., Pullman, G. S. 2006. Genetic control of somatic embryogenesis initiation in loblolly pine and implications for breeding. Tree Genet Genomes 2:1–9.

Majumdar, R., Shao, L., Minocha, R., Long, S., Minocha, S. C. 2013. Ornithine: the overlooked molecule in the regulation of polyamine metabolism. Plant Cell Physiol 54:990–1004.

Mauri, P. V., Manzanera, J. A. 2011. Somatic embryogenesis of holm oak (*Quercus ilex* L.): Ethylene production and polyamine content. Acta Physiol Plant 33:717–723; 2011.

Miguel, C., Gonçalves, S., Tereso, S., Marum, L., Maroco, J., and Oliveira, M.M. 2004. Somatic embryogenesis from 20 open-pollinated families of Portuguese plus trees of maritime pine. Plant Cell Tiss Org Cult 76:121–130.

Minocha, R., Majumdar, R., Minocha, S. C. 2014. Polyamines and abiotic stress in plants: a complex relationship. Front. Plant Sci. 5, doi.org/10.3389/fpls.2014.00175.

Minocha, R., Minocha, S. C., Long, S. 2004. Polyamines and their biosynthetic enzymes during somatic embryo development in red spruce (Picea rubens SARG.). In Vitro Cell. Dev. Biol.-Plant 40:572-580.

Minocha, R., Shortle, W. C., Long, S. L., Minocha, S. C. 1994. A Rapid and reliable procedure for extraction of cellular polyamines and inorganic ions from plant tissues. J Plant Growth Regul 13:187-193.

Minocha, R., Smith, D. R., Reeves, C., Steele, K. D., Minocha, S. C. 1999. Polyamine levels during the development of zygotic and somatic embryos of *Pinus radiata*. Physiol Plant 105:155–164.

Montalbán, I. A., De Diego, N., Moncalean, P. 2012. Enhancing initiation and proliferation in radiata pine (*Pinus radiata* D. Don) somatic embryogenesis through seed family screening, zygotic embryo staging and media adjustments. Acta Physiologiae Plantarum 34: 451–460.

Nakagawa, R., Ogita, S., Kubo, T., Funada, R. 2006. Effect of polyamines and L-ornithine on the development of proembryogenic masses of *Cryptomeria japônica*. Plant Cell, Tissue and Organ Culture 85: 229–234.

Niemi, K., Sutela, S., Häggman, H., Scagel, C., Vuosku, J., Jokela, A., Sarjala, T. 2006. Changes in polyamine content and localization of *Pinus sylvestris* ADC and *Suillus variegatus ODC* mRNA transcripts during the formation of mycorrhizal interaction in *in vitro* cultivation system. Journal of Experimental Botany 57:2795–2804.

Niskanen, A-M., Lu, J., Seitz, S., Keinonen, K., von Weissenberg, K., Pappinen, A. 2004. Effect of parent genotype on somatic embryogenesis in Scots pine (*Pinus sylvestris*). Tree Physiology 24:1259–1265.

Noceda, C., Salaj, T., Pérez, M., Viejo, M., Cañaç, M., Salaj, J., Rodriguez, R. 2009. DNA demethylation and decrease on free polyamines is associated with the embryogenic capacity of *Pinus nigra* Arn. cell culture. Trees 23:1285–1293.

Page, A. F., Minocha, R., Minocha, S. C. 2012. Living with high putrescine: expression of ornithine and arginine biosynthetic pathway genes in high and low putrescine producing poplar cells. Amino Acids 42:295–308.

Park, Y. S. 2002. Implementation of conifer somatic embryogenesis in clonal forestry: technical requirements and deployment considerations. Ann. For. Sci. 59:651–656.

Park, Y. S., Barret, J. D., Bonga, J. M. 1998. Application of somatic embryogenesis in high-value clonal forestry: deployment, genetic control, and stability of cryopreserved clones. In Vitro Cell. Dev. Biol.-Plant 34:231-239.

Park, Y. S., Lelu-Walter, M.-A., Harvengt, L., Trontin, J.-F., MacEacheron, I., Klimaszewska, K., Bonga, J. M. 2006. Initiation of somatic embryogenesis in *Pinus banksiana*, *P. strobus*, *P. pinaster*, and *P. sylvestris* at three laboratories in Canada and France. Plant Cell Tissue and Organ Culture 86:87-101.

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

Pieruzzi, F. P., Dias, L. L. C., Balbuena, T. S., Santa-Catarina, C., Santos, A. L. W., Floh, E. I. S. 2011. Polyamines, IAA and ABA during germination in two recalcitrant seeds: *Araucaria angustifolia* (Gymnosperm) and *Ocotea odorifera* (Angiosperm). Annals of botany (Print) 108:337-345.

Pullman, G. S., Bucalo, K. 2014. Pine somatic embryogenesis: analyses of seed tissue and medium to improve protocol development. New Forests 45:353–377.

R Development Core Team (2015) R: A language and environment for statistical computing: reference index version 2.8.0. Vienna foundation for statistical computing. Disponível em: http://www.r-project.org (Acesso em 15 de agosto de 2016).

Reis, R. S., Vale, E. M., Heringer, S. A., Santa-Catarina, C., Silveira, V. 2016. Putrescine induces somatic embryo development and proteomic changes in embryogenic callus of sugarcane. J Proteomics 130:170–179.

Salo, H. M., Sarjala, T., Jokela, A., Häggman, H., Vuosku, J. 2016. Moderate stress responses and specific changes in polyamine metabolism characterize Scots pine somatic embryogenesis. Tree Physiol 36:392–402.

Santa-Catarina, C., Silveira, V., Balbuena, T. S., Viana, A. M., Estelita, M. E. M., Handro, W., Floh, E. I. S. 2006. IAA, ABA, polyamines and free amino acids associated with zygotic embryo development of *Ocotea catharinensis*. Plant Growth Regul 49:237-247.

Santa-Catarina, C., Silveira, V., Scherer, G. F. E., Floh, E. I. S. 2007. Polyamine and nitric oxide levels relate with morphogenetic evolution in somatic embryogenesis of *Ocotea catharinensis*. Plant Cell Tiss Organ Cut 90:93-101.

Sarker, K. K., Kabir, A. H., Sharmin, S. A., Nasrin, Z., Alam, M. F. 2007. Improved somatic embryogenesis using L-asparagine in wheat (*Triticum aestivum* L.). Sjemenarstvo 24:187–196.

Schlogl, P. S., dos Santos, A. L. W., Vieira, L. N., Floh, E. I. S., Guerra, M. P. 2012a. Gene expression during early somatic embryogenesis in Brazilian pine (*Araucaria angustifolia* (Bert) O. Ktze). Plant Cell Tiss Org 108: 173-180.

Schlogl, P. S., dos Santos, A. L. W., Vieira, L. N., Floh, E. I. S., Guerra, M. P. 2012b. Cloning and expression of embryogenesis-regulating genes in *Araucaria angustifolia* (Bert.) O. Kuntze (Brazilian Pine). Genet Mol Biol 35:172-181.

Shevyakova, N. I., Rakitin, V. Y., Stetsenko, L. A., Aronova, E. E., Kuznetsov, V. V. 2006. Oxidative stress and fluctuations of free and conjugated polyamines in the halophyte *Mesembryanthemum crystallinum* L. under NaCl salinity. Plant Growth Regul 50:69–78.

Silveira, V., Floh, E. I. S., Handro, W., Guerra, M. P. 2004. Effect of plant growth regulators on the cellular growth and levels of intracellular protein, starch and polyamines in embryogenic suspension cultures of *Pinus taeda*. Plant Cell, Tissue and Organ Culture 76:53-60.

Silveira, V., Santa-Catarina, C., Balbuena, T. S., Moraes, F. M. S., Ricart, C. A. O., Sousa, M. V., Guerra, M. P., Handro, W., Floh, E. I. S. 2008. Endogenous abscisic acid levels and comparative proteome during seed development of *Araucaria angustifolia* (Bert.) O. Ktze. Biol Plantarum 52:101-104.

Silveira, V., Santa-Catarina, C., Tun, N. N., Scherer, G. F. E., Handro, W., Guerra, M. P., Floh, E. I. S. 2006. Polyamine effects on the endogenous polyamine contents, nitric oxide release, growth and differentiation of embryogenic suspension cultures of *Araucaria angustifolia* (Bert.) O. Ktze. Plant Science 171:91-98.

Slocum, R. D. 2005. Genes, enzymes and regulation of arginine biosynthesis in plants. Plant Physiol Bioch 43:729–745.

Smertenko, A. P., Bozhkov, P. V., Filonova, L. H., von Arnold, A., Hussey, P. J. 2003. Re-organisation of the cytoskeleton during developmental programmed cell death in *Picea abies* embryos. The Plant Journal 33:813–824.

Stasolla, C., Belmonte, M. F., van Zyl, L., Craig, D. L., Liu, W., Yeung, E. C., Sederoff, R. R. 2004. The effect of reduced glutathione on morphology and gene expression of white spruce (*Picea glauca*) somatic embryos. Journal of Experimental Botany 55:695-709.

Steiner, N., Santa-Catarina, C., Silveira, V., Floh, E. I. S., Guerra, M. P. 2007. Polyamine effects on growth and endogenous hormones levels in *Araucaria angustifolia* embryogenic cultures. Plant Cell Tiss Organ Cult 89:55-62.

Steiner, N., Vieira, F. N., Maldonado, S., Guerra, M. P. 2005. Effect of carbon source on morphology and histodifferentiation of *Araucaria angustifolia* embryogenic cultures. Brazilian archives of biology and technology 48:895-903.

Vieira, L. N., Santa-Catarina, C., Fraga, H. P. F., dos Santos, A. L. W., Steinmacher, D. A., Schlogl, P. S., Silveira, V., Steiner, N., Floh, E. I. S., Guerra, M. P. 2012. Glutathione improves early somatic embryogenesis in *Araucaria angustifolia* (Bert) O. Kuntze by alteration in nitric oxide emission. Plant Science 195:80-87.

von Arnold, S., Sabala, I., Bozhkov, P., Dyachok, J., Filanova, L. 2002. Developmental pathways of somatic embryogenesis. Plant Cell Tissue Organ Cult 69:233-49.

Vuosku, J., Jokela, A., Läärä, E., Sääskilahti, M., Muilu, R., Sutela, S., Altabella, T., Sarjala, T., Häggman, H. 2006. Consistency of polyamine profiles and expression of arginine decarboxylase in mitosis during zygotic embryogenesis of Scots pine. Plant Physiology 142:1027-1038.

Vuosku, J., Suorsa, M., Ruottinen, M., Sutela, S., Muilu-Mäkelä, R., Julkunen-Tiitto, R., Sarjala, T., Neubauer, P., Häggman, H. 2012. Polyamine metabolismo during exponential growth transition in Scots pine embryogenic cell culture. Tree Physiol 32:1274–1287.

Considerações finais e perspectivas

CONSIDERAÇÕES FINAIS E PERSPECTIVAS

O presente trabalho está inserido na temática que envolve a compreensão dos processos de embriogênese zigótica e somática em *A. angustifolia*. Diversas pesquisas têm sido realizadas visando a abordagem de aspectos básicos relacionados à competência, determinação e diferenciação celular da embriogênese, em associação com o aperfeiçoamento de um protocolo para a aplicação da propagação clonal massal neste sistema vegetal. Neste contexto, as pesquisas tem recorrido à utilização de parâmetros morfo-fisiológicos, bioquímicos e moleculares, num estudo comparativo entre a embriogênese zigótica e somática. Dentro desta perspectiva, diferentes vias de sinalização celular foram estudadas, onde as PAs e aminoácidos foram identificados como importantes ao longo do desenvolvimento da semente, e em linhagens celulares com alto potencial embriogênico da *A. angustifolia.* Neste contexto insere-se o presente trabalho, que objetivou aprofundar o conhecimento destes grupos de substâncias na modulação dos processos de embriogênese zigótica e somática de *A. angustifolia*. Estudos nesta linha de pesquisa foram realizados em espécies modelo, como *Arabidospsis thaliana*, *Medicago sativa*, *Nicotiana tabacum*, e em algumas coníferas de interesse econômico. Neste trabalho, de maneira inédita, foi utilizado um sistema constituído de uma arbórea nativa, que possui sementes recalcitrantes, de difícil propagação por métodos tradicionais, e considerada ameaçada de extinção.

Os recentes avanços obtidos com as novas plataformas de sequenciadores de ácidos nucléicos vem permitindo, com grande eficiência, o estudo do transcriptoma através da técnica de RNA-seq de espécies sequenciadas e não sequenciadas, ou então, com um grande genoma como é o caso da *A. angustifolia*. Um extenso banco de dados, utilizando esta metodologia, foi obtido para embriogênese zigótica e somática no presente sistema de estudo, possibilitando um maior conhecimento da regulação molecular do desenvolvimento embrionário *in vivo* e *in vitro* (Elbl *et al.*, 2015). Adicionalmente, estudos integrados do transcriptoma e do proteoma (dos Santos *et al*., 2016) puderam predizer a função de genes, caracterizar os processos biológicos envolvidos, e o estudo das redes metabólicas relacionadas aos reguladores, como aqueles selecionados para o presente trabalho. Associados a estes estudos, no presente trabalho, a determinação da atividade das enzimas das vias metabólicas, a utilização de aminoácidos marcados, e a expressão gênica das enzimas de biossíntese foram ferramentas fundamentais para elucidação da participação das PAs e aminoácidos na evolução dos processos da

embriogênese *in vitro* e *in vivo*. Adicionalmente, foi possível confirmar a possibilidade de utilização das PAs como marcador bioquímico para a capacidade embriogênica, em consonância com outros trabalhos desenvolvidos.

Os resultados, apresentados no Capítulo I e publicados no periódico *Tree Physiology* (de Oliveira *et al.*, 2016), elucidaram, utilizando parâmetros bioquímicos e moleculares, o metabolismo e a participação das PAs e aminoácidos nos vários estádios de desenvolvimento do embrião zigótico. Foi verificado que: a) dois genes diferencialmente expressos que podem codificar para enzimas chaves na via de biossíntese de PAs, sendo o gene *AaADC* para a regulação da biossíntese de Put e *AaSAMDC* para biossíntese de Spd/Spm; b) a via preferencial de biossíntese de Put é realizada através da enzima ADC; e c) diferentes perfis de aminoácidos associados à via de PAs. A arginina, diferente do observado para outras coníferas, não foi o principal aminoácido identificado durante a embriogênese zigótica de *A. angustifolia*. Os resultados indicam que a arginina estaria menos relacionada com a incorporação de proteínas de reserva na semente, e numa maior integração com a biossíntese de NO ou PAs. A citrulina foi o principal aminoácido observado nos estádios cotiledonar e maturo dos embriões zigóticos, o qual pode estar associado ao metabolismo oxidativo, como um produto da via de NO. Para comprovar estas hipóteses, novos estudos envolvendo a análise da emissão de NO, bem como a incorporação de precursores (arginina e ornitina), devem ser realizados, obtendo informações relevantes sobre a participação da citrulina no metabolismo oxidativo durante o desenvolvimento embrionário.

Estudos anteriores revelaram que linhagens celulares de *A. angustifolia* com diferentes potenciais embriogênicos, apresentam distintos perfis de PAs, sobretudo nos conteúdos de Put (Jo *et al.*, 2014). Entretanto, os mecanismos que controlam o metabolismo de PAs em diferentes linhagens celulares ainda não foram completamente determinados em coníferas. Nesse contexto, o uso de precursores marcados, como a ^{14}C-Arg e ^{14}C-Orn, permitiram obter um maior conhecimento sobre a participação da arginina e da ornitina tanto no metabolismo da via da Ornitina/Arginina como na regulação da Put, em linhagens celulares com diferentes potenciais embriogênicos. Ressalta-se que esse tipo de estudo é inédito para coníferas.

Os resultados apresentados no Capítulo II, demonstraram que a arginina tem maior participação no metabolismo de PAs do que a ornitina, nas duas linhagens testadas. Em linhagens responsivas, o efeito da aplicação exógena de arginina parece ativar os genes

relacionados ao catabolismo de PAs, enquanto que para a linhagem bloqueada, parece haver um efeito acumulativo das PAs. Futuros estudos envolvendo as atividades das enzimas relacionadas ao catabolismo de PAs permitirão uma análise mais detalhada para confirmar a participação do catabolismo de PAs em culturas embriogênicas de *A. angustifolia*. As análises de expressão gênica e atividade enzimática da ADC e ODC, no presente trabalho, foram avaliados após 48h de incubação com os precursores marcados. Entretanto, não foi possível observar uma relação entre esses dois perfis, sugerindo que pode haver uma regulação pós-traducional. A maior acumulação de Put foi observado após 168h e 336h de incubação, indicando que esses pontos devem ser levados em conta para futuras análises dos perfis de expressão gênica e atividade enzimática. Estudos com inibidores da ADC, ODC e SAMDC também serão efetivos para uma melhor compreensão da participação dessas enzimas na evolução das culturas embriogênicas de *A. angustifolia*.

Além de regular os níveis de PAs, a arginina e ornitina também alteram o conjunto total de aminoácidos. A citrulina foi o principal aminoácido identificado nas culturas embriogênicas, sendo alterada em linhagens responsivas, quando da suplementação da arginina exógena. Este resultado vem corroborar a possível participação da arginina na biossíntese de NO, uma vez que citrulina é um dos produtos dessa via. Estudos mais detalhados, envolvendo as análises de emissão de NO devem ser realizados, confirmando a via de biossíntese em questão, e seu papel durante a embriogênese somática. Pelos dados obtidos, observou-se que a taxa de conversão da ornitina em arginina pode ser maior do que o inverso, ou seja, a conversão da arginina em ornitina. Estudos envolvendo a análise da atividade enzimática da arginase e o ciclo da ureia, são pontos importantes a serem estudados, apontando para a importância do metabolismo do nitrogênio nas culturas embriogênicas de *A. angustifolia*.

A embriogênese somática é um processo complexo, onde o sucesso de cada etapa depende do desempenho adequado na etapa anterior. O estabelecimento de culturas embriogênicas nesta espécie é genótipo-dependente, entretanto, o potencial embriogênico é apenas observado quando da etapa de maturação. Estudos anteriores apontam as PAs como candidatos a marcadores bioquímicos para a seleção de linhagens com potencial embriogênico, previamente à etapa de maturação (Jo *et al.*, 2014). Esta temática é abordada no o Capítulo III, onde é proposta a utilização dos perfis de PAs e aminoácidos como possíveis marcadores bioquímicos para a seleção de matrizes com maior potencial embriogênico. Três metabólitos (Put total, ornitina e asparagina) identificados nas sementes

das matrizes foram associados às respostas observadas *in vitro*. As abordagens utilizadas neste trabalho, ou seja, a análise dos perfis bioquímicos das matrizes e sua associação com as respostas observadas *in vitro*, permitiram abrir novas perspectivas para o estabelecimento de marcadores bioquímicos para seleção de matrizes com maior potencial embriogênico em *A. angustifolia*. Entretanto, uma análise estatística mais aprofundada é necessária para estabelecer uma correlação entre os parâmetros bioquímicos observados nas sementes, e as respostas observadas *in vitro*, bem como expandir para um número maior de matrizes testadas em diferentes populações de *A. angustifolia*.

Em conjunto, os dados obtidos no presente trabalho representam um avanço no conhecimento dos mecanismos que regulam a biossíntese de PAs durante a embriogênese zigótica e em culturas embriogênicas de *A. angustifolia*. Ressalta-se que os resultados obtidos neste trabalho são inéditos, considerando-se a espécie em estudo.

Os resultados apresentados elucidaram pontos relevantes sobre o metabolismo das PAs, entretanto, futuros estudos a nível proteico, ou seja, que envolvam as análises das principais enzimas relacionadas às PAs e aminoácidos, deverão ser conduzidos para complementar as informações obtidas no presente estudo. Adicionalmente, outros metabólitos, como carboidratos, estão sendo avaliados e poderão ser integrados às PAs e aminoácidos no estabelecimento de marcadores bioquímicos para o sistema *A. angustifolia.*

Referências bibliográficas

Elbl, P., Campos, R. A., Lira, B. S., Andrade, S. C. S., Jo, L., dos Santos, A. L. W, Coutinho, L. L., Floh, E. I. S., Rossi, M. 2015. Comparative transcriptome analysis of early somatic embryo formation and seed development in Brazilian pine, *Araucaria angustifolia* (Bertol.) Kuntze. Plant Cell Tiss Organ Cult 120:903–915.

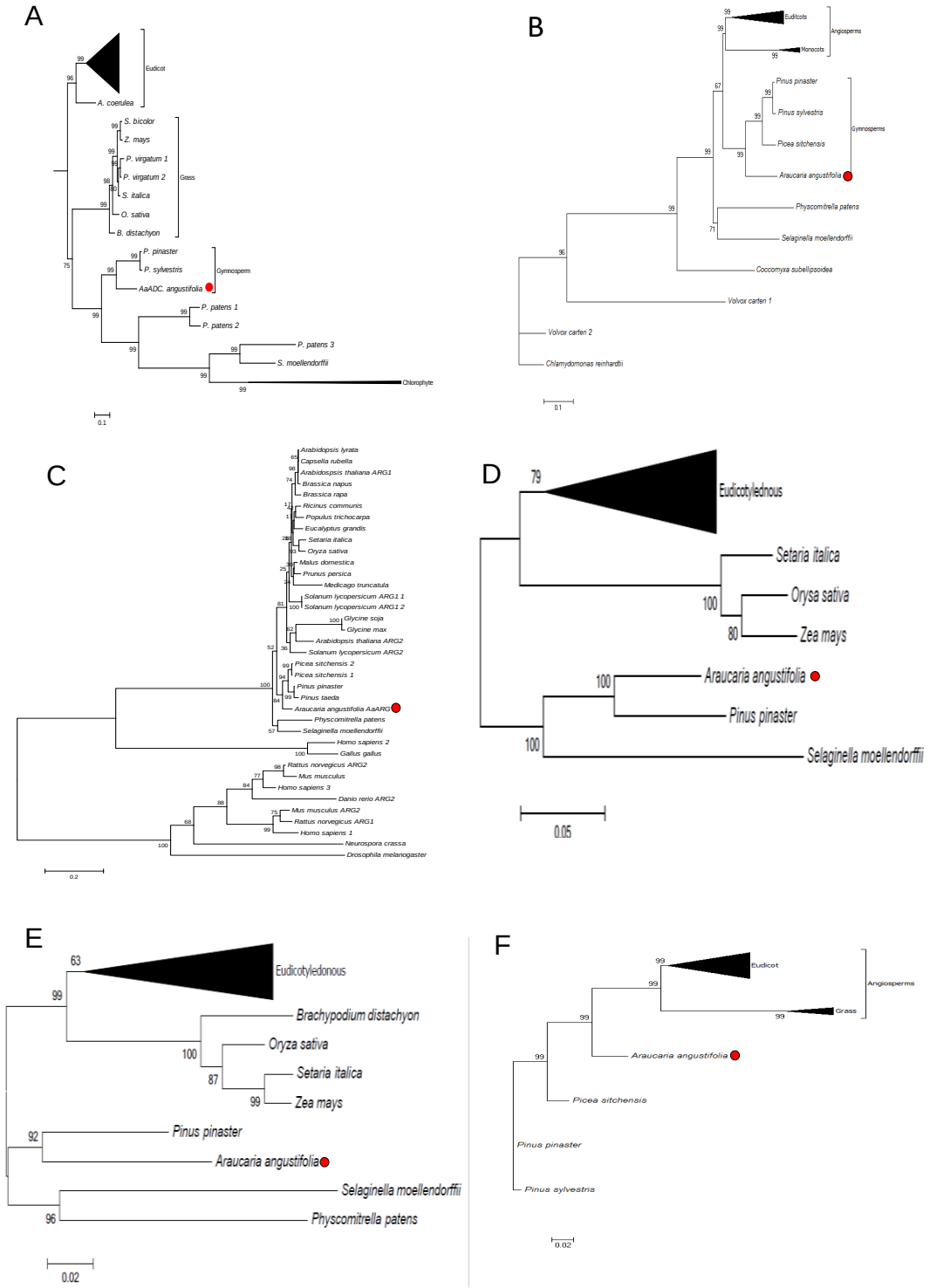
de Oliveira, L. F., Elbl, P., Navarro, B. V., Macedo, A. F., Dos Santos, A. L. W., Floh, E. I. S. 2016. Elucidation of the polyamine biosynthesis pathway during Brazilian pine (*Araucaria angustifolia*) seed development. Tree Physiol 1-15, doi:10.1093/treephys/tpw107.

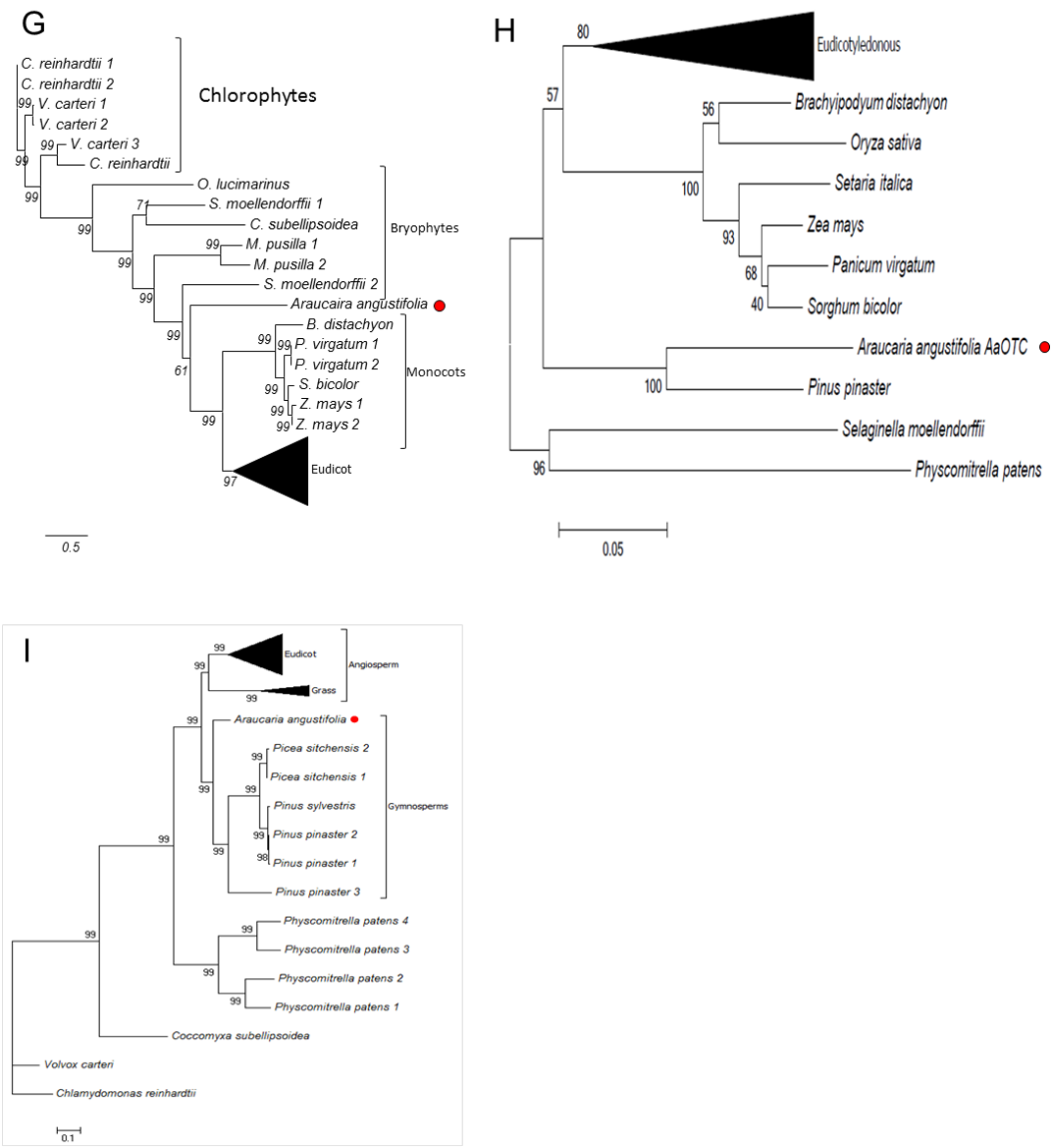
Jo, L., dos Santos, A. L. W., Bueno, C. A., Barbosa, H. R., Floh, E. I. S. 2014. Proteomic analysis and polyamines, ethylene and reactive oxygen species levels of *Araucaria angustifolia* (Brazilian pine) embryogenic cultures with different embryogenic potential. Tree Physiol 34:94–104.

dos Santos, A. L. W., Elbl, P., Navarro, B. V., de Oliveira, L. F., Salvato, F., Balbuena, T. S., Floh, E. I. S. 2016. Quantitative proteomic analysis of *Araucaria angustifolia* (Bertol.) Kuntze cell lines with contrasting embryogenic potential. J Proteomics 130:180–189.

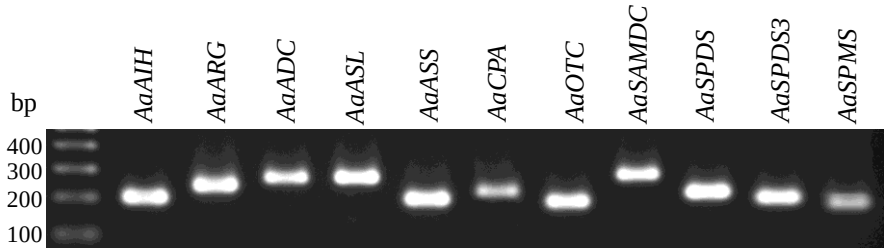
Anexos

ANEXO I – referente ao material suplementar do Capítulo I

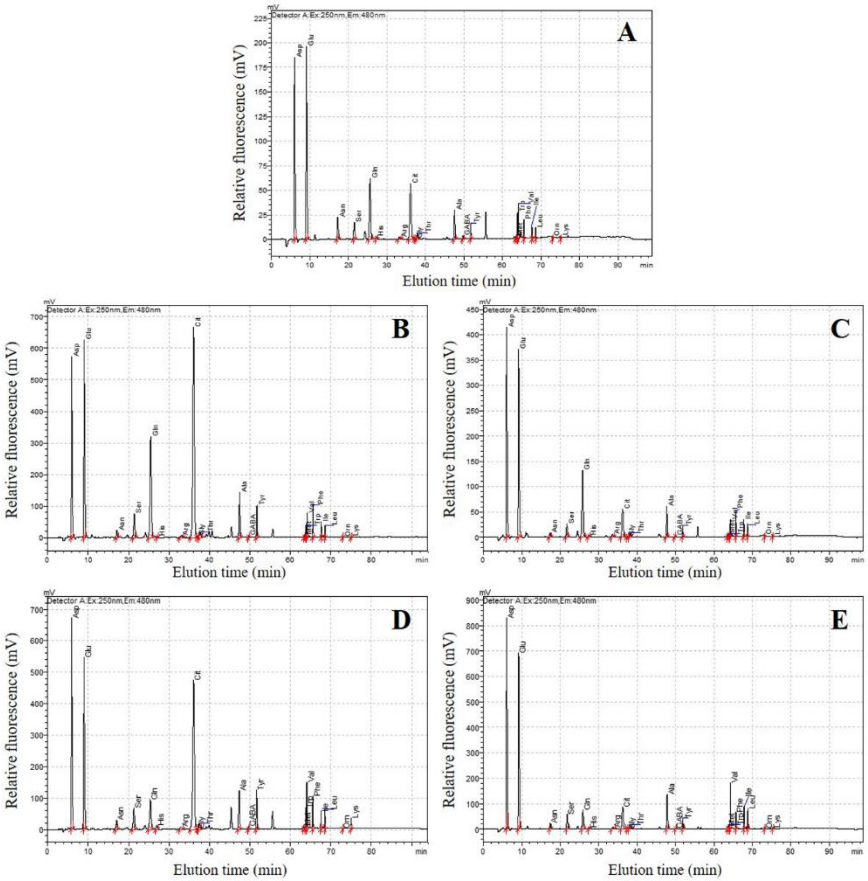




Supplementary Figure S1. Phylogenetic tree based on ornithine/arginine (Orn/Arg) and polyamine (PA) biosynthetic pathway associated protein sequences from *Araucaria angustifolia* and other species. (A) *AaADC* (arginine decarboxylase), (B) *AaAIH* (agmatine deiminase), (C) *AaARG* (arginase), (D) *AaASL* (argininosuccinate lyase), (E) *AaASS* (arginosuccinate synthase), (F) *AaCPA* (*N*-carbamoylputrescine amidase), (G) *AaODC* (ornithine decarboxylase), (H) *AaOTC* (ornithine carbamoyltransferase), (I) *AaSAMDC* (*S*-adenosylmethionine decarboxylase) of *Araucaria angustifolia* (red point). Genetic distances were inferred using the neighbor-joining method and 1500 replicates bootstrap calculations. Database and accession numbers are listed in Supplementary Table S2.



Supplementary Figure S2. Amplicons obtained by qPCR using specific primers for 11 genes analyzed. *AaAIH* (agmatine deiminase), *AaARG* (arginase), *AaADC* (arginine decarboxylase), *AaASL* (argininosuccinate lyase), *AaASS* (arginosuccinate synthase), *AaCPA* (*N*-carbamoylputrescine amidase), *AaOTC* (ornithine carbamoyltransferase), *AaSAMDC* (*S*-adenosylmethionine decarboxylase), *AaSPDS* (spermidine synthase), *AaSPDS3* (spermidine synthase 3), *AaSPMS* (spermine synthase). bp = base pairs.



Supplementary Figure S3. Separation profiles of 21 amino acids by HPLC in three stages of *Araucaria angustifolia* seed development: (A) megagametophyte containing globular embryos, (B) isolated cotyledonal embryos, (C) megagametophyte at cotyledonal stage, (D) isolated mature embryos and (E) megagametophyte at mature stage. For run conditions see section *Determination of free amino acids*, in the text.

Supplementary Table S1. List of primer sequences used in the qPCR analysis of the ornithine/arginine (Orn/Arg) and polyamine (PA) biosynthetic pathway associated candidate genes.

Gene Name	Gene symbol	Primer sequence (Foward/Reverse)
Agmatine deiminase	*AaAIH*	CCCCCACCGAGCACAAGTTC CCTGACAGAAGAAGAAGCAAATG
Arginase	*AaARG*	CCTTGTTCCCTACCTTC GAGCCTCCTCTTCGTCC
Arginine decarboxylase	*AaADC*	GGTGGAGGGCTTGGCATC CGAAAACGAGGAGGGAATGG
Argininosuccinate lyase	*AaASL*	GCAGGACATCAATGGCAGC CGCACATCAGTACAGACTTG
Arginosuccinate synthase	*AaASS*	GCTCCTTCCCATTTACTG CTACAGCAGAGACCGCAATC
N-carbamoylputrescine amidase	*AaCPA*	CCCTCTCTACTTCTCCTC GTGCCTCCCTTACCAAC
Ornithine decarboxylase	*AaODC*	GTGTACTTAGCATAGGGGTTG GGCGGAGTCGTCAGTC
Ornithine carbamoyltransferase	*AaOTC*	CTCTCTCCTCCCTTTCTC GCTTCTGCTTATTACAACAC
S-adenosylmethionine decarboxylase	*AaSAMDC*	CTCTTTGCTTTCCTCTTCCC GACCTCTCTCTCCTTGTGG
Spermidine synthase	*AaSPDS*	CAGGAGGTGTGGTGTGTAC GATGGGATGACGGAAGTC
Spermidine synthase 3	*AaSPDS3*	CTGGTGGTGTGGTAATC CTGGTGGTCCTTCTGTTG
Spermine synthase	*AaSPMS*	CCGTTCTCTGAGTTACCG CATCTTCCTTCCTCCCC
Translational initiation factor 4B	*AaEIFA4B-L**	CAGTCGCCTCCTGTCTTG CCGTCGTCTGGTGAAAATG
Protein phosphatase 2A	*AaPP2A**	GATGAAGGTCAATGTAGAGGG GGTGGGGCTTATTTTGCTTTG

* Reference genes (Elbl et al. 2015b)

Supplementary Table S2. Sequences used for construction of phylogenetic trees of ADC, ODC, SAMDC, ARG, AIH, CPA and SPDS/SPDS3/SPMS. Public Databases: Phytozome (https://phytozome.jgi.doe.gov/), NCBI (http://www.ncbi.nlm.nih.gov/) and SustainPineDB (http://www.scbi.uma.es/sustainpinedb/home_page).

ADC

Species	Accession number
Aquilegia coerulea	>Aquca_007_00366.1.aco.22048541/1-761
Arabidopsis lyrata	>fgenesh2_kg.7__616__AT4G34710.1.aly.16045265/1-708
Arabidopsis lyrata	>scaffold_304290.1.aly.16064915/1-703
Arabidopsis thaliana	>AT2G16500.1.ath.19641078/1-703
Arabidopsis thaliana	>AT4G34710.1.ath.19644176/1-712
Brachypodium distachyon	>Bradi1g50067.1.p.bdi.31126691/1-711
Brassica rapa	>Brara.A00352.1.p.bra.30636204/1-668
Brassica rapa	>Brara.A00353.1.p.bra.30638017/1-649
Brassica rapa	>Brara.H01183.1.p.bra.30649572/1-544
Brassica rapa	>Brara.K00332.1.p.bra.30627519/1-690
Boechera stricta	>Bostr.18351s0111.1.p.adr.30676794/1-708
Boechera stricta	>Bostr.7867s1331.1.p.adr.30657412/1-714
Citrus clementina	>Ciclev10027873m.ccl.20814377/1-754
Capsella grandiflora	>Cagra.21579s0001.1.p.cgr.28911844/1-704
Capsella grandiflora	>Cagra.2350s0020.1.p.cgr.28908818/1-719
Carica papaya	>evm.model.supercontig_150.13.cpa.16409465/1-739
Capsella rubella	>Carubv10004246m.cru.20894900/1-718
Capsella rubella	>Carubv10013090m.cru.20898330/1-704
Cucumis sativus	>Cucsa.109090.1.csa.16959795/1-718
Citrus sinensis	>orange1.1g004438m.csi.18122160/1-754
Coccomyxa subellipsoidea	>24487.csu.27392165/1-727
Eucalyptus grandis	>Eucgr.G01735.1.egr.23587294/1-739
Eutrema salsugineum	>Thhalv10022578m.esa.20201976/1-717
Eutrema salsugineum	>Thhalv10024529m.esa.20193536/1-726
Fragaria vesca	>mrna00390.1-v1.0-hybrid.fve.27249518/1-709
Fragaria vesca	>mrna01668.1-v1.0-hybrid.fve.27264899/1-718
Glycine max	>Glyma.04G007700.1.p.gma.30489524/1-698
Glycine max	>Glyma.06G007500.1.p.gma.30553706/1-692
Gossypium raimondii	>Gorai.002G149300.1.gra.26792821/1-727
Gossypium raimondii	>Gorai.005G248700.1.gra.26806383/1-598
Gossypium raimondii	>Gorai.012G154100.1.gra.26828727/1-716
Linum usitatissimum	>Lus10031079.lus.23166796/1-724
Linum usitatissimum	>Lus10035464.lus.23170676/1-409
Malus domestica	>MDP0000228682.m.22664620/1-728
Malus domestica	>MDP0000813339.m.22674091/1-731
Manihot esculenta	>cassava4.1_002501m.mes.17978043/1-725
Manihot esculenta	>cassava4.1_002558m.mes.17961126/1-719
Mimulus guttatus	>Migut.B00171.1.p.mgu.28947896/1-715
Mimulus guttatus	>Migut.N01279.1.p.mgu.28923729/1-680
Micromonas pusilla	>203222.mpu.27342443/1-633
Medicago truncatula	>Medtr4g072020.1.mtr.31114568/1-731
Oryza sativa	>LOC_Os04g01690.3.osa.24103810/1-624
Oryza sativa	>LOC_Os06g04070.1.osa.24142420/1-703
Physcomitrella patens	>Phpat.005G013600.1.p.ppa.28244423/1-690
Physcomitrella patens	>Phpat.006G071900.1.p.ppa.28260166/1-669
Physcomitrella patens	>Phpat.016G006200.1.p.ppa.28266743/1-772
Prunus persica	>ppa002034m.ppe.17647482/1-726
Pinus pinaster	>sp_v3.0_unigene3382_SustainPineDB
Pinus sylvestris	>gi|312162104|gb|ADQ37299.1| putative arginine decarboxylase Pinus sylvestris
Populus trichocarpa	>Potri.004G163300.1.ptr.26990843/1-731
Panicum virgatum	>Pavir.Fb01397.1.p.pvi.30278599/1-609
Panicum virgatum	>Pavir.J13691.1.p.pvi.30207719/1-696
Panicum virgatum	>Pavir.J33899.1.p.pvi.30294369/1-697
Phaseolus vulgaris	>Phvul.009G002500.1.pvu.27147988/1-685
Ricinus communis	>30131.m007139.rco.16818962/1-724
Sorghum bicolor	>Sobic.010G021800.1.p.sbi.28365908/1-702
Setaria italica	>Si007956m.sit.19703305/1-703
Setaria italica	>Si016654m.sit.19688367/1-624
Solanum lycopersicum	>Solyc01g110440.2.1.sly.27301477/1-608
Solanum lycopersicum	>Solyc10g054440.1.1.sly.27280340/1-708
Selaginella moellendorffii	>estExt_Genewise1.C_00954.smo.15404224/1-628
Solanum tuberosum	>PGSC0003DMP400002989.stu.24417817/1-736
Solanum tuberosum	>PGSC0003DMP400046356.stu.24412448/1-721
Theobroma cacao	>Thecc1EG006773t1.tca.27461487/1-733
Theobroma cacao	>Thecc1EG020310t1.tca.27453328/1-652
Vitis vinifera	>GSVIVT01024167001.vvi.17832430/1-456
Zea mays	>GRMZM2G374302_P01.zma.31049040/1-640
Zea mays	>GRMZM2G396553_P01.zma.31039215/1-695

ODC

Species	Accession number
Solanum lycopersicum	SlODC
Solanum tuberosum	PGSC0003DMP400017584|Stuberosum
Phaseolus vulgaris	27146485_peptide|Pvulgaris
Phaseolus vulgaris	27145300_peptide|Pvulgaris
Populus trichocarpa	27028028_peptide|Ptrichocarpa
Populus trichocarpa	27021043_peptide|Ptrichocarpa
Glicyne max	26304035_peptide|Gmax
Glicyne max	26301702_peptide|Gmax
Orysa sativa	24138147_peptide|Osativa
Panicum virgatum	23801844_peptide|Pvirgatum
Panicum virgatum	23801504_peptide|Pvirgatum
Mediago truncatula	23009883_peptide|Mtruncatula

Brachypodium distachyon	21814144_peptide|Bdistachyon
Brachypodium distachyon	21812353_peptide|Bdistachyon
Brachypodium distachyon	21811893_peptide|Bdistachyon
Zea mays	20846696_peptide|Zmays
Zea mays	20835306_peptide|Zmays
Zea mays	20833089_peptide|Zmays
Citrus clementina	20811303_peptide|Cclementina
Citrus clementina	20805615_peptide|Cclementina
Setaria italica	19712981_peptide|Sitalica
Setaria italica	19712438_peptide|Sitalica
Sorghum bicolor	1970632_peptide|Sbicolor_v1.0
Sorghum bicolor	1958650_peptide|Sbicolor_v1.0
Sorghum bicolor	1958451_peptide|Sbicolor_v1.0
Sorghum bicolor	1958440_peptide|Sbicolor_v1.0
Citrus sinensis	18124488_peptide|Csinensis
Citrus sinensis	18124432_peptide|Csinensis
Citrus sinensis	18113170_peptide|Csinensis
Manihot esculenta	17990135_peptide|Mesculenta
Ricinus communis	16822132_peptide|Rcommunis
Ricinus communis	16822131_peptide|Rcommunis

SAMDC

Species	Accession number
Physcomitrella patens_1	>Phpat.020G016900.1.p.ppa.28234481/1392
Physcomitrella patens_2	>Phpat.023G076800.1.p.ppa.28237534/1392
Physcomitrella patens_3	>Phpat.009G046500.1.p.ppa.28241103/1419
Physcomitrella patens_4	>Phpat.015G068700.1.p.ppa.28254396/1413
Panicum virgatum_1	>Pavir.Gb01229.1.p.pvi.30221358/1398
Panicum virgatum_2	>Pavir.Aa01378.1.p.pvi.30250817/1405
Panicum virgatum_3	>Pavir.Bb02216.1.p.pvi.30298923/1395
Panicum virgatum_4	>Pavir.Ga01281.1.p.pvi.30303966/1398
Setaria italica_1	>Si017413m.sit.19690438/1401
Setaria italica_2	>Si010282m.sit.19696127/1397
Setaria italica_3	>Si030103m.sit.19712552/1395
Aquilegia coerulea	>Aquca_006_00324.1.aco.22036632/1359
Solanum lycopersicum_1	>Solyc02g089610.1.1.sly.27287857/1363
Solanum lycopersicum_2	>Solyc05g010420.1.1.sly.27298169/1361
Solanum lycopersicum_3	>Solyc01g010050.2.1.sly.27303723/1365
Solanum tuberosum	>PGSC0003DMP400013315.stu.24387245/1361
Manihot esculenta	>cassava4.1_021499m.mes.17962765/1365
Ricinus communis_1	>29673.m000902.rco.16805295/1359
Ricinus communis_2	>30138.m003848.rco.16819218/1361
Gossypium raimondii_1	>Gorai.004G045700.1.gra.26776667/1370
Gossypium raimondii_2	>Gorai.013G102500.1.gra.26788102/1356
Theobroma cacao	>Thecc1EG011372t1.tca.27461032/1360
Arabidopsis thaliana_1	>AT3G02470.1.ath.19659716/1367
Arabidopsis thaliana_2	>AT5G15950.1.ath.19666704/1363
Brassica rapa_1	>Brara.J01921.1.p.bra.30612568/1369
Brassica rapa_2	>Brara.C02987.1.p.bra.30618651/1252
Brassica rapa_3	>Brara.A03903.1.p.bra.30637532/1261
Capsella rubella_1	>Carubv10017502m.cru.20886329/1363
Capsella rubella_2	>Carubv10001243m.cru.20911116/1363
Glycine max_1	>Glyma.02G128000.1.p.gma.30509668/1356
Glycine max_2	>Glyma.08G255800.1.p.gma.30538266/1360
Glycine max_3	>Glyma.01G071300.1.p.gma.30543364/1354
Glycine max_4	>Glyma.18G278800.1.p.gma.30557762/1360
Medicago truncatula_1	>Medtr0076s0020.1.mtr.31069344/1354
Medicago truncatula_2	>Medtr8g069905.1.mtr.31071063/1371
Medicago truncatula_3	>Medtr7g018290.2.mtr.31082731/1361
Medicago truncatula_4	>Medtr0003s0660.1.mtr.31105782/1407
Phaseolus vulgaris	>Phvul.008G022100.1.pvu.27154921/1357
Chlamydomonas reinhardtii	>Cre03.g205900.t1.2.cre.30787393/1391
Volvox carteri	>Vocar20012220m.vca.23139161/1386
Coccomyxa subellipsoidea	>31759.csu.27392659/1306
Pinus pinaster_1	>Pinus_pinaster_sp_v3.0_unigene17140
Pinus pinaster_2	>Pinus_pinaster_sp_v3.0_unigene17208
Pinus pinaster_3	>Pinus_pinaster_sp_v3.0_unigene11132
Pinus sylvestris	>gi|312162110|gb|ADQ37302.1| putative Sadenosyl methionine decarboxylase Pinus sylvestris
Picea sitchensis_1	>gi|116790324|gb|ABK25576.1| unknown Picea sitchensis
Picea sitchensis_2	>gi|148909291|gb|ABR17745.1| unknown Picea sitchensis

ARG

Species	Accession number
Arabidopsis thaliana	AT4G08870.1.ath.19646719/1-345
Arabidopsis thaliana	AT4G08900.1.ath.19646298/1-343
Boechera stricta	Bostr.9686s0015.1.p.adr.30679817/1-343
Brachypodium distachyon	Bradi5g02160.1.p.bdi.31146479/1-343
Brassica rapa	Brara.C02579.1.p.bra.30617985/1-342
Capsella grandiflora	Cagra.8074s0001.1.p.cgr.28913009/1-295
Capsella rubella	Carubv10001337m.cru.20907478/1-345
Capsella rubella	Carubv10001349m.cru.20907471/1-343
Selaginella moellendorffii	e_gw1.0.671.1.smo.15407590/1-335
Picea sitchensis	gi|116784304|gb|ABK23295.1|_unknown_Picea_sitchensis
Picea sitchensis	gi|116792976|gb|ABK26575.1|_unknown_Picea_sitchensis
Pinus taeda	gi|12802155|gb|AAK07744.1|AF130440_1_arginase_Pinus_taeda
Glycine max	Glyma.01G140200.1.p.gma.30543616/1-339
Glycine max	Glyma.17G131300.1.p.gma.30480954/1-339
Gossypium raimondii	Gorai.009G265900.1.gra.26771335/1-350
Gossypium raimondii	Gorai.009G265900.3.gra.26771337/1-310
Zea mays	GRMZM2G174671_P04.zma.31000403/1-341
Linum usitatissimum	Lus10030288.lus.23153032/1-339
Medicago truncatula	Medtr4g024960.1.mtr.31107156/1-339
Mimulus guttatus	Migut.B01616.1.p.mgu.28947261/1-339
Fragaria vesca	mrna03912.1-v1.0-hybrid.fve.27247647/1-383
Panicum virgatum	Pavir.Gb01213.1.p.pvi.30221415/1-414
Panicum virgatum	Pavir.Ib03192.1.p.pvi.30238114/1-379
Physcomitrella patens	Phpat.005G007400.1.p.ppa.28244031/1-356
Phaseolus vulgaris	Phvul.010G081600.1.pvu.27140967/1-339

Pinus pinaster	Pinus_pinaster_sp_v3.0_unigene23824
Populus trichocarpa	Potri.002G146200.1.ptr.27024475/1-339
Populus trichocarpa	Potri.014G067700.1.ptr.27033257/1-334
Setaria italica	Si010532m.sit.19695117/1-341
Sorghum bicolor	Sobic.006G004500.1.p.sbi.28403766/1-341
Solanum lycopersicum	Solyc01g091160.2.1.sly.27302769/1-339
Solanum lycopersicum	Solyc01g091170.2.1.sly.27303765/1-339
Theobroma cacao	Thecc1EG036209t1.tca.27457040/1-340
Eutrema salsugineum	Thhalv10028793m.esa.20197913/1-332

AIH

Species	Accession number
Physcomitrella patens	>Phpat.014G085000.1.p.ppa.28248246/1-438
Selaginella moellendorffii	>fgenesh1_pm.C_scaffold_14000038.smo.15417560/1-369
Panicum virgatum	>Pavir.Gb01492.1.p.pvi.30223861/1-298
Setaria italica	>Si010376m.sit.19696825/1-374
Sorghum bicolor	>Sobic.006G111700.1.p.sbi.28404216/1-376
Zea mays	>GRMZM2G064159_P02.zma.31004524/1-415
Aquilegia coerulea	>Aquca_006_00330.1.aco.22036860/1-397
Solanum lycopersicum	>Solyc12g038970.1.1.sly.27308372/1-376
Solanum tuberosum	>PGSC0003DMP400001290.stu.24379059/1-376
Manihot esculenta	>cassava4.1_009901m.mes.17960237/1-375
Ricinus communis	>30174.m009045.rco.16822686/1-377
Gossypium raimondii	>Gorai.001G065900.1.gra.26820172/1-375
Theobroma cacao	>Thecc1EG014443t1.tca.27450842/1-374
Arabidopsis lyrata	>fgenesh2_kg.6__786__AT5G08170.1.aly.16035061/1-384
Arabidopsis thaliana	>AT5G08170.1.ath.19670786/1-384
Glycine max	>Glyma.17G083400.1.p.gma.30479429/1-377
Phaseolus vulgaris	>Phvul.003G180900.1.pvu.27144658/1-375
Chlamydomonas reinhardtii	>Cre01.g009350.t1.2.cre.30789624/1-431
Volvox carteri_1	>Vocar20008389m.vca.23126364/1-437
Volvox carteri_2	>Vocar20001248m.vca.23130630/1-427
Coccomyxa subellipsoidea	>35944.csu.27387373/1-350
Pinus pinaster	>Pinuspinaster_sp_v3.0_unigene7574
Pinus sylvestris	>gi|312162106|gb|ADQ37300.1| putative agmatine iminohydrolase [Pinus sylvestris]
Picea sitchensis	>gi|148906450|gb|ABR16378.1| unknown [Picea sitchensis]

CPA

Species	Accession number
Ricinus communis	>30055.m001577.rco.16816208/1255
Arabidopsis thaliana	>AT2G27450.1.ath.19643270/1300
Boechera stricta	>Bostr.27991s0004.1.p.adr.30677583/1300
Brachypodium distachyon	>Bradi3g44960.1.p.bdi.31155302/1303
Brassica rapa	>Brara.D01638.1.p.bra.30620593/1298
Capsella rubella	>Carubv10023735m.cru.20903819/1300
Citrus clementina	>Ciclev10028933m.ccl.20813923/1302
Eucalyptus grandis	>Eucgr.J00500.1.egr.23598285/1300
Carica papaya	>evm.model.supercontig_195.1.cpa.16412260/1325
Arabidopsis lyrata	>fgenesh2_kg.4__704__AT2G27450.2.aly.16059967/1323
Picea sitchensis	>gi|116781124|gb|ABK21974.1| unknown Picea sitchensis
Pinus sylvestris	>gi|312162108|gb|ADQ37301.1| putative Ncarbamoylputrescine amidohydrolase Pinus sylvestris
Glycine max_3	>Glyma.08G074800.1.p.gma.30540414/1300
Glycine max_2	>Glyma.12G185700.1.p.gma.30546448/1300
Glycine max_1	>Glyma.13G315800.1.p.gma.30501204/1300
Gossypium raimondii	>Gorai.006G112500.1.gra.26831211/1301
Zea mays	>GRMZM2G073950_P01.zma.30990896/1301
Vitis vinifera	>GSVIVT01036111001.vvi.17841142/1298
Oryza sativa	>LOC_Os02g33080.1.osa.24134702/1302
Linum usitatissimum	>Lus10020627.lus.23172365/1303
Medicago truncatula	>Medtr2g086600.1.mtr.31063467/1302
Fragaria vesca	>mrna21763.1v1.0hybrid.fve.27266317/1302
Citrus sinensis	>orange1.1g022174m.csi.18111145/1302
Panicum virgatum_1	>Pavir.Aa01978.2.p.pvi.30250001/1299
Panicum virgatum_2	>Pavir.Ab01810.1.p.pvi.30232693/1277
Solanum tuberosum	>PGSC0003DMP400045714.stu.24409783/1301
Phaseolus vulgaris	>Phvul.005G123100.1.pvu.27148851/1300
Pinus pinaster	>Pinus_pinaster_sp_v3.0_unigene18248
Populus trichocarpa	>Potri.004G201400.1.ptr.26989521/1302
Prunus persica	>ppa009250m.ppe.17664882/1301
Setaria italica	>Si017557m.sit.19688695/1370
Sorghum bicolor	>Sobic.004G166500.1.p.sbi.28373628/1299
Solanum lycopersicum	>Solyc11g068540.1.1.sly.27295149/1301
Theobroma cacao	>Thecc1EG041385t2.tca.27425773/1303
Eutrema salsugineum	>Thhalv10017003m.esa.20180524/1300

SPDS/SPDS3/SPMS

Species	Accession number
Malus sylvestris_ACL5	gi|658002242
Arabidopsis lyrata_SPDS3	gi|297792679
Arabidopsis lyrata_ACL5	gi|297812137
Ashbya gossypii	gi|302307177
Arabidopsis thaliana_ACL5	gi|18419941
Arabidopsis thaliana_SPDS1	gi|145336078
Arabidopsis thaliana_SPDS2	gi|15223115
Arabidopsis thaliana_SPDS3	gi|334188357
Brassica rapa_SPMS_1	gi|685272254
Brassica rapa_SPMS_2	gi|685372574
Capsella rubella	gi|565433632
Chlamydomonas reinhardtii_SPDS	gi|159489717
Citrus sinensis_SPMS	gi|568874832
Coccomyxa subellipsoidea_SPDS	gi|545364814
Coffea arabica_SPDS	gi|6094335
Danio rerio_SPDS	gi|242117904
Datura stramonium_SPDS	gi|6094327
Drosophila melanogaster_SPDS	gi|24645443
Eucalyptus grandis_SPMS	gi|702243059
Glycine max_2_ACL5	gi|359807232

Glycine max_1_SPDS3	gi|356516027
Gossypium raimondii_2	Gorai.004G115100
Gossypium raimondii_1	Gorai.008G269900
Homo sapiens_SPDS	gi|63253298
Kluyveromyces lactis	gi|50303991
Linum usitatissimum_1	Lus10003398.g
Linum usitatissimum_2	Lus10019016.g
Macaca mulatta_SPDS	gi|383873318
Magnaporthe oryzae_SPDS_1	gi|389628860
Magnaporthe oryzae_SPDS_2	gi|440485934
Mallus domestica_SPMS	gi|658016874
Medicago truncatula_2_SPDS	gi|357480843
Medicago truncatula_1_SPDS	gi|357463867
Micromonas pusilla_1	gi|255086215
Micromonas pusilla_2	gi|303284801
Mus musculus_SPDS	gi|6678131
Neurospora crassa_SPDS	gi|85100119
Nicotiana sylvestris_SPDS	gi|700584686
Oryza sativa_2	gi|115445231
Oryza sativa_1	gi|115471679
Ostreococcus lucimarinus_SPDS	gi|145352221
Pan troglodytes_SPDS	gi|114553926
Panax ginseng_SPDS	gi|251831262
Phaseolus vulgaris	gi|593268760
Physcomitrella patens_1	Phpat.017G018400.1
Physcomitrella patens_2	Phpat.002G013200.1
Picea sitchensis	gi|116785514
Pinus sylvestris_SPMS	gi|312162114
Pinus sylvestris_SPDS	gi|312162112
Pisum sativum_2_SPDS2	gi|12229957
Pisum sativum_1_SPDS1	gi|12229958
Populus tomentosa_SPDS	gi|357433153
Populus trichocarpa_1	gi|566190032
Populus trichocarpa_2	gi|118484280
Populus trichocarpa_3	gi|566205156
Prunus persica_1	gi|596134444
Prunus persica_2	gi|596001294
Rattus norgegicus_SPDS	gi|16758208
Ricinus communis_SPMS	gi|255551457
Ricinus communis_1_SPDS1	gi|255587587
Ricinus communis_3_SPDS1	gi|255545020
Saccharomyces cerevisiae_SPDS	gi|6325326
Selaginella moellendorffii_1	gi|302761430
Selaginella moellendorffii_2	gi|302784574
Solanum lycopersicum_2	gi|350537177
Solanum lycopersicum_1	gi|350534638
Theobroma cacao_SPDS3	gi|590673798
Xenopus tropicalis_SPDS	gi|301620173
Canis lupus_SPMS	gi|345806962
Bos taurus_SPMS	gi|78369282
Rattus norvegicus_SPMS	gi|76559933
Mus musculus	gi|26340516
Homo sapiens_SPMS	gi|21264341
Drosophila melanogaster_isoform_A	gi|24663238
Drosophila melanogaster_isoform_B	gi|21358269
Apis mellifera_SPMS	gi|110750077
Ectocarpus siliculosus_SPDS/SPMS	gi|298709403
Micromonas sp._ACL5	gi|255073361
Physcomitrella patens_ACL5	gi|168019612
Thermus thermophilus	gi|499486278
Saccharomyces cerevisiae_SPMS	gi|151941307
Cryptomeria japonica_SPMS	gi|34146773

Supplementary Table S3. Functional categories and terms for ornithine/arginine (Orn/Arg) biosynthetic pathway associated sequences.

Level	GO ID	Terms	Category	Score	Sequences
6	GO:0044106	cellular amine metabolic process	biological_process	7	AaADC, AaCPA, AaSAMDC, AaAIH, AaSPDS, AaSPDS3, AaSPMS
3	GO:0071704	organic substance metabolic process	biological_process	7	AaSAMDC, AaSPMS, AaSPDS, AaSPDS3, AaADC, AaCPA, AaAIH
4	GO:0044249	cellular biosynthetic process	biological_process	7	AaSAMDC, AaSPDS, AaSPDS3, AaADC, AaCPA, AaAIH, AaSPMS
2	GO:0008152	metabolic process	biological_process	7	AaSAMDC, AaSPMS, AaCPA, AaSPDS, AaSPDS3, AaADC, AaAIH
8	GO:0042401	cellular biogenic amine biosynthetic process	biological_process	7	AaADC, AaCPA, AaAIH, AaSAMDC, AaSPDS, AaSPDS3, AaSPMS
8	GO:0006595	polyamine metabolic process	biological_process	7	AaADC, AaCPA, AaSAMDC, AaAIH, AaSPDS, AaSPDS3, AaSPMS
4	GO:1901564	organonitrogen compound metabolic process	biological_process	7	AaSPDS, AaSPDS3, AaADC, AaCPA, AaSAMDC, AaAIH, AaSPMS
3	GO:0044237	cellular metabolic process	biological_process	7	AaSAMDC, AaSPDS, AaSPDS3, AaADC, AaCPA, AaAIH, AaSPMS
2	GO:0009987	cellular process	biological_process	7	AaSAMDC, AaADC, AaSPMS, AaSPDS, AaSPDS3, AaCPA, AaAIH
3	GO:0006807	nitrogen compound metabolic process	biological_process	7	AaSPDS, AaSPDS3, AaADC, AaCPA, AaSAMDC, AaAIH, AaSPMS
9	GO:0006596	polyamine biosynthetic process	biological_process	7	AaADC, AaCPA, AaAIH, AaSAMDC, AaSPDS, AaSPDS3, AaSPMS
3	GO:0009058	biosynthetic process	biological_process	7	AaSAMDC, AaSPMS, AaSPDS, AaSPDS3, AaADC, AaCPA, AaAIH
7	GO:0006576	cellular biogenic amine metabolic process	biological_process	7	AaADC, AaCPA, AaSAMDC, AaAIH, AaSPDS, AaSPDS3, AaSPMS
4	GO:1901576	organic substance biosynthetic process	biological_process	7	AaSAMDC, AaSPMS, AaSPDS, AaSPDS3, AaADC, AaCPA, AaAIH
5	GO:0044271	cellular nitrogen compound biosynthetic process	biological_process	7	AaADC, AaCPA, AaAIH, AaSAMDC, AaSPDS, AaSPDS3, AaSPMS
6	GO:0009309	amine biosynthetic process	biological_process	7	AaADC, AaCPA, AaAIH, AaSAMDC, AaSPDS, AaSPDS3, AaSPMS
4	GO:0034641	cellular nitrogen compound metabolic process	biological_process	7	AaADC, AaCPA, AaSAMDC, AaAIH, AaSPDS, AaSPDS3, AaSPMS
5	GO:1901566	organonitrogen compound biosynthetic process	biological_process	7	AaSPDS, AaSPDS3, AaADC, AaCPA, AaAIH, AaSAMDC, AaSPMS
5	GO:0009308	amine metabolic process	biological_process	7	AaADC, AaCPA, AaSAMDC, AaAIH, AaSPDS, AaSPDS3, AaSPMS
2	GO:0044699	single-organism process	biological_process	6	AaSPMS, AaADC, AaSAMDC, AaSPDS, AaSPDS3, AaAIH
2	GO:0050896	response to stimulus	biological_process	6	AaSPDS, AaSPDS3, AaADC, AaSAMDC, AaSPMS, AaCPA
3	GO:0044710	single-organism metabolic process	biological_process	5	AaSAMDC, AaCPA, AaSPDS, AaSPDS3, AaADC
3	GO:0016043	cellular component organization	biological_process	5	AaADC, AaSPMS, AaSPDS, AaSPDS3, AaSAMDC
3	GO:0044707	single-multicellular organism process	biological_process	5	AaSPMS, AaSPDS, AaSPDS3, AaADC, AaAIH
2	GO:0032502	developmental process	biological_process	5	AaSPMS, AaSPDS, AaSPDS3, AaADC, AaAIH
3	GO:0044763	single-organism cellular process	biological_process	5	AaADC, AaSAMDC, AaSPMS, AaSPDS, AaSPDS3
4	GO:0007275	multicellular organismal development	biological_process	5	AaSPMS, AaSPDS, AaSPDS3, AaADC, AaAIH

5	GO:0048731	system development	biological_process	5	AaSPDS, AaSPDS3, AaADC, AaSPMS, AaAIH
2	GO:0032501	multicellular organismal process	biological_process	5	AaSPMS, AaSPDS, AaSPDS3, AaADC, AaAIH
2	GO:0071840	cellular component organization or biogenesis	biological_process	5	AaADC, AaSPMS, AaSPDS, AaSPDS3, AaSAMDC
2	GO:0065007	biological regulation	biological_process	5	AaSPDS, AaSPDS3, AaADC, AaSAMDC, AaSPMS
3	GO:0048856	anatomical structure development	biological_process	5	AaSPDS, AaSPDS3, AaADC, AaSPMS, AaAIH
2	GO:0051704	multi-organism process	biological_process	4	AaSPDS, AaSPDS3, AaSAMDC, AaADC
5	GO:0006082	organic acid metabolic process	biological_process	4	AaSPDS, AaSPDS3, AaADC, AaSAMDC
6	GO:0043436	oxoacid metabolic process	biological_process	4	AaSPDS, AaSPDS3, AaADC, AaSAMDC
7	GO:0019752	carboxylic acid metabolic process	biological_process	4	AaSPDS, AaSPDS3, AaADC, AaSAMDC
3	GO:0044238	primary metabolic process	biological_process	4	AaSPMS, AaSPDS, AaSPDS3, AaSAMDC
5	GO:0044283	small molecule biosynthetic process	biological_process	4	AaSAMDC, AaSPDS, AaSPDS3, AaADC
5	GO:0009791	post-embryonic development	biological_process	4	AaSPDS, AaSPDS3, AaADC, AaAIH
4	GO:0044711	single-organism biosynthetic process	biological_process	4	AaSAMDC, AaSPDS, AaSPDS3, AaADC
4	GO:0044281	small molecule metabolic process	biological_process	4	AaSAMDC, AaSPDS, AaSPDS3, AaADC
6	GO:0016053	organic acid biosynthetic process	biological_process	4	AaSPDS, AaSPDS3, AaADC, AaSAMDC
8	GO:0046394	carboxylic acid biosynthetic process	biological_process	4	AaSPDS, AaSPDS3, AaADC, AaSAMDC
3	GO:0006950	response to stress	biological_process	4	AaSPDS, AaSPDS3, AaADC, AaSAMDC
3	GO:0044767	single-organism developmental process	biological_process	4	AaSPMS, AaAIH, AaSPDS, AaSPDS3
3	GO:0051234	establishment of localization	biological_process	3	AaADC, AaSAMDC, AaSPMS
9	GO:0008652	cellular amino acid biosynthetic process	biological_process	3	AaSPDS, AaSPDS3, AaSAMDC
5	GO:0010087	phloem or xylem histogenesis	biological_process	3	AaSPMS, AaSPDS, AaSPDS3
6	GO:0000902	cell morphogenesis	biological_process	3	AaSPMS, AaSPDS, AaSPDS3
10	GO:0008295	spermidine biosynthetic process	biological_process	3	AaSPDS, AaSPDS3, AaSPMS
5	GO:0060560	developmental growth involved in morphogenesis	biological_process	3	AaSPMS, AaSPDS, AaSPDS3
4	GO:0009888	tissue development	biological_process	3	AaSPMS, AaSPDS, AaSPDS3
3	GO:0009628	response to abiotic stimulus	biological_process	3	AaSPMS, AaADC, AaSAMDC
3	GO:0006955	immune response	biological_process	3	AaSPDS, AaSPDS3, AaADC
3	GO:0009607	response to biotic stimulus	biological_process	3	AaSPDS, AaSPDS3, AaADC
9	GO:0008216	spermidine metabolic process	biological_process	3	AaSPDS, AaSPDS3, AaSPMS
5	GO:0032989	cellular component morphogenesis	biological_process	3	AaSPMS, AaSPDS, AaSPDS3
8	GO:0006520	cellular amino acid metabolic process	biological_process	3	AaSPDS, AaSPDS3, AaSAMDC
4	GO:0006952	defense response	biological_process	3	AaSPDS, AaSPDS3, AaADC
2	GO:0051179	localization	biological_process	3	AaADC, AaSAMDC, AaSPMS
4	GO:0016049	cell growth	biological_process	3	AaSPMS, AaSPDS, AaSPDS3
4	GO:0010817	regulation of hormone levels	biological_process	3	AaSPDS, AaSPDS3, AaSPMS
4	GO:0009653	anatomical structure morphogenesis	biological_process	3	AaSPMS, AaSPDS, AaSPDS3
4	GO:0048869	cellular developmental process	biological_process	3	AaSPMS, AaSPDS, AaSPDS3
6	GO:0048513	organ development	biological_process	3	AaSPDS, AaSPDS3, AaSPMS
6	GO:0010089	xylem development	biological_process	3	AaSPMS, AaSPDS, AaSPDS3
2	GO:0040007	growth	biological_process	3	AaSPMS, AaSPDS, AaSPDS3
4	GO:0071554	cell wall organization or biogenesis	biological_process	3	AaSPDS, AaSPDS3, AaSPMS
4	GO:0044403	symbiosis, encompassing mutualism through parasitism	biological_process	3	AaSPDS, AaSPDS3, AaSAMDC
5	GO:0045087	innate immune response	biological_process	3	AaSPDS, AaSPDS3, AaADC
4	GO:0045229	external encapsulating structure organization	biological_process	3	AaSPDS, AaSPDS3, AaSPMS
3	GO:0042221	response to chemical stimulus	biological_process	3	AaCPA, AaADC, AaSAMDC
7	GO:0009826	unidimensional cell growth	biological_process	3	AaSPMS, AaSPDS, AaSPDS3
2	GO:0002376	immune system process	biological_process	3	AaSPDS, AaSPDS3, AaADC
5	GO:0030154	cell differentiation	biological_process	3	AaSPMS, AaSPDS, AaSPDS3
3	GO:0044419	interspecies interaction between organisms	biological_process	3	AaSPDS, AaSPDS3, AaSAMDC
4	GO:0006810	transport	biological_process	3	AaADC, AaSAMDC, AaSPMS
3	GO:0065008	regulation of biological quality	biological_process	3	AaSPDS, AaSPDS3, AaSPMS
4	GO:0048589	developmental growth	biological_process	3	AaSPMS, AaSPDS, AaSPDS3
5	GO:0044765	single-organism transport	biological_process	3	AaADC, AaSAMDC, AaSPMS
4	GO:0051707	response to other organism	biological_process	3	AaSPDS, AaSPDS3, AaADC
5	GO:0071555	cell wall organization	biological_process	3	AaSPDS, AaSPDS3, AaSPMS
7	GO:0048608	reproductive structure development	biological_process	2	AaADC, AaAIH
4	GO:0051641	cellular localization	biological_process	2	AaADC, AaSAMDC
4	GO:0003006	developmental process involved in reproduction	biological_process	2	AaADC, AaAIH
5	GO:0051649	establishment of localization in cell	biological_process	2	AaADC, AaSAMDC
4	GO:1901700	response to oxygen-containing compound	biological_process	2	AaADC, AaSAMDC
3	GO:0050789	regulation of biological process	biological_process	2	AaADC, AaSAMDC
4	GO:0008104	protein localization	biological_process	2	AaADC, AaSAMDC
9	GO:0048316	seed development	biological_process	2	AaADC, AaAIH
10	GO:0009069	serine family amino acid metabolic process	biological_process	2	AaSPDS, AaSPDS3
6	GO:0015031	protein transport	biological_process	2	AaADC, AaSAMDC
6	GO:0061458	reproductive system development	biological_process	2	AaADC, AaAIH
5	GO:0051701	interaction with host	biological_process	2	AaSPDS, AaSPDS3
6	GO:0046907	intracellular transport	biological_process	2	AaADC, AaSAMDC
4	GO:0009314	response to radiation	biological_process	2	AaSPMS, AaSAMDC
6	GO:0010050	vegetative phase change	biological_process	2	AaSPDS, AaSPDS3
4	GO:0035821	modification of morphology or physiology of other organism	biological_process	2	AaSPDS, AaSPDS3
6	GO:0052249	modulation of RNA levels in other organism involved in symbiotic interaction	biological_process	2	AaSPDS, AaSPDS3
8	GO:0009616	virus induced gene silencing	biological_process	2	AaSPDS, AaSPDS3
6	GO:0042742	defense response to bacterium	biological_process	2	AaSPDS, AaSPDS3
6	GO:0044003	modification by symbiont of host morphology or physiology	biological_process	2	AaSPDS, AaSPDS3
6	GO:0051607	defense response to virus	biological_process	2	AaSPDS, AaSPDS3
5	GO:0009416	response to light stimulus	biological_process	2	AaSPMS, AaSAMDC
6	GO:0009411	response to UV	biological_process	2	AaSPMS, AaSAMDC
3	GO:0022414	reproductive process	biological_process	2	AaADC, AaAIH
4	GO:0010033	response to organic substance	biological_process	2	AaADC, AaSAMDC
5	GO:0071702	organic substance transport	biological_process	2	AaADC, AaSAMDC
8	GO:0006605	protein targeting	biological_process	2	AaADC, AaSAMDC
5	GO:0051817	modification of morphology or physiology of other organism involved in symbiotic interaction	biological_process	2	AaSPDS, AaSPDS3
4	GO:0043170	macromolecule metabolic process	biological_process	2	AaSPMS, AaSAMDC
7	GO:0052018	modulation by symbiont of RNA levels in host	biological_process	2	AaSPDS, AaSPDS3
7	GO:0006886	intracellular protein transport	biological_process	2	AaADC, AaSAMDC
4	GO:0006790	sulfur compound metabolic process	biological_process	2	AaSPDS, AaSPDS3
5	GO:0045184	establishment of protein localization	biological_process	2	AaADC, AaSAMDC
2	GO:0000003	reproduction	biological_process	2	AaADC, AaAIH
3	GO:0002252	immune effector process	biological_process	2	AaSPDS, AaSPDS3
9	GO:0000096	sulfur amino acid metabolic process	biological_process	2	AaSPDS, AaSPDS3
5	GO:0009615	response to virus	biological_process	2	AaSPDS, AaSPDS3
6	GO:0042545	cell wall modification	biological_process	2	AaSPDS, AaSPDS3
9	GO:0009445	putrescine metabolic process	biological_process	2	AaADC, AaCPA
9	GO:1901605	alpha-amino acid metabolic process	biological_process	2	AaSPDS, AaSPDS3

8	GO:0010154	fruit development	biological_process	2	AaADC, AaAIH
10	GO:0009446	putrescine biosynthetic process	biological_process	2	AaADC, AaCPA
6	GO:0034613	cellular protein localization	biological_process	2	AaADC, AaSAMDC
4	GO:0061024	membrane organization	biological_process	2	AaADC, AaSAMDC
3	GO:0033036	macromolecule localization	biological_process	2	AaADC, AaSAMDC
5	GO:0070727	cellular macromolecule localization	biological_process	2	AaADC, AaSAMDC
4	GO:0009611	response to wounding	biological_process	2	AaADC, AaSAMDC
5	GO:0009617	response to bacterium	biological_process	2	AaSPDS, AaSPDS3
14	GO:0048767	root hair elongation	biological_process	1	AaSPMS
9	GO:0010264	myo-inositol hexakisphosphate biosynthetic process	biological_process	1	AaSAMDC
5	GO:0006066	alcohol metabolic process	biological_process	1	AaSAMDC
5	GO:1901617	organic hydroxy compound biosynthetic process	biological_process	1	AaSAMDC
7	GO:0071396	cellular response to lipid	biological_process	1	AaADC
5	GO:0010467	gene expression	biological_process	1	AaSAMDC
9	GO:0072330	monocarboxylic acid biosynthetic process	biological_process	1	AaADC
6	GO:0010468	regulation of gene expression	biological_process	1	AaSAMDC
6	GO:0009914	hormone transport	biological_process	1	AaSPMS
10	GO:0009793	embryo development ending in seed dormancy	biological_process	1	AaAIH
4	GO:0021700	developmental maturation	biological_process	1	AaSPMS
7	GO:0071407	cellular response to organic cyclic compound	biological_process	1	AaADC
6	GO:0009414	response to water deprivation	biological_process	1	AaADC
10	GO:0009695	jasmonic acid biosynthetic process	biological_process	1	AaADC
6	GO:0048468	cell development	biological_process	1	AaSPMS
9	GO:0009863	salicylic acid mediated signaling pathway	biological_process	1	AaADC
5	GO:0009415	response to water stimulus	biological_process	1	AaADC
8	GO:0072594	establishment of protein localization to organelle	biological_process	1	AaSAMDC
7	GO:0000271	polysaccharide biosynthetic process	biological_process	1	AaSPMS
8	GO:0010015	root morphogenesis	biological_process	1	AaSPMS
9	GO:0043481	anthocyanin accumulation in tissues in response to UV light	biological_process	1	AaSPMS
5	GO:1901362	organic cyclic compound biosynthetic process	biological_process	1	AaSAMDC
5	GO:0009825	multidimensional cell growth	biological_process	1	AaSPMS
4	GO:0051716	cellular response to stimulus	biological_process	1	AaADC
9	GO:0009738	abscisic acid mediated signaling pathway	biological_process	1	AaADC
7	GO:0046173	polyol biosynthetic process	biological_process	1	AaSAMDC
4	GO:0010035	response to inorganic substance	biological_process	1	AaADC
6	GO:0009751	response to salicylic acid stimulus	biological_process	1	AaADC
2	GO:0023052	signaling	biological_process	1	AaADC
7	GO:0045088	regulation of innate immune response	biological_process	1	AaADC
7	GO:0048759	xylem vessel member cell differentiation	biological_process	1	AaSPMS
7	GO:0048364	root development	biological_process	1	AaSPMS
8	GO:0010363	regulation of plant-type hypersensitive response	biological_process	1	AaADC
6	GO:0016051	carbohydrate biosynthetic process	biological_process	1	AaSPMS
5	GO:1901565	organonitrogen compound catabolic process	biological_process	1	AaSAMDC
13	GO:0048765	root hair cell differentiation	biological_process	1	AaSPMS
4	GO:0048519	negative regulation of biological process	biological_process	1	AaSAMDC
5	GO:0050776	regulation of immune response	biological_process	1	AaADC
8	GO:0042402	cellular biogenic amine catabolic process	biological_process	1	AaSAMDC
3	GO:0044700	single organism signaling	biological_process	1	AaADC
6	GO:0019058	viral life cycle	biological_process	1	AaSAMDC
7	GO:0043588	skin development	biological_process	1	AaSPMS
6	GO:0009639	response to red or far red light	biological_process	1	AaSPMS
4	GO:1901360	organic cyclic compound metabolic process	biological_process	1	AaSAMDC
6	GO:0009699	phenylpropanoid biosynthetic process	biological_process	1	AaSAMDC
7	GO:0019079	viral genome replication	biological_process	1	AaSAMDC
8	GO:0032787	monocarboxylic acid metabolic process	biological_process	1	AaADC
5	GO:0019438	aromatic compound biosynthetic process	biological_process	1	AaSAMDC
8	GO:0009926	auxin polar transport	biological_process	1	AaSPMS
3	GO:0044764	multi-organism cellular process	biological_process	1	AaSAMDC
7	GO:0009805	coumarin biosynthetic process	biological_process	1	AaSAMDC
4	GO:0090066	regulation of anatomical structure size	biological_process	1	AaSPMS
6	GO:0009804	coumarin metabolic process	biological_process	1	AaSAMDC
6	GO:0090407	organophosphate biosynthetic process	biological_process	1	AaSAMDC
5	GO:0010941	regulation of cell death	biological_process	1	AaADC
9	GO:0009694	jasmonic acid metabolic process	biological_process	1	AaADC
5	GO:0048532	anatomical structure arrangement	biological_process	1	AaSPMS
4	GO:0002682	regulation of immune system process	biological_process	1	AaADC
5	GO:0009790	embryo development	biological_process	1	AaAIH
6	GO:0009753	response to jasmonic acid stimulus	biological_process	1	AaADC
5	GO:0043480	pigment accumulation in tissues	biological_process	1	AaSPMS
5	GO:0006796	phosphate-containing compound metabolic process	biological_process	1	AaSAMDC
6	GO:0019751	polyol metabolic process	biological_process	1	AaSAMDC
7	GO:0060918	auxin transport	biological_process	1	AaSPMS
5	GO:0044550	secondary metabolite biosynthetic process	biological_process	1	AaSAMDC
8	GO:0033517	myo-inositol hexakisphosphate metabolic process	biological_process	1	AaSAMDC
5	GO:0014070	response to organic cyclic compound	biological_process	1	AaADC
4	GO:0043476	pigment accumulation	biological_process	1	AaSPMS
7	GO:0033365	protein localization to organelle	biological_process	1	AaSAMDC
7	GO:0016482	cytoplasmic transport	biological_process	1	AaSAMDC
6	GO:0034285	response to disaccharide stimulus	biological_process	1	AaSAMDC
4	GO:0019222	regulation of metabolic process	biological_process	1	AaSAMDC
6	GO:0010605	negative regulation of macromolecule metabolic process	biological_process	1	AaSAMDC
4	GO:1901615	organic hydroxy compound metabolic process	biological_process	1	AaSAMDC
9	GO:0009867	jasmonic acid mediated signaling pathway	biological_process	1	AaADC
5	GO:0009651	response to salt stress	biological_process	1	AaADC
7	GO:0072657	protein localization to membrane	biological_process	1	AaADC
8	GO:0032958	inositol phosphate biosynthetic process	biological_process	1	AaSAMDC
4	GO:0009266	response to temperature stimulus	biological_process	1	AaADC
7	GO:0009626	plant-type hypersensitive response	biological_process	1	AaADC
7	GO:0032870	cellular response to hormone stimulus	biological_process	1	AaADC
10	GO:0010053	root epidermal cell differentiation	biological_process	1	AaSPMS
4	GO:0006970	response to osmotic stress	biological_process	1	AaADC
5	GO:0019637	organophosphate metabolic process	biological_process	1	AaSAMDC
7	GO:0048507	meristem development	biological_process	1	AaSPMS
5	GO:0009059	macromolecule biosynthetic process	biological_process	1	AaSPMS
12	GO:0048764	trichoblast maturation	biological_process	1	AaSPMS
6	GO:0009737	response to abscisic acid stimulus	biological_process	1	AaADC
5	GO:0009620	response to fungus	biological_process	1	AaADC

9	GO:0006612	protein targeting to membrane	biological_process	1	AaADC
4	GO:0009812	flavonoid metabolic process	biological_process	1	AaSAMDC
4	GO:0006793	phosphorus metabolic process	biological_process	1	AaSAMDC
9	GO:0010014	meristem initiation	biological_process	1	AaSPMS
7	GO:0009744	response to sucrose stimulus	biological_process	1	AaSAMDC
4	GO:0046483	heterocycle metabolic process	biological_process	1	AaSAMDC
8	GO:0016458	gene silencing	biological_process	1	AaSAMDC
5	GO:0060429	epithelium development	biological_process	1	AaSPMS
5	GO:0060255	regulation of macromolecule metabolic process	biological_process	1	AaSAMDC
8	GO:0008544	epidermis development	biological_process	1	AaSPMS
6	GO:0009733	response to auxin stimulus	biological_process	1	AaADC
10	GO:0006623	protein targeting to vacuole	biological_process	1	AaSAMDC
6	GO:0031347	regulation of defense response	biological_process	1	AaADC
7	GO:0043478	pigment accumulation in response to UV light	biological_process	1	AaSPMS
5	GO:0007165	signal transduction	biological_process	1	AaADC
4	GO:0006979	response to oxidative stress	biological_process	1	AaADC
5	GO:0033993	response to lipid	biological_process	1	AaADC
6	GO:0043067	regulation of programmed cell death	biological_process	1	AaADC
10	GO:0042398	cellular modified amino acid biosynthetic process	biological_process	1	AaSAMDC
7	GO:0010224	response to UV-B	biological_process	1	AaSAMDC
3	GO:0009605	response to external stimulus	biological_process	1	AaSPMS
7	GO:0010218	response to far red light	biological_process	1	AaSPMS
5	GO:0018130	heterocycle biosynthetic process	biological_process	1	AaSAMDC
8	GO:0043479	pigment accumulation in tissues in response to UV light	biological_process	1	AaSPMS
6	GO:0046165	alcohol biosynthetic process	biological_process	1	AaSAMDC
8	GO:0071446	cellular response to salicylic acid stimulus	biological_process	1	AaADC
6	GO:0009723	response to ethylene stimulus	biological_process	1	AaADC
4	GO:0006725	cellular aromatic compound metabolic process	biological_process	1	AaSAMDC
4	GO:1901575	organic substance catabolic process	biological_process	1	AaSAMDC
7	GO:0010629	negative regulation of gene expression	biological_process	1	AaSAMDC
5	GO:0016032	viral process	biological_process	1	AaSAMDC
9	GO:0072666	establishment of protein localization to vacuole	biological_process	1	AaSAMDC
5	GO:0044723	single-organism carbohydrate metabolic process	biological_process	1	AaSPMS
5	GO:0080134	regulation of response to stress	biological_process	1	AaADC
3	GO:0009056	catabolic process	biological_process	1	AaSAMDC
4	GO:0050794	regulation of cellular process	biological_process	1	AaADC
4	GO:0008219	cell death	biological_process	1	AaADC
5	GO:0009892	negative regulation of metabolic process	biological_process	1	AaSAMDC
4	GO:0005975	carbohydrate metabolic process	biological_process	1	AaSPMS
6	GO:0008361	regulation of cell size	biological_process	1	AaSPMS
5	GO:0061025	membrane fusion	biological_process	1	AaSAMDC
8	GO:0071215	cellular response to abscisic acid stimulus	biological_process	1	AaADC
4	GO:0071495	cellular response to endogenous stimulus	biological_process	1	AaADC
8	GO:0072665	protein localization to vacuole	biological_process	1	AaSAMDC
5	GO:0009813	flavonoid biosynthetic process	biological_process	1	AaSAMDC
4	GO:0080167	response to karrikin	biological_process	1	AaADC
4	GO:0019748	secondary metabolic process	biological_process	1	AaSAMDC
6	GO:0042538	hyperosmotic salinity response	biological_process	1	AaADC
3	GO:0043473	pigmentation	biological_process	1	AaSPMS
4	GO:0048583	regulation of response to stimulus	biological_process	1	AaADC
6	GO:0009310	amine catabolic process	biological_process	1	AaSAMDC
7	GO:0007034	vacuolar transport	biological_process	1	AaSAMDC
6	GO:0022622	root system development	biological_process	1	AaSPMS
5	GO:0033554	cellular response to stress	biological_process	1	AaADC
7	GO:0043647	inositol phosphate metabolic process	biological_process	1	AaSAMDC
6	GO:0030855	epithelial cell differentiation	biological_process	1	AaSPMS
8	GO:0009755	hormone-mediated signaling pathway	biological_process	1	AaADC
9	GO:0006598	polyamine catabolic process	biological_process	1	AaSAMDC
6	GO:0080135	regulation of cellular response to stress	biological_process	1	AaADC
6	GO:0071310	cellular response to organic substance	biological_process	1	AaADC
7	GO:0097306	cellular response to alcohol	biological_process	1	AaADC
5	GO:0006972	hyperosmotic response	biological_process	1	AaADC
3	GO:0016265	death	biological_process	1	AaADC
4	GO:0007154	cell communication	biological_process	1	AaADC
5	GO:0007389	pattern specification process	biological_process	1	AaSPMS
8	GO:0009933	meristem structural organization	biological_process	1	AaSPMS
5	GO:0009698	phenylpropanoid metabolic process	biological_process	1	AaSAMDC
8	GO:0071395	cellular response to jasmonic acid stimulus	biological_process	1	AaADC
8	GO:0009932	cell tip growth	biological_process	1	AaSPMS
5	GO:0009409	response to cold	biological_process	1	AaADC
5	GO:0016192	vesicle-mediated transport	biological_process	1	AaSAMDC
6	GO:0034050	host programmed cell death induced by symbiont	biological_process	1	AaADC
5	GO:0044802	single-organism membrane organization	biological_process	1	AaADC
5	GO:0032535	regulation of cellular component size	biological_process	1	AaSPMS
9	GO:0009913	epidermal cell differentiation	biological_process	1	AaSPMS
8	GO:0090150	establishment of protein localization to membrane	biological_process	1	AaADC
7	GO:0048469	cell maturation	biological_process	1	AaSPMS
5	GO:0005976	polysaccharide metabolic process	biological_process	1	AaSPMS
5	GO:0009725	response to hormone stimulus	biological_process	1	AaADC
5	GO:0097305	response to alcohol	biological_process	1	AaADC
5	GO:0070887	cellular response to chemical stimulus	biological_process	1	AaADC
6	GO:1901701	cellular response to oxygen-containing compound	biological_process	1	AaADC
5	GO:0012501	programmed cell death	biological_process	1	AaADC
5	GO:0009743	response to carbohydrate stimulus	biological_process	1	AaSAMDC
11	GO:0010054	trichoblast differentiation	biological_process	1	AaSPMS
3	GO:0009719	response to endogenous stimulus	biological_process	1	AaADC
7	GO:0016197	endosomal transport	biological_process	1	AaSAMDC
9	GO:0006575	cellular modified amino acid metabolic process	biological_process	1	AaSAMDC
7	GO:0048588	developmental cell growth	biological_process	1	AaSPMS
2	GO:0003824	catalytic activity	molecular_function	7	AaAIH, AaSPDS, AaSPDS3, AaSPMS, AaSAMDC, AaADC, AaCPA
2	GO:0005488	binding	molecular_function	4	AaSAMDC, AaSPDS, AaSPDS3, AaSPMS
3	GO:0005515	protein binding	molecular_function	4	AaSAMDC, AaSPDS, AaSPDS3, AaSPMS
5	GO:0004766	spermidine synthase activity	molecular_function	3	AaSPDS, AaSPDS3, AaSPMS
5	GO:0016768	spermine synthase activity	molecular_function	3	AaSPDS, AaSPDS3, AaSPMS
4	GO:0016765	transferase activity, transferring alkyl or aryl (other than methyl) groups	molecular_function	3	AaSPDS, AaSPDS3, AaSPMS
3	GO:0016740	transferase activity	molecular_function	3	AaSPDS, AaSPDS3, AaSPMS
3	GO:0016787	hydrolase activity	molecular_function	2	AaAIH, AaCPA
5	GO:0016831	carboxy-lyase activity	molecular_function	2	AaSAMDC, AaADC

4	GO:0016810	hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds	molecular_function	2	AaAIH, AaCPA
4	GO:0016830	carbon-carbon lyase activity	molecular_function	2	AaSAMDC, AaADC
3	GO:0016829	lyase activity	molecular_function	2	AaSAMDC, AaADC
5	GO:0016813	hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds, in linear amidines	molecular_function	1	AaAIH
5	GO:0016811	hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds, in linear amides	molecular_function	1	AaCPA
6	GO:0050126	N-carbamoylputrescine amidase activity	molecular_function	1	AaCPA
6	GO:0047632	agmatine deiminase activity	molecular_function	1	AaAIH
6	GO:0004014	adenosylmethionine decarboxylase activity	molecular_function	1	AaSAMDC
5	GO:0010487	thermospermine synthase activity	molecular_function	1	AaSPMS
6	GO:0008792	arginine decarboxylase activity	molecular_function	1	AaADC
2	GO:0005623	cell	cellular_component	7	AaSPDS, AaSPDS3, AaADC, AaAIH, AaSAMDC, AaSPMS, AaCPA
2	GO:0043226	organelle	cellular_component	7	AaSPDS, AaSPDS3, AaADC, AaAIH, AaSAMDC, AaSPMS, AaCPA
3	GO:0044464	cell part	cellular_component	7	AaSPDS, AaSPDS3, AaADC, AaAIH, AaSAMDC, AaSPMS, AaCPA
5	GO:0044424	intracellular part	cellular_component	7	AaSPDS, AaSPDS3, AaADC, AaAIH, AaSAMDC, AaSPMS, AaCPA
4	GO:0005622	intracellular	cellular_component	7	AaSPDS, AaSPDS3, AaADC, AaAIH, AaSAMDC, AaSPMS, AaCPA
6	GO:0043229	intracellular organelle	cellular_component	7	AaSPDS, AaSPDS3, AaADC, AaAIH, AaSAMDC, AaSPMS, AaCPA
7	GO:0043231	intracellular membrane-bounded organelle	cellular_component	7	AaSPDS, AaSPDS3, AaADC, AaAIH, AaSAMDC, AaSPMS, AaCPA
3	GO:0043227	membrane-bounded organelle	cellular_component	7	AaSPDS, AaSPDS3, AaADC, AaAIH, AaSAMDC, AaSPMS, AaCPA
8	GO:0005634	nucleus	cellular_component	6	AaADC, AaAIH, AaSAMDC, AaSPDS, AaSPDS3, AaSPMS
6	GO:0005737	cytoplasm	cellular_component	4	AaSPDS, AaSPDS3, AaSPMS, AaCPA
7	GO:0044444	cytoplasmic part	cellular_component	4	AaSPDS, AaSPDS3, AaSPMS, AaCPA
8	GO:0005829	cytosol	cellular_component	3	AaSPDS, AaSPDS3, AaSPMS
8	GO:0009536	plastid	cellular_component	3	AaSPDS, AaSPDS3, AaCPA
2	GO:0016020	membrane	cellular_component	2	AaSPDS, AaSPDS3
9	GO:0009507	chloroplast	cellular_component	2	AaSPDS, AaSPDS3
5	GO:0005886	plasma membrane	cellular_component	2	AaSPDS, AaSPDS3
4	GO:0071944	cell periphery	cellular_component	2	AaSPDS, AaSPDS3

Supplementary Table S4. Functional categories and terms for polyamine (PA) biosynthetic pathway associated sequences.

Level	GO ID	Term	Category	Score	Sequences
2	GO:0009987	cellular process	biological_process	4	AaARG, AaASS, AaOTC, AaASL
3	GO:0071704	organic substance metabolic process	biological_process	4	AaARG, AaASS, AaOTC, AaASL
3	GO:0044238	primary metabolic process	biological_process	4	AaARG, AaASS, AaOTC, AaASL
3	GO:0044710	single-organism metabolic process	biological_process	4	AaARG, AaASS, AaOTC, AaASL
4	GO:1901564	organonitrogen compound metabolic process	biological_process	4	AaARG, AaASS, AaOTC, AaASL
4	GO:0046483	heterocycle metabolic process	biological_process	4	AaASL, AaOTC, AaARG, AaASS
4	GO:0034641	cellular nitrogen compound metabolic process	biological_process	4	AaASL, AaOTC, AaARG, AaASS
4	GO:1901360	organic cyclic compound metabolic process	biological_process	4	AaARG, AaASL, AaOTC, AaASS
3	GO:0006807	nitrogen compound metabolic process	biological_process	4	AaARG, AaASS, AaOTC, AaASL
2	GO:0008152	metabolic process	biological_process	4	AaARG, AaASS, AaOTC, AaASL
4	GO:0006725	cellular aromatic compound metabolic process	biological_process	4	AaARG, AaASL, AaOTC, AaASS
4	GO:0044281	small molecule metabolic process	biological_process	4	AaARG, AaASS, AaOTC, AaASL
3	GO:0044237	cellular metabolic process	biological_process	4	AaARG, AaASS, AaOTC, AaASL
7	GO:0019752	carboxylic acid metabolic process	biological_process	3	AaARG, AaASS, AaOTC
4	GO:0044249	cellular biosynthetic process	biological_process	3	AaASS, AaASL, AaOTC
4	GO:1901576	organic substance biosynthetic process	biological_process	3	AaASS, AaASL, AaOTC
6	GO:0090407	organophosphate biosynthetic process	biological_process	3	AaASL, AaOTC, AaASS
5	GO:0019438	aromatic compound biosynthetic process	biological_process	3	AaASL, AaOTC, AaASS
3	GO:0009058	biosynthetic process	biological_process	3	AaASS, AaASL, AaOTC
8	GO:0009117	nucleotide metabolic process	biological_process	3	AaASL, AaOTC, AaASS
9	GO:0009165	nucleotide biosynthetic process	biological_process	3	AaASL, AaOTC, AaASS
6	GO:0043436	oxoacid metabolic process	biological_process	3	AaARG, AaASS, AaOTC
8	GO:0006520	cellular amino acid metabolic process	biological_process	3	AaARG, AaASS, AaOTC
5	GO:0019637	organophosphate metabolic process	biological_process	3	AaASL, AaOTC, AaASS
4	GO:0006793	phosphorus metabolic process	biological_process	3	AaASL, AaOTC, AaASS
5	GO:1901362	organic cyclic compound biosynthetic process	biological_process	3	AaASL, AaOTC, AaASS
5	GO:0018130	heterocycle biosynthetic process	biological_process	3	AaASL, AaOTC, AaASS
8	GO:1901293	nucleoside phosphate biosynthetic process	biological_process	3	AaASL, AaOTC, AaASS
7	GO:0006753	nucleoside phosphate metabolic process	biological_process	3	AaASL, AaOTC, AaASS
5	GO:0044271	cellular nitrogen compound biosynthetic process	biological_process	3	AaASL, AaOTC, AaASS
5	GO:0006796	phosphate-containing compound metabolic process	biological_process	3	AaASL, AaOTC, AaASS
5	GO:0006139	nucleobase-containing compound metabolic process	biological_process	3	AaASL, AaOTC, AaASS
6	GO:0034654	nucleobase-containing compound biosynthetic process	biological_process	3	AaASL, AaOTC, AaASS
5	GO:0006082	organic acid metabolic process	biological_process	3	AaARG, AaASS, AaOTC
6	GO:0055086	nucleobase-containing small molecule metabolic process	biological_process	3	AaASL, AaOTC, AaASS
5	GO:1901566	organonitrogen compound biosynthetic process	biological_process	3	AaASS, AaASL, AaOTC
5	GO:0044282	small molecule catabolic process	biological_process	2	AaARG, AaASS
6	GO:0016054	organic acid catabolic process	biological_process	2	AaARG, AaASS
3	GO:0009056	catabolic process	biological_process	2	AaARG, AaASS
4	GO:0044248	cellular catabolic process	biological_process	2	AaARG, AaASS
10	GO:1901606	alpha-amino acid catabolic process	biological_process	2	AaARG, AaASS
5	GO:0072521	purine-containing compound metabolic process	biological_process	2	AaASL, AaOTC
8	GO:0046395	carboxylic acid catabolic process	biological_process	2	AaARG, AaASS
10	GO:0006164	purine nucleotide biosynthetic process	biological_process	2	AaASL, AaOTC
4	GO:1901575	organic substance catabolic process	biological_process	2	AaARG, AaASS
10	GO:0009064	glutamine family amino acid metabolic process	biological_process	2	AaARG, AaASS
4	GO:0044712	single-organism catabolic process	biological_process	2	AaARG, AaASS
5	GO:1901565	organonitrogen compound catabolic process	biological_process	2	AaARG, AaASS
9	GO:0009063	cellular amino acid catabolic process	biological_process	2	AaARG, AaASS
11	GO:0006525	arginine metabolic process	biological_process	2	AaARG, AaASS
9	GO:1901605	alpha-amino acid metabolic process	biological_process	2	AaARG, AaASS
9	GO:0006163	purine nucleotide metabolic process	biological_process	2	AaASL, AaOTC
6	GO:0072522	purine-containing compound biosynthetic process	biological_process	2	AaASL, AaOTC
10	GO:0048467	gynoecium development	biological_process	1	AaOTC
7	GO:0048827	phyllome development	biological_process	1	AaOTC
4	GO:0006952	defense response	biological_process	1	AaARG
2	GO:0050896	response to stimulus	biological_process	1	AaARG
12	GO:0035670	plant-type ovary development	biological_process	1	AaOTC
11	GO:0009068	aspartate family amino acid catabolic process	biological_process	1	AaASS
6	GO:0061458	reproductive system development	biological_process	1	AaOTC
5	GO:0048731	system development	biological_process	1	AaOTC

4	GO:0051707	response to other organism	biological_process	1	AaARG
9	GO:0048437	floral organ development	biological_process	1	AaOTC
7	GO:0048569	post-embryonic organ development	biological_process	1	AaOTC
11	GO:0048440	carpel development	biological_process	1	AaOTC
12	GO:0006527	arginine catabolic process	biological_process	1	AaARG
3	GO:0048856	anatomical structure development	biological_process	1	AaOTC
5	GO:0009620	response to fungus	biological_process	1	AaARG
12	GO:0006526	arginine biosynthetic process	biological_process	1	AaASS
9	GO:0009445	putrescine metabolic process	biological_process	1	AaARG
6	GO:0042742	defense response to bacterium	biological_process	1	AaARG
7	GO:0006576	cellular biogenic amine metabolic process	biological_process	1	AaARG
2	GO:0051704	multi-organism process	biological_process	1	AaARG
10	GO:0006570	tyrosine metabolic process	biological_process	1	AaARG
6	GO:0048367	shoot system development	biological_process	1	AaOTC
8	GO:0009908	flower development	biological_process	1	AaOTC
2	GO:0044699	single-organism process	biological_process	1	AaOTC
2	GO:0000003	reproduction	biological_process	1	AaOTC
8	GO:0046394	carboxylic acid biosynthetic process	biological_process	1	AaASS
5	GO:0009617	response to bacterium	biological_process	1	AaARG
11	GO:0009084	glutamine family amino acid biosynthetic process	biological_process	1	AaASS
3	GO:0044767	single-organism developmental process	biological_process	1	AaOTC
11	GO:0006566	threonine metabolic process	biological_process	1	AaASS
4	GO:0044711	single-organism biosynthetic process	biological_process	1	AaASS
9	GO:0008652	cellular amino acid biosynthetic process	biological_process	1	AaASS
13	GO:0048481	ovule development	biological_process	1	AaOTC
10	GO:0006591	ornithine metabolic process	biological_process	1	AaARG
6	GO:0044106	cellular amine metabolic process	biological_process	1	AaARG
2	GO:0032501	multicellular organismal process	biological_process	1	AaOTC
11	GO:0009065	glutamine family amino acid catabolic process	biological_process	1	AaARG
11	GO:0006560	proline metabolic process	biological_process	1	AaARG
6	GO:0016053	organic acid biosynthetic process	biological_process	1	AaASS
6	GO:0048513	organ development	biological_process	1	AaOTC
5	GO:0009791	post-embryonic development	biological_process	1	AaOTC
8	GO:0006595	polyamine metabolic process	biological_process	1	AaARG
12	GO:0006567	threonine catabolic process	biological_process	1	AaASS
7	GO:0048608	reproductive structure development	biological_process	1	AaOTC
9	GO:0048438	floral whorl development	biological_process	1	AaOTC
4	GO:0007275	multicellular organismal development	biological_process	1	AaOTC
3	GO:0022414	reproductive process	biological_process	1	AaOTC
4	GO:0003006	developmental process involved in reproduction	biological_process	1	AaOTC
5	GO:0009308	amine metabolic process	biological_process	1	AaARG
10	GO:0009066	aspartate family amino acid metabolic process	biological_process	1	AaASS
10	GO:1901607	alpha-amino acid biosynthetic process	biological_process	1	AaASS
9	GO:0009072	aromatic amino acid family metabolic process	biological_process	1	AaARG
6	GO:0050832	defense response to fungus	biological_process	1	AaARG
2	GO:0032502	developmental process	biological_process	1	AaOTC
3	GO:0006950	response to stress	biological_process	1	AaARG
3	GO:0009607	response to biotic stimulus	biological_process	1	AaARG
3	GO:0044707	single-multicellular organism process	biological_process	1	AaOTC
5	GO:0044283	small molecule biosynthetic process	biological_process	1	AaASS
2	GO:0003824	catalytic activity	molecular_function	4	AaASL, AaASS, AaARG, AaOTC
2	GO:0005488	binding	molecular_function	3	AaASS, AaOTC, AaARG
3	GO:0043167	ion binding	molecular_function	3	AaASS, AaOTC, AaARG
3	GO:0036094	small molecule binding	molecular_function	2	AaASS, AaOTC
4	GO:0043168	anion binding	molecular_function	2	AaASS, AaOTC
3	GO:0016874	ligase activity	molecular_function	1	AaASS
7	GO:0030554	adenyl nucleotide binding	molecular_function	1	AaASS
6	GO:0008783	agmatinase activity	molecular_function	1	AaARG
5	GO:0016743	carboxyl- or carbamoyltransferase activity	molecular_function	1	AaOTC
5	GO:0046872	metal ion binding	molecular_function	1	AaARG
7	GO:0050897	cobalt ion binding	molecular_function	1	AaARG
5	GO:0031406	carboxylic acid binding	molecular_function	1	AaOTC
4	GO:0043169	cation binding	molecular_function	1	AaARG
4	GO:0043177	organic acid binding	molecular_function	1	AaOTC
5	GO:0016813	hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds, in linear amidines	molecular_function	1	AaARG
6	GO:0046914	transition metal ion binding	molecular_function	1	AaARG
5	GO:0001883	purine nucleoside binding	molecular_function	1	AaASS
6	GO:0032553	ribonucleotide binding	molecular_function	1	AaASS
4	GO:0016879	ligase activity, forming carbon-nitrogen bonds	molecular_function	1	AaASS
4	GO:0016810	hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds	molecular_function	1	AaARG
5	GO:0016842	amidine-lyase activity	molecular_function	1	AaASL
4	GO:0001882	nucleoside binding	molecular_function	1	AaASS
7	GO:0032555	purine ribonucleotide binding	molecular_function	1	AaASS
6	GO:0004056	argininosuccinate lyase activity	molecular_function	1	AaASL
3	GO:0016787	hydrolase activity	molecular_function	1	AaARG
4	GO:0016840	carbon-nitrogen lyase activity	molecular_function	1	AaASL
6	GO:0016597	amino acid binding	molecular_function	1	AaOTC
9	GO:0005524	ATP binding	molecular_function	1	AaASS
5	GO:0004055	argininosuccinate synthase activity	molecular_function	1	AaASS
4	GO:1901265	nucleoside phosphate binding	molecular_function	1	AaASS
3	GO:0097367	carbohydrate derivative binding	molecular_function	1	AaASS
5	GO:0035639	purine ribonucleoside triphosphate binding	molecular_function	1	AaASS
3	GO:0016740	transferase activity	molecular_function	1	AaOTC
5	GO:0032549	ribonucleoside binding	molecular_function	1	AaASS
3	GO:1901363	heterocyclic compound binding	molecular_function	1	AaASS
3	GO:0016829	lyase activity	molecular_function	1	AaASL
6	GO:0017076	purine nucleotide binding	molecular_function	1	AaASS
4	GO:0016741	transferase activity, transferring one-carbon groups	molecular_function	1	AaOTC
3	GO:0097159	organic cyclic compound binding	molecular_function	1	AaASS
8	GO:0032559	adenyl ribonucleotide binding	molecular_function	1	AaASS
6	GO:0004053	arginase activity	molecular_function	1	AaARG
6	GO:0032550	purine ribonucleoside binding	molecular_function	1	AaASS
5	GO:0000166	nucleotide binding	molecular_function	1	AaASS
2	GO:0005623	cell	cellular_component	4	AaARG, AaASL, AaOTC, AaASS
2	GO:0043226	organelle	cellular_component	4	AaARG, AaASL, AaOTC, AaASS
3	GO:0044464	cell part	cellular_component	4	AaARG, AaASL, AaOTC, AaASS
5	GO:0044424	intracellular part	cellular_component	4	AaARG, AaASL, AaOTC, AaASS
6	GO:0005737	cytoplasm	cellular_component	4	AaARG, AaASL, AaOTC, AaASS
7	GO:0044444	cytoplasmic part	cellular_component	4	AaARG, AaASL, AaOTC, AaASS

4	GO:0005622	intracellular	cellular_component	4	AaARG, AaASL, AaOTC, AaASS
6	GO:0043229	intracellular organelle	cellular_component	4	AaARG, AaASL, AaOTC, AaASS
8	GO:0009536	plastid	cellular_component	4	AaARG, AaASL, AaOTC, AaASS
9	GO:0009507	chloroplast	cellular_component	4	AaARG, AaASL, AaOTC, AaASS
7	GO:0043231	intracellular membrane-bounded organelle	cellular_component	4	AaARG, AaASL, AaOTC, AaASS
3	GO:0043227	membrane-bounded organelle	cellular_component	4	AaARG, AaASL, AaOTC, AaASS
3	GO:0044422	organelle part	cellular_component	3	AaASL, AaOTC, AaASS
10	GO:0044434	chloroplast part	cellular_component	3	AaASL, AaOTC, AaASS
10	GO:0009532	plastid stroma	cellular_component	3	AaASL, AaOTC, AaASS
7	GO:0044446	intracellular organelle part	cellular_component	3	AaASL, AaOTC, AaASS
9	GO:0044435	plastid part	cellular_component	3	AaASL, AaOTC, AaASS
11	GO:0009570	chloroplast stroma	cellular_component	3	AaASL, AaOTC, AaASS
8	GO:0005829	cytosol	cellular_component	1	AaOTC
8	GO:0005739	mitochondrion	cellular_component	1	AaARG

Supplementary Table S5. Contents (nmol.g^{-1} FW) of amino acids and polyamines identified in three stages of *Araucaria angustifolia* seed development: GZE – megagametophyte containing globular embryos, CZE – isolated cotyledonal embryos, MZE – isolated mature embryos, CZE MG – megagametophyte at cotyledonal stage, and MZE MG – megagametophyte at mature stage. Put (putrescine), Spd (spermidine), Spm (spermine).

	GZE	CZE	MZE	CZMG	MZMG
			Polyamines (free and conjugated forms)		
Put (Free)	58.33 ± 9.17 c	73.06 ± 3.49 b	220.07 ± 52.99 a	29.86 ± 4.96 d	33.62 ± 2.15 d
Spd (Free)	57.29 ± 7.47 c	624.16 ± 47.20 a	669.85 ± 141.95 a	189.36 ± 10.70 b	204.98 ± 6.77 b
Spm (Free)	41.67 ± 5.05 d	690.44 ± 40.54 a	373.90 ± 78.90 b	157.67 ± 6.78 c	136.92 ± 14.79 c
Put (Conj.)	5.20 ± 1.13 d	19.22 ± 1.99 c	71.35 ± 5.18 a	14.37 ± 0.66 c	30.38 ± 3.72 b
Spd (Conj.)	4.14 ± 0.32 d	82.58 ± 18.35 b	198.50 ± 41.05 a	34.51 ± 1.71 c	90.23 ± 14.49 b
Spm (Conj.)	0.31 ± 0.08 c	88.81 ± 4.82 a	98.80 ± 8.91 a	34.26 ± 2.41 b	46.63 ± 5.13 b
			Total polyamines		
Put	63.53 ± 10.24 c	92.28 ± 2.36 b	291.42 ± 9.6 a	44.23 ± 4.43 d	64.00 ± 4.03 c
Spd	61.43 ± 7.15 e	706.74 ± 19.12 b	868.35 ± 22.68 a	223.87 ± 9.17 d	295.21 ± 25.58 c
Spm	41.98 ± 5.12 d	779.24 ± 15.65 a	472.69 ± 15.86 b	191.93 ± 6.84 c	183.55 ± 19.32 c
Total	166.94 ± 18.19 d	1578.27 ± 34.35 a	1632.46 ± 27.15 a	460.03 ± 20.34 c	542.76 ± 43.53 b
Put/(Spd+Spm)	0.61 ± 0.06 a	0.06 ± 0.00 e	0.22 ± 0.01 b	0.11 ± 0.01 d	0.13 ± 0.02 c
Agmatine	15.72 ± 1.25 c	17.98 ± 4.03 c	36.54 ± 2.78 b	38.91 ± 3.17 b	53.81 ± 3.11 a
			Free aminoacids		
Aspartic acid	631.19 ± 66.44 b	2104.98 ± 426.26 a	2019.28 ± 170.09 a	1209.89 ± 102.07 b	2296.69 ± 239.11 a
Glutamic acid	784.34 ± 83.51 c	2798.93 ± 610.38 a	1990.51 ± 116.31 ab	1253.88 ± 151.67 bc	2257.14 ± 257.57 a
Asparagine	128.53 ± 19.53 ab	135.68 ± 26.52 ab	154.16 ± 6.97 a	43.54 ± 2.18 c	94.9 ± 13.46 b
Serine	110.34 ± 23.64 c	527.38 ± 119.48 a	388.42 ± 26.19 ab	140.27 ± 8.72 c	284.15 ± 34.17 b
Glutamine	298.64 ± 67.42 c	1789.34 ± 379.13 a	474.52 ± 11.02 b	572.5 ± 54.61 b	294.36 ± 35.37 c
Histidine	17.2 ± 1.84 c	34.37 ± 7.17 b	84.51 ± 3.54 a	19.09 ± 2.31 c	43.21 ± 4.82 b
Arginine	38.07 ± 9.84 d	207.34 ± 23.94 a	145.14 ± 10.07 b	85.26 ± 4.08 c	84.91 ± 8.69 c
Citrulline	351.1 ± 20.19 c	4815.27 ± 955.48 a	2675.76 ± 317.9 b	328.59 ± 9.69 c	438.81 ± 65.24 c
Glycine	2.32 ± 0.09 d	22.69 ± 3.68 a	12.29 ± 1.36 b	5.79 ± 0.29 c	2.27 ± 0.14 d
Threonine	35.51 ± 4.4 c	115.71 ± 23.61 a	104.38 ± 7.24 ab	41.99 ± 2.53 c	79.67 ± 10.28 b
Alanine	133.89 ± 28.42 c	643.17 ± 124.83 a	477.61 ± 30.32 ab	209.94 ± 21.61 c	424.74 ± 55.82 b
γ-aminobutyric acid	28.77 ± 0.95 c	144.34 ± 15.4 a	60.84 ± 9.75 b	24.12 ± 4.6 c	22.84 ± 2.76 c
Tyrosine	25.6 ± 2.35 c	176.25 ± 37.1 a	173.14 ± 15.14 a	32.39 ± 1.59 c	50.31 ± 7.24 b
Methionine	6.89 ± 0.1 d	22.78 ± 5.11 b	30.39 ± 2.64 a	17.5 ± 0.89 c	22.97 ± 1.66 b
Tryptophan	31.44 ± 3.26 b	54.18 ± 12.79 a	71.09 ± 5.79 a	9.6 ± 0.8 d	16.72 ± 1.75 c
Valine	52.44 ± 6.51 c	118.44 ± 24.36 b	193.42 ± 10.54 a	45.87 ± 1.98 c	196.9 ± 25.3 a
Phenylalanine	29.63 ± 3.76 c	151.17 ± 27.66 a	123.81 ± 12.92 a	60.66 ± 3.36 bc	71.08 ± 10.27 b
Isoleucine	21.26 ± 1.68 c	82.22 ± 18.54 ab	79.03 ± 7.9 b	46.67 ± 4.4 c	110.47 ± 15.4 a
Leucine	24.55 ± 2.47 d	80.46 ± 18.29 b	106.26 ± 6.32 ab	45.02 ± 0.67 c	119.49 ± 17.14 a
Ornithine	60.41 ± 1.62 bc	85.43 ± 5.2 a	74 ± 5.36 ab	65.6 ± 8.01 bc	40.83 ± 1.7 d
Lysine	74.77 ± 19.66 d	288.92 ± 28 b	547.89 ± 61.73 a	100.78 ± 4.75 cd	108.09 ± 9.42 c
Total	2886.91 ± 325.46 e	14399.05 ± 2866.45 a	9986.45 ± 757.09 b	4358.94 ± 369.65 d	7060.56 ± 817.31 c

Values represent medians ± SD from three biological replicates. Statistically significant differences ($p<0.05$) between samples within rows are indicated with different letters according to Tukey's test.

Supplementary Table S6. Relative gene expression of ornithine/arginine (Orn/Arg) and polyamine (PA) biosynthetic pathway associated genes in three different developmental stages of *Araucaria angustifolia* seeds. GZE – megagametophyte containing globular embryos, CZE – isolated cotyledonal embryos, MZE – isolated mature embryos, CZE MG – megagametophyte at cotyledonal stage, and MZE MG – megagametophyte at mature stage.

	GZE	CZE	MZE	CZMG	MZMG
AaASL	1.00 ± 0.11 b	0.51 ± 0.02 c	1.15 ± 0.17 b	0.83 ± 0.06 bc	2.09 ± 0.32 a
AaOTC	1.00 ± 0.09 a	0.85 ± 0.04 a	0.73 ± 0.06 a	0.86 ± 0.07 a	0.34 ± 0.03 b
AaASS	1.00 ± 0.04 ab	0.55 ± 0.04 c	0.93 ± 0.07 b	0.49 ± 0.02 c	1.19 ± 0.08 a
AaARG	1.00 ± 0.06 a	0.54 ± 0.04 b	0.19 ± 0.02 cd	0.32 ± 0.02 c	0.13 ± 0.01 d
AaADC	1.00 ± 0.04 b	2.26 ± 0.09 a	1.19 ± 0.1 b	1.10 ± 0.16 bc	0.49 ± 0.04 c
AaAIH	1.00 ± 0.08 a	0.19 ± 0.01 d	0.51 ± 0.03 cd	0.67 ± 0.08 bc	0.86 ± 0.01 ab
AaCPA	1.00 ± 0.14 c	1.18 ± 0.08 c	2.71 ± 0.28 b	1.33 ± 0.12 c	7.09 ± 0.29 a
AaSAMDC	1.00 ± 0.12 b	1.78 ± 0.14 a	0.86 ± 0.10 b	1.79 ± 0.28 a	0.57 ± 0.06 b
AaSPDS	1.00 ± 0.06 a	0.37 ± 0.07 b	0.25 ± 0.02 bc	0.36 ± 0.08 bc	0.10 ± 0.01 c
AaSPDS3	1.00 ± 0.09 ab	0.54 ± 0.02 c	0.68 ± 0.06 bc	1.07 ± 0.14 a	0.37 ± 0.02 c
AaSPMS	1.00 ± 0.09 a	0.48 ± 0.06 b	0.73 ± 0.11 ab	0.80 ± 0.19 ab	0.06 ± 0.00 c

Values represent medians ± SE from three biological replicates. The medians were calculated from means of two technical replicates and normalized against the GZE sample relative expression. Statistically significant differences ($p<0.05$) between samples within rows are indicated with different letters according to Tukey's test.

Supplementary Table S7. Values of CPM (counts per minute) from decarboxylation of [^{14}C]-arginine or [^{14}C]-ornithine, through enzymatic activity of arginine decarboxylase (ADC) or ornithine decarboxylase (ODC) in three different developmental stages of *Araucaria angustifolia* seeds. GZE – megagametophyte containing globular embryos, CZE – isolated cotyledonal embryos, MZE – isolated mature embryos, CZE MG – megagametophyte at cotyledonal stage, and MZE MG – megagametophyte at mature stage.

	GZE	CZE	MZE	CZE MG	MZE MG
ADC	77.0 ± 1.9	255.7 ± 14.5	152.3 ± 23.0	136.0 ± 13.5	122.3 ± 18.9
ODC	23.6 ± 5.4	39.4 ± 2.9	25.7 ± 8.0	13.0 ± 2.0	18.7 ± 3.5

Values represent medians ± SD from three biological replicates.

Supplementary Table S8. Pearson's correlations between metabolites and ornithine/arginine (Orn/Arg) and polyamine (PA) biosynthetic pathway associated genes in three different developmental stages of *Araucaria angustifolia* seeds. Correlation coefficient r (p-values <0.01 are given in bold).

GZE – megagametophyte containing globular embryos

	Put	Spd	Spm	Agmatine	Aspartic acid	Glutamate acid	Glutamine	Arginine	Citrulline	Gaba	Methionine	Ornithine	*AaASAL*	*AaOTC*	*AaASS*	*AaARG*	*AaADC*	*AaAIH*	*AaCPA*	*AaSAMDC*	*AaSPDS*	*AaSPDS3*	*AaSPMS*
Put																							
Spd	0.42 (0.410)																						
Spm	0.88 (0.021)	-0.07 (0.902)																					
Agmatine	-0.24 (0.649)	0.78 (0.066)	-0.67 (0.143)																				
Aspartic acid	-0.27 (0.598)	0.76 (0.080)	-0.70 (0.122)	**1.00 (0.000)**																			
Glutamate acid	-0.18 (0.737)	0.82 (0.046)	-0.62 (0.185)	**1.00 (0.000)**	**0.99 (0.000)**																		
Glutamine	0.72 (0.107)	**0.93 (0.007)**	0.30 (0.561)	0.50 (0.310)	0.47 (0.347)	0.56 (0.252)																	
Arginine	**0.96 (0.003)**	0.66 (0.152)	0.70 (0.118)	0.05 (0.923)	0.01 (0.979)	0.11 (0.830)	0.89 (0.018)																
Citrulline	0.10 (0.852)	**0.95 (0.004)**	-0.39 (0.449)	**0.94 (0.005)**	**0.93 (0.007)**	**0.96 (0.002)**	0.76 (0.078)	0.38 (0.455)															
Gaba	0.01 (0.988)	0.91 (0.011)	-0.47 (0.348)	**0.97 (0.001)**	**0.96 (0.002)**	**0.98 (0.000)**	0.70 (0.122)	0.30 (0.569)	**1.00 (0.000)**														
Methionine	-0.89 (0.018)	-0.79 (0.063)	-0.56 (0.245)	-0.23 (0.656)	-0.20 (0.708)	-0.29 (0.571)	**-0.96 (0.003)**	**-0.98 (0.000)**	-0.54 (0.264)	-0.47 (0.352)													
Ornithine	-0.01 (0.988)	-0.91 (0.011)	0.47 (0.348)	**-0.97 (0.001)**	**-0.96 (0.002)**	**-0.98 (0.000)**	-0.70 (0.122)	-0.30 (0.569)	**-1.00 (0.000)**	**-1.00 (0.000)**	0.47 (0.352)												
AaASAL	0.97 (0.001)	0.20 (0.702)	**0.96 (0.002)**	-0.45 (0.369)	-0.48 (0.330)	-0.39 (0.439)	0.54 (0.264)	0.87 (0.025)	-0.13 (0.809)	-0.22 (0.678)	-0.76 (0.078)	0.22 (0.678)											
AaOTC	0.79 (0.064)	-0.23 (0.657)	**0.99 (0.000)**	-0.79 (0.063)	-0.81 (0.051)	-0.75 (0.088)	0.14 (0.796)	0.58 (0.233)	-0.54 (0.272)	-0.61 (0.197)	-0.42 (0.413)	0.61 (0.197)	0.91 (0.013)										
AaASS	0.08 (0.880)	-0.87 (0.024)	0.55 (0.263)	**-0.99 (0.000)**	**-0.98 (0.001)**	**-1.00 (0.000)**	-0.63 (0.176)	-0.21 (0.689)	**-0.98 (0.000)**	**-1.00 (0.000)**	0.39 (0.450)	**1.00 (0.000)**	0.30 (0.559)	0.68 (0.138)									
AaARG	0.77 (0.072)	0.90 (0.014)	0.38 (0.464)	0.43 (0.390)	0.40 (0.432)	0.49 (0.325)	**1.00 (0.000)**	**0.92 (0.009)**	0.71 (0.114)	0.64 (0.169)	**-0.98 (0.001)**	-0.64 (0.169)	0.61 (0.200)	0.21 (0.684)	-0.57 (0.236)								
AaADC	0.61 (0.195)	-0.46 (0.357)	0.92 (0.010)	-0.91 (0.011)	**-0.93 (0.008)**	-0.89 (0.019)	-0.11 (0.840)	0.36 (0.484)	-0.73 (0.103)	-0.78 (0.064)	-0.18 (0.730)	0.78 (0.064)	0.78 (0.070)	**0.97 (0.001)**	0.84 (0.038)	-0.03 (0.956)							
AaAIH	**0.93 (0.006)**	0.07 (0.899)	**0.99 (0.000)**	-0.57 (0.239)	-0.60 (0.209)	-0.52 (0.295)	0.43 (0.400)	0.79 (0.060)	-0.26 (0.617)	-0.35 (0.499)	-0.67 (0.148)	0.35 (0.499)	**0.99 (0.000)**	**0.95 (0.003)**	0.43 (0.395)	0.49 (0.318)	0.85 (0.030)						
AaCPA	0.17 (0.744)	-0.82 (0.044)	0.62 (0.188)	**-1.00 (0.000)**	**-0.99 (0.000)**	**-1.00 (0.000)**	-0.56 (0.248)	-0.12 (0.823)	**-0.96 (0.002)**	**-0.98 (0.000)**	0.30 (0.565)	**0.98 (0.000)**	0.39 (0.444)	0.74 (0.090)	**1.00 (0.000)**	-0.49 (0.320)	0.88 (0.020)	0.51 (0.300)					
AaSAMDC	0.26 (0.618)	-0.77 (0.074)	0.69 (0.130)	**-1.00 (0.000)**	**-1.00 (0.000)**	**-1.00 (0.000)**	-0.48 (0.332)	-0.03 (0.957)	**-0.93 (0.006)**	**-0.96 (0.002)**	0.21 (0.688)	**0.96 (0.002)**	0.47 (0.345)	0.80 (0.055)	**0.98 (0.000)**	-0.41 (0.416)	**0.92 (0.009)**	0.59 (0.221)	**1.00 (0.000)**				
AaSPDS	0.72 (0.104)	-0.32 (0.531)	**0.97 (0.002)**	-0.84 (0.035)	-0.86 (0.027)	-0.81 (0.052)	0.04 (0.936)	0.50 (0.318)	-0.61 (0.195)	-0.68 (0.134)	-0.33 (0.527)	0.68 (0.134)	0.86 (0.028)	**1.00 (0.000)**	0.75 (0.089)	0.12 (0.820)	**0.99 (0.000)**	**0.92 (0.009)**	0.80 (0.054)	0.85 (0.030)			
AaSPDS3	-0.28 (0.592)	**-0.99 (0.000)**	0.21 (0.687)	-0.87 (0.026)	-0.85 (0.034)	-0.90 (0.016)	-0.87 (0.025)	-0.54 (0.264)	**-0.98 (0.000)**	**-0.96 (0.002)**	0.69 (0.130)	**0.96 (0.002)**	-0.06 (0.917)	0.37 (0.466)	**0.93 (0.006)**	-0.83 (0.043)	0.59 (0.221)	0.08 (0.880)	0.90 (0.015)	0.85 (0.030)	0.46 (0.359)		
AaSPMS	**0.96 (0.002)**	0.15 (0.783)	**0.98 (0.001)**	-0.50 (0.311)	-0.53 (0.275)	-0.45 (0.375)	0.50 (0.317)	0.84 (0.037)	-0.18 (0.727)	-0.27 (0.600)	-0.72 (0.104)	0.27 (0.600)	**1.00 (0.000)**	**0.93 (0.008)**	0.36 (0.487)	0.56 (0.246)	0.81 (0.051)	**1.00 (0.000)**	0.44 (0.380)	0.52 (0.289)	0.89 (0.018)	0.00 (0.998)	

CZE – isolated cotyledonal embryos

	Put	Spd	Spm	Agmatine	Aspartic acid	Glutamate acid	Glutamine	Arginine	Citrulline	Gaba	Methionine	Ornithine	*AaASAL*	*AaOTC*	*AaASS*	*AaARG*	*AaADC*	*AaAIH*	*AaCPA*	*AaSAMDC*	*AaSPDS*	*AaSPDS3*	*AaSPMS*
Put																							
Spd	**0.93 (0.008)**																						
Spm	0.45 (0.375)	0.75 (0.087)																					
Agmatine	0.76 (0.082)	**0.95 (0.004)**	**0.92 (0.009)**																				
Aspartic acid	0.70 (0.120)	0.92 (0.010)	**0.95 (0.004)**	**1.00 (0.000)**																			
Glutamate acid	0.68 (0.137)	0.90 (0.013)	**0.96 (0.002)**	**0.99 (0.000)**	**1.00 (0.000)**																		
Glutamine	0.60 (0.204)	0.86 (0.029)	**0.98 (0.000)**	**0.98 (0.001)**	**0.99 (0.000)**	**1.00 (0.000)**																	
Arginine	**1.00 (0.000)**	**0.96 (0.003)**	0.53 (0.283)	0.81 (0.049)	0.76 (0.077)	0.74 (0.089)	0.67 (0.142)																
Citrulline	0.60 (0.204)	0.86 (0.029)	**0.98 (0.000)**	**0.98 (0.001)**	**0.99 (0.000)**	**0.99 (0.000)**	**1.00 (0.000)**	0.67 (0.142)															
Gaba	**1.00 (0.000)**	**0.96 (0.003)**	0.53 (0.283)	0.81 (0.049)	0.76 (0.077)	0.74 (0.089)	0.67 (0.142)	**1.00 (0.000)**	0.67 (0.142)														
Methionine	0.62 (0.185)	0.87 (0.024)	**0.98 (0.001)**	**0.98 (0.000)**	**0.99 (0.000)**	**1.00 (0.000)**	**1.00 (0.000)**	0.69 (0.127)	**1.00 (0.000)**	0.69 (0.127)													
Ornithine	**1.00 (0.000)**	**0.96 (0.003)**	0.53 (0.283)	0.81 (0.049)	0.76 (0.077)	0.74 (0.089)	0.67 (0.142)	**1.00 (0.000)**	0.67 (0.142)	**1.00 (0.000)**	0.69 (0.127)												
AaASAL	0.38 (0.459)	0.01 (0.991)	-0.66 (0.155)	-0.32 (0.538)	-0.39 (0.441)	-0.42 (0.407)	-0.51 (0.303)	0.29 (0.573)	-0.51 (0.303)	0.29 (0.573)	-0.49 (0.329)	0.29 (0.573)											
AaOTC	-0.58 (0.225)	-0.84 (0.035)	**-0.99 (0.000)**	**-0.97 (0.001)**	**-0.99 (0.000)**	**-0.99 (0.000)**	**-1.00 (0.000)**	-0.65 (0.158)	**-1.00 (0.000)**	-0.65 (0.158)	**-1.00 (0.000)**	-0.65 (0.158)	0.53 (0.278)										
AaASS	-0.32 (0.537)	-0.65 (0.162)	**-0.99 (0.000)**	-0.86 (0.027)	-0.90 (0.015)	-0.91 (0.011)	**-0.95 (0.004)**	-0.40 (0.426)	**-0.95 (0.004)**	-0.40 (0.426)	**-0.94 (0.005)**	-0.40 (0.426)	0.76 (0.082)	**0.96 (0.003)**									
AaARG	-0.87 (0.024)	-0.62 (0.186)	0.05 (0.923)	-0.34 (0.515)	-0.26 (0.618)	-0.23 (0.658)	-0.13 (0.800)	-0.82 (0.045)	-0.13 (0.801)	-0.82 (0.045)	-0.16 (0.763)	-0.82 (0.045)	-0.79 (0.064)	0.11 (0.839)	-0.19 (0.721)								
AaADC	-0.59 (0.222)	-0.24 (0.646)	0.46 (0.354)	0.09 (0.868)	0.17 (0.752)	0.20 (0.711)	0.29 (0.574)	-0.51 (0.303)	0.29 (0.573)	-0.51 (0.303)	0.27 (0.608)	-0.51 (0.303)	**-0.97 (0.001)**	-0.32 (0.540)	-0.58 (0.226)	0.91 (0.012)							
AaAIH	0.39 (0.449)	0.70 (0.119)	**1.00 (0.000)**	0.90 (0.016)	**0.93 (0.008)**	**0.94 (0.006)**	**0.97 (0.002)**	0.47 (0.348)	**0.97 (0.002)**	0.47 (0.348)	**0.96 (0.002)**	0.47 (0.348)	-0.71 (0.116)	**-0.97 (0.001)**	**-1.00 (0.000)**	0.12 (0.825)	0.52 (0.289)						
AaCPA	-0.33 (0.527)	-0.66 (0.157)	**-0.99 (0.000)**	-0.87 (0.026)	-0.90 (0.014)	-0.91 (0.011)	**-0.95 (0.004)**	-0.41 (0.417)	**-0.95 (0.004)**	-0.41 (0.417)	**-0.94 (0.005)**	-0.41 (0.417)	0.75 (0.085)	**0.96 (0.003)**	**1.00 (0.000)**	-0.18 (0.732)	-0.58 (0.233)	**-1.00 (0.000)**					
AaSAMDC	0.81 (0.051)	**0.97 (0.001)**	0.89 (0.018)	**1.00 (0.000)**	**0.99 (0.000)**	**0.98 (0.001)**	**0.96 (0.003)**	0.86 (0.028)	**0.96 (0.003)**	0.86 (0.028)	**0.96 (0.002)**	0.86 (0.028)	-0.24 (0.650)	**-0.95 (0.004)**	-0.82 (0.048)	-0.41 (0.413)	0.00 (0.995)	0.85 (0.030)	-0.82 (0.046)				
AaSPDS	0.39 (0.445)	0.71 (0.118)	**1.00 (0.000)**	0.90 (0.015)	**0.93 (0.007)**	**0.94 (0.005)**	**0.97 (0.001)**	0.47 (0.345)	**0.97 (0.001)**	0.47 (0.345)	**0.96 (0.002)**	0.47 (0.345)	-0.70 (0.118)	**-0.98 (0.001)**	**-1.00 (0.000)**	0.11 (0.829)	0.52 (0.292)	**1.00 (0.000)**	**-1.00 (0.000)**	0.86 (0.029)			
AaSPDS3	-0.90 (0.015)	**-1.00 (0.000)**	-0.80 (0.059)	**-0.97 (0.002)**	**-0.94 (0.005)**	**-0.93 (0.006)**	-0.89 (0.016)	**-0.93 (0.006)**	-0.89 (0.016)	**-0.93 (0.006)**	-0.91 (0.013)	**-0.93 (0.006)**	0.07 (0.898)	0.88 (0.020)	0.70 (0.118)	0.56 (0.244)	0.17 (0.751)	-0.75 (0.084)	0.71 (0.114)	**-0.99 (0.000)**	-0.76 (0.082)		
AaSPMS	0.65 (0.161)	0.89 (0.018)	**0.97 (0.001)**	**0.99 (0.000)**	**1.00 (0.000)**	**1.00 (0.000)**	**1.00 (0.000)**	0.72 (0.108)	**1.00 (0.000)**	0.72 (0.108)	**1.00 (0.000)**	0.72 (0.108)	-0.46 (0.364)	**-1.00 (0.000)**	**-0.93 (0.008)**	-0.19 (0.713)	0.23 (0.655)	**0.95 (0.004)**	**-0.93 (0.007)**	**0.97 (0.001)**	**0.95 (0.003)**	**-0.92 (0.010)**	

MZE – isolated mature embryos

	Put	Spd	Spm	Agmatine	Aspartic acid	Glutamate acid	Glutamine	Arginine	Citrulline	Gaba	Methionine	Ornithine	*AaASAL*	*AaOTC*	*AaASS*	*AaARG*	*AaADC*	*AaAIH*	*AaCPA*	*AaSAMDC*	*AaSPDS*	*AaSPDS3*	*AaSPMS*
Put																							
Spd	0.77 (0.071)																						
Spm	-0.83 (0.042)	-0.29 (0.583)																					
Agmatine	0.61 (0.198)	-0.03 (0.959)	**-0.95 (0.004)**																				
Aspartic acid	**-0.99 (0.000)**	-0.84 (0.037)	0.76 (0.078)	-0.52 (0.288)																			
Glutamate acid	**-1.00 (0.000)**	-0.73 (0.102)	0.87 (0.026)	-0.67 (0.148)	**0.98 (0.000)**																		
Glutamine	-0.83 (0.043)	-0.28 (0.586)	**1.00 (0.000)**	**-0.95 (0.004)**	0.76 (0.079)	0.86 (0.026)																	
Arginine	**-0.99 (0.000)**	-0.69 (0.131)	0.89 (0.017)	-0.71 (0.116)	**0.97 (0.001)**	**1.00 (0.000)**	0.89 (0.017)																
Citrulline	-0.72 (0.105)	**-1.00 (0.000)**	0.21 (0.690)	0.11 (0.842)	0.79 (0.060)	0.67 (0.145)	0.21 (0.693)	0.63 (0.181)															
Gaba	-0.91 (0.013)	**-0.97 (0.001)**	0.51 (0.301)	-0.22 (0.681)	**0.95 (0.004)**	0.87 (0.024)	0.51 (0.304)	0.84 (0.035)	**0.95 (0.004)**														
Methionine	**-0.96 (0.003)**	-0.55 (0.255)	**0.96 (0.003)**	-0.82 (0.047)	**0.92 (0.010)**	**0.97 (0.001)**	**0.96 (0.003)**	**0.99 (0.000)**	0.49 (0.329)	0.74 (0.093)													
Ornithine	**-1.00 (0.000)**	-0.76 (0.079)	0.84 (0.037)	-0.63 (0.182)	**0.99 (0.000)**	**1.00 (0.000)**	0.84 (0.037)	**0.99 (0.000)**	0.71 (0.116)	0.90 (0.016)	**0.96 (0.002)**												
AaASAL	**0.99 (0.000)**	0.84 (0.037)	-0.76 (0.078)	0.52 (0.288)	**-1.00 (0.000)**	**-0.98 (0.000)**	-0.76 (0.079)	**-0.97 (0.001)**	-0.79 (0.060)	**-0.95 (0.004)**	**-0.92 (0.010)**	**-0.99 (0.000)**											
AaOTC	0.80 (0.058)	0.23 (0.654)	**-1.00 (0.000)**	**0.97 (0.002)**	-0.73 (0.102)	-0.84 (0.037)	**-1.00 (0.000)**	-0.87 (0.025)	-0.16 (0.765)	-0.46 (0.354)	**-0.94 (0.005)**	-0.81 (0.051)	0.73 (0.102)										
AaASS	**0.99 (0.000)**	0.66 (0.156)	-0.91 (0.012)	0.74 (0.096)	**-0.96 (0.002)**	**-1.00 (0.000)**	-0.91 (0.012)	**-1.00 (0.000)**	-0.60 (0.212)	-0.82 (0.045)	**-0.99 (0.000)**	**-0.99 (0.000)**	**0.96 (0.002)**	0.89 (0.018)									
AaARG	0.66 (0.154)	0.04 (0.947)	**-0.97 (0.002)**	**1.00 (0.000)**	-0.57 (0.233)	-0.71 (0.113)	**-0.97 (0.002)**	-0.75 (0.086)	0.04 (0.935)	-0.28 (0.595)	-0.85 (0.031)	-0.68 (0.141)	0.57 (0.234)	**0.98 (0.001)**	0.78 (0.069)								
AaADC	**0.99 (0.000)**	0.85 (0.030)	-0.74 (0.091)	0.50 (0.317)	**-1.00 (0.000)**	**-0.98 (0.001)**	-0.74 (0.092)	**-0.96 (0.002)**	-0.81 (0.050)	**-0.95 (0.003)**	-0.91 (0.013)	**-0.99 (0.000)**	**1.00 (0.000)**	0.71 (0.117)	**0.95 (0.003)**	0.55 (0.259)							
AaAIH	**0.99 (0.000)**	0.67 (0.142)	-0.90 (0.014)	0.72 (0.107)	**-0.97 (0.002)**	**-1.00 (0.000)**	-0.90 (0.015)	**-1.00 (0.000)**	-0.61 (0.194)	-0.83 (0.039)	**-0.99 (0.000)**	**-0.99 (0.000)**	**0.97 (0.002)**	0.88 (0.022)	**1.00 (0.000)**	0.76 (0.079)	**0.96 (0.002)**						
AaCPA	0.28 (0.588)	0.83 (0.043)	0.30 (0.557)	-0.59 (0.221)	-0.38 (0.453)	-0.21 (0.686)	0.31 (0.553)	-0.16 (0.766)	-0.87 (0.025)	-0.66 (0.151)	0.01 (0.979)	-0.26 (0.617)	0.38 (0.452)	-0.35 (0.490)	0.12 (0.826)	-0.54 (0.274)	0.41 (0.418)	0.14 (0.792)					
AaSAMDC	**0.95 (0.004)**	0.54 (0.267)	**-0.96 (0.002)**	0.83 (0.043)	-0.91 (0.011)	**-0.97 (0.001)**	**-0.96 (0.002)**	**-0.98 (0.000)**	-0.47 (0.342)	-0.73 (0.100)	**-1.00 (0.000)**	**-0.96 (0.003)**	0.91 (0.011)	**0.94 (0.005)**	**0.99 (0.000)**	0.86 (0.029)	0.90 (0.015)	**0.99 (0.000)**	-0.03 (0.959)				
AaSPDS	**0.93 (0.007)**	0.49 (0.324)	**-0.98 (0.001)**	0.86 (0.029)	-0.89 (0.019)	**-0.95 (0.003)**	**-0.97 (0.001)**	**-0.97 (0.001)**	-0.42 (0.407)	-0.69 (0.131)	**-1.00 (0.000)**	**-0.94 (0.006)**	0.89 (0.019)	**0.96 (0.002)**	**0.98 (0.001)**	0.89 (0.018)	0.87 (0.024)	**0.97 (0.001)**	-0.09 (0.869)	**1.00 (0.000)**			
AaSPDS3	-0.09 (0.859)	0.56 (0.252)	0.64 (0.174)	-0.85 (0.034)	-0.01 (0.980)	0.17 (0.753)	0.64 (0.172)	0.22 (0.674)	-0.62 (0.189)	-0.34 (0.513)	0.38 (0.451)	0.12 (0.827)	0.01 (0.980)	-0.68 (0.140)	-0.26 (0.618)	-0.81 (0.050)	0.04 (0.935)	-0.24 (0.649)	**0.93 (0.008)**	-0.40 (0.436)	-0.45 (0.369)		
AaSPMS	0.58 (0.232)	-0.07 (0.893)	**-0.94 (0.006)**	**1.00 (0.000)**	-0.48 (0.331)	-0.63 (0.178)	**-0.94 (0.006)**	-0.68 (0.141)	0.15 (0.777)	-0.17 (0.743)	-0.79 (0.061)	-0.59 (0.215)	0.48 (0.331)	**0.95 (0.003)**	0.70 (0.118)	**0.99 (0.000)**	0.46 (0.362)	0.69 (0.131)	-0.62 (0.187)	0.80 (0.056)	0.83 (0.039)	-0.87 (0.025)	

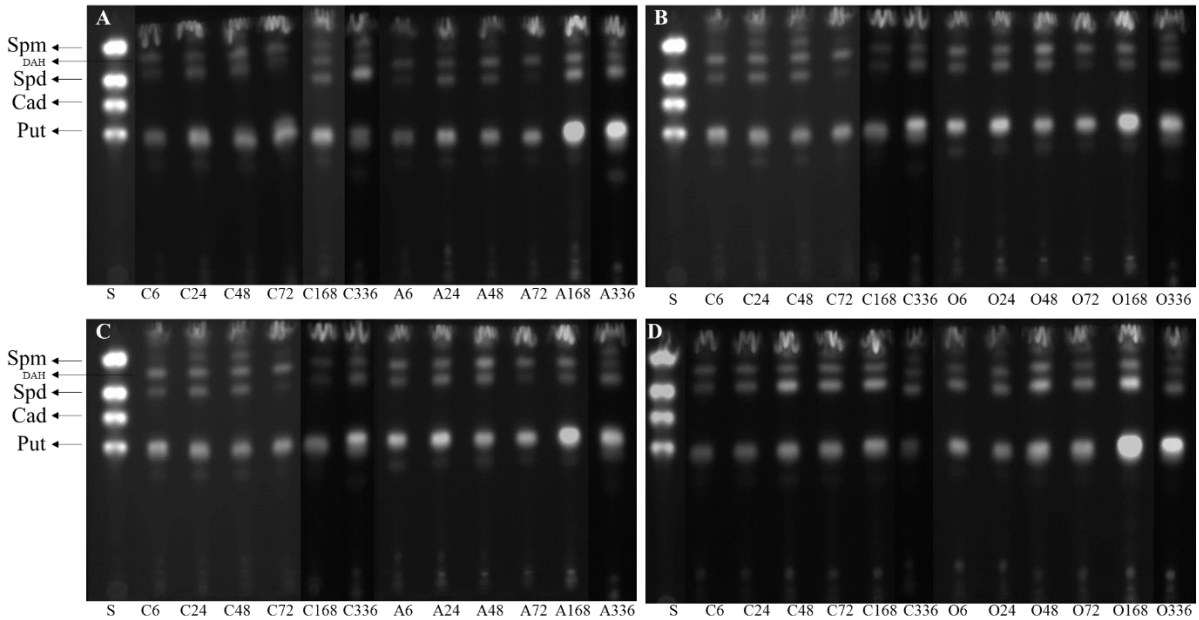
CZE MG – megagametophyte at cotyledonal stage

	Put	Spd	Spm	Agmatine	Aspartic acid	Glutamate acid	Glutamine	Arginine	Citrulline	Gaba	Methionine	Ornithine	*AaASAL*	*AaOTC*	*AaASS*	*AaARG*	*AaADC*	*AaAIH*	*AaCPA*	*AaSAMDC*	*AaSPDS*	*AaSPDS3*	*AaSPMS*
Put																							
Spd	**0.98 (0.001)**																						
Spm	**0.96 (0.002)**	**1.00 (0.000)**																					
Agmatine	0.08 (0.887)	-0.13 (0.811)	-0.20 (0.708)																				
Aspartic acid	**0.96 (0.002)**	0.89 (0.017)	0.86 (0.030)	0.34 (0.512)																			
Glutamate acid	**1.00 (0.000)**	**0.96 (0.002)**	**0.94 (0.006)**	0.16 (0.766)	**0.98 (0.001)**																		
Glutamine	**0.99 (0.000)**	**1.00 (0.000)**	**0.99 (0.000)**	-0.09 (0.869)	0.91 (0.012)	**0.97 (0.001)**																	
Arginine	**0.96 (0.003)**	0.88 (0.020)	0.85 (0.034)	0.35 (0.491)	**1.00 (0.000)**	**0.98 (0.001)**	0.90 (0.014)																
Citrulline	0.60 (0.212)	0.75 (0.089)	0.79 (0.061)	-0.76 (0.082)	0.36 (0.483)	0.53 (0.282)	0.72 (0.108)	0.34 (0.504)															
Gaba	**0.97 (0.001)**	**1.00 (0.000)**	**1.00 (0.000)**	-0.17 (0.744)	0.87 (0.025)	**0.95 (0.004)**	**1.00 (0.000)**	0.86 (0.028)	0.78 (0.070)														
Methionine	-0.08 (0.884)	-0.28 (0.596)	-0.34 (0.504)	**0.99 (0.000)**	0.19 (0.717)	0.01 (0.992)	-0.24 (0.650)	0.21 (0.693)	-0.85 (0.033)	-0.32 (0.536)													
Ornithine	0.63 (0.177)	0.46 (0.354)	0.40 (0.433)	0.82 (0.046)	0.82 (0.048)	0.69 (0.126)	0.50 (0.314)	0.83 (0.043)	-0.25 (0.640)	0.42 (0.404)	0.72 (0.105)												
AaASAL	0.79 (0.060)	0.65 (0.158)	0.60 (0.209)	0.67 (0.148)	**0.93 (0.008)**	0.84 (0.036)	0.68 (0.134)	**0.93 (0.007)**	-0.02 (0.976)	0.62 (0.190)	0.55 (0.263)	**0.97 (0.001)**											
AaOTC	0.81 (0.048)	0.68 (0.136)	0.63 (0.183)	0.64 (0.171)	**0.94 (0.005)**	0.86 (0.028)	0.71 (0.114)	**0.95 (0.004)**	0.02 (0.971)	0.65 (0.165)	0.52 (0.296)	**0.96 (0.002)**	**1.00 (0.000)**										
AaASS	0.73 (0.102)	0.85 (0.032)	0.89 (0.019)	-0.63 (0.179)	0.52 (0.294)	0.67 (0.148)	0.83 (0.042)	0.50 (0.310)	**0.98 (0.000)**	0.87 (0.023)	-0.74 (0.092)	-0.07 (0.891)	0.16 (0.765)	0.19 (0.714)									
AaARG	**1.00 (0.000)**	**0.99 (0.000)**	**0.98 (0.001)**	0.01 (0.987)	**0.94 (0.005)**	**0.99 (0.000)**	**1.00 (0.000)**	**0.94 (0.006)**	0.65 (0.164)	**0.98 (0.000)**	-0.14 (0.786)	0.58 (0.228)	0.75 (0.085)	0.77 (0.071)	0.77 (0.073)								
AaADC	0.90 (0.015)	**0.97 (0.002)**	**0.98 (0.000)**	-0.37 (0.468)	0.75 (0.088)	0.86 (0.029)	**0.96 (0.003)**	0.74 (0.095)	0.89 (0.018)	**0.98 (0.001)**	-0.51 (0.302)	0.23 (0.666)	0.44 (0.378)	0.48 (0.341)	**0.95 (0.003)**	**0.92 (0.008)**							
AaAIH	0.91 (0.011)	0.81 (0.051)	0.77 (0.076)	0.48 (0.336)	**0.99 (0.000)**	**0.94 (0.005)**	0.83 (0.040)	**0.99 (0.000)**	0.21 (0.687)	0.78 (0.066)	0.34 (0.510)	0.90 (0.016)	**0.97 (0.001)**	**0.98 (0.001)**	0.38 (0.459)	0.88 (0.020)	0.64 (0.174)						
AaCPA	0.23 (0.668)	0.42 (0.411)	0.48 (0.334)	**-0.95 (0.003)**	-0.04 (0.937)	0.14 (0.785)	0.38 (0.457)	-0.06 (0.911)	**0.92 (0.010)**	0.46 (0.360)	**-0.99 (0.000)**	-0.61 (0.197)	-0.41 (0.415)	-0.38 (0.456)	0.83 (0.039)	0.29 (0.577)	0.63 (0.178)	-0.20 (0.711)					
AaSAMDC	0.76 (0.081)	0.87 (0.023)	0.91 (0.013)	-0.59 (0.215)	0.56 (0.251)	0.70 (0.120)	0.85 (0.030)	0.54 (0.265)	**0.98 (0.001)**	0.90 (0.016)	-0.71 (0.115)	-0.02 (0.963)	0.21 (0.696)	0.24 (0.647)	**1.00 (0.000)**	0.80 (0.056)	**0.97 (0.002)**	0.42 (0.403)	0.81 (0.053)				
AaSPDS	**0.95 (0.003)**	**0.99 (0.000)**	**1.00 (0.000)**	-0.23 (0.665)	0.84 (0.037)	**0.93 (0.008)**	**0.99 (0.000)**	0.83 (0.041)	0.81 (0.051)	**1.00 (0.000)**	-0.37 (0.467)	0.37 (0.469)	0.57 (0.233)	0.60 (0.205)	0.90 (0.015)	**0.97 (0.001)**	**0.99 (0.000)**	0.75 (0.089)	0.51 (0.304)	**0.92 (0.010)**			
AaSPDS3	0.84 (0.037)	0.71 (0.113)	0.66 (0.154)	0.61 (0.202)	**0.95 (0.003)**	0.88 (0.021)	0.74 (0.093)	**0.96 (0.003)**	0.06 (0.908)	0.68 (0.139)	0.48 (0.337)	**0.95 (0.003)**	**1.00 (0.000)**	**1.00 (0.000)**	0.23 (0.655)	0.80 (0.056)	0.51 (0.299)	**0.99 (0.000)**	-0.34 (0.507)	0.28 (0.590)	0.64 (0.174)		
AaSPMS	**-1.00 (0.000)**	**-0.97 (0.001)**	**-0.95 (0.004)**	-0.12 (0.817)	**-0.98 (0.001)**	**-1.00 (0.000)**	**-0.98 (0.001)**	**-0.97 (0.001)**	-0.56 (0.251)	**-0.96 (0.003)**	0.03 (0.955)	-0.67 (0.146)	-0.82 (0.045)	-0.84 (0.036)	-0.69 (0.127)	**-0.99 (0.000)**	-0.88 (0.022)	**-0.93 (0.007)**	-0.18 (0.735)	-0.73 (0.102)	**-0.94 (0.006)**	-0.86 (0.027)	

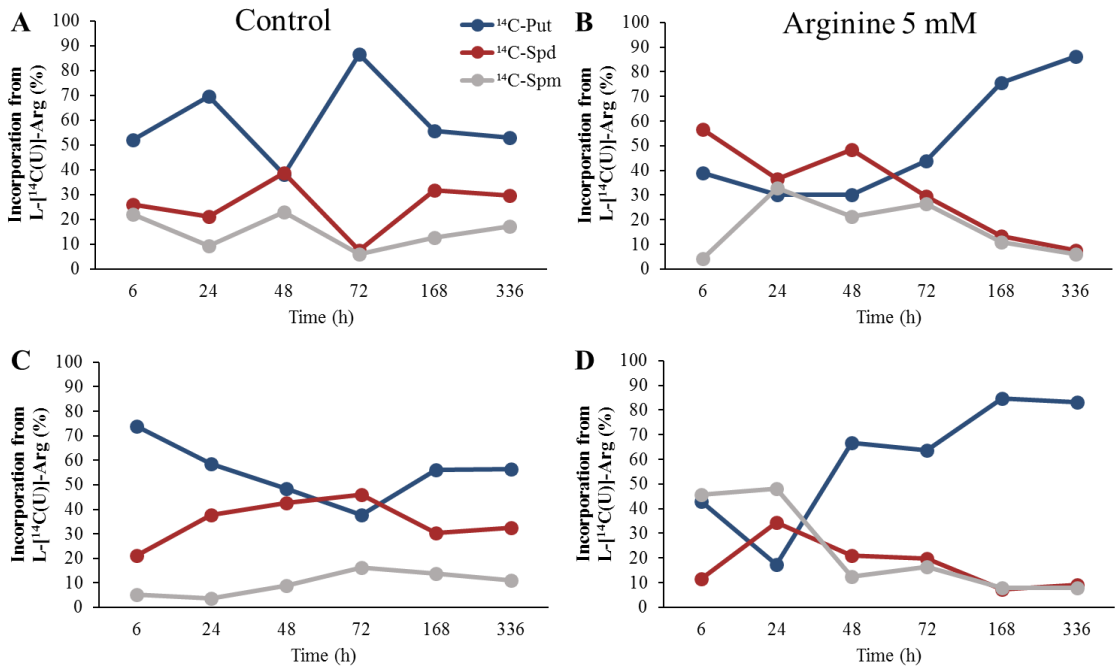
MZE MG – megagametophyte at mature stage

	Put	Spd	Spm	Agmatine	Aspartic acid	Glutamate acid	Glutamine	Arginine	Citrulline	Gaba	Methionine	Ornithine	*AaASAL*	*AaOTC*	*AaASS*	*AaARG*	*AaADC*	*AaAIH*	*AaCPA*	*AaSAMDC*	*AaSPDS*	*AaSPDS3*	*AaSPMS*
Put																							
Spd	-0.41 (0.425)																						
Spm	-0.31 (0.548)	**0.99 (0.000)**																					
Agmatine	-0.42 (0.404)	-0.66 (0.156)	-0.73 (0.100)																				
Aspartic acid	**-0.98 (0.000)**	0.24 (0.647)	0.14 (0.789)	0.57 (0.234)																			
Glutamate acid	**-0.98 (0.000)**	0.24 (0.647)	0.14 (0.789)	0.57 (0.234)	**1.00 (0.000)**																		
Glutamine	**-0.98 (0.000)**	0.24 (0.647)	0.14 (0.789)	0.57 (0.234)	**1.00 (0.000)**	**1.00 (0.000)**																	
Arginine	**-0.98 (0.000)**	0.24 (0.647)	0.14 (0.789)	0.57 (0.234)	**1.00 (0.000)**	**1.00 (0.000)**	**1.00 (0.000)**																
Citrulline	**-0.98 (0.000)**	0.24 (0.647)	0.14 (0.789)	0.57 (0.234)	**1.00 (0.000)**	**1.00 (0.000)**	**1.00 (0.000)**	**1.00 (0.000)**															
Gaba	**-0.98 (0.000)**	0.24 (0.647)	0.14 (0.789)	0.57 (0.234)	**1.00 (0.000)**	**1.00 (0.000)**	**1.00 (0.000)**	**1.00 (0.000)**	**1.00 (0.000)**														
Methionine	**-0.98 (0.000)**	0.24 (0.647)	0.14 (0.789)	0.57 (0.234)	**1.00 (0.000)**	**1.00 (0.000)**	**1.00 (0.000)**	**1.00 (0.000)**	**1.00 (0.000)**	**1.00 (0.000)**													
Ornithine	**-0.98 (0.000)**	0.24 (0.647)	0.14 (0.789)	0.57 (0.234)	**1.00 (0.000)**	**1.00 (0.000)**	**1.00 (0.000)**	**1.00 (0.000)**	**1.00 (0.000)**	**1.00 (0.000)**	**1.00 (0.000)**												
AaASAL	-0.34 (0.515)	-0.72 (0.103)	-0.79 (0.061)	**1.00 (0.000)**	0.49 (0.319)	0.49 (0.319)	0.49 (0.319)	0.49 (0.319)	0.49 (0.319)	0.49 (0.319)	0.49 (0.319)	0.49 (0.319)											
AaOTC	0.49 (0.329)	**-1.00 (0.000)**	**-0.98 (0.001)**	0.59 (0.220)	-0.33 (0.529)	-0.33 (0.529)	-0.33 (0.529)	-0.33 (0.529)	-0.33 (0.529)	-0.33 (0.529)	-0.33 (0.529)	-0.33 (0.529)	0.66 (0.153)										
AaASS	0.35 (0.499)	0.72 (0.110)	0.78 (0.066)	**-1.00 (0.000)**	-0.51 (0.306)	-0.51 (0.306)	-0.51 (0.306)	-0.51 (0.306)	-0.51 (0.306)	-0.51 (0.306)	-0.51 (0.306)	-0.51 (0.306)	**-1.00 (0.000)**	-0.65 (0.161)									
AaARG	0.57 (0.239)	**-0.98 (0.001)**	**-0.96 (0.003)**	0.51 (0.306)	-0.42 (0.411)	-0.42 (0.411)	-0.42 (0.411)	-0.42 (0.411)	-0.42 (0.411)	-0.42 (0.411)	-0.42 (0.411)	-0.42 (0.411)	0.58 (0.224)	**1.00 (0.000)**	-0.57 (0.234)								
AaADC	0.23 (0.655)	0.79 (0.059)	0.85 (0.032)	**-0.98 (0.001)**	-0.40 (0.432)	-0.40 (0.432)	-0.40 (0.432)	-0.40 (0.432)	-0.40 (0.432)	-0.40 (0.432)	-0.40 (0.432)	-0.40 (0.432)	**-0.99 (0.000)**	-0.74 (0.095)	**0.99 (0.000)**	-0.67 (0.148)							
AaAIH	**0.92 (0.010)**	-0.74 (0.094)	-0.67 (0.149)	-0.02 (0.964)	-0.83 (0.040)	-0.83 (0.040)	-0.83 (0.040)	-0.83 (0.040)	-0.83 (0.040)	-0.83 (0.040)	-0.83 (0.040)	-0.83 (0.040)	0.07 (0.896)	0.79 (0.059)	-0.06 (0.915)	0.85 (0.032)	-0.17 (0.740)						
AaCPA	**-0.94 (0.006)**	0.06 (0.916)	-0.04 (0.933)	0.72 (0.110)	**0.98 (0.001)**	**0.98 (0.001)**	**0.98 (0.001)**	**0.98 (0.001)**	**0.98 (0.001)**	**0.98 (0.001)**	**0.98 (0.001)**	**0.98 (0.001)**	0.65 (0.165)	-0.14 (0.785)	-0.66 (0.156)	-0.24 (0.646)	-0.56 (0.245)	-0.72 (0.110)					
AaSAMDC	0.08 (0.885)	**-0.94 (0.005)**	**-0.97 (0.001)**	0.87 (0.024)	0.10 (0.854)	0.10 (0.854)	0.10 (0.854)	0.10 (0.854)	0.10 (0.854)	0.10 (0.854)	0.10 (0.854)	0.10 (0.854)	0.91 (0.011)	0.91 (0.012)	-0.91 (0.012)	0.86 (0.027)	**-0.95 (0.004)**	0.47 (0.347)	0.28 (0.590)				
AaSPDS	0.41 (0.422)	**-1.00 (0.000)**	**-0.99 (0.000)**	0.66 (0.158)	-0.24 (0.643)	-0.24 (0.643)	-0.24 (0.643)	-0.24 (0.643)	-0.24 (0.643)	-0.24 (0.643)	-0.24 (0.643)	-0.24 (0.643)	0.72 (0.105)	**1.00 (0.000)**	-0.71 (0.111)	**0.98 (0.000)**	-0.79 (0.060)	0.74 (0.093)	-0.06 (0.912)	**0.94 (0.005)**			
AaSPDS3	0.87 (0.024)	-0.80 (0.055)	-0.74 (0.095)	0.08 (0.887)	-0.77 (0.071)	-0.77 (0.071)	-0.77 (0.071)	-0.77 (0.071)	-0.77 (0.071)	-0.77 (0.071)	-0.77 (0.071)	-0.77 (0.071)	0.17 (0.750)	0.85 (0.032)	-0.16 (0.768)	0.90 (0.015)	-0.27 (0.602)	**1.00 (0.000)**	-0.64 (0.169)	0.56 (0.253)	0.80 (0.054)		
AaSPMS	**-0.94 (0.005)**	0.07 (0.892)	-0.03 (0.957)	0.70 (0.118)	**0.99 (0.000)**	**0.99 (0.000)**	**0.99 (0.000)**	**0.99 (0.000)**	**0.99 (0.000)**	**0.99 (0.000)**	**0.99 (0.000)**	**0.99 (0.000)**	0.64 (0.176)	-0.16 (0.761)	-0.64 (0.167)	-0.26 (0.624)	-0.55 (0.259)	-0.73 (0.102)	**1.00 (0.000)**	0.27 (0.612)	-0.07 (0.888)	-0.65 (0.159)	

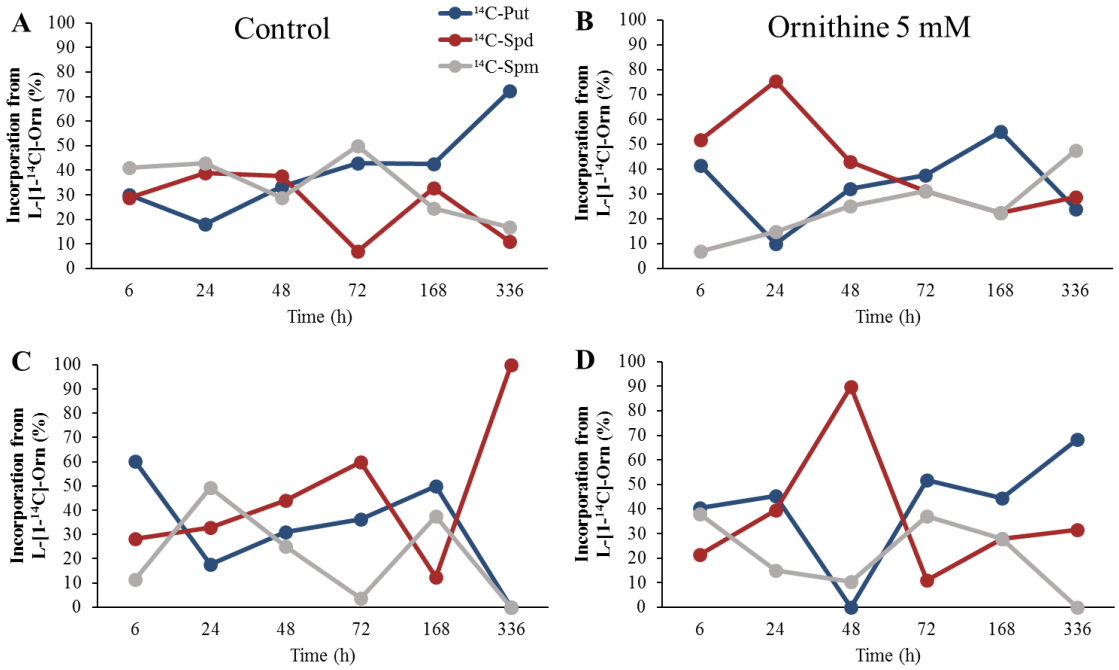
ANEXO II – Referente ao material suplementar do Capítulo II.



Supplementary Figure S1. Separation of dansyl-polyamines from radioisotope incorporation samples on TLC plates, as visualized under UV light, in Responsive (**A, B**) and Blocked (**C, D**) cells. S=standards, Put=putrescine, Spd=spermidine, Spm=spermine, DAH=diaminoheptane (internal standard), C=control, A=cells supplied with 5 mM Arg, O=cells supplied with 5 mM Orn. Numbers indicate the time of incubation in hours.



Supplementary Figure S2. Percentage of incorporation from L-[^{14}C(U)]-Arg into polyamines (^{14}C-putrescine, ^{14}C-spermidine and ^{14}C-spermine), in Responsive (**A, B**) and Blocked (**C, D**) cells of *Araucaria angustifolia* at different times of incubation in a liquid MSG medium supplemented with, or without, 5 mM cold Arg.



Supplementary Figure S3. Percentage of incorporation from L-[1-^{14}C]-Orn into polyamines (^{14}C-putrescine, ^{14}C-spermidine and ^{14}C-spermine), in Responsive (**A, B**) and Blocked (**C, D**) cells of *Araucaria angustifolia* at different times of incubation in a liquid MSG medium supplemented with, or without, 5 mM cold Orn.

Supplementary Table S1. Amount of radioactivity (CPM x 10^3) g^{-1} FW in amino acids in Responsive cells of *Araucaria angustifolia* during cell proliferation in a liquid MSG medium supplemented with, or without, 5 mM Arginine or Ornithine at different times.

^{14}C-Labeled substrate	Cold sub.	Metabolite	6 h	24 h	48 h	72 h	168 h	336 h
L-Arginine	Control	Arginine	12.4 ± 2.4	202.5 ± 3.0	58.5 ± 1.5	60.0 ± 6.0	69.8 ± 8.3	29.3 ± 5.3
	5 mM		**4.5 ± 1.5 ***	**72.0 ± 12.0 ***	**531.5 ± 42.8 ***	**130.5 ± 19.5 ***	**207.0 ± 4.5 ***	**221.0 ± 15.8 ***
	Control	Ornithine	18.1 ± 1.9	91.5 ± 3.0	109.5 ± 16.5	78.0 ± 12.0	25.5 ± 3.0	18.0 ± 4.5
	5 mM		11.0 ± 2.3	**55.5 ± 7.5 ***	**14.5 ± 2.3 ***	**18.0 ± 4.5 ***	**15.0 ± 1.5 ***	27.0 ± 3.0
	Control	Citruline	9.6 ± 1.4	10.5 ± 1.5	0.0 ± 0.0	15.5 ± 2.3	15.8 ± 2.3	10.5 ± 1.5
	5 mM		12.0 ± 3.0	13.0 ± 2.3	13.5 ± 1.5	11.3 ± 3.8	0.0 ± 0.0	**2.3 ± 0.4 ***
	Control	Proline	0.0 ± 0.0	17.3 ± 2.3	4.5 ± 1.5	15.8 ± 5.3	12.8 ± 2.3	8.3 ± 2.3
	5 mM		10.1 ± 1.9	0.0 ± 0.0	6.0 ± 0.8	**0.4 ± 0.1 ***	0.0 ± 0.0	9.0 ± 1.5
	Control	GABA	26.7 ± 11.9	12.8 ± 3.8	0.0 ± 0.0	18.0 ± 3.0	6.0 ± 1.5	0.8 ± 0.2
	5 mM		7.5 ± 1.5	3.8 ± 0.8	13.5 ± 3.0	**5.3 ± 0.8 ***	3.8 ± 0.8	**4.5 ± 1.4 ***
L-Ornithine	Control	Arginine	14.3 ± 2.0	18.0 ± 3.0	33.8 ± 5.3	13.5 ± 1.5	15.0 ± 1.5	23.0 ± 0.9
	5 mM		**6.0 ± 1.5 ***	**7.5 ± 1.5 ***	27.5 ± 0.9	13.0 ± 0.9	11.0 ± 0.9	**5.3 ± 0.8 ***
	Control	Ornithine	7.5 ± 3.4	18.0 ± 4.5	38.0 ± 5.3	40.0 ± 2.3	11.5 ± 0.9	7.5 ± 1.5
	5 mM		6.8 ± 0.8	17.0 ± 2.3	**67.5 ± 1.5 ***	34.0 ± 3.8	**31.0 ± 3.8 ***	**21.5 ± 0.9 ***
	Control	Citruline	0.0 ± 0.0	7.5 ± 1.5	6.0 ± 1.5	3.0 ± 0.8	2.5 ± 0.6	6.5 ± 0.9
	5 mM		0.0 ± 0.0	0.0 ± 0.0	9.0 ± 1.5	9.0 ± 3.0	6.0 ± 1.5	**2.8 ± 0.6 ***
	Control	Proline	1.9 ± 0.4	9.0 ± 0.0	9.8 ± 2.3	1.5 ± 0.4	4.5 ± 1.5	2.5 ± 0.6
	5 mM		0.0 ± 0.0	7.5 ± 1.5	0.0 ± 0.0	**7.5 ± 0.0 ***	1.5 ± 0.4	**7.5 ± 1.5 ***
	Control	GABA	0.0 ± 0.0	1.5 ± 0.4	1.5 ± 0.4	0.0 ± 0.0	27.8 ± 5.3	1.5 ± 0.4
	5 mM		7.5 ± 1.5	0.0 ± 0.0	**6.4 ± 1.1 ***	6.8 ± 1.5	0.0 ± 0.0	3.5 ± 0.9

Values represent medians ± SD from three biological replicates. Asterisks indicate that the values for Arginine 5 mM or Ornithine 5 mM are significantly different (Student's *t*-test; $P < 0.01$) from control at a given time.

Supplementary Table S2. Amount of radioactivity (CPM x 10^3) g^{-1} FW in amino acids in Blocked cells of *Araucaria angustifolia* during cell proliferation in a liquid MSG medium supplemented with, or without, 5 mM Arginine or Ornithine at different times.

^{14}C-Labeled substrate	Cold sub.	Metabolite	6 h	24 h	48 h	72 h	168 h	336 h
L-Arginine	Control	Arginine	32.0 ± 3.2	147.0 ± 1.5	208.5 ± 43.5	396.0 ± 3.0	72.5 ± 5.3	21.8 ± 5.3
	5 mM		21.8 ± 2.3	**100.0 ± 0.9 ***	**585.0 ± 15.0 ***	**749.0 ± 26.3 ***	**433.5 ± 19.5 ***	**220.5 ± 3.0 ***
	Control	Ornithine	75.1 ± 7.3	58.5 ± 10.5	78.0 ± 6.0	147.5 ± 5.3	54.5 ± 6.8	55.5 ± 7.5
	5 mM		**15.8 ± 3.8 ***	**12.0 ± 1.5 ***	94.5 ± 12.0	**130.0 ± 0.9 ***	69.0 ± 9.0	53.0 ± 3.8
	Control	Citruline	2.4 ± 0.7	18.0 ± 3.0	10.5 ± 1.5	3.0 ± 0.4	7.5 ± 0.4	3.0 ± 0.4
	5 mM		0.5 ± 0.2	**3.8 ± 0.8 ***	12.8 ± 0.8	4.5 ± 1.1	9.8 ± 2.3	**8.3 ± 2.3 ***
	Control	Proline	0.4 ± 0.3	9.8 ± 2.3	0.0 ± 0.0	15.0 ± 3.0	3.8 ± 0.8	3.8 ± 0.8
	5 mM		0.5 ± 0.2	12.0 ± 1.1	15.0 ± 3.0	9.0 ± 1.5	**6.0 ± 0.4 ***	5.5 ± 0.9
	Control	GABA	0.1 ± 0.0	0.5 ± 0.1	9.0 ± 0.4	28.5 ± 4.5	1.5 ± 0.4	9.0 ± 3.0
	5 mM		0.0 ± 0.0	**9.0 ± 0.4 ***	0.0 ± 0.0	**11.3 ± 0.8 ***	**5.0 ± 0.9 ***	**3.0 ± 0.4 ***
L-Ornithine	Control	Arginine	6.5 ± 1.8	7.0 ± 0.9	585.0 ± 15.0	61.5 ± 4.5	17.5 ± 3.8	0.0 ± 0.0
	5 mM		6.8 ± 0.8	7.5 ± 1.5	**16.5 ± 1.5 ***	54.0 ± 3.0	30.0 ± 6.0	17.0 ± 0.9
	Control	Ornithine	1.1 ± 0.1	12.8 ± 2.3	94.5 ± 12.0	49.5 ± 0.4	20.5 ± 0.9	0.0 ± 0.0
	5 mM		**3.0 ± 0.4 ***	**34.5 ± 4.5 ***	**47.5 ± 9.8 ***	**111.0 ± 4.5 ***	**47.3 ± 8.3 ***	**22.0 ± 3.8 ***
	Control	Citruline	2.6 ± 0.8	2.5 ± 0.6	12.8 ± 0.8	10.0 ± 2.3	6.0 ± 1.5	3.8 ± 0.8
	5 mM		**9.0 ± 1.5 ***	**9.0 ± 1.5 ***	0.0 ± 0.0	5.3 ± 0.8	4.5 ± 1.5	8.3 ± 2.3
	Control	Proline	0.0 ± 0.0	2.0 ± 0.4	15.0 ± 3.0	7.5 ± 1.5	2.5 ± 0.6	7.5 ± 1.5
	5 mM		1.3 ± 0.2	0.0 ± 0.0	11.3 ± 0.8	9.0 ± 3.0	8.5 ± 2.3	**1.5 ± 0.4 ***
	Control	GABA	3.4 ± 0.7	2.3 ± 0.4	0.0 ± 0.0	3.8 ± 0.8	5.3 ± 0.8	6.0 ± 1.5
	5 mM		2.3 ± 0.2	1.5 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	3.0 ± 0.4

Values represent medians ± SD from three biological replicates. Asterisks indicate that the values for Arginine 5 mM or Ornithine 5 mM are significantly different (Student's *t*-test; $P < 0.01$) from control at a given time.

Supplementary Table S3. Changes in the contents (nmol.g^{-1} FW) of free amino acids identified in Responsive cells of *Araucaria angustifolia* during cell proliferation in a liquid MSG medium supplemented with, or without, 5 mM Arginine or Ornithine.

	6 h			24 h			48 h		
Free amino acids	**Control**	**Arginine 5 mM**	**Ornithine 5 mM**	**Control**	**Arginine 5 mM**	**Ornithine 5 mM**	**Control**	**Arginine 5 mM**	**Ornithine 5 mM**
Aspartic acid	66.71 ± 3.44 b	**88.58 ± 4.73 a**	73.55 ± 0.00 b	9.49 ± 3.74 b	15.76 ± 3.46 ab	**40.62 ± 13.68 a**	7.21 ± 0.35 b	**29.88 ± 1.90 a**	**34.34 ± 4.85 a**
Glutamic acid	730.81 ± 26.44 b	671.27 ± 0.24 b	**798.76 ± 4.06 a**	136.75 ± 50.86 b	278.73 ± 60.82 b	**788.33 ± 170.82 a**	136.47 ± 8.76 b	**601.29 ± 42.24 a**	**839.41 ± 122.41 a**
Asparagine	309.19 ± 2.41 a	**249.53 ± 5.76 b**	**209.17 ± 2.94 c**	79.56 ± 25.96 a	27.11 ± 6.11 a	63.43 ± 21.68 a	32.79 ± 1.78 b	**115.58 ± 4.38 a**	**129.15 ± 16.57 a**
Serine	252.01 ± 5.12 b	**274.69 ± 6.28 a**	**288.78 ± 0.61 a**	26.37 ± 1.65 b	59.29 ± 8.24 b	**148.51 ± 15.25 a**	29.32 ± 1.38 c	**123.76 ± 4.08 b**	**169.77 ± 21.06 a**
Glutamine	6441.29 ± 65.47 b	**7351.18 ± 147.83 a**	6757.84 ± 95.14 b	1087.41 ± 163.50 b	1773.51 ± 205.97 b	**5194.87 ± 728.97 a**	1004.06 ± 54.23 c	**3405.70 ± 167.72 b**	**4912.03 ± 620.36 a**
Histidine	78.46 ± 2.31 a	74.15 ± 4.61 a	82.95 ± 1.57 a	13.81 ± 4.03 b	41.13 ± 8.31 ab	**73.23 ± 25.94 a**	15.08 ± 1.08 b	**83.19 ± 2.94 a**	**88.78 ± 10.92 a**
Glycine	266.73 ± 11.64 a	**229.76 ± 3.38 b**	**204.56 ± 9.10 b**	33.83 ± 6.70 b	47.59 ± 7.37 ab	**70.74 ± 12.65 a**	29.43 ± 0.64 c	**68.95 ± 1.99 b**	**88.55 ± 6.04 a**
Arginine	108.50 ± 2.78 c	**482.65 ± 61.18 a**	**188.63 ± 4.66 b**	53.77 ± 16.67 b	**5892.24 ± 1980.69 a**	37.30 ± 10.15 b	7.60 ± 0.49 c	**8733.50 ± 1709.08 a**	**661.11 ± 74.27 b**
Citrulline	9.12 ± 0.37 c	**25.13 ± 3.81 b**	**39.59 ± 0.58 a**	10.01 ± 3.34 b	**229.76 ± 62.52 a**	7.59 ± 2.28 b	2.11 ± 0.05 c	**350.13 ± 17.14 a**	**119.03 ± 17.75 b**
Threonine	95.68 ± 0.78 a	99.54 ± 2.48 a	100.66 ± 0.92 a	8.83 ± 2.86 b	24.44 ± 4.69 ab	**43.27 ± 14.10 a**	6.18 ± 0.23 b	**45.86 ± 1.73 a**	**38.81 ± 4.20 a**
Alanine	2030.91 ± 65.80 a	**1629.92 ± 65.70 b**	**1787.85 ± 7.18 b**	161.45 ± 54.92 b	336.34 ± 74.86 ab	**761.98 ± 255.62 a**	200.12 ± 14.51 c	**792.17 ± 19.93 b**	**1145.90 ± 125.08 a**
γ-aminobutyric acid	1941.83 ± 5.14 a	**1708.57 ± 15.37 b**	**1619.65 ± 52.29 b**	106.25 ± 42.28 a	114.11 ± 29.38 a	124.04 ± 34.53 a	69.42 ± 0.87 b	**296.48 ± 6.74 a**	**340.18 ± 20.75 a**
Tyrosine	86.15 ± 3.09 b	**86.72 ± 0.85 a**	**111.62 ± 1.72 a**	9.95 ± 2.88 a	20.89 ± 4.95 a	37.15 ± 13.09 a	8.01 ± 0.44 c	**36.09 ± 1.95 b**	**51.65 ± 6.69 a**
Tryptophan	29.93 ± 0.81 b	28.77 ± 0.97 b	**37.51 ± 1.13 a**	4.05 ± 0.84 a	8.28 ± 1.60 a	13.38 ± 4.53 a	2.99 ± 0.12 c	**11.53 ± 0.63 b**	**23.54 ± 1.08 a**
Methionine	17.76 ± 1.69 a	16.01 ± 0.94 a	20.41 ± 1.51 a	5.19 ± 1.18 b	8.54 ± 1.53 ab	**16.35 ± 4.69 a**	6.50 ± 0.23 b	**22.93 ± 0.50 a**	**21.87 ± 2.70 a**
Valline	153.92 ± 2.49 a	**133.93 ± 3.83 b**	152.18 ± 3.12 a	17.14 ± 4.50 a	26.96 ± 5.39 a	49.65 ± 16.85 a	13.83 ± 0.38 c	**52.01 ± 2.47 b**	**81.31 ± 10.20 a**
Phenylalanine	92.26 ± 0.78 c	**97.36 ± 1.81 b**	**130.74 ± 1.03 a**	15.80 ± 4.00 a	20.34 ± 3.69 a	30.92 ± 10.06 a	9.49 ± 0.30 c	**37.47 ± 0.63 b**	**97.41 ± 3.24 a**
Isoleucine	67.75 ± 2.28 b	**57.13 ± 1.78 c**	**74.58 ± 1.28 a**	8.35 ± 1.79 a	12.22 ± 1.88 a	18.57 ± 5.83 a	6.75 ± 0.27 c	**19.90 ± 0.64 b**	**28.82 ± 3.14 a**
Leucine	81.64 ± 4.25 a	**67.71 ± 1.66 b**	82.18 ± 1.62 a	11.55 ± 2.75 a	15.73 ± 3.05 a	23.23 ± 7.03 a	9.53 ± 0.63 c	**33.72 ± 1.63 b**	**55.98 ± 7.73 a**
Ornithine	85.54 ± 1.35 c	**139.39 ± 2.49 b**	**5463.19 ± 237.70 a**	78.98 ± 5.92 b	**78.55 ± 10.86 c**	**3929.86 ± 198.82 a**	65.61 ± 7.85 b	81.09 ± 1.94 b	**3302.08 ± 260.25 a**
Lysine	361.90 ± 10.15 b	**490.55 ± 26.70 a**	**434.38 ± 7.44 a**	144.60 ± 3.48 a	149.17 ± 14.20 a	132.12 ± 7.73 a	129.69 ± 2.71 c	**177.60 ± 1.72 b**	**197.72 ± 5.45 a**

Values represent medians ± SD from three biological replicates. Statistically significant differences ($p<0.01$) within rows at a given time are indicated with different letters according to Tukey's test. Numbers in bold denote significant difference between treatment and control at a given time ($p<0.01$).

(cont.)

	72 h			168 h			336 h		
Free amino acids	**Control**	**Arginine 5 mM**	**Ornithine 5 mM**	**Control**	**Arginine 5 mM**	**Ornithine 5 mM**	**Control**	**Arginine 5 mM**	**Ornithine 5 mM**
Aspartic acid	10.21 ± 0.66 c	**30.99 ± 1.54 a**	**18.69 ± 3.09 b**	3.70 ± 0.16 b	**11.32 ± 2.91 a**	3.65 ± 0.25 b	3.72 ± 1.46 b	1.60 ± 0.19 b	**12.04 ± 3.06 a**
Glutamic acid	167.07 ± 11.95 c	**455.80 ± 28.38 a**	**294.00 ± 50.72 b**	89.79 ± 1.39 b	**195.76 ± 50.26 a**	**59.89 ± 3.78 c**	20.33 ± 2.48 b	30.27 ± 4.40 b	**124.33 ± 29.25 a**
Asparagine	318.47 ± 22.76 a	292.25 ± 19.89 a	**172.42 ± 30.13 b**	56.99 ± 2.26 b	**135.29 ± 31.68 a**	64.11 ± 3.25 b	50.67 ± 21.43 b	**23.62 ± 3.29 a**	**221.56 ± 54.14 a**
Serine	40.94 ± 2.67 b	**100.68 ± 6.16 a**	62.97 ± 10.72 b	35.87 ± 1.39 b	**105.41 ± 21.49 a**	**20.04 ± 1.22 c**	60.94 ± 2.52 b	65.54 ± 10.54 b	**353.71 ± 86.58 a**
Glutamine	4299.95 ± 304.84 b	**7130.68 ± 455.07 a**	**6319.22 ± 261.52 a**	562.18 ± 33.52 c	**3452.41 ± 892.23 a**	**1703.84 ± 94.23 b**	191.54 ± 85.66 a	76.50 ± 9.56 a	191.14 ± 52.21 a
Histidine	22.66 ± 1.05 c	**77.98 ± 2.12 b**	**46.61 ± 4.09 a**	8.59 ± 1.26 b	**55.08 ± 14.04 a**	14.28 ± 1.28 b	21.07 ± 3.03 b	**10.23 ± 1.55 c**	**60.75 ± 14.48 a**
Glycine	42.35 ± 1.31 b	**75.07 ± 5.99 a**	51.22 ± 4.93 b	31.26 ± 1.30 a	28.34 ± 1.20 a	28.49 ± 0.61 a	49.85 ± 17.30 a	31.39 ± 12.35 a	59.18 ± 15.54 a
Arginine	30.53 ± 1.58 c	**5253.75 ± 472.32 a**	**312.75 ± 54.68 b**	13.00 ± 0.10 c	**4561.46 ± 615.99 a**	**133.55 ± 9.34 b**	10.06 ± 1.05 c	**1716.05 ± 321.90 a**	**244.35 ± 54.75 b**
Citrulline	4.93 ± 0.27 c	**243.06 ± 43.36 a**	**48.54 ± 8.07 b**	2.23 ± 0.01 c	**171.45 ± 40.06 a**	**14.09 ± 0.42 b**	5.97 ± 2.54 b	**94.89 ± 20.98 a**	**115.24 ± 26.81 a**
Threonine	16.46 ± 1.02 b	**63.45 ± 4.49 a**	25.12 ± 3.97 b	9.49 ± 0.17 b	**57.02 ± 2.65 a**	**7.84 ± 0.22 c**	22.87 ± 6.87 b	18.44 ± 3.93 b	**90.14 ± 25.35 a**
Alanine	336.00 ± 18.83 b	**723.33 ± 44.16 a**	512.64 ± 99.71 ab	195.39 ± 16.38 b	**446.69 ± 42.14 a**	131.37 ± 6.63 b	366.90 ± 84.40 a	341.07 ± 48.47 a	794.03 ± 198.40 a
γ-aminobutyric acid	216.77 ± 6.84 b	**324.30 ± 32.38 a**	**165.19 ± 3.51 c**	44.26 ± 6.03 b	**76.21 ± 24.26 a**	58.16 ± 1.02 b	36.70 ± 1.43 b	28.28 ± 6.28 b	**90.05 ± 31.10 a**
Tyrosine	24.70 ± 2.00 b	**55.15 ± 4.02 a**	37.95 ± 6.94 ab	7.11 ± 0.23 c	**32.64 ± 6.11 a**	**15.79 ± 0.95 b**	14.63 ± 1.85 b	13.35 ± 1.18 b	**95.39 ± 22.70 a**
Tryptophan	3.29 ± 0.24 b	**11.19 ± 0.63 a**	**12.26 ± 0.93 a**	4.06 ± 0.09 c	**21.89 ± 0.85 a**	**6.91 ± 0.35 b**	11.66 ± 4.36 b	5.11 ± 0.33 b	**33.24 ± 7.03 a**
Methionine	11.56 ± 0.94 b	**24.67 ± 1.79 a**	12.89 ± 2.43 b	12.20 ± 0.26 b	**45.87 ± 11.76 a**	**6.91 ± 0.80 c**	7.12 ± 1.03 c	**20.06 ± 1.39 b**	**78.81 ± 18.58 a**
Valline	31.82 ± 2.14 b	**69.50 ± 4.02 a**	**52.42 ± 7.24 a**	15.35 ± 0.41 b	**49.99 ± 3.22 a**	16.24 ± 1.04 b	34.71 ± 5.31 b	22.46 ± 1.29 b	**89.48 ± 19.05 a**
Phenylalanine	24.15 ± 1.85 b	**79.16 ± 5.52 a**	**112.66 ± 16.47 a**	23.85 ± 0.68 c	**114.19 ± 2.62 a**	**35.92 ± .76 b**	43.36 ± 1.81 b	**20.44 ± 1.84 c**	**236.05 ± 54.13 a**
Isoleucine	11.57 ± 0.63 b	**26.40 ± 1.47 a**	17.04 ± 2.14 b	8.55 ± 0.15 b	**27.97 ± 0.93 a**	10.09 ± 0.43 b	9.55 ± 3.87 b	**11.20 ± 0.87 a**	**62.12 ± 14.96 a**
Leucine	18.73 ± 1.12 c	**48.79 ± 3.08 a**	**31.46 ± 4.29 b**	10.38 ± 0.34 b	**46.86 ± 10.62 a**	14.42 ± 2.23 b	8.36 ± 3.33 b	**11.71 ± 1.07 a**	**45.82 ± 10.06 a**
Ornithine	118.04 ± 12.03 b	158.16 ± 14.09 b	**2618.45 ± 137.08 a**	77.99 ± 5.18 b	103.26 ± 20.10 b	**1800.43 ± 37.51 a**	76.94 ± 2.59 b	75.21 ± 6.51 b	**1359.95 ± 447.21 a**
Lysine	142.69 ± 8.19 a	197.08 ± 9.47 a	195.32 ± 28.54 a	112.41 ± 1.41 b	**163.97 ± 11.41 a**	138.50 ± 8.10 ab	126.51 ± 4.09 a	127.43 ± 6.36 a	141.56 ± 13.50 a

Values represent medians ± SD from three biological replicates. Statistically significant differences ($p<0.01$) within rows at a given time are indicated with different letters according to Tukey's test. Numbers in bold denote significant difference between treatment and control at a given time ($p<0.01$).

Supplementary Table S4. Changes on contents (nmol.g^{-1} FW) of free amino acids identified in Blocked cells of *Araucaria angustifolia* during cell proliferation in a liquid MSG medium supplemented with, or without, 5 mM Arginine or Ornithine.

	6 h			24 h			48 h		
Free amino acids	**Control**	**Arginine 5 mM**	**Ornithine 5 mM**	**Control**	**Arginine 5 mM**	**Ornithine 5 mM**	**Control**	**Arginine 5 mM**	**Ornithine 5 mM**
Aspartic acid	56.23 ± 0.52 a	**49.97 ± 2.51 b**	53.78 ± 0.96 ab	27.13 ± 0.46 ab	21.52 ± 0.80 b	30.24 ± 2.88 a	35.50 ± 5.34 a	**5.60 ± 0.84 c**	**14.72 ± 0.68 b**
Glutamic acid	1308.32 ± 42.17 a	**995.65 ± 74.69 b**	1219.60 ± 21.69 a	950.25 ± 3.09 a	756.91 ± 179.89 a	1100.23 ± 164.71 a	1411.07 ± 210.42 a	**202.39 ± 25.67 c**	**607.61 ± 25.63 b**
Asparagine	69.26 ± 3.71 a	**49.77 ± 1.80 b**	73.75 ± 2.05 a	39.95 ± 1.35 a	32.72 ± 7.21 a	50.63 ± 7.30 a	64.45 ± 9.38 a	**11.79 ± 1.36 c**	**33.68 ± 1.76 b**
Serine	245.99 ± 1.52 a	**180.05 ± 16.81 b**	244.23 ± 7.84 a	117.85 ± 5.15 b	**112.58 ± 5.05 a**	**165.45 ± 6.50 a**	178.98 ± 24.91 a	**33.81 ± 4.44 c**	**106.22 ± 5.18 b**
Glutamine	6829.53 ± 97.64 a	**5893.25 ± 405.77 b**	**5935.62 ± 98.84 b**	3052.97 ± 52.24 a	2824.45 ± 742.56 a	3727.62 ± 495.15 a	3988.32 ± 555.31 a	**571.77 ± 70.85 c**	**1803.77 ± 89.89 b**
Histidine	83.33 ± 7.37 a	81.44 ± 9.57 a	104.47 ± 5.12 a	50.06 ± 4.89 a	57.05 ± 15.68 a	75.93 ± 12.34 a	106.83 ± 13.62 a	**25.24 ± 2.25 c**	**57.01 ± 4.16 b**
Glycine	183.49 ± 7.45 b	**112.82 ± 2.98 c**	**221.85 ± 2.99 a**	116.74 ± 14.52 a	82.50 ± 11.47 a	132.96 ± 23.10 a	160.54 ± 10.98 a	**31.65 ± 4.01 c**	**109.92 ± 4.34 b**
Arginine	82.13 ± 1.42 c	**1129.21 ± 162.12 a**	**212.43 ± 1.53 b**	41.55 ± 0.06 c	**1661.51 ± 239.55 a**	**250.58 ± 29.55 b**	96.70 ± 12.39 c	**4078.70 ± 1185.01 a**	**424.95 ± 24.29 b**
Citrulline	12.13 ± 0.19 c	**56.53 ± 7.69 b**	**103.00 ± 1.06 a**	6.07 ± 0.30 b	**74.16 ± 12.74 a**	**83.67 ± 13.05 a**	14.72 ± 2.08 c	**186.82 ± 69.09 a**	**69.43 ± 3.78 b**
Threonine	90.34 ± 4.40 ab	76.59 ± 3.97 b	103.55 ± 7.54 a	41.42 ± 0.63 b	46.78 ± 1.14 b	**58.75 ± 4.26 a**	57.09 ± 7.92 a	**18.23 ± 2.44 b**	40.41 ± 2.31 a
Alanine	1715.94 ± 23.96 a	**1206.59 ± 138.24 b**	1631.18 ± 45.86 a	1068.68 ± 19.02 ab	823.53 ± 64.30 b	1160.99 ± 134.34 a	2011.54 ± 275.64 a	**326.58 ± 38.48 c**	**895.64 ± 45.44 b**
γ-aminobutyric acid	565.24 ± 21.38 a	**256.89 ± 62.12 b**	523.63 ± 0.84 a	273.47 ± 11.39 a	**171.54 ± 45.57 b**	264.33 ± 45.17 a	504.62 ± 0.87 a	**79.88 ± 27.32 c**	**390.54 ± 15.91 b**
Tyrosine	120.95 ± 3.13 b	**96.08 ± 3.89 c**	**138.93 ± 1.91 a**	54.32 ± 1.56 a	51.51 ± 11.00 a	78.77 ± 10.98 a	79.42 ± 9.92 a	**16.21 ± 1.33 c**	**47.47 ± 2.49 b**
Tryptophan	57.76 ± 1.03 a	**42.67 ± 0.85 b**	59.75 ± 5.10 a	23.25 ± 0.63 a	23.94 ± 6.08 a	37.55 ± 6.16 a	39.03 ± 4.65 a	**9.88 ± 1.35 c**	**26.27 ± 1.34 b**
Methionine	14.26 ± 0.64 b	15.86 ± 0.76 ab	**20.93 ± 2.89 a**	10.77 ± 0.73 a	12.88 ± 2.75 a	17.15 ± 1.36 a	23.86 ± 3.88 a	**6.63 ± 0.09 c**	**16.80 ± 1.15 b**
Valline	147.62 ± 5.08 b	**108.85 ± 3.86 c**	**177.00 ± 7.90 a**	68.59 ± 2.48 b	**65.90 ± 4.41 a**	**96.98 ± 4.23 a**	96.60 ± 12.91 a	**23.26 ± 2.36 c**	**64.21 ± 3.31 b**
Phenylalanine	153.32 ± 3.30 b	**122.30 ± 3.64 c**	**206.69 ± 8.31 a**	59.55 ± 0.93 b	62.20 ± 3.91 b	**112.65 ± 9.29 a**	91.12 ± 12.25 a	**22.21 ± 1.93 b**	73.48 ± 3.79 a
Isoleucine	66.24 ± 0.93 b	**47.88 ± 2.61 c**	**99.36 ± 2.86 a**	32.91 ± 0.84 a	30.97 ± 8.06 a	49.85 ± 7.60 a	43.05 ± 5.32 a	**11.61 ± 1.13 c**	**30.48 ± 1.47 b**
Leucine	88.16 ± 0.99 b	**69.48 ± 4.17 c**	**121.78 ± 2.90 a**	51.66 ± 1.16 a	48.89 ± 12.26 a	78.42 ± 12.71 a	66.63 ± 8.08 a	**18.43 ± 1.55 b**	50.88 ± 2.11 a
Ornithine	71.62 ± 9.00 b	**87.50 ± 13.63 a**	**4597.65 ± 127.64 a**	78.87 ± 2.02 b	**83.11 ± 4.85 a**	**2462.48 ± 444.12 a**	108.60 ± 10.77 b	**73.87 ± 2.21 c**	**1856.06 ± 68.04 a**
Lysine	301.19 ± 21.96 a	194.26 ± 97.27 a	381.92 ± 41.36 a	177.66 ± 12.77 a	167.61 ± 13.71 a	244.40 ± 36.75 a	221.79 ± 2.62 a	**124.77 ± 11.93 b**	238.87 ± 1.98 a

Values represent medians ± SD from three biological replicates. Statistically significant differences ($p<0.01$) within rows at a given time are indicated with different letters according to Tukey's test. Numbers in bold denote significant difference between treatment and control at a given time ($p<0.01$).

(cont.)

	72 h			168 h			336 h		
Free amino acids	**Control**	**Arginine 5 mM**	**Ornithine 5 mM**	**Control**	**Arginine 5 mM**	**Ornithine 5 mM**	**Control**	**Arginine 5 mM**	**Ornithine 5 mM**
Aspartic acid	8.43 ± 0.40 a	7.30 ± 0.96 a	9.52 ± 1.27 a	6.59 ± 0.89 a	3.79 ± 0.82 a	7.91 ± 1.56 a	0.79 ± 0.27 b	**4.13 ± 0.27 a**	3.36 ± 1.36 ab
Glutamic acid	243.91 ± 16.69 b	273.15 ± 30.96 ab	**352.32 ± 22.83 a**	211.34 ± 24.02 a	**70.78 ± 15.41 b**	188.06 ± 35.57 a	12.09 ± 0.71 b	**25.27 ± 2.22 a**	**21.83 ± 9.02 a**
Asparagine	19.46 ± 1.88 a	21.52 ± 1.98 a	23.94 ± 2.61 a	17.26 ± 1.34 ab	9.37 ± 1.66 b	21.76 ± 4.04 a	1.66 ± 0.05 c	**16.95 ± 0.62 a**	**12.78 ± .57 b**
Serine	46.03 ± 4.30 a	46.76 ± 6.85 a	56.72 ± 3.84 a	55.78 ± 5.87 a	**23.64 ± 4.47 b**	54.62 ± 11.13 a	5.42 ± 0.33 b	**36.79 ± 1.44 a**	**34.00 ± 1.06 a**
Glutamine	782.49 ± 71.95 b	**1190.78 ± 157.83 a**	**1330.00 ± 43.85 a**	348.83 ± 39.06 b	255.79 ± 49.84 b	**644.29 ± 134.33 a**	3.36 ± 0.09 c	**77.87 ± 1.58 a**	**29.37 ± 1.78 b**
Histidine	25.93 ± 1.83 b	**41.78 ± 1.43 a**	**41.72 ± 5.33 a**	18.11 ± 2.07 a	18.32 ± 3.83 a	33.74 ± 6.69 a	4.78 ± 0.19 c	**19.35 ± 0.75 a**	**16.18 ± .11 b**
Glycine	48.44 ± 5.89 a	52.44 ± 2.39 a	63.11 ± 5.48 a	33.43 ± 5.24 a	19.98 ± 3.06 a	38.10 ± 11.45 a	16.41 ± 3.00 b	**42.51 ± 1.31 a**	**37.35 ± 8.86 a**
Arginine	41.11 ± 2.90 c	**5068.33 ± 1056.85 a**	**456.97 ± 29.65 b**	31.43 ± 2.72 c	**3630.68 ± 497.31 a**	**512.29 ± 103.10 b**	7.26 ± 2.03 c	**1762.62 ± 136.22 a**	**192.55 ± 49.27 b**
Citrulline	3.95 ± 0.10 c	**231.98 ± 1.60 a**	**41.95 ± 3.33 b**	3.66 ± 0.53 c	**246.05 ± 4.27 a**	**39.91 ± 8.51 b**	0.75 ± 0.13 c	**107.99 ± 5.26 a**	**17.79 ± 6.16 b**
Threonine	19.45 ± 1.43 b	**28.51 ± 2.03 a**	**27.58 ± 3.44 a**	21.25 ± 1.93 a	17.41 ± 3.19 a	30.08 ± 5.69 a	4.26 ± 0.24 b	**24.06 ± 1.20 a**	**21.61 ± 1.04 a**
Alanine	439.32 ± 48.11 a	488.05 ± 60.73 a	616.46 ± 49.14 a	516.15 ± 38.81 a	222.79 ± 51.26 a	546.42 ± 147.17 a	22.34 ± 0.82 c	**266.20 ± 8.95 a**	**194.92 ± 5.19 b**
γ-aminobutyric acid	100.50 ± 2.51 b	90.28 ± 14.74 b	**190.24 ± 25.30 a**	105.41 ± 26.24 a	43.03 ± 13.00 a	115.70 ± 18.24 a	12.56 ± 3.33 b	**59.34 ± 2.51 a**	**52.17 ± 19.39 a**
Tyrosine	19.24 ± 1.88 c	**25.53 ± 1.70 b**	**31.74 ± 1.29 a**	23.87 ± 1.86 ab	12.88 ± 2.02 b	29.80 ± 5.67 a	4.57 ± 0.36 b	**16.74 ± 1.19 a**	**18.89 ± 1.50 a**
Tryptophan	11.36 ± 0.92 b	15.41 ± 1.70 b	**23.19 ± 1.82 a**	17.45 ± 1.04 ab	10.24 ± 1.66 b	24.01 ± 4.25 a	1.83 ± 0.32 b	**11.42 ± 0.53 a**	**10.69 ± 3.77 a**
Methionine	9.87 ± 1.01 a	11.63 ± 1.17 a	10.95 ± 0.53 a	12.87 ± 1.74 a	7.30 ± 0.84 a	12.56 ± 2.33 a	9.93 ± 0.76 a	6.93 ± 0.81 a	6.72 ± 1.77 a
Valline	31.22 ± 3.17 b	37.13 ± 2.21 ab	**45.83 ± 3.39 a**	35.50 ± 2.35 ab	20.62 ± 3.53 b	45.79 ± 8.21 a	7.44 ± 0.27 b	**37.77 ± 1.36 a**	**37.84 ± 1.53 a**
Phenylalanine	29.67 ± 2.62 b	37.94 ± 3.86 b	**71.73 ± 7.26 a**	45.31 ± 2.90 a	**17.11 ± 3.42 b**	60.19 ± 10.42 a	20.37 ± 0.25 b	21.38 ± 1.30 ab	**23.98 ± 0.97 a**
Isoleucine	15.20 ± 1.08 a	17.58 ± 1.87 a	23.61 ± 3.40 a	18.67 ± 1.15 ab	10.60 ± 1.30 b	27.10 ± 4.51 a	6.10 ± 0.62 b	**16.27 ± 0.56 a**	**17.13 ± 0.70 a**
Leucine	25.31 ± 2.16 a	30.36 ± 2.81 a	34.97 ± 2.98 a	25.98 ± 1.43 a	14.87 ± 2.24 a	35.32 ± 6.07 a	8.26 ± 0.34 b	**22.73 ± 1.16 a**	**23.20 ± 0.72 a**
Ornithine	66.80 ± 3.90 b	63.90 ± 9.23 b	**1191.49 ± 124.66 a**	73.05 ± 8.24 b	**77.81 ± 0.53 a**	**566.14 ± 238.79 a**	73.74 ± 12.01 b	**74.36 ± 7.72 a**	**364.80 ± 96.94 a**
Lysine	124.05 ± 12.16 a	129.46 ± 22.73 a	182.59 ± 36.11 a	151.56 ± 24.34 a	147.74 ± 14.14 a	143.27 ± 33.91 a	142.64 ± 16.08 b	**231.33 ± 15.41 a**	192.37 ± 25.05 ab

Values represent medians ± SD from three biological replicates. Statistically significant differences ($p<0.01$) within rows at a given time are indicated with different letters according to Tukey's test. Numbers in bold denote significant difference between treatment and control at a given time ($p<0.01$).

ANEXO III – Referente ao material suplementar do Capítulo III.

Tabela Suplementar S1. Padrão e conteúdo (nmol.g^{-1} MF) de PAs (livres e conjugadas) e aminoácidos presentes em megagametófitos de sementes imaturas de quatro diferentes matrizes de *A. angustifolia*, coletadas em dezembro de 2013. Put – putrescina, Spd – espermidina, Spm – espermina.

	Matriz A	**Matriz B**	**Matriz C**	**Matriz D**
		PAs (Livres e Conjugadas)		
Put (livre)	13.92 ± 0.05	17.78 ± 4.50	18.02 ± 0.05	20.23 ± 1.27
Spd (livre)	31.05 ± 1.74	38.10 ± 9.67	41.47 ± 8.06	43.25 ± 1.40
Spm (livre)	26.37 ± 2.66	39.59 ± 10.09	29.27 ± 0.74	25.62 ± 1.05
Put (conj.)	5.48 ± 0.75	15.04 ± 1.27	11.55 ± 2.90	16.11 ± 0.71
Spd (conj.)	3.72 ± 0.13	4.11 ± 0.83	4.40 ± 0.96	5.15 ± 1.07
Spm (conj.)	3.26 ± 0.54	3.72 ± 0.84	3.57 ± 0.73	3.07 ± 0.64
		PAs totais		
Put	19.39 ± 0.79	32.82 ± 5.45	29.57 ± 2.84	36.34 ± 0.68
Spd	34.77 ± 1.87	42.22 ± 9.15	45.88 ± 8.17	48.40 ± 1.06
Spm	29.63 ± 3.14	43.31 ± 9.70	32.84 ± 1.47	28.70 ± 0.70
Put/(Spd+Spm)	0.30 ± 0.03	0.39 ± 0.03	0.38 ± 0.05	0.47 ± 0.00
		Aminoácidos livres totais		
Ácido aspártico	92.8 ± 13.11	343.69 ± 56.25	219 ± 30.77	225.58 ± 62.69
Glutamato	245.34 ± 31.66	494.6 ± 78.61	367.12 ± 51.6	569.01 ± 128.65
Asparagina	135.91 ± 10.59	114.51 ± 17.96	84.91 ± 11.04	44.32 ± 9.25
Serina	37.05 ± 0.88	326.59 ± 58.2	60.18 ± 8.19	110.92 ± 25.25
Glutamina	120.49 ± 14.96	423.17 ± 68.97	179.43 ± 28.85	237 ± 63.83
Histidina	25.39 ± 0.81	47.99 ± 7.61	27.03 ± 3.17	29.57 ± 4.32
Arginina	24.39 ± 4.09	34.37 ± 6.63	28.96 ± 3.62	26.48 ± 4.35
Citrulina	9.46 ± 1.57	11.81 ± 0.7	10.69 ± 0.78	10.45 ± 1.12
Glicina	14.11 ± 0.39	36.48 ± 4.67	14.62 ± 1.61	27.5 ± 5.99
Treonina	18.34 ± 1.46	33.96 ± 6.45	24.37 ± 3.62	26.09 ± 5.14
Alanina	34.39 ± 3.14	89.67 ± 12.92	90.14 ± 11.66	134.4 ± 27.24
GABA	20.82 ± 0.59	29.57 ± 1.35	36.27 ± 4.94	39.4 ± 4.72
Tirosina	14.79 ± 1.26	19.19 ± 2.76	8.45 ± 0.83	15.94 ± 3.08
Metionina	10.01 ± 1.2	14.36 ± 0.68	12.48 ± 0.16	19.11 ± 2.89
Triptofano	47.2 ± 3.65	115.39 ± 17.13	72.4 ± 9.34	97.15 ± 17.04
Valina	17.18 ± 0.97	22.84 ± 5.77	20.98 ± 3.49	13.35 ± 1.16
Fenilalanina	30 ± 3.48	53.47 ± 7.98	27.57 ± 3.38	41.32 ± 6.34
Isoleucina	16.92 ± 0.57	45.69 ± 6.18	27.68 ± 1.12	41.05 ± 6.68
Leucina	20.74 ± 1.03	37.34 ± 3.41	25.59 ± 1.02	33.52 ± 5.68
Ornitina	152.67 ± 31.96	255.48 ± 48.7	191.85 ± 17.72	252.48 ± 19.84
Lisina	309.12 ± 81.13	401.6 ± 16.19	340.43 ± 22.38	421.46 ± 30.14
Total aminoácidos	1397.11 ± 156.97	2951.76 ± 312.27	1870.14 ± 180.85	2416.1 ± 387.64

Média ± desvio padrão, n=3.

Tabela Suplementar S2. Padrão e conteúdo (nmol.g^{-1} MF) de PAs (livres e conjugadas) e aminoácidos presentes em megagametófitos de sementes imaturas de três diferentes matrizes de *A. angustifolia*, coletadas em dezembro de 2014. Put – putrescina, Spd – espermidina, Spm – espermina.

	Matriz A	**Matriz B**	**Matriz D**
		PAs (Livres e Conjugadas)	
Put (livre)	6.02 ± 1.25	8.21 ± 0.89	8.99 ± 1.01
Spd (livre)	19.59 ± 4.43	22.21 ± 3.63	23.14 ± 3.88
Spm (livre)	16.40 ± 3.09	19.79 ± 3.07	18.30 ± 2.96
Put (conj.)	15.05 ± 3.19	11.56 ± 1.07	30.40 ± 5.48
Spd (conj.)	20.40 ± 6.44	12.72 ± 1.73	7.02 ± 0.76
Spm (conj.)	3.58 ± 1.32	7.22 ± 0.98	1.58 ± 0.27
		PAs totais	
Put	21.07 ± 2.09	19.77 ± 1.66	39.39 ± 4.73
Spd	40.00 ± 7.13	34.93 ± 2.69	30.16 ± 4.36
Spm	19.98 ± 2.35	27.02 ± 2.16	19.88 ± 2.92
Put/(Spd+Spm)	0.36 ± 0.08	0.32 ± 0.01	0.80 ± 0.19
		Aminoácidos livres totais	
Ácido aspartico	174.98 ± 45.06	269.80 ± 46.44	274.57 ± 41.18
Glutamato	508.30 ± 138.52	392.03 ± 71.37	431.77 ± 72.07
Asparagina	239.96 ± 42.50	73.72 ± 13.37	80.28 ± 12.39
Serina	34.43 ± 7.10	24.84 ± 5.22	28.07 ± 4.52
Glutamina+Histidina	132.62 ± 82.66	101.25 ± 2.62	173.99 ± 42.08
Arginina	9.20 ± 4.36	10.39 ± 1.28	13.96 ± 2.92
Citrulina	18.64 ± 8.27	17.85 ± 3.02	15.04 ± 2.49
Glicina	9.71 ± 6.37	28.58 ± 1.66	17.27 ± 2.89
Treonina	21.21 ± 5.27	20.94 ± 1.47	19.11 ± 1.50
Alanina	21.36 ± 3.63	17.70 ± 3.04	18.73 ± 1.99
GABA	54.97 ± 7.50	54.60 ± 9.29	82.80 ± 11.78
Tirosina	18.64 ± 2.17	7.09 ± 0.78	10.84 ± 1.21
Metionina	36.75 ± 9.78	27.80 ± 3.05	25.69 ± 1.27
Triptofano	23.93 ± 2.80	14.94 ± 1.87	32.53 ± 4.42
Valina	63.74 ± 12.00	30.69 ± 4.13	32.36 ± 3.88
Fenilalanina	26.92 ± 5.81	23.61 ± 2.75	34.17 ± 3.04
Isoleucina	19.92 ± 3.86	14.49 ± 1.32	17.79 ± 1.21
Leucina	23.17 ± 4.02	18.87 ± 1.58	20.16 ± 1.40
Ornitina	89.00 ± 0.69	42.92 ± 6.78	154.36 ± 0.64
Lisina	115.66 ± 6.51	102.90 ± 6.66	127.93 ± 11.97
Total aminoácidos	1643.10 ± 297.26	1295.03 ± 157.20	1611.42 ± 222.58

Média ± desvio padrão, n=3

Tabela Suplementar S3. Padrão e conteúdo (nmol.g^{-1} MF) de PAs (livres e conjugadas) de linhagens celulares, induzidas a partir de sementes imaturas de quatro diferentes matrizes de *A. angustifolia*, coletadas em dezembro de 2013. As análises foram realizadas durante a fase de proliferação celular, após 14 dias de cultivo em meio de cultura MSG (Becwar *et al.*, 1989). Put – putrescina, Spd – espermidina, Spm – espermina. NES.MPE^{-1} – número médio de embriões somáticos por 100 mg de massas proembriogênicas, ESs – embriões somáticos estádio II.

Linhagem celular	***PAs Livres***			***PAs Conjugadas Solúveis***			***PAs Totais***			***Razão***	**NES.MPE^{-1}**	**Frequência ESs**
	Put	**Spd**	**Spm**	**Put**	**Spd**	**Spm**	**Put**	**Spd**	**Spm**	**Put/(Spd+Spm)**		
AI.02	1.12 ± 0.27	0.14 ± 0.01	0.02 ± 0.00	0.26 ± 0.18	0.06 ± 0.01	0.00 ± 0.00	1.38 ± 0.09	0.20 ± 0.03	0.02 ± 0.00	6.14 ± 0.78	45.50 ± 10.47	100%
AI.04	0.86 ± 0.03	0.16 ± 0.01	0.01 ± 0.00	0.02 ± 0.02	0.02 ± 0.00	0.00 ± 0.00	0.88 ± 0.02	0.17 ± 0.01	0.01 ± 0.00	4.67 ± 0.28	0.00 ± 0.00	0%
AI.05	0.68 ± 0.09	0.17 ± 0.01	0.02 ± 0.00	0.32 ± 0.23	0.03 ± 0.01	0.00 ± 0.00	1.00 ± 0.17	0.19 ± 0.02	0.02 ± 0.00	4.73 ± 0.53	0.50 ± 1.00	25%
AI.06	0.52 ± 0.04	0.07 ± 0.01	0.01 ± 0.00	0.06 ± 0.04	0.01 ± 0.01	0.00 ± 0.00	0.58 ± 0.01	0.09 ± 0.02	0.01 ± 0.00	6.06 ± 1.19	8.75 ± 6.95	100%
AI.07	0.27 ± 0.01	0.14 ± 0.02	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.01	0.00 ± 0.00	0.27 ± 0.01	0.15 ± 0.02	0.02 ± 0.00	1.71 ± 0.24	0.50 ± 0.58	50%
AI.08	1.41 ± 0.16	0.12 ± 0.00	0.01 ± 0.00	0.01 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	1.42 ± 0.15	0.12 ± 0.00	0.02 ± 0.00	10.51 ± 1.02	8.50 ± 3.11	100%
AI.12	1.45 ± 0.32	0.08 ± 0.01	0.01 ± 0.00	0.00 ± 0.00	0.01 ± 0.01	0.01 ± 0.01	1.45 ± 0.32	0.09 ± 0.02	0.02 ± 0.01	12.84 ± 0.68	0.00 ± 0.00	0%
AI.14	0.58 ± 0.06	0.12 ± 0.01	0.02 ± 0.00	0.00 ± 0.00	0.02 ± 0.01	0.00 ± 0.00	0.58 ± 0.06	0.14 ± 0.01	0.02 ± 0.00	3.74 ± 0.56	10.25 ± 7.23	100%
AI.15	1.45 ± 0.15	0.15 ± 0.01	0.02 ± 0.00	0.01 ± 0.02	0.00 ± 0.01	0.00 ± 0.00	1.46 ± 0.13	0.16 ± 0.01	0.02 ± 0.00	8.10 ± 0.39	0.25 ± 0.50	25%
AI.16	0.32 ± 0.03	0.11 ± 0.01	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.32 ± 0.03	0.11 ± 0.01	0.01 ± 0.00	2.64 ± 0.16	0.25 ± 0.50	25%
AI.17	1.18 ± 0.26	0.11 ± 0.02	0.02 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.18 ± 0.26	0.11 ± 0.02	0.02 ± 0.00	9.10 ± 0.68	0.00 ± 0.00	0%
CI.03	0.36 ± 0.06	0.14 ± 0.01	0.03 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.36 ± 0.06	0.14 ± 0.01	0.03 ± 0.00	2.06 ± 0.25	0.75 ± 0.50	75%
CI.05	0.35 ± 0.03	0.19 ± 0.02	0.03 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.35 ± 0.03	0.19 ± 0.03	0.03 ± 0.00	1.58 ± 0.09	0.00 ± 0.00	0%
DI.01	0.62 ± 0.12	0.13 ± 0.01	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.62 ± 0.12	0.14 ± 0.01	0.01 ± 0.00	4.28 ± 0.72	0.00 ± 0.00	0%
DI.03	0.31 ± 0.05	0.10 ± 0.01	0.01 ± 0.00	0.02 ± 0.01	0.02 ± 0.01	0.00 ± 0.00	0.32 ± 0.04	0.12 ± 0.00	0.01 ± 0.00	2.43 ± 0.28	0.50 ± 1.00	25%
DI.04	0.56 ± 0.11	0.36 ± 0.05	0.02 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.56 ± 0.11	0.36 ± 0.05	0.02 ± 0.00	1.46 ± 0.19	15.00 ± 8.45	100%
DI.09	0.99 ± 0.17	0.18 ± 0.04	0.02 ± 0.00	0.00 ± 0.00	0.02 ± 0.03	0.00 ± 0.00	0.99 ± 0.17	0.20 ± 0.01	0.02 ± 0.00	4.45 ± 0.55	0.00 ± 0.00	0%

Média ± desvio padrão, n=3.

Tabela Suplementar S4. Padrão e conteúdo (nmol.g^{-1} MF) de PAs (livres e conjugadas) de linhagens celulares, induzidas a partir de sementes imaturas de três diferentes matrizes de *A. angustifolia*, coletadas em dezembro de 2014. As análises foram realizadas durante a fase de proliferação celular, após 14 dias de cultivo em meio de cultura MSG (Becwar *et al.*, 1989). Put – putrescina, Spd – espermidina, Spm – espermina. NES.MPE^{-1} – número médio de embriões somáticos por 100 mg de massas proembriogênicas, ESs – embriões somáticos estádio II.

Linhagem celular	*PAs Livres*			*PAs Conjugadas Solúveis*			*PAs Totais*			*Razão*	NES.MPE^{-1}	Frequência ESs
	Put	Spd	Spm	Put	Spd	Spm	Put	Spd	Spm	Put/(Spd+Spm)		
AII.130	0.26 ± 0.03	0.13 ± 0.00	0.02 ± 0.00	0.37 ± 0.08	0.15 ± 0.03	0.02 ± 0.00	0.63 ± 0.07	0.28 ± 0.03	0.05 ± 0.00	1.93 ± 0.19	24.50 ± 4.43	100%
AII.64	1.32 ± 0.20	0.23 ± 0.02	0.03 ± 0.00	1.07 ± 0.17	0.19 ± 0.01	0.03 ± 0.01	2.39 ± 0.33	0.41 ± 0.02	0.06 ± 0.01	5.12 ± 1.00	4.00 ± 0.82	100%
AII.77	0.29 ± 0.04	0.16 ± 0.00	0.03 ± 0.00	0.29 ± 0.04	0.10 ± 0.01	0.01 ± 0.00	0.58 ± 0.08	0.25 ± 0.01	0.04 ± 0.00	1.98 ± 0.32	14.00 ± 6.16	100%
BII.123	0.35 ± 0.05	0.12 ± 0.01	0.01 ± 0.00	0.72 ± 0.13	0.11 ± 0.02	0.01 ± 0.00	1.07 ± 0.11	0.23 ± 0.02	0.02 ± 0.00	4.49 ± 0.75	4.25 ± 2.63	100%
BII.144	0.71 ± 0.20	0.13 ± 0.03	0.02 ± 0.00	0.25 ± 0.03	0.15 ± 0.03	0.02 ± 0.00	0.96 ± 0.21	0.28 ± 0.04	0.04 ± 0.01	2.96 ± 0.26	0.00 ± 0.00	0%
BII.150	0.32 ± 0.07	0.36 ± 0.01	0.05 ± 0.00	0.37 ± 0.06	0.21 ± 0.04	0.03 ± 0.01	0.69 ± 0.06	0.57 ± 0.05	0.09 ± 0.01	1.06 ± 0.01	26.67 ± 3.68	100%
BII.20	0.15 ± 0.02	0.03 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.08 ± 0.01	0.01 ± 0.00	0.16 ± 0.02	0.11 ± 0.00	0.01 ± 0.00	1.34 ± 0.17	4.50 ± 3.87	75%
BII.29	0.64 ± 0.13	0.12 ± 0.02	0.01 ± 0.00	0.61 ± 0.14	0.09 ± 0.01	0.01 ± 0.00	1.25 ± 0.21	0.21 ± 0.03	0.02 ± 0.00	5.47 ± 0.68	4.00 ± 3.16	100%
BII.47	0.21 ± 0.04	0.05 ± 0.00	0.01 ± 0.00	0.36 ± 0.07	0.07 ± 0.01	0.01 ± 0.00	0.57 ± 0.11	0.12 ± 0.01	0.02 ± 0.00	4.14 ± 0.62	1.75 ± 0.96	100%
BII.75	0.20 ± 0.02	0.12 ± 0.02	0.02 ± 0.00	0.27 ± 0.03	0.13 ± 0.02	0.02 ± 0.00	0.48 ± 0.05	0.25 ± 0.04	0.03 ± 0.00	1.69 ± 0.12	48.25 ± 21.70	100%
BII.78	0.97 ± 0.23	0.20 ± 0.03	0.02 ± 0.00	0.97 ± 0.08	0.17 ± 0.03	0.01 ± 0.00	1.93 ± 0.15	0.37 ± 0.04	0.03 ± 0.00	4.94 ± 0.51	28.67 ± 2.62	100%
DII.100	0.24 ± 0.03	0.08 ± 0.01	0.03 ± 0.00	0.29 ± 0.01	0.18 ± 0.02	0.02 ± 0.00	0.53 ± 0.04	0.26 ± 0.01	0.05 ± 0.00	1.74 ± 0.16	0.00 ± 0.00	0%
DII.12	0.19 ± 0.01	0.08 ± 0.01	0.01 ± 0.00	0.35 ± 0.03	0.11 ± 0.02	0.03 ± 0.00	0.53 ± 0.04	0.19 ± 0.00	0.04 ± 0.00	2.29 ± 0.16	0.00 ± 0.00	0%
DII.125	0.95 ± 0.01	0.09 ± 0.02	0.01 ± 0.00	1.17 ± 0.12	0.10 ± 0.02	0.02 ± 0.00	2.13 ± 0.12	0.18 ± 0.03	0.03 ± 0.01	10.09 ± 1.45	29.75 ± 6.60	100%
DII.140	0.64 ± 0.06	0.15 ± 0.00	0.01 ± 0.00	0.44 ± 0.10	0.09 ± 0.02	0.00 ± 0.00	1.08 ± 0.05	0.24 ± 0.02	0.01 ± 0.00	4.30 ± 0.20	0.00 ± 0.00	0%
DII.144	0.44 ± 0.04	0.09 ± 0.02	0.01 ± 0.00	0.56 ± 0.06	0.13 ± 0.02	0.02 ± 0.00	1.00 ± 0.06	0.23 ± 0.03	0.03 ± 0.00	3.91 ± 0.30	22.25 ± 8.62	100%
DII.33	0.16 ± 0.01	0.17 ± 0.03	0.04 ± 0.01	0.27 ± 0.06	0.23 ± 0.02	0.02 ± 0.00	0.44 ± 0.06	0.41 ± 0.04	0.06 ± 0.01	0.94 ± 0.10	0.00 ± 0.00	0%
DII.37	0.27 ± 0.03	0.13 ± 0.02	0.02 ± 0.00	0.47 ± 0.04	0.17 ± 0.02	0.02 ± 0.00	0.74 ± 0.05	0.30 ± 0.01	0.03 ± 0.00	2.20 ± 0.03	2.25 ± 1.26	100%
DII.97	0.70 ± 0.02	0.11 ± 0.02	0.01 ± 0.00	0.00 ± 0.00	0.13 ± 0.03	0.01 ± 0.00	0.70 ± 0.02	0.24 ± 0.02	0.02 ± 0.00	2.73 ± 0.13	0.25 ± 0.50	25%

Média ± desvio padrão, n=3.

ANEXO IV – DIVULGAÇÃO DE RESULTADOS NO PERÍODO DE DOUTORADO

Artigos publicados

Leandro F. de Oliveira, Paula Elbl, Bruno V. Navarro, Amanda F. Macedo, André L. W. dos Santos, Eny I. S. Floh (2017) Elucidation of the polyamine biosynthesis pathway during Brazilian pine (*Araucaria angustifolia*) seed development. *Tree Physiology* 37(1):116-130 DOI:10.1093/treephys/tpw107.

L.F. de Oliveira, A.F. Macedo, A.L.W. dos Santos, E.I.S. Floh (2015) Polyamine levels, Arginine and Ornithine Decarboxylase Activity in Embryogenic Cultures of *Araucaria angustifolia* (Bert.) O. Kuntze. *Acta Horticulturae* 1083, DOI: 10.17660/ActaHortic.2015.1083.54.

Elbl P*, Navarro BV*, **de Oliveira LF***, Almeida J, Mosini AC, dos Santos ALW, et al. (2015) Identification and Evaluation of Reference Genes for Quantitative Analysis of Brazilian Pine (*Araucaria angustifolia* Bertol. Kuntze) Gene Expression. *PLoS ONE* 10(8): e0136714. doi:10.1371/journal.pone.0136714. ***Contribuição equivalente.***

André Luis Wendt dos Santos, Paula Elbl, Bruno Viana Navarro, **Leandro Francisco de Oliveira**, Fernanda Salvato, Tiago Santana Balbuena, Eny Iochevet Segal Floh. (2016) Quantitative proteomic analysis of *Araucaria angustifolia* (Bertol.) Kuntze cell lines with contrasting embryogenic potential, *Journal of Proteomics* 130:1, dx.doi.org/10.1016/j.jprot.2015.09.027.

Bruno V. Navarro, Paula Elbl, Amanda P. de Souza, Vinícius J. Carvalho, **Leandro F. de Oliveira**, Amanda F. Macedo, André L. W. dos Santos, Marcos S. Buckeridge, Eny I. S. Floh. Carbohydrate-mediated responses during zygotic and early somatic embryogenesis of an endangered conifer species. *Submitted.*

Resumos publicados em congressos

de Oliveira, L.F.; dos Santos, A.L.W.; Macedo, A. F.; Floh, E. I. S. 2013. Polyamine levels and arginine and ornithine decarboxylase activity in embryogenic cultures of *Araucaria angustifolia* (Bert.) O. Kuntze. In: 8th International Symposium on In Vitro Culture and Horticultural Breeding, Coimbra.

de Oliveira, L.F.; Macedo, A. F.; dos Santos, A.L.W.; Floh, E. I. S. 2013. Influence of L-Arginine and L-Ornithine on the Biosynthesis of Polyamines and Early Somatic Embryo Development of *Araucaria angustifolia* (Bert.) O. Kuntze. In: Congresso Brasileiro de Fisiologia Vegetal, 2013, Poços de Caldas, MG, Brasil.

Elbl, P. M.; **de Oliveira, L.F.**; dos Santos, A.L.W.; Rossi, M.; Floh, E. I. S. 2014. Transcriptional regulation of auxin signaling transduction pathway in *Araucaria angustifolia* (Bertol.) Kuntze: a comparative analysis between early somatic (SEM) and globular zygotic (GZE) ambryos. In: Third IUFRO Working Party 2.09.02: Somatic Embryogenesis and Other Vegetative Propagation Technologies, 2014, Vitoria-Gasteiz, Espanha.

de Oliveira, L.F.; Meneguzzi, A; Macedo, A. F.; dos Santos, A.L.W.; Floh, E. I. S. 2014. Influence of megagametophyte free amino acids and polyamines profile in the induction of embryogenic cell lines in Brazilian pine (*Araucaria angustifolia* Bertol. Kuntze). In: Third International Conference of the IUFRO Working Party 2.09.02: Somatic Embryogenesis and Other Vegetative Propagation Technologies, 2014, Vitoria-Gasteiz, Espanha.

de Oliveira, L.F.; Macedo, A. F.; dos Santos, A.L.W.; Floh, E. I. S. 2014. Polyamines modulation during pro-embryogenic masses proliferation in Brazilian pine (*Araucaria angustifolia*). In: Third International Conferenece on Polyamines: Biochemical, Physiological and Clinical Perspectives, 2014, Ubatuba, Brasil.

Elbl, P. M.; Souza, D. T.; Rosado, D.; Navarro, B. V.; **de Oliveira, L.F.**; dos Santos, A.L.W.; Rossi, M.; Floh, E. I. S. 2015. The origin of *Araucaria angustifolia* auxin-related genes and their crucial role in the embryo formation. In: 11th International Congress of Plant Molecular Biology, 2015, Foz do Iguaçu, Brasil.

Navarro, B. V.; **de Oliveira, L.F.**; Elbl, P. M.; Piovezani, A. R.; dos Santos, A.L.W.; Souza, A. P.; Buckeridge, M. S.; Floh, Eny I. S. 2015. Co-variation network related to carbohydrates and polyamines metabolism during zygotic embryogenesis of *Araucaria angustifolia*. In: 11th International Congress of Plant Molecular Biology, 2015, Foz do Iguaçu, Brasil.

Navarro, B. V.; dos Santos, A.L.W.; Elbl, P. M.; **de Oliveira, L.F.**; Macedo, A. F.; Souza, A. P.; Buckeridge, M. S.; Floh, Eny I. S. 2015. Quantitative proteomic associated with carbohydrate biochemical profiles of *Araucaria angustifolia* cell lines with diferencial embryogenic potential. In: XV Brazilian Congress of Plant Physiology, 2015, Foz do Iguaçu, Brasil.

Navarro, B. V.; Elbl, P.; dos Santos, A.L.W.; **de Oliveira, L.F.**; Demarco, D.; Buckeridge, M. S.; Floh, E. I. S. 2016. Cell-to-cell trafficking patterns of Brazilian Pine (*Araucaria angustifolia* Bertol. Kuntze) cell lines with contrasting embryogenic potential. In: IUFRO 2.09.02 Somatic embryogenesis and other vegetative propagation technologies, 2016, La Plata, Argentina.

de Oliveira, L.F.; Macedo, A. F.; Navarro, B. V.; Elbl, P.; dos Santos, A.L.W.; Floh, E. I. S. 2016. Polyamines biosynthesis in embryogenic cultures of *Araucaria angustifolia*. In: IUFRO 2.09.02 Somatic Embryogenesis and other vegetative propagation technologies, 2016, La Plata, Argentina.