ASPECTOS MORFOLÓGICOS, FISIOLÓGICOS E BIOQUÍMICOS NA PROPAGAÇÃO *IN VITRO* E NO ARMAZENAMENTO DE SEMENTES EM ESPÉCIES ARBÓREAS NATIVAS DA MATA ATLÂNTICA

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Tese apresentada ao Centro de Ciências e Tecnologias Agropecuárias da Universidade Estadual do Norte Fluminense Darcy Ribeiro, como parte das exigências para obtenção do título de Doutora em Produção Vegetal

Orientadora: Profª. Drª. Claudete Santa Catarina

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A Deus;

Aos meus pais, Jeremias Nunes da Costa e Angela Maria Rangel Costa;

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RESUMO

COSTA, Poliana Rangel, D.Sc., Universidade Estadual do Norte Fluminense Darcy Ribeiro. Fevereiro de 2018. Aspectos morfológicos, fisiológicos e bioquímicos na propagação *in vitro* e no armazenamento de sementes em espécies arbóreas nativas da Mata Atlântica. Orientador: Profª. Drª. Claudete Santa Catarina.

O presente estudo visou estabelecer e melhorar a propagação de espécies arbóreas nativas da Mata Atlântica, como a *Paubrasilia echinata* e *Cedrela odorata,* a partir de análises dos aspectos morfológicos, fisiológicos e bioquímicos na propagação *in vitro* e no armazenamento de sementes. Sementes imaturas de *P. echinata* foram inoculadas em diferentes concentrações de ácido 2,4-diclorofenoxiacético (2,4-D) isoladamente e em combinação com 6-benziladenina (BA) para indução de calos, que posteriormente foram multiplicados em 10 µM de 2,4-D e em sua associação com 1 e 5 µM de BA. Os tratamentos de maturação consistiram em diferentes combinações de BA, glutamina, ácido abscísico (ABA), polietilenoglicol (PEG) e maltose. A análise do conteúdo endógeno de poliaminas livres (PAs) foi realizada em calos maturados em tratamentos controle e 25 µM de ABA. Segmentos nodais axilares e cotiledonares foram utilizados como fonte de explante para indução, desenvolvimento e avaliação de conteúdo de PAs de brotações, sob a influência de diferentes concentrações de BA. Microestacas

obtidas de brotações de segmentos nodais cotiledonares induzidas em diferentes concentrações de BA, foram enraizadas *in vitro* em diferentes concentrações de ácido indolbutírico (AIB). Adicionalmente, analisou-se a influência do tamanho (0,5 e 1 cm) e da origem (segmento nodal axilar e cotiledonar) das microestacas sobre enraizamento *in vitro*, aclimatização e crescimento das mudas. Sementes de *C. odorata*, foram armazenadas a 25 e 6 °C, durante 24 meses. Ao longo deste período foram realizadas análises da germinação, índice de velocidade de germinação, tempo médio de germinação, plântulas normais, conteúdo de água e conteúdo de PAs. As sementes imaturas de *P. echinata* possibilitaram a obtenção de calos em todos os tratamentos contendo reguladores de crescimento. O tratamento com 25 µM de ABA permitiu a formação de embriões somáticos nos estádios iniciais (globular, cordiforme e torpedo) de desenvolvimento e modulou o metabolismo de PAs, reduzindo significativamente o conteúdo de putrescina (Put). Segmentos nodais cotiledonares incubados em 20 µM de BA possibilitaram o maior comprimento dos brotos, com aumento significativo no conteúdo de Put, o qual pode estar associado à promoção do alongamento das brotações. Microestacas obtidas de segmentos nodais cotiledonares incubados em diferentes concentrações de BA induziram raízes na ausência ou presença de AIB. Microestacas com 0,5 e 1 cm, independente da origem, podem ser usadas para a produção de mudas micropropagadas de *C. odorata*. O armazenamento de sementes de *C. odorata* a 6 °C é eficiente para manter a viabilidade por 24 meses. A redução da viabilidade em sementes armazenadas a 25 °C pode estar relacionada com uma redução significante nos teores de Spd e Spm no tempo em que não houve germinação. A partir dos dados obtidos pode-se concluir que é possível o estabelecimento de metodologias alternativas de propagação para espécies arbóreas nativas ameaçadas de extinção da Mata Atlântica, bem como, identificar condições adequadas para o armazenamento de sementes para estas espécies.

Palavras-chave: ácido abscísico; benziladenina; *Caesalpinia echinata; Cedrela odorata*; cedro; cultura *in vitro*; pau-brasil; *Paubrasilia echinata*; poliaminas.

ABSTRACT

COSTA, Poliana Rangel, D.Sc., Universidade Estadual do Norte Fluminense Darcy Ribeiro. February, 2018. Morphological, physiological and biochemical aspects of *in vitro* propagation and seed storage in native tree species from Atlantic Forest. Advisor: Profª. Drª. Claudete Santa Catarina.

The present study aimed to establish and improve the propagation of native tree species of the Atlantic Forest, such as *Paubrasilia echinata* and *Cedrela odorata*, from analyzes of morphological, physiological and biochemical aspects of *in vitro* propagation and seed storage. Immature *P. echinata* seeds were inoculated at different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) alone and in combination with 6-benzyladenine (BA) for callus induction, which were then multiplied by 10 µM of 2,4-D and its association with 1 and 5 µM BA. The maturation treatments consisted of different combinations of BA, glutamine, abscisic acid (ABA), polyethylene glycol (PEG) and maltose. Endogenous content of free polyamines (PAs) was analyzed in callus matured in control and 25 µM ABA treatments. Axillary and cotyledonary nodal segments were used as explant source for the induction, development and evaluation of PAs contents, under the influence of different concentrations of BA. Microcuttings obtained from shoots of cotyledonary nodal segments induced at different BA concentrations were rooted *in vitro* at different concentrations of indolebutyric acid (IBA). In addition, the influence

of size (0.5 and 1 cm) and the origin (axillary and cotyledonary nodal segments) of the microcuttings on *in vitro* rooting, acclimatization and plantlets growth were analyzed. Seeds of *C. odorata* were stored at 25 and 6 °C for 24 months. During this period, germination, germination rate index, average germination time, normal seedlings, water content and PA content were analyzed. The immature seeds of *P. echinata* made it possible to obtain callus in all treatments containing growth regulators. Treatment with 25 µM of ABA allowed the formation of somatic embryos in the early stages (globular, heart and torpedo) of development and modulated the metabolism of PAs, significantly reducing putrescine (Put) content. Cotyledonary nodal segments incubated at 20 µM BA allowed the highest shoot length, with a significant increase in Put content, which may be associated with the promotion of shoot elongation. Microcuttings obtained from cotyledonary nodal segments incubated at different BA concentrations induced roots in the absence or presence of IBA. Microcuttings with 0.5 and 1 cm, regardless of origin, can be used for the production of *C. odorata* micropropagated plantlets. Seed storage of *C. odorata* at 6 °C is efficient to maintain viability for 24 months. The reduction of viability in seeds stored at 25 °C may be related to a significant reduction in the Spd and Spm contents at the time when there was no germination. From the data obtained it can be concluded that it is possible to establish alternative propagation methodologies for native tree species threatened with extinction of the Atlantic Forest, as well as to identify suitable conditions for the storage of seeds for these species.

Keywords: abscisic acid; benzyladenine; *Caesalpinia echinata*; *Cedrela odorata*; cedar; *in vitro* culture; Brazil wood; *Paubrasilia echinata*; polyamines.

1. INTRODUÇÃO

A conservação da biodiversidade representa um dos maiores desafios da atualidade. Com a destruição acelerada das florestas tropicais, grande parte da biodiversidade presente nestes ecossistemas está se perdendo, antes mesmo que se tenha inteiro conhecimento de sua riqueza natural (Borém e Oliveira-Filho, 2002). Atividades antrópicas relacionadas a expansão agrícola e pecuária, ocupação populacional desordenada e extração madeireira, contribuíram para intensificação da devastação das florestas tropicais, principalmente da Mata Atlântica, um dos biomas mais ameaçados e ricos do planeta (Myers et al., 2000; Colombo e Joly, 2010).

Com o intenso processo de desmatamento realizado ao longo de anos grande parte das áreas florestais tornaram-se fragmentadas, ocasionando a redução de habitats disponíveis às espécies, aumentando o grau de isolamento entre as populações e consequentemente reduzindo o fluxo gênico, podendo assim acarretar perdas de variabilidade genética e, eventualmente, a extinção de espécies (Pinto et al., 2006). Em adição, o pouco conhecimento acerca da biodiversidade biológica do bioma Mata Atlântica representa um dos grandes obstáculos para sua preservação, sendo, portanto, de fundamental importância a realização de estudos que possibilitem conhecer a resposta das espécies em diversos âmbitos, em especial das espécies vegetais que se encontram em risco de extinção, como *Cedrela odorata* L. e *Paubrasilia echinata* E. Gagnon, H. C. Lima e G. P. Lewis (Varty, 1998; Carvalho et al., 2006; Mark e Rivers, 2017).

Neste sentido, pesquisas referentes à propagação de espécies arbóreas nativas mostram-se de grande importância, pois promovem a produção de mudas para serem aplicadas em reflorestamentos. Dentre as formas de propagação, a seminífera é a mais aplicada, pois a semente é a unidade de propagação natural para a maioria das espécies de plantas superiores, constituindo o insumo básico nos programas de recuperação e conservação de ecossistemas (Santos, 2000; Carvalho et al., 2006). Para que se possa produzir mudas ao longo do ano, é necessário que se tenha disponibilidade de sementes viáveis. Para tanto, é necessário a realização do armazenamento conforme as exigências de cada espécie. Assim, estudos que determinem as melhores condições de armazenamento, promovendo a manutenção da viabilidade das sementes por maior período de tempo são essenciais.

Em adição, o estabelecimento de metodologias de propagação alternativas às convencionais (por exemplo, a estaquia), como a propagação *in vitro*, vem se mostrando uma importante ferramenta para os programas de conservação de espécies arbóreas nativas, pois possibilitam a produção em larga escala de plantas, livres de doenças em curto espaço de tempo (Ulisses et al., 2013). As respostas morfogenéticas durante o cultivo *in vitro* são afetadas por vários fatores, como tipo de explante, meio de cultura e principalmente pelo tipo e concentração dos reguladores de crescimento, sendo o balanço entre auxinas e citocininas fundamental para várias respostas (Oliveira et al., 2013).

A análise de compostos bioquímicos durante a morfogênese *in vitro* e no armazenamento de sementes pode ser uma importante ferramenta para entender os mecanismos bioquímicos relacionados com as respostas morfogenéticas *in vitro* e com a manutenção e, ou perda da viabilidade das sementes. Dentre estes compostos, as poliaminas (PAs) possuem papel importante nas plantas, visto que estas desempenham importantes funções no crescimento e desenvolvimento, tais como a divisão celular, a regulação da morfogênese vegetal, a embriogênese zigótica e somática, germinação; o crescimento radicular, organogênese *in vitro* e viabilidade de sementes (Galston e Sawhney, 1990; Santa-Catarina et al., 2004; Silveira et al., 2006; Steiner et al., 2007; Baron et al., 2008; Aragão et al., 2015; Rios et al., 2015; Sousa et al., 2016).

Neste sentido, os estudos relacionando os aspectos morfológicos, bioquímicos e fisiológicos durante a morfogênese *in vitro* e o armazenamento de

sementes em espécies arbóreas como *C. odorata* e *P. echinata* são fundamentais tanto para entender os mecanismos básicos associados a resposta morfogenética *in vitro* e conservação de sementes, quanto para a conservação destas espécies arbóreas ameaçadas de extinção.

2. REVISÃO DE LITERATURA

2.1. Mata Atlântica

A Mata Atlântica é a segunda maior floresta pluvial tropical do continente Americano (Aragão et al., 2011). Este bioma é composto por um conjunto de formações florestais caracterizadas como Floresta Ombrófila Densa, Floresta Ombrófila Mista, Floresta Estacional Semidecidual, Floresta Estacional Decidual, Floresta Ombrófila Aberta e também agrega ecossistemas como restingas, manguezais e Campos de Altitude (Fundação SOS Mata Atlântica, 2018).

Originalmente a Mata Atlântica abrangia uma área equivalente a 1.315.460 km^2, estendendo-se ao longo dos estados do Rio Grande do Sul, Santa Catarina, Paraná, São Paulo, Goiás, Mato Grosso do Sul, Rio de Janeiro, Minas Gerais, Espírito Santo, Bahia, Alagoas, Sergipe, Paraíba, Pernambuco, Rio Grande do Norte, Ceará e Piauí. Entretanto, atualmente restam apenas 12,5 % da área original deste bioma, somando todos os fragmentos de floresta nativa acima de três hectares (Fundação SOS Mata Atlântica, 2018). Este fato deve-se ao intenso processo de devastação, iniciado com a colonização do Brasil e continuado durante os diversos ciclos econômicos seguintes (mineração, pecuária, agricultura, extração madeireira e ocupação desordenada) (Rodrigues et al., 2009; Cardoso, 2016).

Em virtude de sua riqueza biológica e níveis de ameaça, a Mata Atlântica, é considerada um *hotspot* mundial, ou seja, uma das prioridades para a conservação de biodiversidade em todo o mundo (Myers et al., 2000; Cardoso,

2016; Fundação SOS Mata Atlântica, 2018). No entanto, a conservação e a recuperação desse hotspot constituem um grande desafio, visto que as estratégias, ações e intervenções necessárias esbarram em dificuldades impostas pelo estado fragmentado deste bioma e do pouco conhecimento a respeito de seus ecossistemas e suas espécies (Barbedo, 2006; Pinto et al., 2006).

Outra dificuldade para a recuperação de áreas degradadas é a baixa disponibilidade de mudas de espécies nativas (Rodrigues et al., 2009). Este fato deve-se, em parte, à baixa viabilidade de sementes de algumas espécies, e também pela escassez de conhecimento sobre a biologia reprodutiva de espécies florestais nativas (Walker et al., 2015). Portanto, estudos referentes à propagação destas espécies arbóreas, em especial as ameaçadas de extinção como *Paubrasilia echinata* e *Cedrela odotara*, são importantes para promoção da conservação e restauração de áreas degradadas.

2.2. Aplicação do cultivo *in vitro* na propagação de espécies arbóreas

A propagação *in vitro* pode ser uma importante alternativa para a propagação vegetativa de espécies arbóreas nativas, pois possibilita a obtenção de mudas selecionadas produzidas em um espaço reduzido (comparado com as formas de propagação convencionais), com alta taxa de propagação e uma produção contínua (George e Debergh, 2008; Oliveira et al., 2013; Ulisses et al., 2013).

Nos últimos anos, o cultivo *in vitro* de espécies lenhosas tem sido desenvolvido principalmente com a finalidade de clonar materiais selecionados, para a produção em larga escala visando reflorestamento, revitalização de áreas degradadas e produção bioenergética. Desta forma, a organogênese e embriogênese somática vêm sendo estudadas para várias espécies de interesse econômico, incluindo arbóreas nativas ameaçadas de extinção (Santa-Catarina et al., 2001; Peña-Ramírez et al., 2010; Aragão et al., 2011; Pelegrini et al., 2013; Aragão et al., 2016).

No entanto, existem algumas limitações ao estabelecimento das culturas *in vitro*, tais como a baixa velocidade de multiplicação, a recalcitrância em relação à indução da resposta morfogenética, a contaminação por microrganismos e a dificuldade de enraizamento (Jiménez, 2001; Oliveira et al., 2013). Fazendo-se

necessário o desenvolvimento de estudos que possibilitem estabelecer e, ou aprimorar os protocolos para o cultivo *in vitro* conforme a espécie de estudo.

2.2.1. A embriogênese somática em arbóreas

A embriogênese somática é o processo morfogenético no qual células somáticas dão origem a embriões somáticos, passando por estádios de desenvolvimento similares aos de embriões zigóticos (von Arnold et al., 2002; Fehér, 2015). Este processo morfogenético apresenta duas vias de regeneração. Desta forma, o embrião somático pode ser originado diretamente do explante (embriogênese somática direta) ou se desenvolver a partir de células de calo (embriogênese somática indireta) (Dodeman et al., 1997; Arnold et al., 2002).

A modulação da embriogênese somática para espécies arbóreas pode ser realizada de acordo com um sistema tecnológico de dois ciclos, o primeiro denominado ciclo de indução e proliferação e o segundo ciclo de maturação (Fig. 1) (Santa-Catarina et al., 2012).

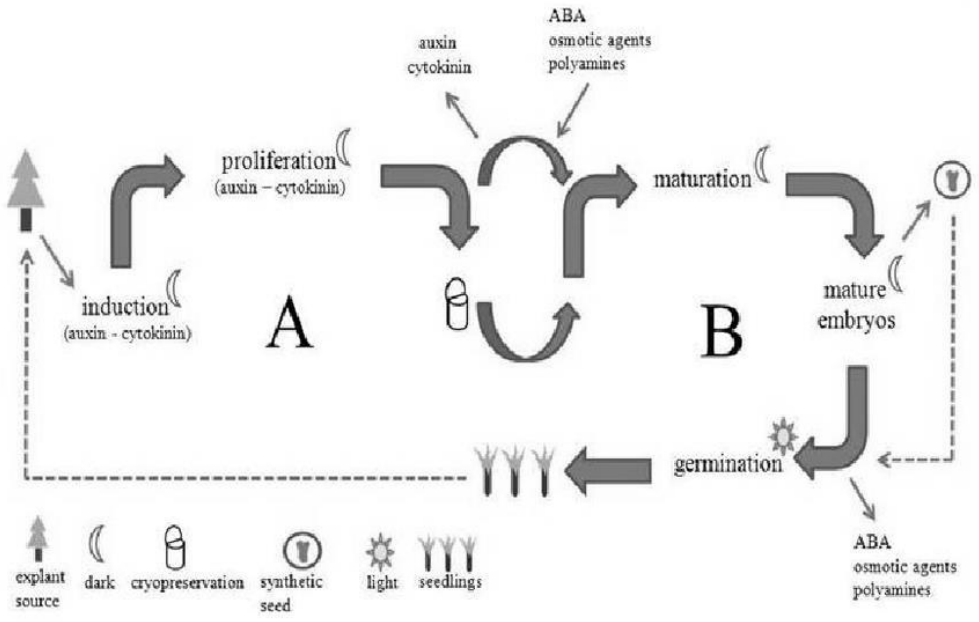

Figura 1. Modulação da embriogênese somática em espécies arbóreas, mostrando os ciclos de indução e multiplicação (A) e maturação (B). (Fonte: Santa-Catarina et al., 2012).

Estes ciclos podem ser divididos em quatro fases: (a) indução de culturas embriogênicas, em meio de cultura contendo auxinas (mais frequente) e citocininas

(menos frequentes); (b) multiplicação das culturas embriogênicas em meios de cultura sem ou com concentrações reduzidas de auxinas; (c) maturação das culturas embriogênicas em meio de cultura contendo agentes promotores da maturação, como ácido abscísico (ABA) e, ou agentes osmóticos como polietilenoglicol (PEG) e maltose; (d) germinação dos embriões somáticos e conversão em plântulas, que pode ocorrer em meio isento de reguladores de crescimento vegetal (Senger et al., 2001; von Arnold et al., 2002; Stasolla e Yeung, 2003; Steiner et al., 2008).

As culturas embriogênicas podem ser induzidas a partir da exposição das células somáticas do explante aos sinais existentes no meio de cultura, como os reguladores de crescimento vegetal, ou em resposta ao estresse induzido pela inoculação na condição *in vitro*, resultando na desdiferenciação e diferenciação das células para nova rota morfogenética, devido a totipotencialidade das células vegetais (Fehér, 2015).

A seleção do tipo de explante a ser utilizado é essencial obter sucesso quanto a resposta morfogenética alvo. Isto porque vários tecidos da mesma planta e, ou o mesmo tecido em diferentes estádios de desenvolvimento podem diferir em sua resposta quando cultivados *in vitro* (Tautorus et al., 1991). Vários tipos de explantes, como pecíolos, folhas, raízes, meristemas de brotações, sementes, cotilédones e embriões zigóticos têm sido utilizados para a obtenção da embriogênese somática. Entretanto, devido à sua natureza embrionária os embriões zigóticos imaturos são mais comumente utilizados para a obtenção da embriogênese somática em arbóreas (Finer, 1995; Gaj, 2004).

Além do tipo de explante, os sinais químicos presentes no meio de cultura, como os reguladores de crescimento vegetal, são fundamentais para a indução da resposta morfogenética. Dentre os reguladores de crescimento, aplicados no processo de indução da embriogênese somática, as auxinas e citocininas são mais frequentemente utilizadas, pelo fato de estarem envolvidas na regulação do ciclo celular e promoverem divisões celulares (Gaj, 2004; Fehér, 2015). O indutor mais amplamente aplicado na fase de indução é um herbicida auxínico, o ácido 2,4-diclorofenoxiacético (2,4-D). Este fato deve-se à sua eficácia na promoção do estabelecimento e da proliferação de culturas embriogênicas, sendo geralmente metabolizado de forma mais lenta pelas células do que outras auxinas (von Arnold et al., 2002; Fehér, 2015).

Na indução acontece a proliferação das células a partir do explante formando os calos e, ou culturas embriogênicas nos quais pode ser observada a presença de células com características embriogênicas e não embriogênicas, coexistentes (Santa-Catarina et al., 2012). Em coníferas esses tipos celulares podem ser identificados ao serem corados com carmim acético e azul de Evans. Células embriogênicas apresentam forte reação ao carmim acético e fraca reação ao azul de Evans, enquanto o padrão de coloração oposto indica a presença de células do suspensor (Durzan, 2008). Esta metodologia não é aplicada a angiospermas por não haver uma morfologia diferenciada como em coníferas. Uma forma de diferenciar células embriogênicas de não embriogênicas em angiospermas foi demonstrada em culturas de *Ocotea catharineneses* (Santa-Catarina et al., 2004) e *Theobroma cacao* (Santos et al., 2004) a partir da análise de expressão do gene 'somatic embryogenesis receptor kinase' (SERK) e seus homólogos, o qual marca a presença de células embriogênicas.

De forma geral, após a indução, ocorre a multiplicação das culturas embriogênicas em meio de cultura com redução ou remoção da auxina, visto que a manutenção da concentração inicial de auxina utilizada na fase de indução pode inibir o desenvolvimento de embriões somáticos (von Arnold et al., 2002). Durante o estágio de multiplicação, o processo da embriogênese somática pode ser estabelecido a partir de suspensões celulares, iniciadas pela inoculação de culturas embriogênicas em frascos contendo meio líquido, que permanecerão sob agitação, podendo ser utilizados biorreatores (Tautorus et al., 1991). A utilização de biorreatores e a automatização de operações em suspensões celulares possibilitam a produção em larga escala de embriões somáticos uniformes, reduzindo os custos de produção e as taxas de contaminação (Santa-Catarina et al., 2012).

Após a multiplicação, as culturas embriogênicas são transferidas para fase de maturação, visando à obtenção de embriões somáticos. A principal estratégia de maturação de culturas embriogênicas é o uso de promotores da maturação, como reguladores de crescimento, sendo o ABA o mais utilizado, e, ou agentes osmóticos, tais como PEG e maltose (Stasolla e Yeung, 2003; Shoji et al., 2006). Estes promotores inibem a divisão celular e atuam na sincronização da maturação dos embriões somáticos (Santa-Catarina et al., 2012).

O ABA é um fator importante na promoção, no amadurecimento e no desenvolvimento normal de embriões somáticos em várias espécies (Senger et al.,

2001; von Arnold et al., 2002; Kikuchi et al., 2006; Vahdati et al., 2008; Normah et al., 2013; Fraga et al., 2016; Naz et al., 2016), incluindo *Juglans regia* (Vahdati et al., 2008), *Quercus suber* (García-Martín, 2005) e *Hevea brasiliensis* (Etienne et al., 1993). Na embriogênese zigótica, o ABA se acumula nos estádios intermediários do desenvolvimento embrionário, impedindo a germinação precoce. Além disso, a ativação de genes responsivos ao ABA (*rab* genes) promove a acumulação de RNA mensageiro que controla, entre outros processos, o acúmulo de compostos de reserva, tais como proteínas, lipídios e amido, e também as proteínas abundantes na embriogênese tardia (late embryogenesis abundant; LEA), que são relacionadas à aquisição da tolerância à dessecação (Attree e Fowke, 1993; Normah et al., 2013). Também é sugerido que o ABA exógeno atue na inibição da poliembrionia por clivagem, promovendo assim, o desenvolvimento de embriões somáticos individuais (Tautorus et al., 1991).

A combinação de ABA com um agente osmótico, como PEG ou maltose, pode ser uma estratégia para promoção do desenvolvimento de embriões somáticos a partir das culturas embriogênicas. Entre os agentes osmóticos utilizados, o PEG é preferido por ser um agente osmótico não plasmolisante, o qual não entra na célula vegetal enquanto a água é retirada, levando a uma pressão de turgência reduzida e a uma potencialidade osmótica intracelular mais negativa (Attree et al., 1991; Attree e Fowke, 1993). Em *Picea glauca* o tratamento com PEG proveu melhor qualidade dos embriões somáticos em comparação ao manitol, sendo observados níveis elevados de ascorbato reduzido (GSSG) e declínio constante na razão de glutationa (GSH) e GSSG (GSH/GSSG), que são padrões similares aos observados em embriões somáticos (Belmonte et al., 2005). Além disso, nesta mesma espécie o PEG foi relacionado ao aumento de expressão de genes envolvidos na formação do plano do corpo embrionário e no controle de meristemas apicais do caule e raiz (Stasolla et al., 2003).

A maltose é uma fonte de carbono e também é utilizada como um agente osmótico na maturação de culturas embriogênicas (Shoji et al., 2006). Sua utilização é preferida em relação à sacarose, pois a quebra da maltose ocorre de forma mais lenta que a sacarose, e assim o potencial osmótico do meio é mantido por um período mais longo, necessário para induzir a maturação dos embriões somáticos (Yildirim et al., 2006). Além disso, a maltose desempenha um papel importante: a estabilização das membranas, sendo o carboidrato mais

frequentemente observado durante a resposta ao estresse (Hoekstra e Golovina,1999). Em algumas espécies arbóreas como *Pinus elliottii* e *Pinus taeda* (Salajova et al., 1999), *H. brasiliensis* (Blanc et al., 2002), *Prunus avium* (Reidiboym-Talleux et al., 1998) e *Araucaria angustifolia* (Steiner et al., 2008) a maltose promoveu maior número e melhor qualidade de embriões somáticos em comparação com outros açúcares.

Os estádios de desenvolvimento dos embriões somáticos são similares aos dos embriões zigóticos. O primeiro estádio de desenvolvimento observado é o globular, no qual ocorre uma série de divisões celulares formando um embrião esférico com simetria radial. O estádio globular é seguido por um estádio oblongo, que sinaliza a mudança do crescimento isodiamétrico para a simetria bilateral, coincidindo com o início da formação de células que originarão o primórdio radicular seguido, nas Magnoliopsidas, pelo desenvolvimento dos cotilédones, caracterizando o estádio cordiforme. Em seguida ocorre o estádio torpedo, com o alongamento celular ao longo do eixo do embrião e subsequente desenvolvimento dos cotilédones, dando origem ao embrião cotiledonar (Zimmerman, 1993; Dodeman et al., 1997; von Arnold et al., 2002).

A conversão dos embriões somáticos em plântulas, a posterior sobrevivência e o crescimento dependem das condições fornecidas na maturação. Geralmente a conversão ocorre em meio de cultura livre de reguladores de crescimento vegetal. No entanto, existem casos em que a auxina e a citocinina estimulam a germinação. Além disso, muitas vezes é necessária uma mudança acentuada no meio basal e, ou também é necessário incluir compostos extras, como a glutamina e caseína hidrolisada para esta resposta germinativa (von Arnold et al., 2002).

A embriogênese somática apresenta grande aplicabilidade em estudos referentes aos parâmetros bioquímicos e fisiológicos do processo embriogênico (Silveira et al., 2004; Silveira et al., 2006; Santa-Catarina et al., 2007). Adicionalmente, possibilita a obtenção de compostos biologicamente ativos, como, por exemplo, a produção de neolignanas obtidas a partir de embriões somáticos de *O. catharinensis* (Funasaki et al., 2009) e guggulsterona obtidas a partir de culturas em suspensão celular de *Commiphora wightii* (Mathur e Ramawat, 2007). Apresenta ainda grande potencial para a propagação e conservação de espécies arbóreas nativas como *Cedrela odorata* (Peña-Ramírez et al., 2011), *Ocotea*

odorifera (Santa-Catarina et al., 2001), *Ocotea porosa* (Pelegrini et al., 2013) e *A. angustifolia* (Silveira et al., 2006; Steiner et al., 2016).

Na conservação *ex situ* de espécies ameaçadas a embriogênese somática pode ser associada a outras técnicas como a criopreservação e a tecnologia de sementes sintéticas (Oliveira et al., 2013; Santa-Catarina et al., 2012). A criopreservação é uma técnica eficaz apara a conservação em longo prazo de embriões somáticos, que pode ser definida como a suspensão das funções metabólicas de uma célula viva através de redução de temperatura para -196 °C, temperatura do nitrogênio líquido (Jain e Minocha, 2000). Seus protocolos incluem componentes criogênicos (agentes de crioproteção e baixa temperatura e não-criogênicos (tratamento antes e depois do armazenamento do explante). Além disso, alguns protocolos incluem o encapsulamento de explantes, incluindo agentes de crioproteção e a desidratação subsequente antes do congelamento (Santa-Catarina et al., 2012).

O principal requisito da criopreservação é a redução do conteúdo de água das células, para evitar a formação de cristais de gelo, assegurando que os embriões somáticos possam se recuperar facilmente após o armazenamento em nitrogênio líquido (Guan et al., 2016). Em relação às espécies arbóreas, a criopreservação foi aplicada com sucesso em culturas embriogênicas *de Mangifera indica* (Wu et al., 2006), *Quercus robur* (Martinez et al., (2003), *Q. suber* (Valladares et al.,2004), *Picea abies* (Varis et al., 2017), *A*. *angustifolia* (Fraga et al., 2016) e *Quercus ilex* (Barra-Jiménez et al., 2015).

A semente sintética imita a semente zigótica, na qual o embrião é representado pelo explante (embriões somáticos, gemas axilares, segmento apical caulinar e segmentos nodais) e o endosperma é representado pela cápsula composta por hidrogel biodegradável, fontes de carbono, minerais e reguladores de crescimento (Santa-Catarina et al., 2012; Rihan et al., 2017). Os agentes de encapsulação podem ser alginato de sódio, alginato de potássio, pectato de sódio e carragenina (Guan et al., 2016). O alginato de sódio é o principal composto utilizado no encapsulamento por apresentar as propriedades de geleificação desejadas, baixo custo, simples aplicação e a ausência de toxicidade (Santa-Catarina et al., 2012).

As principais vantagens das sementes sintéticas são os menores custos em comparação aos volumes produzidos, a possibilidade de usar biorreatores para

automatizar o processo, a possibilidade de armazenamento e a semeadura direta no campo com redução dos custos (Santa-Catarina et al., 2012). Esta tecnologia, usando como explante embriões somáticos, foi investigada em várias espécies arbóreas incluindo *M. indica* (Ara et al., 1999), *Paulownia elongata* (Ipekci e Gozukirmizi, 2003), *P. glauca* e *Picea mariana* (Lulsdorf et al., 1993), *Pinus patula* (Malabadi e Staden, 2009), *Q. suber* (Pintos et al., 2008) e *Acca sellowiana* (Cangahuala-Inocente et al., 2007).

2.2.2. Organogênese *in vitro* em espécies arbóreas

O processo de organogênese *in vitro* geralmente é realizado a partir da brotação de gemas ou meristemas preexistentes obtidos diretamente que são estimulados a crescer e proliferar (Giri et al., 2004; George e Debergh, 2008).

Esta regeneração pode ocorrer de forma direta, na qual brotações adventícias surgem diretamente dos tecidos do explante, ou indireta, havendo desdiferenciação do explante que resulta na formação de calos, para posterior desenvolvimento de brotações adventícias (Giri et al., 2004). Todavia, para finalidade de micropropagação clonal a regeneração indireta é indesejável, pois pode resultar em variação somaclonal (Larkin e Scowcroft, 1981).

A obtenção de organogênese *in vitro* ainda é um processo empírico, no qual é necessário testar para cada espécie, ou mesmo para cada variedade dentro de uma espécie, múltiplos fatores externos e internos que afetam a resposta morfogenética (Carvalho et al., 2006; Oliveira et al., 2013). Estes fatores envolvem a fonte de explante e a habilidade dos tecidos em responder a mudanças hormonais e ambientais durante o cultivo, a composição mineral do meio de cultura (suas vitaminas e fonte de carbono) e a ação dos regulares de crescimento vegetal (Carvalho et al., 2006; Oliveira et al., 2013).

O primeiro passo da organogênese consiste no estabelecimento *in vitro* de brotações a partir dos explantes, sem que ocorra a contaminação. A escolha do material vegetal é fundamental para a resposta morfogenética. Em arbóreas, os explantes geralmente são obtidos a partir de plântulas germinadas *in vitro*, por serem cultivadas em condições assépticas e por seu estádio juvenil, possuindo assim maior capacidade de crescimento e resposta aos reguladores de crescimento vegetal (Morais et al., 2012; Oliveira et al., 2013). Os segmentos nodais são os explantes mais comumente usados, pois permitem, em condições adequadas, o desenvolvimento das gemas axilares existentes e a proliferação de brotações

(Nunes et al., 2002; Ribas et al., 2005; Hubner et al., 2007; Rocha et al., 2007; Junior et al., 2012; Aragão et al., 2016; Aragão et al., 2017).

O segundo passo consiste em estabelecer as condições de cultivo do explante, que são peculiares a cada espécie. Sendo a constituição e concentração de nutrientes do meio de cultura um dos principais fatores de influência nas respostas morfogenéticas. O meio de cultura MS (Murashige e Skoog, 1962) é provavelmente o mais utilizado na micropropagação de espécies arbóreas nativas. (Vila et al., 2004; Rocha et al., 2007; García-Gonzáles et al., 2011; Pelegrini et al., 2013; Aragão et al., 2017). Entretanto, outros meios de cultura como o WPM (Woody Plant Medium) (Lloyd e McCown, 1981) também vêm sendo amplamente empregados no cultivo de espécies lenhosas. Este meio de cultura difere do MS por possuir 25 % das concentrações dos íons nitrato e amônia, além de conter mais potássio e íons sulfato (Bassan et al., 2006; Werner et al., 2010; Martínez et al., 2017).

A adição de reguladores de crescimento ao meio de cultura muitas vezes é necessária para promover a multiplicação, o crescimento e o desenvolvimento das brotações (Medeiros, 2006; Morais et al., 2012; Oliveira et al., 2013). Sendo que as respostas morfogenéticas são dependentes do tipo e da concentração dos reguladores de crescimentos utilizados. Skoog e Miller (1957) demonstraram que a razão entre auxina/citocinina no meio de cultura era responsável pela resposta organogenética em tecidos medulares de tabaco. Assim, meios de cultura contendo concentrações maiores de citocinina promoviam a formação de brotações, enquanto meios de cultura contendo níveis maiores de auxinas induziam a formação de raízes. Quando as concentrações destes reguladores de crescimento eram equimolares, estes autores verificaram a formação de calos.

Desta forma, as citocininas são aplicadas com maior frequência na indução de brotações em explantes de espécies florestais, sendo a 6-benziladenina (BA) a mais comumente utilizada (Oliveira et al., 2013). Entretanto, outras citocininas como a zeatina, a cinetina, isopenteniladenina e thidiazuron (TDZ) também são utilizadas (Ribas et al., 2005; Schottz et al., 2007; Gomes et al., 2010). Além disso, as citocininas também podem ser associadas a auxinas (Santos et al., 2006; Hubner et al., 2007), como observado em *Maclura tinctoria*, em que combinação de BA com o ácido naftalenoacético (ANA) promoveu maior indução de brotações (Gomes et al., 2010).

A concentração da citocinina usada também é um fator importante na indução de brotações. Segundo Huetteman e Preece (1993), as citocininas de amino-purina têm faixas de atividade efetivas similares (1-10 µM) e os experimentos frequentemente são conduzidos comparando combinações fatoriais dessas citocininas em concentrações similares. Porém, quando utilizada a citocinina TDZ, esta faixa similar de concentração pode resultar em formação excessiva de calo e inibir o crescimento dos brotos. Desta forma, as concentrações de TDZ a serem aplicadas devem ser menores quando comparadas às outras citocininas (Huetteman e Preece, 1993).

As brotações obtidas a partir da organogênse *in vitro* são segmentadas em microestacas, geralmente no tamanho de 2-2,5 cm de comprimento, e transferidas para etapa de enraizamento *in vitro* ou *ex vitro* (Oliveira et al., 2013; Govil et al., 2017). No enraizamento *in vitro*, as microestacas são normalmente cultivadas em meio de cultura suplementado com auxinas responsáveis pela promoção de indução de raízes adventícias, sendo posteriormente transferidas para um meio livre de reguladores de crescimento ou diretamente transferidas para a aclimatização (George e Debergh, 2008; Rocha et al., 2008; Oliveira et al., 2013).

Assim como nas etapas anteriores as condições de cultivo, como o meio de cultura e os reguladores de crescimento, influenciam o processo de enraizamento *in vitro*. Em algumas espécies, a alta concentração de sais que compõem o meio de cultura MS pode inibir o enraizamento, mesmo em presença de auxinas. Desta forma, a incubação das microestacas em concentrações reduzidas do meio de cultura MS, ou, o uso de meios de cultura com menor concentração em nitrogênios como WPM, White e Knop pode promover melhores resultados de enraizamento (Souza e Pereira, 2007).

No enraizamento *ex vitro* as microestacas são enraizadas diretamente no substrato, juntamente com a etapa de aclimatização. Sendo que, para a maioria das espécies há necessidade de aplicação da auxina na base das microestacas antes da transferência para o substrato (George e Debergh, 2008; Rocha et al., 2008; Oliveira et al., 2013). Do ponto de vista econômico, o enraizamento *ex vitro* apresenta uma considerável redução dos custos de mão de obra e de infraestrutura, necessárias no enraizamento *in vitro*. Além de conferir qualidades adicionais como sistema de enraizamento funcional que é refletido no bom desenvolvimento da parte aérea (Cuzzuol et al., 1996; Oliveira et al., 2013).

Dentre as auxinas utilizadas para o enraizamento *in vitro* e *ex vitro*, o ácido indolbutírico (AIB) tem sido amplamente empregado por sua baixa fitotoxicidade para os explantes, proporcionando resultados positivos no enraizamento (Millán-Orozco et al., 2011; Oliveira et al., 2013). O processo de formação de raízes é regulado pela relação quantitativa entre os níveis de auxina e citocinina e também pela participação de outros hormônios como ácido jasmônico, ácido giberélico e brassinosteróides (Fu e Harberd, 2003; Müssig et al., 2003; Bao et al., 2004; Steffens et al., 2006; Kim et al., 2007; George e Debergh, 2008; Gutierrez et al., 2012).

O alto nível de auxinas endógenas é necessário para a formação de raízes adventícias, pois estas agem em nível celular para a formação dos meristemas radiculares, estimulando a divisão e o subsequente alongamento das células, culminando com a formação das raízes (Souza e Pereira, 2007). Adicionalmente, a exposição das microestacas à auxina exógena estimula o aumento do nível endógeno da auxina ácido indol-3-acético (AIA), que age como um ativador de genes, proporcionando a formação do primórdio radicular. Além disso, a aplicação da auxina sintética favorece a conjugação entre o AIA endógeno e aminoácidos que promovem a síntese de proteínas específicas para a formação de raízes, como GmAKR1, PCNT115 e CmACO (Souza e Pereira, 2007; Hur et al., 2009; Liu et al., 2013).

A aclimatização, é caracterizada pela transferência das mudas ou microestacas para a condição *ex vitro*. Esta etapa deve ser realizada de forma cuidadosa, pois pode resultar em perda significativa de material propagado, uma vez que na condição *in vitro* o cultivo é realizado em alta umidade relativa e baixa intensidade luminosa (George e Debergh, 2008). Quando transferidas para a condição *ex vitro* as mudas ou microestacas, terão que desenvolver mecanismos para controlar a perda de água e gás, ativar os mecanismos para controlar a perda de água pelas células modulando a condutância estomática e aumentar a taxa fotossintética em condições atmosféricas com maior quantidade de CO_2 mais abundante. (Rocha et al., 2008). Desta forma, é importante que no início da aclimatização o material propagado seja transferido para condição com alta umidade, que pode ser obtida com uso de câmaras de nebulização, ou com o uso de bandejas cobertas por filme PVC transparente. Em ambas as condições, a

umidade será reduzida gradualmente, permitindo que as mudas se adaptem às condições *ex vitro* (George e Debergh, 2008).

O substrato é outro fator importante para o enraizamento e aclimatização *ex vitro*, sendo fundamental o uso de substrato que possua uma boa aeração e elevada capacidade de retenção de água (Oliveira et al., 2013). Ao fim do período de aclimatização as mudas são transferidas para casa de vegetação, dando continuidade ao seu desenvolvimento e posteriormente são levadas a campo (George e Debergh, 2008).

2.3. Armazenamento de sementes de espécies arbóreas

A necessidade de conservação das florestas tropicais e o fortalecimento da política ambiental promoveram um aumento na demanda de sementes de espécies nativas, que constituem o insumo básico nos programas de recuperação e conservação de ecossistemas (Carvalho et al., 2006). A estratégia de conservação da biodiversidade envolve os métodos *in situ* e *ex situ* (Carvalho et al., 2006). A conservação de sementes é a forma mais comum de conservação *ex situ*, já que a semente é a unidade de propagação natural para a maioria das espécies de plantas superiores (Santos, 2000). Esta conservação é realizada através de bancos de germoplasma de sementes, possibilitando a manutenção dos recursos genéticos de sementes por meses, décadas ou séculos (Wang et al., 1993; Walters et al., 2005). A complexidade das técnicas utilizadas no armazenamento das sementes depende, fundamentalmente, da finalidade da conservação e da longevidade requerida (Medeiros, 2006).

A longevidade das sementes, ou seja, o período que uma semente pode viver é determinada geneticamente. Porém, o período que uma semente realmente vive dentro do seu período de longevidade é denominado viabilidade (Carvalho e Nakagawa, 2000). Para que essa viabilidade seja mantida ao longo do armazenamento é necessário conhecer o comportamento fisiológico da espécie a ser armazenada, que pode ser influenciado pelas condições do ambiente de armazenamento, como temperatura, umidade relativa do ar, tipo de embalagem e sanidade.

Dentre estes fatores, a umidade das sementes e a temperatura de armazenamento são decisivos para a manutenção da viabilidade das sementes durante o período de armazenamento (Sautu et al., 2006). O grau de umidade é a

característica mais estreitamente relacionada com a deterioração da semente, uma vez que está diretamente relacionado com a ativação metabólica. Neste sentido, o armazenamento deve ser conduzido de forma a reduzir ao máximo o metabolismo da semente. (Marcos Filho, 2005). Por outro lado, a temperatura está diretamente relacionada à velocidade de reações químicas, à aceleração da respiração e consequentemente ao desenvolvimento de microrganismos (Marcos Filho, 2005).

A depender da espécie, as sementes apresentam diferentes potenciais de armazenamento de acordo com a tolerância à redução da umidade. Assim, a tolerância à dessecação é um fenômeno necessário ao ciclo de vida da planta, como uma estratégia de adaptação que permite a sobrevivência da semente e assegura a disseminação da espécie (Medeiros, 2006). Conforme o comportamento no armazenamento, as sementes foram inicialmente classificadas por Roberts (1973) em ortodoxas ou recalcitrantes. Mais tarde, Ellis et al. (1990) introduziram o conceito de intermediárias. Contudo, Bonner (1990) sugere uma outra classificação em quatro grupos: ortodoxas verdadeiras, subortodoxas, temperadas recalcitrantes e tropicais recalcitrantes.

As sementes *ortodoxas verdadeiras* são caracterizadas por tolerarem a redução de umidade abaixo de 10 % e o armazenamento a temperaturas abaixo de 0°C, mantendo sua viabilidade por longos períodos, superiores a 6 anos. Enquanto que, as sementes subortodoxas podem ser armazenadas sob as mesmas condições das ortodoxas verdadeiras, porém com manutenção da viabilidade por curtos períodos, no máximo 6 anos, pois têm em sua constituição alto teor de lipídios (Bonner, 1990).

Os gêneros de árvores que são ortodoxas verdadeiras de grande importância econômica nos trópicos incluem *Acácia, Eucalipto, Casuarina e Gmelina* (Bonner, 1990). A maioria dos trabalhos referentes à classificação das sementes de espécies arbóreas baseia-se apenas na tolerância a dessecação e temperatura de armazenamento, sendo o tempo de armazenamento avaliado inferior a seis anos. Estes estudos têm sido realizados para várias espécies descritas como ortodoxas, tais como *Aspidosperma polyneuron*, *Hymenaea courbaril*, *Lafoensia pacari*, *Ceiba speciosa*, *Maclura tinctoria*, *Myrsine umbellata*, *Rudgea viburnoides*, *Aspidosperma cylindrocarpon*, *Myroxylon peruiferum*, *Lecythis pisonis*, *Lithraea molleoides*, *Schinus terebinthifolius*, *Bowdichia virgilioides*, *Anadenanthera colubrina*, *Solanum granuloso-leprosum* (Carvalho et al., 2006),

Casearia sylvestris, Eremanthus incanus (Nery et al.,2014) e *P. echinata* (Mello et al., 2013).

Em relação às sementes recalcitrantes, a classificação baseia-se no grau de tolerância à dissecação e nas condições climáticas da região (Bonner, 1990). Assim, sementes classificadas como temperadas recalcitrantes são caracterizadas pela sensibilidade à dessecação a baixos níveis de umidade, porém podem ser armazenadas por vários anos, sob temperatura próxima ao congelamento (Bonner, 1990). A medida que as sementes recalcitrantes de espécies tropicais devem ser armazenadas com alta umidade e trocas gasosas, apresentando assim, maior sensibilidade a dessecação e a baixas temperaturas (Bonner, 1990), como observado nas espécies arbóreas tropicais *Allophylus edulis*, *Ixora warmingii*, *Aulomyrcia venulosa Calophyllum brasiliense*, *Calyptranthes lucida*, *Cupania vernalis*, *Eugenia handroana* e *Talauma ovata* (José et al., 2007; Carvalho et al., 2006).

Diante do exposto, a determinação de condições ótimas para o armazenamento de sementes de espécies arbóreas nativas, é de grande importância. Pois, apesar de geralmente florescerem e frutificarem periodicamente, pode haver variações na época de ocorrência, na sua duração e intensidade (Oliveira, 2012). Visto que, a temperatura ambiente, chuvas e ação dos seus respectivos dispersores (abelhas, morcegos, aves e outros) influenciam na produção de sementes (Medeiros, 2006). Desta forma, o armazenamento adequado de sementes, reduzindo a perda de viabilidade das sementes é fundamental para programas de produção de mudas e de reflorestamento, pois possibilita a disponibilidade de material fora da época de produção de sementes (Souza et al., 2011).

2.4. As PAS e seu papel na morfogênese *in vitro* e no armazenamento de sementes de espécies arbóreas

As PAs são compostos de baixa massa molecular, alifáticos e policatiônicos que carregam cargas positivas sobre átomos de nitrogênio, uma propriedade que facilita interações eletrostáticas com macromoléculas com cargas negativas, como DNA, RNA, fosfolipídios, componentes da parede celular e proteínas (Wallace et al., 2003). Em células vegetais, as principais PAs encontradas são a diamina putrescina (Put), a triamina espermidina (Spd) e a tetramina espermina (Spm), as

quais podem ocorrer na forma livre ou conjugada, estando ligadas a ácidos fenólicos e outros compostos de baixa massa molecular ou a macromoléculas tais como proteínas e ácidos nucleicos (Kaur-Sawhney et al., 2003). Durante o metabolismo de PAs, a Put é sintetizada a partir dos aminoácidos arginina e ornitina pela ação das enzimas arginina descarboxilase (ADC) e ornitina descarboxilase (ODC), respectivamente. Para a produção de Spd e Spm, são necessários grupos aminopropil provenientes do aminoácido metionina a partir da rota da S-adenosil-metionina (SAM), pela ação da SAM descarboxilase (SAMDC). Com isso, a Spd é formada a partir da Put pela adição de um grupo aminopropil pela ação da enzima Spd sintase (SPDS) e a Spd é convertida em Spm pela adição de grupo aminopropil, pela ação da Spm sintase (SPMS). O catabolismo de Put, Spd e Spm é feito pela ação das enzimas diamina oxidase (DAO) e PA oxidase (PAO) (Kusano et al., 2008). A vias de síntese e degradação das PAs são demonstradas nas Figuras 2 e 3, respectivamente.

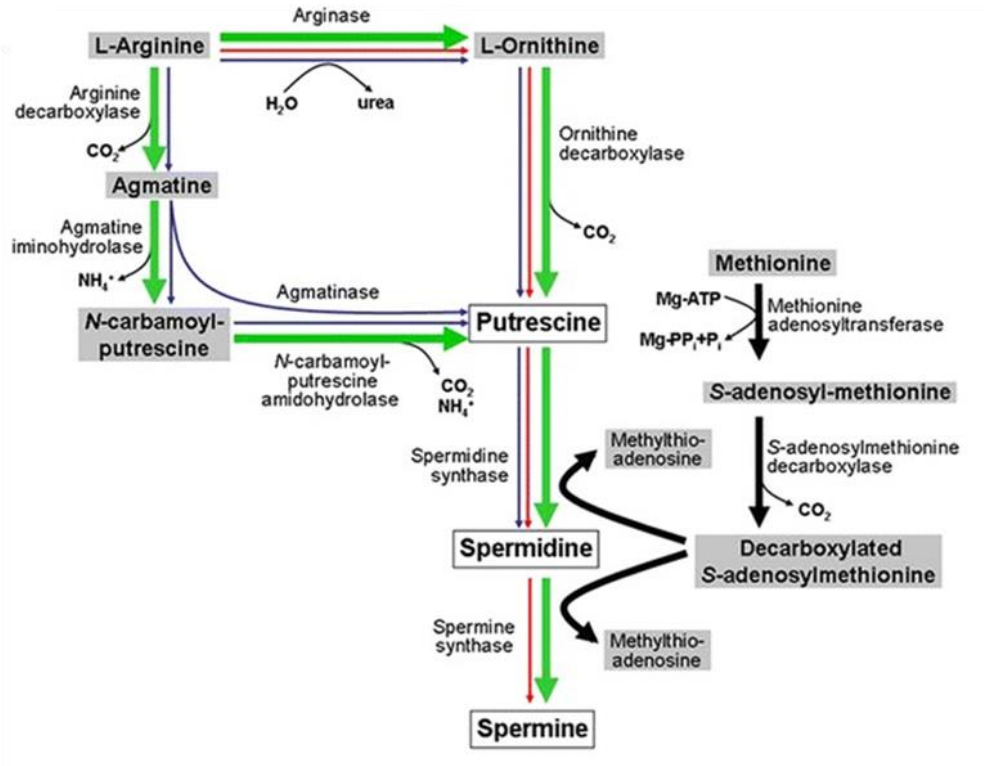

Figura 2. Biossíntese de PAs. As rotas das plantas são indicadas por setas verdes em negrito. Rotas bacterianas e animais, são indicadas por setas azuis e vermelhas, respectivamente. (Fonte: Kusano et al., 2008).

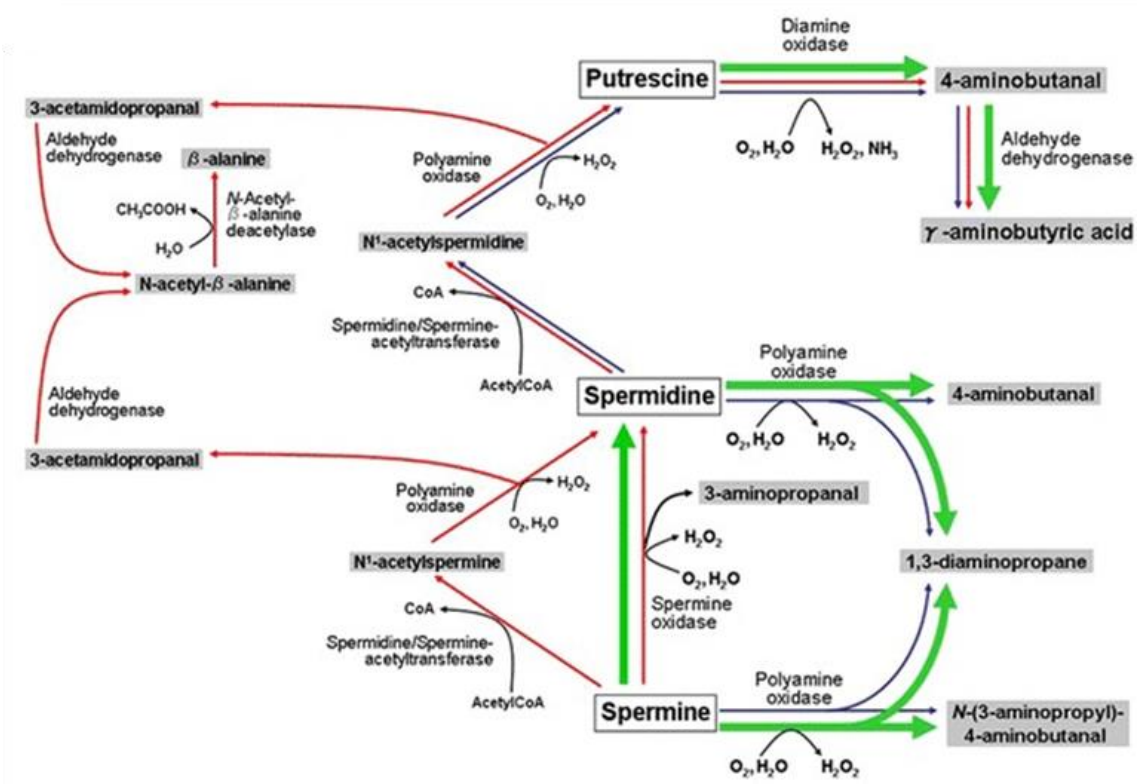

Figura 3. Degradação de PAs. As rotas das plantas são indicadas por setas verdes em negrito. Rotas bacterianas e animais, são indicadas por setas azuis e vermelhas, respectivamente. (Fonte: Kusano et al., 2008).

As PAs desempenham papeis importantes nos processos de desenvolvimento de plantas, tais como divisão celular, regulação da morfogênese, organogênese, embriogênese zigótica e somática, desenvolvimento e amadurecimento de frutos, germinação, crescimento radicular e viabilidade de sementes (Galston e Sawhney, 1990; Santa-Catarina et al., 2004; Silveira et al., 2004; Santa-Catarina et al., 2006; Silveira et al., 2006; Steiner et al., 2007; Baron et al., 2008; Silveira et al., 2013; Aragão et al., 2015; Rios et al., 2015; Aragão et al., 2016; Aragão et al., 2017). Além de estarem associadas a estresses bióticos e abióticos (Galston et al., 1997; Baron et al., 2008).

Alterações no conteúdo de PAs podem estar relacionadas aos estádios de desenvolvimento do embrião somático. Em *Acca sellowiana,* foi mostrado que o conteúdo de Put livre aumentou durante todos os estádios de desenvolvimento de embriões somáticos, enquanto o aumento no Spd livre foi observado somente nos estádios de desenvolvimento embrionário mais avançados, como torpedo, pré-

cotiledonar e cotiledonar (Cangahuala-Inocente et al., 2014). Resultado similar foi observado em *Pinus radiata*, havendo aumento constante do teor de Spd e, uma pequena redução ou nenhuma alteração significativa no conteúdo de Put durante o desenvolvimento dos embriões somáticos (Minocha et al., 1999).

Estudos demonstraram que PAs exógenas também influenciam a resposta morfogenética durante a embriogênese somática. A adição de Spd e Spm ao meio de cultura reduziu o crescimento de culturas embriogênicas de *O. catharinensis,* permitindo a evolução morfogênica do embrião somático do estádio globular até o cotiledonar. Além disso, a adição de PAs (Put, Spd e Spm) promoveu o aumento do conteúdo endógeno de PAs e induziu a liberação de NO em embriões somáticos de *O. catharinensis* (Santa-Catarina et al., 2007). Em *A. angustifolia*, a adição de Spd e Spm reduziu o crescimento celular e a liberação de NO, enquanto a adição de Put aumentou a liberação de NO, promovendo a manutenção da polaridade das PEMs durante a embriogênese somática (Silveira et al., 2006). Nesta mesma espécie, a suplementação do meio de cultura com Put e Spm aumentou os níveis endógenos de AIA e ABA, mostrando uma relação direta entre os níveis de PAs e ABA para a resposta morfogenética relacionada ao crescimento das culturas embriogênicas (Steiner et al., 2007).

Adicionalmente, estudos mostram o envolvimento das PAs no desenvolvimento de brotações (Desai e Mehta, 1985; Heloir et al., 1996; Mader, 1999; Kumar et al., 2007; Viu et al., 2009). Dentre os trabalhos desenvolvidos com espécies arbóreas, pode-se citar o referente a *Cedrela fissilis*, no qual foi demonstrado o aumento dos níveis endógenos de PAs totais e Put durante o processe de indução e desenvolvimento de brotações a partir de segmentos nodais apicais e cotiledonares (Aragão et al., 2016).

Em *Hemerocallis* sp. foi observado que as PAs estão envolvidas na morfogênese e nos padrões oxidativos de células e tecidos. A adição de Put isoladamente ou associada a Spd ou Spm aumentou a porcentagem de indução e o alongamento das brotações, enquanto a associação das três PAs proporcionou a formação de microplantas no calo. Além disso, a adição de Spm ao meio de cultura pode atuar como antioxidante celular na cultura de calos desta espécie (Debiasi et al., 2007). Segundo Viu et al. (2009), a Put tem sido correlacionada com a promoção de divisões celulares necessárias ao crescimento e desenvolvimento vegetal, sendo considerada um marcador bioquímico no processo de diferenciação

de células e tecidos durante a organogênese *in vitro*. Além disso, o aumento no conteúdo das PAs, Put e Spd durante o desenvolvimento de brotos foi relacionado à inibição da biossíntese de etileno (Bais e Ravishankar, 2002).

As PAs também influenciam o crescimento de raízes primárias e formação de raízes laterais, processos que envolvem intensa atividade mitótica e são dependentes de fatores fisiológicos endógenos (Souza e Pereira, 2007). Em *J. regia*, um aumento na síntese de PAs foi observado logo após a transferência das brotações para o meio de enraizamento (Heloir et al., 1996). Enquanto que, em *P. avium* o incremento no conteúdo endógeno de Put e Spd foi observado no processo de indução de raízes (Biondi et al., 1990).

Alterações no conteúdo de PAs livres durante o desenvolvimento de embriões zigóticos foram descritas para algumas espécies arbóreas, como *O. catharinensis*, sendo observada a redução do conteúdo de Put e o aumento do conteúdo de Spm durante o desenvolvimento do embrião globular para cotiledonar (Santa-Catarina et al., 2006). Sugerindo que a Put tem importante atuação no início da embriogênese zigótica, quando há uma elevada taxa de divisão celular, enquanto a concentração mais elevada de Spm é essencial do meio até ao fim do desenvolvimento do embrião, quando o crescimento é devido principalmente ao alongamento celular (Santa-Catarina et al., 2006). Comportamento similar foi observado no desenvolvimento de sementes de Pinus taeda, no qual os níveis de Put foram predominantes no desenvolvimento dos embriões zigóticos até o estádio cotiledonar, seguido por um aumento dos níveis de Spd em sementes maduras (Silveira et al., 2004).

As PAs também estão relacionadas com a germinação de sementes. Em *C. fissilis* foi mostrado uma redução no conteúdo de Spd e Spm nas fases iniciais da germinação até o momento da protrusão radicular, e um aumento de Put e Spd no período de desenvolvimento da plântula, quando há uma intensa divisão celular (Aragão et al., 2015). Enquanto que, em *Enterolobium schomburgkii*, o tratamento prévio com Put proporcionou o aumento da germinação em sementes submetidas ao estresse salino (Braga et al., 2009). Resultado semelhante foi observado em sementes de *Adenanthera pavonina*, sendo mostrada a atenuação do impacto do estresse hídrico induzido pelo uso de PEG sobre a germinação quando as sementes foram submetidas às soluções acrescidas de PAs (Fonseca e Perez, 2003).

2.5. Espécies de estudo

2.5.1. *Paubrasilia echinata* (Lam.) E. Gagnon, H. C. Lima e G. P. Lewis

Descrito como a árvore nacional do Brasil, a espécie *P. echinata*, conhecida popularmente como pau-brasil, apresenta grande importância biológica, histórica e cultural para o país (Aguiar et al., 2005; Rocha, 2011). Originalmente descrita como *Caesalpinia echinata* por Lamarck em 1785, recentemente a espécie foi renomeada, passando a ser descrita como *Paubrasilia echinata* (Lam.) E. Gagnon, H. C. Lima e G. P. Lewis (Gagnon et al., 2016). A alteração no gênero, baseou-se em análises filogenéticas a partir de cinco plastídios e um marcador ribossômico nuclear, com amostras taxonômicas densas, incluindo 172 (84 %) das espécies representantes de todos os gêneros anteriormente descritos no grupo *Caesalpinia* (Gagnon et al., 2016).

Endêmica do Brasil, esta espécie pode ser encontrada em várias áreas da Mata Atlântica nos estados de Pernambuco, Bahia, Espírito Santo e Rio de Janeiro (Rocha, 2011). Esta árvore possui porte médio a grande, com 5-15 m de altura, havendo relatos de indivíduos com 30 m no período colonial. O tronco e os ramos apresentam acúleos pontiagudos, que podem estar ausentes em espécimes mais maduros e nos ramos maiores e mais velhos. Além disso, o tronco possui coloração castanha quase negra e cerne vermelho, exsudando uma seiva vermelha quando ferido (Rezende et al., 2004; Gagnon et al., 2016).

Suas folhas são compostas, bipinadas, com 10-15 cm de comprimento. As inflorescências nascem em racemos próximo ao ápice dos ramos, ou ocasionalmente apresentam origem axilar, com 15-40 flores. Suas flores são bissexuadas, zigomorfas, de coloração amarela, ocorrendo na pétala mediana uma mancha central de coloração vermelha sangrenta (Cardoso et al., 1998). Os frutos são vagens lenhosas, comprimidos nas laterais, cobertos por espinhos lenhosos longos e afiados, de coloração marrom e deiscência de forma explosiva elástica. São observadas de 1-2 sementes por frutos, sendo estas achatadas, obovadas ovadas e de coloração marrom (Oliveira, 2012; Gagnon et al., 2016).

Em decorrência de sua beleza e raridade, o pau-brasil apresenta alto potencial ornamental sendo cultivado em ruas e parques, e às vezes em plantações (Barbedo, 2006; Aragão et al., 2011). A exploração indiscriminada do pau-brasil é um dos clássicos exemplos de distúrbio causado à Mata Atlântica, iniciado logo

após a ocupação da costa brasileira pelos colonizadores portugueses, para a extração do corante vermelho presente em sua madeira. A extração do pau-brasil para obtenção de corante cessou em meados dos anos 1800, em decorrência da produção do corante sintético (Martinelli e Moraes, 2013; Gagnon et al., 2016). Entretanto, a extração da madeira para a confecção de arcos para instrumentos de corda ganhou notoriedade, pois arcos feitos com madeira de pau-brasil são considerados insubstituíveis (Martinelli e Moraes, 2013; Gagnon et al., 2016).

Assim, a distribuição original foi reduzida a pequenos remanescentes na atualidade, ocasionando a redução da variabilidade genética com fluxo gênico cada vez menor entre as subpopulações (Rocha, 2011; Martinelli e Moraes, 2013; Gagnon et al., 2016). E, atualmente esta espécie encontra-se na lista vermelha da International Union for Conservation of Nature (IUCN), sendo caracterizada como uma espécie ameaçada de extinção (IUCN, 2018). Neste sentido, é de grande importância a aplicação de estratégias de conservação, como a criação de unidades de conservação e o desenvolvimento de pesquisas referentes à sua propagação (Martinelli e Moraes, 2013).

Estudos realizados até o momento com o cultivo *in vitro* de pau-brasil mostram que não houve a indução de embriões somáticos para esta espécie. Entretanto, a indução de calogênese foi obtida a partir de folíolos jovens utilizados como explantes (Werner et al., 2009; Werner et al., 2010). Em relação a organogênese *in vitro* para esta espécie foi mostrado que segmentos nodais foram responsivos à indução de brotações quando cultivados em meio de cultura suplementado com 2,5 µM de BA (Aragão et al., 2011). Adicionalmente, estudos mostram que as sementes de pau-brasil são tolerantes ao armazenamento a baixas temperaturas (-18 °C), desde que o conteúdo de água não seja superior a 12,7% (Hellmann et al., 2006). Recentemente, Mello et al. (2013) demonstraram que sementes de pau-brasil se mantêm viáveis após cinco anos de armazenamento a -18 °C, apresentando valores elevados de germinação (~60%) com elevado desenvolvimento de plântulas normais (~40%).

2.5.2. *Cedrela odorata* L.

C. odorata pertence à família Meliaceae, sendo popularmente conhecida como cedro madeira, mogno brasileiro, cedro americano, cedro da América Central, entre outros (Varty, 1998). Esta espécie é típica das regiões neotropicais,

localizando-se nas florestas subtropicais e tropicais úmidas e estacionais (Cervi et al., 2008). No Brasil, a *C. odorata* está distribuída nos biomas Amazônia, Caatinga, Cerrado e Mata Atlântica. Sendo localizada nos Estados do Acre, Amazônia, Bahia, Ceará, Distrito Federal, Espírito Santo, Goiás, Maranhão, Minas Gerais, Mato Grosso do Sul, Mato Grosso, Paraíba, Pernambuco, Paraná, Rio de Janeiro, Rondônia, São Paulo e Santa Catarina (Martinelli e Moraes, 2013).

Esta espécie cresce em solos bem drenados e tolera longos períodos de seca, mas não suporta áreas com precipitações superiores a 3000 mm anuais. Assim, apesar de ser comum observar elevada densidade de plântulas próxima a árvores após a liberação das sementes, a mortalidade dessas plântulas é elevada no meio da estação chuvosa (Cervi et al., 2008; Martins et al., 2008).

Caracterizada por ser uma arbórea de grande porte, a *C. odorata* pode atingir 40 m de altura e 120 cm de diâmetro a altura do peito, com tronco retilíneo. A copa densa, apresenta ramos eretos ou tortuosos, gemas pubescentes, ramos jovens geralmente pubescentes e glabros quando maduros. As folhas apresentam 20-42 cm de comprimento, são compostas, apresentando 10-28 folíolos alternos a opostos com lâmina oblonga, lanceolada ou falcada possuindo face abaxial glabra ou glabrata e face adaxial glabra ou esparsamente pubérula, com tricomas concentrados na nervura principal (Cavers et al., 2004; Cervi et al., 2008; Flores et al., 2017).

A *C. odorata* é uma espécie monoica e, predominantemente, alógama e protogínica (flores femininas abrem-se antes das masculinas), florescendo no início da estação chuvosa. As pequenas flores branco-esverdeadas estão reunidas em numerosas inflorescências terminais. O fruto é uma cápsula ovoide, deiscente, lenhoso e de coloração marrom-avermelhada. Demoram de nove a dez meses para se desenvolverem e amadurecem durante a estação chuvosa do ano seguinte. As sementes elipsoides, aladas em uma das pontas, medem 2-3 cm de comprimento, sem arilo de coloração castanho-claro (Cervi et al., 2008; Martins et al., 2008; Flores et al., 2017).

A madeira de *C. odorata* é considerada uma das mais importantes do mundo, sendo descrita como macia e, portanto, fácil de trabalhar, de boa resistência mecânica e moderadamente resistente ao ataque de pragas. É utilizada na produção de móveis finos, portas, janelas, instrumentos musicais, na construção civil, e fabricação de barcos e canoas, entre outras (Navarro et al., 2004; Martins et

al., 2008; Passos et al., 2008; Orwa et al., 2009; Batista et al., 2011). Além disso, a planta é melífera e praticamente todas as suas partes apresentam propriedades medicinais, sendo utilizada no combate à malária, infecção intestinal intoxicação, má digestão (Omar et al., 2003; Bieski et al., 2015).

Embora a *C. odorata* apresente ampla área de distribuição, a espécie não é muito comum, ocorrendo de forma dispersa na floresta. Este fato deve-se tanto às práticas predatórias de exploração madeireira como pela perda de habitat (Cavers et al., 2003; Martins et al., 2008). Desta forma, atualmente a *C. odorata*, encontra-se incluída na lista vermelha da IUCN, sendo caracterizada como uma espécie vulnerável (IUCN, 2018). Neste sentido, metodologias de propagação para esta espécie necessitam ser estabelecidas, visando à sua reposição em seus habitats, ou ainda, a produção de mudas com fins econômicos.

Estudos relacionados ao cultivo *in vitro* de *C. odorata* foram desenvolvidos. A indução de embriões somáticos foi obtida a partir do cultivo de embriões somáticos imaturos, obtidos nove semanas após a antese. Os calos foram induzidos em meio de cultura MS, suplementado com dicamba e a conversão dos embriões foi obtida na presença de ABA, correspondendo a 12 %. As plantas estabelecidas em solo apresentaram desenvolvimento funcional dos sistemas radicular e fotossintético com 75 % de sobrevivência após transferência para o campo (Peña-Ramírez et al., 2011).

Em relação à organogênse foram avaliados a influência das citocininas, a auxina e os diferentes meios de cultura na indução e enraizamento de brotações, bem como a influência da maturidade do explante (Pérez et al., 2002; Rodríguez et al., 2003; Peña-Ramírez et al., 2010). Rodríguez et al. (2003) descrecevem a concentração de 0.5 mg $mg.L^{-1}$ de BA como ótima para indução de brotos a partir de segmentos nodais obtidos de plântulas de *C. odorata* germinadas *in vitro*, apresentando em média de 2,5 brotos por explante, medindo 3,5 cm. Enquanto que, García-Gonzáles et al. (2011) relatam a optimização do desenvolvimento de brotos utilizando o mesmo tipo de explante, que é obtido a partir da associação de 2 $mg.L^{-1}$ de BA com 3 $mg.L^{-1}$ de ANA. Obtendo-se 100 % de indução de brotos, sendo em média 3,9 brotos por explante com comprimento médio de 3,93 cm.

Peña-Ramírez et al. (2010) demonstraram o desenvolvimento de brotações adventícias a partir de segmentos de hipocótilo incubados em meio TY17 (Gonzalez-Rodríguez e Peña-Ramirez, 2007), suplementado com 20 % de água de

coco. Além da obtenção de mudas micropropagadas a partir de material vegetal rejuvenescido de indivíduos elite maduros, obtidas com a enxertia *ex vitro* de galhos maduros em plantas com 3 meses de idade.

Em relação ao armazenamento, foi observado que as sementes de *C. odorata* apresentam perda da viabilidade mais rapidamente quando armazenadas em temperatura ambiente (25 °C) em comparação a temperaturas mais baixas (7-8 °C). Entretanto, apesar de a temperatura reduzida favorecer a viabilidade das sementes por 9 meses, o seu vigor foi afetado, havendo redução da porcentagem de plântulas normais de 79 %, antes do armazenamento, para 23 % aos 9 meses de armazenamento (Batista et al., 2011).

Até o momento não há relatos do efeito do tipo de segmento nodal utilizado como explante e a relação da PAs na organogênese *in vitro* para esta espécie. Adicionalmente, não existem estudos relacionando o armazenamento de sementes e o conteúdo de PAs livres, fundamentais para entender os mecanismos associados com a manutenção e, ou perda da viabilidade.

3. OBJETIVOS

3.1. Objetivo geral

O objetivo deste trabalho foi estudar os aspectos morfológicos, fisiológicos e bioquímicos da propagação *in vitro* em *P. echinata* e *C. odorata* e no armazenamento de sementes em *C. odorata*.

3.2. Objetivos específicos

- Realizar estudos morfológicos, histoquímicos e bioquímicos durante a indução e o desenvolvimento da embriogênese somática a partir de sementes imaturas de *P. echinata*;
- Avaliar os efeitos da BA e do tipo de explante (segmentos nodais axilares e cotiledonares) na organogênese *in vitro* e no conteúdo endógeno de PAs livres, e os efeitos do AIB e do tamanho das microestacas no enraizamento *in vitro* em *C. odorata*;
- Avaliar o efeito da temperatura e do tempo de armazenamento sobre a viabilidade e o conteúdo de PAs em sementes de *C. odorata*.

4. TRABALHOS

4.1. MORPHOLOGICAL, HISTOCHEMICAL AND BIOCHEMICAL ASPECTS OF SOMATIC EMBRYOGENESIS INDUCTION FROM IMMATURE SEEDS OF *Paubrasilia echinata* (LAM.) E. GAGNON, H.C. LIMA & G. P. LEWIS (FABACEAE): THE NATIONAL TREE OF BRAZIL

RESUMO

Paubrasilia echinata (pau-brasil) é uma árvore endêmica do Brasil que tem um grande valor cultural, histórico e econômico. Pau-brasil está atualmente listado como uma espécie ameaçada de extinção, enfatizando a importância de realizar pesquisas relacionadas à sua conservação. O objetivo do presente trabalho foi realizar estudos morfológicos, histoquímicos e bioquímicos durante a indução e estabelecimento da embriogênese somática a partir de sementes imaturas de *P. echinata*. Para a indução de calo, foram utilizadas diferentes concentrações de

Trabalho submetido: Costa, P. R.; Sousa, K. R.; Silveira, V.; Steiner, N.; Santa-Catarina, C. (2018). Morphological, histochemical and biochemical aspects of somatic embryogenesis induction from immature seeds of *Paubrasilia echinata* (Lam.) E. Gagnon, H.C. Lima & G. P. Lewis (Fabaceae): the national tree of Brazil. *Physiology and Molecular Biology of Plants*, submitted.

ácido 2,4-diclorofenoxiacético (2,4-D), combinadas ou não com 6-benziladenina (BA). O calo foi multiplicado a partir de duas subculturas em meio de cultura com 10 µM de 2,4-D. Posteriormente, foi incubado em tratamentos de maturação contendo diferentes combinações de BA, glutamina, ácido abscísico (ABA), polietileno glicol (PEG) e maltose. As sementes imaturas podem ser usadas como explantes para a indução de calo. Todos os tratamentos contendo 2,4-D induziram a formação de calos, com a maior indução observada com o tratamento com 2,4-D mais BA. O tratamento com 25 µM de ABA permitiu a formação de embriões somáticos em estádios iniciais de desenvolvimento, incluindo os estádios globular, cordiforme e torpedo. O tratamento com ABA modulou as PAs endógenas por meio de uma redução significativa dos conteúdos do Put livre, o que pode estar relacionado ao desenvolvimento do embrião somático. Este é o primeiro estudo a mostrar o desenvolvimento do embrião somático em estádios iniciais, a partir de sementes imaturas de pau-brasil. Estas descobertas fornecem uma base importante para estudos adicionais para melhorar a embriogênese somática, otimizar a maturação e conversão de embriões somáticos em plantas e promover a conservação do germoplasma desta espécie.

Palavras-chave: ácido abscísico; *Caesalpinia echinata*; cultura *in vitro*; poliaminas.

ABSTRACT

Paubrasilia echinata (pau-brasil) is a tree endemic to Brazil that has great cultural, historical and economic value. Pau-brasil is currently listed as an endangered species, emphasizing the importance of conducting research related to its conservation. The aim of the present study was to perform morphological, histochemical and biochemical studies during somatic embryogenesis induction and establishment from immature seeds of *P. echinata*. For callus induction, different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) were used, combined or not with 6-benzyladenine (BA). The callus was multiplied by two subcultures in culture medium with 10 µM 2,4-D. Subsequently, it was incubated

in maturation treatments containing different combinations of BA, glutamine, abscisic acid (ABA), polyethylene glycol (PEG) and maltose. Immature seeds can be used as explants for the induction of callus. All treatments containing 2,4-D induced callus formation, with the highest induction observed with 2,4-D plus BA treatment. Treatment with 25 µM ABA allowed the formation of somatic embryos at earlier stages of development, including the globular, heart and torpedo stages. ABA treatment modulated endogenous free PAs via a significant reduction of free Put contents, which could be related to somatic embryo development. This is the first study to show somatic embryo development at earlier stages from immature seeds of pau-brasil. These findings provide an important foundation for further studies to improve somatic embryogenesis, to optimize maturation and conversion of somatic embryos into plants, and to promote the germplasm conservation of this species.

Keywords: abscisic acid; *Caesalpinia echinata*; *in vitro* culture; polyamines.

1. INTRODUCTION

Caesalpinia echinata, which was originally described by Lamarck in 1785, was recently placed in its own monospecific genus after being renamed *Paubrasilia echinata* (Lam.) E. Gagnon, H.C. Lima & G. P. Lewis (Gagnon et al., 2016). The Latinization of this genus is a tribute to the common name pau-brasil, thus acknowledging the importance of this species to Brazil (Gagnon et al., 2016). Pau-brasil is an endemic species in Brazil and is predominantly found in the states of Rio Grande do Norte, Paraíba, Alagoas, Pernambuco, Bahia, Espírito Santo, and Rio de Janeiro (Martinelli and Moraes, 2013; Gagnon et al., 2016). Currently, this species is classified as endangered on the red list of threatened species of the International Union for Conservation of Nature (IUCN) (IUCN, 2017), reflecting its predatory exploitation for more than 500 years. The selective cutting of this species was initially motivated by the red dye extracted from its wood, resulting in the nearly

complete destruction of natural stands. Persistent subpopulations are affected by deforestation as a result of timber exploitation because this tree remains highly sought after by violin arch makers due to its unique characteristics (Martinelli and Moraes, 2013).

Information regarding the flowering, seed production and seed storage behavior of Brazilian native species is limited, which is a barrier to its improved propagation and conservation (Pilatti et al., 2011). In natural environments, pau-brasil seeds germinate immediately after shedding (Hellmann et al., 2006), reducing their viability in less than three months when stored at room temperature (~ 22 °C) (Barbedo et al., 2002; Mello et al., 2013). However, these seeds tolerate desiccation and maintain viability for at least 18 months when stored at 7 °C and five years when maintained at -18 °C (Barbedo et al., 2002; Mello et al., 2013).

Conventional propagation methods employing cuttings were developed for this species using indole-3-butyric acid (IBA) and α-naphthalene acetic acid; however, the percentage of root formation was low (16 %) (Endres et al., 2007). An increase in the rooting (39 %) of cuttings was achieved using a hydroponic system and pre-treatment with IBA (Valeri et al., 2012). However, the percentage of root formation remained low. Thus, biotechnological tools such as *in vitro* propagation and cryopreservation are alternative methods that will potentially facilitate *ex situ* germplasm conservation studies to protect native species, particularly over-exploited and endangered species in their natural habitats (Pilatti et al., 2011). *In vitro* culture technologies, including *in vitro* germination, micropropagation, somatic embryogenesis, zygotic embryo culture, and callus culture systems, have been successfully developed for many native and/or economically important Brazilian species (Pilatti et al., 2011).

Somatic embryogenesis is an analogous process to zygotic embryogenesis, in which somatic cells under inductive conditions generate embryogenic cells that undergo a series of morphological and biochemical alterations, resulting in the formation of somatic embryos (Tautorus et al., 1991; Jiménez, 2001; Sugiyama, 2015). Callus and cell suspension cultures are employed for somatic embryogenesis, and various biotechnological applications have been developed to exploit the economic value of native plant biodiversity requiring *in vitro* conservation (Pilatti et al., 2011). The application of *in vitro* culture techniques to endemic Brazilian species has increased, but the results are still incipient compared to those

for crop species (Pilatti et al., 2011). Somatic embryogenesis involves several phases, including (a) induction with auxins, such as 2,4-dichlorophenoxyacetic acid (2,4-D), which can be combined with cytokinins, the most widely used plant growth regulators (PGRs) in this phase; (b) multiplication; (c) maturation using compounds that promote the maturation of embryogenic cultures, such as abscisic acid (ABA), polyethylene glycol (PEG), maltose and activated charcoal; and (d) the conversion of somatic embryos into somatic seedlings (Senger et al., 2001; von Arnold et al., 2002; Steiner et al., 2008).

In addition to PGRs, polyamines (PAs) are important signals that act in several important cellular processes, including somatic embryogenesis, during growth and development (Bais and Ravishankar, 2002; Wallace et al., 2003; Baron and Stasolla, 2008; Reis et al., 2016). PAs are an important group of low-molecular-weight, aliphatic, polycationic compounds that carry positive charges on nitrogen atoms, a property that facilitates electrostatic interactions with macromolecules such as DNA, RNA, phospholipids, cell wall components, and proteins (Wallace et al., 2003). Putrescine (Put), spermidine (Spd), and spermine (Spm) are the major PAs in plants (Kaur-Sawhney et al., 2003). The modulation of PA metabolism during somatic embryogenesis plays an important role in *in vitro* somatic embryo development (Kevers et al., 2000; Kaur-Sawhney et al., 2003; Sakhanokho et al., 2005; Tun et al., 2006; Santa-Catarina et al., 2007; Steiner et al., 2007; Wu et al., 2009; Silveira et al., 2013; Reis et al., 2016). The effects of PAs on the regulation of endogenous nitric oxide levels (Santa-Catarina et al., 2007) to maintain the growth and polarity of somatic embryos (Silveira et al., 2006) and increase endogenous ABA levels (Steiner et al., 2007) have been thoroughly documented.

Some studies investigating the *in vitro* culture of *C. echinata* have been reported (Werner et al., 2009; Werner et al., 2010; Aragão et al., 2011). The effects of 6-benzyladenine (BA) on shoot induction from nodal and internodal explants were tested during organogenesis development, for which nodal segments are better explants (Aragão et al., 2011). Initial studies involving *in vitro* callus induction were developed, and calluses were obtained using young leaves as explants (Werner et al., 2009; Werner et al., 2010). However, somatic embryo development has not been previously reported. Thus, the objective of the present study was to perform morphological, histochemical and biochemical studies during somatic embryogenesis induction and development from immature seeds of *P. echinata*.

2. MATERIALS AND METHODS

2.1. Plant material

Immature fruits of *P. echinata* (Fig. 1a,b) were collected from nine trees at day 55 after the beginning of flowering. The trees are located in the city of Campos dos Goytacazes, Rio de Janeiro, Brazil (21° 45'58.8"S and 41° 16'19.0"W). The basal portions (Fig. 1c) of the immature seeds containing zygotic embryos at the early cotyledonary stage (Fig. 1d) were isolated from the fruits and used as explants for somatic embryogenesis induction.

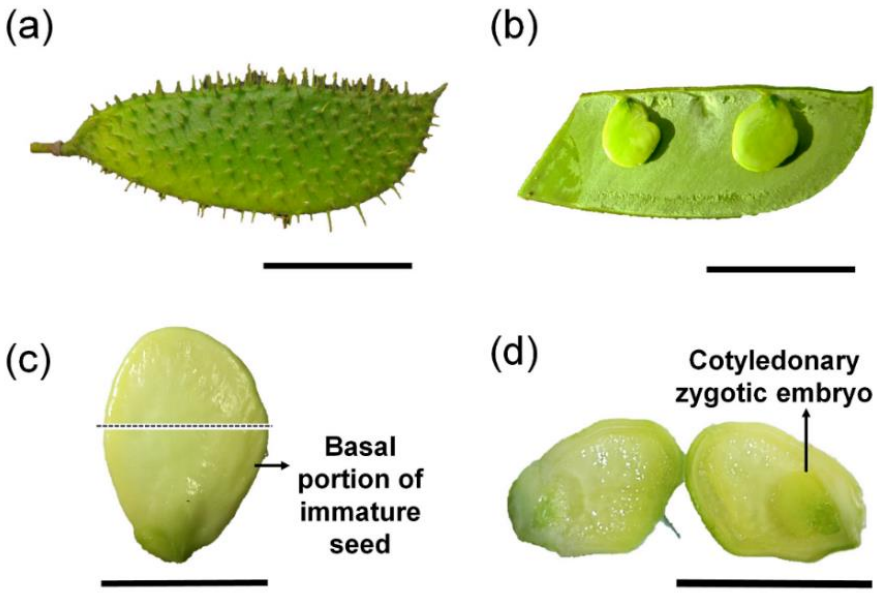

Figure 1. Morphological aspects of immature fruit and seeds of *P. echinata* after 55 days of flowering, showing the external view of fruits with spines on surface (**a**), the immature seeds inside the fruits (**b**) isolated to obtain the basal portion (**c**) containing the zygotic embryo at early cotyledonary stage (**d**), which was used as explant for callus induction. Bars: a, b = 30 mm; c = 6 mm; d = 8 mm.

Fifty immature fruits were randomly collected at this time, showing an average weight of 3 g, length of 60.21 mm, width of 23.72 mm, and thickness of 3.76 mm. An average of 1.4 seeds per fruit was observed, with immature seeds having a weight of 0.04 g, length of 7.93 mm, width of 5.98 mm and thickness of 3.2 mm. Using a stereomicroscope (Carl Zeiss, Jena, Germany), it was possible to observe that immature seeds presented zygotic embryos at the cotyledonary stage, measuring 5.88 mm in length, 3.0 mm in width, and 1.3 mm in thickness.

2.2. Disinfestation of immature fruits

To initiate the callus induction process, the immature fruits were washed with neutral detergent and water. Then, the fruits were surface-disinfested by immersion in 70 % ethanol (v/v) for 1 min. Subsequently, the fruits were immersed for 1 h in a 100 % commercial Qboa® bleach solution (Anhembi-SA, São Paulo, Brazil) containing 2-2.5 % active chlorine. Finally, the fruits were washed three times with autoclaved distilled water in a laminar flow chamber to remove the excess active chlorine.

2.3. Effects of auxin and cytokinin on callus induction

After disinfestation, the immature fruits were opened to extract the immature seeds. The seeds were sectioned in a half portion, and the basal portion (Fig. 1c), containing immature zygotic embryos at the early cotyledonary stage, were inoculated into a test tube (150 × 25 mm) containing 10 mL of MS culture medium (Murashige and Skoog, 1962) (PhytoTechnology Laboratories, Shawnee Mission, KS, USA) supplemented with 30 g L^{-1} sucrose (Vetec, Rio de Janeiro, Brazil), 2 g L^{-1} Phytagel® (Sigma-Aldrich, St. Louis, MO, USA), and different concentrations (0, 10, 50 and 100 µM) of 2,4-D (Sigma-Aldrich), as well as 10 µM 2,4-D combined with different concentrations (0, 1 and 5 µM) of BA (Sigma-Aldrich). The pH of the culture medium was adjusted to 5.7 prior to the addition of Phytagel, and subsequently autoclaved at 121 °C and 1.5 atm for 15 min.

After inoculation, the explants were maintained in a culture room in the dark at 25 ± 2 °C. Five replicates with 24 explants each (one explant per test tube), with 120 explants per treatment, were used. The morphologies of the callus induced under different treatments were monitored during incubation. After 50 days, callus

induction (%) and the type of callus (%), according to their color and consistency, were analyzed for each treatment.

2.4. Callus multiplication

Sixty-day-old callus obtained during the induction phase was multiplied under two conditions. Callus induced in 2,4-D (0, 10, 50 and 100 µM) was transferred to MS culture medium supplemented with 10 µM 2,4-D. Callus induced in medium containing 10 µM 2,4-D combined with BA (1 and 5 µM) was transferred into MS culture medium supplemented with the same PGR concentrations applied for the induction of callus (10 µM 2,4-D combined with 1 and 5 µM BA). In both conditions, the culture medium was supplemented with 30 g L^{-1} sucrose and 2 g L^{-1} Phytagel®. The pH of the culture medium was adjusted to 5.7 and subsequently autoclaved at 121 °C and 1.5 atm for 15 min.

Two subcultures, with a 30-day interval between each, were performed, maintained in a culture room in the dark at 25 ± 2 °C. The first subculture was performed in test tubes (150 × 25 mm) containing 10 mL of culture medium. The second subculture was performed in sterile Petri dishes (90 x 15 mm) containing 25 mL of culture medium. At the end of second subculture, morpho-histological analyses were performed, and the brown callus-type was used in the maturation treatments, analyzing the effects of BA, ABA, PEG, maltose, and glutamine in the development of somatic embryos.

2.5. Effects of maturation treatments in the development of somatic embryos

Brown callus was used in two distinct maturation treatments to examine the effects of BA and glutamine (a), and ABA, PEG, and maltose (b) on the induction of somatic embryos. Both treatments were performed simultaneously.

2.5.1. The effects of BA and glutamine

Brown callus obtained from multiplication treatments containing 10 µM 2,4-D combined with BA (1 and 5 µM) were subjected to treatments with BA (0, 1 and 5 µM); glutamine (0 and 100 µM), and the combination of 1 µM BA with 100 µM glutamine; and 5 µM BA with 100 µM glutamine on MS culture medium supplemented with 30 g L^{-1} sucrose and 2 g L^{-1} Phytagel®. The pH of the culture

medium was adjusted to 5.7 prior to the addition of Phytagel® and autoclaved at 121 °C and 1.5 atm for 15 min.

Five colonies (200 mg of fresh matter from each colony) of callus were inoculated into each sterile Petri dish (90 x 15 mm) containing 25 mL of culture medium, with five Petri dishes per treatment. After inoculation, the cultures were maintained in the dark at 25 ± 2 °C for 14 days and subsequently transferred into the light with a 16-h photoperiod and a light intensity of 22 µmol m^2 s^{-1}, at 25 ± 2 °C. Morphology was monitored throughout the incubation period for somatic embryo development, and histological analysis of the callus was performed at 60, 120 and 180 days of incubation.

2.5.2. The effects of ABA, PEG, and maltose

Brown callus obtained from multiplication treatment with 10 µM 2,4-D was transferred to the treatments with ABA (0, 5, 25 and 50 µM), PEG (0, 6 and 9 %), maltose (0 and 6 %) and the combination of 25 µM ABA with 6 % PEG and 6 % maltose, all in MS culture medium supplemented with 30 g L^{-1} sucrose and 2 g L^{-1} Phytagel®. The pH of all culture media was adjusted to 5.7 and later autoclaved at 121 °C and 1.5 atm for 15 min. The ABA was filter-sterilized through a 0.2 µm PVDF membrane (Millipore, São Paulo, SP, Brazil) in a laminar flow chamber prior to its addition to the autoclaved culture medium.

Five colonies (200 mg of fresh matter each) of callus were inoculated into each sterile Petri dish (90 x 15 mm) containing 25 mL of culture medium, with five Petri dishes per treatment. After inoculation, embryogenic callus under different treatments was maintained in the dark for 14 days at 25 ± 2 °C and subsequently transferred into the light with a 16-h photoperiod and a light intensity of 22 µmol m^2 s^{-1} at 25 ± 2 °C.

The morphologies of the callus were monitored during incubation to observe the development of somatic embryos, and histological analysis was performed at 60, 120, and 180 days. At 180 days of incubation, samples (200 mg of fresh matter each, in triplicate) of callus on MS culture medium without PGRs (control) and 25 µM ABA were collected for PA analysis.

2.6. Morphological and histological analyses

The morphological analysis of callus was performed using a stereomicroscope and an AxioCam ERc5s 0.5 X digital camera (Carl Zeiss) at induction (at 50 days of incubation) in the brown callus-type after 30 days of second subculture of multiplication and in the callus subjected to different maturation treatments at 60, 120, and 180 days of incubation.

For histological studies, the brown callus-type from multiplication and the callus submitted to the maturation treatments at 60, 120 and 180 days of incubation were fixed in 4 % (v/v) formaldehyde and 2.5 % (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2). For light microscopy analyses, the samples were washed twice for 30 min each with 0.1 M sodium cacodylate buffer and dehydrated twice (during 1 h each) in increasing ethanol series (30, 50, 70, 90, and 100 %). After dehydration, the samples were infiltrated with HistoResin® (Leica, Wetzlar, Germany) and 100 % ethanol (1:1, v/v) for 12 h and subsequently in 100 % HistoResin® for 24 h, followed by embedding in HistoResin® (Silveira et al., 2013).

Longitudinal sections (5 µm thick) were obtained using a microtome (Leica Microsystems Inc., Buffalo Grove, IL, USA). For structural characterization, the sections were stained for 30 s with 1 % Toluidine blue (TB-O) solution containing 1 % sodium borate (O'brien et al., 1964). Pectic substances were observed after staining with ruthenium red (0.02 %) for 5 min in the dark (Chamberlain, 1932). The sections were stained with 0.02 % Coomassie brilliant blue R250 (CBB) in acidified ethanolic Clarke solution for 24 h to detect total proteins according to the methodology of Gahan (Gahan, 1984). Neutral polysaccharides were stained using Schiff's periodic acid (PAS) solution (O'Brien and McCully, 1981). The sections were first oxidized in 1 % aqueous periodic acid solution for 20 min, washed three times in distilled water for 10 min each, and subsequently stained with Schiff's reagent for 30 min. The sections were then washed in water for 20 min to remove the excess color (O'Brien and McCully, 1981). After staining, the sections were air-dried and mounted using Canada's balsam. Images were captured using an AxioCam MRC5 digital camera (Carl Zeiss) coupled to a light Axioplan optical microscope (Carl Zeiss).

2.7. Determination of free PAs

Free PAs were analyzed according to Santa-Catarina et al. (2006). The samples (200 mg of fresh matter each, in triplicate) of callus incubated over 180 days at two concentrations (0 and 25 µM) of ABA were ground in 1.2 mL of 5 % perchloric acid (Merck, Darmstadt, Germany) and incubated at 4 °C for 1 h. Subsequently, the samples were centrifuged for 20 min at 20.000 g at 4 °C, and the supernatants, which contained the free PAs, were collected. Free PAs were determined directly from the supernatants via derivatization with dansyl chloride (Merck) and identified by performing high-performance liquid chromatography (HPLC, Shimadzu, Kyoto, Japan) using a 5 µm C18 reverse-phase column (Shimadzu Shin-pack CLC ODS).

The HPLC column gradient was developed by mixing increasing proportions of absolute acetonitrile (Merck) with 10 % acetonitrile in water (pH 3.5), adjusted with HCl (Merck). The gradient of absolute acetonitrile was programmed to 65 % over the first 10 min, 65 to 100 % between 10 min and 13 min, and 100 % between 13 min and 21 min, with a 1 mL min^{-1} flow at 40 °C. Free PA concentrations were determined using a fluorescence detector with excitation at 340 nm and emission at 510 nm. Peak areas and retention times were measured by comparison using the standard PAs Put, Spd, and Spm (Sigma-Aldrich).

2.8. Statistical analyses

All experiments were performed using a completely randomized design. The data were analyzed using analysis of variance (ANOVA) ($P < 0.05$) followed by the Student-Newman-Keuls (SNK) test (Sokal and Rohlf, 1995) using the ASSISTAT program version 7.7 beta (Silva, 2014).

3. RESULTS

3.1. Effects of auxin and cytokinin on callus induction

After 50 days of incubation of the basal portion seeds on induction medium, callus induction was observed in all treatments with PGRs, and the highest percentage was obtained using 10 µM 2,4-D combined with BA (1 and 5 µM) (Fig. 2). Moreover, callus formation was not detected in the absence of PGRs. The contamination of explants was lower in all treatments at less than 7 % (data not shown).

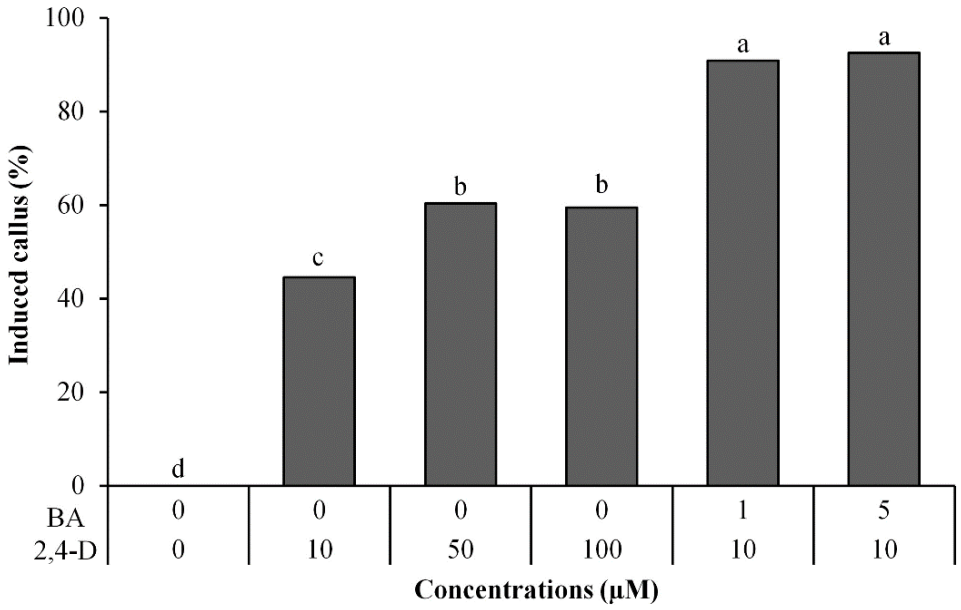

Figure 2. Callus induction (%) from immature *P. echinata* seeds after 50 days of incubation in treatments containing 2,4-dichlorophenoxyacetic acid (2,4-D) alone or in combination with benzyladenine (BA). Means followed by different letters are significantly different according to the SNK test ($P < 0.05$). CV = coefficient of variation. (n = 120, CV = 12.10 %).

Variations in the consistency (friable or compact) and colors of the induced callus were observed (Fig. 3). In general, friable callus was beige (Fig. 3a) and brown (Fig. 3b), whereas compact callus was white (Fig. 3c) and dark brown (Fig. 3d).

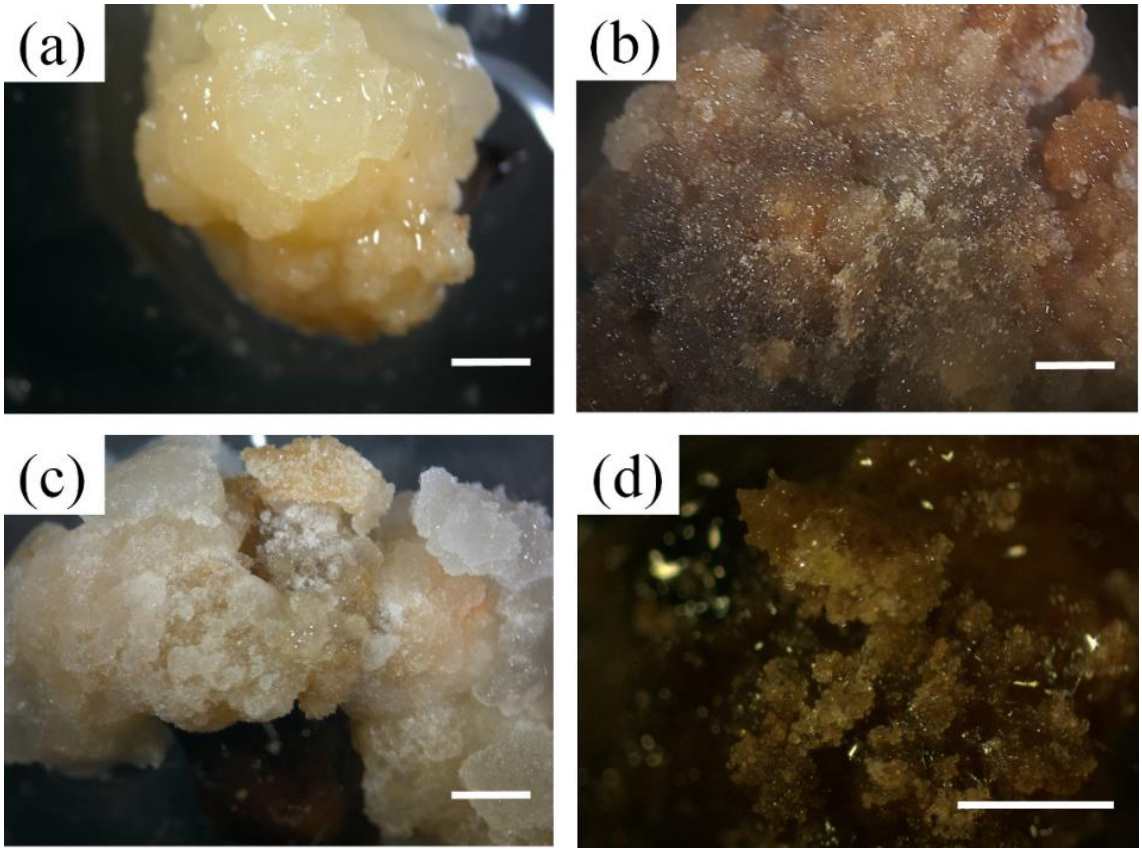

Figure 3. Morphological features of friable beige (**a**), friable brown (**b**), compact white (**c**) and compact dark-brown (**d**) callus obtained from immature *P. echinata* seeds after 50 days of incubation in callus induction treatments. Bars: 1 mm.

With respect to each type of callus induced with the application of PGRs, no significant difference was observed between the treatments applied when the friable beige callus was analyzed (Fig. 4). For the friable brown callus, the highest percentages were obtained by combining 10 µM 2,4-D with BA (1 and 5 µM), while the percentages of white and dark brown compact callus were the same for all treatments applied (Fig. 4).

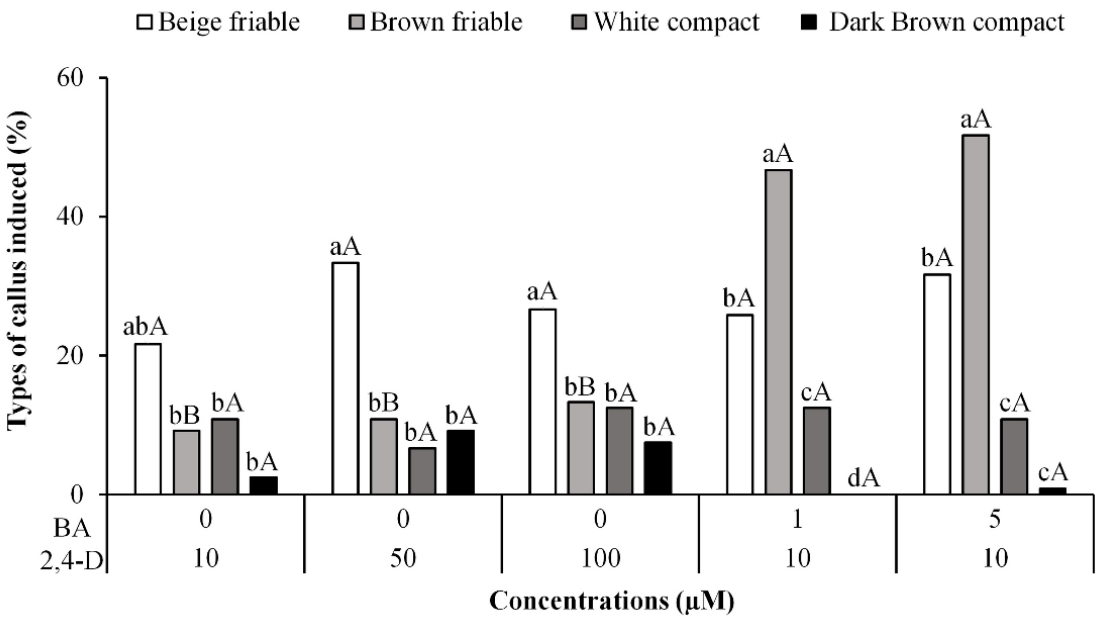

Figure 4. Different types of callus induced (%) from immature *P. echinata* seeds after 50 days of incubation in treatments containing 2,4-dichlorophenoxyacetic acid (2,4-D), combined or not with benzyladenine (BA). Lowercase letters indicate significant differences between the four callus types for each treatment. Capital letters indicate significant differences for each callus type under different treatments. Means followed by different letters are significantly different according to the SNK test ($P < 0.05$). CV = coefficient of variation. (n = 120, CV = 49.18 %).

3.2. Callus multiplication

After the two subcultures of induced callus, the majority of the four types of obtained callus presented a brown color and both friable and compact regions (Fig. 5a).

Histological analysis of the friable regions of this callus, when stained with TB-O, showed the presence of cells with different morphologies. In the superficial region, the cells were largest, and some of them had an elongated shape (Fig. 5b, stars). In the inner region of the callus, two distinct cell groups were observed. One was composed of larger cells that were similar in size to those in the superficial region (Fig. 5b, asterisks), and the other cell group was formed by small thin-walled cells organized in a compact group (Fig. 5b-c, dotted area). These cells exhibited features of meristematic cells, which can divide and differentiate into somatic embryos under the appropriate stimuli. In addition, TB-O-stained cells demonstrated

an orthochromatic reaction in the cytoplasm and a metachromatic reaction in the cell wall, indicating the presence of acidic polysaccharides (Fig. 5b-c).

A positive reaction to ruthenium red revealed the extracellular matrix containing pectic substances (Fig. 5d). Compact cell groups, composed of cells with small size and clearly separated from the larger adjacent cells, reacted with ruthenium red (Fig. 5d). In addition, the cells of callus exhibited positive PAS staining, indicating the presence of neutral polysaccharides in the cell wall, while the presence of starch grains was not observed (Fig. 5e). The positive reaction of cells to CBB indicated the presence of proteins in the cytoplasm and nucleus (Fig. 5f).

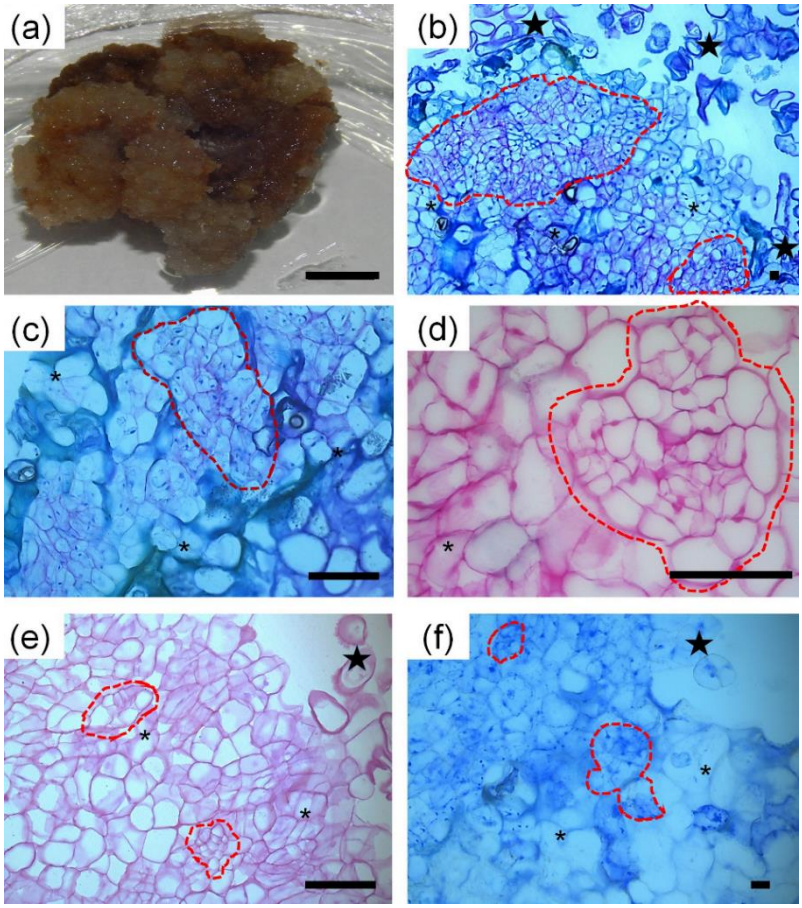

Figure 5. Morphological aspects of *P. echinata* embryogenic callus (**a**) after two subcultures in multiplication medium showing the histological features of friable brown regions, presenting large and some elongated cells in the superficial region (**b**, stars) and the inner region (**b**, asterisks) of callus, as well as groups of compact cells (**b-e**, dotted areas). The cells positively reacted to TB-O (**b**), ruthenium red (**c**), PAS (**e**) and CBB (**f**). TB-O = Toluidine blue; PAS = Schiff's periodic acid; CBB = Coomassie brilliant blue. Bars: **a** = 2 mm; **b**-**f** = 20 µM.

3.3. Effects of maturation treatments in the development of somatic embryos

3.3.1. Effects of BA and glutamine

The incubation of brown callus in free-PGR culture medium (control), as well as in culture medium supplemented with glutamine and BA, induced the formation of heterogeneous callus, showing friable and compact regions with white, beige, brown, dark-brown and green colors. Histological analysis revealed white (Fig. 6a-b), beige (Fig. 6c-d), brown (Fig. 6e-f) and dark-brown (Fig. 6g-h) regions of callus presenting little or no cell division depending on the treatment applied. Cells were larger, vacuolated, elongated and dispersed, and they lacked the presence of small cells with characteristics of meristematic cells. However, histological analyses of the green regions revealed small and aggregated cells, which were less vacuolated, primarily in the inner region (Fig. 6i-j). Although it was not observed, histodifferentiation of these cells into somatic embryos under the treatments used.

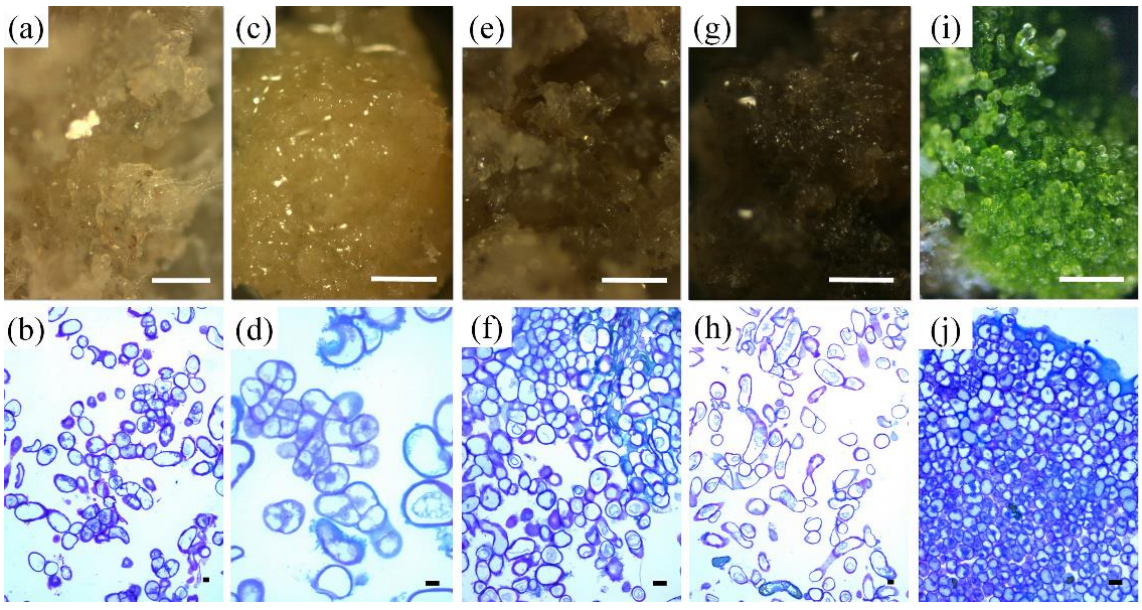

Figure 6. Morphological and histological aspects by TB-O stain of the white (**a-b**), beige (**c-d**), brown (**e-f**), dark-brown (**g-h**) and green (**i-j**) regions of *P. echinata* callus at 60 days of incubation in culture medium containing benzyladenine (BA) and glutamine. These aspects were similar to those of callus obtained under different treatments (data not showed). Bars: **a, c, e, g** = 0.5 mm; **b, d, f, h, j** = 20 µm.

3.3.2. Effects of ABA, PEG, and maltose

Brown callus subcultured in 10 µM 2,4-D when transferred to free-PGRs culture medium (control) or in culture medium supplemented with ABA, PEG, and maltose, promoted the formation of heterogeneous callus, presenting friable and compact regions with different colors, including beige, brown, dark-brown, and green.

Among the ABA concentrations applied, 25 µM ABA was the only ABA treatment that promoted the histodifferentiation of cells from callus in somatic embryos at early developmental stages. After 60 days of incubation of brown callus in 25 µM ABA, the formation of two distinct regions was observed, one green (Fig. 7a) and the other brown (Fig. 7f) colors. The green regions presented a nodular morphology (Fig. 7a) characteristic of embryogenic callus. The histomorphological analysis of this area revealed the presence of a meristematic zone (Fig. 7b) located below cells of larger diameter and arranged on the surface of the callus. The meristematic zone was formed by small cells organized in compact groups (Fig. 7c, dotted area) and had a dense cytoplasm, large central nuclei, predominantly heterochromatic regions and a visible nucleolus (Fig. 7c). Cells from the brown regions of the callus were larger, elongated and highly vacuolated, with characteristics of non-meristematic cells (Fig. 7d). In addition, compared to cells from the green region (Fig. 7a-c), cells from the brown region were less aggregated and exhibited retraction in the plasmatic membrane and a reduction in cytoplasmic content based on a weak reaction to TB-O (Fig. 7e).

Cellulosic compounds were observed in the cell wall based on positive PAS reactions, which also revealed a few of small starch grain in cells on the surfaces of the callus and in some meristematic cells in the green region (Fig. 7d). Cells of brown regions exhibited a positive reaction to PAS in the cell wall, particularly in elongated cells (Fig. 7h). Cells from the green regions had more proteins in the cytoplasm and nuclei based on strong positive reactions with CBB (Fig. 7e) than the cells in the brown regions, in which the cytoplasm and nuclei reacted weakly to CBB (Fig. 7i).

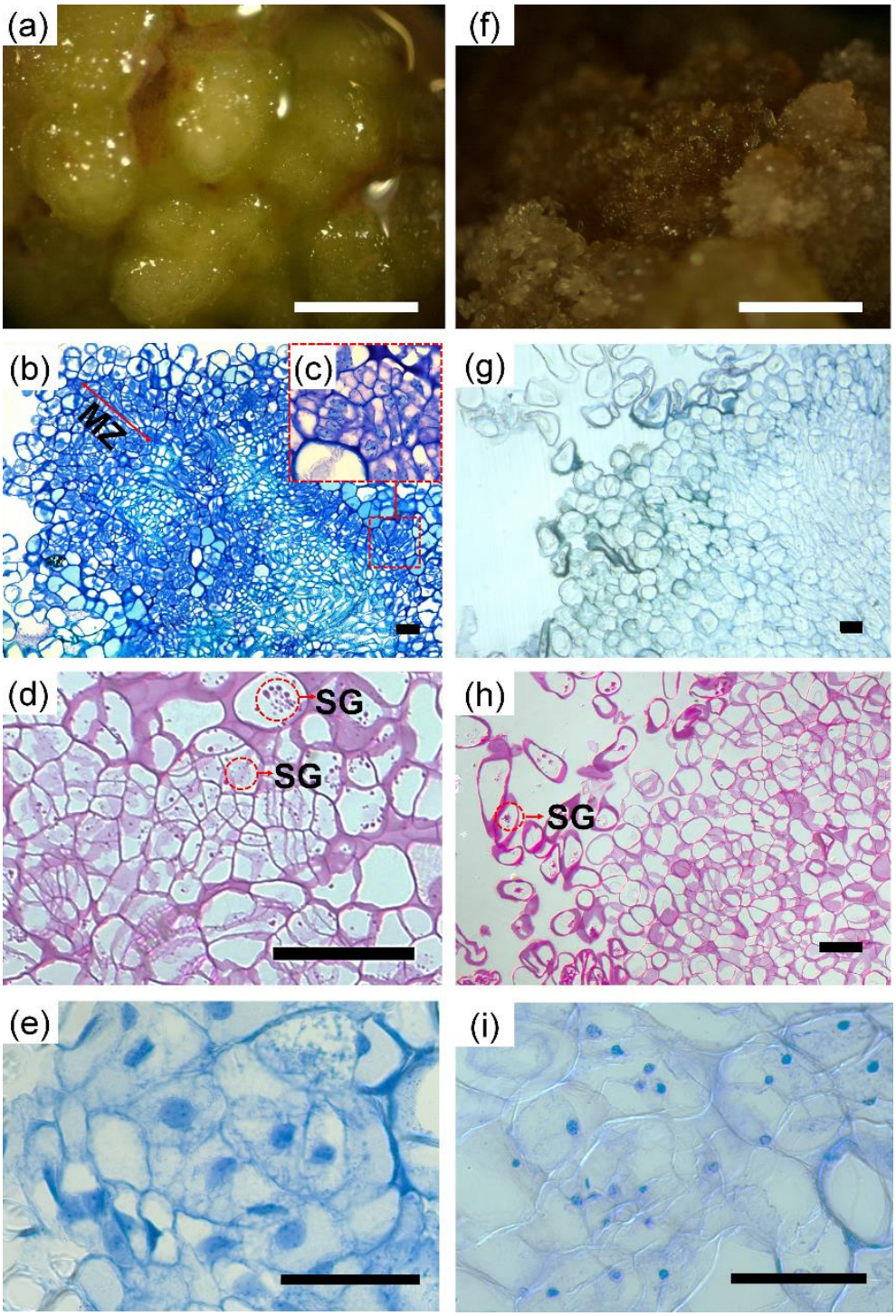

Figure 7. Morphological and histochemical aspects of *P. echinata* callus after 60 days of incubation in 25 µM ABA treatment, showing the green region with nodular appearance (**a**) with a meristematic zone (MZ) (**b**) formed by compact group of small cells (**c**, dotted area), positively reacted to PAS (**d**), showing the presence of small starch grains (SG) and positive reaction to CBB (**e**). The brown region of this callus (**f**) is formed by cells with non-meristematic characteristics, which presented a weak reaction in the cytoplasm with TB-O (**g**), PAS (**h**) and CBB (**i**). TB-O = toluidine blue; PAS = periodic acid of Schiff; CBB = Coomassie bright blue. Bars: **a, e** = 0.5 mm; **b, c, d, f, g, h** = 20 µm.

The green regions of the callus obtained with 25 µM ABA treatment observed after incubation for 60 days (Fig. 8a) allowed the evolution and differentiation of somatic embryos at the globular stage at 120 days (Fig. 8b) and somatic embryos at different stages of development at 180 days (Fig. 8c). However, the green regions observed in callus obtained with the control treatment (without ABA) showed only cellular proliferation without somatic embryo development (Fig. 8d-f).

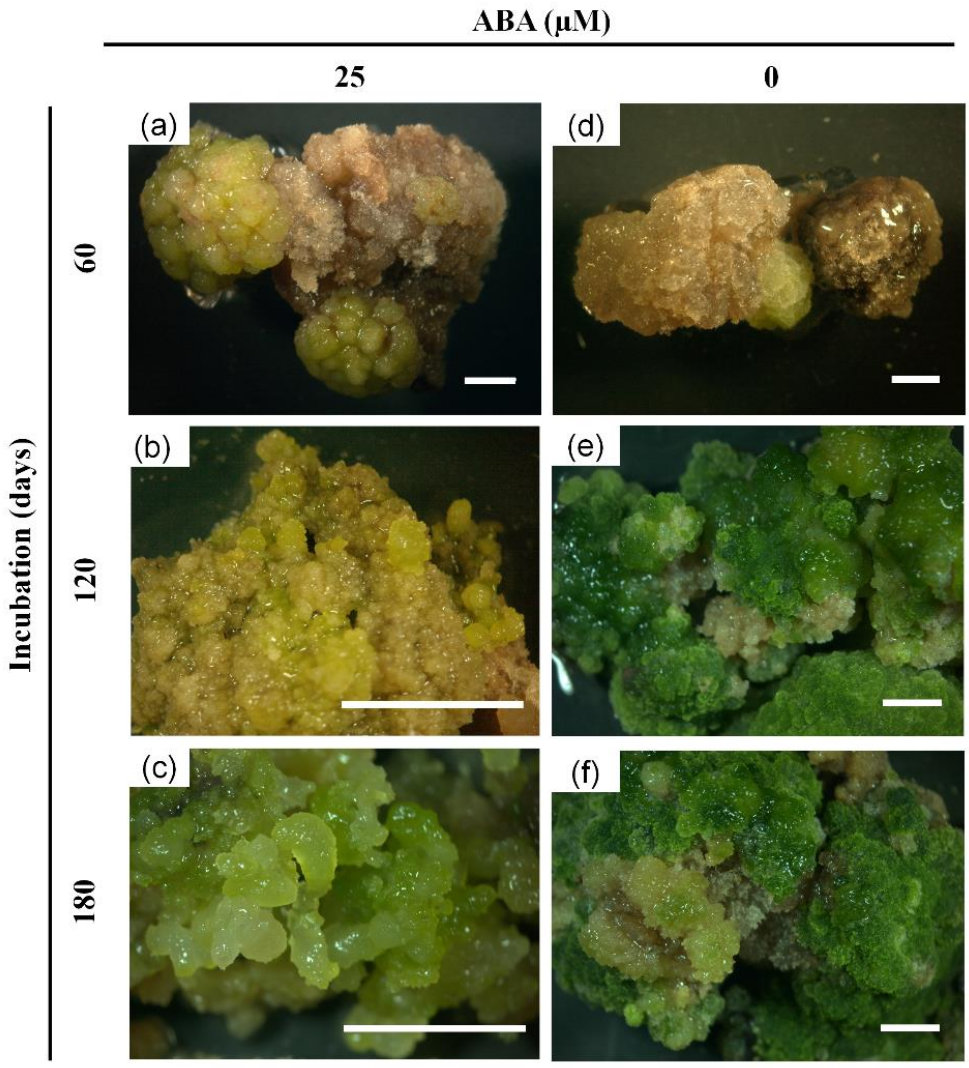

Figure 8. Morphological features of *P. echinata* callus at 60 (**a, d**), 120 (**b-e**) and 180 (**c-f)** days of incubation without (control) and with 25 µM ABA maturation treatments, showing the formation of somatic embryos at globular on 120 days (**b**) and at different developmental stages at 180 days (**f**) of incubation in ABA treatment. Bars = 1 mm.

After incubation for 120 days, the green regions of the callus in 25 µM ABA treatment showed nodular aspects at the surface of the callus (Fig. 9a). By histological analysis, it was observed the presence of cells with meristematic characteristics started to divide and differentiate into somatic pro-embryos (Fig. 9b-c, arrows). Positive PAS reactions indicated the presence of neutral polysaccharides, which was more evident in the cells of pro-embryos that presented starch grains at the beginning of development (Fig. 9d-e). The peripheral cells surrounding the somatic pro-embryos showed the presence of cells with larger starch grains (Fig. 9d-e). In addition to PAS, positive CBB reactions were intense in small and isodiametric cells, with a higher abundance of proteins in the cytoplasm and nuclei (Fig. 9f).

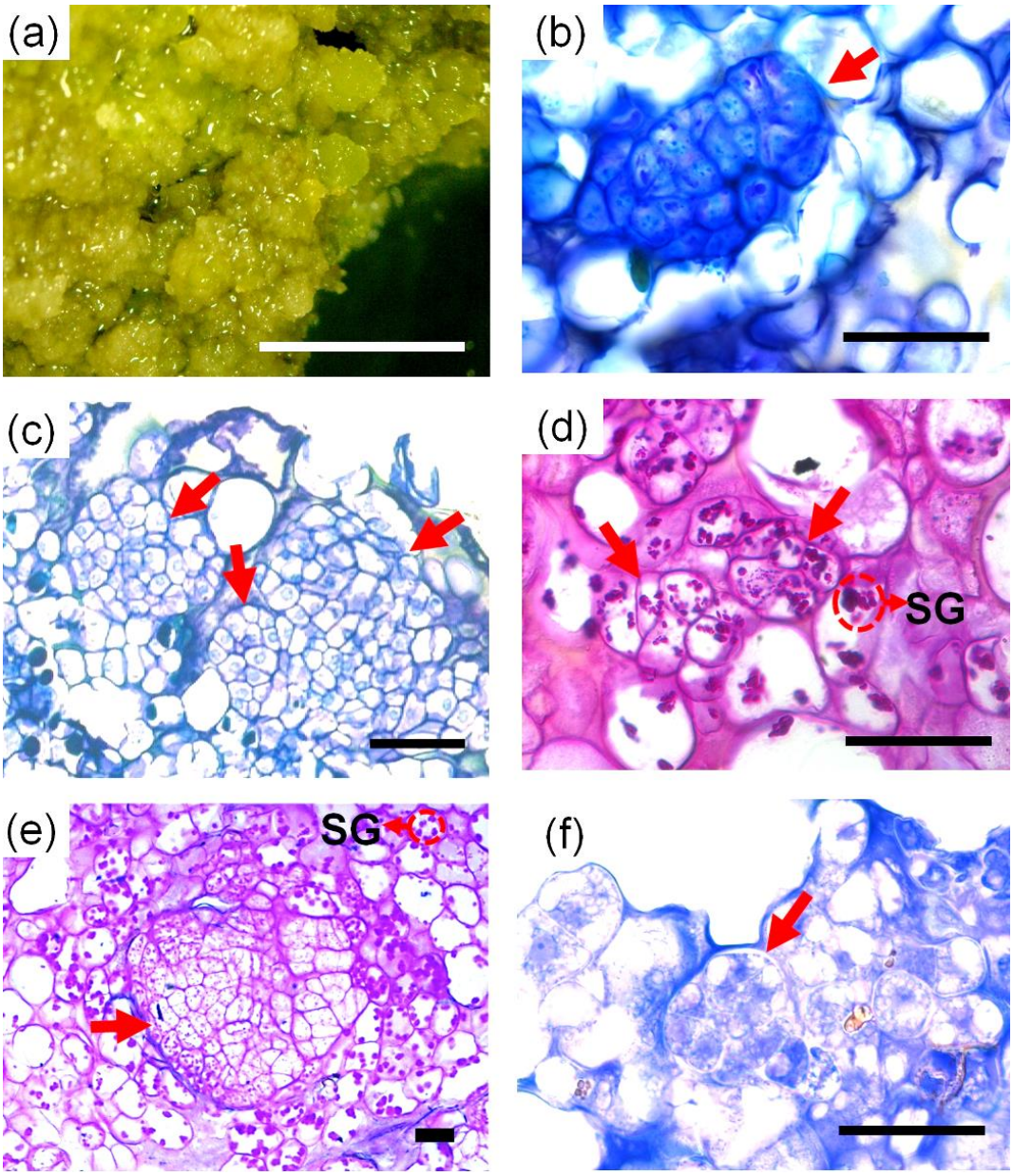

Figure 9. Morphological (**a**) and histochemical (**b-f**) features of cells from the green regions of *P. echinata* callus after incubation for 120 days on 25 µM ABA treatment, showing the formation of pro-embryos (red arrows) in sections stained with TB-O (**b, c**), PAS (**d, e**) and CBB (**f**). Details of starch grains (SG), which were present in larger quantities in initial pro-embryos (**d-e**, red arrow) and in cells surrounding the embryos (**e**). TB-O = Toluidine blue stain; PAS = Schiff's periodic acid stain; CBB = Coomassie brilliant blue stain. Bars: **a** = 0.5 mm; **b-f** = 20 µm.

After incubation for 180 days in 25 µM ABA, the presence of somatic embryos at earlier developmental stages was observed in the green nodular regions of the callus. Following successive divisions, pro-embryos gave rise to somatic embryos at the globular (Fig. 10a-b), transitional globular-heart (Fig. 10c-d), heart

(Fig. 10e-h) and torpedo (Fig. 10i-j) stages, similar to observations for zygotic embryos in dicotyledons. The protoderm (Fig. 10f-g) and procambium (Fig. 10f-h) were observed in the somatic embryo at the heart stage (Fig. 10i).

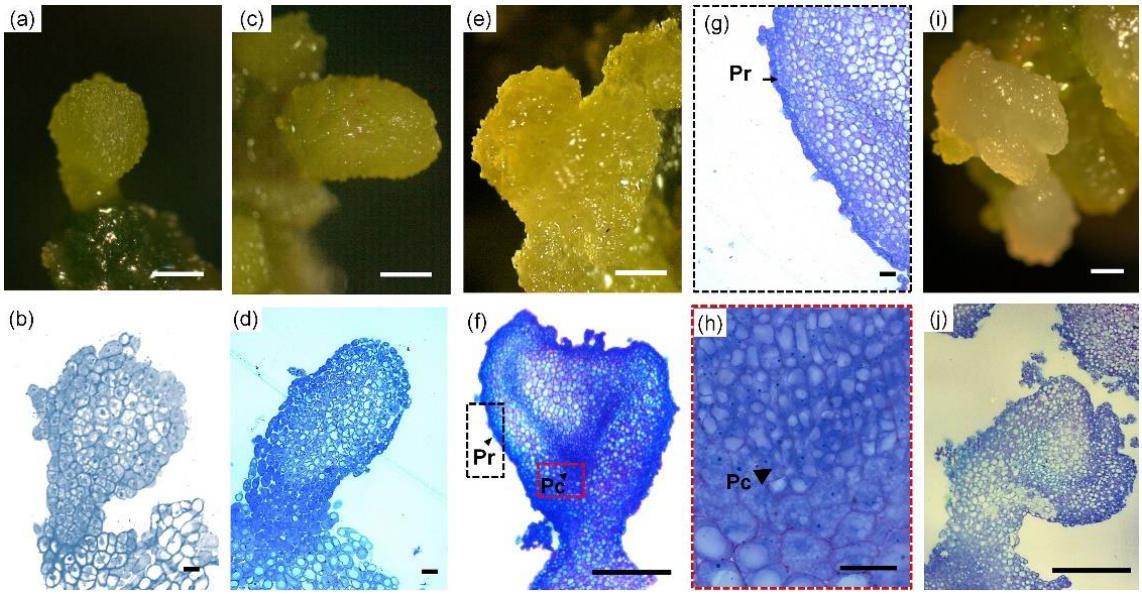

Figure 10. Morphological and histological features of *P. echinata* somatic embryo differentiation and development in callus incubated in 25 µM ABA treatment, showing somatic embryos at the globular (**a-b**), transient globular-heart (**c-d**), heart (**e-h**) detailing protoderm (Pr) and procambium (Pc) cells, and torpedo (**i-j**) stages. Histological sections were stained with TB-O. Bars: **a, c, e, f, i, j** = 0.5 mm; **b, d, g, h** = 20 µm.

3.4. Effects of ABA treatment on endogenous free PA content

Analyses of free PAs were performed using samples from the green regions of callus incubated for 180 days in 0 (control) and 25 µM ABA treatments. The 25 µM ABA treatment induced the formation of somatic embryos at earlier developmental stages from green nodular regions. However, the green regions of callus in the control treatment induced cellular proliferation without somatic embryo development. The ABA treatment affected the content of total free PA (Fig. 11a) and the free PA ratio (Fig. 11b), which were higher in callus incubated in the control treatment and significantly reduced in callus treated with 25 µM ABA. The ABA treatment also affected the endogenous contents of free Put, which were significantly lower with 25 µM ABA treatment than in the control (Fig. 11c), showing that ABA can reduce endogenous Put during and allow somatic embryo formation

at an earlier stage of development (Fig. 11c). In contrast, the endogenous content of free Spd and Spm was not affected by the ABA treatment (Fig. 11c).

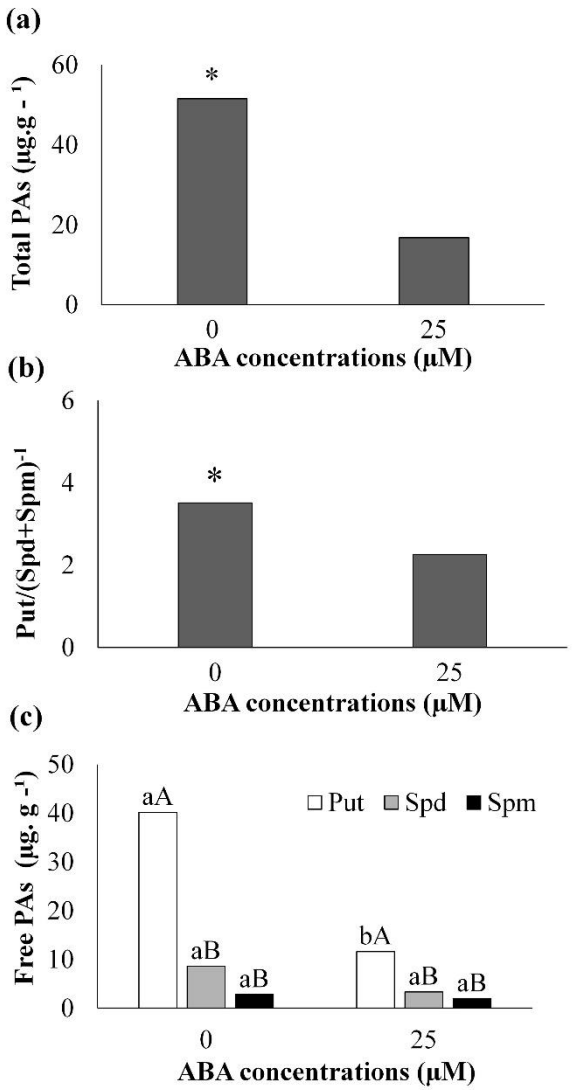

Figure 11. Contents of total free PAs (**a**), PAs ratio of Put/(Spd+Spm) (**b**) and free Put, Spd and Spm contents (**c**) in callus of *P. echinata* after 180 days of incubation without (control) and with 25 µM ABA treatments. Asterisks indicate significant differences for total free PAs and the PA ratio between ABA treatments (0 or 25 µM ABA). Lowercase letters indicate significant differences for each PA (Put, Spd, and Spm) between the two ABA (0 and 25 µM) treatments. Capital letters indicate significant differences for Put, Spd, and Spm between each ABA (0 or 25 µM ABA) treatment. Means followed by different letters are significantly different according to the SNK test ($P < 0.05$). CV = coefficient of variation. (n = 3; CV of total PAs = 18.84 %; CV of PA ratio = 16.21 %; CV of free Put, Spd and Spm = 30.07 %).

4. DISCUSSION

The use of immature seeds of *P. echinata*, which contain zygotic embryos at the cotyledonary stage, enabled callus induction under all concentrations of tested PGRs (Fig. 2). Traditionally, somatic embryo formation in several species involves treating cells with auxins, of which 2,4-D is the most commonly applied, with the auxins occasionally being combined with cytokinins (Jiménez and Thomas, 2006; Nogueira et al., 2007; Braybrook and Harada, 2008; Avilés-Viñas et al., 2013; Fehér, 2015). The effects of 2,4-D on the induction of pau-brasil callus has been previously examined, and it was shown that auxins (Werner et al., 2009) as well as the combination of auxins (2,4-D or IBA) and cytokinins (BA) are essential to induce callus in young leaflet explants (Werner et al., 2010). However, the callus did not demonstrate the evolution of pro-embryo structures to somatic embryo development in this species (Werner et al., 2009; Werner et al., 2010).

Differences in colors (beige, white, brown, and dark-brown colors) and consistency (friable and compact) of callus induced were observed (Fig. 3). After multiplication of these callus-types for two subcultures, there was a predominance of brown callus (Fig. 5a) with meristematic regions (Fig. 5b-f, dotted areas). The presence of this type of cell is important because once in the somatic embryogenesis pathway, the competent state is attributed to the presence of meristematic cells, which acquire the competence to develop into somatic embryos under adequate stimulation (Fehér et al., 2003). Thus, morphological analysis provides inter- and intracellular characteristics that enable the distinction between cell types and provide clues to inherent developmental capacities for somatic embryogenesis and morphogenesis for many species (Steiner et al., 2016).

In addition, the histochemical analysis of brown friable regions of callus during the multiplication phase revealed the presence of pectic substance (Fig. 5d), polysaccharides (Fig. 5e), and proteins (Fig. 5f). Pectic substances are important

for intercellular adhesion and contribute to the mechanical properties of cell walls, such as porosity, elasticity, adhesion, and ion binding (Verdeil et al., 2001; Pilarska et al., 2013). Additionally, studies have proposed that the extracellular matrix may be involved in recognizing embryogenic cells and regulating early embryogenic stages because weakly methylated pectins and arabinogalactan proteins may play important roles in cell-to-cell adhesion and plant morphogenesis, respectively (Verdeil et al., 2001; Pilarska et al., 2013). Moreover, polysaccharides are associated with cell elongation and cell expansion capacity, which is one of the mechanisms responsible for the formation of embryogenic cells (George and Debergh, 2008). These data show that it is possible to establish a somatic embryogenesis system from using immature seeds as explants and ABA to promote the formation of somatic embryos at earlier stages.

The importance of ABA in the promotion and maturation of somatic embryogenesis has previously been suggested for several species (Normah et al., 2013). Among all the treatments tested for somatic embryo development in *P. echinata*, the 25 µM ABA induced the formation of callus characterized by green nodular regions (Fig. 7) containing meristematic cell groups, which later resulted in the formation of pro-embryos (Fig. 9) and subsequently somatic embryos at earlier developmental stages (Fig. 10). Thus, the addition of 25 µM ABA was primordial for somatic embryo formation at earlier development stages in *P. echinata*, showing that it is possible to establish a somatic embryogenesis system using immature seeds as explants and to use ABA to promote the formation of somatic embryos at earlier stages. The application of ABA during somatic embryogenesis maturation is more commonly reported in conifers because this PGR is generally associated with osmotic agents such as PEG or maltose, which induce the alteration of osmotic potential to similar conditions observed during the early stages of zygotic embryo development (Steiner et al., 2008). In addition, in *Daucus carota*, different stress conditions promote an increase in endogenous ABA levels, stimulating the acquisition of embryogenic competence from somatic cells and the development of somatic embryogenesis. ABA also plays an important role during somatic embryo initiation in *Arabidopsis*, indicating that the presence of this PGR is related to local auxin biosynthesis, auxin polar transport, and subsequently the establishment of auxin response patterns (Su et al., 2013). In *Nicotiana plumbaginifolia* cultures, ABA is required to promote the formation of pre-globular structures (Senger et al., 2001).

Moreover, the addition of ABA into maturation medium stimulates the development of normal somatic embryos in *Juglans regia* (Vahdati et al., 2008) and higher numbers of somatic embryos with well-organized morphologies in *Podocarpus lambertii* (Fraga et al., 2016). However, in some species, such as *Podophyllum peltatum* (Kim et al., 2007), the pre-incubation of embryogenic callus in ABA followed by subculture in PGR-free medium increases the high frequency of somatic embryos (Kim et al., 2007).

Studies suggest that ABA is also involved in the accumulation of storage proteins (Stasolla and Yeung, 2003), such as late embryogenesis abundant (LEA) proteins, PA synthesis, and cellular expansion control (Steiner et al., 2008), as well as increased production of storage reserves (proteins, triglycerides and lipids) and increased sucrose absorption or starch synthesis (Higgins et al., 1982; Feirer et al., 1989; Fernando and Gamage, 2000; Stasolla and Yeung, 2003). In the present study, histochemical analyses showed that 25 µM ABA treatment induced the presence of starch grains in meristematic cells of green regions, which was more evident during the initial formation of pro-embryos (Fig. 9d). Similarly, the presence and accumulation of starch have been observed in cells of embryogenic callus in *Gentiana punctata* (Mikula et al. 2004) and to precede the development of somatic embryos in *Bactris gasipaes* (Steinmacher et al. 2011). Starch is considered a primary source of energy for cell growth and proliferation, supplying energy for the somatic embryo development (Pinto et al., 2010; da Silva et al., 2015). Thus, starch grains may be used for somatic embryos during evolution in *P. echinata*.

In addition, a positive reaction to CBB was observed in the cytoplasm and nuclei of meristematic type-cells and in pro-embryos at different evolutionary stages following the treatment with 25 µM ABA (Fig. 9f). The proteins are important to regulate cell expansion and to generate biophysical characteristics required for seedling morphogenesis (Jiménez, 2001), and they can be consumed during the induction of somatic embryogenesis and are available for the re-differentiation of somatic cells into competent embryogenic cells (Cangahuala-Inocente et al., 2009). This phenomenon has also been observed in zygotic embryos of *Araucaria angustifolia* as cytological characteristics during embryo development (Rogge-Renner et al., 2013).

Additionally, compared to the control treatment, the effects of ABA on endogenous free PAs, especially the significant reduction of the content of free Put

in callus incubated in 25 µM ABA treatment, allowed the development of somatic embryos at different stages (Fig. 11c). Put is related to the control of cell cycle progression at the G1/S and G2/M transition phases in *Catharanthus roseus* suspension culture (Maki et al., 1991) and induces cell division in *Prunus dulcis* cv. Nonpareil protoplasts (Wu and Kuniyuki, 1985). The exogenous application of PAs modulates the growth and development of somatic embryos in *Gossypium hirsutum* (Sakhanokho et al., 2005; Cheng et al., 2016), *A. angustifolia* (Steiner et al., 2007) and *Panax ginseng* (Kevers et al., 2000). Exogenous Put is also associated with the multiplication of embryogenic cultures in some trees such as *Ocotea catharinensis* (Santa-Catarina et al., 2007) and *A. angustifolia* (Silveira et al., 2006). In embryogenic cultures of *A. angustifolia*, the exogenous Put may increase nitric oxide content and promote the growth of pro-embryogenic masses (PEMs) (Silveira et al., 2006), as demonstrated by the addition of exogenous nitric oxide in this species, thus maintaining multiplication without differentiation (Osti et al., 2010). Additionally, in this same species, exogenous Put promotes an increase in the endogenous content of the auxin indole 3-acetic acid (Steiner et al., 2007). Thus, a decrease in the endogenous levels of free Put in *P. echinata* callus induced by ABA treatment may reduce cell growth, facilitating the evolution of embryogenic cells to somatic embryos through the different observed developmental stages. In this context, the development of somatic embryos at the torpedo stage in *P. echinata* may be related to the modulation of endogenous PAs induced by ABA. Further studies involving the exogenous application of PAs will be developed to promote the maturation of somatic embryos at the cotyledonary stages and the conversion of somatic embryos to somatic seedlings.

An overview of the main steps and primary results of the present study is shown in Figure 12. The results of the present study represent the first evidence of somatic embryo development in immature seeds and provide information for establishing somatic embryogenesis in *P. echinata*, a tree from the Brazilian Atlantic Rain Forest with great ecological, economic and cultural importance. This information will be important for further studies related to germplasm conservation and alternative methods of *P. echinata* propagation.

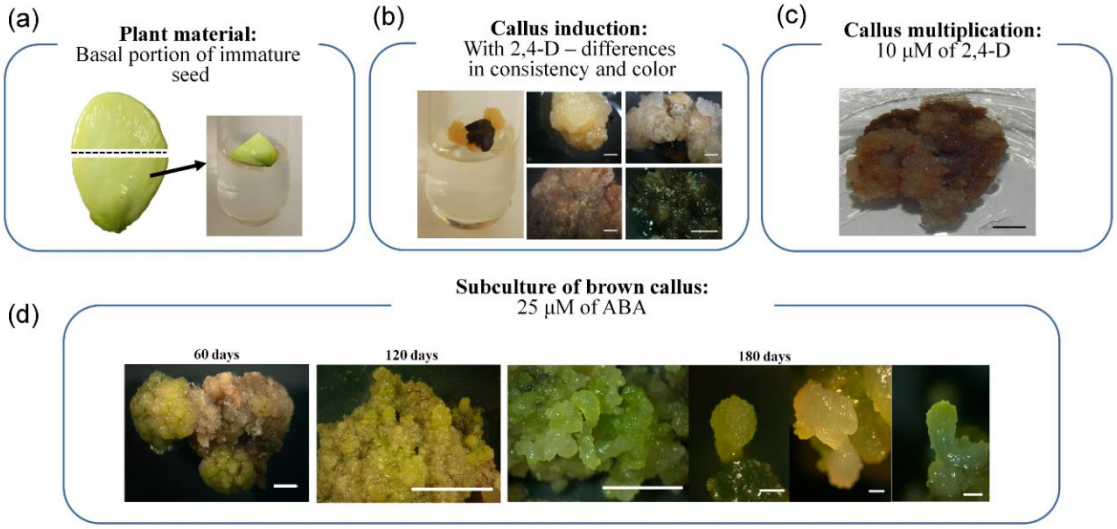

Figure 12. Steps of somatic embryogenesis of *P. echinata* from immature seeds. The basal portion of the immature seed, used as explant (**a**), was inoculated in culture medium with different concentrations (0, 10, 50 and 10 µM) 2,4-D for callus induction (**b**). The callus obtained during the induction phase were subsequently multiplied using 10 µM 2,4-D, which was performed in two subcultures, to obtain brown callus containing regions of friable and compact consistency, described as embryogenic callus (**c**). The embryogenic callus incubated with 25 µM ABA treatment induced the formation of green nodular regions at 60 days, allowing the formation of globular somatic embryos at 120 days, and somatic embryos at different earlier stages (globular, heart and torpedo) of development after 180 days of incubation (**d**). Further studies investigating the progression of somatic embryos into the cotyledonary stages using other PGRs, such as PAs, and their subsequent conversion into somatic plants are required for this species

5. CONCLUSION

Thus, we conclude that immature seeds of *P. echinata* are suitable explants for the induction of somatic embryogenesis and that the use of 2,4-D is essential for callus induction and multiplication, resulting in the formation of cells with meristematic characteristics. Treatment with ABA (25 µM) promoted the development of somatic embryos at the globular, heart and torpedo stages, which could be related to the modulation of endogenous free PAs, by the significant reduction of free Put content. Finally, it is important to emphasize that the data

presented herein refer to an initial study of somatic embryogenesis in *P. echinata*, and further studies are necessary to optimize the somatic embryogenesis process, particularly the maturation and conversion of somatic embryos into plants.

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4.2. THE RELATIONSHIP OF BENZYLADENINE AND POLYAMINES ON SHOOT DEVELOPMENT AND OF THE INDOL BUTYRIC ACID ON MICROCUTTING ROOTING IN *Cedrela odorata* L.

RESUMO

Cedrela odorata L. (Meliaceae) é uma das árvores florestais mais valiosas nos trópicos, sendo amplamente apreciada pela madeira de alta qualidade. Devido à exploração indiscriminada, esta espécie está atualmente em perigo. A cultura *in vitro* pode ser um método alternativo de propagação, produzindo mudas que podem ser usadas tanto na conservação quanto para fins comerciais. Na propagação *in vitro*, as citocininas são moléculas envolvidas na indução de brotos e podem modular o metabolismo endógeno de compostos relacionados com respostas morfogenéticas, como as poliaminas (PAs). Em várias espécies, o uso de auxinas é necessário para a indução do enraizamento adventício. Desta forma, o presente estudo tem como objetivo avaliar os efeitos da 6-benziladenina (BA) e do tipo explante na organogênese *in vitro* e no conteúdo endógeno de PAs livres, e o efeito do ácido indolbutírico (AIB) e tamanho do explante no enraizamento de microestacas de *C. odorata*. A adição de BA e o tipo de explante influenciaram a resposta morfogenética. Segmentos nodais cotiledonares incubados em 20 µM de

BA possibilitaram o maior alongamento das brotações, verificando-se um aumento significativo no conteúdo da PA putrescina, a qual pode estar associada à promoção do alongamento dos brotos. As microestacas obtidas a partir de segmentos nodais cotiledonares incubados em meio de cultura com BA (1-20 µM) induziram raízes tanto na ausência como na presença de AIB. Microestacas com 0,5 e 1 cm obtidas de brotações induzidas em meio de cultura com 20 µM de BA a partir de segmentos nodais apicais e cotiledonares, e enraizadas em 5 µM de AIB apresentaram uma resposta efetiva no enraizamento, aclimatização e crescimento subsequente na casa de vegetação, e podem ser usadas para a produção de mudas micropropagadas de *C. odorata*.

Palavras-chave: benziladenina; cedro; cultura *in vitro*; Meliaceae; micropropagação.

ABSTRACT

Cedrela odorata L. (Meliaceae) is one of the most valuable forest tree in the tropics, being widely appreciated for the wood of high quality. Due to overexploitation, this species is currently endangered. *In vitro* culture can provide an alternative method of propagation, producing plantlets that can be used in conservation as well as in commercial purposes. On *in vitro* propagation, cytokinins are essential molecules involved in shoot induction, and can modulate the endogenous metabolism of compounds related to morphogenesis responses, such as polyamines (PAs). In several species, the use of auxins is necessary for adventitious rooting. In this way, the present study aims to evaluate the effects of 6-benzyladenine (BA) and explant type (axillary and cotyledonary nodal segments) on *in vitro* organogenesis and endogenous content of free PAs, and the effect of indolebutyric acid (IBA) and size of explant on microcutting rooting *in C. odorata*. The addition of BA and type of explant influenced the morphogenetic response. Cotyledonary nodal segments incubated in 20 µM BA promoted the higher

elongation of shoots, with a significant increase in the contents of PA putrescine, which could be associated to the promotion of shoot elongation. Microcuttings obtained from cotyledonary nodal segments incubated in BA (1-20 µM) induced root both in the absence and in presence of IBA. Microcuttings with 0.5 and 1 cm obtained from shoots induced in culture medium with 20 µM BA from axillary and cotyledonary nodal segments, and rooted with 5 µM IBA showed an effective response in the rooting, acclimatization and subsequent growth at greenhouse, and could be used in the production of *C. odorata* micropropagated plantlets.

Keywords: benzyladenine; cedar; *in vitro* culture; Meliaceae; micropropagation.

1. INTRODUCTION

Cedrela odorata L. (Meliaceae), popularly known as cedar, spanish cedar, cigar-box wood, red cedar, acajou-bois, acajou rouge, cedro rojo, occurs in the neotropical region. In Brazil this species occurs naturally in Atlantic Forest, Amazon rainforest, Caatinga and Cerrado biomes. Due to the high quality, the *C. odorata* wood is among the most commercialized and valued in the world (Cavers et al., 2003; Luchi, 2011; Martinelli and Moraes, 2013). However, the population of *C. odorata* has declined significantly due to its exploitation over than 200 years, and currently, this species is included in the red list of threatened species from the International Union for Conservation of Nature (IUCN), classified as vulnerable (Martinelli and Moraes, 2013; Mark and Rivers, 2017).

Seeds and vegetative methods can be used to propagate this species. *C. odorata* seeds showed a reduction in viability during nine months of storage. Before storage, the seeds presented 99 % of germination and 79 % of normal seedlings. After nine months of storage at low temperature (7-8 °C), germination was reduced to 88 % and normal seedlings to 23 %. When the seeds were stored at room temperature (24-26 °C), there was no germination at six months storage. However, at three months, no normal seedlings were observed (Batista et al., 2011). By

vegetative propagation, *C. odorata* cuttings treated with 0.2 % of IBA showed 64 % rooting using sand as substrate (Maldonado, 1991). In addition, studies on the grafting were also developed for this species, aiming to confer resistance to the *Hypsipyla grandella* (Insecta: Pyralidae), which destroys the axillary meristems and produces structural deformations in the trees (Grijpma and Roberts, 1976).

Studies using biotechnological techniques, such as micropropagation and somatic embryogenesis, were applied in the propagation of *C. odorata* (Maruyama et al., 1989; Rodríguez et al., 2003; Pérez et al., 2005; Peña-Ramírez et al., 2011) and for several other endangered species (Golle et al., 2009; Pijut et al., 2011). However, there are no reports relating the effect of explant type (axial and cotyledonary nodal segments) and the relationship of endogenous PAs on *in vitro* organogenesis for this species. As well as, there are no reports about the influence of microcuttings size on *in vitro* rooting and acclimatization of *C. odorata* plantlets. This approach could be used to study the metabolism, differentiation, and morphogenesis of plant cells, providing information about biochemical and physiological events during the modulation of *in vitro* morphogenesis.

Usually, several wood species are *in vitro* propagated by direct organogenesis (Maruyama et al., 1989; Pérez et al., 2005; Parimalan et al., 2011; Pijut et al., 2012; Aragão et al., 2016; Canatto et al., 2016). The induction of *in vitro* shoots is usually accomplished by the addition of one cytokinin into the culture medium, being the 6-benzyladenine (BA) the most commonly used for this purpose (Pijut et al., 2011; Pijut et al., 2012), breaking axillary dominance and stimulating shoot proliferation (Giri et al., 2004; Mohebalipour et al., 2012). The addition of BA into the culture medium may also modulate the endogenous metabolism of some compounds that play important roles during *in vitro* organogenesis, such as PAs, exhibiting a synergistic effect to enhance differentiation during micropropagation (Vasudevan et al., 2008; Arun et al., 2014; Aragão et al., 2016).

The PAs are low-molecular weight, aliphatic, polycationic compounds carrying positive charges on nitrogen atoms, a property which facilitates electrostatic interactions with macromolecules with negative charges, such as DNA, RNA, phospholipids, cell wall components, and proteins (Wallace et al., 2003). PAs have been described as important signals acting in several cellular processes during growth and development (Bais and Ravishankar, 2002; Wallace et al., 2003; Baron et al., 2008). In plants, putrescine (Put), spermidine (Spd) and spermine (Spm) are

the main PAs, being related to several physiological and developmental process (Santa-Catarina et al., 2007; Pieruzzi et al., 2011; Dutra et al., 2013; Tiburcio et al., 2014), including organogenesis (Parimalan et al., 2011; Arun et al., 2014; Vondráková et al., 2015; Aragão et al., 2016). The investigations of changes in the PA contents during *in vitro* shoot induction and development in *C. odorata* could be important to understand the mechanism by which plant cells become competent for the development of specific morphogenetic routes, which might contribute to the success of *in vitro* morphogenesis and genetic transformation in tree species.

Rooting and acclimatization are fundamental steps for the production of micropropagated plants. *In vitro* rooting is characterized by the induction of adventitious roots in microcuttings, usually achieved with an auxin being applied into the culture medium (Oliveira et al., 2013). Among the auxins, the IBA has been widely used due its low phytotoxicity to the explants, providing positive results in *in vitro* rooting (Millán-Orozco et al., 2011; Oliveira et al., 2013). The *in vitro* rooting therefore aims to obtain complete plantlets for subsequent transplantation and acclimatization for *ex vitro* conditions (Oliveira et al., 2013).

The acclimatization is the most critical step of the regeneration process. In this step, micropropagated plantlets are exposed to changes in the environmental conditions when transferred to an *ex vitro* condition, passing from a heterotrophic to an autotrophic state. In these new conditions, the plantlets will have to activate the mechanisms related to the control of water and gaseous changes by cells to be adapted in the new condition, modulating the stomatal conductance, and increase the photosynthetic rate under conditions of a more abundant CO_2 atmosphere (Rocha et al., 2008).

In this sense, the objective of this work was to evaluate the effects of BA and type (axillary and cotyledonary nodal segments) of explants on *in vitro* organogenesis and on endogenous content of free PAs, and the effects of IBA and size of microcuttings on *in vitro* rooting in *C. odorata*.

2. MATERIAL AND METHODS

2.1. Plant material

Mature seeds of *C. odorata*, obtained from the Sementes Caiçara® located in Brejo Alegre, São Paulo State, Brazil (21°10'S and 50°10'W) were used for *in vitro* germination. Axillary and cotyledonary nodal segments obtained from seedlings germinated *in vitro* were used for *in vitro* organogenesis experiment. For rooting and acclimatization, microcuttings (0.5 and 1 cm) obtained from shoots induced from axillary and cotyledonary nodal segments were used.

2.2. *In vitro* germination

To *in vitro* germination, seeds were surface-disinfested according to Aragão et al. (2016). The seeds were washed with 250 mL distilled water containing few drops of liquid detergent, followed by immersion in 70 % ethanol (Merck, Darmstadt, Germany) for 1 min, followed by 60 min in commercial bleach Qboa® (Ahembi SA, São Paulo, Brazil), containing 2-2.5 % of active chlorine, supplemented with 100 $mg.L^{-1}$ the fungicide Derosal 500 SC (Bayer, São Paulo, Brazil) (active ingredient carbendazim).

The seeds were subsequently washed, three times of 10 min each, in sterile distilled water in a laminar flow, and inoculated in the MS (Murashige and Skoog, 1962) (PhytoTechnology Laboratories, Shawnee Mission, USA) and Woody Plant Medium (WPM) (Lloyd and McCown, 1981) (Phytotechnology Lab) culture media. Both culture media were supplemented with 30 $g.L^{-1}$ sucrose (Vetec, Rio de Janeiro, Brazil) and 2 $g.L^{-1}$ Phytagel® (Sigma-Aldrich, St. Louis, USA). The pH of the culture media was adjusted to 5.8 before the addition of Phytagel® and subsequently, autoclaved at 121 °C, 1.5 atm, for 15 min.

After inoculation, the seeds were maintained in a culture room, at 25 ± 2 °C with a 16-h photoperiod at light intensity of 22 µmol m^2s^{-1}. Ten replicates of ten seeds each, with 100 seeds per treatment, were used. After 30 days of inoculation, the percentage of germinated seeds and normal seedlings development were evaluated.

2.3. Effects of BA and type of explant on induction and development of shoots

Sixty-days-old *in vitro* germinated seedlings (Fig. 1a) were used to obtain axillary and cotyledonary nodal segments (1-1.5 cm) (Fig. 1b), used on *in vitro* organogenesis experiments. The segments were isolated and inoculated in culture glass bottle (250 mL) (Aapace, São Paulo, Brazil) containing 30 mL of MS culture medium, supplemented with 20 $g.L^{-1}$ sucrose, 2 $g.L^{-1}$ Phytagel® and different concentrations (0, 1, 5, 10, 15, 20 and 25 µM) of BA (Sigma-Aldrich). The pH of culture medium was adjusted to 5.8 before being autoclaved at 121 °C, 1.5 atm, for 15 min.

After inoculation, the explants were maintained in the growth room at 25 ± 2 °C with a 16-h photoperiod at light intensity of 22 µmol m^2s^{-1}. Each treatment consisted of eight replicates, being each replicate characterized by a culture glass bottle containing 5 explants, with a total of 40 explants per treatment. At 60 days of incubation, the number and length (cm) of shoots were analyzed.

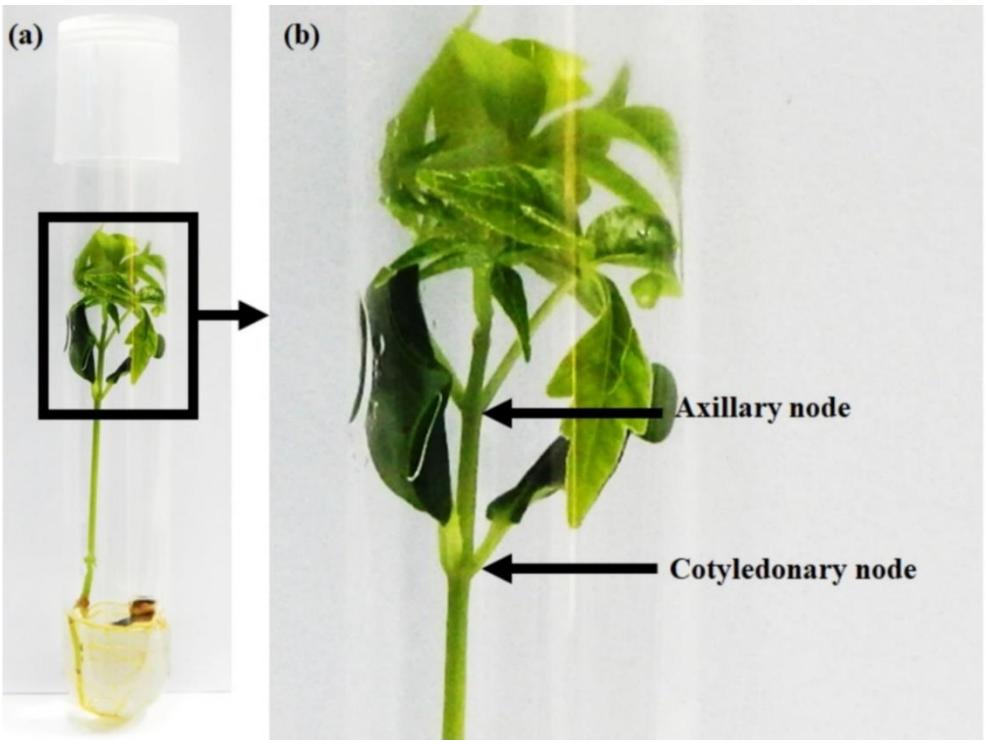

Figure 1. Sixty-days-old *in vitro* germinated seedlings (**a**) used to obtain axillary and cotyledonary nodal segments (**b**), used on *in vitro* organogenesis experiments.

2.4. Effects of IBA on *in vitro* microcuting rooting from shoots induced in different BA concentrations

To determine the effects of IBA on *in vitro* rooting, sixty-day-old shoots obtained from cotyledonary nodal segments incubated in different concentrations of BA (0, 1, 5, 10, 15, 20 and 25 µM) were used to obtain the microcuttings (~1.5 cm). The microcuttings isolated were inoculated in culture glass bottle (250 mL) containing 30 mL of MS culture medium supplemented with 20 $g.L^{-1}$ sucrose, 2 $g.L^{-1}$ Phytagel® and different concentrations (0, 5 and 10 µM) of IBA (Sigma-Aldrich). The pH of culture medium was adjusted to 5.8 before being autoclaved at 121 °C, 1.5 atm, for 15 min. After inoculation, the explants were mantained in the growth room at 25 ± 2 °C with a 16-h photoperiod, at light intensity of 22 µmol m^2s^{-1}. Each treatment consisted of 4 replicates, being each replicate characterized by a culture glass bottle containing 3 explants, with a total of 12 explants per treatment. The percentage of root induction was evaluated at 30 days of incubation.

2.5. Effects of microcuttings size and origin of explant on *in vitro* rooting and aclimatization

Sixty-day-old shoots obtained from axillary and cotyledonary nodal segments cultivated in 20 µM BA were sectioned to obtain the microcutings (0.5 and 1 cm). These microcuttings were inoculated, according to their origin and size, in culture glass bottle (250 mL) containing 30 mL of MS culture medium supplemented with 20 $g.L^{-1}$ sucrose, 2 $g.L^{-1}$ Phytagel® and 5 µM IBA. The pH of culture medium was adjusted to 5.8 before autoclavage at 121°C, 1.5 atm, for 15 min. Each treatment consisted of 16 replicates, being each replicate characterized by a culture glass bottle containing 7 nodal segments, with a total of 112 explants per treatment. At 20 days of incubation, the percentage of rooted and non-rooted (with the presence of green leaves) microcuttings were evaluated.

The plantlets (rooted microcuttings) and microcuttings (non-rooted) obtained at the end of the *in vitro* rooting were transferred to the pre-acclimatization, maintaining the same treatments (size and origin of the microcuttings). The plantlets and microcuttings were transferred to plastic cups (50 mL), containing vermiculite and commercial Basaplant® substrate (Base substratos, Pará Holambra, Brasil) (1:1; v/v), and then were organized in plastic trays covered with a PVC film to keep a high humidity. These samples were keep in the pre-acclimatization room with a 16-h photoperiod at light in tensity of 22 µmol m^2s^{-1} at 25 ± 2 °C. After 15 days, the humidity into the tray was gradually reduced until 25 days, when the plantlets and microcutings were maintained in the trays without PVC film. The survival of plantlets during pre-acclimatization was evaluated at 45 days from 7 replicates, containing 5 plantlets each, with a total of 35 plantlets per treatment. The percentage of rooted microcuttings on *ex vitro* condition after 45 days of pre-acclimatization was obtained from 9 replicates, with 5 microcuttings each, in a total of 45 microcuttings per treatment.

In addition, the analysis of volume (cm^3) and length (cm) of roots, the fresh (FM) and dry matter (DM) of roots and aerial parts, as well as the number of leaves. The analyzes were performed from five replicates, with five explants each, totaling 25 plantlets per treatment, being plantlets originated from axial and cotyledonary microcuttings with initial size of 0.5 and 1 cm.

For the analysis of FM, the aerial and radicular parts were separated and weighed in a precision scale, replace in paper bags (11x12 cm) and dried in a oven

with forced ventilation at 70 °C, during 72 h. After, the samples were weighed again in a precision balance to determine the DM. The volume of roots was determined according to Frazão et al. (2010). The roots were placed in a graduated cylinder, containing a known volume of water. By the difference, the direct response of the root volume was obtained. The length of the roots and the aerial part was realized with the aid of a graduated rule.

2.6. Growth of plantlets in the greenhouse

The rooted plantlets obtained in the pre-acclimatization, with at least 1.5 cm length, were transferred to the greenhouse. The plantlets were transferred to plastic cups (200 mL) containing 100 % Basaplant® substrate, and kept in the pre-acclimatization room with a 16-h photoperiod at light in tensity of 22 μmol m^2s^{-1} at 25 ± 2 °C, for 15 days.

After, the plantlets were ttransferred to the greenhouse for 60 days, from October to December 2016. In this period, the maximum and minimum temperature registered in the Campos dos Goytacazes, RJ - Brazil (21º45'15"S and 41º19'28"W) were 28 °C and 22 °C, respectively (INMET, 2016). Five replicates, being each replicate characterized by 5 rooted plantlets, with a total of 25 plantlets was used to each treatment, considereing the origin (axillary and cotyledonary nodal segments) and size (0.5 and 1 cm) of microcuttings.

After 60 days in the greenhouse, the percentage of survival of plantlets, and the FM and DM of aerial and radicular parts of the plantlets were evaluated. In addition, the length (cm), stem diameter (cm^2), number of leaves, leaf area (cm^2) and specific leaf area ($cm^2.g^{-1}$) were obtained from the aerial part. In relation to the radicular part, the root length (cm), surface area (cm^2) and volume of roots (cm^3) were also evaluated. The Dickson quality index (DQI), total FM and total DM were also evaluated.

The FM and DM of aerial and radicular parts of the plantlets were evaluated, as described in item 2.5. The length of the aerial part was performed using a ruler, and the diameter of stem was determined using a digital pachymeter. The leaf area was determined using a bench meter LI-3100 (LI-COR, Lincoln, USA), and specific leaf area ($cm^2.g^{-1}$) was obtained dividing the values of leaf area by DM of leaves (Magalhães and Ferri, 1979). To analyze the root system of plantlets at 60 days in the greenhouse, the complete root system (without substrate) of each plantlet was

placed in an acrylic cube (30 x 40 cm) containing distilled water and analyzed using WinRhizo Pro 2012b software (Regent Instruments Inc., Quebec, Canada) connected to a professional Epson XL 10000 scanner with a resolution of 400 dpi. The volume (cm^3), surface area (cm^2) and length (cm) of roots were obtained. The length of roots was considered those with size equal or greater than 1 cm.

The DQI was determined according to Dickson et al. (1960), using the formula: total DM/ [(areial part lenght / stem diameter) + (aerial part DM / root DM)].

2.7. Free PA determination

Analyses of free PAs were performed from three samples (200 mg FM each) of shoots before (time 0) and after 7, 30 and 60 days of incubation in 0 and 20 µM BA treatments, according to Santa-Catarina et al. (2006). The samples were macerated in 1.2 mL of 5 % perchloric acid (Merck, Darmstadt, Germany), and incubated at 4 °C, for 1 h. After, the samples were centrifuged for 20 min at 20.000 g at 4 °C, and the supernatant, containing the free PAs, were collected.

Free PAs were determined directly from the supernatant by derivatization with dansyl chloride (Merck) and identified by high performance liquid chromatografy (HPLC - Shimadzu, Kyoto, Japan) using a 5 µm-C18 reverse-phase column (Shimadzu Shin-pack CLC ODS). The HPLC column gradient was developed by mixing increasing proportions of absolute acetonitrile (Merck) to 10 % acetonitrile in water (pH 3.5), adjusted with HCl (Merck). The gradient of absolute acetonitrile was programmed to 65 % over the first 10 min, from 65 to 100 % between 10 and 13 min, and 100 % between 13 and 21 min, at 1 $mL.min^{-1}$ flow, at 40 °C. The free PAs concentration was determined using a fluorescence detector at excitation of 340 nm and emission of 510 nm. Peak areas and retention times were measured by comparison with the standard PAs Put, Spd and Spm (Sigma-Aldrich).

2.8. Statistical analyzes

All of the experiments were performed using a completely randomized design. The data were analyzed by analysis of variance (ANOVA) ($P < 0.05$) followed by the Student–Newman–Keuls (SNK) test (Sokal and Rohlf, 1995) using the ASSISTAT program Version 7.7 beta (Silva, 2014). When required, the arc-sine$\sqrt{x}/100$ data transformation was applied in order to obtain data normality.

3. RESULTS

3.1. *In vitro* germination

The percentage of germination (Fig. 2a) considering the root protrusion, and normal seedlings development (Fig. 2b) was not affected by the type of culture medium. The normal seedlings was characterized by a well developed root system and aerial part (Fig. 2b). In seedlings from both culture media, the axillary nodal part presented variations of length, being smaller than the cotyledonary nodal ones. Due to this, a length of 1 to 1.5 cm was standardized for both type of segments (axillary and cotyledonary nodal) in the experiments of *in vitro* organogenesis.

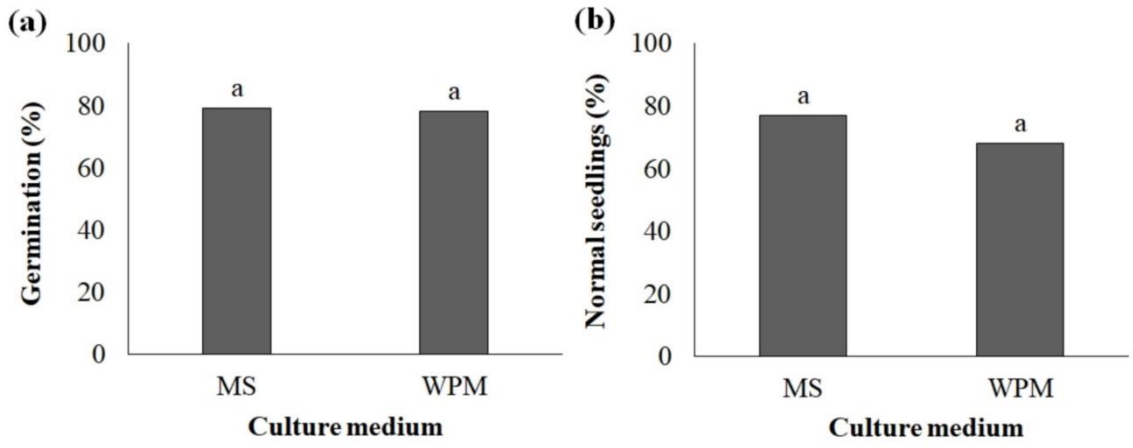

Figure 2. Percentage of germination (**a**) and normal seedlings development (**b**) of *C. odorata* at 30 days of incubation in the MS and WPM culture media. Means followed by the same letter are not significantly different according to the SNK test ($P < 0.05$). Data of germination were transformed by arc-sine$\sqrt{x}/100$. CV = coefficient of variation. (n = 125, CV germination = 17.06 %; CV normal seedlings = 24.81 %).

3.2. Effects of BA and type of explant on induction and development of shoots

The axillary nodal segments showed reduction in the percentage of shoot indcution when incubated in the higher concentration (25 µM) of BA, while no singnificant differences was observed for the percentage of shoot induction from cotyledonary nodal segments (Fig. 3a). Comparing the type of explants, the concentrations of 5, 15 and 25 µM BA showed significant differences for shoot induction, with a significant lower percentage of induction in axillary nodal segments (Fig. 3a).

In relation to the number of shoots per explants, there were no significant differences between the different concentrations of BA and types of explants used (Fig. 3b). On the other hand, the BA concentrations affected signicantly the length of shoots (Fig. 3c). In the shoots obtained from axillary nodal segments it was only observed significant differences in 25 µM BA treatment, presenting the shortest length of shoots, while shoots obtained from cotyledonary nodal segments showed the highest length at 20 µM BA treatment (Fig. 3c). In this sense, the 20 µM is the best concentration of BA for shoot multiplication using cotyledonary and axillary nodal segments.

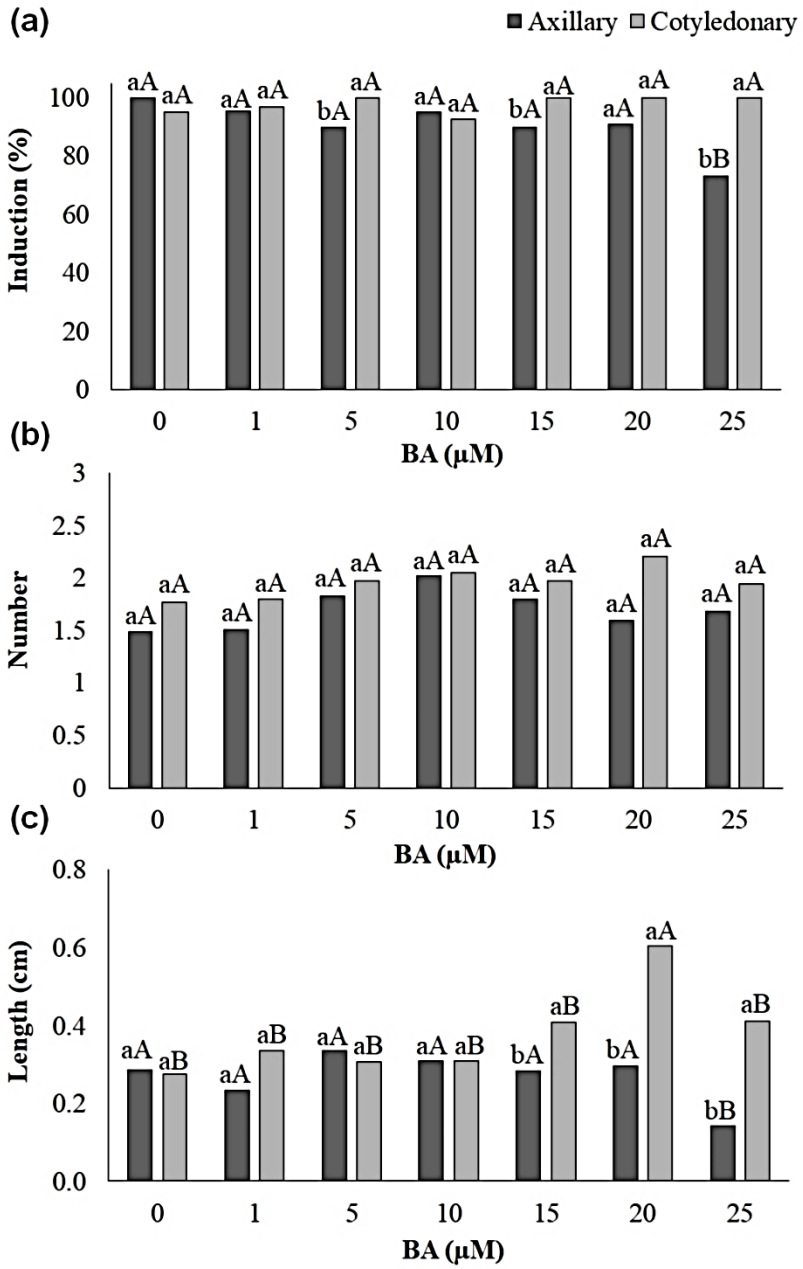

Figure 3. Induction (%) (**a**), number (**b**) and length (cm) (**c**) of shoots from axillary and cotyledonary nodal segments of *C. odorata* at 60 days of incubation in different concentrations (0, 1, 5, 10, 20 and 25 µM) of BA. Lowercase letters compare significant differences between the types of explant (axillary and cotyledonary nodal segments) in each BA concentration (0, 1, 5, 10, 20 or 25 µM). Capital letters compare significant differences for the different concentrations (0, 1, 5, 10, 20 and 25 µM) of BA in each type of explant (axillary or cotyledonary nodal). Means followed by different letters are significantly different according to the SNK test ($P < 0.05$). Data of induction (**a**), number (**b**) and length (**c**) of shoots were transformed by arc-sine$\sqrt{x}/100$. CV = coefficient of variation. (n = 40; CV of shoot induction = 5.22 %; CV of number of shoot = 10.87 %; CV of length of shoot = 17.72 %).

For PAs analysis, the morphology of shoot development was monitored during the 60 days of incubation. The beginning of shoot development was observed on the 7th day of incubation in all treatments, followed by shoot growth and developmental at 30 and 60 days of culture, respectively (Fig. 4).

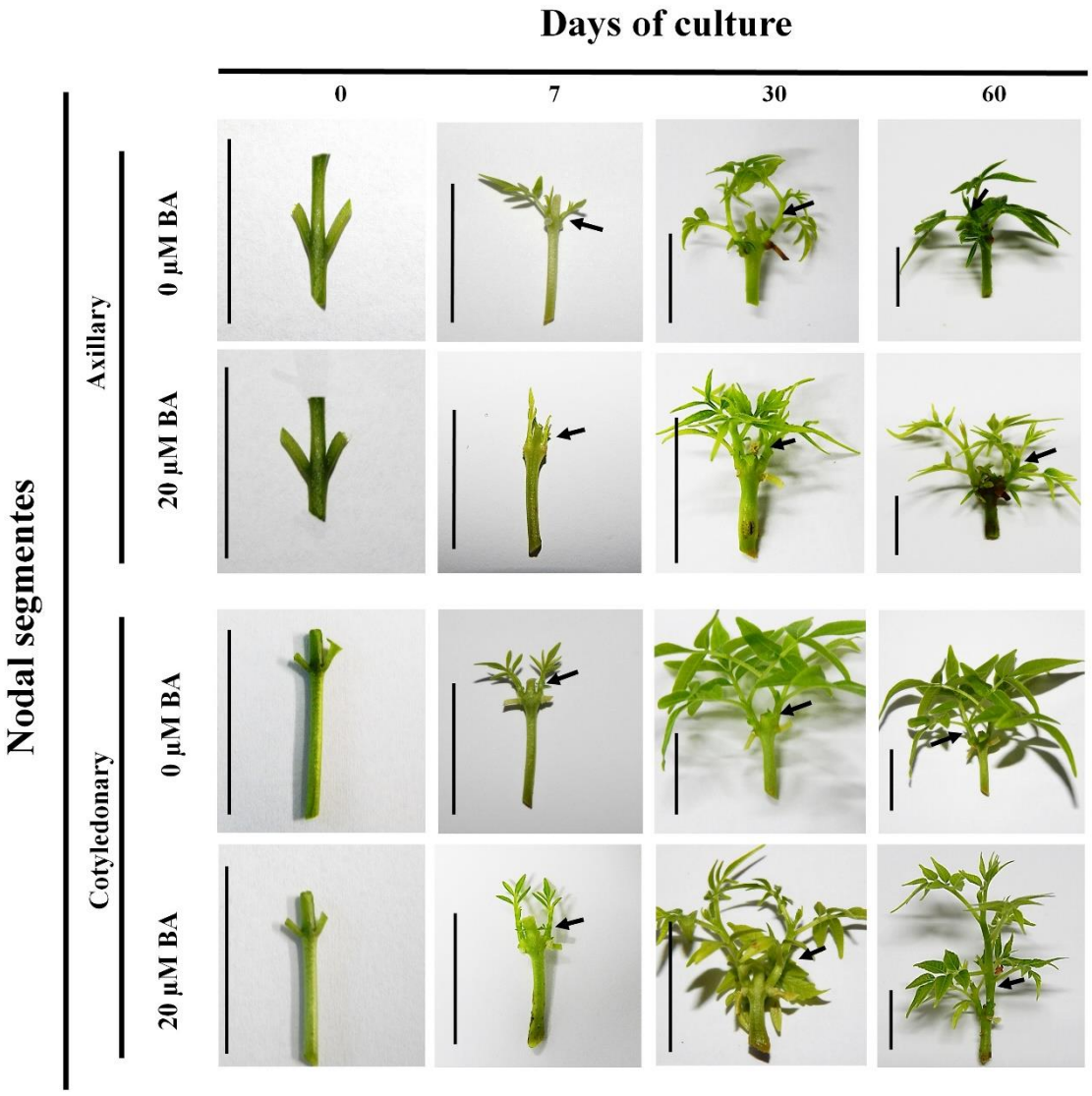

Figure 4. Morphological aspects of shoot development from axillary and cotyledonary nodal segments before (time 0) and after 7, 30 and 60 days of incubation in 0 and 20 µM BA treatments. Arrows indicate the shoots. Bars = 1.5 cm.

3.3. Effects of BA and type of explant on endogenous free PAs contents

Based on morphological analysis (Fig. 4) the content of free PAs was determined from samples of axillary and cotyledonary nodal segments before (time 0) and after 7, 30 and 60 days of incubation in BA (0 and 20 µM). Shoots from both, axillary (Fig. 5a) and cotyledonary (Fig. 5b) nodal segments, presented a higher total free PAs contents when inoculated in 20 µM BA compared with shoots incubated in the control treatment (Fig. 5a, c). In shoots from axillary nodal segments, this increase was observed from the 7th day of culture (Fig. 5a), and at 30th day of culture in shoots from cotyledonary nodal segments (Fig. 5b).

Analyzing the ratio of PAs [Put/(Spd+Spm)]$^{-1}$, no significant differences was observed between the concentrations of BA (0 and 20 µM) applied throughout the incubation period (0, 7, 30 and 60 days) for shoots from axillary nodal segments (Fig 5b). In relation to cotyledonary nodal segments, the values of PAs [Put/(Spd+Spm)$^{-1}$] ratio (Fig. 5d) were significant higher in shoots incubated in BA treatment compared to control (without BA), with the highest contents observed at 30 and 60 days of cultivation.

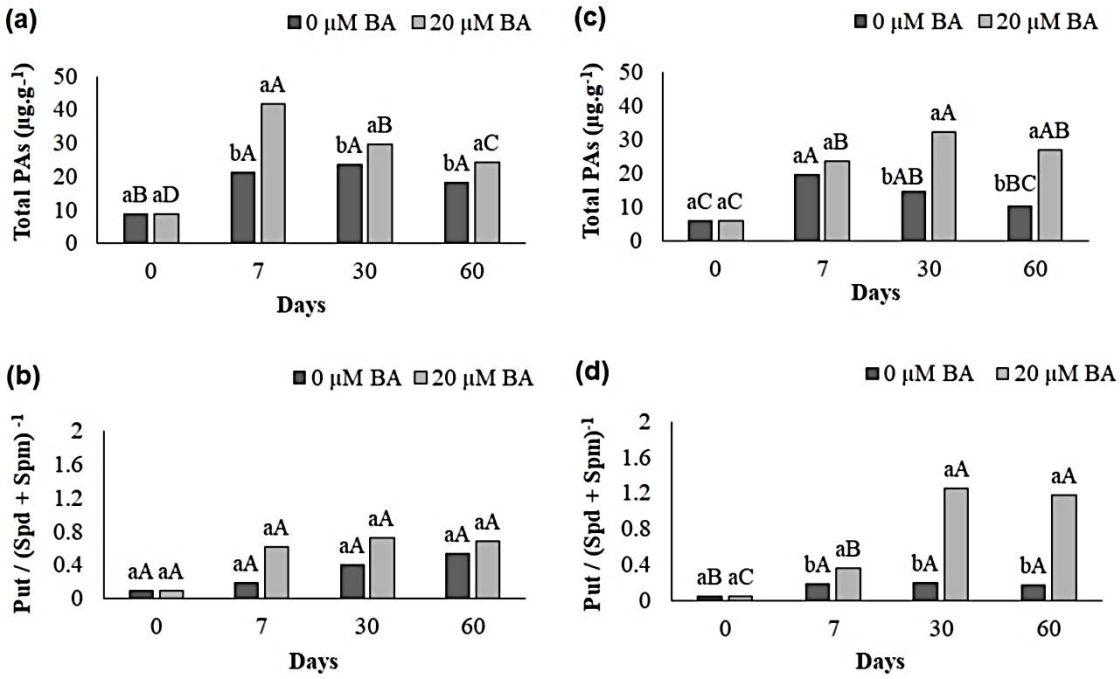

Figure 5. Content (µg.g^{-1} FM) of endogenous total free PAs (**a, c**) and free PAs [Put/(Spd+Spm)$^{-1}$] ratio (**b, d**) during shoot development from axillary (**a, b**) and cotyledonary (**c, d**) nodal segments before (time 0) and after 7, 30 and 60 days of incubation in different concentration (0 and 20 µM) of BA. Lowercase letters compare significant differences between BA concentrations (0 and 20 µM) in each time of incubation (0, 7, 30 and 60 days). Capital letters compare significant differences between each BA concentration (0 or 20 µM) in all times (0, 7, 30 and 60 days) of incubation. Treatments followed by different letters are significantly different according to the SNK test ($P < 0.05$). Data of PAs ratio from cotyledonary nodal segments (**d)** were transformed by arc-sine$\sqrt{x}/100$. CV = coefficient of variation. (n = 3; CV of total PAs in axillary = 13.90 %; CV of PAs ratio in axillary = 37.21 %; CV of total PAs in cotyledonary = 18.36 %; CV of PAs ratio in cotyledonary = 15.58 %).

The endogenous content of free Put were significantly higher in shoots from axillary (Fig. 6a) and cotyledonary (Fig. 6b) nodal segments grown in 20 µM of BA at 7, 30 and 60 days of incubation, compared to those from control. These results suggests that BA induce an increase in the Put contents in both type of explants in this species.

Significant higher content of free Spd was observed in shoots from axillary nodal segments at 7 and 60 days of incubation in the presence of BA compared with control treatment (Fig. 6c). On the other hand, no significant differences was observed in the endogenous contents of Spd in shoots from cotyledonary nodal segments incubated in both treatments (Fig. 6d). No significant differences were observed for endogenous content of Spm in shoots from axillary (Fig. 6e) and

cotyledonary (Fig. 6f) nodal segments throughout the incubation period (0, 7, 30 and 60 days) and BA (0 and 20 µM) treatments.

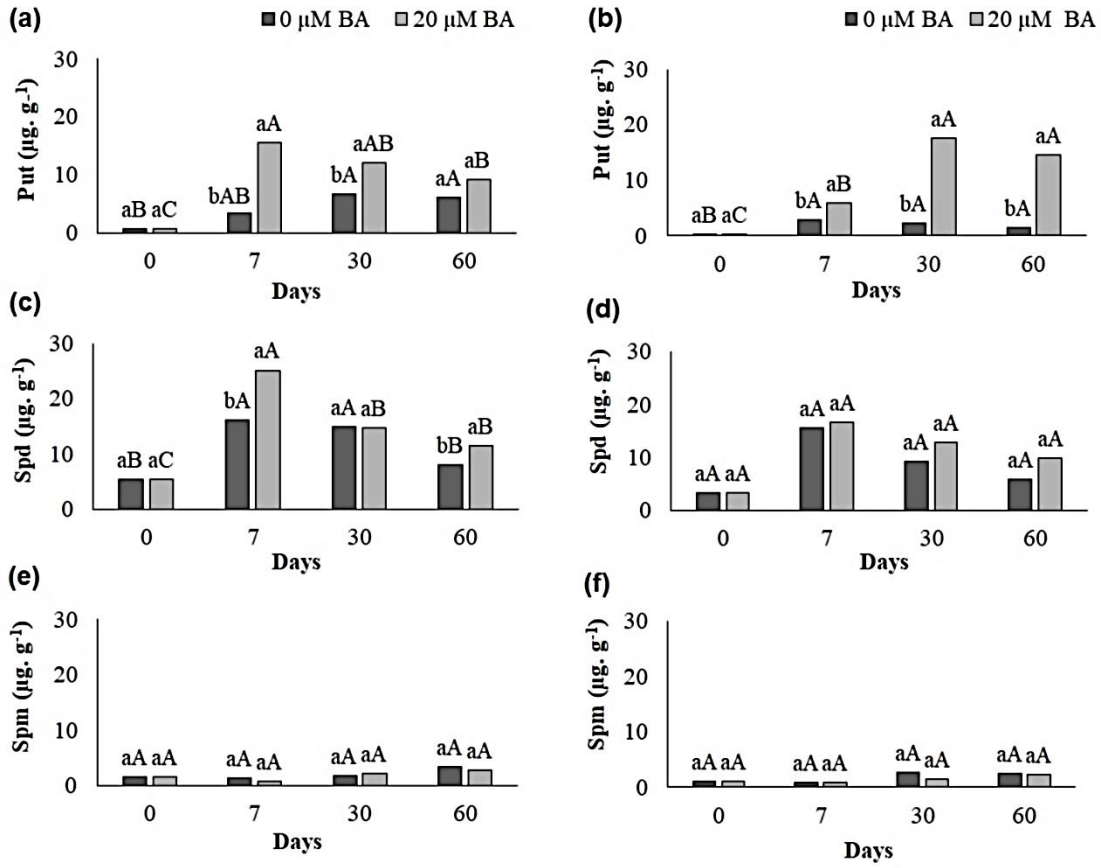

Figure 6. Content (µg.g^{-1} FM) of endogenous free putrescine (**a, b**), spermidine (**c, d**) and spermine (**e, f**) during shoot development from axillary (**a, c, e**) and cotyledonary (**b, d, f**) nodal segments before (time 0) and after 7, 30 and 60 days of incubation in different concentration (0 and 20 µM) of BA. Lowercase letters compare significant differences between the different concentrations (0 and 20 µM) of BA in each time (0, 7, 30 or 60 days) of incubation. Capital letters compare significant differences between each BA concentration (0 or 20 µM) in all times (0, 7, 30 and 60 days) of incubation. Treatments followed by different letters are significantly different according to the SNK test ($P < 0.05$). Data of Spm in axillary nodal segment (**e)** and Put (**b**) and Spm (**f**) in cotyledonary nodal segments were transformed by arc-sine$\sqrt{x}/100$. CV = coefficient of variation. (n = 3; CV of free Put in axillary = 37.28 %; CV of free Spd in axillary = 15.43 %; CV of free Spm in axillary = 15.17 %; CV of free Put in cotyledonary = 13.42 %; CV of free Spd in cotyledonary = 26.55 %; CV of free Spm in cotyledonary = 12.85 %).

3.4. Effects of IBA on *in vitro* microcuting rooting from shoots induced in different BA concentrations

Microcuttings measuring approximately 1.5 cm were obtained of shoots induced from cotyledonary nodal segments incubated in different concentrations (0, 1, 5, 10, 15, 20 and 25 µM) of BA. It has been observed that the concentration of BA used to produce shoots affect significantly the rooting of microcuttings acording the IBA treatment (Fig. 7).

Without IBA addition into the culture medium (control), the microcuttings from shoots cultivated with 1-20 µM BA presented the best percentages of rooting.The incubation of microcuttings in 5 µM IBA showed a significant lower root induction only from microcuttings obtained in shoots grown in the 25 µM of BA. However, the use of 10 µM AIB showed a significant lower values of root induction in microcuttings obtained from shoots cultivated in the concentrations from 10 to 25 µM BA (Fig. 7). This results showed that higher concentration (25 µM) of BA used on growth of shoots affect significantly the rooting of microcuttings in all concentration of IBA used.

In addition, microcuttings from shoots obtained in the best condition for elongation, i.e., in the 20 µM BA treatment, and incubated in all concentrations of IBA showed a significant reduction in the root induction only at the higher concentration (10 µM) IBA treatment (Fig. 7). In this sense, the best concentration of BA for shoot development were 20 µM. The concentration of 5 µM IBA can be applied for root induction in the microcuttings.

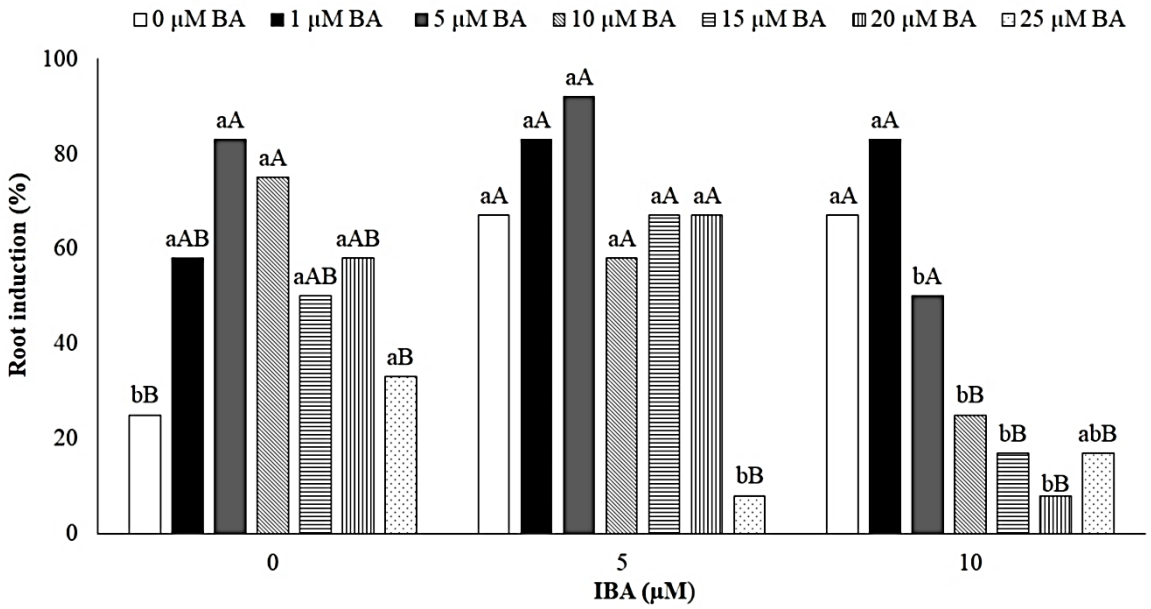

Figure 7. *In vitro* root induction (%) in microcuttings of *C. odorata* at 30 days of incubation in different concentrations (0, 5 and 10 µM) IBA. The microcuttings were obtained from shoots developed from cotyledonary nodal segments grown at different concentrations (0, 1, 5, 10, 15, 20 and 25 µM) of BA. Lower case letters compare significant differences between all concentrations (0, 5 and 10 µM) of IBA used for rooting in each concentration (0, 1, 5, 10, 15, 20 and 25 µM) of BA used for shoots growth. Capital letters compare significant differences for each concentration (0, 5 and 10 µM) of IBA used for rooting with all concentrations (0, 1, 5, 10, 15, 20 and 25 µM) of BA used for shoots growth. Means followed by different letters are significantly different according to the SNK test ($P < 0.05$). Data were transformed by arc-sine$\sqrt{x}/100$. CV = coefficient of variation. (n = 12, CV = 20.80 %).

3.5. Effects of the type of explant and size of microcuttings on *in vitro* rooting

The influence of origin (axillary and cotyledonary nodal segments) of shoots and size (0.5 and 1 cm) of microcuttings on *in vitro* rooting were evaluated (Fig. 9a) using 5 µM of IBA. The results showed no significant effects on root induction between the applied treatments, comparing the type of explant used to obtain the shoots as well as the size of microcuttings used (Fig. 8a).

However, 41-52 % of microcuttings measuring 0.5 and 1 cm of length obtained from axillary and cotyledonary nodal segments did not develop roots on *in vitro* rooting (Fig. 8b; Fig. 9c), but keep the leaves with a similar aspects of the rooted microcuttings (Fig. 9b). In this sense, these microcuttings were also transferred to the aclimatization step to verify if they are able to develop roots in the *ex vitro* condition.

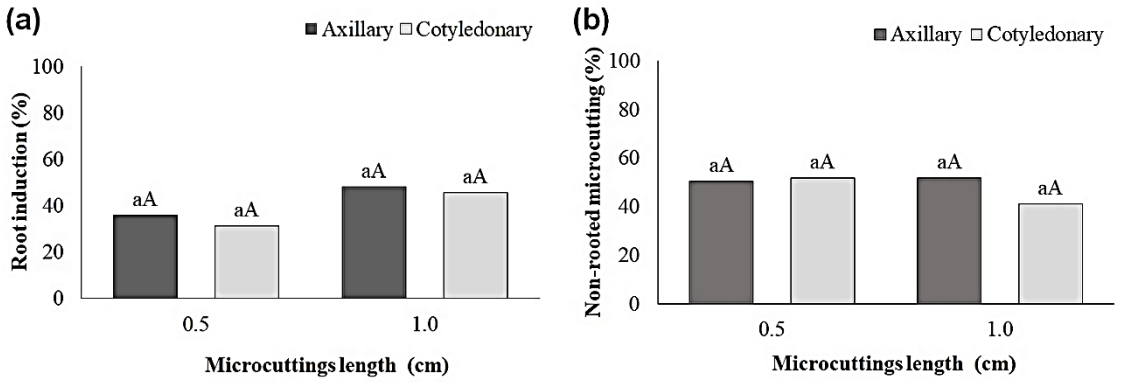

Figure 8. Percentage of *in vitro* root induction (**a**) and non-rooted microcuttings (**b**) with 0.5 and 1 cm obtained from shoots developed from axillary and cotyledonary nodal segments in 20 µM BA, at 20 days of incubation in culture medium containing 5 µM IBA. Lowercase letters compare significant differences between the shoots origin (nodal axillary and cotyledonary segment) in each size (0.5 or 1 cm) of microcuttings. Capital letters compare significant differences for each type of shoot (axillary or cotyledonary nodal segments) in the sizes (0.5 and 1 cm) of microcuttings. The treatments followed by different letters are significantly different according to the SNK test ($P < 0.05$). Data of non-rooted microcuttings (**b**) were transformed by arc-sine$\sqrt{x}/100)$. CV = coefficient of variation. (n = 112, CV of root induction= 35.53 %; CV of non-rooted microcuttings = 22.59 %).

Figure 9. *In vitro* root induction (**a**) and the morphological aspect of rooted (**b**) and non-rooted (**c**) microcuttings transferred to pre-acclimatization step. Bars =0.5 cm.

3.6. Acclimatization

The seedlings obtained during *in vitro* rooting showed a high percentage of survival (95-100 %) after 45 days of pre-acclimatization, without significant effects in relation to shoot origin and explants size (Fig. 10a).

The microcuttings with 0.5 cm obtained from shoots induced by cotyledonary nodal segments showed the highest percentage of *ex vitro* root induction (~57 %) during pre-acclimatization. While the microcuttings of axillary nodal segments measuring 0.5 cm had the lowest percentage rooting of *ex vitro* (~4 %). Both microcuttings, axillary and cotyledonary, measuring 1 cm presented the same percentage of root induction in *ex vitro* condition during pre-acclimatization (Fig. 10b).

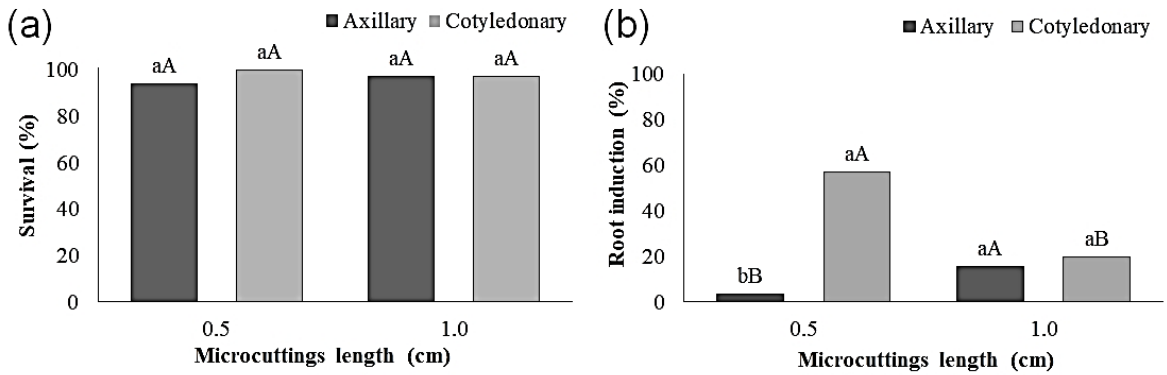

Figure 10. Survival (%) of plantlets obtained after *in vitro* rooting of microcuttings (**a**) and *ex vitro* roots induction (%) of microcuttings (**b**) at 45 days of pre-acclimatization. The treatments consisted of the size (0.5 and 1 cm) and the origin (Axillary nodal seed and cotyledonary) of the microcuttings used on *in vitro* rooting. Lowercase letters compare significant differences between the different types of shoots origin (axillary and cotyledonary nodal segments) in each size (0.5 or 1 cm) of microcuttings. Capital letters compare significant differences for each type of shoot origin (axillary or cotyledonary nodal segments) in both size (0.5 and 1 cm) of microcuttings. The treatments followed by different letters are significantly different according to the SNK test ($P < 0.05$). Data of survival of plantlets (**a**) and root induction of microcuttings (**b**) transformed by arc-sine$\sqrt{x}/100$. CV = coefficient of variation. (n of survival = 35; n of root induction = 45; CV of survival = 5.43 %; CV of root induction = 34.10 %).

The analysis of the root system of the plantlets obtained at 45 days of pre-acclimatization showed that those plantlets originated by 0.5 cm microcuttings obtained from shoots cotyledonary nodal presented the lowest root volume (Fig. 11a). However, in relation to root length, no significant difference was observed between treatments (Fig. 11b). Plantlets produced from 1 cm microcuttings obtained from shoots originated by cotyledonary nodal segments presented the higher content of FM in comparison with 0.5 cm microcuttings of the same origin (Fig. 11c).

DM content showed no significant differences for both size (0.5 and 1 cm) of microcuttings used (Fig. 11d).

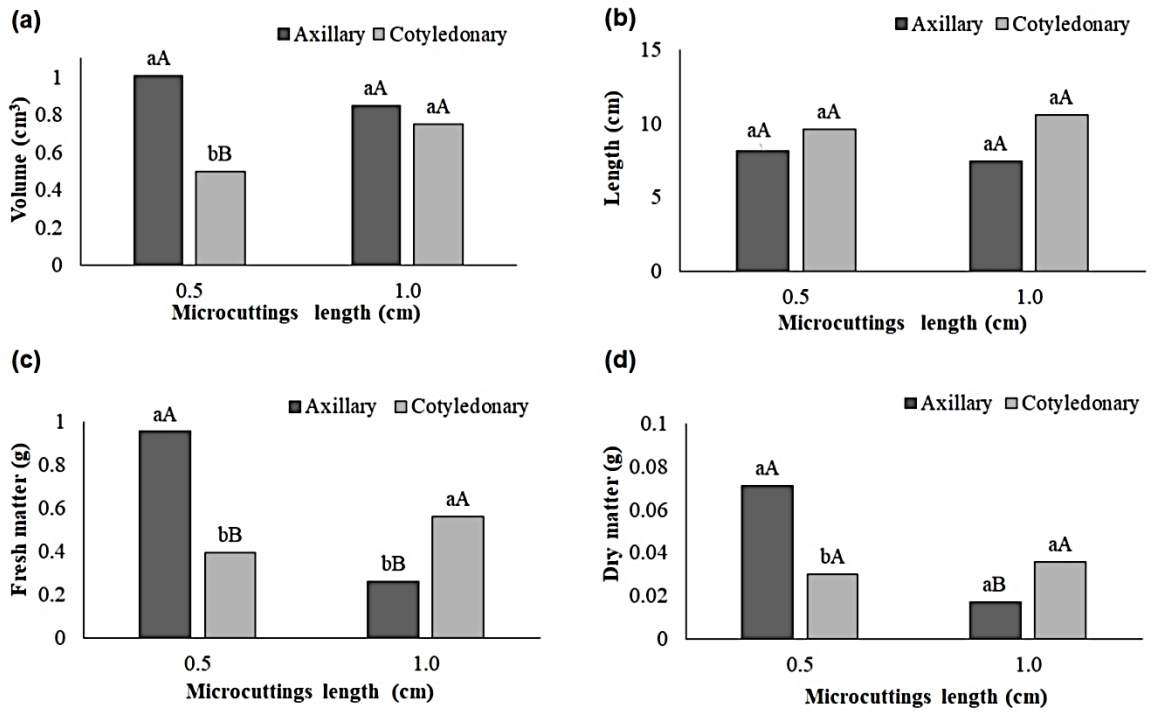

Figure 11. Values of volume (cm^3) (**a**), length (cm) (**b**), FM (g) (**c**) and DM (g) (**d**) from the root system of *C. odorata* plantlets at 45 days of pre-acclimatization. Lowercase letters compare significant differences between the different types of shoots origin (axillary and cotyledonary nodal segments) according to each size (0.5 and 1 cm) of microcuttings. Capital letters compare significant differences for each type of shoot origin (axillary and cotyledonary nodal segments) in both size (0.5 and 1 cm) of microcuttings. The treatments followed by different letters are significantly different according to the SNK test ($P < 0.05$). Data of FM (**c**) and DM (**d**) were transformed by arc-sine$\sqrt{x}/100$. CV = coefficient of variation. (n = 25; CV of volume = 10.07 %; CV of length = 11.06 %; CV of FM = 9.13 %; CV of DM = 27.70 %).

In relation to the aerial part analyzes from plantlets, no significant differences were observed between the treatments for the number of leaves (Fig. 12a). Plantlets originated from 0.5 cm microcuttings obtained from shoots of axillary nodal segments presented a larger aerial part length compared to 1 cm microcuttings of the same origin. The opposite pattern was observed in cotyledonary nodal segments, in which 1 cm microcuttings have originated plantlets with greater length of aerial part in relation to 0.5 cm microcuttings (Fig. 12b).

Concerning the FM content of the aerial part, there was no significant difference between the treatments (Fig. 12c). However, plantlets originating from 0.5 cm microcuttings obtained from shoots axillar nodal segments presented more content of DM in comparison with plantlets originated 1 cm microcuttings of the same origin, as well in compared with plantlets obtained from 0.5 microcuttings originated of shoots cotyledonary nodal segments (Fig. 12d). As for the plantlets obtained from cotyledonary nodal segments, the 1 cm microcuttings showed a higher DM content in comparison to those obtained from 0.5 cm microcuttings (Fig. 12d).

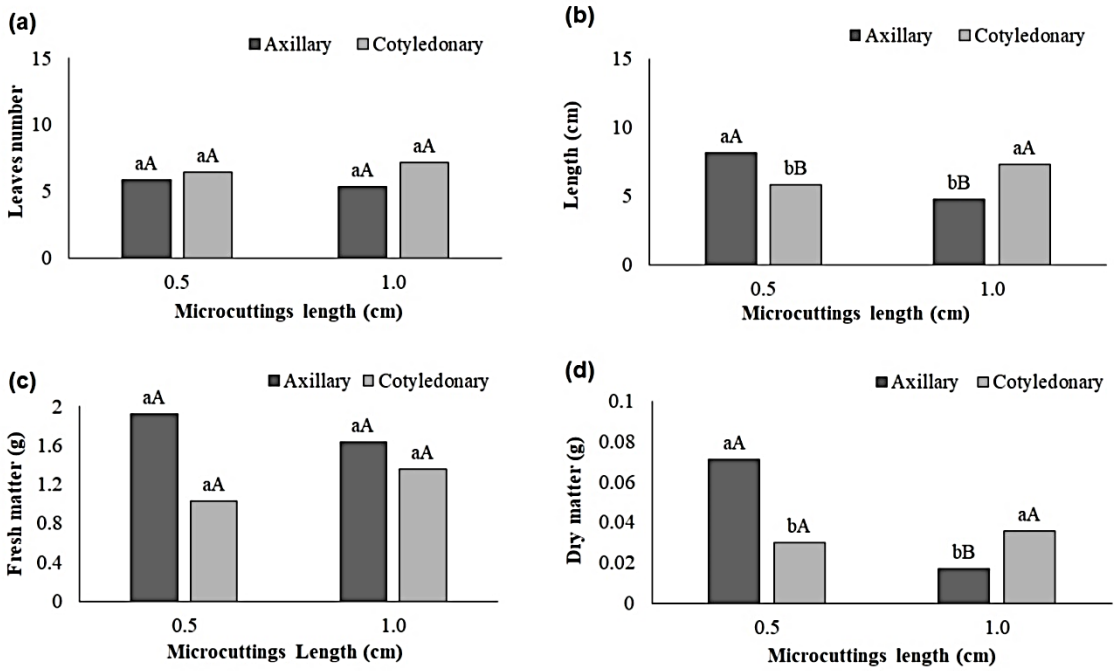

Figure 12. Analysis of number of leaves (**a**), length (cm) (**b**), FM (g) (**c**) and DM (g) (**d**) of the aerial part of *C. odorata* plantlets at 45 days of pre-acclimatization. Lowercase letters compare significant differences between the types of shoots origin (axillary and cotyledonary nodal segments) in each size (0.5 or 1 cm) of microcuttings. Capital letters compare significant differences for each type of shoots origin (axillary or cotyledonary nodal segments) in both sizes (0.5 and 1 cm) of microcuttings. The treatments followed by different letters are significantly different according to the SNK test ($P < 0.05$). CV = coefficient of variation. (n = 25; CV of leaves number = 11.20 %; CV of aerial part length = 10.81 %; CV of FM = 14.64 %; CV of DM = 27.07 %).

After pre-acclimatization, the plantlets obtained from microcuttings measuring 0.5 and 1 cm from shoots produced by axillary and cotyledonary nodal segments were transferred to the greenhouse. At 60 days of cultivation, the survival and growth (Figs. 13 and 14) of plantlets were evaluated, and the morphological aspects were showed (Fig 15).

No significant differences was observed in the percentage of survival of plantlets comparing the origin of shoots (axillary and cotyledonary nodal segments) and size (0.5 and 1 cm) of microcuttings used (Fig. 13a). Plantlets obtained from microcuttings with 1 cm from shoots by axillary nodal segments produces lowest volume, length and surface area root compared to other treatments (Fig. 13b-d).

With regard to FM, plantlets obtained from microcuttings with 0.5 cm from shoots of axillary nodal segments showed a significant higher FM compared to microcuttings with 1 cm (Fig. 13d). However, the plantlets obtained in microcuttings from shoot of cotyledonary nodal segments presented a similar FM for both size (0.5 and 1 cm) used (Fig. 13d). However, no significant difference was observed between plantlets produced from microcuttings with 1 cm obtained from shoots of both type origins (axillary and cotyledonary nodal segments) (Fig. 13d). Regarding the DM of root, plantlets obtained from microcuttings with 1 cm from shoots by axillary nodal segments showed the lowest values compared to plantlets from microcuttings with 0.5 cm from shoots of axillary nodal segments and those obtained from microcuttings with 0.5 and 1 cm from shoots from cotyledonary nodal segments (Fig. 13e).

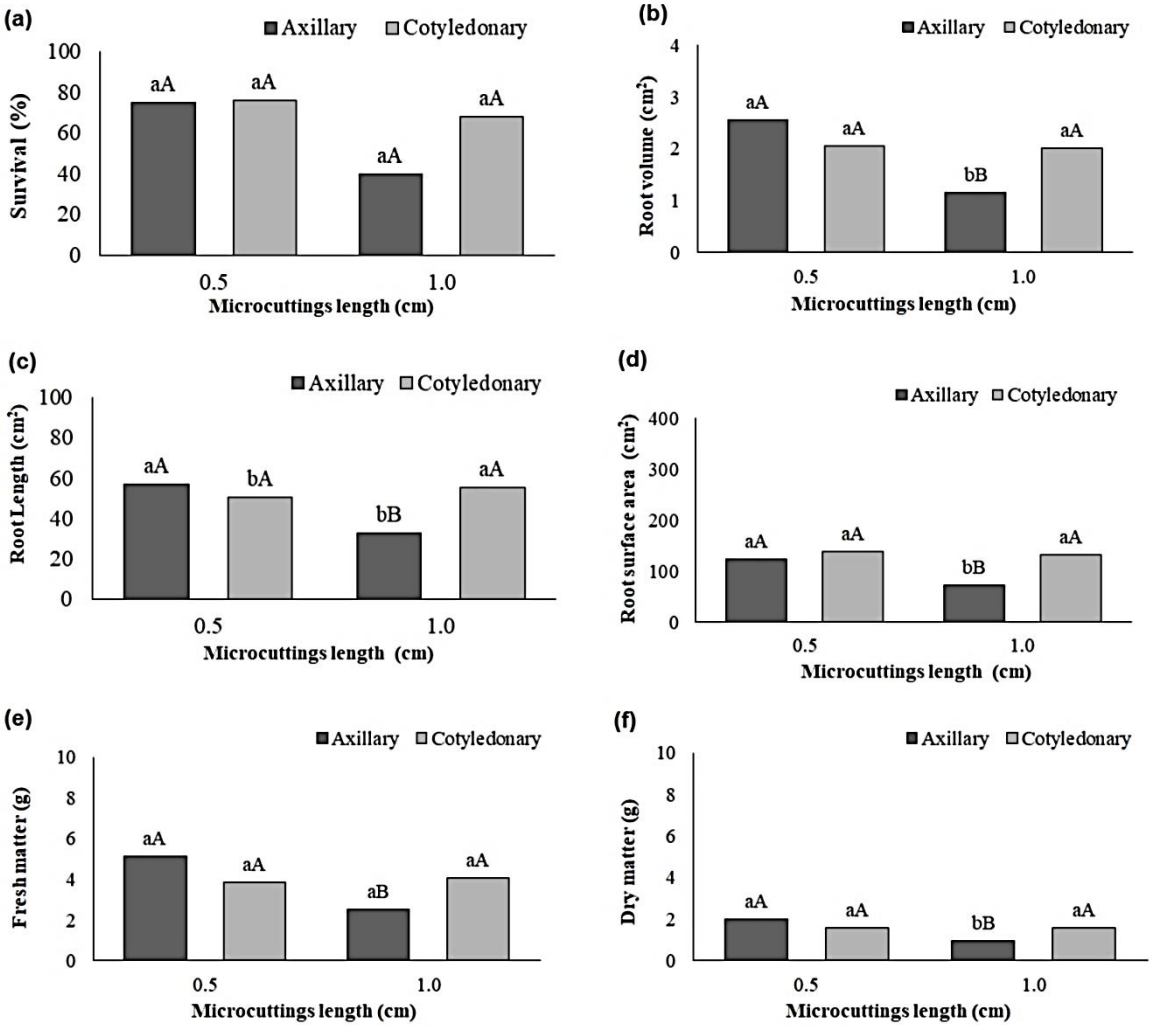

Figure 13. Percentage of survival (**a**) and analyses of volume (cm^3) (**b**), length (cm) (**c**), surface area (cm^2) (**d**), FM (g) (**e**) and DM (g) (**f**) in root system of *C. odorata* plantlets at 60 days in greenhouse. Lowercase letters compare significant differences between the different types of shoots origin (axillary and cotyledonary) in each size (0.5 or 1 cm) of microcutting. Capital letters compare significant differences for each type of shoots origin (axillary or cotyledonary) comparing both size (0.5 and 1 cm) of microcuttings. The treatments followed by different letters are significantly different according to the SNK test ($P < 0.05$). CV = coefficient of variation. (n = 25; CV of survival = 23.78 %; CV of root volume = 22.22 %; CV of root length = 25.89 %; CV of surface area = 20.22 %; CV of FM = 24.43 %; CV of DM = 24.88 %).

In relation to the growth data of the aerial part, plantlets obtained in microcuttings with 1 cm from axillary shoots presented the lowest values for number of leaves (Fig. 14a), specific leaf area (Fig. 14c), stem diameter (Fig. 14d), FM (Fig. 14f) and DM (Fig 14g). For leaf area (Fig. 14b) no significant difference was observed when comparing the size of the microcuttings (0.5 and 1 cm) in each type

of nodal segments. However, plants obtained from 1 cm microcuttings from axillary nodal segments showed a smaller leaf area in relation to plants obtained from 1 cm microcuttings from cotyledonary nodal segments (Fig. 14b). Regarding the length of the aerial part, there was no significant difference between the treatments (Fig. 14e).

In relation to the total DM content of plantlets, considering the aerial part and the root system, the plantlets obtained by 1 cm microcuttings from axillary shoots presented the lowest values compared with plantlets obtained from 0.5 cm microcuttings of the same origin (Fig. 14h). The plantlets originated from cotyledonary nodal segments showed no significant difference in the total FM content, ideally of the size of the microcuttings (Fig. 14i).

The Dickson quality index (DQI) is a quality indication of the plantlets that take into account the robustness and the phytomass distribution equilibrium. Plantlets originated from 1 cm microcuttings obtained by shoots of axillary nodal segments showed the lowest DQI (Fig. 14j).

The morphological aspects of micropropagated plantlets obtained using microcuttings with 0.5 and 1 cm from shoots produced by axillary and cotyledonary nodal segments, at 60 days of incubation in the greenhouse can be observed in the Fig. 15.

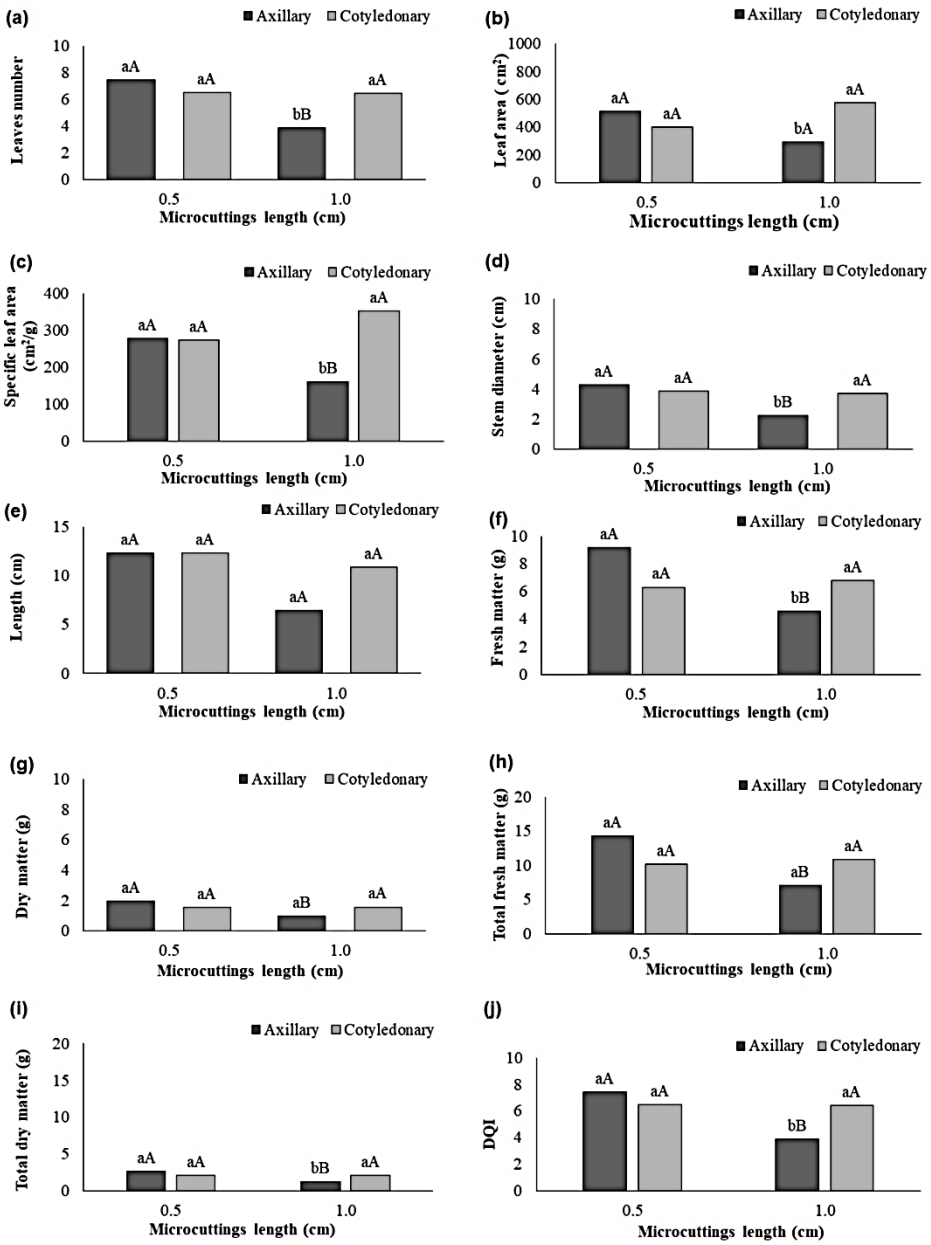

Figure 14. Number of leaves number (**a**), leaf area (cm^2) (**b**), specific leaf area (cm^2) (**c**), stem diameter (cm) (**d**), length (cm) (**e**), FM (g) (**f**) and DM (g) (**g**) in aerial part, and total FM (**h**), total DM (**i**) and DQI (**h**) of *C. odorata* plantlets at 60 days in the greenhouse. Lowercase letters compare significant differences between the different types of shoots origin (axillary and nodal) in each size (0.5 and 1 cm) of microcuttings. Capital letters compare significant differences for each type of shoots origin (axillary and cotyledonary) in both lengths (0.5 and 1 cm) of microcuttings. Means followed by different letters are significantly different according to the SNK test ($P < 0.05$). Data of leaf area (**b**) and specific leaf area (**c**) were transformed by arc-sine$\sqrt{x}/100$). CV = coefficient of variation. (n = 25; CV of number of leaves = 24.31 %; CV of leaf area = 20.52 %; CV of specific leaf area = 15.95 %; CV of stem diameter = 22.78 %; CV of length= 28.91 %; CV of FM = 33.18 %; CV of DM= 31.22%; CV total FM = 28.94 %; CV total DM = 28.73 %; CV of DQI = 18.63 %).

Figure 15. Morphological aspects of micropropagated plantlets from microcuttings with 0.5 cm and 1 cm obtained of shoots produced by axillary and cotyledonary nodal segments, at 60 days of cultivation in the greenhouse. Bars = 12 cm.

4. DISCUSSION

The *in vitro* propagation has several steps, and *in vitro* germination can be used to obtain the explants for organogenesis experiments. The MS culture medium is the most commonly used for *in vitro* germination and micropropagation experiments in several species (Nunes et al., 2002; Cheepala et al., 2004; Handa et al., 2005; Faisal et al., 2006; Buendía-González et al., 2007; Rocha et al., 2007; Prakash and Van Staden, 2008; Parimalan et al., 2011; Siddique et al., 2015;

Aragão et al., 2016). As no significant differences was observed between the MS and WPM culture medium in the percentage of germination (Fig. 2) and seedling development, the MS culture medium was chosen to be used for *in vitro* germination.

The *in vitro* multiplication of woody species from axillary shoots explants has been achieved for several forest tree species, being necessary the addition of cytokinin for shoot development in some species (Aragão et al. 2016 de França Bezerra et al. 2014; Hubner et al. 2007; Junior et al. 2012; Martínez et al. 2017; Parimalan et al. 2011; Ribas et al. 2005; Rocha et al. 2007; Rossato et al. 2016). The addition of BA into the culture medium influenced the length of shoots in *C. odorata*, being the concentrations of 5 and 20 µM the best treatment to provide larger shoots in axillary and cotyledonary nodal segments, respectively (Fig. 3c). These results shows that BA addition is necessary for shoot elongation in *C. odorata*, being concentration-dependent of explant used. The efficacy of BA on *in vitro* multiplication of plant species is related to the action of this cytokinin in the modulation of size, activity and maintenance of axillary meristems by means of controlling the balance between cell division and differentiation, as well as controlling cell cycle progression, influencing the transition between the G1/S and G2/M phases during mitosis (Uzelac et al., 2012).

In addition, the grow of axillary meristem depends on the proportion of auxin and cytokinins content, since auxin has an inhibitory effect on the growth of axillary shoots, whereas cytokinin promotes its growth (Shimizu-Sato and Mori, 2001). The auxin produced is in the shoot apex and is transported to the base of plant, forming a concentration gradient along the stem (Faria et al., 2007). In the present study, the cotyledonary nodal segments incubated at 20 µM BA were able to promote shoots with longer length compared to axillary nodal ones (Fig 3c). In this sense, we can suggest that the cotyledonary nodal segments of *C. odorata* have a favorable balance between auxin/cytokinin to promote the shoot elongation compared with the axillary nodal segments at 20 µM of BA. Similar results were obtained for *Cedrela fissilis*, in which cotyledonary nodal segments had a longer length of shoots compared to axillary nodal segments, when cultivated in culture medium supplemented with 2.5 µM BA (Aragão et al., 2011). In *Sesbania drummondii*, the axillary and cotyledonary nodes proliferated into multiple shoots when incubated on MS medium supplemented with 4.4-22.2 µM BA, and the cotyledonary nodes

explant-type allowed the production of a higher number of shoots per explant and also longer shoots (Cheepala et al., 2004). Moreover, in *Prosopis laevigata* different explants were tested for shoot induction, nevertheless, only the cotyledonary nodal type developed multiple shoots, which could be used to establish a system of clonal propagation of this specie (Buendía-González et al., 2007). Similar results were observed for *Erythrina velutina* in which the cotyledonary nodal type of explant had the highest capacity of regeneration in purchase to the nodal segment (Costa et al., 2017).

Besides of cytokinin, PAs are also an important factor related to *in vitro* morphogenesis control in plants, once they act in various physiological processes in plants, including cell division, differentiation, and elongation (Kakkar et al., 2000; Bais and Ravishankar, 2002; Kaur-Sawhney et al., 2003). PAs can act on growth by increasing the rate of cell growth, once inhibition of its biosynthesis can blocks this process (Kusano et al., 2008). The application of the cytokinin BA during shoot development in *C. odorata* influenced the endogenous content of free PAs in shoots from axillary and cotyledonary nodal segments, increasing the contents of endogenous free Put during incubation, compared to shoots incubated in the control treatment (Fig. 6). These alterations in Put content BA-induced can be related with the more elongation of shoots observed, especially in shoots from cotyledonary nodal segments incubated in 20 µM BA (Fig. 6). In *C. fissilis*, the BA addition into culture medium induced an increase in Put contents in shoots from axillary and cotyledonary nodal segments at the end of incubation, being related to the intense cell division that results in the growth of new shoots (Aragão et al., 2016). Moreover, in *Scirpus mucronatus* the endogenous Put synthesis was considered essential for the shoots elongation (Lee et al., 1996). In addition, it has been showed that exogenous addition of Put promoted the highest number and length of shoots per explant in *Bixa orellana* (Parimalan et al., 2011), *Gossypium hirsutum* cv. SVPR2 (Ganesan and Jayabalan, 2006), *Cichorium intybus* (Bais et al., 2000), and in *C. fissilis* (Aragão et al., 2017). In *C. fissilis*, the exogenous addition of 2.5 mM Put induced the elongation of shoots from cotyledonary nodal segments and affects the abundance of proteins related to growth using a proteomic approach (Aragão et al., 2017).

The success for a large-scale of vegetative propagation of woody plants depends on the ability of microcuttings to develop roots, and the quality of the root

system developed is directly related to the success of acclimatization (Millán-Orozco et al., 2011). Usually, the use of auxin is important to the induction of roots. Among the auxin, the IBA increases the kinetics of root formation, as well as provides an increase in the number and length of roots (Millán-Orozco et al., 2011). The different concentrations (0, 1, 5, 10, 15, 20 and 25 μM) of BA applied in the shoot induction and development influenced the rooting of microcuttings obtained from cotyledonary nodal segments, indicating alterations in the endogenous balance of auxins and cytokinins (Fig. 7). In addition, it was observed that the microcuttings can develop roots in the absence of IBA, suggesting the microcuttings contain the endogenous content of auxin necessary to induce this response. The rooting obtained in microcuttings at 5 μM BA was similar to the observed in the control treatment (without IBA) for most of BA concentrations used in the shoot induction, with increased root induction in microcutting induced without IBA and reduction rooting at 25 μM (Fig. 7). Previous studies with this species corroborate with the data presented here, with the induction of roots in a culture medium free of auxins (Millán-Orozco et al. 2011; Rodríguez et al. 2003). By the results observed in the present work (Fig. 7), we suggested the use of 5 μM IBA to root induction in microcuttings of *C. odorata*. Previous studies for this species carried out by Millán-Orozco et al. (2011) showed that lower concentration of IBA (1 $mg.L^{-1}$ corresponding to 4.9 μM) improve the rooting, as well as, increase the number and length of roots.

Usually, the size of microcuttings used in the rooting process are longer than 1 cm in several species (Cheepala et al., 2004; Erig et al., 2004; Buendía-González et al., 2007; Rocha et al., 2008; Santana et al., 2008; Peña-Ramírez et al., 2010; Costa et al., 2017). In this way, the smaller shoots are usually discarded. Therefore, the rooting of microcuttings with size equal or smaller than 1 cm, and subsequent acclimatization of the plantlets obtained, would allowed a better use of these shoots induced, as well as, an increase the quantities of plantlets provided by micropropagation. In this sense, we tested the effects of the size of microcuttings in the rooting induction. The results obtained here demonstrate that it is possible to perform *in vitro* rooting of microcuttings with 0.5 and 1 cm, regardless of their origin (shoots induced from axillary and cotyledon nodal segments) in *C. odorata* (Fig. 8a). In addition, the adequate acclimatization process of microcuttings in *C. odorata* depends on the induction of roots *in vitro* before the transference to the acclimatization step, since microcuttings with 0.5 and 1 cm of axillary origin and with

1 cm of cotyledonary origin when transferred to the *ex vitro* conditions presented rooting less than 20 % (Fig.10). Further studies related to *ex vitro* rooting are necessary to be performed for this species.

5. CONCLUSION

Based on the data presented, it was possible to conclude that the germination of *C. odorata* seeds and subsequent formation of normal seedlings are not influenced by the culture medium MS and WPM, under the conditions tested. The addition of 20 µM BA should be used for shoot elongation, being the largest shoots obtained from cotyledonary nodal segments compared to axillary. BA also influenced the endogenous content of free PAs during the development of shoots from axillary and cotyledonary nodal segments, increasing the Put content during incubation. The rooting of microcuttings was influenced by the concentration of BA in which the shoots were induced. The concentration of 5µM of IBA can be used for the *in vitro* rooting of microcuttings obtained from all concentrations of BA. Microcuttings measuring 0.5 cm obtained from shoots using axillary and cotyledonary nodal segments can be used to produce plantlets of *C. odorata*. The data obtained in this study are relevant for micropropagation of *C. odorata*.

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4.3. *Cedrela odorata* L. SEED STORAGE: PHYSIOLOGICAL AND BIOCHEMICAL ANALYSIS

RESUMO

A conservação de sementes de espécies florestais nativas é essencial para programas de recuperação e conservação de ecossistemas, bem como para aplicações econômicas, uma vez que a semente é a unidade de propagação natural para a maioria das espécies de plantas superiores. O objetivo deste estudo foi avaliar o efeito de diferentes temperaturas (6 e 25 °C) e tempos de armazenamento (0, 4, 8, 12, 16, 20 e 24 meses) sobre a germinação, o vigor das sementes e o conteúdo endógeno de poliaminas (PAs) em *Cedrela odorata* L., uma espécie arbórea nativa ameaçada de extinção. O armazenamento a 6 °C proporcionou a manutenção da viabilidade das sementes, sem redução significativa da germinação. Por outro lado, as sementes armazenadas a 25 °C mostraram uma redução significativa na germinação ao longo do período de armazenamento. A redução na viabilidade das sementes armazenadas a 25 ºC pode estar relacionada com a redução significativa no conteúdo de Spd e Spm após 20 e 24 meses de armazenamento, quando não foi observada a germinação das sementes.

Palavras-chave: cedro; Meliaceae; poliaminas; viabilidade da semente.

ABSTRACT

Seed conservation of native forest species is essential for ecosystem recovery and conservation programs, as well as for economic applications, since, seed is the natural propagation unit for most species of higher plants. The objective of this study was to evaluate the effect of different temperatures (6 and 25 °C) and storage times (0, 4, 8, 12, 16, 20 and 24 months) on germination, seed vigor and endogenous content of polyamines (PAs) in *Cedrela odorata* L., an endangered native tree species. Storage at 6 °C provide the maintenance of seed viability, without significant reduction on seed germination. On the other hand, seeds stored at 25 °C showed a significant reduction in germination along the storage period. The reduction of viability of seeds stored at 25 °C could be related with a significant reduction in the Spd and Spm contents in the time that seeds showed no germination.

Keywords: cedar; Meliaceae; polyamines; seed viability.

1. INTRODUCTION

Seeds are the basic input in programs of reforestation and recovery of degraded areas and conservation of ecosystems, as well as, germplasm conservation (Sena et al., 2016). However, in the case of native tree species, establishing strategies for conservation becomes even more complex due to the difficulty of accesses the seeds, the reduced and irregular seed production, the reduced number of trees in the natural area and a limited knowledge regarding seed storage conditions (Pilatti et al., 2011). In this sense, the establishment of conditions to storage of seeds is important, especially for those of endangered species, as *Cedrela odorata* L. (Meliaceae). This species occurs naturally in the Brazilian Atlantic Forest and presents a high economic value of wood, being currently in the Red List of the International Union for Conservation of Nature (IUCN), in the vulnerable category (Martinelli and Moraes, 2013; IUCN, 2018). Due to its economic and ecological relevance, and its rapid growth in the yield, the use of this species is indicated for the composition of heterogeneous reforestation, agroforestry systems and recovery of degraded areas (Navarro et al., 2004). However, studies concerning the establishment of seed storage conditions were developed.

Adequate storage of seed allows the maintenance of the physiological, physical and sanitary quality of the seeds, thus reducing their deterioration (Batista et al., 2011). Several factors may influence the maintenance of seed viability, and the understanding of the relationships between the temperature, relative air humidity and seed water content during storage is fundamental for the development of efficient forms of storage (Sena et al., 2016). Among the factors, the storage behavior of seeds is an important factor for studies to establish the best storage conditions.

According to the storage behavior, seeds are classified as orthodox or recalcitrant (Roberts, 1973). The orthodox seeds are characterized by the fact that

they survive the desiccation at very low values of water content, between 5 % and 7 %, without losing the viability, while the recalcitrant seeds are very sensitive to desiccation (Medeiros, 2006). The intermediates seeds are able to survive to a moderate desiccation, until they reach around 12 % moisture (wet basis) and are sensitive to storage at temperatures below 15 °C (Ellis et al., 1990). The storage conditions should aim to reduce the speed and intensity of seed deterioration to maintain seed viability (Krohn and Malavasi, 2004; Alves et al., 2008). Seeds of *C. odorata* are classified as orthodox (Leão et al., 2011), and previous studies showed no influences of paper or plastic bags, regardless of storage condition (refrigerator and environment), on viability of *C. odorata* seeds during storage (Batista et al., 2011). These authors have been showed that low temperatures (7 and 8 °C) and reduced water content are ideal to maintain the viability for longer time. However, no biochemical changes were performed during seed storage in this species.

During storage, some changes in the levels of biomolecules can occur in seeds, being already demonstrated the influence of compounds, such as carbohydrates, polyamines (PAs), amino acids and proteins, with the maintenance of seed viability (Bernal-Lugo and Leopold, 1992; Garcia et al., 2006; Sousa et al., 2016). PAs are low-molecular-weight, aliphatic, polycationic compounds that carry positive charges on nitrogen atoms, and can exert a broad spectrum of biological activities, such as the regulation of gene expression, signal modulation, membrane stabilization, cell differentiation and proliferation, cell death, DNA and protein synthesis (Kakkar et al., 2000; Kusano et al., 2008; Alcázar and Tiburcio, 2017). In this sense, PAs are involved in several process of growth in plants, such as seed development, germination (Santa-Catarina et al., 2006; Pieruzzi et al., 2011) and the maintenance or loss of viability of the seeds during storage in native trees (Sousa et al., 2016) and cultivated species (Mikitzel and Knowles, 1989; Anguillesi et al., 1990; Zhu and Zeng, 1993; Bonneau et al., 1994; Kondo et al., 2001). However, the effects of storage conditions on biochemical alterations during seed storage in *C. odorata* are not still showed, being relevant these studies for future use in research field on seed technology and physiology, as well as, for germplasm conservation in this species. In this sense, the objective of this work was to evaluate the effect of temperature and storage time on vigor and PAs contents in *C. odorata* seeds.

2. MATERIALS AND METHODS

2.1. Plant material

For the experiment, it was used mature seeds of *C. odorata* obtained in August 2014 from Sementes Caiçara nursery, located in Brejo Alegre, São Paulo State, Brazil (21°10'S and 50°10'W).

2.2. Seed imbibition curve during germination

The analysis of the imbibition curve during germination of *C. odorata* seeds was performed prior to storage. For germination, seeds were surface-disinfested according to Brasil (2013). First, seeds were washed with water and few drops of detergent, followed by washing under running water. Then, seeds were replaced in a water solution containing 1 % of commercial bleach (Qboa® - Anhembi SA, São Paulo, Brazil) containing 2.0 - 2.5 % active chlorine, for 5 min. Following, seeds were washed three times with distilled water, and were distributed on Germitest® paper sheets (J ProLab, São José dos Pinhais, Brazil) moistened with distilled water in the volume corresponding to 2.5 times its weight (Brasil, 2013). Seeds were incubated in a Biochemical Oxygen Demand (BOD)-type germination chamber at 25 °C ± 2 °C with 16 h-light photoperiod and light intensity of 22 µmol m^2s^{-1} (Brasil, 2013).

The fresh matter (FM) dry matter (DM) and water content (WC) were analyzed in seeds before (time 0) and after 2, 4, 6, 8 and 14 days of imbibition.

For FM analysis, six biological replicates, with 20 seeds each, were weighed in a precision balance. Then, the seeds were dried at 105 °C in a chamber with forced air circulation (Ethik technology, São Paulo, Brazil) for 24 h. After, samples were weighed again and the DM was obtained. The water content (WC) was obtained according to Aragão et al. (2015), by the difference between FM and DM.

2.3. Effects of temperature and time of storage on germination and PAs contents

To analyze the effects of temperature and time of storage on germination, seeds without wings were stored in polyethylene bags (12 x 8.5 cm), and kept in two temperatures,at 6 ± 2°C (refrigerator) and 25 ± 2°C (laboratory room), being protected from light.

For germination, samples (four replicates, with 50 seeds each) of seeds before (time 0) and after 4, 8, 12, 16, 20 and 24 months of storage in each temperature were taken, being disinfested and germinated according to Brasil (2013). After being surface-disinfested, seeds were placed in a Germitest paper, moistened with distilled water in the volume corresponding to 2.5 times its weight (Brasil, 2009), and maintained in BOD-type germination chamber at 25 °C ± 2 °C, with 16 h light photoperiod and light intensity of 22 µmol m^2 s^{-1}.

The germination (%), germination rate index (GRI), average germination time (AGT), abnormal and normal seedling (%) and water content (%) development were analysed in all storage times and temperatures.

The seed germination was analyzed daily to obtain the germination rate index (GRI), using the methodology recommended by Maguire (1962), and the average germination time (AGT), calculated according to methodology proposed by Labouriau (1983). In additon, at 28 days, the analysis of germination (%), as well as normal seedlings (i.e., seedlings with well-formed root system and aerial part) and of abnormal seedlings (i.e., seedling that presented bad formation of the root system or aerial part, as well as, the seeds that germinated but died due contamination) were evaluated acording to Brasil (2013). The water content (%) was obtained from four samples of 2.5 g of seeds.

In addition, samples (200 mg FM of seeds without teguments, in triplicate) were collected at each time of analysis and treatments for free PAs analysis.

2.4. Free PAs determination

The free PAs analyses was performed according to Santa-Catarina et al. (2006). The samples were ground in 1.2 mL of 5 % perchloric acid (Merck, Darmstadt, Germany), and incubated at 4 °C for 1 h. After, the samples were centrifuged for 20 min at 20.000 x g at 4 °C, and the supernatant, containing the free PAs, was collected.

Free PAs were determined directly from the supernatant by derivatization with dansyl chloride (Merck) and identified by high performance liquid chromatografy (HPLC, Shimadzu, Kyoto, Japan) using a 5-µm C18 reverse-phase column (Shimadzu Shin-pack CLC ODS). The HPLC column gradient was developed by mixing increasing proportions of absolute acetronitrile (Merck) to 10 % acetonitrile in water (pH 3.5), adjusted with HCl (Merck). The gradient of absolute acetonitrile was programmed to 65 % over the first 10 min, from 65 to 100 % for between 10 min and 13 min, and 100 % for between 13 min and 21 min, at 1 mL min^{-1} flow at 40 °C. The free PAs concentration was determined using a fluorescence detector at excitation of 340 nm and emission at 510 nm. Peak areas and retention times were measured by comparison with the standard PAs Put, Spd and Spm (Sigma-Aldrich).

2.5. Statistical analyzes

All of the experiments were performed using a completely randomized design. The data were analyzed by analysis of variance (ANOVA) ($P < 0.05$) followed by the Student–Newman–Keuls (SNK) test (Sokal and Rohlf, 1995) using the ASSISTAT program Version 7.7 beta (Silva, 2014). When required, the arc-sine$\sqrt{x}/100$ data transformation was applied in order to obtain data normality.

3. RESULTS

3.1. Imbibition curve during germination

During *C. odorata* seed imbibition, a triphasic development pattern was observed (Fig. 1b). When the root protrusion was observed on the sixth day of imbibition (Fig. 1a). The first phase (PI) began at the mature seed and lasted until the second day of imbibition when rapid water absorption occurred. The second phase (PII) began on day two and was characterized by a reduction and stabilization of water absorption, and it was completed on day sixth of incubation when radicle protrusion occurred. The third phase (PIII) began after day seven and was characterized by continued water absorption and post-germinative events.

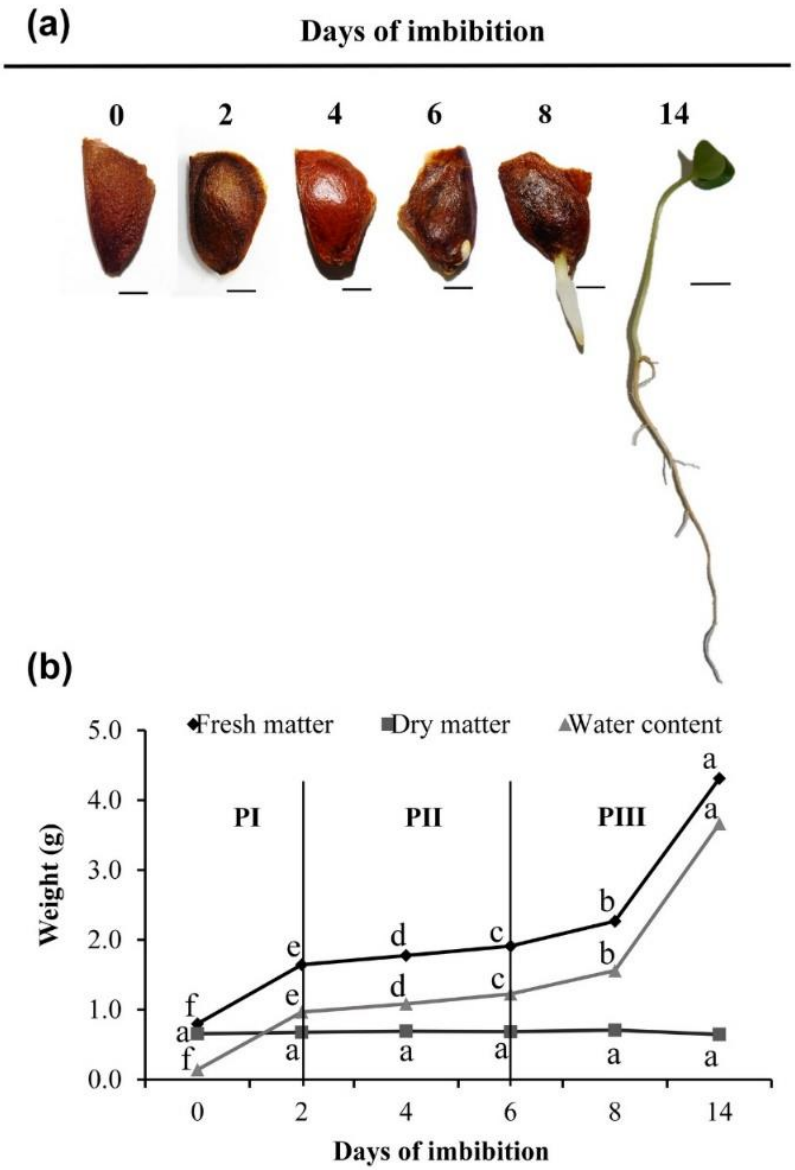

Figure 1. Morphological aspects of *C. odorata* seeds during germination and early seedling development (**a**) and FM, DM and water content before (0 day) and after 2, 4, 6, 8 and 14 days of imbibition (**b**). Imbibition phases: PI - characterized by the rapid absorption of water and reactivation of metabolism; PII - characterized by slow water absorption and completed with radicle protrusion; PIII - characterized by continuous water absorption and seedling growth. Lowercase letters denote significant differences in each analysis (FM, DM and water content) during the days of imbibition. Means followed by different letters are significantly different ($P < 0.05$) according to the SNK test. Data of FM were transformed by arc-sine$\sqrt{x}/100$. (n = 120; CV FM = 4.38 %; CV DM = 6.72 %; CV water content = 6.44 %). DM = dry mass; FM = fresh mass; CV coefficient of variation. Bars in (**a**) = 0.5 cm.

3.2. Effects of temperature and time of storage on physiological alterations

The seeds of *C. odorata* initially presented 13.75 % of water content. During storage at 25 °C, a significant reduction of water content was observed at 4 °C months, without significant changes until 16 months of storge (Fig. 2a). While the seeds stored at 6 °C showed a homogeneous reduction of water content during storage (Fig. 2a). At the end of storage, at 24 months, the seeds storaged in both treatments showed about 8 % of water content (Fig. 2a).

The germination (Fig. 2b) and normal seedlings (Fig. 2c) were significant affected by the temperature of storage. Seeds stored at 6 °C did not showed a significant reduction in germination and normal seedlings during 24 months of storage (Fig. 2b).

Seeds stored at 25 °C, showed a significant reduction in the germination from 8 months of storage (Fig. 2b), and from 16 months of storage for normal seedling produced, without germination of seeds at 20 months of storage (Fig. 2c).

The GRI, which refers to the number of germinated seeds per day during the germination, was also affected by the storage time and temperatures.The storage of seeds at 6 °C keep a higher GRI compared to those stored at 25 °C, throughout the time of storage (Fig. 2d). In addition, seeds stored at 6 °C did not presented a significant GRI difference during storage, while seeds stored at 25 °C showed a constant reduction (Fig. 2d).

Before being stored (time 0) the seeds of *C. odorata* presented an AGT, which refers to the average time in days that the seeds take to germinate, equivalent to 12 days (Fig. 2e). During storage time at 6 °C, the seeds germinated between 8 and 10 days after the imbibition (Fig. 2e). However, in the storage at 25 °C, the AGT was significantly reduced during storage, being zero in seeds at 20 months of storage (Fig. 2e). This fact is due to the absence of germination on 20 months of storage at 25 °C.

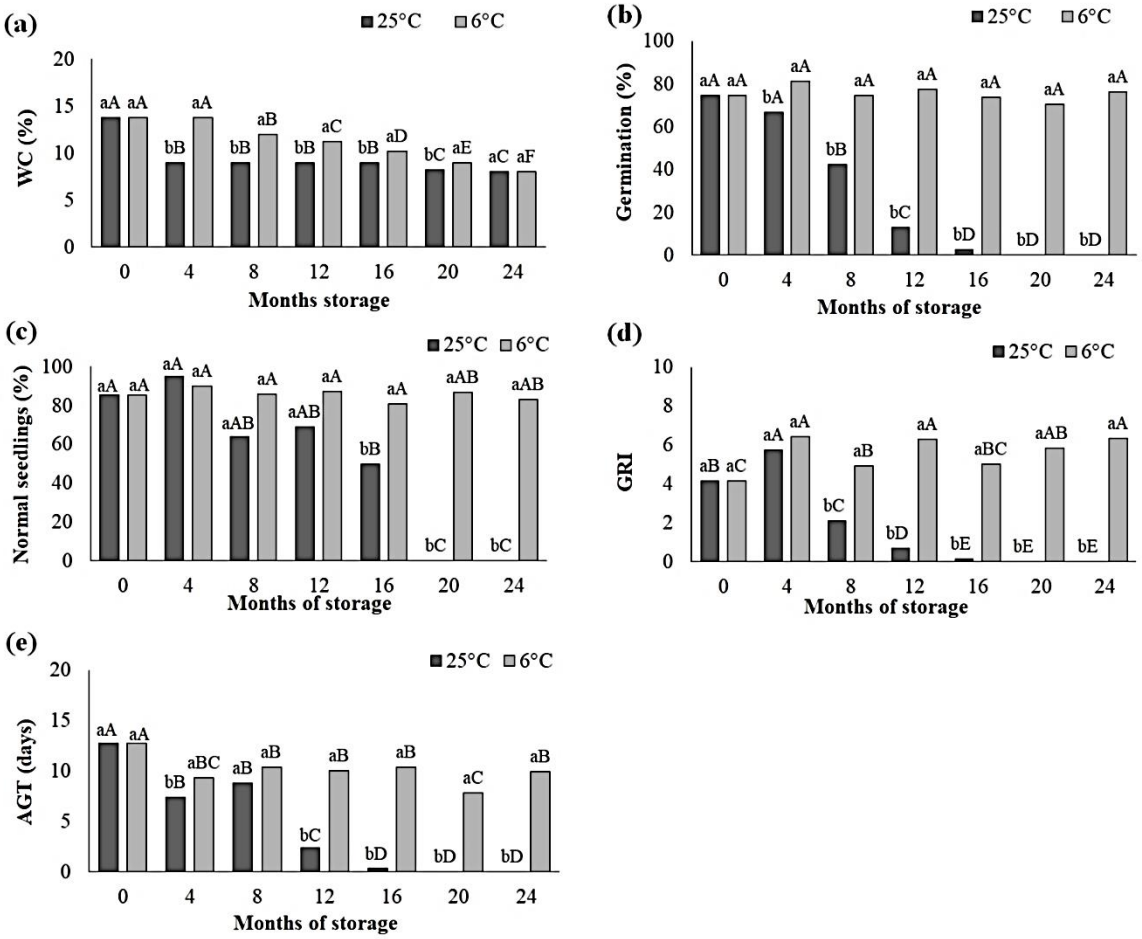

Figure 2. Water content (%) (**a**), germination (%) (**b**), normal seedlings (%) (**c**), germination rate index (**d**) and average germination time (**e**) in seeds of *Cedrela odorata* before (time 0) and after 4, 8, 12, 16, 20 and 24 months of storage at 6 and 25 °C. Lowercase letters diferents indicate significant differences for both temperatures (6 and 25 °C) in each storage time (0, 4, 8, 12, 16, 20 or 24 months). Capital letters diferents indicate significant differences in each temperature (6 or 25 °C) during storage (0, 4, 8, 12, 16, 20 and 24 months). Means followed by different letters are significantly different ($P < 0.05$) according to the SNK test. Data of WC (**a**), germination (**b**), normal seedlings (**c**),GRI) (**d**) andAGT (**e**) were transformed by arc-sine$\sqrt{x}/100$. CV coefficient of variation; WC = water content; GRI = germination rate index; AGT = average germination time (n = 200; CV of WC = 1.91 %; CV of Germination = 7.74 %; CV of normal seedlings = 20.10 %; CV of GRI = 5.32 %; CV of AGT = 7.47 %).

3.3. Effects of temperature and time of storage on PAs contents

Seeds stored at 25 °C showed a significant reduction in the content of total free PAs at 24 months of storage, whereas, seeds stored at 6 °C showed a significant reduction at 4 months of storage, without significant differences until the end of storage (Fig. 3a). Regarding the ratio of PAs [Put/(Spd + Spm)], no significant

difference was observed for the temperature tested, as well as during the time of incubation (Fig. 3b).

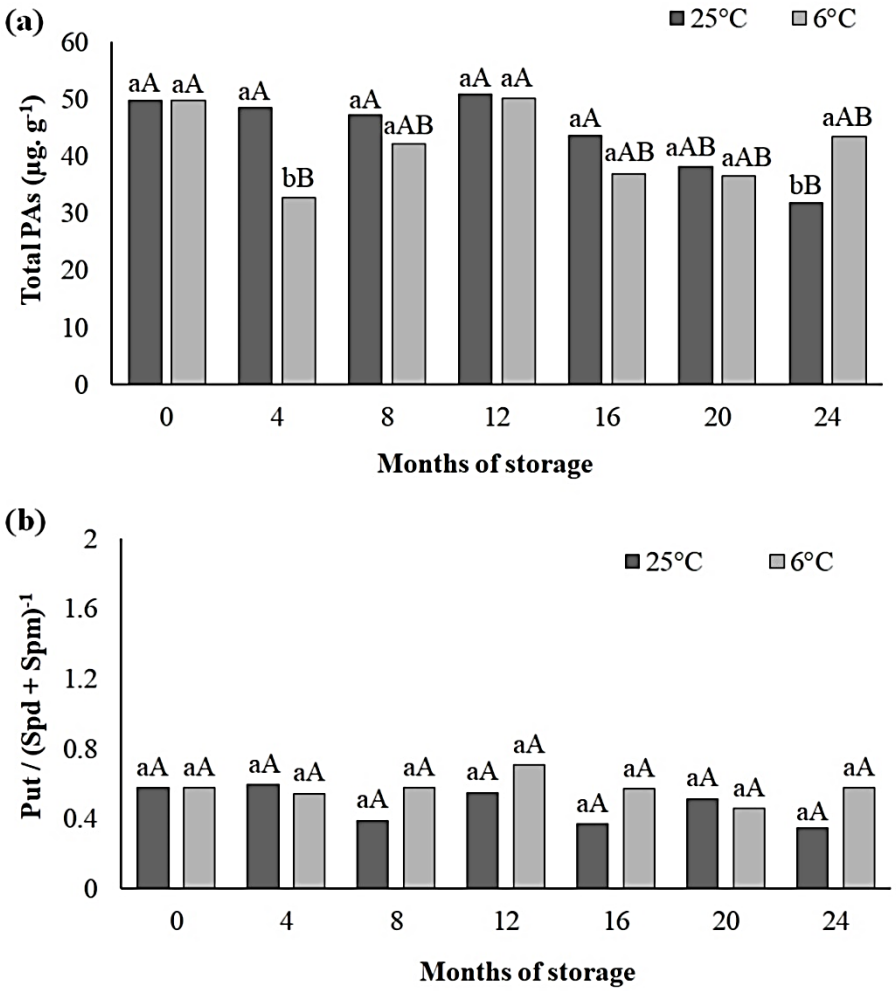

Figure 3. Contents (µg.g^{-1}) of total free PAs (**a**) and PAs ratio [Put/(Spd+Spm)] (**b**) in seeds of *Cedrela odorata* before (time 0) and after 4, 8, 12, 16, 20 and 24 months of storage at 6 and 25 °C. Lowercase letters indicate significant differences for temperatures (6 and 25 °C) in each time of storage (0, 4, 8, 12, 16, 20 or 24 months). Capital letters indicate significant differences in each temperature (6 or 25 °C) during the storage (0, 4, 8, 12, 16, 20 and 24 months). Means followed by different letters are significantly different ($P < 0.05$) according to the SNK test. Data of total free PAs (**a**) and PAs ratio [Put/(Spd+Spm)] (**b**) were transformed by arc-sine$\sqrt{x}/100$. CV = Coeficient of variation. (n = 3; CV of total PAs = 8.39 %; CV of PAs ratio = 16.23 %).

Comparing the contents of different PAs, it was observed that temperature and time of storage did not affected significantly the contents of free Put (Fig. 4a). In relation to Spd levels, a significant reduction at 24 months in seeds stored at 25

°C and at 4 months in seeds stored at 6 °C was observed (Fig. 4b). As regards the Spm contents, a significant reduction was observed in seeds stored at 25 °C from the 20 months of storage, while the storage at 6 °C provided a significant reduction on Spm contents at 4 months (Fig. 4c).

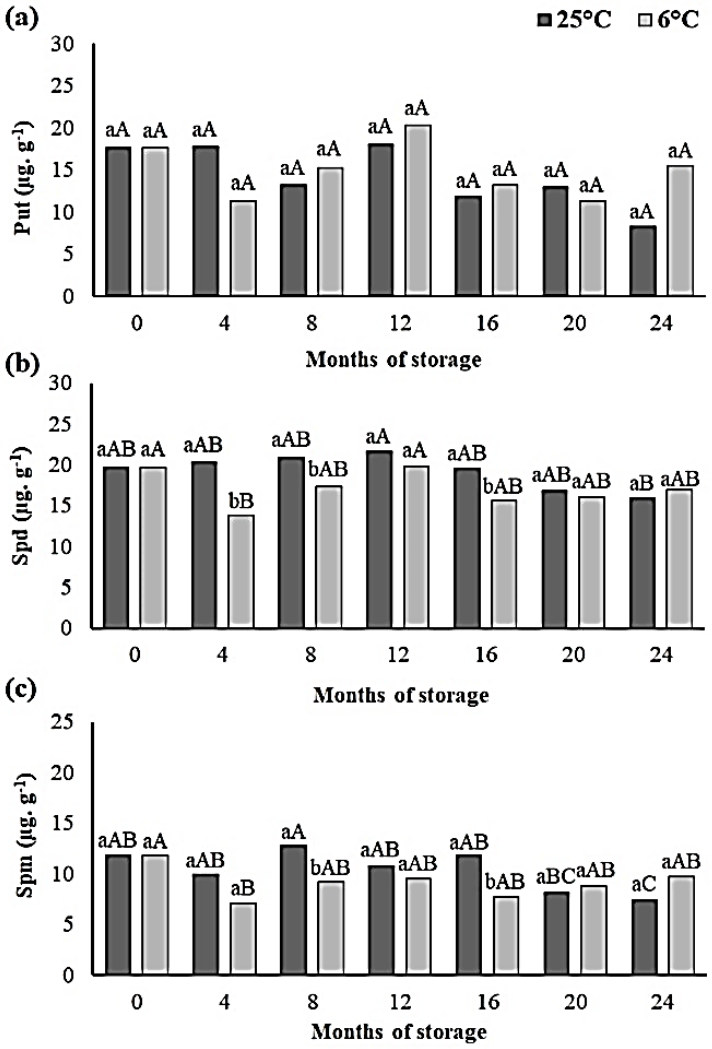

Figure 4. Content (µg.g^{-1}) of free Put (**a**), Spd (**b**) and Spm (**c**) in seeds of *Cedrela odorata* before (time 0) and after 4, 8, 12, 16, 20 and 24 months of storage at 6 and 25 °C. Lowercase letters indicate significant differences for temperatures (6 and 25 °C) in each time of storage (0, 4, 8, 12, 16, 20 or 24 months). Capital letters indicate significant differences in each temperature (6 or 25 °C) during the storage (0, 4, 8, 12, 16, 20 and 24 months). Means followed by different letters are significantly different ($P < 0.05$) according to the SNK test. CV = coefficient of variation. Put = putrescine; Spd = spermidine; Spm = spermine. (n = 3; CV of Put = 30.36 %; CV of Spd = 11.09 %; CV of Spm = 17.98 %).

4. DISCUSSION

Seed germination is a complex process that starts with water absorption by the quiescent seed and ends with the radicle protrusion, being all of the subsequent events classified as post-germinative (Bewley, 1997). Germination in *C. odorata* ends with radicle protrusion on the 6th day of imbibition (Fig. 1b), being observed a triphasic pattern of imbibition (Bewley and Black, 1994). According to Bove et al. (2001) the phase I is characterized by a rapid imbibition, known as physical imbibition, in which the basal metabolic recovery occurs. In the phase II, when the level of hydration exceeds 60 %, the imbibition is stabilized and new physiological mechanisms enable cell expansion along the embryonic axes until occurs the radicular protrusion, characterizing the beginning of phase III (Bove et al., 2001). At this phase, storage nutrients accumulated in the embryo's cotyledon and/or endosperm start to be mobilized before completion of germination and are used in the post-germination steps to sustain the young plant in its early growth stages, before it becomes autotrophic (Bove et al., 2001). *Cedrela fissilis* seeds also presented triphasic pattern of imbibition, being the phase I obtained at the second day of imbibition, while the germination process with root protrusion was obtained on the 7th day of imbibition, characterizing the end of phase II and the beginning of phase III (Aragão et al., 2015)

The storage conditions are determinant for the maintenance of seed viability. However, the longevity of the seeds varies according to the different species (Marcos Filho 2005). The seeds of *C. odorata* presented 13.75 % of water content before storage, reducing to 8 % at 24 months, regardless of temperature used (Fig. 2). These results suggests that in both temperature, seeds reduced the water during storage, reaching the same value at 24 months.

In addition, the physiological analyzes carried out along the storage showed that the temperature of 6 °C was effective to maintain the vigor of the seeds of *C. odorata* for two years (Fig. 3). While at 25 °C the vigor of seeds reduced from the 8th month, with no germination at 20th month of storage (Fig. 3). Batista et al. (2011) demonstrated a reduction in vigor of *C. odorata* seeds during nine months of storage in two temperatures, at 24-26 °C and 7-8 °C. Before storage, the seeds presented 99 % of germination and 79 % of normal seedlings. After six months of storage at 24-26 °C the germination was not observed, however at three months no formation of normal seedlings was observed (Batista et al., 2011). These results were similar to those observed in the present work when stored at 25 °C. On the other hand, *C. odorata* seeds stored at 7-8 °C showed significantly reduced germination during storage, and at 9 months the seeds presents 88 % of germination and 23 % of normal seedlings formed (Batista et al., 2011). On the contrary, in our conditions (at 6 °C in polyethylene bags) the seeds of *C. odorata* were able to maintain the viability until 24 months.

The causes of deterioration and death of seed have not been fully elucidated, there may be several interacting mechanisms involved in the aging process, that can be studied and understood by analyzing the composition of proteins, carbohydrates, and biogenic amines (Dobiesz et al., 2017). PAs, especially Put, Spd and Spm contribute to the processes of plant growth and development (Dobiesz et al., 2017). The content of PAs in mature seeds vary according to species, being related to development and germination seed (Silveira et al., 2004; Santa-Catarina et al., 2006; Dias et al., 2009; Pieruzzi et al., 2011; Aragão et al., 2015; Rios et al., 2015; Sousa et al., 2016).

Seeds of *C. odorata* stored at 25 °C showed a reduction in the total content of free PAs (Fig. 3a) and free Spd (Fig. 4b) at 24 months of storage, as well as a reduction in the Spm contents (Fig. 4c) at 20 and 24 months of storage. In these times, the seeds stored at this temperature lost the vigor observed by the non germination (Fig. 3b). In this way, it is possible that the reduction in Spd and Spm at contents is related to loss of seed vigor in this seeds during storage in a non-adequate temperature. Santa-Catarina et al. (2006) suggested that Spd and/or Spm participated at the end of seed maturation *Ocotea odorifera,* whereas a higher content of Put was associated with the histodifferentiation of embryo, at the beginning of seed development. Similar results were observed in *Araucaria*

angustifolia , in which the highest levels occurring in the embryonic axis at the early stages, when Put and Spd were most abundant, and with seed development, the contents of Spd increased while Put decreased (Astarita et al., 2003).

In addition, during germination of *C. fissilis* seeds, the contents of free Spm decreased significantly while free Put increased during seedling growth, showing the importance of Put to growth and development of seedling (Aragão et al., 2015). Besides a great number of studies on PAs during germination, fewer studies related the evolvement of PAs during seed aging and storage (Mikitzel and Knowles, 1989; Zhu and Zeng, 1993; Bonneau et al., 1994; Kondo et al., 2001; Sousa et al., 2016; Dobiesz et al., 2017). In *Japonica rice* variety CV Tapei 309, the content of free PAs was related to the germination potential of different lots of seed (Bonneau et al., 1994). The content of free Put, Spd and Spm was higher in lots of seed that have a low germination potential compared to those with a higher germination potential (Bonneau et al., 1994). A similar response was observed in seeds of yellow lupin (*Lupinus luteus*) stored for 29 years. The content of Spd occurred in highest quantities compared to Put and Spm in the seed stored at -14 °C and 0°C, with germination higher than 90 %. However, seeds stored under laboratory conditions (with temperature about +20 °C) did not germinate, and the content of Spm, Spd, and Put practically doubled (Dobiesz et al., 2017). In addition, seeds of *C. fissilis* stored during 12 months at 4°C did not reduced significantly the percentage of emergence of seedlings along the storage, as well as, did not affected significantly the contents of Put, Spd, Spm, and total free PAs (Sousa et al., 2016). On the other hand, the opposite was observed in seeds of *Cariniana legalis*, which presented a significant reduction in the seedling emergence along the storage, as well as, a significant reduction in the contents of free Put, Spd, Spm, and total free PAs in the first month of storage (Sousa et al., 2016).

During *C. fissilis* seed aging, the seeds aged at 50 ºC presented the lost of viability and a significantly decreased on free Spd and Spm contents compared to seeds aged at 41 ºC, which did not lost the viability (Sousa et al., 2018). The PAs Spd and Spm have a protective effects due to longer chains and a number of positive charges once have more amine groups in these molecules (Bouchereau et al., 1999; Velikova et al., 2000). In this sense, the characteristics of these PAs allow the ability of higher neutralizing and membrane-stabilizing effects and can provide the protection of against reactive oxygen species damage (Velikova et al., 2000; Gill

and Tuteja, 2010). In addition, further studies should be developed with other biochemical compounds, such as proteins and carbohydrates that can be affected by the temperature during seed storage *C. odorata*, since 25 °C does not promote the maintenance of seed vigor during the 24 months of storage.

5. CONCLUSION

According to the data presented we concluded that seeds of *C. odorata* presented a triphasic pattern of imbibition during germination process, occurring the germination at 6 day of imbibition. The storage of seeds at 6 °C is efficient for maintaining the viability of the seeds for a period of 24 months. The reduction of viability of seeds stored at 25 °C could be related with a significant reduction in the Spd and Spm contents in the time that seeds showed no germination.

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5. RESUMO E CONCLUSÕES

A partir deste estudo foi possível a obtenção de informações inéditas e de relevância para as espécies em estudo, a *P. echinata* e *C. odorata*, referentes a morfogênese *in vitro* e armazenamento de sementes.

Em *P. echinata* foram estabelecidas condições fases importantes para o desenvolvimento da embriogênse somática, obtendo-se resultados inéditos para a espécie. O uso de sementes imaturas como fonte de explante para a indução de calos embriogênicos foi fundamental, visto que trabalhos anteriores utilizaram segmentos foliares e não obtiveram êxito na indução e obtenção de embriões somáticos. Além disso, o uso de 2,4-D foi essencial para a indução e multiplicação de calos, resultando na formação de células com características meristemáticas comparativamente aos calos obtidos no tratamento combinando 2,4-D com a citocinina BA. Adicionalmente, o uso de ABA mostrou-se essencial no desenvolvimento de embriões somáticos em estádios de desenvolvimento iniciais, possivelmente, pela modulação do conteúdo endógeno de PAs livres. Observou-se uma redução significativa do conteúdo de Put livre nos calos mantidos com 25 µM de ABA, onde ocorreu o desenvolvimento de embriões somáticos nos estádios globular, cordiforme e torpedo, comparativamente ao tratamento sem ABA (controle). Neste sentido, o presente estudo foi o primeiro a relatar o desenvolvimento de embriões somáticos para *P. echinata* e pode ser utilizado como base para estudos futuros que visam otimizar o processo de embriogênese

somática, particularmente relacionado com a maturação e conversão de embriões somáticos em plantas. Assim sendo, estas são novas perspectivas para a propagação de *P. echinata* e quando aprimoradas poderão ser uma alternativa promissora para a produção de mudas visando à sua conservação.

No estudo da organogênese *in vitro* em *C. odorata* verificou-se que a aplicação de BA influenciou o processo organogenético nesta espécie, influenciando no alongamento das brotações induzidas a partir de segmentos nodais apicais e cotiledonares, sendo o maior alongamento destas observado em segmentos nodais cotiledonares inoculados em 20 µM de BA. Esta citocinina também afetou o conteúdo de PAs durante o processo de indução e desenvolvimento das brotações, sendo a Put identificada como a principal PA envolvida na regulação da organogênese *in vitro* nesta espécie, atuando no alongamento das brotações. Além disso, observou uma interação significativa entre a concentração de BA utilizada para o desenvolvimento das brotações e as concentrações de auxina AIB aplicada no enraizamento *in vitro* de microestacas. A concentração de 5 µM de AIB é indicada para o enraizamento *in vitro* de microestacas obtidas a partir de brotos multiplicados em 0, 1, 5, 10, 15 e 20 µM de BA. É possível produzir mudas de microestacas medindo 0,5 e 1 cm, obtidas de brotos desenvolvidos a partir de segmentos nodais axilar e cotiledonar. Os estudos realizados para esta espécie demonstram aplicações práticas e viáveis para a produção de mudas, as quais podem ser aplicadas em atividades de reflorestamento. Futuros estudos visando o enraizamento *ex vitro* podem ser realizados para esta espécie visando aprimorar o enraizamento, o qual pode reduzir significativamente os custos na produção de mudas, obtendo-se o enraizamento das microestacas *ex vitro* simultaneamente ao processo de aclimatização, otimizando o processo com a redução de etapas.

No armazenamento de sementes de *C. odorata* verificou-se que a temperatura de 6 °C foi eficiente para a manutenção da viabilidade das sementes por 24 meses comparativamente à temperatura de 25 ºC. O armazenamento a 6 °C proporcionou uma redução gradual do conteúdo de água das sementes, passando de 13,75 % antes do armazenamento para 8 % aos 24 meses. Além disso, não foi observada redução na porcentagem de germinação e de plântulas normais durante o armazenamento. As sementes armazenadas a 25 °C apresentaram significativa redução da germinação a partir dos quatro meses de

armazenamento, não sendo observada mais a germinação aos 20 meses de armazenamento. A redução na viabilidade das sementes armazenadas a 25 ºC pode estar relacionada com a redução significativa no conteúdo de Spd e Spm após 20 e 24 meses de armazenamento, quando não foi observada a germinação das sementes.

A partir dos dados obtidos neste trabalho pode-se concluir que é possível o estabelecimento de metodologias alternativas de propagação para espécies arbóreas nativas ameaçadas de extinção da Mata Atlântica, bem como, identificar condições adequadas para o armazenamento de sementes para estas espécies.

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