EFEITO DE POLIAMINAS NA MICROPROPAGAÇÃO E ANÁLISE PROTEÔMICA DURANTE O DESENVOLVIMENTO DE BROTAÇÕES E ARMAZENAMENTO DE SEMENTES EM ARBÓREAS NATIVAS AMEAÇADAS DE EXTINÇÃO

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UNIVERSIDADE ESTADUAL DO NORTE FLUMINENSE DARCY RIBEIRO - UENF

CAMPOS DOS GOYTACAZES - RJ
MARÇO - 2017

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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 Doutor em Produção Vegetal".

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Aprovada em 29 de Março de 2017.

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Ao Senhor meu Deus;
À minha amada avó Rita “Dona Ritinha”;
À minha amada mãe Rita Luzie;
À minha tia Antônia de Maria.

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RESUMO

ARAGÃO, Victor Paulo Mesquita. D.Sc. Universidade Estadual do Norte Fluminense Darcy Ribeiro. Março de 2017. Efeito de poliaminas na micropropagação e análise proteômica durante o desenvolvimento de brotações e armazenamento de sementes em arbóreas nativas ameaçadas de extinção. Orientador: Drª. Claudete Santa Catarina.

O objetivo deste trabalho foi estabelecer condições para o desenvolvimento da organogênese direta em *Cariniana legalis* e *Cedrela fissilis* e identificar proteínas diretamente relacionadas com o desenvolvimento de brotações *in vitro* em *C. fissilis* e com a manutenção ou perda da viabilidade em sementes de *C. legalis.* Em *C. fissilis* foi testado o efeito de diferentes concentrações (0; 0,5; 1; 2,5 e 5 mM) das poliaminas (PAs) putrescina (Put), espermidina (Spd) e espermina (Spm) no desenvolvimento da organogênese direta, utilizando segmentos nodais apicais e cotiledonares. Foi analisado o conteúdo de PAs endógenas em explantes nodais cotiledonares antes (tempo 0) e após 3, 6, 10, 20 e 30 dias de incubação com 2,5 mM de Put por cromatografia líquida de alta performance. A abundância diferencial de proteínas nos tempos 0 (explantes antes da inoculação) e em brotações após 30 dias de incubação em 2,5 mM de Put foi analisada por espectrometria de massas (MS/MS). Em *C.legalis* foram testadas diferentes concentrações e combinações de 6-benziladenina (BA), ácido α-naftalenoacético (ANA) e ácido giberélico (GA_3), bem como Put, Spd e Spm no desenvolvimento da organogênese direta a partir de explantes nodais apicais e cotiledonares. Para o

enraizamento de brotações micropropagadas de *C. legalis* foi utilizado ácido indol-3-butírico (AIB). Sementes de *C. legalis* foram armazenadas durante 12 meses e dados de germinação foram obtidos nos tempos 0 (antes) e após 3, 6 e 12 meses de armazenamento. Nestes mesmos tempos foi analisada a abundância diferencial de proteínas via eletroforese bidimensional (2-DE) e MS/MS. Em *C. fissilis*, o tratamento com 2,5 mM de Put promoveu maior comprimento das brotações. Este tratamento possibilitou alterações no conteúdo endógeno de PAs livres totais, Put livre e na abundância diferencial de proteínas nas brotações, com alterações em proteínas associadas com processos de estresse e energia, bem como divisão celular. Em *C. legalis*, o desenvolvimento de brotações foi possível sem a adição de reguladores de crescimento vegetal. Explantes nodais cotiledonares proporcionaram maior comprimento das brotações comparativamente com os nodais apicais. O enraizamento das brotações micropropagadas foi possível sem adição de AIB, entretanto, em baixa percentagem. Em *C. legalis*, o armazenamento de sementes durante 12 meses reduziu significativamente a germinação, o índice de velocidade de germinação e o teor de umidade das sementes. Verificou-se que o tempo de armazenamento afetou significativamente a abundância de proteínas associadas principalmente a processos biológicos, metabólicos, celulares, de oxidação-redução, metabólicos de carboidratos, bem como proteínas de reserva, de envelhecimento e organização ou biogênese da parede celular, de resposta a estresse e estímulos e proteólise. Estes estudos de morfogênese *in vitro* e armazenamento de sementes possibilitaram informações relevantes sobre as sinalizações bioquímicas e fisiológicas nestes eventos, assim como para o conhecimento aplicado, possibilitando estabelecer as melhores condições para a propagação *in vitro* das espécies de estudo.

Palavras-chave: Poliaminas, organogênese, proteômica, armazenamento de sementes.

ABSTRACT

ARAGÃO, Victor Paulo Mesquita. D.Sc. Universidade Estadual do Norte Fluminense Darcy Ribeiro. March, 2017. Effect of polyamines on micropropagation and proteomic analysis during shoot development and seed storage in native endangered wood plants. Advisor: Drª. Claudete Santa Catarina.

The aim of this work was to establish conditions for the development of direct organogenesis in *Cariniana legalis* and *Cedrela fissilis* and identify proteins directly related to shoot development *in vitro* in *C. fissilis* and to maintenance or loss of seed viability in *C. legalis*. In *C. fissilis*, were tested the effects of different concentrations (0, 0.5, 1, 2.5 and 5 mM) of polyamines (PAs) putrescine (Put), spermidine (Spd) and spermine (Spm) on the development of direct organogenesis using apical and cotyledonary nodal explants. The contents of endogenous PAs were analyzed in cotyledonary nodal explants before (time 0) and after 3, 6, 10, 20 and 30 days of incubation in 2.5 mM Put by high performance liquid chromatography. The differential abundance of proteins in time 0 (explants before inoculation) and in shoots after 30 days of incubation with 2.5 mM Put was analyzed by mass spectrometry (MS/MS). In *C.legalis*, different concentrations and combinations of 6-benzyladenine (BA), α-naphthaleneacetic acid (ANA) and gibberellic acid (GA_3), as well as Put, Spd and Spm in the development of direct organogenesis from apical and cotyledonary nodal explants were tested. For rooting of micropropagated shoots in *C. legalis,* the indole-3-butyric acid (IBA) was used. Seeds of *C. legalis* were storaged during 12 months

and germination data were obtained at time 0 (before) and after 3, 6 and 12 months of storage. The differential abundance of proteins was analyzed through two-dimensional electrophoresis (2-DE) and MS/MS. In *C. fissilis*, the treatment of 2.5 µM Put promoted greater shoot length. This treatment promoted changes in the endogenous contents of total free PAs, free Put and the differential abundance of proteins in shoots, with alterations in proteins associated with stress and energy processes, such as cell division. In *C. legalis*, the shoot development was possible without the addition of plant growth regulators. Cotyledonary nodal explantes provided greater shoot length compared to apical nodal. The rooting of micropropagated shoots was possible without IBA, however, in low percentage. In *C. legalis*, seed storage during 12 months significantly reduced germination, germination speed index and seed moisture content. The time of storage affected significantly the abundance of proteins associated mainly to biological, metabolic, cellular, oxidation-reduction and carbohydrate metabolic processes, as well as storage protein, aging and cell wall organization or biogenesis, response to stress and stimulus and proteolysis. These studies with *in vitro* morphogenesis and seed storage provided relevant information related to biochemical and physiological signaling in these events, as well as the applied knowledge, enabling to establish the best conditions for *in vitro* propagation of the study species.

Keyword: Polyamines, organogenesis, proteomics, seed storage.

1. INTRODUÇÃO

A intensiva exploração da Mata Atlântica tem levado a destruição de seus recursos vegetais, sendo sua área de vegetação original substituída por monoculturas como cana-de-açúcar, café e cacau (Colombo e Joly, 2010). Atividades antrópicas relacionadas à expansão agrícola e pecuária, ocupação populacional desordenada e extração madeireira, também contribuíram para intensificação da devastação da Mata Atlântica, tornando-a um dos biomas com maiores riscos de extinção do mundo (Myers et al., 2000; Colombo e Joly, 2010), incluindo-a na lista dos *hotspots* mundiais de conservação da biodiversidade (Colombo e Joly, 2010). Tais processos de devastação e degradação têm levado várias espécies arbóreas nativas à ameaça de extinção, dentre elas *Cariniana legalis* (Martius) O. Kuntze e *Cedrela fissilis* Vell. (IUCN, 2016)

O estabelecimento de metodologias alternativas às convencionais (via seminífera ou estaquia), como a propagação *in vitro*, é fundamental para a conservação de espécies arbóreas nativas de alto valor econômico e ecológico (Pence, 2010; Sarasan, 2010). Desta forma, a cultura de tecidos vegetais, através da micropropagação, pode ser uma ferramenta para a multiplicação de várias espécies arbóreas dentro de um curto período de tempo (Giri et al., 2004; Pijut et al., 2012).

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). No entanto, outros compostos podem promover a regeneração de brotações *in vitro*, como as poliaminas (PAs), que atuam no controle e frequência das divisões celulares e síntese de moléculas, associadas a eventos de síntese de ácidos nucleicos e proteínas, modulando a morfogênese vegetal (Gaspar et al., 2003).

Entretanto, o controle da morfogênese *in vitro* em espécies arbóreas é um processo complexo, em especial por que algumas não respondem aos estímulos utilizados para a indução e obtenção da resposta morfogenética (Oliveira et al., 2013). Apesar da necessidade do uso de reguladores do crescimento e desenvolvimento vegetal para a obtenção das diferentes vias morfogênicas *in vitro*, pouco se sabe sobre os mecanismos bioquímicos e moleculares que atuam na totipotência celular (Vogel, 2005). Neste sentido, análises bioquímicas do perfil de PAs, bem como de proteínas diferencialmente abundantes, pela proteômica comparativa, podem ser ferramentas importantes para a obtenção de informações a respeito dos mecanismos pelos quais as células vegetais tornam-se competentes para o desenvolvimento da morfogênese *in vitro*. Estes estudos permitirão ainda a identificação de potenciais proteínas marcadoras do desenvolvimento da organogênese *in vitro*, possibilitando melhorar os protocolos de propagação para a produção de mudas, que possam ser utilizadas em programas de reflorestamento ou recuperação de áreas degradadas.

Adicionalmente, a demanda por sementes de espécies nativas tem aumentado nos últimos anos, pois elas constituem a base dos programas de recuperação e conservação dos ecossistemas (Sarmento e Villela, 2010). Todavia, durante o período de armazenamento, a deterioração é inevitável e resulta na perda da viabilidade e vigor de sementes (McDonald, 1999). Até o momento, poucos são os estudos que analisam os mecanismos moleculares de deterioração das sementes (Sathish et al., 2015). Porém, pesquisam apontam que a produção de radicais livres durante o armazenamento é uma das principais causas para a ruptura das membranas celulares, danos em proteínas e ácidos nucleicos, que, finalmente, resultam na deterioração de organelas celulares e envelhecimento da semente (Bailly, 2004; Kibinza et al., 2011). Segundo Davies

(2005), as proteínas são os principais alvos de radicais livres, devido à sua abundância nos sistemas biológicos. Neste sentido, a identificação de proteínas diferencialmente abundantes, via proteômica comparativa, permite sua associação com diferentes eventos fisiológicos que ocorrem nas células, tecidos e órgãos, em um determinado processo de desenvolvimento ou evento fisiológico (Kormuťák et al., 2006). Desta forma, estudos em nível celular sobre a abundância diferencial de proteínas durante o armazenamento de sementes podem ser úteis para o fornecimento de informações acerca dos processos que levam à perda da viabilidade das sementes em espécies arbóreas. Tais conhecimentos podem ser usados como base para o melhoramento genético de espécies nativas, visando à redução da velocidade de deterioração e consequente conservação da qualidade fisiológica e vigor por um período de tempo maior.

Ademais, a análise de alterações no perfil de proteínas diferencialmente abundantes, durante o armazenamento de sementes possibilitará identificar possíveis proteínas envolvidas na manutenção ou perda da capacidade germinativa e, assim, contribuir com informações básicas para novas alternativas de armazenamento e para futuros programas de conservação *ex situ* e melhoramento genético em espécies arbóreas.

2. REVISÃO BIBLIOGRÁFICA

2.1. Mata Atlântica

A Mata Atlântica é um dos biomas mais ameaçados do mundo, compreendendo uma área original de 1,3 milhões de km^2 (Ribeiro et al., 2009; Lira et al., 2012). Atualmente, restam apenas 7,5 % de sua cobertura vegetacional original (Fundação SOS Mata Atlântica, 2016), sendo apontado como um dos principais *hotspots* mundiais, ou seja, uma das prioridades para a conservação da biodiversidade em todo o mundo (Colombo e Joly, 2010). No entanto, a Mata Atlântica ainda apresenta uma elevada biodiversidade, estimando-se que este bioma abriga aproximadamente 1 a 8 % da biodiversidade mundial, contendo 20.000 espécies de plantas com uma taxa de 40% de endemismo (Myers et al., 2000). Esta grande biodiversidade é atribuída principalmente a grandes variações de relevo, regimes pluviométricos e unidades fitogeográficas na área florestal. Dentre os fatores que levaram à redução da área florestal encontram-se, principalmente, o desmatamento, a expansão da agricultura e a ocupação populacional desordenada. Devido a estas práticas, atualmente a Mata Atlântica é constituída de remanescentes florestais fragmentados, em sua grande maioria menores que 100 ha (Fig. 1) (Fundação SOS Mata Atlântica, 2016). Entretanto, o desmatamento ainda ocorre neste bioma. Entre os anos de 2014 e 2015 foram desmatados 184 Km² de remanescentes florestais nos 17 Estados da Mata

Atlântica, um aumento de 1% em relação ao período anterior (2013-2014) (Fundação SOS Mata Atlântica, 2016).

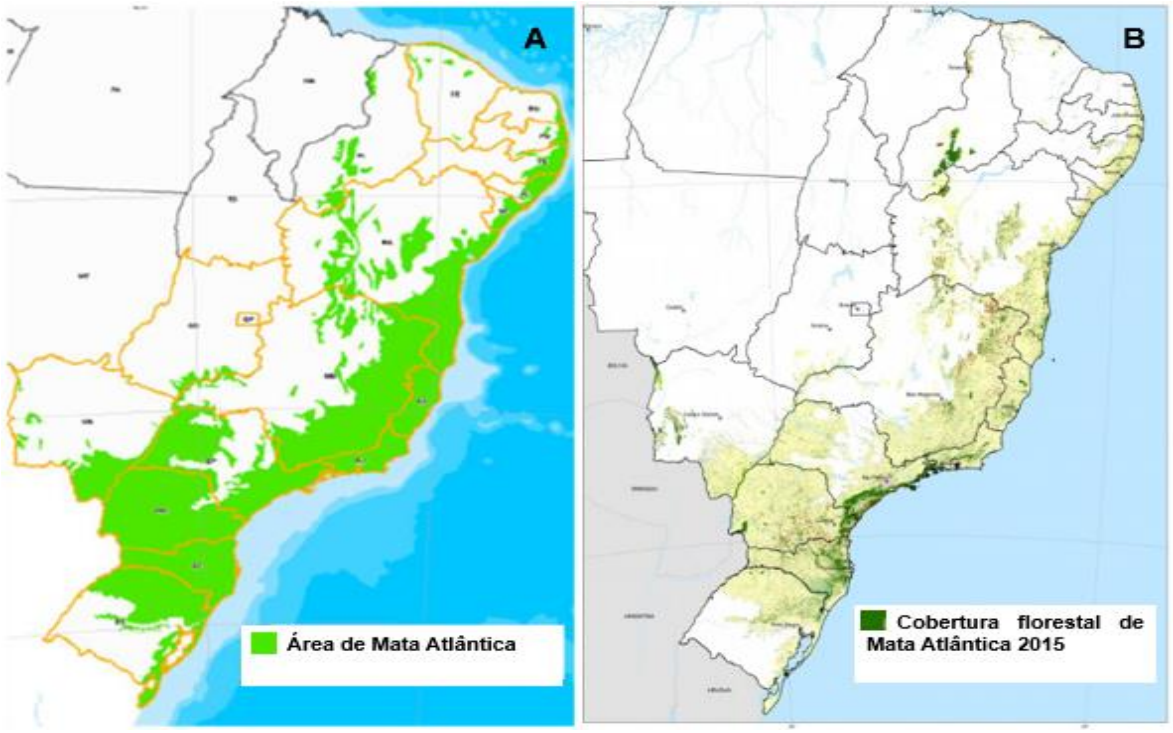


Figura 1: Mapas ilustrando a cobertura florestal original (A) e a cobertura florestal em 2015 da Mata Atlântica (B). Adaptado de Atlas dos remanescentes da Mata Atlântica período 2014 - 2015. Fonte: SOS Mata Atlântica (2016).

No Sudeste brasileiro, especialmente no Estado do Rio de Janeiro, a devastação da Mata Atlântica é avançada, restando 30,7% da área original, com uma taxa de desmatamento estimada em 27 ha por ano (Fundação SOS Mata Atlântica, 2016). Adicionalmente, na região Norte Fluminense restam apenas 13% da cobertura original da Mata Atlântica, sendo esta devastação resultado principalmente da ação antrópica na área florestal no Estado do Rio de Janeiro (Abreu et al., 2014). Os poucos remanescentes florestais da região Norte do Estado encontram-se altamente fragmentados, em áreas geralmente inferiores a 1000 ha e estão localizados, em sua grande maioria, em propriedades privadas (Carvalho et al., 2006a). Em particular, no município de Campos dos Goytacazes, localizado no extremo Norte do Estado do Rio de Janeiro, restam apenas 3% de cobertura original de Mata Atlântica representados por remanescentes florestais

menores que 100 ha (Carvalho et al., 2006a). Esta devastação é principalmente consequência do intenso desmatamento iniciado no século XIX para a implementação de monoculturas de cana-de-açúcar (Carvalho et al., 2006a).

Esta fragmentação florestal tem impactos graves na diversidade genética de espécies arbóreas, bem como em outros organismos associados com ecossistemas florestais (Laurance, 2012; Tambarussi et al., 2015). Desta forma, devido ao valioso patrimônio ecológico e genético de seus recursos naturais, a conservação e o repovoamento de áreas degradadas mostram-se como atividades emergenciais (Fundação SOS Mata Atlântica, 2016). Contudo, uma das maiores dificuldades para a recuperação de áreas degradadas nas regiões afetadas é a baixa disponibilidade de mudas de espécies nativas. Este fato deve-se, em parte, à baixa viabilidade de sementes, e também pela escassez de conhecimento sobre a biologia reprodutiva de espécies florestais nativas (Walker et al., 2015).

2.2. Espécies de estudo

Dentre as arbóreas nativas da Mata Atlântica, as espécies *C. legalis* e *C. fissilis* possuem relevância econômica e ecológica.

2.2.1 *Cariniana legalis*

Pertecente à família Lecythidaceae, *C. legalis* é uma arbórea endêmica da Mata Atlântica, conhecida popularmente como jequitibá rosa (Tambarussi et al., 2013). É uma espécie de ocorrência natural nos Estados de Alagoas, Bahia, Espírito Santo, Minas Gerais, Paraíba, Pernambuco, Rio de Janeiro e São Paulo (Fig. 2). A reprodução via seminífera ocorre por volta dos 20 anos de idade e a floração e frutificação, em diferentes épocas, dependendo da região. A dispersão das sementes é anemocórica, sendo o fruto do tipo pixídio lenhoso (Carvalho, 2003).

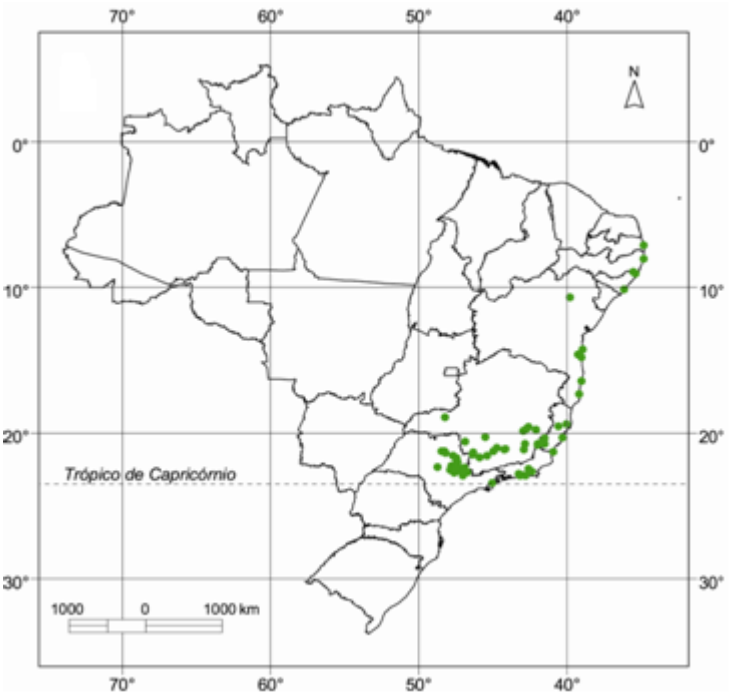


Figura 2: Mapa da distribuição de *C. legalis* no Brasil. Os pontos verdes indicam os locais de ocorrência natural da espécie. Fonte: Carvalho (2003).

C. legalis é uma das maiores árvores da Mata Atlântica, podendo alcançar de 10 a 25 m de altura e 60 a 100 cm de diâmetro a altura do peito (DAP) na fase adulta, exibindo um fuste reto. Esta espécie é semi-heliófila, tolerante ao sombreamento de leve a moderado na fase juvenil. A madeira é moderadamente densa, de alto valor comercial, sendo utilizada na fabricação de móveis em geral e na construção civil (Carvalho, 2003). A germinação das sementes é epígea e abundante nas primeiras semanas após a colheita (Carvalho, 2003). Mas, as sementes perdem a viabilidade à medida que se prolonga o armazenamento.

Devido à sua importância econômica para a produção de madeira, esta espécie foi intensamente explorada, restando apenas um reduzido número de indivíduos no local de ocorrência natural (Kageyama et al., 2003; Tambarussi et al., 2013). Estima-se que *C. legalis* ocorra na floresta em uma densidade populacional de menos de 1 árvore/ha (Tambarussi et al., 2015). Atualmente, *C. legalis* encontra-se ameaçada de extinção, na categoria vulnerável, caracterizada por espécies que tiveram uma redução de 30 % de indivíduos adultos nos últimos

dez anos ou que esta redução está projetada para os próximos dez anos, com um risco de 10 % de redução dos indivíduos adultos neste período (IUCN, 2016).

Neste sentido, estudos com armazenamento de sementes e a micropropagação desta espécie são fundamentais para sua preservação. Estudos iniciais mostraram a perda da viabilidade das sementes durante o armazenamento a 4ºC, verificado pela redução significativa na taxa de emergência de plântulas após 12 meses (Sousa et al., 2016). Segundo os autores, esta redução pode estar associada ao aumento no conteúdo endógeno de Putrescina (Put). Entretanto, estudos sobre alterações no perfil de proteínas, via proteômica comparativa, ainda não foram realizados para esta espécie, e são importantes para entender o papel das proteínas na perda de viabilidade das sementes. Além disso, o estabelecimento de metodologias alternativas de propagação, através de técnicas biotecnológicas, poderá auxiliar a produção de mudas, e ainda não foram realizados para esta espécie.

2.2.2 *Cedrela fissilis*

A espécie *C. fissilis*, conhecida popularmente pelo nome de cedro rosa, é uma arbórea nativa da Mata Atlântica pertencente à família Meliaceae. Sua ocorrência abrange uma ampla distribuição, incluindo os Estados de Alagoas, Amazonas, Bahia, Espírito Santo, Goiás, Maranhão, Mato Grosso, Mato Grosso do Sul, Minas Gerais, Pará, Paraná, Paraíba, Pernambuco, Piauí, Rio de Janeiro, Rio Grande do Sul, Santa Catarina, Rondônia, Sergipe, São Paulo, Tocantins e Distrito Federal (Fig. 3) (Carvalho, 2003).

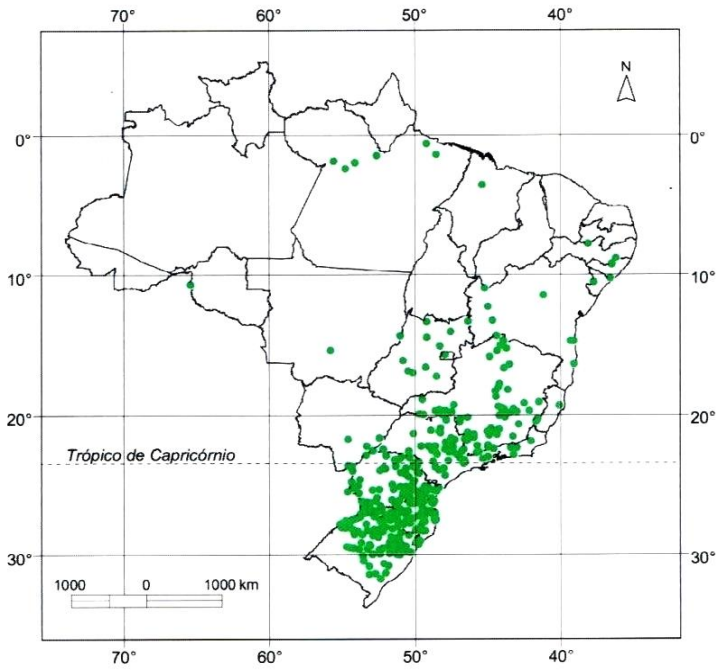


Figura 3: Mapa da distribuição de *C. fissilis* no Brasil. Os pontos verdes indicam os locais de ocorrência natural da espécie. Fonte: Carvalho (2003).

Esta espécie é uma árvore caducifólia, com altura variando entre 10 e 25 m e 40 e 80 cm de DAP. Apresenta tronco cilíndrico, reto ou pouco tortuoso, com fuste de até 15 m (Carvalho, 2003), sendo usada para a fabricação de móveis e na construção civil em geral (Carvalho, 2003). *C. fissilis* também é bastante recomendada para programas de reflorestamento ambiental em sua área de ocorrência natural (Martins e Lago, 2008). Esta espécie inicia seu processo reprodutivo entre 10 e 15 anos de idade, ocorrendo o florescimento e o amadurecimento dos frutos em diferentes épocas, dependendo da região (Carvalho, 2003). O fruto é do tipo cápsula piriforme deiscente, septífraga, abrindo-se até mais da metade por cinco valvas longitudinais, lenhosas, espessas e marrom-escuras, com lenticelas claras, com aproximadamente 30 a 100 sementes por fruto. A dispersão das sementes ocorre pela queda destas no solo, dentro do fruto ou por anemocoria (Alcántara et al., 1997). As sementes desta espécie são classificadas como ortodoxas (Carvalho et al., 2006b), e podem ser conservadas por 12 meses quando armazenadas a 4 ºC em sacos plásticos, sem diferenças significativas na emergência de plântulas (Sousa et al., 2016).

Devido à sua importância econômica e consequente exploração, *C. fissilis* encontra-se atualmente ameaçada de extinção, na categoria em perigo, caracterizada por espécies que sofreram redução de 50 % de indivíduos adultos nos últimos dez anos ou que esta redução está projetada para os próximos dez anos, com probabilidade de redução de pelo menos 20 % dos indivíduos adultos em cinco anos (IUCN, 2016).

Desta forma, estudos de micropropagação são importantes para estabelecer metodologias alternativas de propagação para espécies ameaçadas de extinção, especialmente *C. fissilis*. Para esta espécie foram estabelecidas as melhores condições para a propagação *in vitro* por organogênese direta (Nunes et al., 2002). Também foi estabelecido o enraizamento *ex vitro* das brotações micropropagadas desta espécie em substrato florestal e vermiculita (1:1; v:v), sem a necessidade de uso de auxina, como o ácido indol-3-butírico (AIB) (Ribeiro, 2015). Adicionalmente, alterações bioquímicas, especialmente no conteúdo endógeno de Put, induzido pela citocinina BA adicionada no meio de cultura, foram importantes para o desenvolvimento das brotações nesta espécie (Aragão et al., 2016). Assim, a continuidade dos estudos, visando à obtenção de informações sobre o efeito da adição exógena de outros compostos, como PAs, no conteúdo endógeno destas e no perfil de proteínas diferencialmente abundantes, é importante para entender a sinalização envolvida na organogênese direta e possibilitar a melhoria no potencial de regeneração *in vitro* para esta espécie.

2.3. Biotecnologias associadas à propagação vegetal: organogênese *in vitro*

A regeneração de uma planta completa a partir de tecidos já diferenciados é um evento complexo, que envolve um rigoroso controle hormonal, gênico, bem como controladas condições ambientais para a expressão da resposta morfogenética *in vitro* (Almeida et al., 2015). A micropropagação é um método viável para a propagação em diversas espécies arbóreas (Pijut et al., 2012), e pode ser uma alternativa para a produção clonal de plantas, visando à aplicação em programas de reflorestamento e conservação de germoplasma (Giri

et al., 2004). Esta técnica tem algumas vantagens sobre a propagação convencional, tais como a rápida multiplicação de genótipos de elite, a produção de plantas livres de doença, propagação independente da estação do ano e menor exigência de espaço para a produção, comparativamente a mudas obtidas a partir de métodos convencionais, tais como, estaquia ou sementes (Giri et al., 2004; Shukla et al., 2009). Neste sentido, ferramentas biotecnológicas, como a propagação *in vitro*, podem ser aplicadas para propagar espécies em vias de extinção, tais como *C. legalis* e *C. fissilis*.

O sucesso da regeneração *in vitro* depende de vários fatores como tipo de explante, componentes nutricionais do meio de cultura, reguladores de crescimento vegetal e condições de cultura, assim como os fatores genéticos (Magnusson et al., 2007; Oliveira et al., 2013). Este controle é difícil para espécies com características recalcitrantes para a cultura *in vitro*, como é o caso de muitas espécies arbóreas, as quais não respondem adequadamente aos estímulos utilizados para a indução da resposta morfogenética (Oliveira et al., 2013).

A micropropagação de espécies arbóreas compreende as fases de iniciação e estabelecimento da cultura *in vitro*, multiplicação e alongamento, enraizamento e a aclimatização das brotações (Xavier e Otoni, 2009). Nestas espécies, a multiplicação por meio da proliferação de gemas axilares preexistentes, mediante indução de brotações por organogênese direta tem sido a mais utilizada por promover melhores respostas na propagação *in vitro* (Xavier e Otoni, 2009). Desta forma, segmentos nodais apicais e cotiledonares são explantes de origem caulinar que contêm gemas axilares preexistentes, sendo empregados para o desenvolvimento de brotações *in vitro* em espécies arbóreas (Hong et al., 2004; Pijut et al., 2012). As gemas axilares presentes nestes tipos de explantes possuem determinação para o crescimento vegetativo, necessitando de estímulos e condições ótimas para o crescimento *in vitro.* Assim, a utilização deste tipo de explante na cultura *in vitro* de espécies arbóreas oferece algumas vantagens na produção final de mudas, tais como, facilidade para o crescimento *in vitro*, fidelidade genética, rapidez no processo de micropropagação e bom crescimento das plantas clonais (Xavier e Otoni, 2009).

Agentes de sinalização, tais como reguladores de crescimento vegetal são utilizados para a resposta da morfogênese *in vitro*, sendo citocininas e auxinas os mais utilizados (Lee et al., 2011). Segundo Duclercq et al. (2011), os processos de regulação para promoção da organogênese *in vitro* envolvem eventos de sinalização celular, que promovem mudanças em substâncias associadas à sinalização e atividades de genes reguladores durante a formação de brotos. Neste sentido, para espécies arbóreas, a 6-benziladenina (BA) é a citocinina frequentemente mais usada para promover o desenvolvimento de gemas axilares, quebrando a dominância apical e estimulando a proliferação de brotos (Mohebalipour et al., 2012; Nas et al., 2012). Além desta citocinina, a auxina ácido α-naftalenoacético (ANA), combinada com citocininas como BA e/ou thidiazuron (TDZ), também pode promover o desenvolvimento de brotações *in vitro* (Brijwal et al., 2015; Sivanesan et al., 2016), estimulando a divisão e o alongamento celular (Basuchaudhuri, 2016). Além disso, o ácido giberélico (GA_3) tem sido conhecido por promover o crescimento vegetativo, incluindo a iniciação de órgãos laterais e alongamento, promovendo o desenvolvimento de brotos necessários para a micropropagação (Fleet e Sun, 2005; Shani et al., 2006). Além das auxinas e citocininas, outros compostos podem ser utilizados para promover um aumento da taxa de regeneração de brotações *in vitro*, como as PAs (Zhu e Chen, 2005; Parimalan et al., 2011; Arun et al., 2014).

Durante a micropropagação, o enraizamento e a aclimatização das plântulas são as fases finais, sendo etapas críticas para a formação de uma planta completa. Entre as auxinas, o AIB é a mais comumente utilizada para o enraizamento de brotações (Pacurar et al., 2014). Neste processo, a indução e o desenvolvimento de raízes podem ser alcançados através de vias *in vitro* e *ex vitro*. Estima-se que o enraizamento *ex vitro* reduz os custos de um protocolo de micropropagação em até 71% (Ranaweera et al., 2013). Comparativamente, o enraizamento *ex vitro* se destaca em relação ao *in vitro* porque as brotações são enraizadas e aclimatizadas simultaneamente (Yan et al., 2010). Além disso, as mudas oriundas do enraizamento *ex vitro* apresentam um melhor desenvolvimento do sistema radicular, contribuindo para uma melhor aclimatização e sobrevivência na casa de vegetação (Phulwaria et al. 2012). No

entanto, dependendo da espécie, estratégias que possibilitem a junção entre enraizamento *in vitro* e *ex vitro* podem ser importantes para o sucesso desta fase da micropropagação.

2.4. Poliaminas e a morfogênese *in vitro*

As PAs são aminas alifáticas carregadas positivamente que, devido a esta característica, ligam-se a moléculas com carga negativa como DNA, RNA e proteínas, influenciando diretamente em processos de replicação, transcrição, tradução, divisão e expansão celular (Kusano et al., 2008; Tiburcio et al., 2014). As principais PAs encontradas nos vegetais são a Put, espermidina (Spd) e espermina (Spm) (Bouchereau et al., 1999). 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). Desta forma, 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).

As PAs estão envolvidas em vários processos fisiológicos nas plantas, tais como, embriogênese zigótica e somática, morfogênese, iniciação floral e desenvolvimento, senescência foliar, crescimento do tubo polínico, desenvolvimento e amadurecimento de frutos e resposta a estresses bióticos e abióticos (Kusano et al., 2008; Tiburcio et al., 2014). Estudos realizados em espécies arbóreas mostram que as PAs endógenas atuam em processos de desenvolvimento e germinação de sementes (Santa-Catarina et al., 2006; Pieruzzi et al., 2011; Aragão et al., 2015; Rios et al., 2015), assim como na embriogênese somática (Santa-Catarina et al., 2007; Dutra et al., 2013; Jo et al., 2013; Farias-Soares et al., 2014; Vondráková et al., 2015) e na organogênese *in vitro* (Aragão et al., 2016). Em adição, durante a cultura *in vitro* de arbóreas, o uso de PAs exógenas também tem sido importante para promoção da embriogênese somática

em espécies como *Araucaria angustifolia* e *Picea glehnii* (Silveira et al., 2006; Steiner et al., 2007; Nakagawa et al., 2011) e também na organogênese direta em *Bixa orellana* (Parimalan et al., 2011).

Segundo Debiasi et al. (2007), dentre as PAs, Put está envolvida no estímulo da proliferação celular e regeneração de tecidos, enquanto a combinação de Put, Spd e Spm é importante para o desenvolvimento de brotações. Assim, 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* (Viu et al., 2009). Além disso, as PAs atuam sobre o metabolismo de compostos importantes para o desenvolvimento vegetal como óxido nítrico e ácido abscísico (Silveira et al., 2006; Santa-Catarina et al., 2007; Steiner et al., 2007). Em *A. angustifolia*, a adição de Spd e Spm reduziu o crescimento celular e a liberação de óxido nítrico, enquanto a adição de Put aumentou a liberação de óxido nítrico, a qual foi relacionada com a manutenção da polaridade de massas pró-embriogênicas (PEMs) (Silveira et al., 2006). Nesta mesma espécie, a suplementação do meio de cultura com Put e Spm aumentou os níveis endógenos de ácido indolacético e ácido abscísico, mostrando uma relação direta entre os níveis de PAs e ácido abscísico na melhoria do crescimento das culturas embriogênicas (Steiner et al., 2007). Similarmente ao observado em *A. angustifolia* por Silveira et al. (2006), Spd e Spm reduziram o crescimento celular de culturas embriogênicas de *Ocotea catharinensis* (Santa-Catarina et al., 2007). No entanto, estas PAs aumentaram a liberação de óxido nítrico nas culturas embriogênicas desta espécie, demonstrando um importante papel durante o crescimento e evolução morfogenética dos embriões somáticos (Santa-Catarina et al., 2007).

Como as PAs alteram as concentrações destes compostos, pode-se sugerir que elas possam influenciar também o seu próprio metabolismo, alterando os níveis celulares e auxiliando, consequentemente, a indução e o desenvolvimento de brotações *in vitro* em espécies arbóreas. Estudos em *C. fissilis* mostram que as PAs, especialmente a Put, pode estar relacionada com a resposta morfogênética durante a organogênese direta (Aragão et al., 2016). A

adição de BA ao meio de cultura aumentou os níveis endógenos de PAs totais e Put, sendo relacionado com o crescimento e desenvolvimento das brotações nesta espécie (Aragão et al., 2016). Segundo Kakkar et al. (2000), a capacidade das PAs na indução da resposta morfogenética *in vitro* pode estar associada com a competência que estas biomoléculas apresentam de induzir divisões e alongamento celular, promovendo a regeneração em culturas vegetais.

2.5. Estudos proteômicos associados à competência para a organogênese *in vitro*

Abordagens proteômicas que permitem a identificação de proteínas específicas durante o desenvolvimento da organogênese *in vitro* são importantes para o estabelecimento de possíveis proteínas marcadoras associadas à competência organogênica em plantas. A identificação de proteínas em amostras biológicas complexas tem sido tradicionalmente realizada utilizando eletroforese bidimensional (2-DE). Esta técnica separa proteínas tanto por ponto isoelétrico quanto por peso molecular, produzindo um mapa de alta resolução, onde as proteínas individuais são excisadas dos géis e sequenciadas (Klose e Kobalz, 1995). Enquanto a 2-DE continua a ser uma poderosa ferramenta, a proteômica chamada *shotgun* emergiu como uma técnica para o estudo de proteínas em larga escala devido ao seu rendimento e sensibilidade superiores a 2-DE (Dowell et al., 2008). Na técnica *shotgun,* uma amostra complexa de proteínas é enzimaticamente digerida, fragmentada, sequenciada e as proteínas identificadas em bancos de dados específicos. Desta forma, enquanto a técnica *shotgun* identifica um número muito maior de proteínas e também aquelas menos abundantes, através de um sequenciamento de alto rendimento, a 2-DE permite a seleção de proteínas específicas diferencialmente abundantes para serem sequenciadas e identificadas (Dowell et al., 2008).

Usando a abordagem *shotgun*, em cana-de-açúcar (*Saccharum* spp.) proteínas identificadas foram relacionadas com a competência embriogênica das células somáticas e adaptação ao ambiente de estresse durante o cultivo *in vitro* (Heringer et al., 2015). Dentre estas, foram identificadas a proteína lsm sm-like que atua no metabolismo do mRNA em plantas (He e Parker, 2000) e a

peptidilprolyl cis-trans isomerase que tem atividade catalítica. Em adição, proteínas associadas ao estresse também foram identificadas em calos embriogênicos, tais como a cation transport protein chac associada a uma resposta defensiva geral a vários estresses ambientais em plantas; a polyphosphoinositide phosphatase-like isoform 1, associada a eventos de tráfego vesicular e estresse (Zhang et al., 2011) e a copper-transporting p-type ATPase, que é expressa em resposta ao estresse oxidativo e à sinalização hormonal (Shikanai et al., 2003). A expressão destas proteínas sugere que as culturas embriogênicas estão passando por algum tipo de estresse que pode ser responsável pela modulação fisiológica das células, permitindo assim a competência para esta resposta morfogenética (Moon et al., 2015: Heringer et al., 2015).

Na organogênese *in vitro* foi observado em *Vigna radiata* um rigoroso controle na síntese de proteínas diferencialmente abundantes entre explantes responsivos (1º cotilédone) e não responsivos (2º cotilédone) para a organogênese (Ghosh e Pal, 2013). A abundância diferencial de proteínas indicou a estimulação de vias metabólicas associadas com a aquisição da competência morfogenética, especialmente proteínas envolvidas no metabolismo de carboidratos e nitrogênio, proteínas de reserva, processamento e estresse (Ghosh e Pal, 2013). Proteínas associadas ao metabolismo de carboidratos, como as invertases, apresentaram maior abundância em explantes responsivos para a organogênse in *V. radiata,* indicando uma correlação entre a síntese de invertases e a diferenciação das brotações. Da mesma forma, a glutamine synthetase, associada ao metabolismo de nitrogênio foi mais expressa em explantes responsivos. Esta enzima atua na assimilação de amônia nos vegetais e produz glutamina a partir do aminoácido glutamato, e a maior expressão desta enzima em explantes responsivos sugere que maior quantidade de aminoácidos é requerida para a diferenciação das brotações *in vitro* (Ghosh e Pal, 2013). Similarmente, proteínas de reserva que servem como fonte de aminoácidos e para síntese de novas proteínas também foram mais abundantes em explantes responsivos à organogênese em *V. radiata* (Ghosh e Pal, 2013). Estes explantes também apresentaram maior abundância de proteases cisteínicas e proteínas

relacionadas a estresse, tais como, superoxide dismutase e ascorbate peroxidase (Ghosh e Pal, 2013). As proteases cisteínicas estão associadas à hidrólise de proteínas de reserva, mobilização (Schlereth et al., 2000) e diferenciação de tecidos (Ye e Varner, 1996). Em adição, proteínas relacionadas a estresse como as enzimas antioxidantes superoxide dismutase e ascorbate peroxidase, são importantes durante o processo de diferenciação (Panigrahi et al., 2007). A regeneração de brotos a partir de folhas de *Tacitus bellus* também foi dependente da abundância diferencial de enzimas antioxidantes (Mitrović et al., 2012). Durante os estádios iniciais da organogênese nesta espécie houve uma diminuição na abundância da superoxide dismutase e aumento da abundância da catalase, e posteriormente durante o desenvolvimento das brotações houve um aumento da peroxidase e polifenol oxidase. Segundo Mitrović et al. (2012), estas enzimas regulam pontos específicos durante o desenvolvimento das brotações, tais como o início da organogênese (indução) e desenvolvimento das brotações em *T.bellus*.

Em adição, durante a aquisição da competência morfogênica, proteínas envolvidas com resposta ao estresse, ao metabolismo e à divisão celular foram diferencialmente expressas em culturas nodulares de *Vriesea reitzii* (Corredor-Prado et al., 2016). As principais proteínas reguladas neste processo foram heat shock 22 kDa, chaperone protein dnaJ 50, S-adenosylmethionine synthase 3, UDP-arabinopyranose mutase 1 and 14-3-3-like protein E. Segundo Corredor-Prado et al. (2016), estas proteínas regulam o desenvolvimento da organogênese através da modulação de vias específicas de resposta ao estresse, ao metabolismo e à divisão celular, promovendo a aquisição da competência morfogênica em *V. reitzii.*

Estudos proteômicos também têm revelado uma associação entre PAs e o metabolismo de proteínas na resposta morfogenética *in vitro*, especialmente na embriogênese somática. O uso de PAs exógenas induziu alterações proteômicas em classes de proteínas diretamente relacionadas com resposta embriogênica, sendo importante para a indução e o desenvolvimento da embriogênese somática em *Saccharum* spp (Reis et al., 2016). Especificamente, Put promoveu diferenças na abundância de proteínas relacionadas à embriogênese somática, tais como,

arabinogalactan proteins, peroxidases, heat shock proteins (HSPs), glutathione s-transferases, late embryogenesis abundant proteins (LEA) e 14-3-3 proteins. De acordo com Reis et al. (2016), Put é uma importante PA que regula proteínas que desempenham funções importantes na proteção das células contra estresse, contribuindo para a formação de embriões somáticos. Similarmente, em *A. angustifolia*, a análise proteômica revelou proteínas diferencialmente abundantes associadas com a competência embriogênica, revelando que a competência embriogênica nesta espécie é diretamente afetada por proteínas associadas ao metabolismo de PAs (Jo et al., 2013). Na linha celular resposiva para as condições de maturação em *A. angustifolia* (Linha R), foi identificada a S-adenosylmethionine synthase, uma enzima associada à síntese de PAs, enquanto que na linha não responsiva para condições de maturação (Linha B) esta enzima foi ausente (Jo et al., 2013). A observação da enzima S-adenosylmethionine synthase na linha celular R sugere a importância desta enzima no desenvolvimento de embriões somáticos em A*. angustifolia*, bem como o envolvimento de PAs (Jo et al., 2013). Embora vários estudos mostrem a interação de PAs e proteínas na embriogênese somática, na resposta à organogênese *in vitro* pouco se conhece sobre a relação entre PAs e a abundância diferencial de proteínas.

2.6. Proteômica durante o envelhecimento de sementes: necessidade de estudos para a manutenção da viabilidade durante o armazenamento

O armazenamento consiste em armazenar as sementes adequadamente na tentativa de manter a sua qualidade fisiológica máxima, sendo um método de conservação *ex situ* dos recursos genéticos vegetais, usado para preservar a viabilidade e o vigor das sementes (Phartyal et al., 2002; Medeiros, 2006).

Diversas técnicas estão sendo frequentemente estudadas em busca de condições ideais de armazenamento, entretanto, a principal ainda é a redução do metabolismo da semente, tanto por meio da remoção de água como pela diminuição da temperatura de estocagem (Alves et al., 2008). O tipo de embalagem utilizado afeta a perda de água pela semente. De acordo com Baudet (2003), as embalagens para armazenamento de sementes são classificadas

segundo o grau de permeabilidade da água, sendo de três tipos. As embalagens permeáveis, que admitem trocas de vapor da água entre as sementes e o ar atmosférico (sacos de papel); as semipermeáveis, que oferecem certa resistência à troca de umidade (papel multifoliado e saco de polietileno) e as impermeáveis, que não permitem que a umidade do ar exerça influência sobre a semente (frasco de vidro hermeticamente fechado, frascos de metal). No entanto, outros fatores são de grande importância para o armazenamento de sementes visando à manutenção da viabilidade, tais como, temperatura e umidade, os quais devem ser definidos para cada espécie vegetal (Medeiros, 2001). Porém, a deterioração da semente é um processo inevitável e que ocorre mesmo sob as melhores condições de armazenamento, afetando o vigor e a germinação (Rajjou et al., 2008).

Até o momento, não estão elucidados os mecanismos moleculares de deterioração das sementes (Sathish et al., 2015). No entanto, pesquisas apontam que a produção de radicais livres durante o armazenamento é uma das principais causas de danos em membranas celulares, proteínas e ácidos nucleicos, que, finalmente, resulta na deterioração de organelas celulares e envelhecimento da semente (Bailly, 2004; Kibinza et al., 2006).

Em sementes de espécies florestais, o correto armazenamento pode ajudar a controlar a deterioração, a qual é contínua e muitas vezes exibe uma alta velocidade, que resulta na perda da viabilidade e vigor das sementes (Souza et al., 2012). Desta forma, o período em que a viabilidade de sementes de espécies florestais nativas pode ser mantida é muito curto, a depender do tipo de semente (ortodoxa ou recalcitrante). Desta forma, pesquisas sobre armazenamento assumem caráter de extrema importância, pois a redução da viabilidade durante o armazenamento é uma problemática para a propagação destas espécies (Carvalho et al., 2008). Assim, retardar a velocidade de deterioração é fundamental para programas de produção de mudas e de reflorestamento (Souza et al., 2012). Então, a busca pelas melhores condições de armazenamento e pelo conhecimento das alterações em nível fisiológico e bioquímico tem se intensificado nos últimos anos para a preservação da qualidade das sementes (Carvalho et al., 2008; Sasaki et al., 2013; Sousa et al., 2016).

Alterações no proteoma da semente podem ocorrer durante o armazenamento, mesmo na semente com baixo teor de água, levando à expressão de um perfil proteômico alterado, o qual poderá interferir na qualidade e no vigor das sementes armazenadas e na germinação (Rajjou et al., 2008). Estudos proteômicos durante o armazenamento de sementes e na indução do envelhecimento acelerado foram realizados para algumas espécies. Em *Arabidopsis thaliana* foram identificadas proteínas específicas, como, β-mercaptopiruvato sulfurtransferase (MST), uma das responsáveis pela manutenção do vigor das sementes durante o envelhecimento acelerado (Rajjou et al., 2008). A MST catalisa a transferência de enxofre a partir de mercaptopiruvato para aceptores de enxofre, tais como tióis e cianeto (Papenbrock e Schmidt, 2000), contribuindo para a desintoxicação do cianeto celular (Cipollone et al., 2007). O cianeto é frequentemente associado a mecanismos deletérios que levam a morte celular. Além disso, ele pode inibir a atividade de proteínas, como peroxidases (Ellis e Dunford, 1968; Job e Ricard, 1975) e catalases (García et al., 2007), impedindo potencialmente a defesa contra o ataque de espécies reativas de oxigênio (ROS). Adicionalmente, em sementes de *Zea mays* induzidas ao envelhecimento acelerado foram observadas mudanças significativas na expressão de proteínas associadas com estresse, armazenamento de nutrientes e envolvidas com mecanismos de tolerância a dessecação (Wu et al., 2011).

Sementes de *Fagus sylvatica* armazenadas por 13 anos em caixas plásticas a -10 ºC apresentaram altas quantidades de proteínas carboniladas (Kalemba e Pukacka, 2014). A carbonilação é uma reação química que produz compostos orgânicos com grupamento carbonilo (C=O), tais como aldeídos e cetonas. Em proteínas, a reação envolve várias etapas e ocorre devido ao estresse oxidativo. Proteínas carboniladas perdem sua função biológica e são marcadas para proteólise (Kalemba e Pukacka, 2014). De acordo com Kalemba e Pukacka (2014), há uma forte correlação negativa entre o aumento da carbonilação de proteínas e a capacidade germinativa das sementes. Desta forma, sementes de *F. sylvatica* são incapazes de germinar depois de um longo período de armazenamento, devido à perda da tolerância a dessecação e ainda

por causa dos efeitos do armazenamento sobre a síntese e degradação de proteínas (Kalemba e Pukacka, 2014).

Alterações na composição de fosfolipídios e a consequente perda da integridade da membrana celular também estão correlacionadas com a perda da viabilidade em sementes. A fosfolipase D (PLD) é uma enzima que catalisa a hidrólise de fosfolipídios para ácido fosfatídico, e a PLDα é uma isoforma abundante de PLD (Lee et al., 2012). Investigações feitas em sementes transgênicas de soja (*Glycine max*) armazenadas em envelopes de papel a 25º C por 33 meses, mostraram que a PLDα altera o perfil de fosfolipídios e triacilgliceróis nas membranas celulares afetando a capacidade germinativa (Lee et al., 2012). A supressão da atividade da PLD em sementes de soja potencialmente melhora a qualidade das sementes durante o armazenamento a longo prazo (Lee et al., 2012). Duxbury et al. (1991) sugeriram que a degradação da bicamada lipídica durante o envelhecimento é facilitada por enzimas lipolíticas, tais como PLD, que estimula a perda da função das proteínas e proteólise.

Adicionalmente, sementes de álamo (*Populus* × *Canadensis* Moench) armazenadas por 3 meses a 30 ºC perdem a capacidade germinativa (Zhang et al., 2015). Segundo González et al. (2010) a longevidade de sementes de espécies *Populus* é muito curta e limitada a alguns dias ou semanas sob condições de armazenamento artificial. Nestas sementes, a análise proteômica durante o armazenamento revelou que a maioria das proteínas identificadas estava envolvida em processos de metabolismo celular, síntese, energia, defesa e reserva. Os resultados mostraram que o envelhecimento das sementes é um processo dependente de energia, o qual requer síntese e degradação de proteínas, bem como proteínas envolvidas em processos de defesa celular contra estresse, tais como as HSPs e proteínas LEA (Zhang et al., 2015). As proteínas HSPs, quando acumuladas durante o armazenamento de sementes, demonstram maior tolerância à dessecação (Prieto-Dapena et al., 2006). Similarmente, as proteínas LEA, envolvidas na tolerância à dessecação no final do desenvolvimento embrionário, são potenciais marcadores de sementes com baixo vigor e são expressas no momento da aquisição da tolerância à dessecação,

desaparecendo durante a germinação (Zhang et al., 2015). Por outro lado, as HSPs atuam como chaperonas moleculares, desempenhando funções na manutenção da homeostase celular tanto para crescimento quanto em resposta a condições de estresse (Rodziewicz et al., 2014)

Em sementes de *Medicago sativa* armazenadas durante 42 anos em recipientes de polietileno a 20 ºC, a atividade das enzimas peroxidase, catalase e superóxido dismutase foram significativamente mais baixas comparativamente ao controle (sementes não armazenadas) (Cakmak et al., 2010). A diminuição da capacidade germinativa foi associada à baixa atividade destes enzimas (Cakmak et al., 2010) uma vez que estas fazem parte de um aparato enzimático protetor atuando na remoção e redução de ROS que podem causar danos celulares afetando a qualidade das sementes (Hosamani et al., 2013). Neste sentido, a superóxido dismutase catalisa a dismutação de duas moléculas superóxido dentro de oxigênio molecular e do peróxido de hidrogênio (H_2O_2) (Scandalios, 1993), enquanto a peroxidase e a catalase estão envolvidas na remoção de H_2O_2 (Goel et al., 2003). Em adição, a ascorbato peroxidase reduz H_2O_2 a água, usando ascorbato como um doador de elétrons, sendo sua atividade resultado de estresse oxidativo (Nakano e Asada, 1981). Por outro lado, em sementes de *Oriza sativa* cultivares BRS Ourominas e BRSMG Caravera houve aumento da atividade das enzimas catalase e ascorbato peroxidase durante o armazenamento em sacos de papel em câmara fria a 5 ºC (Marques et al., 2014). O aumento na atividade da catalase tem sido relacionado com a produção de H_2O_2 em condições de estresse oxidativo (Nasr et al., 2011). Assim, a redução da atividade desta enzima em sementes reduz a capacidade respiratória, diminuindo o fornecimento de energia (ATP) e prejudicando a germinação (Demirkaya et al., 2010).

Diante do exposto, é possível verificar que várias proteínas são reguladas durante o armazenamento na tentativa de manter o vigor e o potencial germinativo das sementes. Por outro lado, muitas das proteínas têm suas funções comprometidas devido a danos causados principalmente por estresse oxidativo. Portanto, para compreender melhor a função destas e outras proteínas durante o envelhecimento e a germinação de sementes, estudos sobre a abundância

diferencial de proteínas são necessários para melhor elucidação das funções biológicas que cada proteína exerce nestes eventos.

Em espécies arbóreas, especialmente *C. legalis*, o adequado armazenamento de sementes é um fator determinante para a manutenção da viabilidade. As sementes desta espécie sob condições de armazenamento a 4º C em sacos plásticos, reduzem o potencial germinativo e exibem alterações em compostos bioquímicos importantes para a viabilidade, tais como carboidratos, PAs e aminoácidos (Sousa et al., 2016). Entretanto, não foram ainda realizados estudos proteômicos durante o armazenamento de sementes nesta espécie.

3. OBJETIVOS

3.1. Geral

Estabelecer as melhores condições para o desenvolvimento da organogênese direta a partir de explantes nodais apicais e cotiledonares em *C. legalis* e *C. fissilis* e identificar proteínas diretamente relacionadas com o desenvolvimento de brotações *in vitro* em *C. fissilis* e com a manutenção ou perda da viabilidade em sementes de *C. legalis.*

3.2. Específicos

- Determinar os efeitos de PAs exógenas na indução e no desenvolvimento de brotações e da modulação de Put nos níveis endógenos de PAs e na abundância de proteínas pela análise *shotgun* durante o desenvolvimento de brotações em *C. fissilis;*
- Estabelecer as melhores condições para germinação *in vitro* de sementes, desenvolvimento da organogênese a partir de explantes nodais apicais e cotiledonares sob efeito de diferentes reguladores de crescimento vegetal (BA, ANA, GA_3 e PAs) e enraizamento de brotações micropropagadas de *C. legalis*;
- Investigar os efeitos do armazenamento de sementes de *C. legalis* por 12 meses através de abordagens proteômicas utilizando eletroforese bidimensional (2-DE), para identificar proteínas diferencialmente abundantes associadas com a perda e/ou manutenção da viabilidade das sementes.

4. TRABALHOS

4.1. PUTRESCINE PROMOTES CHANGES IN ENDOGENOUS POLYAMINE LEVELS AND PROTEIN ABUNDANCE TO REGULATE ORGANOGENESIS IN *Cedrela fissilis* VELLOZO (MELIACEAE)

RESUMO

Melhorias na organogênese *in vitro* são essenciais para otimizar o desenvolvimento de brotações e compreender os processos fisiológicos básicos. A adição de poliaminas (PAs) dentro do meio de cultura tem sido utilizada para modular a organogênese em plantas. Assim, este trabalho avaliou os efeitos de PAs exógenas sobre a organogênese direta de explantes nodais apicais e cotiledonares de *Cedrela fissilis* e os efeitos de putrescina (Put) sobre os níveis de PAs endógenas e na abundância diferencial de proteínas. Os efeitos de Put, espermidina (Spd) e espermina (Spm) exógenas, em diferentes concentrações (0; 0,5; 1; 2,5 e 5 mM) foram testados sobre o desenvolvimento de brotações. Entre as PAs usadas, 2,5 mM de Put exógena significativamente aumentou o comprimento das brotações (5 vezes) comparativamente ao tratamento controle

em brotações a partir de explantes nodais cotiledonares. O tratamento com 2,5 mM de Put significativamente aumentou os níveis endógenos de PAs livres totais e Put livre nas brotações oriundas de explantes nodais cotiledonares comparativamente ao tratamento controle (sem Put). A análise proteômica comparativa a partir de brotações mostrou mudanças significativas na abundância de proteínas após o tratamento com 2,5 mM Put, principalmente proteínas metabólicas e celulares associadas com processos de estresse e energia, bem como divisão celular. Estes resultados mostram que Put atua no metabolismo endógeno de PAs e altera a abundância de proteínas, contribuindo para o desenvolvimento de brotações em *C. fissilis*.

ABSTRACT

Improvements to *in vitro* organogenesis are essential to optimize shoot development and understand basic physiological processes. The addition of polyamines (PAs) into the culture medium has been used to modulate organogenesis in plants. Thus, this work evaluates the effects of exogenous PAs on direct organogenesis from apical and cotyledonary nodal *Cedrela fissilis* explants and the effects of putrescine (Put) on endogenous PA levels and differential protein abundance. The effects of exogenous Put, spermidine (Spd), and spermine (Spm) at different concentrations (0, 0.5, 1, 2.5 and 5 mM) on shoot development were tested. Among the PAs used, 2.5 mM exogenous Put significantly increased shoot length (5-fold) compared to the control treatment in shoots from cotyledonary nodal explants. Treatment with 2.5 mM Put significantly increased endogenous total free PA and free Put levels in shoots grown from cotyledonary nodal explants compared to the control treatment (no Put). Comparative proteomic analysis from shoots showed significant changes in protein abundance after 2.5 mM Put treatment, primarily metabolic and cellular proteins associated with stress and energy processes such as cell division. These

results show that Put functions in endogenous PA metabolism and alters protein abundance, contributing to shoot development in *C. fissilis*.

1. INTRODUCTION

Cedrela fissilis Vellozo (Meliaceae) is a native tree species in the Atlantic Forest that has been exploited because of its high ecological and economic value, primarily its highly prized wood. This species is currently included on the red threatened species list from the International Union for Conservation of Nature (IUCN) and is classified as endangered (IUCN 2016). Thus, appropriate strategies for the biotechnological and sustainable conservation of tropical hardwood tree species require additional research and development. *In vitro* clonal propagation has significant potential to produce and propagate threatened wood species (Pijut et al. 2012). In *C. fissilis*, a micropropagation system has been applied using nodal explants (Nunes et al. 2002), and changes to endogenous polyamine (PA) levels are related to the shoot development response (Aragão et al. 2016).

PAs are low-molecular-weight, aliphatic, polycationic compounds with positively charged nitrogen atoms that facilitate electrostatic interactions with macromolecules such as DNA, RNA, phospholipids, cell wall components, and proteins (Baron and Stasolla 2008). In plants, the three most common PAs are putrescine (Put), spermidine (Spd), and spermine (Spm) (Bouchereau et al. 1999), although other PAs, such as cadaverine and thermospermine might be present (Knott et al. 2007; Takano et al. 2012). The three common PAs are required for cell survival and growth because of molecular interactions with nucleic acids (transcription and translation) and cell membranes (Kusano et al. 2008; Minocha et al. 2014). PAs are involved in several physiological processes in plants, such as zygotic and somatic embryogenesis, morphogenesis, floral initiation and development, leaf senescence, pollen tube growth, fruit development and ripening, and response to abiotic and biotic stresses (Santa-Catarina et al. 2006; Dutra et al. 2013; Silveira et al. 2013; Farias-Soares et al. 2014; Tiburcio et al. 2014; Reis

et al. 2016), including direct organogenesis (Zhu and Chen 2005; Parimalan et al. 2011; Arun et al. 2014; Aragão et al. 2016).

Direct organogenesis is a biotechnological tool used to produce *in vitro* shoots from several trees (Pijut et al. 2012) by applying plant growth regulators (PGRs) to improve the rate of shoot induction and development. Among PGRs, 6-benzyladenine (BA) is the main cytokinin used on tree species during micropropagation to promote axillary bud development, apical dominance breaking, and shoot proliferation (Pijut et al. 2012). Nunes et al. (2002) described a micropropagation system using nodal explants in *C. fissilis* and showed that BA was indispensable for direct shoot induction and multiplication. In addition to cytokinin, other common PGRs used similarly, such as PAs, also improve the rate of shoot induction and development in plants, as demonstrated in *Cucumis sativus*, *Bixa orellana* and *Glycine max* (Zhu and Chen 2005; Parimalan et al. 2011; Arun et al. 2014). It has been suggested that exogenous PAs promote shoot formation by increasing the proliferation and growth of plant cells (Martin-Tanguy 2001). Recently, a study on *C. fissilis* showed addition of BA to the culture medium increased endogenous total free PA and free Put levels, which were potentially related to the growth and development of shoots from nodal explants (Aragão et al. 2016). However, there are no studies describing the effects of exogenous or endogenous PA levels on shoot development in *C. fissilis*.

Comparative proteomics allow the identification of specific proteins during *in vitro* organogenesis, which can establish possible marker proteins associated with organogenic competence. This technique is valuable to identify proteins involved in important physiological events that might control the specific morphogenic routes plant cells take *in vitro* to generate a complete plant (Heringer et al. 2015; Reis et al. 2016). In this sense, exogenous PAs induce changes in protein abundance directly related to embryogenic responses and are important to induce and maintain somatic embryogenesis in *Saccharum* spp (Reis et al. 2016). Similarly, in *Araucaria angustifolia*, proteomics analysis revealed that protein abundance patterns and changes to endogenous PA levels were associated with embryogenic competence, which in this species is directly affected by proteins associated with PA metabolism (Jo et al. 2013). In addition, during direct

organogenesis, proteomic studies revealed that shoots development competence involves multiple proteins with specific functions in morphogenetic acquisition. In *Vigna radiata*, proteomic profiling of explants that were responsive or not responsive to direct organogenesis revealed protein abundance differences (Das et al. 2006). In the same species, the different protein levels observed in these explants might indicate the stimulation of metabolic pathways associated with the acquisition of morphogenic competence, especially proteins involved in cell reprogramming (Ghosh and Pal 2013). Likewise, shoot regeneration from *Tacitus bellus* leaves was dependent on changes in specific protein and enzyme abundance (Mitrović et al. 2012). In addition, during morphogenic competence acquisition, proteins involved in the stress response, metabolism and cell division were expressed differently in nodular *Vriesea reitzii* cultures (Corredor-Prado et al. 2016).

The aim of this study was to determine the effects of exogenous PAs on shoot induction and development, of Put modulation on endogenous PA levels, and on protein abundance changes during shoot development in *C. fissilis*.

2. MATERIALS AND METHODS

2.1. Plant material

Mature *C. fissilis* seeds obtained from the Caiçara Comércio de Sementes Ltda. located in Brejo Alegre, SP, Brazil (21°10'S and 50°10'W) were germinated *in vitro*, and sixty-day-old seedlings were used as the explant source (apical and cotyledonary nodal) for shoot induction and development experiments as described by Aragão et al. (2016).

2.2. The effects of exogenous PAs on *in vitro* shoot induction and development

Apical and cotyledonary nodal explants (± 2 cm) were isolated from sixty-day-old seedlings and cultured in MS (Murashige and Skoog 1962) culture

medium (Phytotechnology Lab, Overland Park, USA) supplemented with 20 g L^{-1} sucrose (Vetec, Rio de Janeiro, Brazil), 2 g L^{-1} Phytagel (Sigma-Aldrich, St. Louis, MO, USA), and Put, Spd or Spm (all Sigma-Aldrich) at different concentrations (0, 0.5, 1, 2.5 and 5 mM). The pH of the culture medium was adjusted to 5.7, and the medium was autoclaved at 121 °C for 15 min. PAs were incorporated into the MS culture medium after filter sterilization using a 0.22 µm filter (Biofil, Guangzhou, China), with the pH adjusted to 5.7. The culture medium (30 mL per glass culture vessel) was aliquoted into 150 mL glass culture vessels (Aapace, São Paulo, Brazil). After inoculation, the explants were incubated in a growth room at 25 ± 2 °C with a 16 h photoperiod and a light intensity of 22 µmol m^2 s^{-1}. Each treatment contained eight replicates, and each replicate included one glass culture vessel containing five explants. For all treatments, the shoot induction (%), shoot length (cm) and number of shoots per explant (cm) were evaluated after 30 days of culture.

2.3. The effects of exogenous Put on endogenous free PA levels and the protein profile

Endogenous PAs and abundance in proteins levels were evaluated in control treatment (without Put) and treatment with 2.5 mM Put, which induced the best shoot development from cotyledonary nodal explants. These explants were cultured in the same conditions described above. For PA analysis, samples were collected before (day 0) and after 3, 6, 10, 20 and 30 days of culture, with three biological samples (200 mg fresh matter – FM - each sample) collected at each incubation time and stored at -20 °C until free PA assessment. Three biological samples (300 mg FM each sample) were collected after 30 days of culture and stored at -70 °C for proteomic analyses.

2.4. Free PA determination

Free PAs were determined according to Santa-Catarina et al. (2006). The samples (explants containing shoots) were ground in 1.2 mL of 5% perchloric acid (Merck, Darmstadt, Germany). After 1 h of incubation at 4 °C, the samples were centrifuged for 20 min at 20,000 x g at 4 °C. Free PAs were collected directly from

the supernatant by derivatization with dansyl chloride (Merck) and identified by 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 created by adding increasing volumes of absolute acetonitrile (Merck) to a 10% aqueous acetonitrile solution (pH 3.5) adjusted with hydrochloric acid (Merck). The gradient of absolute acetonitrile was programmed to 65% for the first 10 min, increasing from 65 to 100% between 10 and 13 min, and maintained at 100% between 13 and 21 min at a 1 mL min^{-1} flow rate and 40 °C. The PA concentration was determined using a fluorescence detector at 340 nm (excitation) and 510 nm (emission). The peak areas and retention times of the samples were measured by comparison with the standard PAs Put, Spd and Spm (Sigma-Aldrich).

The identification and quantification of endogenous PAs was conducted in a completely randomized factorial design. The data were analyzed by analysis of variance (ANOVA) ($p< 0.05$), and the means were compared with the Student–Newman–Keuls (SNK) test (Sokal and Rohlf 1995) using the R program (R Foundation for Statistical Computing, version 3.1.1, 2014, Vienna, Austria).

2.5. Protein extraction and digestion

The extraction of total protein was performed according to Balbuena et al. (2009). Samples were first macerated with liquid nitrogen until a powder was obtained. Next, 1 mL of extraction buffer containing 7 M urea (GE Healthcare, Piscataway, NJ, USA), 2 M thiourea (GE Healthcare), 2% Triton X-100 (GE Healthcare), 1% dithiothreitol (DTT) (Bio-Rad Laboratories, Hercules, CA, USA), 1 mM phenylmethanesulfonyl fluoride (PMSF) (Sigma-Aldrich) and 5 µM pepstatin (Sigma-Aldrich) was added to the samples. Then, the samples were vortexed and incubated on ice for 30 min, followed by centrifugation at 16,000 x g for 20 min at 4 °C. The supernatants were collected, and the protein concentration was measured using a 2-D Quant Kit (GE Healthcare).

For protein digestion, three biological replicates of 100 µg of protein were used for each treatment. Before digestion with trypsin, the samples were precipitated using methanol/chloroform methodology to remove MS-interfering

compounds (Nanjo et al. 2011). Next, the samples were desalted on Amicon Ultra-0.5 3 kDa centrifugal filters (Merck) using 50 mM ammonium bicarbonate (Sigma-Aldrich) pH 8.5 as the washing buffer. This procedure was repeated at least three times, resulting in approximately 50 µL per sample. Protein digestion was performed according to the methodology described by Calderan-Rodrigues et al. (2014). For each biological sample, 25 µL of 0.2% (v/v) RapiGest® (Waters, Milford, CT, USA) was added, and the samples were briefly vortexed and incubated in an Eppendorf Thermomixer® at 80 °C for 15 min. Then, 2.5 µL of 100 mM DTT (Bio-Rad) was added, and the tubes were vortexed and incubated at 60 °C for 30 min under agitation in the thermomixer. Next, 2.5 µL of 300 mM iodoacetamide (GE Healthcare) was added, and the samples were vortexed and incubated in the dark for 30 min at room temperature. The digestion was performed by adding 20 µL of trypsin solution (50 ng/µL; V5111, Promega, Madison, WI, USA) prepared in 50 mM ammonium bicarbonate, and the samples were incubated at 37 °C overnight. For RapiGest® precipitation, 10 µL of 5% (v/v) trifluoroacetic acid (Sigma-Aldrich) was added and incubated at 37 °C for 90 min, followed by a centrifugation step of 30 min at 16,000 x g. Samples were transferred to Total Recovery Vials (Waters).

2.6. Mass spectrometry analysis

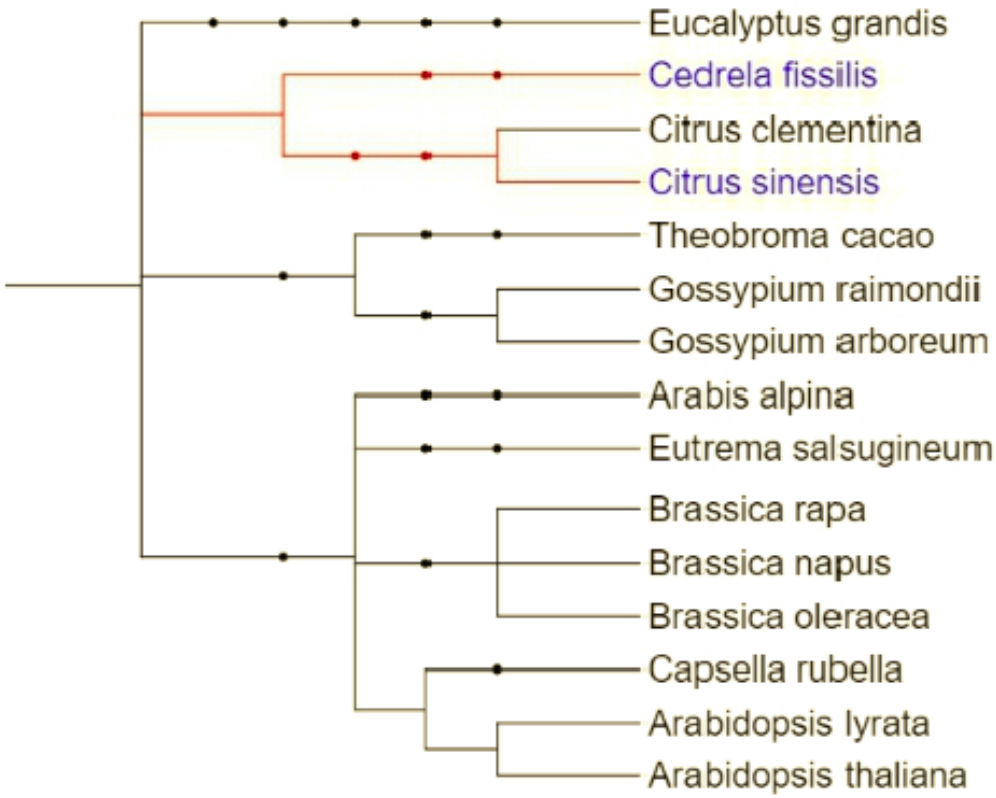
A nanoAcquity UPLC connected to a Synapt G2-Si HDMS mass spectrometer (Waters, Manchester, UK) was used for ESI-LC-MS/MS analysis according to Reis et al. (2016). The chromatography step was performed by injecting 1 µL of digested samples to normalize them before the relative quantification of proteins. To ensure standardized molar values for all conditions, normalization among samples was based on stoichiometric measurements of total ion counts of MS^E scouting runs prior to analyses. Runs consisted of three biological replicates. During separation, the samples were loaded onto a nanoAcquity UPLC 5 µm C18 trap column (180 µm × 20 mm) at 5 µL min^{-1} during 3 min and then on a nanoAcquity HSS T3 1.8 µm analytical reverse phase column (75 µm × 150 mm) at 350 nL/min with a column temperature of 60 °C. For peptide elution, a binary gradient was used, with mobile phase A consisting of water

(Tedia, Fairfield, Ohio, USA) and 0.1% formic acid (Sigma-Aldrich) and mobile phase B consisting of acetonitrile (Sigma-Aldrich) and 0.1% formic acid. Gradient elution began at 7% B, ramped from 7% B to 40% B until 91.12 min, then from 40% B to 99.9% B until 92.72 min, was maintained at 99.9% until 106.00 min, then decreased to 7% B until 106.1 min and maintained 7% B until the end of the experiment at 120.00 min. Mass spectrometry was performed in positive and resolution mode (V mode) at 35,000 FWHM with ion mobility in data-independent acquisition (DIA) mode, and IMS wave velocity was set to 600 m/s. The transfer collision energy ramped from 19 V to 45 V in high-energy mode, and the cone and capillary voltages were 30 V and 2800 V, respectively, with a source temperature of 70 °C. For TOF parameters, the scan time was set to 0.5 s in continuum mode with a mass range of 50 to 2000 Da. The human [Glu1]-fibrinopeptide B (Sigma-Aldrich) at 100 fmol μL^{-1} was used to calibrate the system externally, and lock mass acquisition was performed every 30 s.

2.7. Bioinformatics

Spectra processing and database searching were performed by Progenesis QI for Proteomics Software V.2.0 (Nonlinear Dynamics, Newcastle, UK). The analysis used the following parameters: one missed cleavage, minimum fragment ion per peptide equal to two, minimum fragment ion per protein equal to five, minimum peptide per protein equal to two, fixed modifications of carbamidomethyl (C), variable modifications of oxidation (M) and phosphoryl (STY), a default false discovery rate (FDR) value at a 4% maximum, score greater than five, and maximum mass errors of 10 ppm. For protein identification, we used the *Citrus sinenses* protein sequence database (2016/05) from Uniprot. This database was selected using the PhyloT phylogenetic tree (Letunic and Bork 2016) generated from all plant species with databases available at Uniprot (http://itol.embl.de/tree/2131659112735805147939833#) (Supplementary Figure 1). Label-free relative quantitative analyses were performed based on the ratio of protein ions among contrasting samples. After data processing and to ensure the quality of results, the following protein refinement parameters were used: only proteins present in 3 of 3 biological repetitions. Furthermore, proteins of different

abundances were selected based on a max fold change of at least 1.5 and ANOVA (p< 0.05). Protein description and functional annotation was performed using Blast2Go software v. 3.2 PRO (Conesa et al. 2005).



Supplementary Fig. 1 Phylogenetic placements of *C. fissilis* and *C. sinensis.*

3. RESULTS

3.1. The effects of exogenous PAs on *in vitro* shoot induction and development

In cotyledonary nodal segments, Put addition significantly increased shoot induction at all concentrations compared to the control (Figs. 1a and 2).

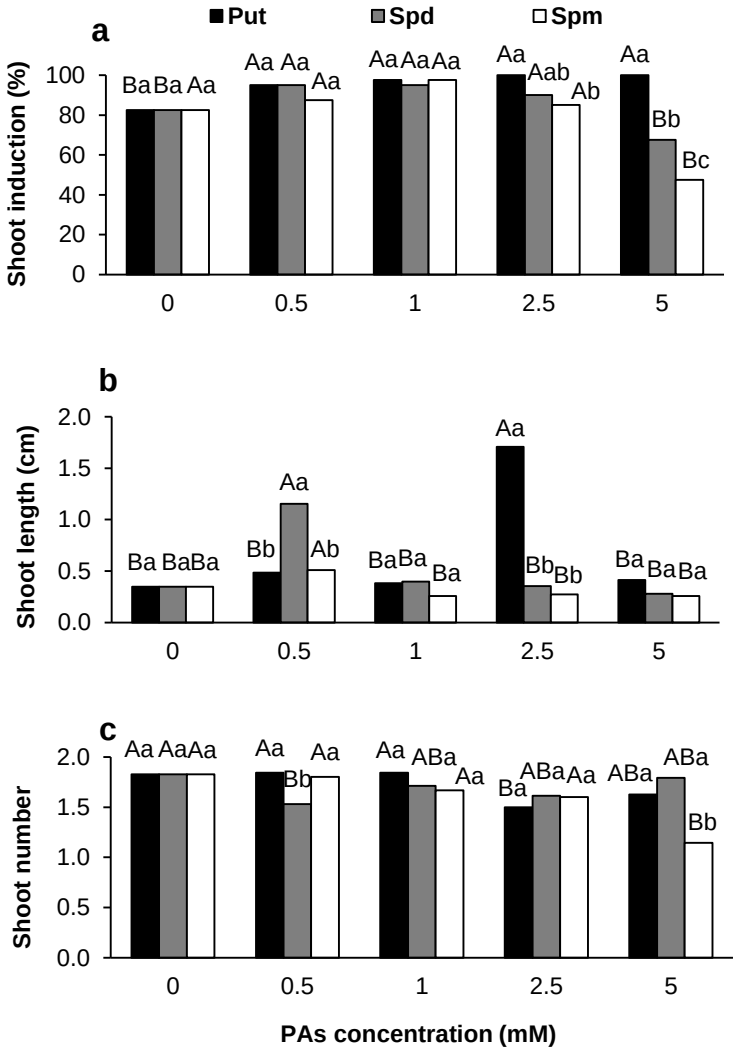


Fig. 1 Effects of the PAs Put, Spd and Spm on the induction (a), length (b) and number (c) of shoots from cotyledonary nodal *C. fissilis* explants after 30 days of culture. The means followed by different letters are significantly different ($p< 0.05$) according to the SNK test. Capital letters indicate significant differences for a single PA at different concentrations. Lowercase letters indicate significant differences between different PAs at the same concentration. CV = coefficient of variation (n = 8; CV shoot induction = 14.1%; CV shoot length = 31.2%; CV shoot number = 12.2%).

In addition, Spd concentrations from 0.5 to 2.5 mM induced a significant increase in shoot induction percentage, whereas the same concentrations of Spm showed no significant changes (Fig. 1a). However, 5 mM Spd and Spm treatment significantly decreased shoot induction compared to the control (Fig. 1a). The maximum shoot length (1.71 cm), 5 times the length of the control (0.35 cm), was obtained after 2.5 mM Put treatment (Figs. 1b and 2). Spd and Spm treatments at

0.5 mM also increased shoot length (approximately 3.5- and 1.5-fold, respectively) compared to the control (Fig. 1b). However, 5 mM Spm reduced the number of shoots per explant compared to other treatments (Fig. 1c).

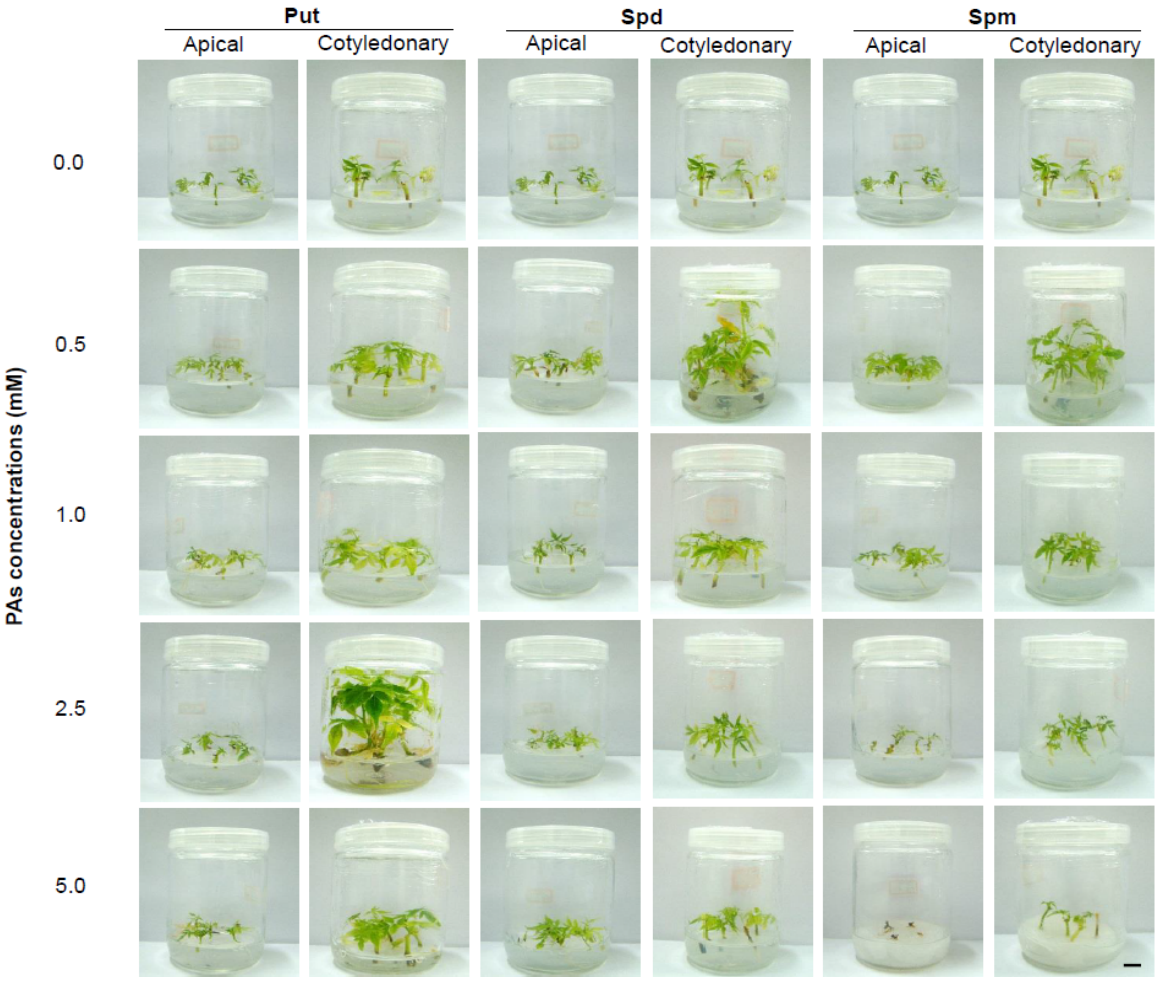
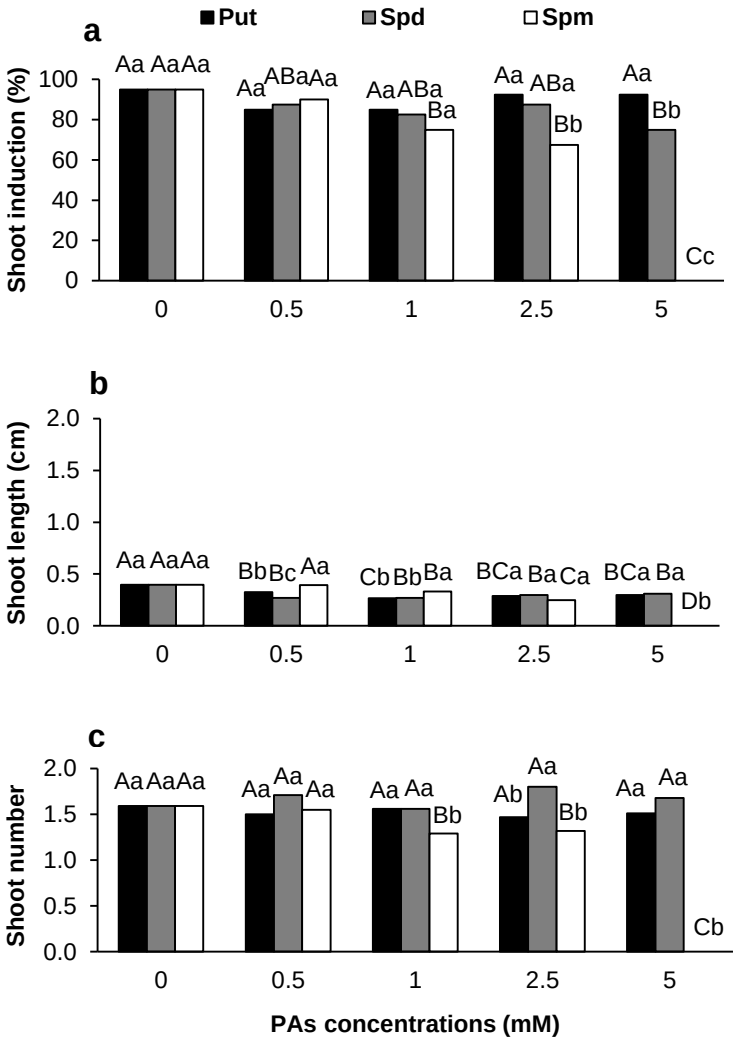


Fig. 2 Aspects of shoots obtained from apical and cotyledonary nodal explants of *C. fissilis* after 30 days of culture with Put, Spd and Spm at different concentrations (0, 0.5, 1, 2.5 and 5 mM). *Bars* = 1 cm.

When apical nodes were used as explants, the PAs added to the culture medium showed no significant increase in the shoot induction percentage, number of shoots per explant, or shoot length compared to the control treatment (Supplementary Fig. 2 and Fig. 2). In addition, 5 mM Spm treatment significantly decreased induction, length and shoot number per explant in this type of explant (Supplementary Fig. 2 and Fig. 2).



Supplementary Fig. 2 Effects of Put, Spd and Spm on the induction (a), length (b) and number (c) of shoots from apical nodal explants of *C. fissilis* after 30 days of culture. The means followed by different letters are significantly different ($p < 0.05$) according to the SNK test. Capital letters indicate significant differences for a single PA at different concentrations. Lowercase letters indicate significant differences between different PAs at the same concentration. CV = coefficient of variation (n = 8; CV shoot induction = 15.5%; CV shoot length = 14.8%; CV shoot number = 12.5%).

3.2. Effects of Put on endogenous free PA levels in shoots from cotyledonary nodal explants

Endogenous free PA was assessed in shoots produced from cotyledonary nodal explants cultured using control or 2.5 mM Put treatments (Fig. 3). Among the analyzed PAs, endogenous free Put levels increased significantly in shoots after 2.5 mM Put treatment compared to the control (without Put) (Fig. 3a). In

addition, the endogenous Spd level (Fig. 3b) was significantly higher after 2.5 mM Put treatment at 10 to 30 days of culture (Fig. 3b). In the control treatment, the endogenous Spd level significantly increased on the third day of culture and remained stable (Fig. 3b). In contrast, at the beginning of the culture (the first three days), endogenous Spm decreased significantly in both treatments and then increased significantly after 30 days of culture with 2.5 mM Put treatment (Fig. 3c) compared to the explant before incubation (time 0).

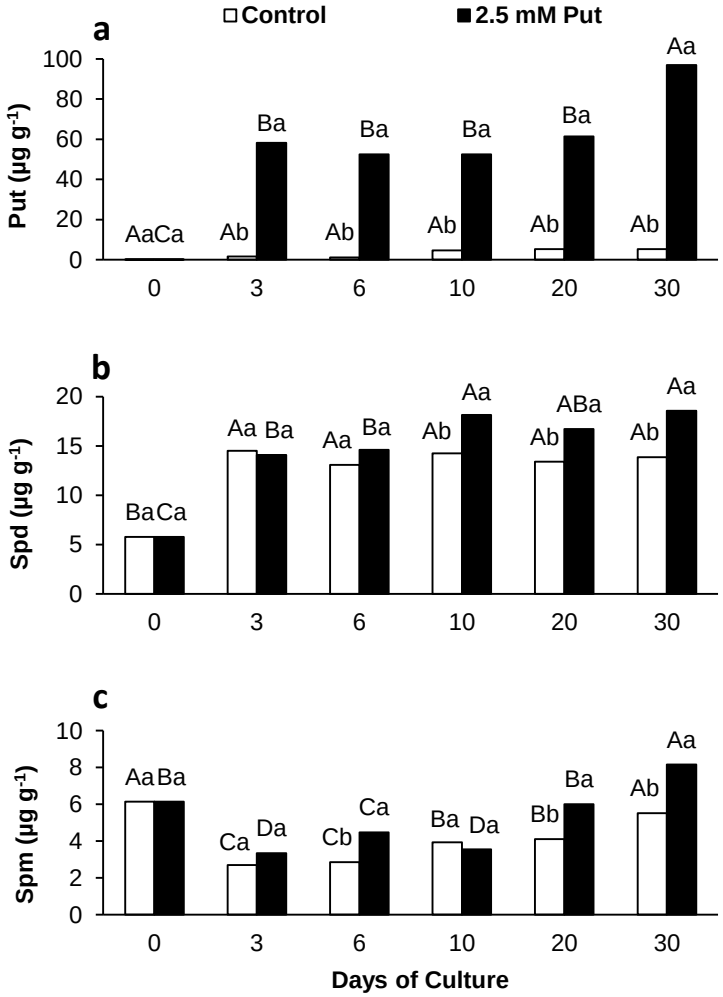


Fig. 3 Endogenous levels (µg g^{-1} FM) of Put (a), Spd (b) and Spm (c) in the shoots of cotyledonary nodal explants of *C. fissilis* cultured in the absence (0 mM) or presence (2.5 mM) of Put before culture (time 0) and after 3, 6, 10, 20 and 30 days of culture. The means followed by different letters are significantly different ($p< 0.05$) according to the SNK test. Capital letters indicate significant differences between days of culture under the same treatment. Lowercase letters indicate significant differences between treatments on each day of culture. CV = coefficient of variation (n = 3; CV Put = 21.4%; CV Spd = 10.8%; CV Spm = 8.9%).

In addition, the explants cultured in 2.5 mM Put showed significantly increased endogenous total free PA levels (Fig. 4), although no significant differences were observed in the control treatment (without Put).

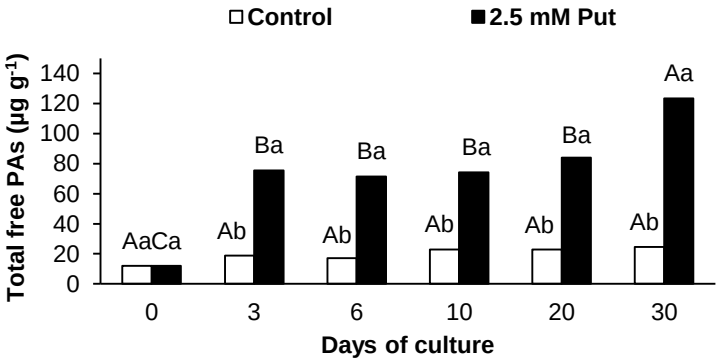


Fig. 4 Endogenous levels (µg g^{-1} FM) of total free PAs in cotyledonary nodal explant shoots from *C. fissilis* incubated in the absence (0 mM) and presence (2.5 mM) of Put before culture (time 0) and after 3, 6, 10, 20 and 30 days of culture. The means followed by different letters are significantly different ($p< 0.05$) according to the SNK test. Capital letters indicate significant differences between days of culture under the same treatment. Lowercase letters indicate significant differences between treatments on each day of culture. CV = coefficient of variation (n = 3; CV Total free PAs = 15.9%).

3.3. Effects of Put on protein abundance changes in micropropagated shoots from cotyledonary nodal explants

Proteomic analysis was performed in shoot samples after 30 days of culture from control and 2.5 mM Put treatments. Significant differences in protein abundance were observed between these treatments. A total of 627 proteins were identified, with 24 proteins being identified as differently abundant. Of these, 4 proteins were up-regulated and 19 were down-regulated in shoots from Put 2.5 mM treatment compared to the control, and one unique protein was identified only in the control treatment (Table 1).

Table 1 Differentially abundance of proteins identified by comparative proteomic analysis in micropropagated shoots from cotyledonary nodal explants of *C. fissilis* incubated in Put 2.5 mM and control treatments at 30 days of culture.

Acession	Peptide count	Score	Protein description	Functional classification	Anova	Fold Change	Regulation
A0A067GFA3	13	112.5	Mitochondrial	Cellular process and metabolic process	0.00455	0.567	DOWN
A0A067DVC5	23	268.2	Actin-7	cell division	0.00104	0.015	DOWN
A0A067FU05	2	11.4	Serine threonine-phosphatase PP2A-3 catalytic subunit	Developmental process, multicellular organismal process, cellular process, single-organism process and metabolic process	0.03454	0.578	DOWN
A0A067EST1	4	25.0	Katanin p60 ATPase-containing subunit A1	Cellular process and cellular component organization or biogenesis	0.01895	0.531	DOWN
A0A067FD00	2	11.4	Acid phosphatase survival	Cellular process and metabolic process	0.03467	0.618	DOWN
A0A067DC18	3	24.2	Pathogenesis-related 1-like	Defense response	0.02271	0.560	DOWN
A0A067EX42	2	15.9	Haloacid dehalogenase-like hydrolase domain-containing At2g33255	Metabolic process	0.00299	0.594	DOWN
A0A067F278	10	74.5	Isocitrate dehydrogenase [NADP]	Cellular process, response to stimulus and metabolic process	0.03179	2.245	UP
A0A067E4I5	2	12.7	Flowering locus K homology domain isoform X2	Developmental process, multicellular organismal process, reproduction and single-organism process, reproductive process	0.01364	0.637	DOWN
A0A067FHE0	9	55.3	ATP-dependent zinc metalloprotease FTSH chloroplastic isoform X1	Cellular process and metabolic process	0.01418	1.516	UP
Q18L98	5	32.7	Retrotransposon Ty1-copia subclasse	Cellular process and metabolic process	0.02914	0.535	DOWN
A0A067EDL0	2	13	26S proteasome non-ATPase regulatory subunit 7 homolog A	Cell division	0.01373	3.578	UP
A0A067FTM1	11	84.1	Cell division cycle 48 homolog	Cellular process	0.00002		Unique control
A0A067G473	4	44.3	Perakine reductase	Metabolic process	0.03479	0.549	DOWN
A0A067EFK3	4	31.6	Peptidyl-prolyl cis-trans isomerase CYP19-3	Cellular process and metabolic process	0.01545	0.448	DOWN
A0A067G0C0	2	11.6	Importin subunit alpha-2-like	NLS-bearing protein import into nucleus	0.00606	0.396	DOWN
A0A067EFQ8	3	23.3	Probable aldo-keto reductase 2	Auxin-activated signaling pathway and oxidation-reduction process	0.02442	0.440	DOWN
A0A067EYP5	2	17.1	Hypothetical protein CISIN_1g035371mg	Unknown	0.02914	0.620	DOWN
A0A067F6B4	3	19.7	Uncharacterized LOC8278573 precursor	Galactose-binding domain-like	0.01406	0.603	DOWN
A0A067GF90	9	85.6	Plastid-lipid-associated chloroplastic	Metabolic process	0.03204	2.067	UP
A0A067FHW1	9	72.5	Ubiquitin-activating enzyme E1 1	Cellular process and metabolic process	0.04988	0.599	DOWN
O65185	2	11.0	Acetyl- carboxylase 1-like	Cellular process, single-organism process and metabolic process	0.00163	0.024	DOWN
A0A067FJ50	3	18.4	Early nodulin 1	Unknown	0.04736	0.582	DOWN
A0A067G7P7	5	32.7	Probable NAD(P)H dehydrogenase (quinone) FQR1-like 1	Cellular process and metabolic process	0.02368	0.533	DOWN

Differentially abundant proteins were selected based on a max fold change of at least 1.5 and ANOVA ($p< 0.05$).

The up- and down-regulated proteins, as well as the unique protein, were classified using Blast2Go into nine groups according to biological processes (Fig. 5). The identified proteins were involved in metabolism (13 proteins), cellular processes (12 proteins), single-organism processes (3 proteins), developmental processes (2 proteins), multicellular organismal processes (2 proteins), reproduction (1 protein), response to stimulus (1 protein), reproductive processes (1 protein) and cellular organization or biogenesis (1 protein) (Fig. 5, Table 1). Of the differently regulated proteins, 7 were not classified under any description or biological process by Blast2Go and were classified according to Uniprot, resulting in the class designated of “others” (Fig. 5).

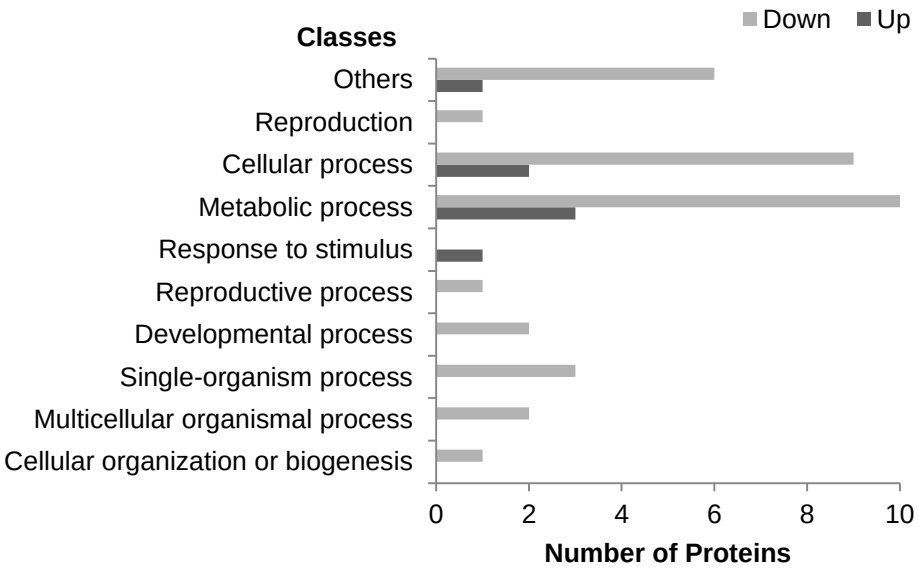


Fig. 5 The functional classification of 24 differentially abundant proteins in micropropagated shoots from cotyledonary nodal explants of *C. fissilis* after 30 days of culture in control and 2.5 Mm Put treatments.

4. DISCUSSION

PAs are involved in a number of developmental processes in plants, such as growth, morphogenesis, and differentiation (Bais and Ravishankar 2002). *In vitro* experiments have shown that PAs might be important in several processes,

including shoot regeneration in plants (Sivanandhan et al. 2011; Vondráková et al. 2015; Aragão et al. 2016). In our work, the significant effects of exogenous Put at improving shoot induction were observed in cotyledonary nodal explant shoots at all concentrations tested (Fig. 1a), inducing maximum shoot length at 2.5 mM (Fig. 1b). In several species, exogenous Put might be associated with shoot morphogenesis. In *Sinningia speciosa*, exogenous Put at a lower concentration (50 mg L^{-1}) increased the number and length of shoots using leaves as explant sources (Park et al. 2012). In addition, in *B. orellana*, treatment with 0.8 and 1 mM Put produced the highest values for shoot length and shoot number per explant, respectively, in shoots arising from nodal explants (Parimalan et al. 2011). Furthermore, in *Cichorium intybus*, the use of a higher concentrations of exogenous Put (40 mM) increased both the induction and length of shoots in axillary shoot cultures (Bais et al. 2001).

Additionally, the effects of exogenous Put on shoot elongation were evident in *C. fissilis* (Fig. 1b) at 2.5 mM, with a significant increase in shoot elongation (5-fold) after 30 days of *in vitro* culture compared to the control (Fig. 2), indicating that Put is an important PA promoting shoot elongation in this species. Higher endogenous free Put levels were associated with both increased shoot induction and shoot length arising from cotyledonary nodal *C. fissilis* explants (Aragão et al. 2016), suggesting that endogenous PA metabolism, especially free Put levels, has an important role in the morphogenetic shoot elongation response in *C. fissilis*. Moreover, exogenous Put 2.5 mM treatment induced an increase in endogenous free Put content (Fig. 3a) in shoots from cotyledonary nodal explants, resulting in more elongated shoots compared to controls. The increase in endogenous Put content is potentially related to the intense cell division necessary for new shoot elongation in this species (Aragão et al. 2016). Put is a relevant PA involved in shoot growth in *Malus sylvestris,* and the regulation of this process might occurs by modulating cellular PA levels (Liu et al. 2009). These authors showed that *M. sylvestris* shoots cultured *in vitro* with exogenous Put (1 and 5 mM) led to an evident increase in endogenous Put levels and shoot length (Liu et al. 2009). High endogenous Put levels have also been observed during shoot development from *Colocasia esculenta* and *Curcuma longa* calluses*,* suggesting that this PA is an

important factor controlling morphogenetic events in organogenesis in these species (Francisco et al. 2008; Viu et al. 2009). In this sense, the higher endogenous Put levels might be considered a valuable candidate biochemical marker of differentiation, especially the organogenesis of shoots and roots (Viu et al. 2009). In *C. fissilis*, high endogenous Put levels promote the development of shoots arising from cotyledonary nodal explants (Aragão et al. 2016), and this work shows that Put is a potential promoter of elongation, as exogenous Put addition increased the endogenous Put levels, inducing longer shoots in *C. fissilis*.

The effect of Put on shoot length enhancement in *C. fissilis* might be attributed to Put's well-known biological role stimulating cell division and elongation (Bais and Ravishankar 2002; Kuznetsov et al. 2002). Thus, we suggest that Put increases the number of cell divisions in shoots arising from cotyledonary nodal explants, resulting in longer shoots than those cultured with the control treatment. In addition, Put also has a regulatory effect on changes in the balance of endogenous indole-3-acetic acid (IAA) during cell growth in embryogenic cultures of *Araucaria angustifólia,* because PAs are able to replace auxin effects (Bais and Ravishankar 2002; Steiner et al. 2007). PAs, which are required for the cell cycle, are subject to concentration changes during the cell cycle (Weiger and Hermann 2014). The cell cycle is divided into defined phases: G1 (first gap), S (synthesis), G2 (second gap), and M (mitosis) (Weiger and Hermann 2014). During the cell cycle, PA levels first increase at the G1/S transition prior to DNA synthesis in S phase, which contributes to the stabilization of DNA and to correct DNA conformation (Maki et al. 1991; Espunya et al. 1999; Scorcioni et al. 2001). The second increase in free PA levels observed at the G2/M phase might be associated partly to the essential role of PAs in stabilizing the β-subunit of CK2, a protein kinase with an important role at the G2/M checkpoint (Espunya et al. 1999).

In this study, the addition of exogenous Put also increased endogenous free Spd (Fig. 3b) and Spm levels (Fig. 3c) at the end of culture compared to the control treatment. According to Niemi et al. (2002), Spd and Spm are PAs associated with cellular differentiation in embryogenic cultures from *Pinus sylvestris*. Thus, the higher Spd and Spm levels observed after 2.5 mM Put

treatment might also be important for cell differentiation and shoot elongation in cotyledonary nodal explants from *C. fissilis*. In addition, PAs not only can act as PGRs but also are important biomolecules that supply carbon and nitrogen for assimilation by plants and are used during growth and development (Couée et al. 2004). Thus, increased PA biosynthesis results in the increased assimilation of both nitrogen and carbon by cells in *Arabidopsis thaliana* seedlings (Majumdar et al. 2016).

Exogenous Put increased total free PA levels in shoots from cotyledonary nodal explants, whereas no significant differences were observed in the absence of Put (Fig. 4). Elevated total free PA levels are important somatic embryogenesis in *Acca sellowiana* (Cangahuala-Inocente et al. 2014) and for direct organogenesis in *C. fissilis* (Aragão et al. 2016). Particularly in *C. fissilis*, elevated total free PA levels were potentially related to shoot growth and development during *in vitro* culture, given that these tissues experience intense cell division activity, differentiation, and elongation (Aragão et al. 2016). In this work, endogenous total free PA accumulation increased when exogenous Put was administered (Fig. 4). Exogenous PAs can be applied to elevate endogenous PA levels, which has already been shown to improve plant regeneration (Takeda et al. 2002).

Proteomics differences have been observed during the *in vitro* organogenesis of different plant species, such as *Asteracantha longifolia, T. bellus, V. radiata* and *V. reitzii* (Panigrahi et al. 2007; Mitrović et al. 2012; Ghosh and Pal 2013; Corredor-Prado et al. 2016), including changes caused by exogenous PAs (Reis et al. 2016). In our study, exogenous Put 2.5 mM enhanced (5x) shoot elongation from cotyledonary nodal explants and modulated the abundance of proteins involved in metabolism processes (Fig. 5), such as haloacid dehalogenase-like hydrolase domain-containing At2g33255 and mitochondrial protein, which were down-regulated, as well ATP-dependent zinc metalloprotease FTSH chloroplastic isoform X1 and plastid-lipid-associated chloroplastic, which were up-regulated (Table 1). These proteins are involved mainly in energy and stress processes. The haloacid dehalogenase-like hydrolase domain-containing At2g33255 is a likely stress-related protein in *Brassica juncea* (Minglin et al. 2005),

whereas mitochondrial proteins are involved in various physiological processes, such as energy metabolism, signal transduction, and the maintenance of mitochondrial morphology (Dudek et al. 2013). According to Jo et al. (2013) the regulation of mitochondrial proteins is important for *in vitro* morphogenetic processes, such as somatic embryogenesis in *A. angustifolia*. In addition, the ATP-dependent zinc metalloprotease FTSH chloroplastic isoform X1 is a protease that degrades plasma membrane and cytosolic proteins and plays an important role in photosynthetic apparatus damage protection (Kosmala et al. 2012). This protease might be important for shoot growth, promoting greater photosynthetic apparatus efficiency *in vitro* cultures. Plastid-lipid-associated chloroplastic are proteins known to accumulate in leaf chloroplasts in response to oxidative and abiotic stress in *Solanaceae* species (Langenkämper et al. 2001). In sugarcane, Put plays important roles protecting the cells against *in vitro* stress (Reis et al. 2016), and this protein can induce protection against *in vitro* stress in *C. fissilis* by up-regulating plastid-lipid-associated chloroplastic.

Comparative proteomics analysis showed that shoot developmental is followed by changes in proteins related to cellular processes (Fig. 5). Treatment with 2.5 mM Put induced the down-regulation of proteins associated with catalytic activity and energy, as such peptidyl-prolyl cis-trans isomerase CYP19-3 and probable NAD(P)H dehydrogenase (quinone), respectively (Table 1). The peptidyl-prolyl cis-trans isomerase CYP19-3 has catalytic activity in the embryogenic sugarcane callus (Heringer et al. 2015). During *in vitro* shoot development in *V. radiata*, the onset of shoot induction is associated with higher levels of this protein (Ghosh and Pal 2013). These authors suggested that protein frame rearrangement occur during shoot organogenesis and are important for this process, acting as folding catalysts. However, dehydrogenase-like protein is an enzyme that oxidizes a substrate through a reduction reaction in which one or more hydrides (H) are transferred to an electron acceptor. Plants express many dehydrogenases involved in several biological processes, particularly somatic embryogenesis (Heringer et al. 2015), and this protein is highly regulated during morphogenetic processes acting to promote (Bian et al. 2010) or inhibit (Jo et al. 2013) somatic embryogenesis.

In class developmental process (Fig. 5), the serine threonine-phosphatase PP2A-3 catalytic subunit was down-regulated (Table 1). This protein is an abundant and ubiquitous member of the plant protein phosphatase P family and serves as the obligate partner of protein kinases in cell growth control circuitry (DeLong 2006). This protein plays diverse roles in transcription, translation, differentiation, cell cycle, and signal transduction in many organisms (Du et al. 2013) and is absent from the embryogenic callus of *Vitis vinifera* because it affects normal development (Zhang et al. 2009). Thus, we suggest an important role for Put in the regulation of this protein due its deleterious effects on *in vitro* morphogenesis, as observed by Zhang et al. (2009) in *V. vinifera*.

The proteins classified in the response to stimulus group (Fig. 5) were up-regulated, including the isocitrate dehydrogenase [NADP] protein involved in stress response (Table 1). According to Corpas et al. (1999), isocitrate dehydrogenase can defend against oxidative stress in *Pisum sativum.* In addition, isocitrate dehydrogenase has been proposed to be a source of 2-oxoglutarate for ammonium assimilation and, therefore, for amino acid synthesis (Gálvez et al. 1999). Thus, up-regulation of this enzyme in shoots after 2.5 mM Put treatment might be important to relieve oxidative stress during shoot development and provide a source of nitrogen for the intense growth of *in vitro* cultures.

5. CONCLUSIONS

To our knowledge, this is the first comprehensive report describing the effects of exogenous PAs on organogenesis from apical and cotyledonary nodal explants from *C. fissilis*, an endangered native tree from the Brazilian Atlantic Forest. Exogenous Put treatment at 2.5 mM increased the length of shoots arising from cotyledonary nodal explants and changed the endogenous levels of free Put and total free PAs, which might be related to shoot elongation. Exogenous Put induced changes in protein abundance necessary for shoot development, altering the protein profile in shoots from cotyledonary nodal explants compared to shoots

from control treatment. Put treatment regulated proteins involved mainly in metabolic, cellular and developmental processes as well as the response to stimulus associated with oxidative and abiotic stress, energy and cell division. This study shows the successful improvement of the micropropagation protocol for *C. fissilis* using PAs to modulate the endogenous PA levels and using proteomic profiling to study shoot development.

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4.2. MICROPROPAGATION OF *Cariniana legalis* (MARTIUS) O. KUNTZE, AN ENDANGERED HARDWOOD TREE FROM THE BRAZILIAN ATLANTIC FOREST

RESUMO

O objetivo deste estudo foi estabelecer as melhores condições para germinação *in vitro*, organogênese e enraizamento de brotações micropropagadas de *Cariniana legalis*. Para a germinação *in vitro* foram testados os efeitos dos meios de cultura Murashige e Skoog (MS) e woody plant medium (WPM). Os efeitos de diferentes combinações e concentrações de 6-benziladenina (BA), ácido α-naftalenoacético (ANA), ácido giberélico (GA_3) e poliaminas (PAs) foram investigados sobre a organogênese direta, e o ácido indol-3-butírico (AIB) foi testado para o enraizamento *ex vitro* de brotações. O meio de cultura WPM resultou em maior germinação de sementes *in vitro* e crescimento de plântulas do que o meio de cultura MS. O desenvolvimento de brotações foi alcançado em meio de cultura WPM sem a adição dos reguladores de crescimento vegetal BA, ANA, e GA_3 ou as PAs putrescina, espermidina e espermina. Os explantes nodais cotiledonares proporcionaram maiores respostas do que os explantes nodais apicais. O enraizamento de brotações micropropagadas pode ser alcançado através do enraizamento *ex vitro* na ausência de AIB, e uma maior indução de

raízes foi encontrada em brotações provenientes de explantes nodais cotiledonares do que aquelas de explantes nodais apicais. Este trabalho é o primeiro a descrever o estabelecimento da micropropagação em *C. legalis*, uma importante espécie arbórea, e os resultados são importantes para o estabelecimento de novas estratégias de conservação de plantas para espécies arbóreas ameaçadas de extinção.

ABSTRACT

The aim of this study was to establish the best conditions for *in vitro* germination, organogenesis and rooting of micropropagated shoots of *Cariniana legalis*. For *in vitro* germination, the effects of Murashige and Skoog (MS) and woody plant medium (WPM) were tested. The effects of different combinations and concentrations of 6-benzyladenine (BA), α-naphthaleneacetic acid (NAA), gibberellic acid (GA_3) and polyamines (PAs) on direct organogenesis were investigated, and indole-3-butyric acid (IBA) was tested for the ex vitro rooting of shoots. The WPM culture medium resulted in higher *in vitro* seed germination and seedling growth than the MS culture. Shoot development was achieved in WPM without adding the plant growth regulators BA, NAA, and GA_3 or the PAs putrescine, spermidine and spermine. Cotyledonary nodal explants provided greater responses than apical nodal explants. The rooting of micropropagated shoots can be achieved through ex vitro rooting in the absence of IBA, and a higher root induction was found in shoots arising from cotyledonary nodal explants than in those from apical nodal explants. This work is the first describing the establishment of micropropagation in *C. legalis*, an important wood species, and the results are important for establishing further plant conservation strategies for threatened tree species.

1. INTRODUCTION

Cariniana legalis (Martius) O. Kuntze (Lecythidaceae) is a native tree species of the Brazilian Atlantic Forest, and it currently occurs at a population density of less than 1 tree/ha in the forest [1]. Because of intensive wood exploitation, *C. legalis* is currently included on the red list of endangered species by the International Union for Conservation of Nature (IUCN), and it is classified as vulnerable [2]. Methods of vegetative propagation, such as traditional mini-cuttings or the use of micropropagation as a biotechnological tool, have not been used for this species.

Micropropagation is a viable method for mass propagation, which is the aim of the research that is being performed for many tree species [3]. Therefore, an efficient plant regeneration protocol is a prerequisite for the biotechnological breeding of economically and ecologically important species. Micropropagation offers a rapid means to produce clonal plants for forestation programs and conservation of germplasm [4]. This technique has many advantages over conventional propagation, such as the rapid multiplication of elite genotypes, the production of disease-free plants, and season-independent propagation, requiring less space in comparison with seed-grown saplings [4, 5]. In this sense, biotechnological tools, such as *in vitro* propagation, can be applied to propagate endangered tree species such as *C. legalis*.

Signalling agents such as plant growth regulators (PGRs) are used for micropropagation, and cytokinins and auxins are the most important PGRs [6]. For tree species, 6-benzyladenine (BA) is the cytokinin most often used to promote the development of axillary buds, the breaking of apical dominance and the stimulation of shoot proliferation [7, 8]. Moreover, the auxin α-naphthaleneacetic acid (NAA), combined with cytokinins such as BA and/or thidiazuron, can promote shoot development *in vitro* [9, 10] by stimulating cell division, cell elongation and shoot elongation [11]. In addition, gibberellic acid (GA_3) has long been known to promote vegetative growth, including the initiation of lateral organs and elongation,

furthering the development of more robust micropropagated shoots [12, 13]. Furthermore, polyamines (PAs) are multifunctional aliphatic nitrogen polycationic compounds that interact with macromolecules such as DNA, RNA, phospholipids, cell wall components and proteins [14] . PAs have been implicated in several physiological processes in plants, including organogenesis in tree species [15, 16].

Auxins also have a direct effect on adventitious root induction, and indole-3-butyric acid (IBA) is most often used to promote *in vitro* or *ex vitro* rooting in different tree species [3] because of its higher root-inducing capacity and greater stability in response to light [17]. In relation to *in vitro* rooting, *ex vitro* rooting is an important step in the establishment of propagation methodologies, and it reduces the costs and the time needed for micropropagation [18]. Another relevant advantage of *ex vitro* rooting is that the plantlets produced do not require any additional acclimatization steps; they exhibit lateral roots similar to the natural root system and achieve better survival in comparison to those that are developed from *in vitro* rooting [19, 20].

Thus, the aim of this study was to establish the best conditions for *in vitro* seed germination, organogenesis development from apical and cotyledonary nodal explants and rooting induction in micropropagated shoots of *C. legalis*.

2. MATERIALS AND METHODS

2.1. Plant material

Mature *C. legalis* seeds were obtained from the Caiçara Comércio de Sementes Ltda. located in Brejo Alegre, SP, Brazil (21º10'S and 50º10'W), and they were germinated *in vitro*. Ninety-day-old seedlings were used as the source of apical and cotyledonary nodal explants in the organogenesis experiments. Sixty-day-old micropropagated shoots were used for the rooting experiments.

2.2. Seed disinfestation and germination

Prior to inoculation, the seeds were surface-disinfected according to Aragão [16]. Following, the seeds were cultured on culture medium of Murashige and Skoog (MS) [21] (Phytotechnology Lab, Overland Park, USA) and woody plant medium (WPM)[22] (Phytotechnology Lab), both supplemented with 20 g L^{-1} sucrose (Merck), 1.5 g L^{-1} activated charcoal (Sigma-Aldrich, St. Louis, MO, USA) and 2 g L^{-1} Phytagel® (Sigma-Aldrich). The pH of each culture medium was adjusted to 5.7; the medium was distributed into culture tubes (150 x 25 mm; 10 mL per tube), and then it was autoclaved at 121 °C for 15 min. The seeds were inoculated in the culture tubes and incubated in a growth room with a 16-h photoperiod at a light intensity of 22 µmol m^2 s^{-1}, at 25 ± 2 °C. Twenty replications with five seeds per replication were performed for each treatment. The germination percentage was evaluated throughout the culture period over 10 weeks.

2.3. The effects of cytokinins, auxins and gibberellin on shoot development

Ninety-day-old *C. legalis* seedlings that were germinated *in vitro* were used as a source of explants. Apical and cotyledonary nodal explants (± 2 cm) were isolated and inoculated on WPM (Phytotechnology Lab) supplemented with 20 g L^{-1} sucrose, 1.5 g L^{-1} activated charcoal (Sigma-Aldrich) and 2 g L^{-1} Phytagel (Sigma-Aldrich) and different concentrations of the PGRs (all from Sigma-Aldrich) as follows: BA (0, 5 and 10 µM), NAA (0 and 5 µM) and GA_3 (0 and 10 µM), which were combined or not. The experiment consisted of nine treatments (Table 1).

Table 1 Different treatments with plant growth regulators 6-benzyladenine (BA), α-naphthaleneacetic acid (NAA) and gibberellic acid (GA_3) in apical and cotyledonary nodal explants of *C. legalis.*

BA	0	5	10	0	5	10	0	5	10
GA_3	0	0	0	10	10	10	0	0	0
NAA	0	0	0	0	0	0	5	5	5
	Concentrations (µM)								

The pH of the culture medium was adjusted to 5.7 and distributed into culture tubes (10 mL per tube), and the tubes were autoclaved for 15 min at 121 °C. After inoculation, the explants were incubated in a growth room with a 16-h photoperiod and a light intensity of 22 µmol m^2 s^{-1} at 25±2 °C. Each treatment consisted of six replicates, and each replicate consisted of five explants. The shoot induction (%), number of shoots per explant and shoot length (cm) were analysed after 60 days of culture.

2.4. Effect of PAs on shoot development

The effects of the PAs putrescine (Put), spermidine (Spd) and spermine (Spm) on shoot development were investigated using apical and cotyledonary nodal explants (± 2 cm) isolated from 90-day-old *C. legalis* seedlings germinated *in vitro*. The explants were inoculated on WPM (Phytotechnology Lab) supplemented with 20 g L^{-1} sucrose, 2 g L^{-1} Phytagel (Sigma-Aldrich) and different concentrations (0, 0.5, 1, 2.5 and 5 mM) of each PA (Put, Spd or Spm) (Sigma-Aldrich). The pH of the culture medium was adjusted to 5.7 and autoclaved at 121 °C for 15 min. The PAs were incorporated into the medium by filter sterilisation using a 0.22-µm filter (JetBio-Filtration®, Guangzhou, China) under laminar flow. The culture medium was distributed into culture pots (150 mL) (Aapace, São Paulo, Brazil) containing 30 mL of culture medium per pot.

After the inoculation, the explants were incubated in the growth room with a 16-h photoperiod and a light intensity of 22 µmol m^2 s^{-1} at 25±2 °C. Each treatment consisted of eight replicates, and each replicate consisted of one culture pot containing five explants. The shoot induction (%), number of shoots per explant and shoot length (cm) were recorded after 60 days of culture.

2.5. Effect of IBA on shoot rooting

Sixty-day-old shoots (0.5-1.0 cm length) arising from apical and cotyledonary nodal explants that were cultured on WPM in the absence of PGRs were inoculated into WPM culture medium (Phytotechnology Lab) and supplemented with 20 g L^{-1} sucrose, 2 g L^{-1} Phytagel (Sigma-Aldrich) and different concentrations (0, 250, 500 and 1000 µM) of IBA (Sigma-Aldrich). The pH of the

culture medium was adjusted to 5.7 and distributed into culture tubes (10 mL per tube), followed by autoclaving for 15 min at 121 °C. Following inoculation, the explants were incubated in a growth room with a 16-h photoperiod and a light intensity of 22 µmol m^2 s^{-1} at 25 ± 2 °C; these conditions were maintained over 7 days for root induction.

After this time, the shoots were transferred to *ex vitro* environment conditions, where they were placed in plastic pots (20 mL) containing a 1:1 mixture (v/v) of PlantMax® (DDL Agroindústria, Paulínia, Brazil) and vermiculite for root development. These shoots were maintained in plastic trays covered with a plastic film to maintain the high humidity needed for root development. The shoots were kept in the grow room under the same conditions described above. After 15 days of incubation, the humidity was gradually reduced until 25 days, when the explants were exposed to the grow room atmosphere, and they were considered acclimatized. Each treatment consisted of eight replicates, and each replicate comprised four explants. The root induction (%), number of roots per explant and root length were analysed after 60 days of culture.

2.6. Statistical analysis

All experiments were performed using a completely randomised design. The data were analysed with analysis of variance (ANOVA) ($p< 0.05$) followed by a Student-Newman-Keuls (SNK) test [23] in the R program (R Foundation for Statistical Computing, version 3.1.1, 2014, Vienna, Austria).

3. RESULTS AND DISCUSSION

The percentage of *C. legalis* seed germination was higher in WPM (66%) than MS (25%) culture medium after 9 weeks of culture (Fig. 1). Radicle protrusion, which corresponds to germination, started after 5 weeks of culture in both culture media and stabilised after 9 weeks. *In vitro* seed germination is an important step for obtaining aseptic explants needed for micropropagation studies.

Similar results have been reported for other tree species, such as *Ilex paraguariensis* and *Hancornia speciosa* [24, 25], in WPM. These results may be related to the formulation of WPM [22] , which contains only 25% of the nitrate and ammonium ion concentrations of MS culture medium and consequently has a low concentration of total nitrogen [26]. However, WPM has higher levels of potassium and sulphate ions [27]. The combinations of nutrients in WPM may be important for the seed germination of select species, including *C. legalis*.

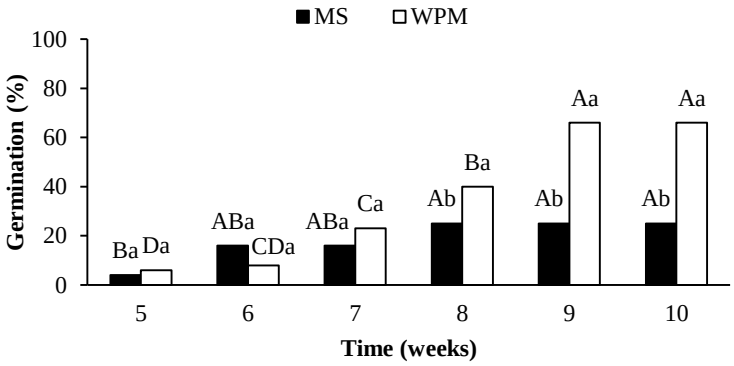

Figure 1: *In vitro* seed germination (%) of *C. legalis* in MS and WPM culture media after 5, 6, 7, 8, 9 and 10 weeks of culture. Means followed by different letters are significantly different ($p< 0.05$) according to the SNK test. Capital letters indicate significant differences among different weeks in the same culture medium. Lowercase letters indicate significant differences between MS and WPM culture media in the same week of culture.

In vitro shoot development in *C. legalis* can occur without the use of PGRs (BA, NAA or GA_3), and the different types and concentrations of PGRs in use did not induce significant increase in the induction, number and length of shoots compared to the control treatment (Fig. 2). Moreover, the cotyledonary nodal explants were more responsive than the apical nodal ones, showing a greater number of shoots per explant (Fig. 2B) and greater shoot lengths (Fig. 2C) in all the tested treatments. Therefore, the GA_3 concentrations used, which were combined or not combined with BA, affected the shoot induction, number and length of shoots in a negative manner (Fig. 2). The effects of cytokinins and auxins, whether combined or not, have been investigated in several tree species [9, 16, 28-31].

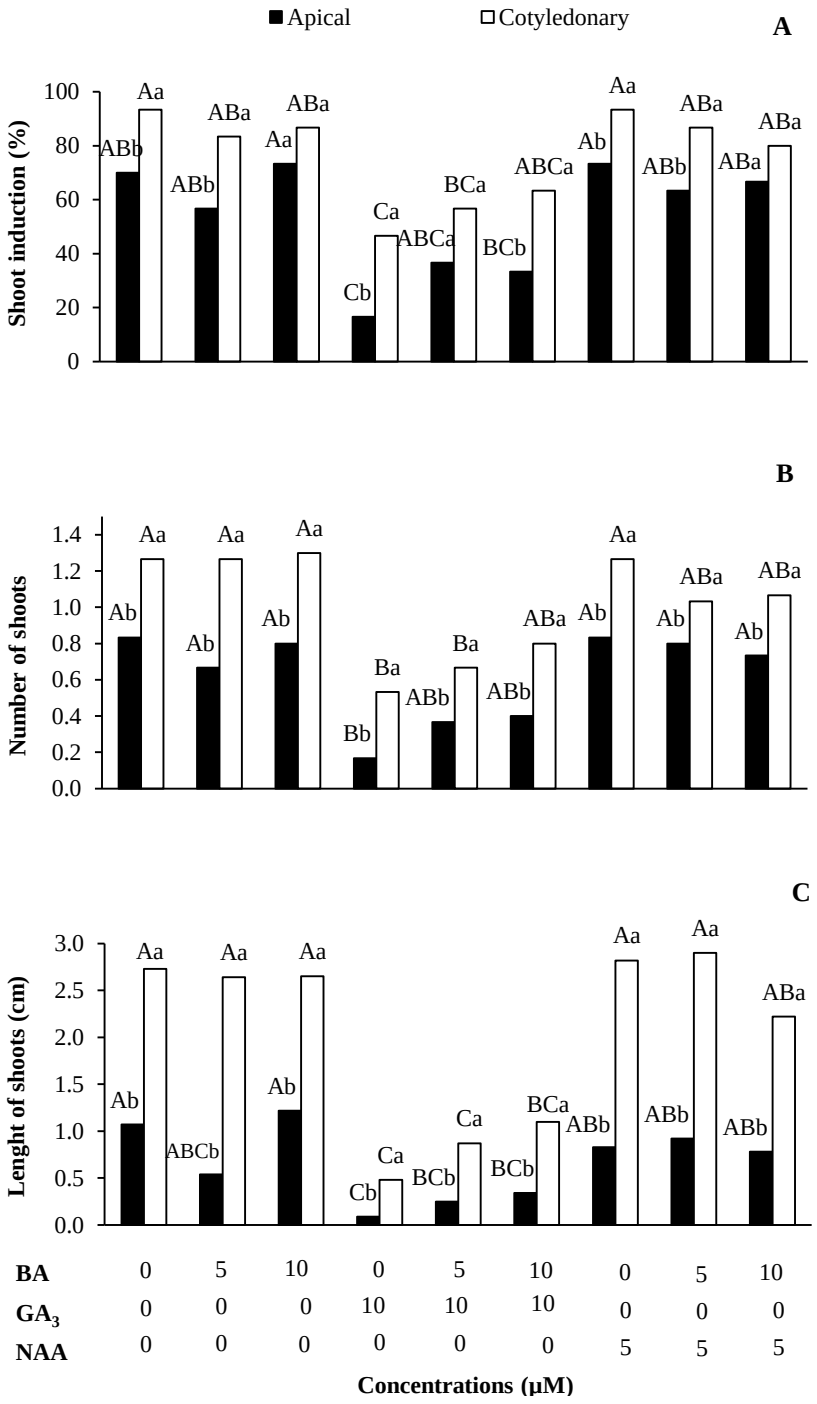

Figure 2: Induction (A), number (B) and length (C) of shoots developed from apical and cotyledonary nodal explants of *C. legalis* under the influence of the plant growth regulators N6-benzyladenine (BA), α-naphthaleneacetic acid (NAA) and gibberellic acid (GA_3) after 60 days of culture. Means followed by different letters are significantly different ($p< 0.05$) according to the SNK test. Capital letters indicate significant differences among the different treatments. Lowercase letters indicate significant differences between the different types of explants in the same treatment.

The greatest number and length of shoots in *Berberis aristata* were recorded in WPM supplemented with 8.88 µM BA in combination with 2.68 µM NAA [9]. Similarly, the maximum induction, number and length of shoots for *Shorea robusta* were obtained in WPM supplemented with 1.0 $mg \cdot L^{-1}$ (4.44 µM) BA combined with 0.5 $mg \cdot L^{-1}$ (2.68 µM) NAA [31]. Additionally, the 7.5 µM BA treatment combined with 0.4 µM NAA in MS culture medium promoted a greater number of shoots in *Aegiphila verticillata* [29]. The 0.1 µM NAA concentration combined with 10 µM BA was sufficient for promoting a greater number of shoots in *Acacia ehrenbergiana*, and any increase in the NAA concentrations beyond this optimum concentration will induce a detrimental effect on the number of shoots [32]. Otherwise, the use of BA alone in WPM promoted the best results in some tree species, such as greater shoot induction in *Salix pseudolasiogyne* with the addition of 2.2 µM BA [28] and a higher shoot induction and maximum number of shoots per explant in *Garcinia xanthochymus* when using 20 µM BA [30]. According to Javed [32], the low concentration of auxin (NAA) required to promote the greater number of shoots in *Acacia ehrenbergiana* indicates the higher endogenous contents of auxin in the explant. Thus, it is necessary to have a higher concentration of exogenous cytokinin to promote an adequate balance between auxin and cytokinin for the development of shoots. In addition, the endogenous and exogenous cytokinin/auxin balance is necessary for the development and elongation of shoots in *Tectona grandis*, a tree species [33]. In this sense, it is possible that the endogenous contents of auxins and cytokinins in *C. legalis* explants can be equilibrated at a rate that can promote shoot development without PGR addition.

In this study, the supplementation of the culture medium with 0.5 mM Spd increased shoot induction significantly (95%) compared to the control (85%) in apical nodal explants (Fig. 3A). PAs are important for seed germination, zygotic and somatic embryogenesis [34-39] and shoot regeneration [15, 16, 40]. Effects of Spd on shoot development have been observed in other species. In *Cucumis sativus,* greater shoot induction was obtained from apical nodal explants incubated with BA in combination with leucine and Spd [41]. Exogenous Spd combined with BA increased shoot induction from cotyledonary nodal explants in *Glycine max* [40]. In

addition, Spd provides a nitrogen source to enhance shoot differentiation, as suggested for *C. sativus* [41] and *G. max* [40]. Thus, exogenous Spd can contribute to the increase in the shoot induction, possibly acting directly on the metabolism of nitrogen, and in the cell cycle, increasing the mitotic divisions necessary for the development of morphogenetic processes. Although the shoot induction increased with exogenous 0.5 mM Spd, the number and length of shoots did not increase by the addition of different PAs from apical nodal explants (Fig. 3 and 4) in *C. legalis*, leading to shoot development without exogenous PA supplementation.

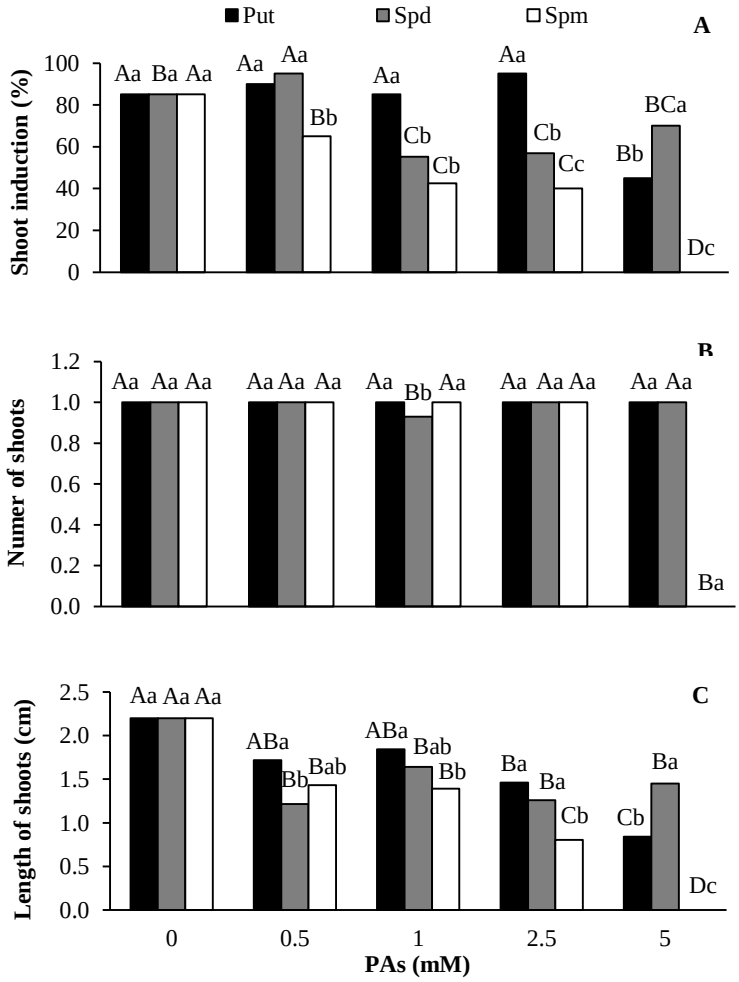

Figure 3: Induction (A), number (B) and length (C) of shoots developed from apical nodal explants of *C. legalis* under the influence of the polyamines (PAs) putrescine (Put), spermidine (Spd) and spermine (Spm) after 60 days of culture. Means followed by different letters are significantly different ($p < 0.05$) according to the SNK test. Capital letters indicate significant differences among the different treatments. Lowercase letters indicate significant differences among the different PAs in the same treatment.

In addition, in cotyledonary nodal explants, the induction of shoots (Fig. 4A) and the number (Fig. 4B) and length (Fig. 4C) of shoot also did not increase significantly by adding different PAs. In this sense, the morphogenetic response of the explant to the exogenous application of different PGRs depends on the endogenous balance of the hormones in the plant tissue (explant), which, in turn, varies with the organ or plant species [42]. Thus, our results showed that direct organogenesis in *C. legalis* can be achieved without PGRs using apical and cotyledonary nodal tissue as a source of explants, with cotyledonary nodal explants being more responsive in terms of the induction, number and length of shoots than the apical tissues (Fig. 2).

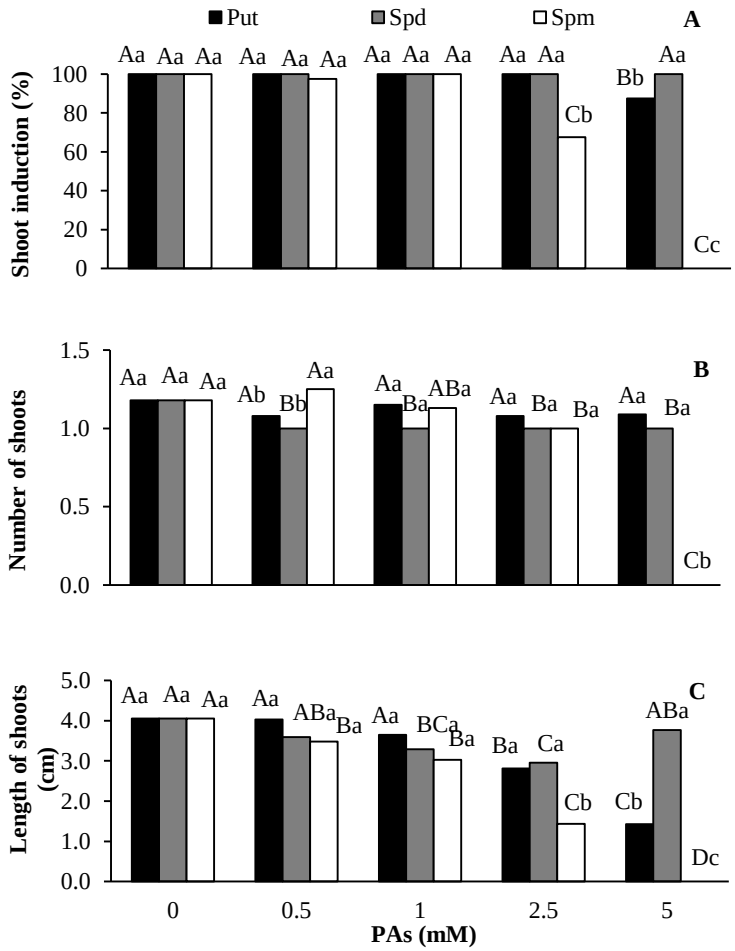

Figure 4: Induction (A), number (B) and length (C) of shoots developed from cotyledonary nodal explants of *C. legalis* under influence of the polyamines (PAs) putrescine (Put), spermidine (Spd) and spermine (Spm) after 60 days of culture. Means followed by different letters are significantly different ($p< 0.05$) according to the SNK test. Capital letters indicate significant differences among the different treatments. Lowercase letters indicate significant differences among the different PAs in the same treatment.

In preliminary experiments, *in vitro* rooting was performed in *C. legalis* using different concentrations (0, 5, 10 and 20 µM) of IBA, with only 7.7% rooting in the 10 µM IBA treatment (data not shown). Thus, we have developed a rooting system for *C. legalis* using an *in vitro* approach for root induction and an *ex vitro* approach for root development. After 7 days of *in vitro* incubation with different IBA concentrations following 60 days of incubation under *ex vitro* conditions, greater root induction was obtained in shoots arising from cotyledonary (75%) than apical (50.3%) nodal explants in the control treatment (Fig. 5A). In addition, a higher number of roots per shoot was observed in the control treatment for both explant types (Fig. 5B). Moreover, a higher root length was recorded in shoots arising from cotyledonary nodal explants, and no significant difference was observed between the control (1.84 cm) and 250 µM IBA (1.93 cm) treatments (Fig. 5C).

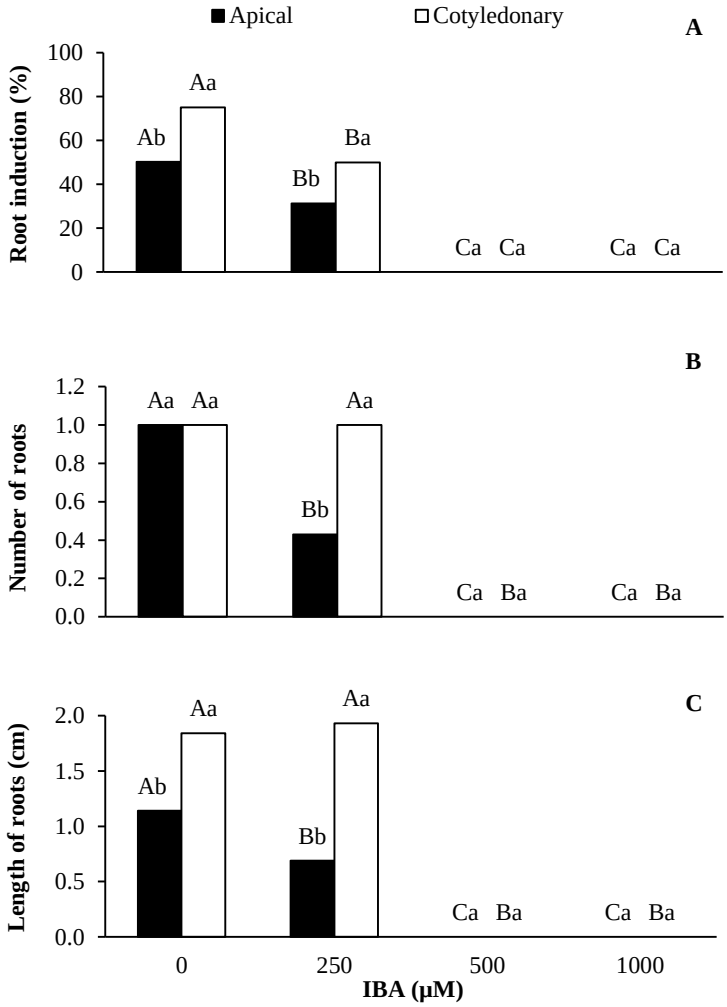

Figure 6: Induction (A), number (B) and length (C) of roots in micropropagated shoots arising from apical and cotyledonary nodal explants of *C. legalis* after 60 days of *ex vitro* incubation. Different IBA concentrations were applied over 7 days of *in vitro* incubation. Means followed by different letters are significantly different ($p< 0.05$) according to the SNK test. Capital letters indicate significant differences among the different treatments. Lowercase letters indicate significant differences between the different types of explants in the same treatment.

Thus, shoots from cotyledonary nodal explants may have had sufficient endogenous auxin content to promote the induction and development of adventitious roots, and the auxin-regulated responses depend on the different levels of this hormone in plants [17].

Ex vitro rooting is more effective than *in vitro* rooting because rooted *ex vitro* plantlets do not require any additional acclimatization step prior to transplantation to regular greenhouse conditions [19]. Additionally, *ex vitro* rooting has been successfully applied to tree species, such as *Embelia ribes* [43], *Tecomella*

undulata [44], *Albizia lebbeck* [45] and *Leptadenia reticulata* [46]. This system is promising because it can reduce the labour and time needed for somatic plantlet establishment from the laboratory to the soil, which is helpful for reducing manipulation costs [47]. It is estimated that *ex vitro* rooting reduces the costs of a micropropagation system by up to 71% [18]. Therefore, our results showed that *ex vitro* rooting without IBA can be a viable alternative for the rooting of micropropagated *C. legalis* shoots.

In summary, the micropropagation of *C.legalis* was achieved using *in vitro* seed germination in WPM culture medium (Fig. 6A). Ninety-day-old seedlings were used as the source of apical and cotyledonary nodal explants (Fig. 6B, C). Sixty-day-old shoots obtained in WPM culture medium free PGRs (Fig. 6D) were transferred to WPM culture medium supplemented with IBA under *in vitro* conditions during 7 days (Fig. 6E). After this time, shoots were rooting in *ex vitro* conditions (Fig. 6F).

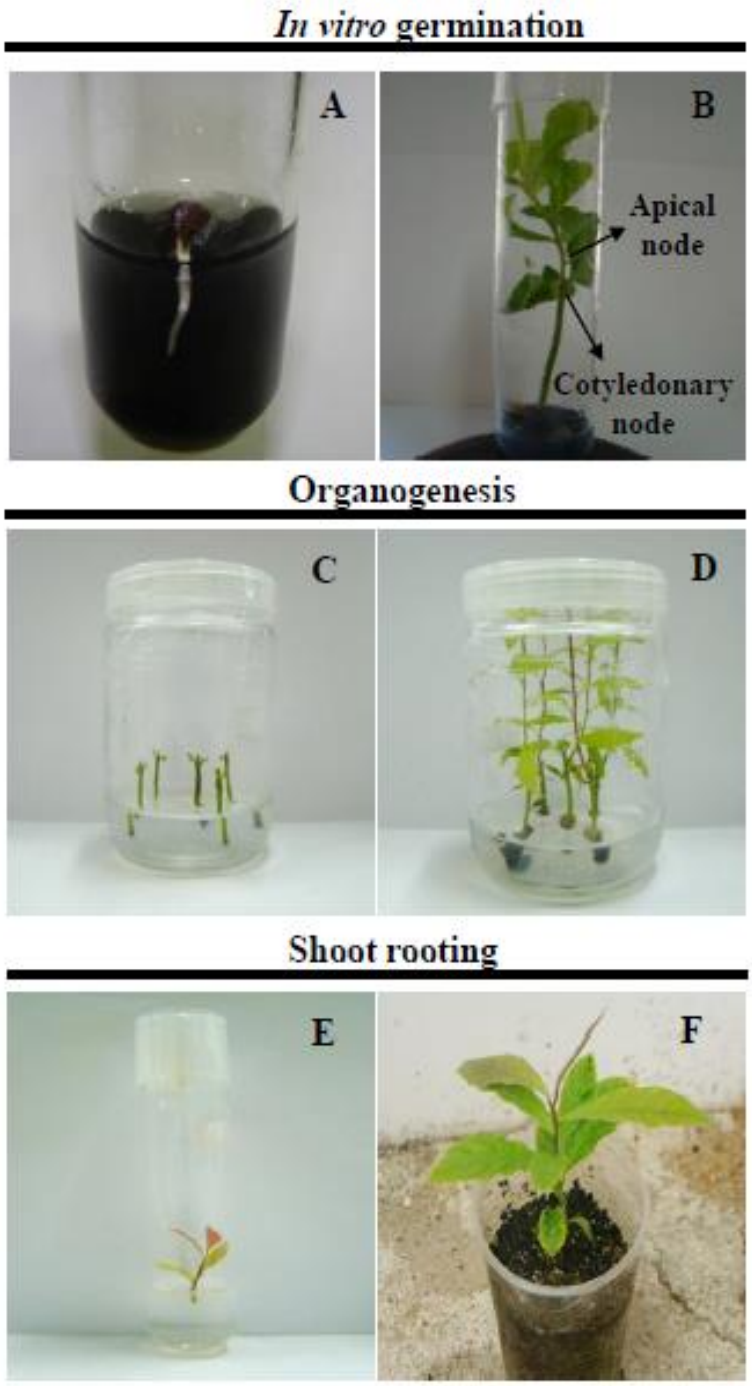

Figure 6: Morphological aspects during the micropropagation of *C. legalis* with *in vitro* seed germination after 3 weeks of culture in WPM culture medium (A), ninety-day-old seedlings used as a source of apical and cotyledonary nodal explants (arrows) (B), nodal explants obtained from seedlings germinated *in vitro* (C), sixty-day-old shoots obtained *in vitro* arising from apical and cotyledonary nodal explants (D), shoots transferred to WPM culture medium supplemented with IBA (0, 250, 500 and 1000 µM) under *in vitro* conditions, during 7 days of incubation (E) and the *ex vitro* rooting of micropropagated shoots, which produced somatic plantlets after 60 days (F).

4. CONCLUSIONS

To our knowledge, this report is the first to present micropropagation research in *C. legalis*, an endangered native hardwood tree species from the Brazilian Atlantic Forest. The best seed germination rate was obtained with WPM. Shoot development was achieved in WPM without adding PGRs, and cotyledonary nodal explants provided greater responses than apical nodal explants. Micropropagated shoots can be rooted *ex vitro* in the absence of IBA. These results are important for establishing further plant conservation strategies for endangered woody, such as *C. legalis*.

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4.3. TIME OF STORAGE AFFECTS THE GERMINATION AND DIFERENTIALLY ABUNDANT PROTEINS IN SEEDS OF *Cariniana legalis* (MART.) O. KUNTZE (LECYTHIDACEAE), AN ENDANGERED TREE SPECIES NATIVE FROM BRAZILIAN ATLANTIC FOREST

RESUMO

Estudos do proteoma durante o armazenamento de sementes são importantes para compreender os processos associados à manutenção da viabilidade. No entanto, as bases bioquímicas da regulação da viabilidade de sementes permanecem mal compreendidas. O presente estudo teve como objetivo investigar os efeitos do armazenamento de sementes em longo prazo na germinação e abundância diferencial de proteínas associadas à manutenção da viabilidade em *Cariniana legalis*. Sementes de *C. legalis* foram armazenadas a 5 ° C em sacos plásticos durante 12 meses. Análises de germinação foram realizadas antes (tempo 0) e após 3, 6 e 12 meses de armazenamento. Eletroforeses bidimensionais (2-DE) foram realizadas, seguidas por análises via espectrometria de massa (MS/MS) para identificar proteínas diferencialmente abundantes. Houve uma redução significativa na germinação de sementes, índice de velocidade de germinação e teor de umidade durante o tempo de armazenamento. A análise por LC-MS/MS revelou 50 proteínas diferencialmente

abundantes entre os tempos analisados, sendo classificadas em nove classes funcionais a: processo biológico, processo metabólico, processo celular, processo de oxidação-redução, processo metabólico de carboidratos, proteína de reserva, envelhecimento e organização ou biogênese da parede celular, resposta a estresse e estímulos e proteólise. A correlação entre envelhecimento de sementes e alterações proteômicas sugere que proteínas específicas podem estar envolvidas em um possível mecanismo de deterioração de sementes, tais como ferritina, germacrene A oxidase, proteína semelhante à maturação de sementes, 2,4-dienol-CoA redutase, ribulose-1,5-bisfosfato carboxilase oxigenase (rubisco), globulina 11S, poligalacturonases, superóxido dismutase, 1cis-peroxiredoxina, proteínas de choque térmico (HSPs) e 14 kDa proteína rica em prolina. Os dados sugerem que o tempo de armazenamento afeta a germinação e a abundância de proteínas, contribuindo para o declínio da viabilidade de sementes em *C. legalis*.

ABSTRACT

Seed proteome studies during seed storage are important to understand the processes associated with viability maintainance. However, the biochemical basis of the seed viability regulation remains poorly understood. The present study aimed to investigate the effects of long-term seed storage on the germination and differentially abundant proteins associated with maintenance viability in *Cariniana legalis*. Seeds of *C. legalis* were stored at 5 °C in plastic bags for 12 months. Analyses of germination were conducted before (time 0) and after 3, 6 and 12 months of storage. Two-dimensional electrophoresis (2-DE) were performed followed by mass spectrometric (MS/MS) analysis to identify differentially abundant proteins. There was a significant reduction in seed germination, seed speed index and moisture content during the time of storage. Analysis by LC-MS/MS revealed 50 differentially abundant proteins among the time analysed, being classified in nine functional classes: biological process, metabolic process,

cellular process, oxidation-reduction process, carbohydrate metabolic process, storage protein, aging and cell wall organization or biogenesis, response to stress and stimulus and proteolysis. The correlation between seed ageing and proteomics changes suggests that specific proteins may be involved in a possible mechanism for seed deterioration, such as ferritin, germacrene A oxidase, seed maturation-like, 2,4-dienoyl-CoA reductase, ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco), 11S globulin, polygalacturonases, superoxide dismutase, 1cys-peroxiredoxin, heat shock proteins (HSPs) and 14 kDa proline-rich protein. Data suggest that the time of storage affect the germination and differentially abundant proteins, contributing to a decline of seed viability in *C. legalis*.

1. INTRODUCTION

The seed gemination is a prerequisite for plants to initiate their life (Holdsworth et al. 2008). Prolonged time of storage induces seed ageing and affects negatively the germination and seedlings growth (Garza-Caligaris et al. 2012). The seed deterioration is a process which results in both, delayed and reduction in germination rate, once it affects the vigor and seed viability (Rajjou et al. 2008). Seed ageing and consequently deterioration, is associated with cellular, metabolic, and chemical alterations, which include the loss of membrane integrity, reduce the energy metabolism, DNA degradation, and protein synthesis (McDonald 1999). In addition, the production of free radicals during storage is a major cause for disruption of cellular membranes and damage to protein and nucleic acid, which ultimately results in deterioration of cell organelles and seed ageing (Bailly 2004; Kibinza et al. 2006). Moreover, seed longevity is affected by storage conditions, including temperature and humidity (Sathish et al. 2015). In this sense, the physiological seed quality depends on the ability to repair damaged molecules and to prevent oxidative damage. Thus, the repair of damaged proteins

spontaneously over time is need to increase longevity and, consequently, germinative potential (Ogé et al. 2008; Châtelain et al. 2013).

Proteomics approaches have been a useful tool for determining the biological roles and functions of individual proteins and identifying the molecular mechanisms that govern seed germination, vigour and viability in response to ageing (Nguyen et al. 2015). This approach has an ecological and agronomical relevance to better understand the mechanisms related to the loss of seed vigour during ageing (Nguyen et al. 2015). Thus, proteome analysis has provided an important tool for the information about the individual proteins involved in specific biological responses and/or processes (Liu et al. 2015). In addition, proteomics analyses is used to examine changes and temporal patterns of protein accumulation in different biological processes (Bove et al. 2005). However, seed ageing is a complex process and proteomic analysis has been performed for understand the role of these biomolecules in maintaining the viability and vigor of seeds. However, the molecular mechanism of seed deterioration and information on the proteome changes in dry seeds during storage are not well understood (Sathish et al. 2015).

In *Arabidopsis thaliana* (L.) Heynh, proteomic analysis showed essential mechanisms for seed vigor, such as translational capacity, mobilization of storage reserves, and detoxification efficiency during ageing (Rajjou et al. 2008). In addition, low abundance of specific proteins in deteriorated seeds was detrimental for seed gemination in *A. thaliana*, because synthesis of new proteins is a prerequisite for seed germination in this species (Rajjou et al. 2008). In *Medicago sativa* L. the long-term storage (42 years) reduced the germination capability, and caused a delay in the speed of seed germination (Cakmak et al. 2010). A decrease in germination ability of seeds were well correlated with both, an increase in lipid peroxidation and decrease in the activities of antioxidants enzymes (Cakmak et al. 2010). During natural ageing, a suppression in the activity of specific proteins in seed improved seed quality during long-term storage in background cultivar and wild type of *Glycine max* (L.) Merr. (Lee et al. 2012). In *Fagus sylvatica* L. seeds, protein synthesis, folding and degradation are the most biochemical features affected in long-term stored seeds. In addition, proteins

associated with the stress response may have contributed to the reduced viability of *F. sylvatica* seeds, especially the desiccation tolerance associated proteins (Kalemba and Pukacka 2014). Similarly, the decrease in seed vigor (ageing) in poplar (*Populus* × *Canadensis Moench*) is an energy-dependent process, which requires protein synthesis and degradation as well as cellular defense (Zhang et al. 2015).

Cariniana legalis (Mart.) O. Kuntze is one of the largest trees of the Brazilian Atlantic Forest, occurring nowadays in a population density of less than 1 tree/ha in the forest (Tambarussi et al. 2015). Due to these factors and intensive wood exploitation, currently *C. legalis* is included in the red list of threatened species of the International Union for Conservation of Nature (IUCN), being classified in the vulnerable category (IUCN 2016). The reduced dispersal of pollen and seeds, due to fragmentation, is associated with reduction of *C. legalis* plants (Leal et al. 2014). In addition, seeds from this species reduce significantly the viability during a year of storage (Sousa et al. 2016). Due to its ecological and economic relevance, the aim of this study was to investigate the effects of seed storage in *C. legalis*, through proteomics approaches using two-dimensional electrophoresis (2-DE), to identify differentially abundant proteins that may be associated to the lose and/or maintenance viability of seeds.

2. MATERIALS AND METHODS

2.1. Plant material

Mature seeds of *C. legalis* were obtained from the Caiçara Comércio de Sementes Ltda. located in Brejo Alegre, São Paulo State, Brazil (21º10'S and 50º10'W).

2.2. Conditions of seed storage

Seeds with 6.8% of humidity were stored at 5 °C in plastic bags according to Sousa et al. (2016). Samples of seeds at time 0 (before storage) and after 3, 6

and 12 months of storage were obtained for germination, germination speed index and moisture content, as well as, for proteomic analysis.

2.3. Analyses of germination, germination speed index and moisture content of the seeds

Germination of seeds before (time 0) and after 3, 6 and 12 months of storage was conducted in a chamber B.O.D. type, at 25 ° C and a 16-h photoperiod. Four replicates of 50 seeds were used, which were disposed on the roll substrate germitest® paper moistened with distilled water equivalent to 2 to 3 times the weight of the substrate. The germinated seeds was analysed after 28 days according to the instructions for forest analysis (Brasília 2013). The germination analysis was expressed as a percentage of normal seedlings. The germination speed index was calculated according to Maguire (1962).

The initial moisture content of the seeds (time 0) and after 3, 6 and 12 months of storage was determined according to Brasil (2009). Approximately, 2 g fresh matter (FM) of seeds were put in a sample container and weighed, and they were then dried at 105 ± 3 °C during 24 hours. After, seeds were trasnferred to a desiccator during 30 min, weighed again. The seed moisture content was calculated as percentage using moisture content = (water content/fresh weight)x100.

2.4. Protein extraction

Soluble proteins were extracted according to methodology described by Balbuena et al. (2009) with modifications, using seeds without integument. Protein extracts were prepared from three biological samples at each time of analysis (0, 3, 6, 9 and 12 months), being 100 mg FM for each biological sample. Samples were first macerated with liquid nitrogen until a powder was obtained. The powdered material was re-suspended in extraction buffer containing 7 M urea (GE Healthcare, Freiburg, Germany), 2 M thiourea (GE Healthcare) , 1% dithiothreitol (DTT) (Bio-Rad Laboratories, Hercules, Ca, USA), 2% Triton-X100 (GE Healthcare), 1mM phenylmethanesulfonyl fluoride (PMSF) (Sigma-Aldrich, Saint Louis, USA), and 5 uM pepstatin (Sigma Aldrich). Samples were vortexed for 5

min at room temperature, incubated for 30 min on ice and centrifuged for 20 min at 16,000 *x* g, at 4 °C. The supernatants were collected and quantified using 2-D Quant Kit (GE Healthcare).

2.5. Two-dimensional electrophoresis (2-DE)

2-DE gels were performed to each biological sample. Sample aliquots containing 700 µg of proteins were used. Each biological sample was precipitated overnight in five volumes of 0.1 M ammonium acetate (Vetec, Rio de Janeiro, Brazil) in methanol (Merck, Darmstadt, Germany) at -20 °C. After, samples were centrifuged at 16,000 *x* g, and the pellets were washed twice with ammonium acetate 0.1 M and once with cold acetone 100% (Merck) plus containing 20 mM DTT (Bio-Rad). Pellets were diluted in 350 µL of rehydration buffer containing 7 M urea, 2 M thiourea, 2% CHAPS (GE Healthcare), 0.5% IPG buffer (pH 3-10) (GE Healthcare), 1% DTT, and 0.002% bromophenol blue (Sigma-Aldrich). Samples were loaded onto 18 cm IPG strips pH 3-10 (GE Healthcare). Isoelectric focusing (IEF) was performed in an Ettan IPGphor 3 (GE Healthcare) at 20 °C. The IEF consisted of a rehydration step of 12 h followed by five other steps: (1) 500 V for 1 h; (2) 1,000 V for 45 min; (3) 8,000 V for 1 h and 45 min; (4) 8,000 V for 2 h and 30 min, and (5) 1,000 V for 6 h. After IEF, samples were reduced for 15 min in equilibration solution containing 1.5 M Tris-HCl (pH 8.8), 6 M urea and 30% glycerol (Sigma-Aldrich), 2% sodium dodecyl sulfate SDS (Sigma-Aldrich), 125 mM DTT, and 1% bromophenol blue (Sigma-Aldrich) and subsequently alkylated in equilibration solution containing 125 mM iodoacetamide (GE Healthcare). The second dimension was performed under denaturing condition in a 12% polyacrylamide gel (SDS-PAGE). Electrophoretic separation was performed at constant current of 25 mA per gel in a vertical Protean II Xi system (BioRad, Richmord, USA). After second dimension, the gels were fixed for 1 h in a fixation solution containing 40% ethanol and 10% acetic acid. After, gels were washed and stained according to the methodology of Neuhoff et al. (1985) using a solution of 0.1% Coomassie brilliant blue G250, 1.2% orthophosphoric acid (85%) and 10% ammonium sulfate.

2.6. Image gels analysis

The 2-DE gels were scanned with Image Scanner III (GE Healthcare, USA) and analyzed by Image Master Platinum software v. 7.0 (GE Healthcare), which allows spot detection, relative quantification, and spot matching among multiple gels. Scanner calibration was performed according to the manufacturer's instructions. Each spot authenticity and contour were automatically determined and then validated by visual inspection, being edited when needed. Three gels were run for each sample, representing three biological replicates to evaluate the number of spots detected and the resolution of the gels. Alignments between gels were carried out automatically using three spots as reference, followed by manual inspection and correction when necessary. The relative abundance of each spot was determined dividing the spot volume by the sum of spot volumes to obtain individual relative spot volume (% vol.). The spots that showed reproducibility were considered for identification. Difference in protein abundance was considered significant statistically for values of $p < 0.05$, and showing at least 2-fold change in the level of abundance for the times evaluated.

2.7. Trypsin digestion

Spots that showed a fold-change greater than 2 were excised from the gels, destained, and subsequently, these spots were vacuum-dried at 30 °C using a CentriVap (Labcomo, Kansas, MO, USA). For spot digestion, it was added 15 µL of trypsin solution (33 ng/ µL; V5111, Promega, Madison, WI, USA) prepared in 50 mM ammonium bicarbonate (Sigma-Aldrich) and incubated for 60 min on ice. The spots were incubated in a Thermomixer® (Eppendorf, Hamburg, Germany) at 58 °C for 30 min. After, it was added 1 µL of 5% trifluoroacetic acid (TFA) (Sigma-Aldrich), 30 µL of 5% formic acid (FA) (Sigma- Aldrich), and 50% (v/v) acetonitrile (Sigma-Aldrich), followed by a vortexing of tubes for 20 s, ultrasonication of 10 min, and another vortexing for 20 s. Samples were concentrated until 10 µL using a CentriVap® (Labconco, Kansas, MO, USA) during 5 min, desalted by C18 Zip Tip (Millipore, Billerico, MA, USA) and transferred to Total Recovery Vials (Waters).

2.8. Protein identification by MS/MS and bioinformatics analysis

A NanoAcquity UPLC connected to a Synapt G2-Si HDMS mass spectrometry (Waters, Manchester, UK) for ESI-LC-MS/MS using a BEH 130 C18 1.7 µm (100 µm × 100 mm) at a flow rate of 500 nL/ min was used for mass spectrometric analysis. For peptide elution, a binary gradient was used, with mobile phase A consisting of water (Tedia, Fairfield, Ohio, USA) and 0.1% formic acid (Sigma-Aldrich) and mobile phase B consisting of acetonitrile and 0.1% formic acid. The gradient started at 7-40% B in 0-33.21 min; 40-85% B in 33.21-37.21 min; 85-85% B in 37.21-41.21 min; 85-7% B in 41.21-43.21 min. Mass spectrometry was performed in positive and resolution mode (V mode), and in data independent acquisition (DIA) mode. Transfer collision energy ramped from 20 V to 35 V in high energy mode, cone and capillary voltage of 30 V and 2800 V, respectively, and source temperature of 60 °C. Spectral acquisition scan rates were set to 0.5. The human [Glu1]-fibrinopeptide B (Sigma-Aldrich) at 100 fmol/µL was used as an external calibrant. Spectra processing and database searching conditions were performed by Protein Lynx Global Service v.3.02 (PLGS, Waters) with the following parameters: Minimun Fragment Ion Matches per Peptide 2, Minimun Fragment Ion Matches per Protein 5, Missed Cleavages 1, Carbamidomethyl (C) as Fixed Modification and Oxidation (M) as Variable Modification , and using the Asterids protein databases (289,542 sequences). Functional classification was performed using Blast2Go software v. 3.0 (Conesa et al. 2005).

3. RESULTS

3.1. Analyses of germination, germination speed index and moisture content of the seeds

There was a significant difference in seed germination of *C. legalis* throughout the storage, with greater reduction observed in times of 6 and 12 months (Fig. 1a). The lowest seed germination was observed at 12 months

(44.5%) compared to seeds before storaged (78%) (Fig. 1a). The germination speed index showed a decrease at 12 months compared to the other months of storage (Fig. 1b). Moreover, there was a significant reduction in seed moisture content during the storage period, being greater at 6 and 12 months of storage (Fig.1c).

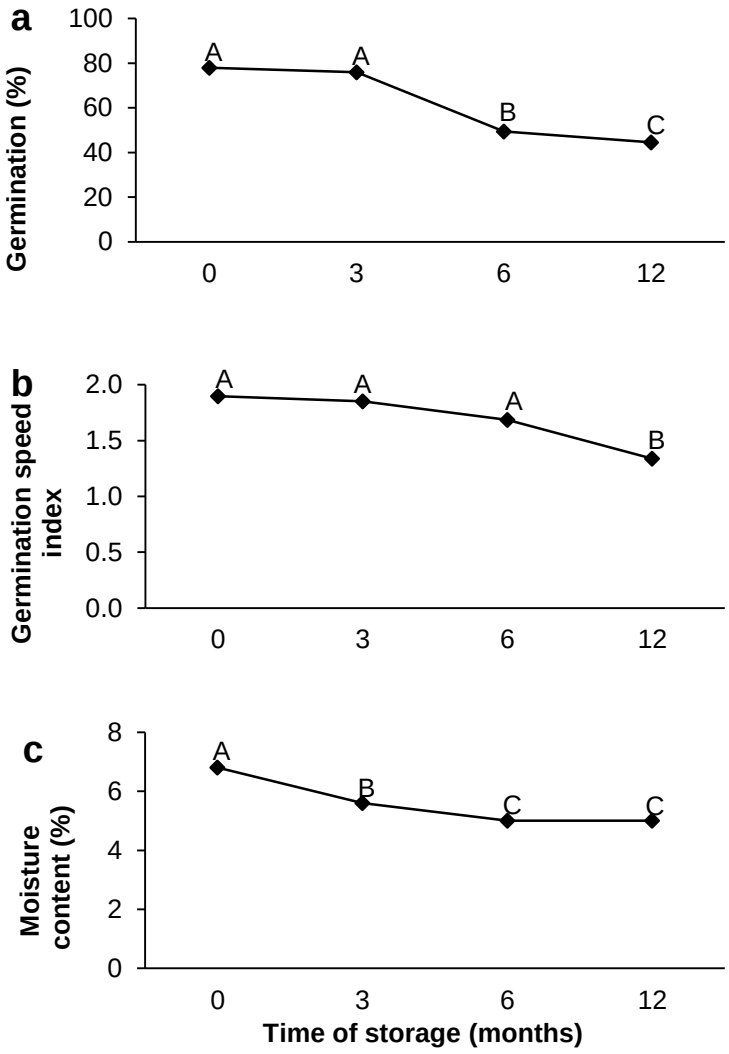


Fig. 1 a Seed germination (%), **b** seed germination speed index, and moisture content of *C. legalis* in control (seeds before storage - month 0) and after 3, 6, and 12 months of seed storage. Means followed by different letters are significantly different (p< 0.05) according to SNK test. CV coefficient of variation (n=4; CVgermination = 4.8%; CVspeed index =7.1%; CVmoisture content = 1.3%).

3.2. Changes in protein abundance

2-DE was performed to compare the differential abundace of proteins during the seed storage of *C. legalis* (Fig. 2). Comparative analysis of the 2-DE maps revealed 86 spots differentially abundants, which were excised from the gels, destained, and identified by mass spectrometry, being indicated in the 2-DE maps by arrows (Fig. 2). Larger differences in the abundance of proteins were observed in seeds at 6 and 12 months of storage (Figs. 2c and 2d). On the other hand, few changes were observed between the control (seeds before storage) and the 3 months of seed storage (Figs. 2a and 2b). It was observed a reduction in the intensity of the spots, and the most changes were observed in proteins with molecular weigth between 20 and 45 kDa (Fig. 2).

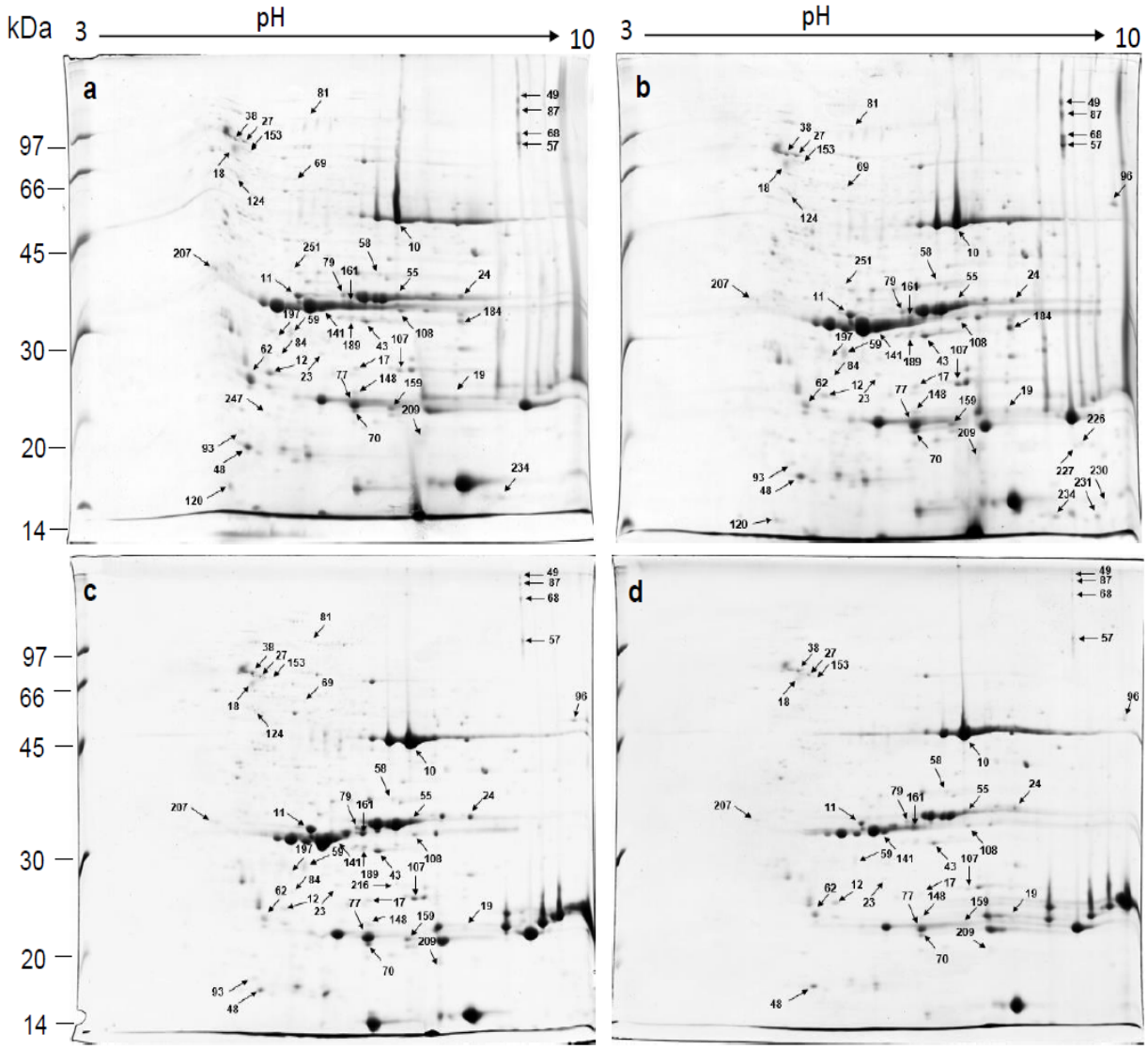
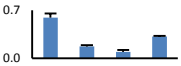
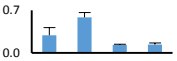
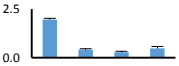
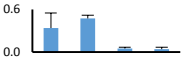
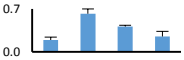
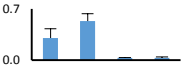
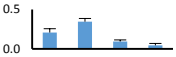
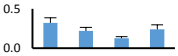
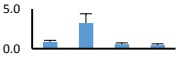
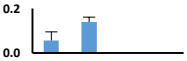
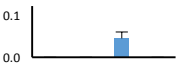
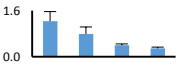
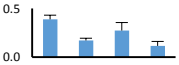
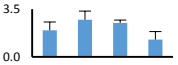
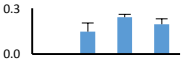
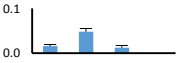


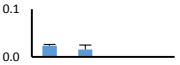
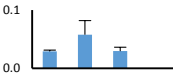
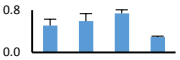
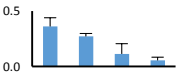
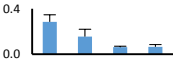
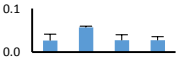
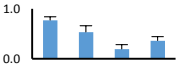
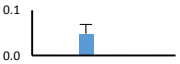
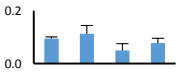
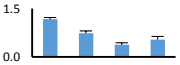
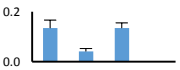
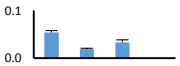
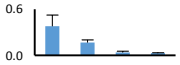
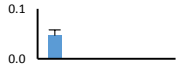
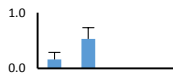
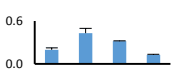
Fig. 2 2-DE gel analysis of proteins from different storage times of seeds of *C. legalis*. **a** Control (seeds before storage - month 0) and after **b** 3, **c** 6 and **d** 12 months of storage. Arrows show the differentially displayed protein spots.

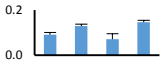
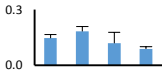
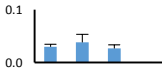
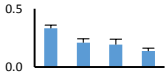
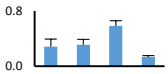
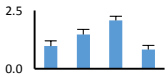
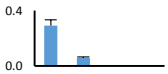
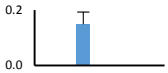
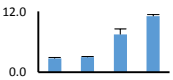
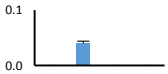
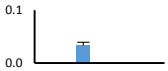
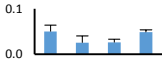
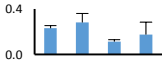
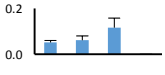
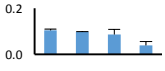
3.3. Protein identification during natural ageing by mass spectrometry and functional classification

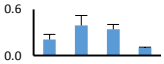
Analysis by LC-MS/MS revealed the identitification of 50 differentially abundant proteins among the months of storage (Table 1).

Table 1 Differentially abundant proteins identified by mass spectrometry during seed storage of *C. legalis*.

Spot[a]	Acession[b]	Protein name and species	Function[c]	Score[e]	Molecular mass Theoretical/ Experimental	pI Theoretical/ experimental	Abundance/Time 0 3 6 12
12	Q84ND2	11S globulin/ *Bertholletia excelsa*	Storage protein	2472.4	52263/25122	6.1/5.5	
57	Q84ND2	11S globulin/ *Bertholletia excelsa*	Storage protein	1385.0	52263/92915	6.1/9.1	
62	Q84ND2	11S globulin/ *Bertholletia excelsa*	Storage protein	2314.3	52263/24402	6.1/5.2	
87	Q84ND2	11S globulin/ *Bertholletia excelsa*	Storage protein	1159.8	52263/126926	6.1/9.0	
70	Q84ND2	11S globulin/ *Bertholletia excelsa*	Storage protein	107.9	52263/21099	6.1/6.7	
49	Q84ND2	11S globulin/ *Bertholletia excelsa*	Storage protein	391.4	52263/143484	6.1/9.0	
68	Q84ND2	11S globulin/ *Bertholletia excelsa*	Storage protein	714.2	52263/102139	6.1/9.1	
108	Q84ND2	11S globulin/ *Bertholletia excelsa*	Storage protein	341.2	52263/33071	6.1/7.4	
141	Q84ND2	11S globulin/ *Bertholletia excelsa*	Storage protein	1306.6	52263/33157	6.1/6.2	
234	K4CMG7	14 kDa proline-rich protein/ *Solanum tuberosum*	Metabolic process, proteolysis and cuticle development	434.8	13623/14556	8.0/8.9	
216	A0A022QI62	1cys-peroxiredoxin/ *Helianthus annuus*	Response to desiccation, maintenance of seed dormancy and oxidation-reduction process	265.9	24965/26482	5.4/7.2	
161	K4CHY6	2,4-dienoyl-CoA reductase, putative/ *Ricinus communis*	Oxidation-reduction process	246.8	31826/33886	9.5/6.6	
159	S8CIM6	4-coumarate:coenzyme A ligase 8/ *Salvia miltiorrhiza*	Metabolic process	49.7	59458/21345	6.7/7.2	
77	S8CK73	Acyl-CoA-binding domain-containing protein 4/ *Glycine max*	Biological process and lipid transport	106.8	26201/21700	9.8/6.7	
96	A5X488	ATP synthase subunit 9, mitochondrial/ *Carthamus tinctorius*	ATP hydrolysis coupled proton transport and ATP synthesis coupled proton transport	1016.3	7566/22867	9.6/9.3	
124	A0A022RK2	ATP synthase subunit beta/ *Erythranthe guttata*	ATP synthesis coupled proton transport and ATP hydrolysis coupled proton transport	665.9	59261/65909	6.1/5.0	

81	D2KLM4	DNA-directed RNA polymerase subunit alpha/ *Olea europaea*	Biological process and translation	29.4	38708/114968	7.3/6.2	
251	K4BVD4	Eugenol synthase 1/ *Petunia hybrida*	Oxidation-reduction process	278.7	34026/39084	5.3/5.8	
197	A0A022PUJ0	Ferritin/ *Erythranthe guttata*	Iron ion transport, cellular iron ion homeostasis and oxidation-reduction process	142.1	28977/29481	5.5/5.6	
43	R9WUG5	Germacrene A oxidase/ *Tanacetum cinerariifolium*	Oxidation-reduction process	33.9	55029/31608	8.8/6.9	
38	M0ZI18	Heat shock cognate 70 kDa protein/ *Petunia hybrida*	Protein folding, protein ubiquitination and response to stress	4144.4	71269/96584	4.9/5.0	
19	S8BRT	Kinesin-like protein/ *Genlisea aurea*	Microtubule-based movement and metabolic process	380.3	22348/22471	5.4/8.0	
27	E0YCS7	Methylketone synthase/ *Solanum lycopersicum*	Unclassified	40.1	29374/94936	5.9/5.1	
153	A0A022RFY1	Mitochondrial HSO70 2 isoform 2/ *Theobroma cacao*	Protein folding and oxidation-reduction process	167.4	72757/83800	5.9/5.3	
55	M1CSE2	NAD(P)-binding rossmann-fold protein/ *Medicago truncatula*	Oxidation-reduction process	287.8	31814/35148	6.1/7.3	
227	F6KNQ6	Peptidyl-prolyl cis-trans isomerase/ *Tagetes patula*	Biological process and protein folding	49.8	17999/1988	9.0/9.1	
23	A0A022Q2J0	Polygalacturonase/ *Actinidia deliciosa*	Cell wall organization or biogenesis and metabolic process	45.6	43321/27466	8.9/6.2	
48	A0A022Q2J0	Polygalacturonase/ *Actinidia deliciosa*	Cell wall organization or biogenesis and metabolic process	71.8	43321/18234	8.9/5.2	
93	A0A022Q2J0	Polygalacturonase/ *Actinidia deliciosa*	Cell wall organization or biogenesis and metabolic process	63.8	43321/19381	8.9/5.1	
84	A0A022Q2J0	Polygalacturonase/ *Actinidia deliciosa*	Cell wall organization or biogenesis and metabolic process	70.6	43321/27254	8.9/5.6	
209	A0A022Q2J0	Polygalacturonase/ *Actinidia deliciosa*	Cell wall organization or biogenesis and metabolic process	50.0	43321/18643	8.9/7.6	
247	A0A022Q2J0	Polygalacturonase/ *Actinidia deliciosa*	Cell wall organization or biogenesis and metabolic process	74.5	43321/20745	8.9/5.5	
184	A0A022Q2J0	Polygalacturonase/ *Actinidia deliciosa*	Cell wall organization or biogenesis and metabolic process	39.3	43321/31635	8.9/8.2	
59	K4BR74	Ppi-phosphofructokinase/ *Glycine max*	Phosphate-containing compound and metabolic process	118.8	10786/30210	7.7/5.8	

58	M1C9K3	Proteasome component (PCI) domain protein isoform 1/ *Theobroma cacao*	Unclassified	18.4	44090/38476	5.0/7.1	
148	M4M296	Ribulose bisphosphate carboxylase large chain/ *Ardisia polysticta*	Photorespiration, carbohydrate metabolic process and oxidation-reduction process	4261.1	52564/23000	6.2/6.7	
69	A0A060D3C1	Ribulose bisphosphate carboxylase large chain/ *Centaurea diffusa*	Photorespiration, carbohydrate metabolic process and oxidation-reduction process	2668.4	52794/65367	6.1/5.8	
18	A0A023M644	Ribulose bisphosphate carboxylase large chain (Fragment)/ *Comastoma pedunculatum*	Photorespiration, carbohydrate metabolic process and oxidation-reduction process	1486.9	22575/88620	5.2/5.0	
24	A0A023M644	Ribulose bisphosphate carboxylase large chain (Fragment)/ *Comastoma pedunculatum*	Photorespiration, carbohydrate metabolic process and oxidation-reduction process	934.3	22575/35117	5.2/8.2	
11	A0A023M64	Ribulose bisphosphate carboxylase large chain (Fragment)/ *Comastoma pedunculatum*	Photorespiration, carbohydrate metabolic process and oxidation-reduction process	1131.4	22575/35148	5.2/5.9	
120	P28377	Ribulose bisphosphate carboxylase large chain (Fragment)/ *Actinidia chinensis*	Photorespiration, carbohydrate metabolic process and oxidation-reduction process	2260.2	51553/15723	6.2/4.9	
231	O99001	Ribulose bisphosphate carboxylase large chain (Fragment)/ *Beaumontia grandiflora*	Photorespiration, carbohydrate metabolic process and oxidation-reduction process	759.0	51497/14556	6.6/9.5	
10	Q9GDL2	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (Fragment)/ *Veronica salicifolia*	Photorespiration, carbohydrate metabolic process and oxidation-reduction process	766.3	48876/50036	7.0/7.3	
226	A0A024HR79	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit(Fragment)/ *Cibirhiza albersiana*	Photorespiration, carbohydrate metabolic process and oxidation-reduction process	2099.8	46554/19363	6.1/9.2	
230	X5CUS2	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit(Fragment)/ *Cuscuta burrellii*	Photorespiration, carbohydrate metabolic process and oxidation-reduction process	1772.4	50401/15401	6.6/9.6	
207	A0A022QGC0	Saposin-like aspartyl protease family protein/ *Theobroma cacao*	Proteolysis and lipid metabolic process	704.8	55708/39632	6.3/4.6	
79	A0A022R5D9	Seed maturation-like protein/ *Sesamum indicum*	Oxidation-reduction process	1933.8	31439/35270	6.0/6.5	
189	A0A022R5D9	Seed maturation-like protein/ *Sesamum indicum*	Oxidation-reduction process	401.3	31439/31829	6.0/6.6	
17	Q6DV41	Superoxide dismutase/ *Camellia sinensis*	Response to stress and oxidation-reduction process	1630.2	25552/25614	8.4/6.7	

107	A0A068UZE8	Superoxide dismutase/ *Coffea canephora*	Response to stress and oxidation-reduction process	286.1	25513/25367	8.5/7.4	

Using the Blast2go program it was possible to classify these 50 proteins into nine functional classes: biological process, metabolic process, cellular process, oxidation-reduction process, carbohydrate metabolic process, storage protein, ageing and cell wall organization or biogenesis, response to stress and stimulus and proteolysis (Fig. 3).

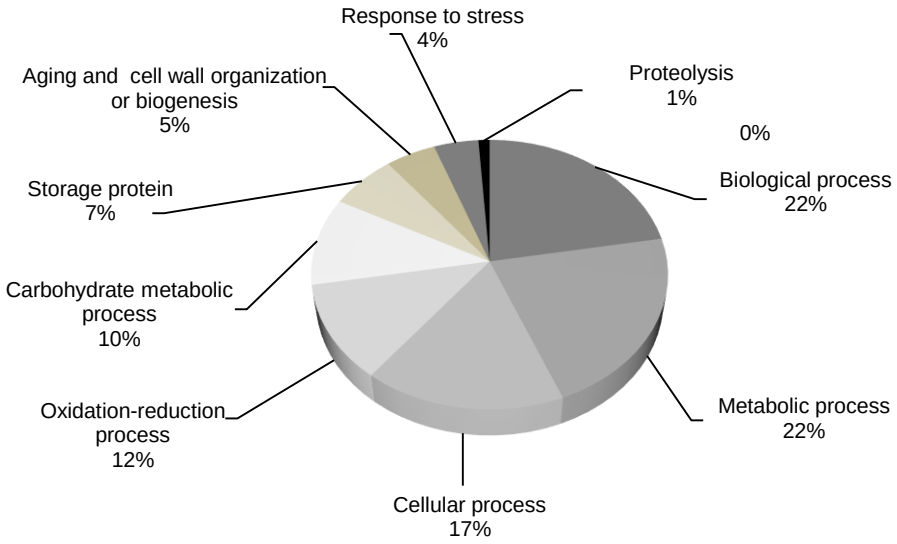


Fig. 3 The functional classes distribution of the 50 proteins differentially abundant during seed storage of *C. legalis.*

4. DISCUSSION

Physiological and biochemal alterations induced by deterioration during seed storage have been studied in tree species (Benedito et al. 2011; Sousa et al. 2016). In this study, seeds of *C. legalis* were significantly affected by the storage time showing a higher reduction of germination, germination speed index and moisture content (Fig. 2). Similarly, the seed storage also affected the seedling

emergence, vigor and changes in biochemical compounds as such polyamines and amino acids in this species (Sousa et al. 2016). According to Sousa et al. (2016) significant differences in seedling emergence and vigor throughout the seed storage of *C. legalis* may be associated with biochemical changes during seed storage, especially alterations in Putrescine contents. In other tree species, such as *Tabebuia aurea* Benth. & Hook.f. ex S.Moore and *Piptadenia moniliformis* Benth, the time of storage reduced the germination as well as the vigor (Cabral et al. 2003; Benedito et al. 2011). The reduction of germination and vigor in seeds of *C. legalis* may be associated with the level of tolerance to seed desiccation, which is low in this species as suggested by Sousa et al. (2016). These data are consistent with our results because the moisture content of seeds was significantly affected by the time of storage. The initial moisture content of seeds (6.8%) (Fig. 1c) problably favored an optimal germination (78%) of seeds before its storage (Fig. 1a). However, a significative change in moisture content from 6.8% to 5% after12 months (Fig. 1c) of storage reduced significantly the germination, from 78 to 44.5% (Fig. 1a). Thus, maintaining the moisture content at optimum level is indispensable to ensure an efficient seed germination in *C. legalis*.

A proteomic approach was used to identify proteins whose accumulation levels are associated with changes in seed vigor in *C. legalis*, and our results clearly showed an association between the abundance of proteins and seed ageing. Fifty differentially abundant proteins (Table 1) were identified, being most of them involved in biological, metabolic, and cellular processes (Fig. 3) and related to seed germination and vigor (Figs. 1). Some of the proteins belonging to specific groups, such as oxidation-reduction process, carbohydrate metabolic process, storage protein, aging and cell wall organization or biogenesis, response to stress and stimulus and proteolysis (Fig. 3) are discussed.

Several proteins identified (12%) were related to oxidation-reduction process in *C. legalis* (Fig. 3). Numerous deleterious oxidative processes can induce disruption and deteriorative changes in cells, being traiggered by several signals, such as the production of reactive oxygen species (ROS), which is associated with dehydration of stored seed tissues (Smirnoff 1993; Leprince et al. 1999; Lee et al. 2010). Therefore, the protection against oxidative processes may

be a major determinant role for the longevity of stored seed (Wilson and McDonald 1986; Lee et al. 2010). Among the proteins, ferritin is a superfamily of iron storage proteins that participates of developmental processes or in response to environmental challenges in plants, to maintain iron homeostasis (Briat et al. 2009). Ferritin protein (spot 197) showed a decrease in the abundace during seed storage of *C. legalis* (Table 1). It is possible that during seed ageing this protein has been used to combat oxidative stress once it is involved in the protection against oxidative stress through their potential detoxification properties of excess iron and hydrogen peroxide (Briat et al. 2009; Theil et al. 2014).

Other proteins associated with oxidative stress response were also differentially abundant in *C. legalis* seeds during the storage. Germacrene A oxidase (spot 43) decreased its abundance at 12 month of storage (Table 1). Germacrene A oxidase is an enzyme that participates in the terpenoid metabolism, which may contribute to the chemical and catalytic diversity in nature (Nguyen et al. 2010). Seed maturation-like protein (sport 79) was present during all the storage period, while this same protein in the spot 189 was not expressed at 12 month (Table 1). In *Pisum sativum* L. seed maturation protein was upregulated and the percentage of germinated seeds was negatively affected by aging (Yao et al. 2012). These authors suggested that this protein has functional significance in controlling the germination process in *P. sativum*, which was also found in our study with *C. legalis*. On the other hand, 2,4-dienoyl-CoA reductase (spot 161) decreased its abundance through storage period (Table 1). The 2,4-dienoyl-CoA reductase is an enzyme related to degradation of unsaturated fatty acids, being important for β-oxidation, acting biochemically on a diversity biological processes such as seed development, germination and post-germinative growth before the establishment of photosynthesis (Goepfert and Poirier 2007). From our data it can be suggested that the differential abundance of antioxidant enzymes in *C. legalis* seeds is a result of seed deterioration, which leads to a loss of viability and vigor, consequently affecting the germination. Thus, these antioxidant enzymes are of great importance for ensuring the vigor and germination of *C. legalis* seeds during the storage period.

In class proteins related to carbohydrate metabolic process was identified ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). Rubisco is an enzyme related to photosynthetic metabolism in plants. However, some functions remain unknown in many physiological processes, such as ageing seeds (Famiani et al. 2012). According to El Amrani et al. (1997) the control of Rubisco production and activity in the different organs of a plant are very complex, with regulation occurring at many levels to growth and development of seedlings. In our study, Rubisco was present in the dry seed, decresasing its abundance in some spots (120, 148, 18, 24, 69 and 11) (Table 1), while an increase throughout the storage period was observed in the spot 10. This enzyme was also observed at specific points (spots 226, 230 and 231) (Table 1) during the storage period. The presence of Rubisco during the storage period of *C. legalis* seeds may be an attempt to keep seed with an optimal concentration of CO_2, thus it will not occur toxicity at the cellular level. According to Famiani et al. (2012) the presence of Rubisco in dry seeds may be related to limited gas exchange, because in many developing seeds, limited gas exchange results in a high concentration of CO_2 which is potentially toxic (Schwender et al. 2004; Famiani et al. 2012). These data suggest that abundance of Rubisco in dry seeds of *C. legalis* during storage may be important for removal of CO_2 accumulated, once Rubisco is important in removing this compound in dry seeds (Famiani et al. 2012). On the other hand, it is also possible that Rubisco could have another function such as that of a transient storage protein as suggested by Walker et al. (2011) for *Prunus avium* (L) L. dry seeds. In addition, Rajjou et al. (2008) studied the seed proteome of *A. thaliana* submitted accelerated aging. Several proteins were oxidized, among them, isoforms of the Rubisco. This enzyme has already been shown to a preferential target of ROS in Arabidopsis (Johansson et al. 2004; Job et al. 2005). These observations point out the hypersensitivity to oxidative stress of this protein (Rajjou et al. 2008).

Moreover, the presence of Rubisco in the cotyledons during radicle protrusion is essential for seedlings establishment in *Citrullus lanatus* (Thunb.) Mansf. with increased synthesis signaled by the elongation of the radicle (Botha and Small 1987). Unlike, in dry seeds of *Beta vulgaris* L. it was not detected the

presence of Rubisco, being synthesized at time of transition from heterotrophy to phototrophy during the growth of seedlings (El Amrani et al. 1997). Taken together, these data show that the presence of Rubisco in dry seeds or *de novo* synthesis during germination is an important event for the establishment of seedlings. Thus, the decreased abundance of most Rubisco forms found in dry seeds of *C. legalis* may be associated to the loss of germination and seedling establishment potentials in this species.

According to Li et al. (2007) storage proteins are accumulated in high amounts during maturation and development of seeds and will break down during imbibition to supply amino acids for seed germination. In our study, 11S globulin (spots 12, 57, 62, 87, 49, 68, 108, 141) showed a decreased in the abundance at 12 months compared to control (0 month) (Table 1). This result is according to Sathish et al. (2015) who reported down-regulation of globulin in aged seeds of *Vigna mungo* (L.) Hepper. In addition, Xin et al. (2011) and Wu et al. (2011) also reported down-regulation of globulin in aged seeds of *Zea mays* L. seeds. During ageing, these proteins might be degraded due to free radical attack (Sathish et al. 2015), and the breakdown of the storage proteins prior to germination can lead to an inefficient amino acids supply for synthesis of new proteins essential for seed germination and seedling establishment (Sathish et al. 2015).

In dry seeds of *A. thaliana* the storage proteins are the major targets of ROS (Job et al. 2005). Furthermore, storage proteins are more sensitive to ageing, being highly oxidized (Nguyen et al. 2015). Both, storage proteins present in dry seeds and the *de novo* synthesis of proteins during germination contribute significantly to germination and seedlings establishment (Rajjou et al. 2008). The results observed in the present study indicate that a decreasing in the abundance of storage proteins of *C. legalis* during seed storage can be related to deterioration, affecting the germination and vigor of seeds. Possibly, with the reduction of these storage proteins, the seed will not have a sufficient source of amino acids to ensure the synthesis of new proteins important for the germination process.

In this study, we also identified proteins related to aging and cell wall organization or biogenesis. Some proteins, such as polygalacturonases, have

been hypothesized to play important roles throughout the life cycle of a plant, including germination, cell expansion, pollen grain maturation, anther dehiscence, abscission, fruit ripening, and pod shatter (Roberts et al. 2002; Kim and Patterson 2006; González-Carranza et al. 2007). Particularly in seeds, the expression of polygalacturonases are located in endosperm cell surrounding the emerging radicle in a germinating seed (González-Carranza et al. 2007). Thus, polygalacturonases could also potentially be involved in cell wall modification, allowing the turgor-driven elongation of the radicle, culminating in radicle protrusion through the endosperm (Sitrit et al. 1999). This enzyme is an important hydrolase for the degradation of pectins and has been reported as responsible for softening the integument seed (Sitrit et al. 1999). The abundance of this protein increase during cell separation, inducing a reduction or remotion of the constraints that the cell wall provides for radicle protrusion (Cosgrove 1999), remodeling and/or loosening of the plant cell wall, thus allowing the plant growth.

In our study, the abundance of polygalacturonases decreased throughout the storage period (spots 48, 93, 84, 209, 247, 184), except the spot 23 (Table 1), suggesting the importance of these enzymes for the germination in *C. legalis*. The decrease in the abundance of this enzyme mainly at the 12º month of storage could affect the germination as well as the vigor (Fig. 1a, b).

In *Schizolobium parahyba* (Vell.) Blake seeds, polygalacturonases are active in dry seeds. The activity of this enzyme increased during the imbibition, being higher in the embryonic axis (Magalhães et al. 2009). These evidences show that polygalacturonases are directly involved in the germination process of this species, being part of the mechanisms by which the radicle can break the physical barrier imposed by the integument, facilitating cell expansion. According to Magalhães et al. (2009) this enzyme causes the loss of cohesion among the cells and dissolve the cell walls, allowing the action of other enzymes in the reserves of the cytoplasm, allowing water uptake, the start of cell division necessary for the root growth and the establishment of a new seedling. These results therefore suggest that a decrease in the abundance of polygalacturonases in dry seeds of *C. legalis* under storage conditions can contribute to the loss of seed viability observed in our study.

During exposure to biotic and abiotic stresses, molecular oxygen undergoes reactions that result in the formation of ROS, which are highly destructive to lipids and nucleic acids, inducing protein denaturation and enzyme inactivation (Sharma and Nath 2015). To prevent damages to cellular components caused by oxidative stress, mechanisms of cell detoxification involve a series of proteins related to stress response (Kibinza et al. 2011).

ROS are produced during seed desiccation, germination, and ageing, leading to cellular damage and seed deterioration and, therefore, decreased seed longevity (Lee et al. 2010). Superoxide dismutase is the first enzyme in the detoxifying process (Lin et al. 1993). This enzyme, catalyse the dismutation of two superoxide radical (O_2^-) and water into H_2O_2 and O_2 (Lee et al. 2010). In our study, the abundance of superoxide dismutase (Spots 17 and 107) decreased during storage, being sharper at the 12º months (Table 1). Similarly, in *M. Sativa* seeds stored for 42 years, the activity of superoxide dismutase also decreased, associating the reduction of germination capacity to the lower activity of this enzyme (Cakmak et al. 2010). In addition, *Z. mays* seeds showed a decrease in the abundance of superoxide dismutase during accelerated ageing, suggesting that antioxidant enzymes play an important role in the maintenance of seed viability (Wu et al. 2011). During accelerated ageing of *Gossypium hirsutum* L. seeds there was a significant decrease in the germination, being the loss of vigor associated to the accumulation of peroxides and reduction in the activity of antioxidant enzymes, as superoxide dismutase (Goel et al. 2003).

The effects of simultaneous over-expression of superoxide dismutase and ascorbate peroxidase increased seed longevity and germination rates under stress conditions in seeds of *Nicotiana tabacum* L. (Lee et al. 2010). A simultaneous over-expression of the Cu/Zn-superoxide dismutase (CuZnSOD) and ascorbate peroxidase (APX) genes in plastids improves seed longevity and germination under various environmental stress conditions attenuating the effects of oxidative stress produced by long time of storage conditions and environmental stresses because CuZnSOD and APX are the first line of defence against oxidative stress in plastids (Lee et al. 2010).

In the present study, a decreased on the abundace of superoxide dismutase protein during seed storage suggests that antioxidant enzymes play an important role in the maintenance of seed viability in *C. legalis*. In addition, 1cys-peroxiredoxin is an enzyme that scavenger ROS, being a stress indicator induced by several stress signals (Komatsu et al. 2010). In seeds, 1cys-peroxiredoxin is involved in oxidative stress responses during dormancy breaking, storage, and seed germination (Pawłowski 2010). Peroxiredoxins are proteins related to the perception of environmental surroundings and play the inhibition of seed germination under unfavorable conditions (Pawłowski 2010). In *C. legalis*, the presence of 1cys-peroxiredoxin at 6 months suggests that the seeds are undergoing an oxidative stress, which may be detrimental to germination (Fig.1a) and vigor (Fig. 1b).

The heat shock proteins (HSPs) act as molecular chaperones stabilizing new synthesized proteins and refolding the stress damaged proteins, and protecting cells against desiccation (Vierling 1991; Sun et al. 2002). According to Prieto-Dapena et al. (2006), when HSPs are accumulated during seed storage, it improves the resistance to aging, delaying the deterioration and, consequently, the loss of viability. In our study, we observed the presence of a HSP (spot 38) that reduced its abundance during the storage period (Table 1). Similarly, the proteomic analysis during accelerated ageing of *Z. mays* seeds revealed HSPs proteins with a decreased abundance over aging, thus impairing the germination (Wu et al. 2011). These data show that when seeds present a low abundance of HSPs, a reduction in the protection against desiccation occurs, which may lead to the decreased in seed vigor and germination. Thus, the reduction in the germination rates and vigor of *C. legalis* seeds may be a process that depends on the activity of specific proteins, requiring the presence and activity of proteins involved in the protection against desiccation such as HSPs. Differently, HSPs showed an increase in the abundance during the aging of *Populus* × *canadensis* seeds (Zhang et al. 2015). It is important to note that proteins involved in tolerance to desiccation, such as HSPs, may increase the abundance in an attempt to maintain the viability and the germination of seeds before the dehydration of tissues, which may be used as potential markers for seeds with a low vigor (Zhang

et al. 2015). In addition, the carbonylation of HSPs in *A. thaliana* seeds under accelerated aging affected the vigor and the germination, suggesting that the important mechanisms for maintaining the vigor and the germination are associated with protein metabolism, which are related to the response of the desiccation (Rajjou et al. 2008).

Natural ageing caused changes in two proteases, 14 kDa proline-rich protein (spot 234) and saposin-like aspartyl protease family protein (spot 207) in *C. legalis* seeds during storage period (Table 1). In Arabidopsis proline-rich proteins are involved in tolerance against abiotic stresses (Priyanka et al. 2010), while in *M. sativa* this protein was amply induced under drought conditions (Deutch and Winicov 1995). According to Jose-Estanyol et al. (1992) the proline-rich proteins are found to act in various processes of plant growth and development in a tissue and cell specific manner. Aspartic proteinases have been extensively studied and characterized and are widely distributed among plants (Simões and Faro 2004). Aspartic proteinases type saposin-like proteins had not its biological function completely established in plants. However, saposin-like proteins interacts with plasmatic membrane causing perturbation in permeabilization and also include roles in proteins degradation and metabolism of lipids. Particularly in the metabolism of lipids induces disruption of the lipid bilayer of cell membranes, thus compromising biological functions (Bryksa et al. 2011). During accelerated aging of *Z. mays* seeds, proteases abundance increased and resulted in seed deterioration (Xin et al. 2011). In this same species, the elevated activity of protease in embryos during accelerated aging may contribute to the loss of seed viability (Wu et al. 2011). Similarly, an increase in the activities of proteases was observed in *Triticum durum* Desf. seeds during accelerated aging, which resulted in a substantial reduction of soluble and storage proteins (Galleschi et al. 2002). In addition, the increase of proteases during accelerated aging *V. mungo* seeds was associated with degradation of storage proteins (Sathish et al. 2015).

5. CONCLUSION

The germination rate, germination speed index and moisture content of *C. legalis* seeds were significantly affected by the time of storage. There is a clear negative correlation between time of storage and seed germination, being moisture an important factor for maintaining the viability of *C. legalis* seeds. The analyses of differentially abundant proteins during the storage of seeds suggest that seed deterioration is regulated by many different proteins including proteins related to oxidation-reduction process, carbohydrate metabolic, storage protein, aging and cell wall organization or biogenesis and response to stress and proteolysis. Based on these results, we suggest that seed storage of *C. legalis* by 12 months causes changes in proteome of the dry seeds, which results in seeds deterioration, contributing to a decline in the germination capacity of *C. legalis* seeds. The correlation between seed ageing and proteomics changes suggests that specific proteins may be involved in the regulation of seed viability and germination and subsequent seedling growth in *C. legalis*.

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5. RESUMO E CONCLUSÕES

A partir deste estudo foi possível a obtenção de informações inéditas e de relevância científica para ambas as espécies de estudo, *C. fissilis* e *C. legalis*. Para *C. fissilis*, Put foi potencialmente identificada como uma PA importante para a regulação da organogênese *in vitro*. Além de promover maior alongamento das brotações, Put modulou o metabolismo de proteínas relacionadas ao desenvolvimento da organogênese *in vitro* desta espécie. Estes resultados são inéditos para esta espécie arbórea ameaçada de extinção e caracterizam-se como o primeiro relato da modulação da organogênese pelo uso de PAs exógenas em *C. fissilis*. Além disso, melhorias no protocolo de micropropagação foram estabelecidas, fornecendo uma aplicação prática dos resultados obtidos para a preservação desta espécie.

Em *C. legalis* foram estabelecidas condições para a micropropagação, pela primeira vez descritas para esta espécie. A obtenção de brotações *in vitro* e o enraizamento foram alcançados sem o uso de reguladores de crescimento vegetal. Os dados obtidos em *C. legalis* abrem novas perspectivas para a propagação desta espécie ameaçada de extinção e mostram-se promissores como uma alternativa para a produção de mudas, tendo em vista a problemática de suas sementes que não toleram o armazenamento por longos períodos, perdendo rapidamente a viabilidade. Este estudo com aplicações teóricas e práticas, tem grande importância ecológica, uma vez que a propagação limitante

desta espécie poderá ser superada com aplicações biotecnológicas na produção de mudas.

Análises proteômicas durante o armazenamento de sementes de *C. legalis*, revelaram mudanças na abundância de proteínas associadas a processos biológicos, metabólicos, celulares, de oxidação-redução, metabólicos de carboidratos, bem como proteína de reserva, envelhecimento e organização ou biogênese da parede celular, resposta a estresse e estímulos e proteólise. Estas mudanças foram associadas à redução significativa da germinação, e sugerem que mudanças no proteoma das sementes estão associadas com a perda da viabilidade. Desta forma, este estudo forneceu conhecimento científico básico e aplicado abrindo perspectivas para estudos de armazenamento de sementes e melhoramento genético nesta espécie. Em estudos futuros, a determinação da melhor embalagem de armazenamento e o estudo detalhado de proteínas específicas associadas à manutenção da viabilidade podem contribuir de forma significativa para a preservação das sementes, assegurando assim um insumo para a propagação e também para o melhoramento genético da espécie relacionado à qualidade fisiológica das sementes.

Desta forma, este trabalho inédito para ambas as espécies, faz uma abordagem científica básica sobre os aspectos da propagação *in vitro* e o armazenamento de sementes em espécies arbóreas, podendo ser amplamente aplicado na ciência de plantas promovendo ganhos práticos tanto na produção de mudas, quanto na melhoria da qualidade de armazenamento das sementes.

Portanto, a partir dos dados obtidos neste trabalho pode 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 de armazenamento de sementes para as espécies arbóreas nativas.

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