ASPECTOS FISIOLÓGICOS E BIOQUÍMICOS EM SEMENTES DE *Cedrela fissilis* VELLOZO (MELIACEAE) MANTIDAS EM DIFERENTES CONDIÇÕES DE ARMAZENAMENTO E SUBMETIDAS AO ENVELHECIMENTO ARTIFICIAL

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CAMPOS DOS GOYTACAZES - RJ
FEVEREIRO - 2018

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Orientadora: Profª. Drª. Claudete Santa Catarina

Coorientador: Prof. Dr. Henrique Duarte Vieira

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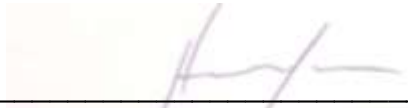

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RESUMO

SOUSA, Kariane Rodrigues. D.Sc. Universidade Estadual do Norte Fluminense Darcy Ribeiro. Fevereiro de 2018. Aspectos fisiológicos e bioquímicos de sementes de *Cedrela fissilis* Vellozo (Meliaceae) mantidas em diferentes condições de armazenamento e submetidas ao envelhecimento artificial.

O objetivo deste trabalho foi analisar os efeitos da temperatura e embalagens nos aspectos fisiológicos e bioquímicos durante o armazenamento e indução do envelhecimento artificial em sementes de *Cedrela fissilis*. Foi testado o efeito das diferentes temperaturas (41, 43, 45, 47 e 50ºC) e tempos (24, 48, 72 e 96 h) para a indução do envelhecimento artificial em sementes. Foi avaliado o efeito da temperatura na germinação, índice de velocidade de germinação (IVG), condutividade elétrica e comprimento, matéria fresca (FM) e seca (MS) da parte aérea e raiz de plântulas. Após a escolha de duas temperaturas (41 e 50ºC), foi utilizada uma abordagem proteômica comparativa e análise do conteúdo de poliaminas (PAs) para avaliar os efeitos da temperatura na germinação e viabilidade das sementes de *C. fissilis*. Para o armazenamento convencional, o efeito da temperatura (4, 12 e 25ºC) e embalagens (papel multifoliado e vidro) na germinação e no conteúdo endógeno de PAs foi avaliado durante 24 meses de armazenamento das sementes. As sementes antes (tempo 0) e após 4, 8, 12, 16, 20 e 24 meses de armazenamento foram utilizadas para análise da qualidade fisiológica das sementes através do teste de germinação, IVG, umidade de semente e conteúdo de PAs. Dentre as temperaturas testadas no envelhecimento artificial,

47°C e, especialmente 50°C, reduziram significativamente a germinação e vigor das sementes comparativamente com 41ºC. Sementes envelhecidas a 50ºC mostraram alteração nas abundâncias de várias proteínas em comparação com aquelas a 41ºC e as não envelhecidas (Time 0), as quais podem estar relacionadas com a redução da germinação e viabilidade. Dentre as proteínas, a redução significativa na abundância da amina oxidase primária em sementes envelhecidas a 50ºC foi relacionada ao acúmulo de Putrescina, a qual pode estar relacionada a danos celulares. No armazenamento convencional, a manutenção das sementes a 4ºC foi mais eficiente em manter a qualidade fisiológica em ambos os tipos de embalagens. A 12ºC, o recipiente de vidro foi a embalagem mais adequada, mas com diminuição da capacidade germinativa. A temperatura de 25°C não foi adequada para armazenar sementes de *C. fissilis* durante longo período. O conteúdo de PAs livres, principalmente Espermidina e Espermina, aumentou significativamente nas sementes armazenadas a 4ºC, sugerindo que essas PAs podem estar relacionadas à manutenção da viabilidade das sementes de *C. fissilis*. Esse é o primeiro trabalho que relaciona o efeito de temperatura na modulação de proteínas diferencialmente abundantes e conteúdo de PAs livres, com importância para compreensão dos eventos relacionados com a perda e/ou manutenção da viabilidade e efeito prático na conservação do potencial germinativo das sementes de *C. fissilis* por até 24 meses de armazenamento.

Palavras-chave: Deterioração, armazenamento de sementes, poliaminas, proteômica.

ABSTRACT

SOUSA, Kariane Rodrigues. D.Sc. Universidade Estadual do Norte Fluminense Darcy Ribeiro. February de 2018. Physiological and biochemical aspects of *Cedrela fissilis* Vellozo (Meliaceae) seeds kept in different storage conditions and submitted to artificial aging.

The aim of this work was to analyze the effects of temperature and package type on physiological and biochemical aspects during the storage and induction of artificial aging in *Cedrela fissilis* seeds. The effects of different temperatures (41, 43, 45, 47 and 50ºC) and times (24, 48, 72 and 96 h) of incubation for the induction of artificial aging in seeds were tested. The effects of temperature on germination, germination speed index (GSI), electrical conductivity and length, fresh matter (FM) and dry matter (DM) of aerial part and root of seedlings were evaluated. After choosing two temperatures (41 and 50ºC), a comparative proteomic approach and analysis of the polyamine content (PAs) were used to evaluate the effects of temperature on germination and viability of *C. fissilis* seeds. For conventional storage, the effects of temperature (4, 12 and 25ºC) and packages (trifoliate paper bags and glass) on germination and endogenous content of PAs were evaluated during 24 months of seed storage. Seeds before (time 0) and after 4, 8, 12, 16, 20 and 24 months of storage were used to analyze the physiological quality of the seeds through the germination test, GSI, seed moisture and PA contents. Among the temperatures tested in artificial aging, 47°C and, especially 50°C, significantly reduced seed germination and vigor compared to 41°C. Seeds aged at 50°C showed a change in

the abundance of several proteins compared to those at 41°C and non-aged seeds (Time 0), which may be related to the reduction of germination and viability. Among the proteins, the significant reduction in the abundance of the primary amine oxidase in seeds aged at 50ºC was related to the Putrescine accumulation, which may be related to cell damage. In the conventional storage, the maintenance of seeds at 4ºC was more efficient to maintain the physiological quality in both types of packages during 24 months. At 12ºC, the glass container was the most suitable package, but with a reduction in germination capacity. The temperature of 25°C was not adequate to store seeds of *C. fissilis* for long periods. The free-PA contents, mainly Spermidine and Spermine, increased significantly in the seeds stored at 4°C, suggesting that these PAs could be related to the maintenance of the viability in *C. fissilis* seeds. This is the first work relating the effects of temperature in the modulation of differentially abundant proteins and free-PAs contents, being important for understanding the events related to the loss and/or maintenance of viability and practical effect on the conservation of the germination potential in *C. fissilis* for up to 24 months of storage.

Keyword: Deterioration, seeds storage, polyamines, proteomics.

1. INTRODUÇÃO

Desde o início da colonização do Brasil, o bioma Mata Atlântica tem sido intensamente explorado, principalmente pela exploração desordenada da madeira, podendo-se observar, atualmente, um cenário de elevada fragmentação e destruição, ameaçando a biodiversidade (Fundação SOS Mata Atlântica, 2017), restando menos de 8,5% de remanescentes florestais acima de 100 hectares (Fundação SOS Mata Atlântica, 2017). Mesmo com a intensa redução na sua área, este bioma ainda possui uma grande diversidade de espécies, sendo classificado com um *hotspot* mundial para a conservação da biodiversidade (Myers et al., 2000).

Muitas espécies da Mata Atlântica, especialmente as arbóreas de interesse econômico para produção de madeira, encontram-se ameaçadas de extinção devido ao intenso extrativismo sem reposição (Martinelli e Moraes, 2013). A necessidade de utilização de sementes viáveis para atender os programas de conservação e de produção florestal levou ao aumento do número de estudos sobre a classificação fisiológica das sementes quanto à capacidade de armazenamento em espécies arbóreas nativas (Carvalho et al., 2006; Carvalho et al., 2008; Aguiar et al., 2010; Antunes et al., 2010). Esses estudos permitem que sejam adotadas condições adequadas de armazenamento de sementes para cada espécie, além da elaboração de programas para a conservação de germoplasma.

A semente é a principal fonte de produção vegetal e o estabelecimento e desempenho bem-sucedido no campo são atribuídos, principalmente, à qualidade da semente utilizada para semeadura (Sathish et al., 2015). Entretanto, em qualquer cultivo a semente tem que ser devidamente armazenada, devido aos

períodos de alternância de produção para várias espécies (Benedito et al., 2011). Neste sentido, no armazenamento deve-se procurar reduzir ao máximo a velocidade e a intensidade do processo de deterioração das sementes, visando manter a sua máxima viabilidade (Krohn e Malavasi, 2004).

As condições fundamentais para o armazenamento das sementes da maioria das espécies são a umidade relativa do ar e a temperatura do ambiente de armazenamento, uma vez que estes fatores influenciam diretamente o metabolismo bioquímico das sementes e a qualidade fisiológica, em particular o vigor (Torres, 2005). Portanto, as embalagens utilizadas, no armazenamento, e temperaturas adequadas, ajudam a diminuir a velocidade do processo de deterioração, mantendo o grau de umidade inicial das sementes armazenadas, com o intuito de diminuir sua respiração (Baudet, 2003; Tonin e Perez, 2006).

A perda da viabilidade da semente pode ser analisada a longo prazo quando armazenadas, ou pode ser simulada por uma técnica conhecida como envelhecimento artificial das sementes. Baseado em diversos trabalhos, o envelhecimento artificial tem sido utilizado por acelerar o processo de envelhecimento da semente, a fim de compreender seus mecanismos (Corte et al., 2010b; Sasaki et al., 2013; Missaoui e Hill, 2015; Sathish et al., 2015; Yin et al., 2015). Deste modo pode-se entender alterações nos parâmetros fisiológicos e bioquímicos induzidas pelo envelhecimento artificial, e relacionar com o envelhecimento natural. Alguns autores afirmam haver correlação entre envelhecimento natural e artificial, sendo os mecanismos promotores da deterioração similares em ambas as situações, variando a velocidade em que ocorrem (Camargo et al., 2000; Corte et al., 2010b).

Estudos têm mostrado que a perda da viabilidade pode estar associada com alterações bioquímicas em sementes armazenadas (Chandrashekar, 2012; Sasaki et al., 2013). Recentemente, foi mostrado que o metabolismo de alguns compostos, como as poliaminas (PAs) pode estar associado com a redução da viabilidade e vigor das sementes em espécies arbóreas, como em *Cariniana legalis* (Sousa et al., 2016). As PAs estão relacionadas ao desenvolvimento e à germinação de sementes de espécies arbóreas, como *Ocotea catharinensis* e *Araucaria angustifolia,* mostrando as variações nos conteúdos destes compostos durante estes processos (Santa-Catarina et al., 2006; Pieruzzi et al., 2011).

A perda da viabilidade das sementes também pode estar associada a alterações no proteôma de sementes (Rajjou et al., 2008; Lu et al., 2016; Natarajan et al., 2016). Em sementes deterioradas de *Arabidopsis thaliana* o dano oxidativo nas sementes foi correlacionado com a perda de vigor e germinação. O aumento da oxidação de proteína (carbonilação) como por exemplo em proteínas de choque térmico (HSPs) e calreticulina pode induzir uma perda de propriedades funcionais destas proteínas (Rajjou et al., 2008). Nesse sentido, estudos visando estabelecer condições que permitam manter a máxima viabilidade das sementes durante o armazenamento, a fim de minimizar a velocidade de deterioração, por meio da conservação apropriada das sementes para cada espécie são importantes (Kissmann et al., 2009). Adicionalmente, conhecer alterações no metabolismo de alguns compostos, como proteínas diferencialmente abundantes e conteúdo de PAs, pode auxiliar na compreensão dos aspectos bioquímicos associados com a perda da germinação. Apesar do crescente interesse associado com estudos proteômicos e conteúdo de PAs em plantas, pesquisas que relacionam o envelhecimento das sementes de espécies arbóreas nativas, especialmente as ameaçadas de extinção, são limitados.

Dentre as espécies ameaçadas de extinção em seu habitat natural da Mata Atlântica encontra-se a *Cedrela fissilis* (Meliaceae), que sofreu forte ação antrópica pela importância econômica na produção da madeira, sendo intensamente explorada comercialmente (Myers et al., 2000; Galindo-Leal e Câmara, 2005; Martinelli e Moraes, 2013). Atualmente, esta espécie encontra-se ameaçada de extinção (IUCN, 2017). *C. fissilis* produz sementes que perdem sua viabilidade após seis meses de armazenamento em condições não controladas e quando armazenadas a 4ºC por 12 meses o seu vigor é afetado (Corvello et al., 1999; Sousa et al., 2016), o que dificulta o estabelecimento de programas de reflorestamento e conservação. Para que se possa vislumbrar, em médio e longo prazo, os programas de conservação desta espécie bem como recuperação de áreas degradadas, ressalta-se a importância de se desenvolver estudos durante o envelhecimento a fim de melhores condições de armazenamento, além do melhor entendimento dos fatores relacionados à deterioração de sementes para esta espécie.

2. REVISÃO BIBLIOGRÁFICA

2.1. Mata Atlântica

A Mata Atlântica é um dos biomas brasileiros que enfrenta um ritmo acelerado de destruição, e devido à sua complexidade biológica foi considerado pela União Internacional para Conservação da Natureza um dos mais ameaçados (IUCN, 2017). Este bioma ocupava uma área de 1,3 milhões de quilômetros quadrados do território brasileiro, e tratava-se da segunda maior floresta tropical úmida do Brasil, só comparável à Floresta Amazônica (Morellato e Haddad, 2000; Ribeiro et al., 2009). No Brasil, originalmente percorria todo o litoral brasileiro, compreendendo sua região costeira. Estendia-se do Rio Grande do Norte até o Rio Grande do Sul (Morellato e Haddad, 2000; Myers et al., 2000). Nas regiões Sul e Sudeste, abrange parte do território da Argentina e do Paraguai (Câmara, 2005). Na Mata Atlântica os desmatamentos, tanto para fins agropecuários como para extração de matéria-prima com finalidade de suprir as necessidades da indústria, têm causado grande pressão sobre os recursos florestais ao longo dos anos (Sena e Gariglio, 2008). Uma longa história de exploração dos recursos naturais como, a intensa extração de madeira, a expansão da agricultura e o crescimento populacional de maneira não sustentável na costa atlântica do Brasil, contribuiu para que a Mata Atlântica se tornasse um dos ecossistemas com maiores riscos de extinção do mundo (Morellato e Haddad, 2000). Extensas áreas foram devastadas sem qualquer conhecimento e grande parte da biodiversidade presente neste ecossistema pode estar se perdendo (Borém e Oliveira-Filho, 2002).

Na escala de paisagem, a maior parte da cobertura da Mata Atlântica está integrada em agromosaicos dinâmicos, incluindo elementos como fragmentos de pequenas florestas, manchas de floresta secundária precoces e tardias e monoculturas de árvores exóticas (Tabarelli et al., 2010). Atualmente sua vegetação encontra-se com 93% de sua área original degradada (Fig. 1) (Fundação SOS Mata Atlântica, 2017). Adicionalmente, dados referentes ao desmatamento no período de 2015 a 2016 representaram aumento de 57,7% em relação ao período de 2012 a 2013 (Fundação SOS Mata Atlântica e Instituto Nacional de Pesquisas Espaciais, 2017), resultando em uma paisagem atualmente fragmentada, de modo que esta floresta encontra-se entre os ecossistemas mais devastados e ameaçados do planeta (Morellato e Haddad, 2000; Câmara, 2005; Galindo-Leal e Câmara, 2005; Fundação SOS Mata Atlântica, 2017).

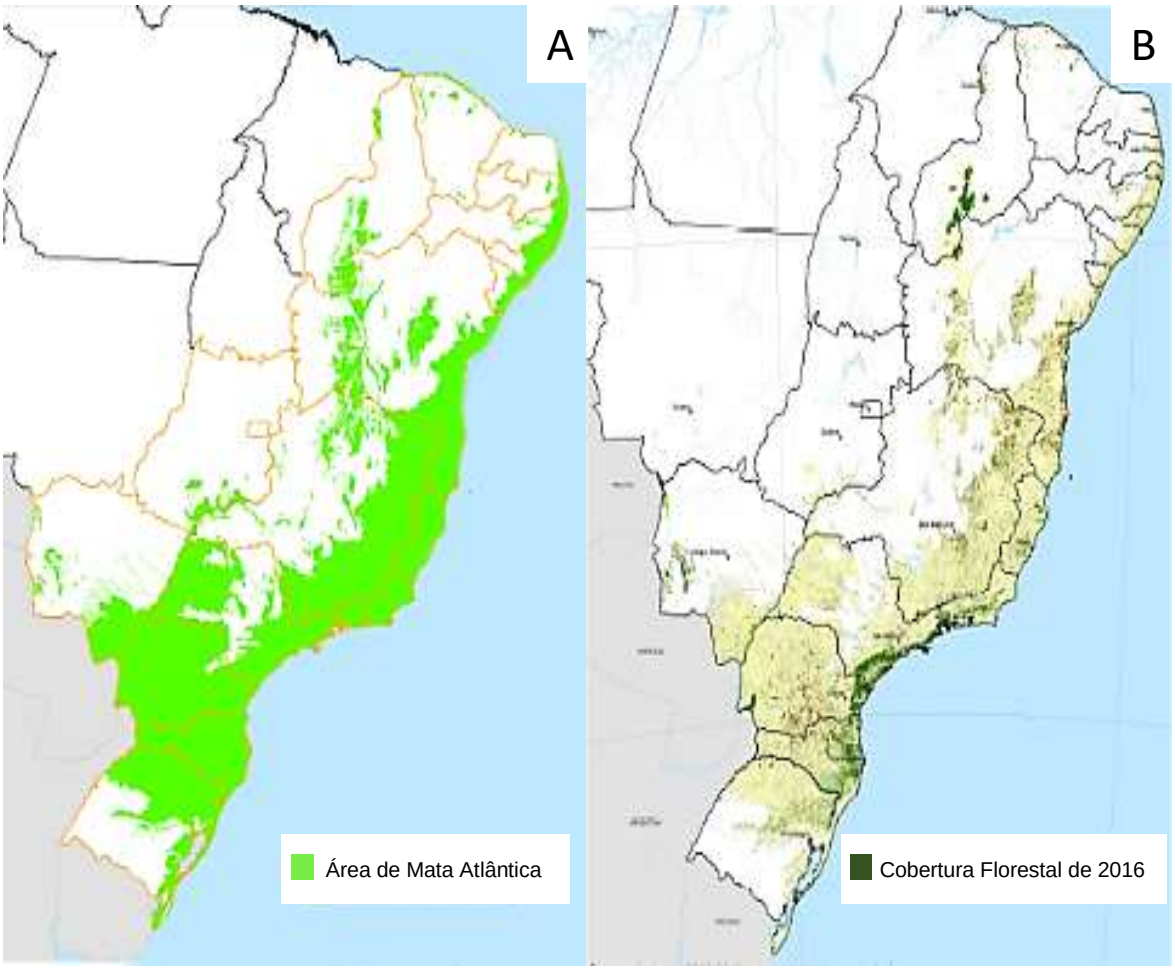

Figura 1: Mapas da área do Bioma da Mata Atlântica, mostrando a cobertura florestal original (A) e a cobertura florestal em 2016 (B). Adaptado do Atlas dos remanescentes da Mata Atlântica período 2015 - 2016. Fonte: Fundação SOS Mata Atlântica (2017).

Na região Sudeste do Brasil, particularmente no Estado do Rio de Janeiro, esse bioma cobria 100% da área. Esse Estado ainda abrange uma porção preservada da Mata Atlântica, especialmente no interior e nas regiões montanhosas (Cysneiros et al., 2015). Em 2017, os remanescentes florestais da Mata Atlântica foram de 20,9% de sua área original (Fundação SOS Mata Atlântica e Instituto Nacional de Pesquisas Espaciais, 2017). Mesmo com essa intensa redução da área, esta floresta é considerada um dos *hotspots* mundiais de biodiversidade, em virtude do elevado número de espécies animais e vegetais que ocorre em seus domínios, sobretudo endêmicas, sendo decretada Reserva da Biosfera pela Unesco e Patrimônio Nacional (Myers et al., 2000; Fundação SOS Mata Atlântica, 2017).

Essa intensa devastação da vegetação causou a perda da biodiversidade genética. A fragmentação do habitat aumenta a limitação da dispersão da semente e também pode afetar os estádios demográficos subsequentes, como o estabelecimento de plântulas (Herrera and García, 2010). Um estudo abrangendo cinco continentes em 35 anos demonstrou que a fragmentação do habitat reduziu a biodiversidade de 13 a 75% e prejudicou as principais funções do ecossistema, diminuindo a biomassa (Haddad et al., 2015). Esse panorama integra um amplo grupo de espécies arbóreas como, espécies tolerantes a sombra (Tabarelli et al., 1999), emergentes (Oliveira et al., 2008) e espécies polinizadoras por animais vertebrados e invertebrados (Girão et al., 2007). Com isso, a diminuição do número de espécies pode ter efeito negativo na reprodução das plantas e dispersão das sementes (Girão et al., 2007; Tabarelli et al., 2010).

Aliado a esses fatores, a escassez de estudos sobre a fisiologia do desenvolvimento, baixa viabilidade das sementes de algumas espécies arbóreas e condições inadequadas de armazenamento, representam obstáculos para produção de mudas viáveis para algumas espécies. Estes resultados indicam uma necessidade urgente de medidas de conservação e restauração de áreas degradadas para melhorar a conectividade desse panorama, o que reduzirá as taxas de ameaça de extinção e ajudará a manter o equilíbrio dos ecossistemas neste bioma (Haddad et al., 2015), e além disso, a conservação *ex situ* de sementes a longo prazo de espécies florestais nativas (Li e Pritchard, 2009).

2.1.1. Características da espécie de estudo

C. fissilis (Meliaceae), conhecida popularmente como cedro rosa, é uma espécie secundária tardia ou clímax exigente de luz (Rodrigues et al., 2003), sendo comumente encontrada na Floresta Ombrófila Densa Submontana da Mata Atlântica, e nas formações Montana e Submontana e Floresta Ombrófila Densa da Floresta Amazônica. Esta espécie é amplamente distribuída no Brasil, de ocorrência nos Estados de Alagoas, Acre, Amazonas, Bahia, Ceará, Distrito Federal, Espírito Santo, Goiás, Maranhão, Minas Gerais, Mato Grosso, Mato Grosso do Sul, Pará, Pernambuco, Piauí, Paraná, Rio de Janeiro, Rondônia, Rio Grande do Sul, Santa Catarina, São Paulo e Tocantins (Fig. 2) (Carvalho, 2003; Martinelli e Moraes, 2013).

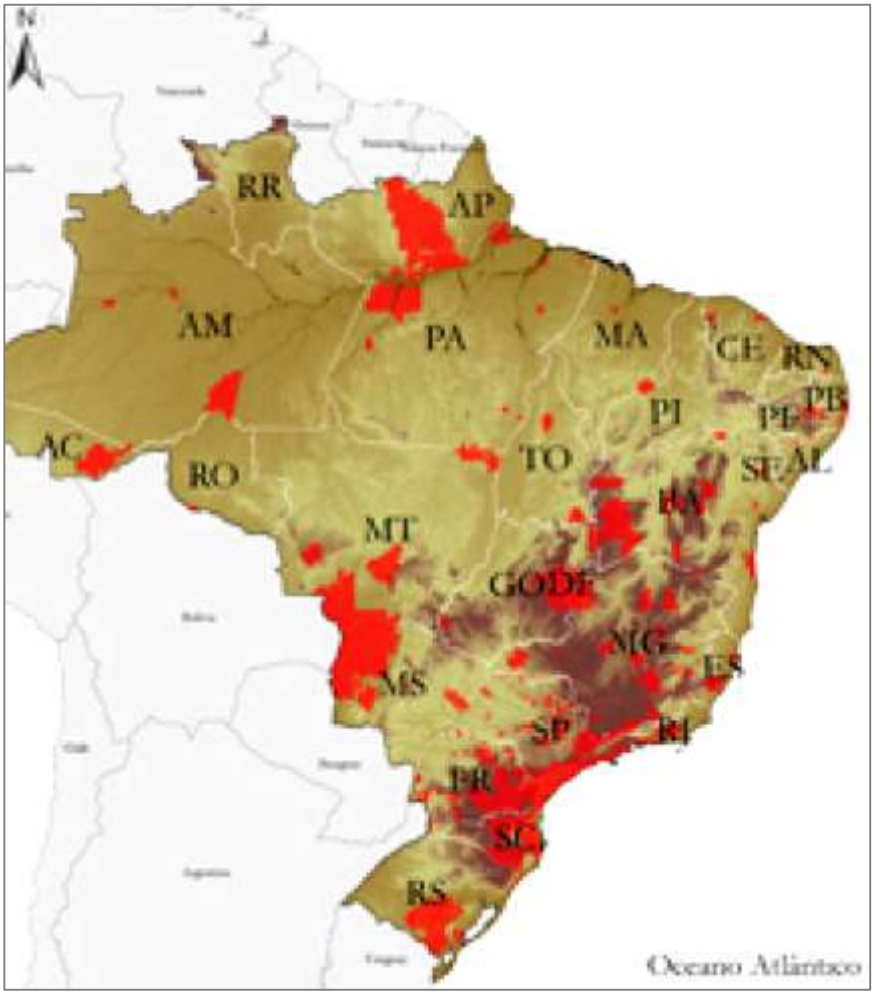

Figura 2: Mapa da distribuição de *C. fissilis* no Brasil. Áreas em vermelho indicam os locais de ocorrência natural da espécie. Fonte: Martinelli e Moraes (2013).

Esta espécie é uma árvore caducifólia, com altura variando entre 10 e 25 m e 40 e 80 cm de DAP, com tronco cilíndrico, reto ou pouco tortuoso, com fuste de até 15 m (Carvalho, 2003). Entre as madeiras leves, o cedro é a que possibilita o uso mais diversificado possível, superado somente pela madeira do pinheiro do Paraná (*A. angustifolia*) com resistência moderada ao ataque de organismos xilófagos. Historicamente, a espécie foi amplamente explorada devido à extração de madeira de alta qualidade, e o alto valor comercial a torna alvo do extrativismo e da exploração indiscriminada (Ruiz Filho et al., 2004). Dentre outras utilidades, a espécie é usada para a fabricação de móveis e na construção civil em geral (Carvalho, 2003). Além disso, é produtora de óleo essencial com propriedades inseticidas, sendo sua casca usada na medicina popular (Maia et al., 2000; Castro Coitinho et al., 2006). A espécie é recomendada para programas de reflorestamento ambiental para recuperação de áreas degradadas em sua área de ocorrência natural (Martins, 2005) como em pastagens degradadas, compostas por gramíneas (Campoe et al., 2014). Além disso, grande parte dos seus habitats foi completamente degradada, tendo sido convertida em áreas urbanas, pastagens, plantações, entre outros (Martinelli e Moraes, 2013). Esses fatores levaram a um declínio populacional da espécie de pelo menos 30% ao longo das últimas três gerações (Martinelli e Moraes, 2013).

O processo reprodutivo ocorre entre dez e quinze anos de idade (Pinheiro et al., 1990) e o florescimento desta espécie acontece normalmente entre os meses de setembro a novembro, ocorrendo o amadurecimento dos frutos entre junho a julho (Carvalho, 2003), com variações entre os locais de ocorrência. O fruto é do tipo cápsula piriforme deiscente, septífraga, com aproximadamente 30 a 100 sementes aladas por fruto. A dispersão se dá pela queda das sementes ou por anemocoria (Alcántara, 1997). As sementes são classificadas como ortodoxa (Carvalho et al., 2006), entretanto se armazenadas em condições ambientais de baixa umidade perdem gradativamente a viabilidade com o tempo (Cherobini et al., 2008; Martins e Lago, 2008), e quando armazenadas em câmara fria elas mantêm a viabilidade por até 3 anos (Piña-Rodrigues e Jesus, 1992; Carvalho, 2003). Estudos recentes mostraram que o armazenamento de sementes por 12 meses a 4ºC não apresentou influência na emergência das plântulas, porém o vigor das sementes diminuiu significativamente (Sousa et al., 2016).

Devido à intensa fragmentação da sua área de ocorrência e exploração da madeira, a espécie encontra-se em risco 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, 2017).

2.2. Deterioração: armazenamento, envelhecimento artificial e vigor de sementes

Em comum a todos os outros seres vivos, as sementes estão sujeitas ao envelhecimento e, culminando na perda da viabilidade. O potencial fisiológico máximo da semente é alcançado perto da maturidade fisiológica, e uma vez iniciada a deterioração, esse processo catabólico não é revertido (Jyoti e Malik, 2013). Dentre as alterações envolvidas na deterioração de semente destacam-se, perda da integridade de membrana, alterações enzimáticas e dos constituintes químicos da célula, redução da atividade metabólica, acúmulo de radicais livres e alterações cromossômicas (Jyoti e Malik, 2013). Assim, após essas perdas resultantes de vários atributos de performance da semente, as manifestações fisiológicas inicias do envelhecimento são percebidas pelo declínio da velocidade de germinação de sementes viáveis seguido da diminuição no tamanho da plântula e aumento da incidência de plântulas anormais (Marcos Filho, 2015). Essas alterações fisiológicas e bioquímicas foram observadas em diversos trabalhos com sementes de espécies agrícolas (Tian et al., 2008; Tilebeni e Golpayegani, 2011; Kharlukhi, 2013; Sasaki et al., 2013; Ratajczak et al., 2015), bem como em sementes de espécies arbóreas nativas (Corte et al., 2010a; Mata Ataíde et al., 2012; Abbade e Takaki, 2014). Entretanto, ainda não é bem compreendido os fatores bioquímicos que acionam a perda da viabilidade das sementes mantidas nas várias condições de armazenamento.

Embora a deterioração durante o envelhecimento da semente não possa ser revertida, esse processo pode ser minimizado. As sementes após a coleta nem sempre são utilizadas imediatamente, e devido a isso devem ser armazenadas para utilização futura, uma vez que muitas espécies apresentam alternância de produção de sementes, caracterizadas por um ano de alta produção, seguido de um ou dois de baixa produção (Carneiro et al., 1993; Benedito et al., 2011). Pesquisas

relacionadas à qualidade fisiológica das sementes têm sido intensificadas ao longo dos anos por estarem sujeitas a uma série de mudanças degenerativas após a maturação (Marcos Filho, 2015). O período em que a semente pode permanecer quiescente é afetado por sua qualidade no momento da coleta, condições ambientais, beneficiamento, procedimentos adotados de secagem, temperatura e embalagens de armazenamento (Abreu et al., 2013; Filho, 2015). Esses fatores citados afetam a viabilidade das sementes e devem ser considerados para determinar as condições adequadas de armazenamento, a fim de manter a germinação e o vigor de sementes por maior tempo.

Uma das alternativas utilizadas para reduzir a velocidade da deterioração de sementes *ex situ* é reduzir seu metabolismo através da remoção de água e redução da temperatura de armazenamento (Roberts, 1973; Spanò et al., 2007). A relação da umidade das sementes e a umidade relativa do ar está estreitamente relacionado à viabilidade e qualidade fisiológica destas, enquanto a temperatura influencia a velocidade dos processos bioquímicos e interfere indiretamente no teor de água e, consequentemente, no seu metabolismo (Torres, 2005). Em sementes de *Swertia chirayita* a temperatura de armazenamento a 4°C manteve a viabilidade das sementes durante 24 meses quando comparado às armazenadas a 15 e 25ºC (Pradhan e Badola, 2012). Além da temperatura, o tipo de embalagem durante o armazenamento tem influência significativa na qualidade fisiológica da semente, uma vez que ajuda a diminuir a velocidade da deterioração, mantendo o teor de água inicial das sementes armazenadas e, diminuindo ou não, a sua taxa de respiração (Tonin e Perez, 2006).

De acordo com Baudet (2003), as embalagens são classificadas conforme o grau de permeabilidade a água, sendo a) permeáveis as que admitem trocas de vapor da água entre as sementes e o ar atmosférico (saco de papel, papelão); b) semipermeáveis, as que oferecem certa resistência à troca de umidade (papel multifoliado, saco de polietileno) e c) impermeáveis, as que não permitem que a umidade do ar exerça influência sobre a semente (frasco de vidro e frascos de metal). O tipo de embalagem mais adequado para o armazenamento depende da espécie alvo, como foi verificado, por exemplo, o saco plástico para *Ocotea porosa* (Tonin e Perez, 2006), e ambos, vidro e saco plástico, para *Piptadenia moniliformis* (Benedito et al., 2011).

Uma das limitações no estudo da deterioração de sementes durante o armazenamento é o fator tempo, que pode variar de meses para sementes recalcitrantes a muitos anos em sementes ortodoxas, para que se observe alterações bioquímicas e fisiológicas relacionadas ao envelhecimento. Um dos procedimentos técnicos utilizados para permitir estudos de deterioração é o uso da indução do envelhecimento artificial das sementes. Neste sentido, o envelhecimento artificial consiste em submeter as sementes à temperatura elevada e alta umidade relativa (±100% UR), simulando o armazenamento, porém estimulando o aumento da velocidade das reações metabólicas, permitindo assim o monitoramento dos processos envolvidos em um tempo menor, onde na velocidade cronológica do armazenamento convencional de sementes pode necessitar de anos a depender da espécie (Zhang et al., 2015). Desta forma, o envelhecimento artificial é utilizado para avaliar o vigor de sementes de diversas espécies, pois em poucos dias obtêm-se informações sobre alterações fisiológicas e bioquímicas sobre o potencial de armazenamento pela análise da germinação e vigor (Marcos Filho, 2015).

Neste sentido, esta metodologia tem sido utilizada para o estudo das alterações fisiológicas e bioquímicas das sementes durante o processo de deterioração visando entender melhor esse processo em várias espécies (Silva et al., 2008; Corte et al., 2010a; Tilebeni e Golpayegani, 2011; Shibata et al., 2012; Moncaleano-Escandon et al., 2013; Abbade e Takaki, 2014). Sasaki et al. (2013) analisaram alterações nos conteúdos de aminoácidos livres em *Oryza sativa* sob indução de envelhecimento artificial a 15 e 70% de umidade relativa (UR) por oito meses. Esses autores sugerem que condições de armazenamento em alta UR influenciam o metabolismo dos aminoácidos, particularmente o aumento de ácido γ-aminobutírico (GABA), deve ser focado para entender o mecanismo molecular de deterioração de sementes. Esses estudos também têm sido realizados em conjunto com o armazenamento convencional em espécies arbóreas. Sementes de *Melanoxylon brauna* foram armazenadas (câmara fria a 5°C) por 12 meses e envelhecidas artificialmente (40 e 45ºC durante 24, 48, 72 e 96 h). Foi mostrado que o envelhecimento artificial a 45ºC por 72 h simula os resultados fisiológicos e bioquímicos da deterioração ocorrida em sementes de *M. brauna* armazenadas por 12 meses, promovendo redução da viabilidade e do vigor (Corte et al., 2010a).

2.3. Estudos proteômicos no envelhecimento de sementes: relação entre manutenção e/ou perda da viabilidade

Embora a deterioração da semente seja inevitável, mesmo em condições adequadas de conservação, muitas modificações moleculares que ocorrem nesse processo ainda não estão bem elucidadas (Sathish, 2015), sendo de relevância ecológica e agronômica entender os mecanismos que regem a perda de vigor das sementes durante o envelhecimento (Nguyen et al., 2015).

Devido à sua abundância nos sistemas biológicos, as proteínas são os principais alvos dos radicais livres (Davies, 2005). Neste sentido, a abordagem proteômica tem sido usada para identificar proteínas cujas alterações nos níveis de abundância estão associadas a alterações no vigor em sementes armazenadas ou envelhecidas artificialmente (Rajjou et al., 2008).

Em sementes de *Triticum aestivum*, a análise proteômica durante o envelhecimento artificial (45°C e 50% UR) revelou que a maioria das proteínas diferencialmente abundantes estava envolvida com metabolismo celular, energia e respostas de defesa/estresse (Lv et al., 2016). Essas proteínas diferencialmente abundantes indicaram que a capacidade reduzida de proteção contra o envelhecimento pode levar a diminuição do incremento das substâncias armazenadas na semente, afetando os suprimentos metabólicos e energéticos, culminando na deterioração da semente (Lv et al., 2016).

Em sementes ortodoxas, a tolerância à dessecação e a manutenção do estado quiescente da semente foram associadas à presença de proteínas abundantes no final da embriogênese (LEA) e às proteínas de choque térmico (HSPs), sugerindo que deve existir uma relação entre proteínas específicas e a manutenção da longevidade das sementes (Rajjou e Debeaujon, 2008). Adicionalmente, em sementes de *A. thaliana*, a oxidação de proteínas HSP 70 foi relacionada com a diminuição progressiva na germinação e vigor de sementes após o envelhecimento artificial, e sugerem que a perda de função desta proteína possa estar relacionada com a perda de vigor da semente (Rajjou et al., 2008). Similarmente, o aumento na abundância de HSFBP, uma proteína da família HSP, durante o envelhecimento em sementes de álamo (*Populus* × *Canadensis* Moench) e *Medicago sativa*, sugeriram que essa proteína pode ser usada como marcador

da diminuição do vigor de sementes nessas espécies (Yacoubi et al., 2011; Zhang et al., 2015).

Além das proteínas LEA e HSPs, a anexina é uma proteína envolvida na sinalização de membrana (Barton et al., 1991; Gerke et al., 2005; Clark et al., 2010). Essa proteína foi importante para a manutenção do vigor em sementes transgênicas de *A. thaliana* (Chu et al., 2012). As sementes transgênicas que expressaram o gene da anexina apresentaram resistência ao tratamento do envelhecimento artificial, enquanto as sementes do tipo selvagem apresentaram redução de germinação, sugerindo que a anexina pode aumentar a tolerância ao envelhecimento (Chu et al., 2012).

Embora vários estudos mostrem a relação do envelhecimento com as modificações bioquímicas, moleculares e genéticas nas sementes de interesse agrícola, poucos estudos abordam sementes de espécies arbóreas nativas, especialmente as ameaçadas de extinção.

2.4. Efeito das poliaminas (PAs) no envelhecimento de sementes

As PAs, putrescina (Put), espermidina (Spd) e espermina (Spm), são compostos orgânicos, alifáticos de baixo peso molecular, existentes em todos os organismos, bactérias, animais e plantas (Kusano et al., 2008). Nas plantas, a Put é sintetizada a partir dos aminoácidos arginina e ornitina pela ação das enzimas arginina descarboxilase (ADC) e ornitina descarboxilase (ODC), respectivamente. A Put é convertida em Spd e esta em Spm por adições sucessivas de grupos aminopropil oriundos do aminoácido metionina, a partir da S-adenosil-metionina (SAM), pela ação da SAM descarboxilase (SAMDC). Assim, a adição de um grupo aminopropil à Put originará a Spd pela ação da Spd sintase, e outro grupo adicionado à Spd originará a Spm pela ação da Spm sintase. O catabolismo de Put, Spd e Spm é feito pela ação das enzimas diamina oxidase (DAO) e PA oxidase (PAO) (Kaur-Sawhney et al., 2003; Kusano et al., 2008; Takano et al., 2012).

As PAs ocorrem na forma livre ou conjugada, com dois ou mais grupos amino carregados positivamente (Kusano et al., 2008). Podem ligar-se a várias macromoléculas, incluindo DNA, RNA, fosfolipídios, componentes da parede celular e proteínas (Kusano et al., 2008; Moschou et al., 2008). Esta característica permite maior capacidade de estabilização da membrana contra os danos causados por espécies reativas de oxigênio (Khan et al., 1992; Ha et al., 1998). Em

plantas, participam de diversos processos no crescimento e desenvolvimento, tais como, regulação, homeostase e sinalização celular, morfogênese, respostas a estresses biótico e abiótico (Alcázar et al., 2010; Pottosin e Shabala, 2014; Tiburcio et al., 2014). Estudo da participação das PAs durante o desenvolvimento embrionário, germinação e armazenamento de sementes tem sido realizado para algumas espécies arbóreas (Santa-Catarina et al., 2006; Pieruzzi et al., 2011; Aragão et al., 2015; Sousa et al., 2016).

O efeito das PAs durante o envelhecimento da semente, na sua maioria tem sido estudado em espécies de interesse econômico, e os resultados sugerem que as alterações podem ser espécie-dependente. Estudos com envelhecimento artificial em *O. sativa* variedade Japonica e Tapei 309 mostram um aumento no conteúdo das PAs Put, Spd e Spm em lotes de sementes que tiveram uma frequência de germinação baixa em comparação com aqueles com elevado potencial germinativo (Bonneau et al., 1994). Contrariamente, sementes de *Allium cepa* tiveram o conteúdo das PAs Put, Spd e Spm reduzidos após um ano, e a aplicação exógena de Put, Spd e Spm aumentou o conteúdo endógeno destas PAs sendo relacionado com a melhoria do vigor das sementes (Basra et al., 1994).

Adicionalmente, foi observado, em sementes de *Triticum durum* armazenadas durante 1 a 8 anos, que as com 1 ano de armazenado apresentaram conteúdo de Put, Spd e Spm maior, enquanto as sementes armazenadas por período de tempo maior exibiram um conteúdo menor destas PAs com o decorrer do envelhecimento e apresentaram redução na viabilidade (Anguillesi et al., 1990). Mikitzel e Knowles (1989), observaram em tubérculos de batata-semente (*Solanum tuberosum*) influência pela idade das sementes, verificando que o envelhecimento foi acompanhado pelo acúmulo endógeno de Put e redução de Spd e Spm. Estes autores sugerem uma conversão menos eficiente de Put para a formação de Spd e Spm com o avanço do envelhecimento do tubérculo, além da atividade reduzida ou síntese de novo de Spd e Spm ou disponibilidade limitada de S-adenosilmetionina (SAM). Ademais, o aumento da concentração de etileno pode estar associada com a possibilidade da SAM ter sido direcionada para a síntese deste composto ao invés de PAs (Mikitzel e Knowles, 1989). Desta forma, uma redução dos níveis de Spd e Spm estaria associada com um aumento concomitante interno de etileno no tubérculo.

Sementes de *C. legalis*, uma espécie arbórea da Mata Atlântica, armazenadas a 4ºC em sacos plásticos por 12 meses apresentaram aumento no conteúdo de Put, o qual foi associado com a redução no vigor e na emergência de plântulas quando comparado às sementes de *C. fissilis* (Sousa et al., 2016). As observações mostradas sugerem que o envolvimento das PAs na manutenção ou perda da viabilidade da semente depende de espécie para espécie, condições de temperatura e embalagem, assim como do tempo de envelhecimento.

3. OBJETIVOS

3.1. Objetivo geral

O objetivo geral deste trabalho foi analisar os efeitos do tempo, temperatura e embalagens nos aspectos fisiológicos e bioquímicos durante o armazenamento e indução do envelhecimento artificial em sementes de *C. fissilis*.

3.2 Objetivos específicos

- Avaliar os efeitos das diferentes temperaturas nos parâmetros fisiológicos durante o envelhecimento artificial em sementes em *C. fissilis*.

- Estudar os efeitos da temperatura na manutenção da viabilidade durante o envelhecimento de sementes de *C. fissilis*, utilizando uma abordagem proteômica comparativa e análise de conteúdo de PAs.

- Estudar os efeitos da temperatura e das embalagens na germinação e na alteração do conteúdo endógeno das PAs durante 24 meses de armazenamento de sementes em *C. fissilis*.

4. TRABALHOS

4.1. EFFECTS OF ARTIFICIAL AGING IN THE GERMINATION AND VIGOR OF *Cedrela fissilis* VELLOZO (MELIACEAE) SEEDS

RESUMO

O efeito do envelhecimento das sementes na germinação ainda não é estudado em várias espécies, como *Cedrela fissili*s, uma espécie nativa da Mata Atlântica brasileira. O objetivo deste trabalho foi avaliar os efeitos da temperatura nos parâmetros fisiológicos durante o envelhecimento artificial de sementes de *C. fissilis*. Para analisar os efeitos da temperatura no envelhecimento, as sementes foram incubadas a 41, 43, 45, 47 e 50ºC durante 24, 48, 72 e 96 h. Em cada período e tratamento foram analisados a germinação, o índice de velocidade de germinação, a condutividade elétrica, bem como o comprimento, a matéria fresca e seca da parte aérea e raiz de plântulas. Nas temperaturas mais elevadas (47 e principalmente 50°C) verificou-se um efeito significativo nos parâmetros fisiológicos, reduzindo a germinação e o crescimento de plântulas. A perda de viabilidade foi observada em sementes incubadas a 50ºC a partir das 72 h. Nossos resultados contribuíram para demonstrar que o envelhecimento artificial pode ser

usado para estudos de alterações bioquímicas durante a perda de viabilidade em *C. fissilis*.

ABSTRACT

The effect of seed aging on germination is not yet studied in several species, such as *Cedrela fissilis*, a native species of the Brazilian Atlantic Forest. The aim of this work was to evaluate the effects of temperature in the physiological parameters during artificial aging of seeds in *C. fissilis*. To analyze the effects of temperature on aging, seeds were incubated at 41, 43, 45, 47 and 50ºC during 24, 48, 72 and 96 h. In each period and treatment, the germination, germination speed index, electrical conductivity, as well as the length, fresh and dry matter of aerial part and roots of seedlings were analyzed. The higher temperatures (47 and mainly 50°C) of artificial aging affected significantly the physiological parameters, reducing the germination and seedling growth. The loss of viability was observed in seeds incubated at 50ºC from 72 h, without germination. Our results contributed to demonstrate that artificial aging could be used to explore further studies relating biochemical changes during the loss of viability in *C. fissilis*.

1. INTRODUCTION

The forest species are of great economic importance due the high quality of their wood and ecological value related to the reforestation of degraded areas (Gris et al., 2012; Mangaravite et al., 2016). Among several forest species, *Cedrela fissilis* Vellozo (Meliaceae) is a native tree of the Atlantic Forest, which produce a wood of high quality, and, due its economic importance, this species is included in the red list of (IUCN) as endangered (IUCN, 2017). The propagation of several endangered species of Atlantic Forest is compromised by the disorderly exploitation and by the reduced studies related to the behavior of its seeds after the harvest (Hong and Ellis, 1998; Mangaravite et al., 2016). In *C. fissilis*, studies related to the deterioration

of seeds during storage are few (Corvello et al., 1999; Sousa et al., 2016), being relevant the establishment of researches that allow the maintenance of seeds with high physiological potential on storage. Therefore, the study of the physiological potential becomes fundamental for a greater maintenance of the germinative capacity and vigor of seeds, and has been developed for some species (Corvello et al., 1999; Masetto et al., 2014; Mayrinck et al., 2016). However, considering the great diversity of Brazilian flora, the available information is still scarce.

The seed deterioration processes can be accessed through the storage of seed or by artificial aging (Rajjou et al., 2008; Nguyen, 2015). The artificial aging is a tool used to determine changes in the physiological potential of seeds under a high temperature and high relative humidity, and can mimic natural aging, applied to study seed longevity and vigor (Rajjou et al., 2008; Zhang et al., 2016). Physiological and biochemical changes occur during seed aging, and the decline in the germination potential and germination speed index (GSI) are more obvious manifestation of aging (Mahjabin et al., 2015). In *Tabebuia roseoalba* seeds, reduction of germination and emergence, shorter length and lower seedling dry weight were associated with a decrease in the physiological quality in aged seeds (Abbade and Takaki, 2014). In addition, studies with *Cariniana legalis* showed a significant reduction of seedling emergency when stored at 4°C in paper bags for 12 months, while in *C. fissilis*, seeds keep the physiological quality at this time of storage (Sousa et al., 2016). However, there are few studies about the physiological alterations related to germination, GSI, moisture content and seedling growth during seeds of *C. fissilis* submitted to artificial aging.

In this sense, the aim of this work was to evaluate the effects of temperature in the physiological parameters during artificial aging of seeds in *C. fissilis*.

2. MATERIAL AND METHODS

2.1. Plant material

Mature seeds, collected in August 2014, were provided by Caiçara nursery located at Brejo Alegre, São Paulo, Brazil (21º10'S and 50º10'W). After arrived at laboratory, seeds were stored in paper bags at 4°C until to perform the experiment.

2.2. Effect of temperature on artificial aging of seeds

For artificial aging, the effect of temperature (41, 43, 45, 47, and 50ºC) was tested for seed germination and seed vigor. Four replicate of seeds (with 50 seeds each) were placed on a wire mesh screen and suspended over 40 mL of water inside a plastic box (11 × 11 × 35 cm). The plastic boxes were incubated in a BOD-type germination chamber (Eletrolab, São Paulo, SP, Brazil) at 41, 43, 45, 47 and 50ºC during 24, 48, 72, and 96 h, and 100% relative humidity. The physiological parameters were evaluated using samples from seeds before (time 0, non-aged seed) and after 24, 48, 72, and 96 h in all tested temperatures.

Analyzes of germination (%), GSI, moisture content (%), electrical conductivity, as well as length, fresh (FM) and dry matter (DM) of aerial part and roots of seedlings were performed from samples of all treatments.

2.2.1. Determination of moisture content

The seed moisture content was determined according to Brasil (2009). The samples (four biological replicates; 2 g FM each) from each treatment were weighed and then dried in a chamber with forced air circulation at 105 °C (Ethik technology, São Paulo, SP, Brazil) for 24 h. The results were expressed as percentage of the FM according to the formula: seed moisture content = (water content/FM) x 100.

2.2.2. Seed germination and GSI

The germination test, used to evaluate seed viability, was performed according to Brasil (2013). Samples of seeds (50 seeds each, in four biological replicates) from each treatment were distributed upon three sheets of germitest® paper (J Prolab, Paraná, PR, Brazil) and moistened with sterile distilled water at a ratio of 2.5 times the water in relation to the weight of dry substrate. After, seeds were incubated in a BOD-type germination chamber at 25ºC and photoperiod of 8 h light, at 40 µmol m^{-2} s^{-1}. The germination was recorded daily during 21 days, and the GSI was determined according to Maguire (1962). Results were expressed as percentage, considering the normal seedlings obtained.

2.2.3. Electrical conductivity

Electrical conductivity was determined according to Vieira et al. (1999). In samples (25 seeds of each samples, in four biological replicates) from each

treatment. Samples of seeds were weighed and soaked in a plastic container with 75 mL of distilled water, and kept in a BOD-type germination chamber at 25°C, during 24 h. After, the electrical conductivity of imbibition solution was measured using a CD-830 conductivimeter (Instrutherm, São Paulo, SP, Brazil). The results were expressed as $\mu S.cm^{-1}\ g^{-1}$.

2.2.4. Determination of length, FM, and DM of aerial parts and roots of seedlings

The determination of length, FM and DM of aerial part and roots of seedlings was carried out. For that, at 21 days of germination, four biological replicates of 10 normal seedlings each, were selected from each treatment. The length, FM and DM were obtained from aerial parts and roots separated. The length (cm) was analyzed using a ruler.

FM (mg) was obtained measuring the weight. Then, aerial part and root, separated, were placed in paper bags and dried at 70°C for 72 h in a chamber with forced air circulation (Ethik). After, the DM (mg) was weighed.

2.3. Statistical analysis

All of the experiments were performed using a completely randomized design. The data were analyzed by analyses of variance (ANOVA $P < 0.05$) followed by Tukey test using the Assistat Software Version 7.7 (Silva and Azevedo, 2016).

3. RESULTS

3.1. Effect of temperature on artificial aging of seeds

The seeds were submitted to five temperatures to evaluate the effects of temperature on germination, GSI, moisture content (Fig. 1A-C), and electrical conductivity (Fig. 2).

The germination decreased significantly in seeds aged at 47 and 50ºC (Fig. 1A). At 47ºC, a reduction in the germination was observed from 48 to 96 h, reaching 56% of germination at the last time. However, at 50ºC, there was a significant reduction of germination in the first 24 h of aging and absence of germination after

72 h. Moreover, the temperatures of 41, 43 and 45ºC did not affect significantly the germination during the 96 h of seed aging (Fig. 1A).

The GSI, which allows to analyses the seed vigor, was significantly affected by the temperature, decreasing from 24 h of incubation in all temperatures tested (Fig. 1B), being a marked decrease in GSI observed in seeds aged at 47 and 50ºC compared to the other temperatures (Fig. 1B).

The moisture content increased after 24 h of aging in seeds in all temperatures (Fig. 1C). Seeds aged at 41, 43 and 45ºC did not change the moisture content from 72 h of incubation, while seeds exposed to 47 and 50ºC showed a significant increase after 72 h of aging.

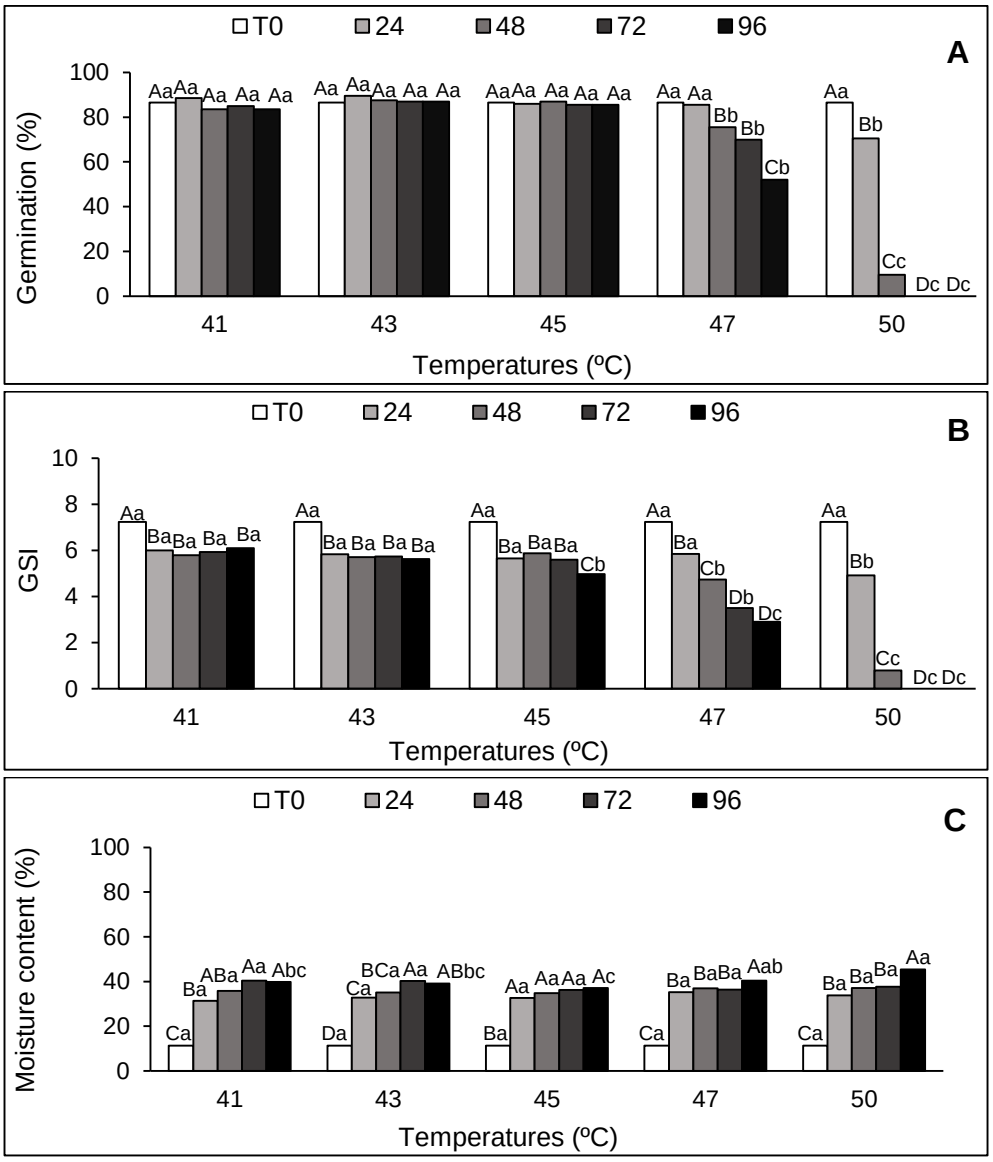

Figure 1. Effect of temperature of aging in the germination (A), GSI (B) and moisture content (C) in seeds of *C. fissilis* before (non-aged seeds, time 0) and after 24, 48, 72, and 96 h of incubation at 41, 43, 45, 47 and 50ºC. Capital letters indicate significant differences comparing the times of incubation (0, 24, 48, 72 and 96 h) at each temperature. Lowercase letters indicate significant differences comparing the temperatures (41, 43, 45, 47 and 50ºC) at each time of incubation. CV = Coefficient of variation (n = 4, CV of germination = 5.31%; CV of GSI = 6.11%; CV of moisture content = 7.38%).

The electrical conductivity was performed in the aged seeds at 41 and 50ºC (Fig. 2), comparing the temperature of 41°C, which did not affect the seed germination, with the 50ºC, which affected significantly the germination (Fig. 1A). The electrical conductivity of seeds showed a significant decrease in both temperatures during first 24 h of aging, no significant differences between the temperatures until 48 h of incubation. A significant difference for electrical conductivity between two temperatures was observed at 72 and 96h, being higher to seeds incubated at 50°C (Fig. 2).

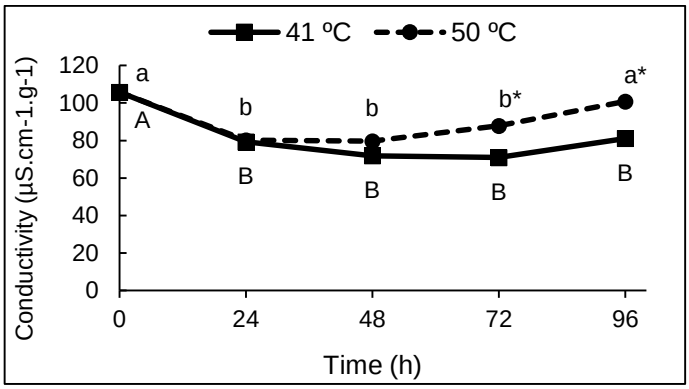

Figure 2. Electrical conductivity (µS/cm.g) in seeds of *C. fissilis* before (non-aged seeds, time 0) and after 24, 48, 72, and 96 h of incubation at 41 and 50ºC. Capital letters indicate significant differences comparing the times of incubation (h) at 41°C. Lowercase letters indicate significant differences comparing the times of incubation (h) at 50°C. The asterisks (*) denote significant differences between the temperatures (41 and 50°C) in each time (h) of incubation. CV = Coefficient of variation (n = 4, CV = 6.79%).

The growth of seedlings was also affected by the temperatures used (Tables 1 and 2). The temperatures and time of incubation affected significantly the length of aerial part and roots of seedlings (Table 1). The length of aerial part reduced significantly during the first 24 hours of seed incubation, except at 43°C where the reduction was observed at 96 h. The greatest reduction of aerial part of seedlings was observed when the seeds were aged at 47 and 50°C. On the other hand, the root length was the most affected parameter analysed, reducing significantly in the first 24 h under all temperatures tested, being the greater reduction observed in the seeds aged at 47 and 50°C.

Table 1. Effect of temperatures in the length of aerial part and root of *C. fissilis* seedlings obtained from seeds before (non-aged seeds, time 0), and after 24, 48, 72 and 96 of incubation at different temperatures.

Seedling Part	Temperature	Incubation (h)				
		0	24	48	72	96
Aerial	41	8.2 Aa	7.3 Ba	7.4 Ba	7.6 Aba	7.6 Aba
	43	8.2 Aa	7.6 Aba	7.8 Aba	7.9 Aa	7.2 Ba
	45	8.2 Aa	7.4 Ba	7.5 Ba	7.4 Bab	7.3 Ba
	47	8.2 Aa	7.4 Ba	7.3 Ba	6.8 BCb	6.5 Cb
	50	8.2 Aa	5.9 Bb	4.0 Cb	0.0 Dc	0.0 Dc
Roots	41	8.5 Aa	7.6 Ba	7.7 Ba	7.4 Ba	7.3 Ba
	43	8.5 Aa	7.4 Bab	7.0 Bab	6.8 Bab	6.7 Bab
	45	8.5 Aa	6.7 Bbc	6.7 Bb	6.6 Bb	6.5 Bb
	47	8.5 Aa	6.6 Bc	6.6 Bb	6.5 Bb	5.7 Cc
	50	8.5 Aa	4.7 Bd	3.8 Cc	0.0 Dc	0.0 Dd

**Capital letters* indicate significant differences between aging times (0, 24, 48, 72 and 96 h) in each temperature (41, 43, 45, 47 and 50 °C). *Lowercase letters* indicate significant differences between temperatures (41, 43, 45, 47 and 50 °C) at each time (0, 24, 48, 72 and 96 h) of incubation. CV = coefficient of variation. (n = 4, CV length of aerial part = 4.74%; CV root length = 6.03%).

Similarly, the FM and DM of the aerial part and root of seedlings decrased from 24 h of incubation at all temperatures tested (Table 2). Artificial aging of the seeds at 47 and 50ºC presented a progressive decrease in FM and DM of the aerial part and root of seedlings. We observed a greater decrease in these parameters when compared to the temperatures in each time analyzed, mainly at 47 and 50ºC (Table).

Table 2. Effect of temperatures in the FM and DM (mg) of aerial part and root of *C. fissilis* seedlings obtained from seeds before (non-aged seeds, time 0), and after 24, 48, 72 and 96 of incubation at different temperatures.

Temperatures	Incubation (h)				
	0	24	48	72	96
Fresh matter of aerial part (mg)					
41	2805.1 Aa	1182.3 Ba	1190.4 Ba	1201.6 Ba	1172.1 Ba
43	2805.1 Aa	1154.1 Ba	1168.0 Ba	1144.1 Ba	1158.8 Ba
45	2805.1 Aa	1177.8 Ba	1151.1 Ba	1133.2 Ba	1050.0 Bab
47	2805.1 Aa	1095.2 Ba	867.5 BCb	823.1 Cb	837.3 Cb
50	2805.1 Aa	568.0 Bb	200.6 Cc	0.0 Cc	0.0 Cc
Dry matter of aerial part (mg)					
41	184.6 Aa	91.0 Ba	89.8 Ba	90.1 Ba	85.4 Ba
43	184.6 Aa	82.8 Ba	82.8 Bab	83.8 Ba	86.2 Ba
45	184.6 Aa	84.7 Ba	84.0 Bab	80.7 Ba	77.8 Ba
47	184.6 Aa	80.8 Ba	65.2 Bb	67.2 Ba	74.8 Ba
50	184.6 Aa	49.4 Bb	64.6 Bb	0.0 Cb	0.0 Cb
Fresh matter of root (mg)					
41	573.2 Aa	273.7 Ba	232.8 Ba	236.7 Ba	204.3 Ba
43	573.2 Aa	232.8 Bab	174.9 Bab	202.1 Bab	193.7 Ba
45	573.2 Aa	196.2 Bbc	178.3 Bab	186.3 Bb	161.1 Ba
47	573.2 Aa	153.1 Bc	131.3 Bb	135.9 Bc	51.9 Cb
50	573.2 Aa	54.3 Bd	38.0 Bc	0.0 Cd	0.0 Cc
Dry matter of root (mg)					
41	48.1 Aa	27.7 Ba	26.2 Ba	25.7 Ba	21.7 Ba
43	48.1 Aa	21.3 Bab	22.9 Bab	21.0 Bab	21.4 Ba
45	48.1 Aa	16.9 Bb	16.1 Bbc	18.2 Bab	16.1 Ba
47	48.1 Aa	15.9 Bb	16.3 Bbc	14.6 Bb	14.5 Ba
50	48.1 Aa	15.0 Bb	10.6 Bc	0.0 Cc	0.0 Cb

**Capital letters* indicate significant differences between aging time (0, 24, 48, 72 and 96 h) in each temperature (41, 43, 45, 47 and 50 °C). *Lowercase letters* indicate significant differences between temperatures (41, 43, 45, 47 and 50 °C) at each time (0, 24, 48, 72 and 96 h) of incubation. CV = coefficient of variation; FM = fresh matter; DM = dry matter. (n = 4, CV fresh matter of aerial part = 8.94%; CV dry matter of aerial part = 13.08%; CV fresh matter of root = 30.53%; CV dry matter of root = 19.44%).

4. DISCUSSION

Humidity and temperature are the two most important factors that determine the rate of seed deterioration (Abass and Shaheed, 2012). In *C. fissilis* the

germination and vigor declined with increased aging periods and temperatures. Under the artificial aging condition employed in this study, the loss of viability and GSI of seeds occurred mainly at 47 and 50ºC (Figs. 1A-B). According to Guedes et al. (2011) is probably due to seed deterioration when subjected to high temperatures and high humidity conditions. In *Dalbergia nigra* seeds, these authors used temperatures of 41 and 45ºC and observed a significant reduction in the viability and vigor of seeds aged at 45ºC, with 8% germination after 96 h, affecting the physiological quality (Guedes et al., 2011). In addition, Borges et al. (1990) aged *C. fissilis* seeds at 40 and 50°C, observing that seeds exposed to 40ºC showed an increase of germination, while at 50ºC reduced the germination in the first 24 h of incubation.

The electrical conductivity analyzed from the seeds aged at 41 and 50ºC showed significant differences in 72 and 96 h of incubation, being higher in seeds kept at 50°C, when they loss the viability and no germination was observed (Fig. 2). An increase in the release of ions in seeds aged at 40 and 45ºC was related with membranes degradation and viability reduction in *M. brauna* (Corte et al.,2010) . In the present work, a significant increase on the conductivity values from seeds incubated at 72 h to 50ºC (Fig. 2), when occurs the absence of germination compared to 41°C, suggests that release of ions in *C. fissilis* could occurs in a slow way. These data are similar to those observed by Corvello et al. (1999) in *C. fissilis* seeds stored for 12 months. These authors suggested that the electrical conductivity can not show efficiently the differences in the physiological quality found in the seed germination and emergence speed index, and the limitation can be attributed to the difficulties in the process of imbibition winged-seeds during the incubation period in *C. fissilis* seeds.

Another parameter affected by the temperatures used was the length of seedlings (Tables 1 and 2). The higher temperatures tested, as 47 and especially at 50°C, affected significantly the growth of seedlings, especially the roots part (Tables 1). Marcos Filho (2015) reported that the seed aging reduces the GSI of viable seeds, as well as decrease the size of seedling. In aged *Triticum aestivum* seeds, Maia et al. (2007) observed a reduced germination at 43ºC after 96 h, besides without significant differences in the length of the primary root. Aged seeds of *Tabernaemontana fuchsiaefolia* at 45ºC showed a significant higher length of root and no significant differences on the growth for aerial part compared to seeds at

41°C (Moraes et al., 2016). In addition, the length of aerial part and root of *Phaseolus aureus* seedlings decreased progressively after aging at 45°C compared to those non-aged seeds (Abass and Shaheed, 2012). In this sense, the evaluation of seedling development submitted to artificial aging must take into account the intrinsic factors such as, differences between species, vigor, seed moisture, conditions of the mother plant and place of seeds production (Negreiros and Perez, 2004).

In addition to the length of *C. fissilis* seedlings, the FM and DM of aerial part and root was affected by the incubation at higher temperatures (47 and 50°C) (Table 2). Seeds of *T. fuchsiaefolia* aged at 41, 43 and 45°C showed a decrease in the vigor with the increase in the temperature and aging time, being the highest DM of seedlings observed in seeds aged at 41°C, which showed the higher vigor compared to 45°C (Moraes et al., 2016). In agreement, *T. aestivum* seeds with a high vigor produced seedlings with a higher DM of aerial part when compared to those from low vigor seeds (Abati et al., 2017). In this sense, the DM is a variable that quantifies seed vigor, being the seedlings with a higher DM considered with greater vigor (Gama et al., 2010). In this way, we verify the same behavior of the parameters length, FM, and DM of seedlings in *C. fissilis* being these variable similar to decrease of the germination and GSI in seeds aged mainly at 47 and 50ºC (Fig. 1; Tables 1 and 2).

Our studies have been showed that the temperatures affected significantly the germination and vigor of seeds in *C. fissilis*, especially at 47 and 50ºC. In this sense, the use of 41 and 50 °C from 48 h is suitable for studies of seed metabolism and biochemical changes due the reduction in seed germination.

5. CONCLUSIONS

The viability and vigor of seeds were significantly affected by the temperature used. The temperatures of 47 and 50ºC, from 48 and 24 h respectively, reduced significantly the germination and seedling growth. The temperature of 50ºC from 72 h of incubation induced the loss of viability in *C. fissilis* seeds. Our results contributed to understand the artificial aging on physiological aspects of seed aging, and can be

used in further studies to explore the biochemical changes during the loss of seed viability in *C. fissilis*.

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4.2. AGEING OF *Cedrela fissilis* VELLOZO (MELIACEAE) SEEDS IS ASSOCIATED WITH PROTEOMIC AND PUTRESCINE PROFILE CHANGES[11]

RESUMO

O envelhecimento das sementes é um processo inevitável, e manter a qualidade fisiológica necessária para manter a viabilidade durante o envelhecimento é um desafio, especialmente em espécies madeireiras em extinção, como *Cedrela fissilis* Vellozo (Meliaceae). Aqui, utilizamos uma abordagem proteômica comparativa e análises do conteúdo de poliaminas (PAs) para estudar os efeitos da temperatura na germinação e viabilidade das sementes de *C. fissilis* durante o envelhecimento. As sementes artificialmente envelhecidas a 41 e 50ºC em diferentes tempos (0 [sementes não-envelhecidas], 24, 48, 72 e 96 h) foram utilizadas para análise de germinação (%), índice de velocidade de germinação, abundância diferencial de proteínas e conteúdo de PAs. As sementes envelhecidas a 50ºC exibiram uma germinação significativamente reduzida e alteração na abundância de proteínas em comparação com às envelhecidas a 41ºC e as não-envelhecidas. Um total de 309 proteínas foram identificadas, sendo 16, 27 e 47 reguladas diferencialmente nas comparações 41ºC/não-envelhecidas, 50ºC/não-envelhecidas e 50ºC/41ºC,

[1] Os dados deste capítulo foram submetidos para publicação:
Sousa, KR; Douétts-Peres, JC; Reis, RS; Passamani, LZ; Vettorazzi, RG; Vieira, HD; Silveira, V; Santa-Catarina, C. (2018). Ageing of *Cedrela fissilis* Vellozo (Meliaceae) seeds is associated with proteomic and putrescine profile changes. Seed Science Research (Submetido).

respectivamente. A redução significativa na abundância da proteína amina oxidase primária, que oxida preferencialmente as diaminas, em sementes envelhecidas a 50ºC, foi potencialmente relacionada ao acúmulo de Put livre, a qual foi relacionada a danos celulares, o que afeta a viabilidade das sementes. Nossas descobertas destacam as novas alterações bioquímicas durante o envelhecimento das sementes de *C. fissilis* e podem ser úteis em futuros estudos sobre a longevidade da semente e a conservação do germoplasma.

ABSTRACT

Seed ageing is an inevitable process, and retaining the physiological qualities necessary for maintaining seed viability during ageing is challenging, especially in endangered wood species, such as *Cedrela fissilis* Vellozo (Meliaceae). Herein, we used a comparative proteomics approach and polyamine (PA) content analyses to study the effects of temperature on the germination and viability of ageing *C. fissilis* seeds. Seeds artificially aged at 41 and 50ºC at different times (0 [non-aged seeds], 24, 48, 72 and 96 h) were sampled for analyses of their germination abilities (%), germination speed index, differential protein abundances and PA contents. Seeds aged at 50ºC exhibited significantly reduced germination and altered protein abundances compared to those of seeds aged at 41ºC and non-aged seeds. A total of 309 proteins were identified, with 16, 27 and 47 being differentially regulated in the 41ºC/non-aged, 50ºC/non-aged and 50ºC/41ºC comparisons, respectively. The reduced abundance of the primary amine oxidase protein, which preferentially oxidizes diamines, in seeds aged at 50°C was potentially related to the accumulation of free Put content, which have been related to cellular damage, and can affects seed viability. Our findings highlight new biochemical alterations during the ageing of *C. fissilis* seeds and may be useful in future studies on seed longevity and germplasm conservation.

1. INTRODUCTION

Seed ageing results in the reduction of germination and induction of biochemical alterations during seed storage, sometimes inducing a total loss of seed viability and vigor (McDonald, 1999). Some aspects of physiological seed qualities have previously been studied. Seeds are subjected to several degenerative changes after maturation, e.g., high temperature and humidity (100%), which may be used as an artificial ageing procedure to study seed metabolic changes (Marcos Filho, 2015). This procedure simulates normal ageing conditions, stimulating an increase in metabolic processes (Marcos Filho, 2015), thus allowing the ageing processes to be monitored in a short period compared to conventional storage, which may require many years.

Studies in several species, such as *Arabidopsis thaliana*, *Vigna mungo*, *Oryza sativa,* and *Cariniana legalis* (Rajjou et al., 2008; Sathish et al., 2015; Sousa et al., 2016; Yin et al., 2017), have investigated the biochemical and physiological changes related to seed deterioration during either artificial or conventional seed storage ageing.

Proteomics approaches have been applied to identify differentially abundant proteins during the seed ageing of some species, and their correlations were related to seed deterioration and loss of viability (Sathish et al., 2015) as well as seed vigor (Zhang et al., 2015). The loss of seed vigor was related to protein changes in mature seeds and to an inability of low-vigor seeds to display a normal proteome during germination in *A. thaliana* (Rajjou et al., 2008). These studies have contributed to better understanding the biochemical changes related to the long conservation period and the seed deterioration process.

Recently, high levels of the polyamine (PA) putrescine (Put) were associated with seed viability and vigor loss in *C. legalis* (Sousa et al., 2016). PAs are low-molecular weight polycationic aliphatic amines that are found in the cells of all living organisms (Kusano et al., 2008). Due to their positive charges, PAs bind macromolecules, such as DNA, RNA, proteins, and phospholipids (Kusano et al., 2008; Moschou et al., 2008), and play important regulatory roles in plant growth and development, including seed development and germination (Astarita et al., 2003; Santa-Catarina et al., 2006; Shu et al., 2012; Yin et al., 2014; Rios et al., 2015).

Studies on seed ageing could improve our knowledge of and provide new insights into understanding seed longevity, an important trait in both ecological and agronomical contexts (Chen et al., 2016). A few studies have determined that seed ageing in tropical tree species, such as *Cedrela fissilis* Vellozo (Meliaceae), a native species in the Brazilian Atlantic Rain Forest, have ecological and economic importance. This species was included in the endangered category (IUCN, 2017) of the red list of endangered species established by the International Union for Conservation of Nature (IUCN). In addition to *C. fissilis* seeds being classified as orthodox (Carvalho et al., 2006), its seeds lost their vigor after storage, with significant reductions in the emergency speed index being observed after 12 months at 4°C (Sousa et al., 2016).

In the present study, we used a comparative proteomics approach and PA content analyses to study the effects of temperature on seed viability maintenance during *C. fissilis* seed ageing.

2. MATERIAL AND METHODS

2.1. Plant material

Mature seeds, collected in August 2014, were provided by Caiçara nursery, located in Brejo Alegre, São Paulo, Brazil (21º10'S and 50º10'W).

2.2. Effects of temperature on artificial seed ageing

For artificial ageing, the effects of temperature on seed germination and seed vigor were tested. Seeds were placed on wire mesh screens and suspended over 40 ml of water inside plastic boxes (11 x 11 x 35 cm). The plastic boxes were then incubated in a biochemical oxygen demand (BOD)-type germination chamber (Eletrolab, São Paulo, Brazil) at 41, 43, 45, 47, and 50ºC for 24, 48, 72, and 96 h under ~100% relative humidity. Non-aged seeds, i.e., seeds before the start of the artificial ageing experiment, was used as time 0 of the ageing temperature treatment. Four biological replicates (50 seeds each) were used for each ageing temperature and incubation time condition.

Samples from non-aged seeds and seeds aged at 41 and 50ºC for 24, 48, 72 and 96 h of incubation were analysed for their moisture content, seed germination (%), germination speed index (GSI), and PAs content. Proteomics analysis was performed on non-aged seeds and seeds aged at 41 and 50ºC after 48 h of incubation.

2.3. Analyses of seed germination, GSI, and moisture content

The germination test for seed viability was conducted according to a protocol provided by Brasil (2013). Four biological replicates (50 seeds each) from each treatment were distributed on Germitest® paper sheets (J ProLab, São José dos Pinhais, Brazil) and then incubated in a BOD-type germination chamber at 25ºC with a photoperiod of 8 h light/16 h dark, at 40 µmol m^{-2} s^{-1}. Germination was recorded daily for 21 days, and the GSI was determined from the daily analysis according to Maguire (1962).

Seed moisture content was determined according to a method established by Brasil (2009). Four biological replicates (2 g fresh matter [FM] from each) of seeds from each treatment were weighed and then dried in a 105°C chamber with forced air circulation (Ethik technology, São Paulo, Brazil) for 24 h. The results were expressed as the percentage of FM according to the following formula: Seed moisture content = (water content/FM) x 100.

2.4. Protein extraction and digestion

Proteomics analysis was performed on non-aged seeds and seeds aged at 41 and 50ºC for 48 h. Three biological replicates (100 mg FM from each) from each treatment were pulverized using a mortar and pestle in liquid nitrogen on ice and macerated with extraction buffer comprising 20 mM Tris-HCl (GE Healthcare, Little Chalfont, UK) pH 6.8, 1% dithiothreitol (DTT; GE Healthcare), 0,1% sodium dodecyl sulfate (SDS; GE Healthcare) and 1 mM phenylmethanesulfonyl fluoride (PMSF; Sigma-Aldrich, St. Louis, USA). Samples were agitated for 30 min and then centrifuged at 16,000 *g* for 10 min at 4ºC. The supernatants were collected, and protein concentrations were measured using the 2-D Quant Kit (GE Healthcare).

For protein digestion, three biological replicates, each containing 100 µg of protein, were used for each treatment. Before digestion, proteins were precipitated with methanol:chloroform to remove any detergent from the samples (Nanjo et al.,

2011). Then, the samples were resuspended in 7 M urea (GE Healthcare) and 2 M thiourea (GE Healthcare) buffer and desalted on Amicon Ultra-0.5 3 kDa centrifugal filters (Merck Millipore, Darmstadt, Germany). The filters were filled to maximum capacity with buffers and centrifuged at 15,000 *g* for 10 min at 20°C. The samples were washed twice with 8 M urea and then twice with 50 mM ammonium bicarbonate (Sigma-Aldrich) pH 8.5, leaving approximately 50 µl per sample after the last wash.

The protein digestion was performed according to methodology described by Calderan-Rodrigues et al. (2014) with modifications. For each 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® (Eppendorf, Hamburg, Germany) at 80°C for 15 min. Then, 2.5 µl of 100 mM DTT was added, and the samples were vortexed and incubated at 60°C for 30 min under agitation (350 rpm). Next, 2.5 µl of 300 mM iodoacetamide (GE Healthcare) was added, and the samples were vortexed and then incubated in the dark for 30 min at room temperature. Then, 5 µl of 100 mM DTT was added to quench excess iodoacetamide. For protein digestion, 20 µl of trypsin solution (50 ng μl^{-1}; V5111, Promega, Madison, USA) prepared in 50 mM ammonium bicarbonate was added, and the mixture was incubated at 37°C for 15 h. For RapiGest® precipitation and trypsin activity inhibition, 10 µl of 5% (v/v) trifluoroacetic acid (TFA; Sigma-Aldrich) was added, and the mixture was incubated at 37°C for 30 min and then centrifuged for 20 min at 16,000 *g*. Samples were transferred to Total Recovery Vials (Waters) for mass spectrometry analysis.

2.4.1. Mass spectrometry analysis

A nanoAcquity UPLC connected to a Synapt G2-Si HDMSE mass spectrometer (Waters) was used for ESI-LC-MS/MS analysis according to the protocol provided by Reis et al. (2016) with modifications. First, to normalize the relative protein quantifications, a chromatography step was performed by loading 500 ng of the digested samples. To ensure standardized molar values for all conditions, normalization among samples was based on stoichiometric measurements of the total ion counts of MS^E scouting runs prior to the analyses using the ProteinLynx Global SERVER v. 3.0 programme (PLGS; Waters). After sample normalization, the $HDMS^E$ runs consisted of three biological replicates for each treatment. During separation, samples were loaded onto the nanoAcquity

UPLC 5 µm C18 trap column (180 µm x 20 mm; Waters) at 5 µl min^{-1} for 3 min and then onto the nanoAcquity HSS T3 1.8 µm analytical reversed phase column (75 µm x 150 mm; Waters) at 400 nl min^{-1}, with a column temperature of 45°C. For peptide elution, a binary gradient was used; mobile phase A consisted of water (Tedia, Fairfield, USA) and 0.1% formic acid (Sigma-Aldrich), and mobile phase B consisted of acetonitrile (Sigma-Aldrich) and 0.1% formic acid. Gradient elution was performed sequentially as follows: 7% B, ramped from 7 to 40% B until 91.12 min, from 40 to 99.9% B until 92.72 min, maintained at 99.9% B until 106 min, decreased to 7% B until 106.1 min and maintained at 7% B until the end of the gradient at 120 min. Mass spectrometry was performed in positive and resolution mode (V mode), 35,000 FWHM, with ion mobility (IMS) ($HDMS^E$), and in data-independent acquisition (DIA) mode. IMS was performed using wave velocity of 600 m s^{-1}, and helium and IMS gas flow of 180 and 90 ml min^{-1}, respectively. The transfer collision energy ramped from 19 to 55 V in high-energy mode, with cone and capillary voltages of 30 and 2750 V, respectively, and a source temperature of 70°C. Regarding 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 as an external standard, and lock mass acquisition was performed every 30 s. Mass spectra acquisition was carried out for 90 min using MassLynx v4.0 software.

2.4.2. Proteomics data analysis

Spectra processing and database search conditions were performed using Progenesis QI for Proteomics software V.2.0 (Nonlinear Dynamics, Newcastle, UK). The analysis utilized the following parameters: Apex3D of 150 counts for low-energy threshold, 50 counts for elevated-energy threshold, and 750 counts for intense threshold; one missed cleavage; minimum fragment ions per peptide equal to two; minimum fragment ions per protein equal to five; minimum peptides per protein equal to two; fixed modifications of carbamidomethyl (C) and variable modifications of oxidation (M) and phosphoryl (STY); default false discovery rate of 1% maximum; peptide score greater than four; and maximum mass errors of 10 ppm. Label-free relative quantitative analyses were performed based on the ratio of protein ion counts among contrasting samples. For protein identification, we used the *Cedrela sinensis* Expressed Sequence Tag (EST) database generated by *de novo*

transcriptome assembly using Trinity (Robertson et al., 2010) compared against the NCBI EST sequence read archive (SRA; SRX096110; downloaded in December 12nd, 2018). To identify a higher number of proteins, MS data were also processed against the orange (*Citrus sinensis*) protein database (UniProt), the species most closely related to *C. fissilis* with a fully sequenced genome. To avoid redundancy, orange proteins showing at least one peptide unique from those found in the EST database analyses were selected for additional analysis. To ensure the quality of the results after data processing, only proteins present in the three runs were accepted and subjected to differential abundance analysis. Proteins were deemed up-regulated if the $\log_2$ value of the fold change (FC) was greater than 1 and deemed down-regulated if the $\log_2$ value of the FC was less than -1, determined using Student's T-Test (two-tailed; $P < 0.05$). The heatmap was created using the means of differentially abundant proteins with the Heatmapper tool (Babicki et al., 2016). Functional annotations were performed using Blast2Go software v.4.1 (Conesa et al., 2005).

2.5. Analysis of free polyamines (PAs)

PA determinations were performed according to a protocol established by Santa-Catarina et al. (2006). Three biological replicates (200 mg FM each) of seeds from each treatment were ground in 1.3 ml of 5% (v/v) perchloric acid (Merck, Darmstadt, Germany) and incubated at 4 °C for 1 h. The samples were then centrifuged at 20,000 *g* for 20 min at 4°C. Diaminoheptane (DAH; Sigma-Aldrich) was used as the internal standard, and PAs were determined directly from the supernatant by derivatization with dansyl chloride (Merck) and identified by HPLC using a 5 µm C18 reverse phase column (Shimadzu Shin-pack CLC ODS) at 1 ml min^{-1} and a column temperature of 40°C. Mobile phase A consisted of 10% acetonitrile in water (pH 3.5), and mobile phase B comprised absolute acetonitrile. The binary gradient began at 65% B for 10 min, ramped to 100% B until 13 min, and was then maintained at 100% B until 21 min. PA concentrations were determined by fluorescence detected at 340 nm (excitation) and 510 nm (emission). Peak areas and retention times were measured by comparison with the PA standards (Sigma-Aldrich) Put, Spd, and Spm.

2.6 Statistical analysis

The experimental design was completely randomized. Analysis of variance (ANOVA $P < 0.05$) followed by Tukey's test were performed using Assistat software Version 7.7 (Silva and Azevedo, 2016).

3. RESULTS

3.1. Effects of artificial ageing temperatures on seed germination, GSI, and moisture content

Among the temperatures tested (41, 43, 45, 47 and 50ºC), 50°C affected germination more significantly than the others, at 48 h of incubation (Fig. 1). Thus, the artificial ageing temperatures 41 and 50ºC were selected for the analyses of germination, GSI, moisture content, proteomics and PAs. Treatment at 41ºC treatment did not affect seed germination, whereas the 50ºC was the temperature that showed the greatest effect on the germination reduction (Fig. 1).

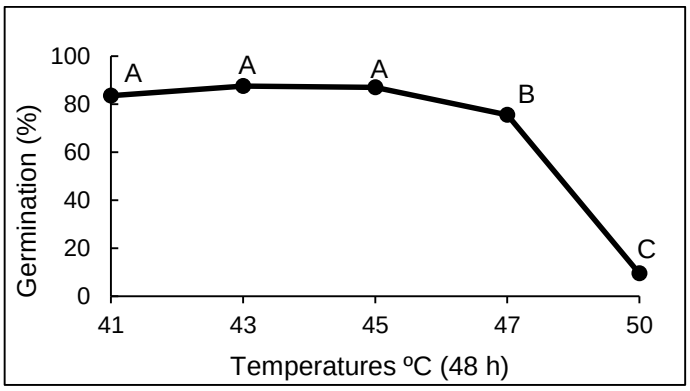

Figure 1. Effects of temperature (41, 43, 45, 47, and 50ºC) on the germination (%) of *C. fissilis* seeds after 48 h of ageing. The results are expressed as the means of four biological replicates. Different letters indicate significant differences ($P < 0.05$) according to Tukey's test (n = 4; coefficient of variation: 5.11%).

Comparing the effects of 41 and 50ºC treatment, the germination (Fig. 2A) and GSI (Fig. 2B) in seeds aged at 50ºC decreased significantly. The reductions in germination and vigor in seeds exposed to 50ºC began at 24 h (Fig. 2A and B), with the lowest seed germination, being observed at 48 h (15%), while no germination was observed at 72 h (Fig. 2A). On the other hand, the moisture contents of the

seeds were significantly increased between 24 and 96 h of exposure at both temperatures (41 and 50°C) (Fig. 2C).

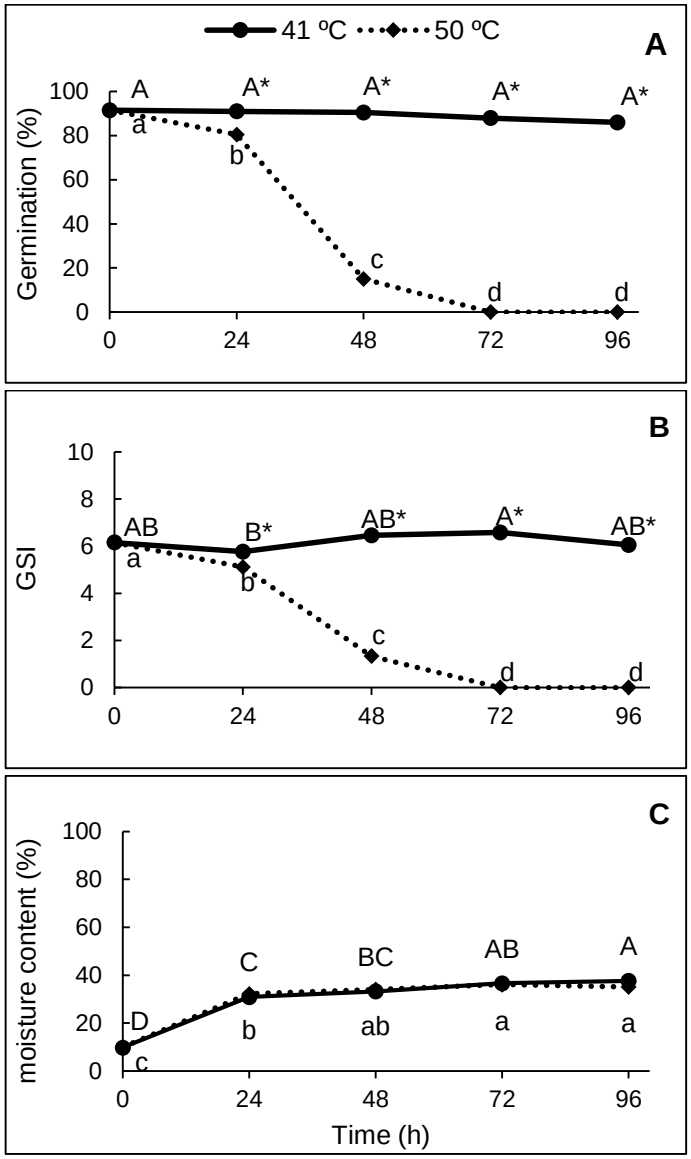

Figure 2. Changes in the (A) germination, (B) GSI, and (C) moisture contents of non-aged seeds (i.e., seeds before the start of the experiment, time 0) and *C. fissilis* seeds after 24, 48, 72, and 96 h of incubation at 41 and 50ºC. Different letters indicate significant differences ($P \leq 0.05$) according to Tukey's test. Capital letters indicate significant differences during the times (0, 24, 48, 72, and 96 h) of seed ageing at 41°C. Lowercase letters indicate significant differences during the times (0, 24, 48, 72, and 96 h) of seed ageing at 50°C. Asterisks (*) indicate significant differences between the temperatures (41 and 50°C) at each incubation time. The results are expressed as the means of four biological replicates. CV = coefficient of variation (n = 4, CV of germination = 4.27**%**; CV of GSI = 8.46%; CV of moisture content = 4.44%).

3.2. Effects of artificial ageing temperatures on the proteomic profile

Proteomics analysis was performed using non-aged seeds (i.e., time 0), seeds aged at 41ºC for 48 h, when the germination percentage was equal to that of

non-aged seeds, and with seeds aged at 50ºC for 48 h, which significantly decreased the germination (Fig. 2A).

In this study, 309 proteins were identified, among which 68 showed differential abundance in at least one comparison between treatments. Among these proteins, comparing the seeds aged at 41°C to non-aged seeds (41ºC/non-aged), 16 proteins were differentially abundant, with six being down-regulated and 10 being up-regulated. When comparing seeds aged at 50ºC with non-aged seeds (50ºC/non-aged), 27 differentially abundant proteins were identified, with 17 being down-regulated and 10 being up-regulated. Comparing seeds aged at 50°C to those aged at 41°C (50ºC/41ºC), a total of 47 differentially abundant proteins were identified, with 24 being down-regulated and 23 being up-regulated (Fig. 3).

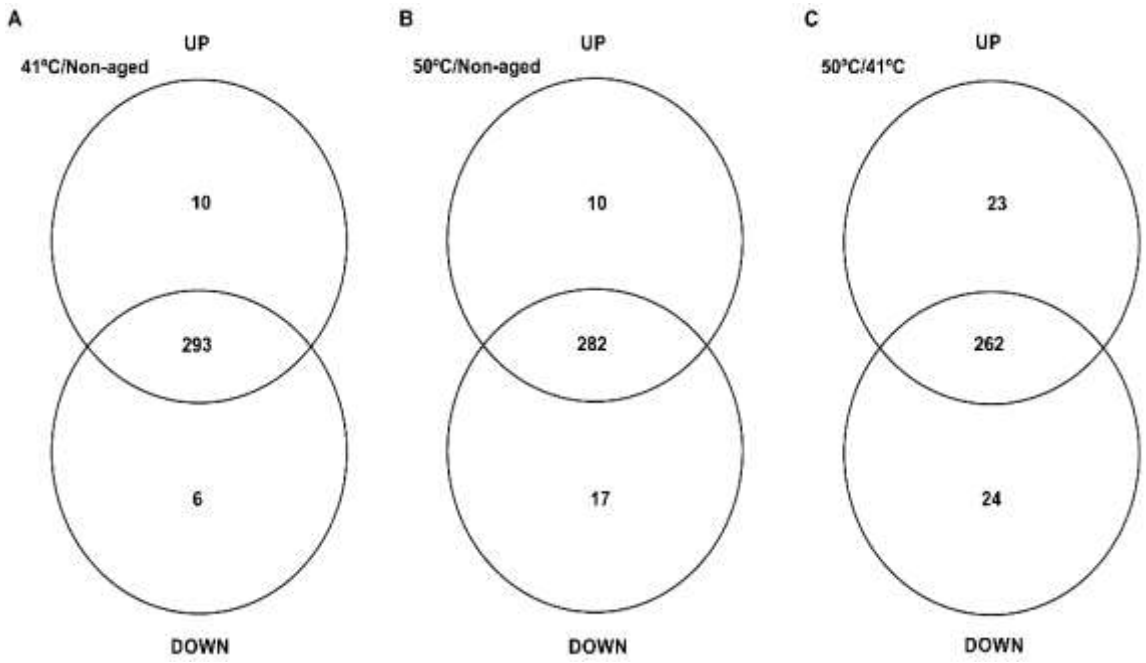

Figure 3. Venn diagram showing the up- and down-regulated proteins in (A) 41°C/non-aged seeds, (B) 50°C/non-aged and (C) 50°C/41°C comparisons of ageing in *C. fissilis* seeds.

Between the regulated proteins, nine were down-regulated and two were up-regulated in both comparisons (50ºC/41ºC and 50ºC/non-aged) and were thus candidate proteins potentially related to the loss of seed viability induced by temperature (Table 1).

Table 1. Regulated proteins in ageing *C. fissilis* seeds highlighted in both comparisons (50ºC/41ºC and 50ºC/non-aged) and other proteins previously associated with the loss of seed viability. Proteins were considered up-regulated if their log_2 fold change (FC) value was greater than 1 and down-regulated if their log_2 FC value was less than -1, as determined by Student's T-Test (two-tailed; $P < 0.05$).

Accession Protein	Description	Peptide count	Score	Average Normalized Total Ion Count (Tic)			Differential abundance		
				non-aged	41ºC	50ºC	41ºC x non-aged	50ºC x non-aged	50ºC x 41ºC
A0A067FEE9	Alcohol dehydrogenase class-3	4	29.5	6675.5	11955.8	381.9	UNCHANGED	DOWN	DOWN
DN22588_c0_g2_i4.p1	Alpha-beta hydrolase superfamily	7	47.1	68235.2	62104.0	23110.8	UNCHANGED	DOWN	DOWN
DN19662_c0_g1_i1.p1	Annexin D1	4	30.1	59283.6	35508.1	15968.2	UNCHANGED	DOWN	DOWN
DN19662_c0_g3_i2.p1	Annexin D1	9	62.9	53850.2	22746.5	14802.3	UNCHANGED	DOWN	UNCHANGED
DN20799_c0_g3_i3.p1	Calreticulin	13	125.4	39685.1	57880.3	2367.6	UNCHANGED	DOWN	DOWN
DN21465_c0_g1_i8.p1	Disulfide isomerase	9	73.7	150662.9	166248.0	57461.2	UNCHANGED	UNCHANGED	DOWN
DN21465_c0_g2_i3.p1	Disulfide-isomerase-like	18	152.0	241209.6	218440.7	71157.8	UNCHANGED	DOWN	DOWN
DN21465_c0_g1_i4.p1	Disulfide-isomerase-like	4	34.1	811626.6	941030.0	364224.9	UNCHANGED	UNCHANGED	DOWN
DN22957_c0_g1_i3.p1	Elongation factor 2	3	18.1	3943.1	3294.6	143.6	UNCHANGED	DOWN	DOWN
A0A067EN97	Heat shock 70 kda	15	96.8	683.4	486.9	1263.7	UNCHANGED	UNCHANGED	UP
A0A067DI64	Heat shock cognate 70 kda 2-like	11	65.9	183.3	322.5	819.2	UNCHANGED	UNCHANGED	UP
DN18982_c0_g2_i1.p1	Nutrient reservoir	2	12.3	43085.2	26941.1	101380.2	UNCHANGED	UP	UP
DN19808_c0_g1_i3.p1	Osmotin	3	26.0	33949.7	13272.9	3651.7	UNCHANGED	DOWN	DOWN
DN23578_c0_g5_i2.p1	Primary amine oxidase-like	5	41.3	32612.1	24626.8	12040.5	UNCHANGED	DOWN	DOWN
DN20196_c0_g1_i3.p1	Probable ribosome-binding factor chloroplastic	2	18.9	5957.9	1681.8	27294.3	UNCHANGED	UP	UP
A0A067H0B5	Serpin-ZX-like	2	11.2	38094.3	23992.0	69080.3	UNCHANGED	UNCHANGED	UP
DN23331_c2_g1_i6.p1	Stromal 70 kda heat shock-related chloroplastic	6	42.8	84410.4	26008.9	76728.8	UNCHANGED	UNCHANGED	UP
DN19867_c1_g2_i4.p1	Triosephosphate isomerase cytosolic	13	110.5	48345.5	23547.2	8471.9	UNCHANGED	DOWN	DOWN

The 50ºC treatment showed a different pattern of down- (in red) and up-regulated (in green) proteins compared to those of non-aged seeds and seeds aged at 41°C, which both presented higher germination (Fig. 4). The heatmap represented demonstrates that the regulated proteins identified in seeds from the non-aged and 41°C treatments shared more similarities than those in seeds aged at 50°C (Fig. 4).

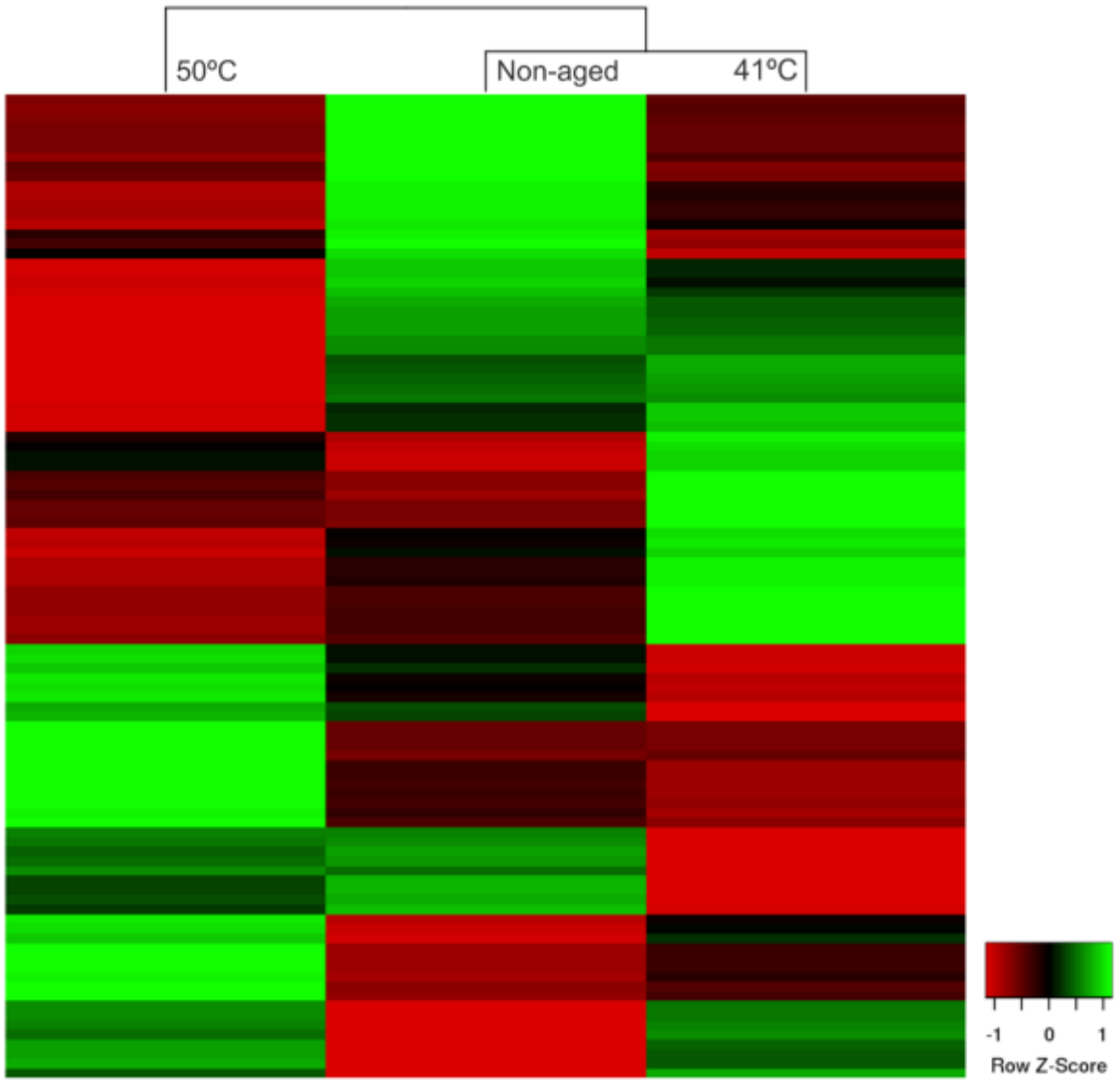

Figure 4. Heatmap of differentially abundant proteins in non-aged seeds (i.e., seeds before the start of the experiment) and *C. fissilis* seeds aged at 41 ºC and 50ºC for 48 h of incubation. Differences in protein abundance are reflected as differences in colour intensity in the heatmap, wherein up-regulated proteins (log_2 FC > 1) are indicated in green, and down-regulated proteins (log_2 FC < - 1) are indicated in red. Differences were considered statistically significant when $P < 0.05$ (ANOVA).

Some differentially regulated proteins, such as the probable ribosome-binding factor chloroplastic (DN20196_c0_g1_i3.p1) and nutrient reservoir (DN18982_c0_g2_i1.p1), were up regulated in both comparisons (50ºC/41ºC and 50ºC/non-aged) and related to the viability loss of the aged seeds (Table 1). In addition, the serpin-ZX-like protein (A0A067H0B5) and three related heat shock proteins (HSP), heat shock 70 kDa (A0A067EN97), heat shock cognate 70 kDa 2-like (A0A067DI64) and stromal 70 kDa heat shock-related chloroplastic (DN23331_c2_g1_i6.p1), were up-regulated in seeds aged at 50ºC, which significantly reduced their germination compared to that of seeds aged at 41ºC, which maintained their germination (comparison 50ºC/41ºC) (Table 1)

On the other hand, some proteins were down-regulated in seeds aged at 50ºC, which significantly reduced their germination compared with those of both non-aged seeds (50ºC/non-aged) and seeds aged at 41ºC (50ºC/41ºC). Among these proteins, annexin D1 (DN19662_c0_g1_i1.p1), calreticulin (DN20799_c0_g3_i3.p1), osmotin (DN19808_c0_g1_i3.p1), alcohol dehydrogenase class-3 (ADHIII) (A0A067FEE9), protein disulfide-isomerase-like (PDI-like) (DN21465_c0_g2_i3.p1), triosephosphate isomerase cytosolic (TIM) (DN19867_c1_g2_i4.p1), alpha-beta hydrolase superfamily (ABH) (DN22588_c0_g2_i4.p1), elongation factor 2 (DN22957_c0_g1_i3.p1), and primary amine oxidase-like (DN23578_c0_g5_i2.p1) exhibited reduced abundances during seed ageing at 50°C (Table 1). Moreover, the abundance of the primary amine oxidase-like protein, which is related to Put catabolism, was unaltered in seeds aged at 41ºC (41ºC/non-aged), and the temperature thus did not significantly affect seed germination.

In addition, the abundance of other PDI (DN21465_c0_g1_i8.p1) and PDI-like (DN21465_c0_g1_i4.p1) proteins were down-regulated in seeds aged at 50ºC compared to those in seeds aged at 41ºC, which maintained their germination (comparison 50ºC/41ºC), and another protein, annexin D1 (DN19662_c0_g3_i2.p1), was also down-regulated in seeds aged at 50ºC compared to that in the non-aged group (50ºC/non-aged) (Table 1).

3.3. Effects of artificial ageing temperatures on endogenous free PA content

As mentioned previously, the abundance of the primary amine oxidase-like protein (DN23578_c0_g5_i2.p1) was down-regulated in seeds aged at 50°C compared to that in seeds aged at 41°C (50ºC/41ºC comparison) and non-aged seeds (50ºC/non-aged comparison) (Table 1). This protein is a copper amine oxidase that preferentially oxidizes the aliphatic diamine Put (Moller and McPherson, 1998; Ghuge et al., 2015). We thus analysed the endogenous free PA content in non-aged seeds and seeds aged at 50ºC and 41ºC for 24, 48, 72 and 96 h (Fig. 5).

As expected, the seeds aged at 50ºC exhibited significantly increased free Put content (Fig. 5A) and decreased free Spd (Fig. 5B) and Spm (Fig. 5C) contents at 96 h of incubation, when no more germination was observed. Moreover, the PA [Put.(Spd + Spm)$^{-1}$] ratio was significantly affected in seeds aged at 41°C for 24 and 48 h, whereas a significant increase was observed in seeds aged from 48 to 96 h of incubation at 50°C (Fig. 5D), indicating the relative importance of Put to total PA content.

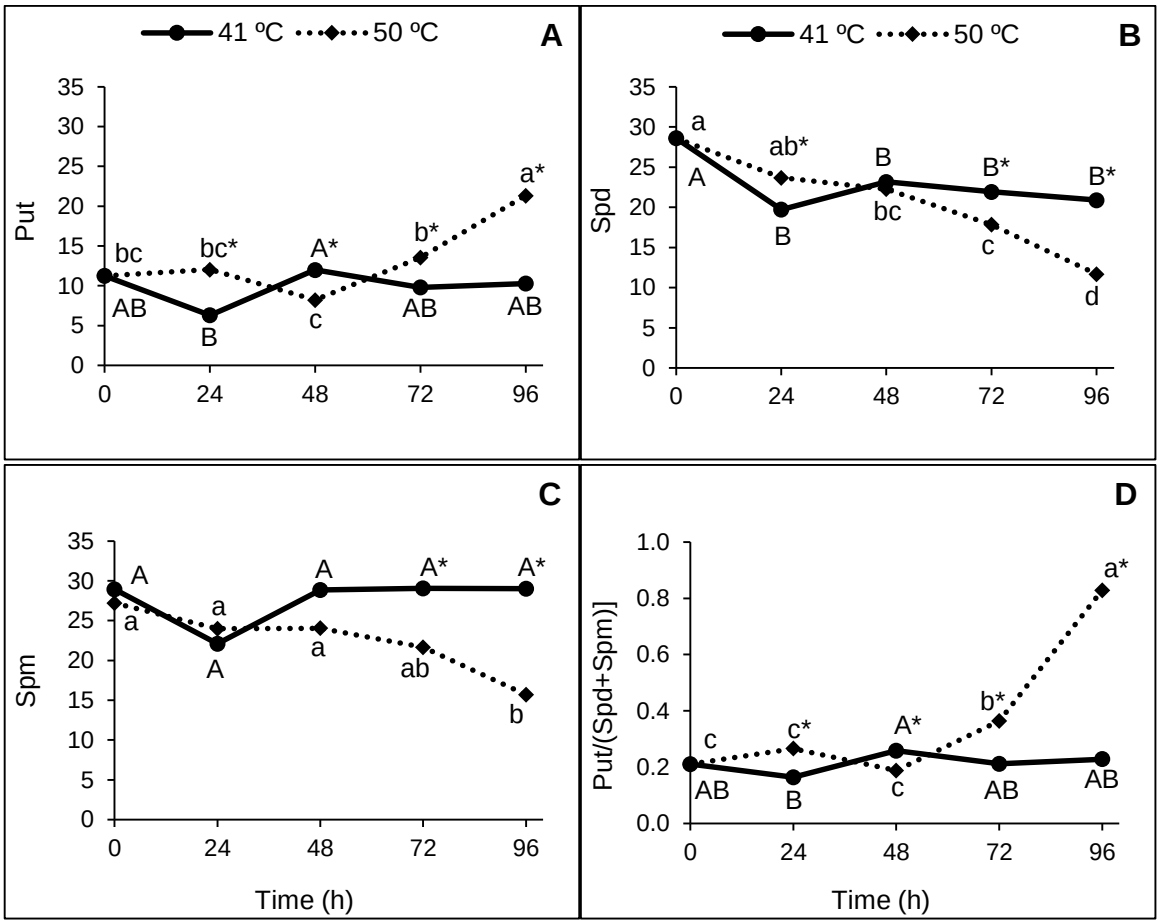

Figure 5. Contents (µg g^{-1} FM) of free (A) Put, (B) Spd, (C) Spm, and (D) PA ratio [Put.(Spd + Spm)$^{-1}$] in non-aged seeds (i.e., seeds before the start of the experiment) and *C. fissilis* seeds aged at 41ºC and 50ºC for 24, 48, 72, and 96 h of incubation. Different letters indicate significant differences ($P \leq 0.05$) according to Tukey's test. Capital letters indicate significant differences during the times (0, 24, 48, 72, and 96 h) of seed ageing at 41°C. Lowercase letters indicate significant differences during the times (0, 24, 48, 72, and 96 h) of seed ageing at 50°C. Asterisks (*) indicate significant differences between temperatures (41 and 50°C) at each incubation time. The results are expressed as the means of three biological replicates. CV = coefficient of variation (n = 3, CV of free Put = 17.95%; CV of free Spd = 9.32%; CV of free Spm = 13.67%; CV of PA ratio = 12.37%).

4. DISCUSSION

Seed ageing is a continuous process that results in decreased germination and vigor, resulting in seed viability loss (Moncaleano-Escandon et al., 2013). *C. fissilis* seeds aged at 50ºC presented significantly decreased germination, seed viability and vigor, whereas seeds treated at 41ºC showed no differences in these parameters (Fig. 2A and B). Artificial ageing is an important tool that has been used to study alterations in the physiological qualities of several species of agronomic

interest, such as *O. sativa* (Zhang et al., 2016), as well as wood species that have ecological importance, such as *Dalbergia nigra* and *Melanoxylon brauna* (Corte et al., 2010; Guedes et al., 2011). Ageing *D. nigra* seeds at 41 and 45°C for 72 h affected their physiological quality by reducing their viability and vigor (Guedes et al., 2011). In *M. brauna*, Corte et al. (2010) evaluated seeds aged naturally at 20ºC for 12 months and seeds aged artificially at 45ºC, revealing that ageing significantly affected their viabilities and vigor in both conditions. In addition, increasing the ageing time at 40ºC significantly affected the germination of *O. sativa* seeds (Zhang et al., 2016). These studies demonstrated that temperature is an important factor modulating viability and vigor during seed ageing in several species and that the temperature that induces seed viability loss is genotype-dependent.

Our study showed that seed ageing significantly affected the seed proteomic profile of *C. fissilis*, identifying proteins that were differentially abundant according to the ageing treatments. Some of these proteins were down- or up-regulated in both comparisons (50ºC/41ºC and 50ºC/non-aged), and others have already been associated with seed viability loss (Table 1). Among the up-regulated proteins, the probable ribosome-binding factor chloroplastic (DN20196_c0_g1_i3.p1) and nutrient reservoir (DN18982_c0_g2_i1.p1) were up regulated in both the 50ºC/41ºC and 50ºC/non-aged comparisons (Table 1). Ribosome-binding factors, also known as plastid-specific ribosomal proteins (PSRPs), are components of the chloroplast ribosome (Bieri et al., 2017). The translation factor pY (previously called PSRP1), involved in the light- and temperature-dependent control of protein synthesis, inactivates chloroplast 70S ribosome monomers and inhibits translation under stress conditions (Sharma et al., 2010; Bieri et al., 2017). Additionally, in *Arabidopsis*, PSRP2 overexpression (35S::PSRP2) negatively affects seed germination under stress conditions (Xu et al., 2013). In addition, nutrient reservoir, another up-regulated protein in both comparisons (50ºC/41ºC and 50ºC/non-aged), is a globulin storage homologue from the cupin family (Gábrišová et al., 2016). Globulins are the most widely distributed proteins found within seeds, occurring not only in dicots and monocots (including cereals and palms) but also in fern spores (Dunwell et al., 2004). Some globulin proteins significantly increase their abundance during the late development of *Triticum aestivum* seeds under high temperature stress (Hurkman, 2009). Additionally, the transcription levels of two globulin-2 genes increase when grain is produced under high temperature conditions (Altenbach et

al., 2009). In this sense, the up-regulated levels of the ribosome-binding factor chloroplastic (DN20196_c0_g1_i3.p1) and nutrient reservoir (DN18982_c0_g2_i1.p1) proteins suggests that these two proteins are associated with viability loss in *C. fissilis* seeds aged at higher temperatures (50°C), which results in significantly reduced germination.

Three related HSP proteins, HSP70 kDa (A0A067EN97), HSP cognate 70 kDa 2-like (A0A067DI64) and stromal 70 kDa HSP-related chloroplastic (DN23331_c2_g1_i6.p1), were also up-regulated in seeds aged at 50ºC compared to that in seeds aged at 41ºC (50ºC/41ºC), which might be related to the reductions in seed germination and viability (Table 1). Artificially aged *O. sativa* seeds increased the abundance of HSP70 kDa in embryos (Zhang et al., 2016). Consistent with this, the increased abundance of a heat shock factor-binding protein was observed during the ageing of poplar (*Populus* x *Canadensis*) and *Medicago sativa* seeds, suggesting that these proteins can be used as potential markers of low seed vigor in these species (Yacoubi et al., 2011; Zhang et al., 2015). Thus, the increased abundance of HSP proteins in seeds aged at 50°C also suggests the involvement of these groups of proteins in the loss of *C. fissilis* viability. Moreover, the serpin-ZX-like protein (A0A067H0B5) was also up-regulated in seeds aged at 50ºC, which significantly reduced their germination compared to that of seeds aged at 41ºC, which maintained their germination (50ºC/41ºC comparison) (Table 1). Serpins (serine proteinase inhibitors) are a family of proteins that inhibit serine proteases (such as trypsin) and play critical roles in controlling proteolysis via the irreversible inhibition of endogenous and exogenous target proteinases. Serpins are thus important for plant growth, development, stress response, and defence against insects and pathogens (Roberts and Hejgaard, 2008). Serpins have also been related to stress-accelerated senescence and plant cell death (Fluhr et al., 2012; Lampl et al., 2013). While serpin proteins have not yet been associated with seed ageing, the up-regulation of this protein observed herein highlights its role in response to higher temperatures and reduced germination.

Some proteins, such as annexin D1 (DN19662_c0_g1_i1.p1; DN19662_c0_g3_i2.p1), exhibited reduced abundance (down-regulation) in *C. fissilis* seeds aged at 50°C compared to that in seeds aged at 41°C and/or non-aged seeds (Table 1). This annexin belongs to a family of calcium-dependent membrane-binding proteins (Barton et al., 1991; Gerke et al., 2005) and is involved in

membrane signalling (Clark et al., 2010). This protein is also important for the maintenance of seed vigor, especially in unfavourable environments (Chu et al., 2012). The abundance of this annexin protein was significantly increased in *Nelumbo nucifera* seeds during heat stress, suggesting that during oxidative stress, annexins may either protect membrane integrity or repair damage (Chu et al., 2012). Transgenic *Arabidopsis* seeds ectopically expressing the annexin gene (NnANN1) exhibited resistance to the artificial ageing treatment, while the wild-type seeds exhibited reduced germination, suggesting that NnANN1 increases ageing treatment tolerance (Chu et al., 2012). In this sense, the decreased abundance of the annexin D1 protein in seeds aged at 50ºC may be related to the reduced vigor and germination of *C. fissilis*, suggesting that this protein is necessary for the maintenance of seed viability.

In addition, a calreticulin (DN20799_c0_g3_i3.p1) protein was down-regulated in seeds aged at 50ºC, which induced germination loss compared to that in non-aged seeds (50ºC/non-aged) and seeds aged at 41ºC (50ºC/41ºC), wherein seed germination reductions were not observed (Table 1). In this sense, the reduced abundance of this protein may be related to the loss of seed viability and reduced germination when seeds are exposed to the 50ºC temperature. The calreticulin protein is related to calcium-binding molecular chaperones and associated with stress (Crofts and Denecke, 1998; Gupta and Tuteja, 2011). Artificially ageing *A. thaliana* seeds increased the stress-induced oxidation of calreticulin (Rajjou et al., 2008). Thus, we suggest that this protein might be important for maintaining the seed viability and germination of *C. fissilis.*

Three PDIs (DN21465_c0_g2_i3.p1, DN21465_c0_g1_i8.p, DN21465_c0_g1_i4.p1) were down-regulated in seeds aged at 50°C (Table 1). These proteins, which are present in all eukaryotic cells, directly donate disulfides to substrate proteins via thioldisulfide exchange reactions. As these proteins participate in the formation, cleavage, and isomerization of disulfide bonds in proteins, they are essential for oxidative protein folding, exhibiting the properties of chaperones (Houston et al., 2005; Selles et al., 2011; Onda, 2013; Freedman et al., 2017). In plants, PDIs contain thioredoxin domains that catalyse protein disulfide bonds, inhibit the aggregation of misfolded proteins, and function in responses to abiotic stresses (Kayum et al., 2017; Wang and Komatsu, 2017). Thus, the down-

regulation of these proteins observed in seeds aged at 50°C suggests that they are associated with the loss of *C. fissilis* seed viability under this treatment.

In addition, the proteins osmotin (DN19808_c0_g1_i3.p1), ADHIII (A0A067FEE9), TIM (DN19867_c1_g2_i4.p1), ABH (DN22588_c0_g2_i4.p1) and elongation factor 2 (DN22957_c0_g1_i3.p1) were also down-regulated in seeds aged at 50°C in both comparisons (50ºC/41ºC and 50ºC/non-aged; Table 1). The osmotin protein accumulates during the adaptation of cells to high osmotic stress, including salt (Singh et al., 1987). The protective efforts of osmotin in plants range from high temperatures to cold and salt to drought (Kumar et al., 2015). ADHIII, also known as glutathione-dependent formaldehyde dehydrogenase, plays a central role in the formaldehyde detoxification of plant xenobiotic metabolism (Achkor et al., 2003). The TIM protein is involved in the gluconeogenesis pathway, which facilitates carbohydrate biosynthesis. Transcription of the TIM gene is stable in rice seedlings grown under 40ºC for up to 12 h, and this gene is thus recommended as a reference gene during reverse transcription quantitative polymerase chain reaction (RT-qPCR) analyses (Wang et al., 2016). Members of the ABH superfamily have widespread functionalities and malleable protein folding, playing catalytic roles in primary and secondary metabolism as esterases, thioesterases, lipases, proteases, dehalogenases, haloperoxidases, and epoxide hydrolases (Mindrebo et al., 2016). Despite mounting evidence of the importance of these enzymes in plant physiology and specialized metabolism, the *A. thaliana* genome alone contains hundreds of uncharacterized ABH-like genes (Mindrebo et al., 2016). Elongation factor 2 catalyses the GTP-dependent ribosomal translocation step during translation elongation and has been shown to be involved in cold responses, playing a critical role in new protein synthesis during the proper transduction of low-temperature signals (Guo et al., 2002). Despite having little evidence of a direct relationship between these proteins and reduced seed viability under high temperatures during artificial *C. fissilis* ageing, this work potentially highlights these proteins in further studies, relating them to the maintenance of aged seed viability.

In addition, the primary amine oxidase-like (DN23578_c0_g5_i2.p1) protein was identified by proteomic analysis as being down-regulated in *C. fissilis* seeds aged at 50ºC, which was associated with reduced germination compared with that of non-aged seeds (50ºC/non-aged) and seeds aged at 41ºC (50ºC/41ºC) (Table 1). This copper amine oxidase degrades PAs via an oxidative deamination process,

preferentially degrading the aliphatic diamine Put (Moller and McPherson, 1998; Ghuge et al., 2015). In plants, primary amine oxidase oxidizes Put, releasing hydrogen peroxide (Tiburcio et al., 2014). Thus, the down-regulation of primary amine oxidase is consistent with the higher amounts of Put observed in *C. fissilis* seeds aged at 50ºC (Fig. 5A). In *Zea mays* root cells, the application of 0.5 mM Put rapidly depolarizes the membrane potential, and high concentrations (5 mM) of this PA can damage the plasma membrane (DiTomaso et al., 1989). In this study, a significant increase in free Put content during ageing at 50ºC (Fig. 5A) was observed, suggesting a relationship between free Put content and the loss of viability and vigor during the ageing of *C. fissilis* seeds. In addition, the contents of free amines were higher in low-viability seeds of japonica rice cv Tapei 309 compared to those in seeds with higher germinations (Bonneau et al., 1994). Put is well known as the first PA to accumulate in cells exposed to abiotic stress (Gupta et al., 2016). This PA most likely acts as a chemical messenger to trigger stress signalling events and stabilize the membrane integrity in the physiological and biochemical responses of *Populus cathayana* to copper stress (Chen et al., 2013). In *C. legalis*, a higher Put content was associated with seed deterioration, possibly due to changes in membrane potential and/or plasma membrane damage, which may have resulted in the reductions in seedling emergence and vigor (Sousa et al., 2016). In this sense, the higher Put contents in *C. fissilis* seeds observed herein may have led to seed deterioration when the seeds were aged at 50ºC.

Because the higher Put content observed herein increased the PA ratio in *C. fissilis* seeds aged at 50°C (Fig. 5D) and was potentially related to the loss of seed viability, Put may be a promising biochemical marker for seed deterioration. Similar results were observed in *C. legalis* seeds during storage (Sousa et al., 2016). According to these authors, the higher PA ratio observed in seeds during storage could be used as a biochemical marker of seed viability loss due to reduced vigor and seedling emergence. Our results suggest that PA homeostasis was affected during *C. fissilis* seed ageing, resulting in the reduced seed germination observed at 50ºC.

In addition, seeds aged at 50ºC, which lost their viability, showed significantly decreased free Spd and Spm contents compared to those in seeds aged at 41ºC (Fig. 5B and C). The PAs Spd and Spm alleviate adverse effects under stress conditions (Duan et al., 2008; Xu et al., 2011). The protective effects of these PAs

might be related to longer chains and a number of positive charges in the amine groups of these molecules (Velikova et al., 2000). This characteristic can allow the Spd and Spm molecules to have higher neutralizing and membrane-stabilizing effects as well as the ability to provide DNA protection against reactive oxygen species (ROS) damage (Khan et al., 1992; Ha et al., 1998). In this sense, we suggest that the reductions in Spd and Spm contents and the increase in Put content in seeds aged at the higher temperature (50ºC) were related to the reduced seed germination observed (Fig. 2A) in *C. fissilis*.

5. CONCLUSION

Ageing *C. fissilis* seeds at 50ºC significantly reduced their germination and vigor compared to those of seeds aged at 41°C and non-aged seeds. Among the differentially regulated proteins, some were related to the loss of seed viability, especially the down-regulated proteins in seeds aged at 50ºC compared to those aged at 41°C and non-aged seeds. Down-regulation of the primary amine oxidase protein was potentially related to the accumulation of free Put content in seeds aged at 50ºC, which may have been harmful to the cells, and to the reduced germination capacity and vigor of the seeds. Moreover, a significant reduction in free Spd and Spm contents in seeds aged at 50°C was also potentially related to the loss of seed viability. These results highlight new biochemical alterations during seed ageing in *C. fissilis* and could be important for future studies on seed longevity maintenance and germplasm conservation.

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4.3. EFFECTS OF TEMPERATURE AND PACKAGE ON GERMINATION AND ENDOGENOUS POLYAMINES CONTENTS DURING SEED STORAGE OF *Cedrela fissilis* VELLOZO (MELIACEAE)

RESUMO

O estabelecimento das melhores condições para o armazenamento é importante para a conservação das sementes. Alterações em biomoléculas, como poliaminas (PAs), ocorrem durante o armazenamento de sementes e podem estar relacionadas com a manutenção ou perda de viabilidade. O objetivo deste trabalho foi estudar os efeitos da temperatura e das embalagens na germinação e conteúdo endógeno de PAs durante 24 meses de armazenamento de sementes em *Cedrela fissilis*, uma arbórea da Mata Atlântica ameaçada de extinção. Para tanto, as sementes foram armazenadas em duas embalagens (sacos de papel trifoliados e frasco de vidro) sob três temperaturas (4, 12 e 25ºC) durante 24 meses. A qualidade fisiológica das sementes foi avaliada através de testes de germinação, índice de velocidade de germinação e teor de água de sementes. O conteúdo de PAs foi avaliado em sementes antes (tempo 0) e após 4, 8, 12, 16, 20 e 24 meses de armazenamento. Verificou-se que o armazenamento a 4ºC foi o mais eficiente na manutenção da qualidade fisiológica das sementes de *C. fissilis* durante 24 meses, em ambos os tipos de embalagens. A 12ºC, o frasco de vidro foi a embalagem mais adequada para armazenamento de sementes, mas com diminuição da capacidade germinativa. A temperatura a 25°C não foi adequada para armazenar sementes de

C. fissilis durante longos períodos nos dois tipos de embalagem. Mudanças no conteúdo de PAs foram observadas em sementes armazenadas a 4 e 12ºC. O conteúdo de PAs livres, principalmente Espermidina e Espermina aumentou significativamente nas sementes armazenadas a 4ºC, sugerindo que essas PAs podem estar associadas à manutenção da viabilidade nas sementes de *C. fissilis*, sem redução significativa na germinação quando mantida nesta condição. Esses resultados destacam novas informações sobre PAs no envelhecimento das sementes, sendo importante para a compreensão de eventos relacionados à perda de viabilidade das sementes em *C. fissilis*, bem como para estabelecer melhores condições de armazenamento de sementes.

ABSTRACT

The establishment of the best conditions for seed storage is important for seed conservation. Alterations in biomolecules, such as polyamines (PAs), occurs during seed storage and can be related with the maintenance or loss of viability. The aim of this work was to study the effects of temperature and packages on germination and endogenous contents of PAs during 24 months of seed storage in *Cedrela fissilis*, an endangered tree from Brazilian Atlantic Forest. Seeds were stored in two packages (trifoliate paper bags and glass container) under three temperatures (4, 12 and 25ºC) for 24 months. Physiological seed quality was assessed through the germination test, germination speed index, and seed moisture content. The PA contents was evaluated in seeds before (time 0) and after 4, 8, 12, 16, 20 and 24 months of storage. The storage at 4ºC was more efficient in maintaining the physiological quality of *C. fissilis* seeds for 24 months in both type of package. At 12ºC, the glass container was the most adequate package for seed storage, but with a reduction in germination capacity. The temperature of 25°C is not a suitable condition to store *C. fissilis* seeds during long periods in both type of package. Changes in PA contents were observed in seeds stored at 4 and 12ºC. The contents of free-PAs, mainly Spermidine and Spermine, increased significantly in the seeds stored at 4ºC, suggesting that this PAs could be related to maintenance of viability in *C. fissilis* seeds, without reduction on germination when kept in this condition.

These results highlight new information about PAs and seed ageing, being important for understanding events related to the loss of seed viability in *C. fissilis,* as well as to establish a better storage condition of seeds.

1. INTRODUCTION

The seeds of most species can be stored from year to year and this practice has been used since the beginning of agriculture to ensure the supply of seeds with a good quality. Thus, seed viability for longer period of storage is essential to preserve the genetic integrity. Indeed, aspects related to the physiological quality of the seed has been one of the most researched interest in recent years because they are subject to a series of degenerative changes after maturation of seeds (Rajjou et al., 2008). Approaches to improve seed conservation are crucial in case of endangered native species, especially in wood species, that can present alternation of seed production, characterized by one year of higher, followed by one or two years of lower seed production (Benedito et al., 2011), as well as loss of viability during storage.

Among the species, the *Cedrela fissilis* Vellozo (Meliaceae) is an endangered native tree from Brazilian Atlantic Forest with a high economic value, especially for wood production, being included in the Red List of threatened species (IUCN, 2017). Seeds from this species is considered orthodox, but they lost the viability when stored in a dry room at 18ºC for 6 months, but can keep the viability if stored at 4ºC up to 12 months if kept in a glass container or plastic bag (Corvello et al., 1999). Although the physiological quality of the seed cannot be improved nor avoided deterioration, this process can be delayed under suitable storage conditions, allowing successful seed conservation program (Pradhan and Badola, 2012). The establishment of best conditions to maintain the seed viability and vigor for a longer time has been developed for several species (Corte et al., 2010b; Chattha et al., 2012; Vange et al., 2016; Nery et al., 2017). The temperature and type of package influences the storage, interfering in the metabolic activity of seeds and affects their longevity, as observed for seeds of several agricultural species (Nery et al., 2017) and native trees (Corte et al., 2010a; Mata Ataíde et al., 2012; Abbade and Takaki,

2014). Thus, depending on the temperature and package used, the long-term storage may lead to considerable reduction on germination and vigor of the seeds. Among the packages, polyethylene bags and glass container are most used, depending on the species, such as *Talisia esculenta* and *Vigna unguiculata* (Kamara et al., 2014; Sena et al., 2016). In addition, the range of temperature from -20 to 10°C can be the best for seed storage, depending on the specie and type of seed according to their tolerance to desiccation, if recalcitrant, orthodox or intermediary (Hong and Ellis, 1998).

The use of appropriate storage conditions can decelerate the physiological and biochemical alterations during seed storage (Gupta et al., 2017). Among the biochemical alterations, changes on endogenous polyamines (PAs) contents could be related with loss or maintenance of seed viability. The PAs, Putrescine (Put), Spermidine (Spd) and Spermine (Spm) are low molecular weight aliphatic cations that are ubiquitous to all living organisms (Kusano et al., 2008). Due to strong electrostatic interactions, the positive charges of PAs bind to the negative charges of macromolecules as DNA, RNA and proteins, to stabilize these molecules (Kusano et al., 2008; Minocha et al., 2014). In plants, PAs act in the growth and developmental processes, seed development and germination, as well as response to stress (Santa-Catarina et al., 2006; Shu et al., 2012; Rios et al., 2015; Gupta et al., 2016).

The relationship of PAs with seed aging has been studied for some species, such as *Triticum durum*, *Allium cepa* and *Cariniana legalis* (Anguillesi et al., 1990; Basra et al., 1994; Sousa et al., 2016). In stored *C. legalis* seeds the high content of free Put was associated with reduction in vigor and emergence of seedlings (Sousa et al., 2016). Previous studies in *C. fissilis* has been showed that seeds maintain the viability when stored at 4°C in plastic bags during 12 months, and endogenous PAs was not affected by these storage conditions in this time tested (Sousa et al., 2016). However, the effects of different packages and temperatures, and a longer time of storage, on seed viability and PAs contents alterations were not developed for this species, and could be relevant to improve the network of these conditions on PAs alteration and seed aging.

In this sense, the aim of this work was to study the effects of temperature and packages on germination and endogenous contents of PAs during 24 months of storage in *C. fissilis* seed.

2. MATERIAL AND METHODS

2.1. Plant material

Mature seeds, collected in August 2014, were provided by Caiçara nursery located at Brejo Alegre, São Paulo, Brazil (21º10'S and 50º10'W), with 21% of moisture content. Before storage, *C. fissilis* seeds were at room temperature until reaching the moisture content of 11%.

2.2. Effect of package and temperature on seed germination and endogenous PAs contents

To analyse the effects of package and temperature, the *C. fissilis* seeds were stored under three different temperatures (4, 12 and 25ºC) and two type of package (the trifoliate paper bags and glass container). The average of relative humidity has ranged from 15%, 88% and 76% for the 4, 12, and 25°C, respectively.

Physiological analyses to determine the vigor and viability were performed with mature dry seeds before (non-aged, time 0) and after 4, 8, 12, 16, 20 and 24 months of storage. For PAs analyses, samples containing 200 mg fresh matter (FM) of seeds of each sample, in triplicate, were collected from each treatment and time of evaluation, and kept at -20ºC until analysis.

2.2.1. Physiological analysis

Physiological evaluations of seed germination, germination speed index (GSI), non-germinated seeds and abnormal seedling developed were developed to access seed viability and vigor. The germination (%) was analyzed according to Brasil (2013). Four biological replicates (with 50 seeds each) from each treatment were distributed upon three sheets of germitest® paper (J Prolab, Paraná, Brazil) moistened with sterile distilled water at a ratio of 2.5 times the dry substrate mass. Seeds were incubated in a BOD-type germination chamber (Eletrolab, São Paulo, Brazil) at 25ºC, with photoperiod of 8 h light/16 h dark, at 40 µmol m^{-2} s^{-1}.

The germination was recorded daily during 21 days, when the percentage of germination, non-germinated seeds and abnormal seedling developed were

obtained. The GSI was determined according to Maguire (1962) by the number of seed germinated, which was evaluated daily.

2.2.2. Seed moisture content determination

The seed moisture content was determined according to Brasil (2009), with modifications. Four biological samples (2 g FM each) of seeds at each time of analysis and each treatment were weighed. Then, the samples were dried at 105°C for 24 h in a chamber with forced air circulation (Ethik technology, São Paulo, Brazil). The results of seed moisture content were expressed as percentage of the FM according to the formula: seed moisture content = (water content/FM) x 100.

2.2.3. Free-PAs determination

The PA determination was performed according to Santa-Catarina et al. (2006). Samples (200 mg FM each, in triplicate) of seeds in each treatment were ground in 1.3 mL of 5% (v/v) perchloric acid (Merck, Darmstadt, Germany), and incubated at 4 °C for 1 h. After, the samples were centrifuged for 20 min at 20,000 *x* g at 4 °C, and the supernatant was collected. The diaminoheptane (DAH; Sigma Aldrich, St. Louis, USA) were used as internal standard. Free PAs were determined directly from the supernatant by derivatization with dansyl chloride (Merck) and identified by HPLC using a 5-µm C18 reverse-phase column (Shimadzu Shin-pack CLC ODS) at 1 mL min^{-1}, at 40 °C. The mobile phase A consisted of 10% acetonitrile in water (pH 3.5) and the mobile phase B of absolute acetonitrile. The binary gradient started at 65% B for 10 min, and then ramped to 100% B up to 13 min, being maintained at 100% B up to 21 min. The PA concentration was determined using a fluorescence detector at 340 nm (excitation) and 510 nm (emission). Peak areas and retention times were measured by comparison with the PAs standard Put, Spd and Spm (Sigma-Aldrich).

2.3. Statistical analysis

The experimental design was completely randomized. The statistical analysis of seed germination, GSI, non-germinated seeds, abnormal seedling, seed moisture content and PA contents from seeds before (time 0) and after 4, 8, 12, 16, 20 and 24 months of storage were analyzed in a factorial scheme 3x2x7 (3 storage

temperatures x 2 package x 7 periods of analysis). The analysis of variance (ANOVA $P < 0.05$) were performed, following by the Tukey test using the Assistat Software Version 7.7 (Silva and Azevedo, 2016).

3. RESULTS

3.1. Effects of temperature and package on physiological analysis during seed storage

The temperature and package affects significantly all physiological parameters analyzed during seed storage of *C. fissilis*. A significant interaction for germination considering the package, temperature and periods of storage, was observed (Supplementary table 1).

Supplementary table 1- Analysis of variance for the analysis of physiological quality in *C. fissilis* seeds before (non-aged, time 0) and after 4, 8, 12,16, 20 and 24 months of storage in two packages (paper bag and glass container) and three temperatures (4, 12 and 25°C).

		Average squares				
SV	**DF**	**G (%)**	**GSI**	**Non-GS**	**AS**	**SMC**
Times	6	15681.09**	88.14**	14725.82**	65.03**	27.37**
Temperature	2	47271.16**	197.83**	48394.66**	415.04**	75.84**
Package	1	2030.09**	10.07**	2016.21**	7.29^{ns}	3.50**
Times*Temperature	12	5013.16**	19.22**	5037.36**	70.11**	11.61**
Times*Package	6	278.42**	3.29**	235.71**	15.375**	4.64**
Temperature*Package	2	2223.02**	6.90**	1816.28**	32.61**	11.61**
Times*Temperature*Package	12	336.19**	1.06**	294.70**	15.32**	3.05**
Residue	126	922.00	7.68	1553.00	610.75	63.15
CV (%)		4.26	5.85	10.57	66.17	7.41

G = germination, GSI = germination speed index, Non-GS = non-germinated seeds, AS= abnormal seedling, SMC= seed moisture content. CV % = coefficient of variation, DF = degree of freedom. **Significant at 1% (P ≤ 0.01) probability level by the F-test, ns = not significant.

Seeds stored at 4ºC, in both type of package (trifoliate paper bags and glass container) showed no significant differences in germination during 24 months, and was able to maintain a higher (92%) germination (Fig. 1A).

Moreover, seeds stored at 12ºC showed a significant reduction on germination from 8th month, for both type of package (Fig. 1B). On the other hand, seeds kept in trifoliate paper bags showed total absence of germination from 20 months (Fig. 1B), showing that glass container was the significantly better package

to store seeds of *C. fissilis* at 12ºC, which still showed 36% of germination at 24 months of storage (Fig 1B).

Seeds stored at 25ºC showed a significant decrease on germination from 8 months, with absence at 12 months of storage, without significant differences between the types of package used (Fig. 1C).

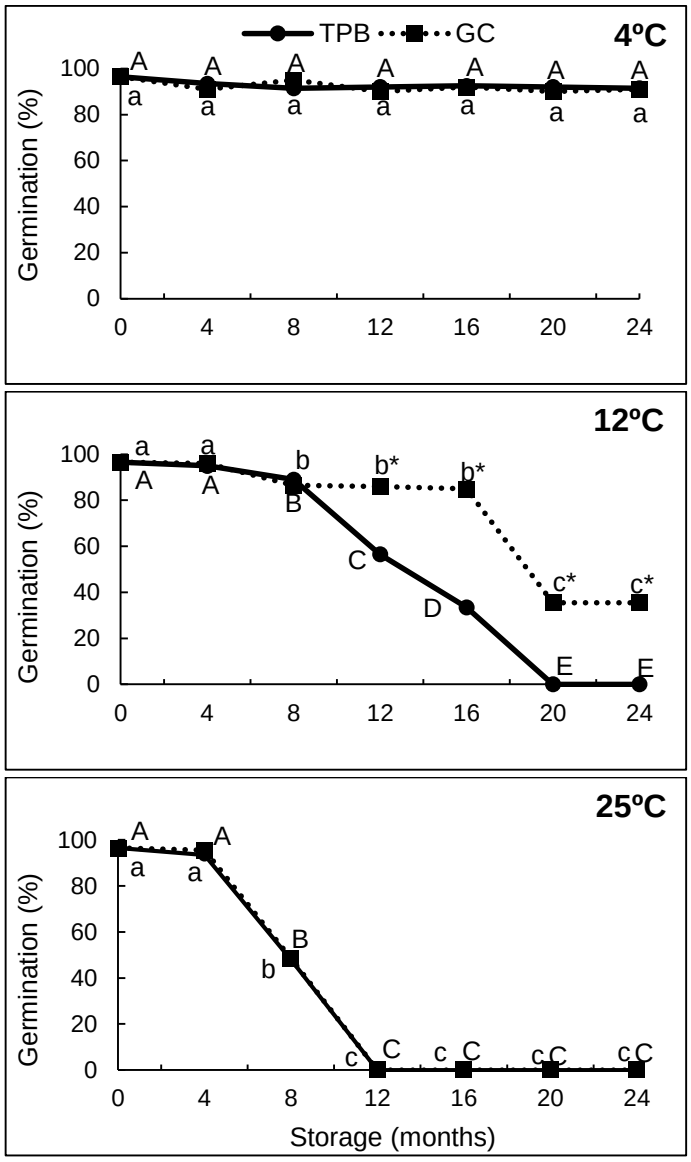

Figure 1. Germination (%) of *C. fissilis* seeds stored in trifoliate paper bags (TPB) and glass container (GC) packages at 4, 12 and 25ºC temperatures, before (time 0) and after 4, 8, 12, 16, 20 and 24 months. *Capital letters* indicate significant differences between months of storage in trifoliate paper bags. *Lowercase letters* indicate significant differences between months of storage in glass containers. The asterisks (*) denote significant differences between the types of package in each month of storage. CV = Coefficient of variation (n = 4, CV of 4ºC = 2.82%; CV of 12ºC = 5.09 %; CV of 25ºC = 6.27%).

The GSI was also significantly affected by the temperature, time of storage and type of package used. The seeds stored at all temperature (4, 12 and 25ºC) in trifoliate paper bags and glass containers presented a decrease in the vigor from 4th month of storage (Fig. 2). Seeds stored at 4ºC, besides no reduction on seed germination (Fig. 1A), showed a decrease in the GSI in both type of package, being a significant higher decrease observed in seeds kept in container glass compared to trifoliate paper bag from 16 to 24 months (Fig 2A).

Seeds storage at 12ºC showed also a significant reduction in the GSI, being the lowest value observed in seeds with 16 months of storage in trifoliate paper bags, with absence of germination at 20 months. The glass container was able to maintain seed vigor when stored at this temperature (12°C), besides the reduction on GSI (Fig. 2B).

On the other hand, seeds stored at 25ºC presented a significant reduction on GSI, with lower values in the 8th month of storage for both types of packages, without significant differences between them (Fig. 2C).

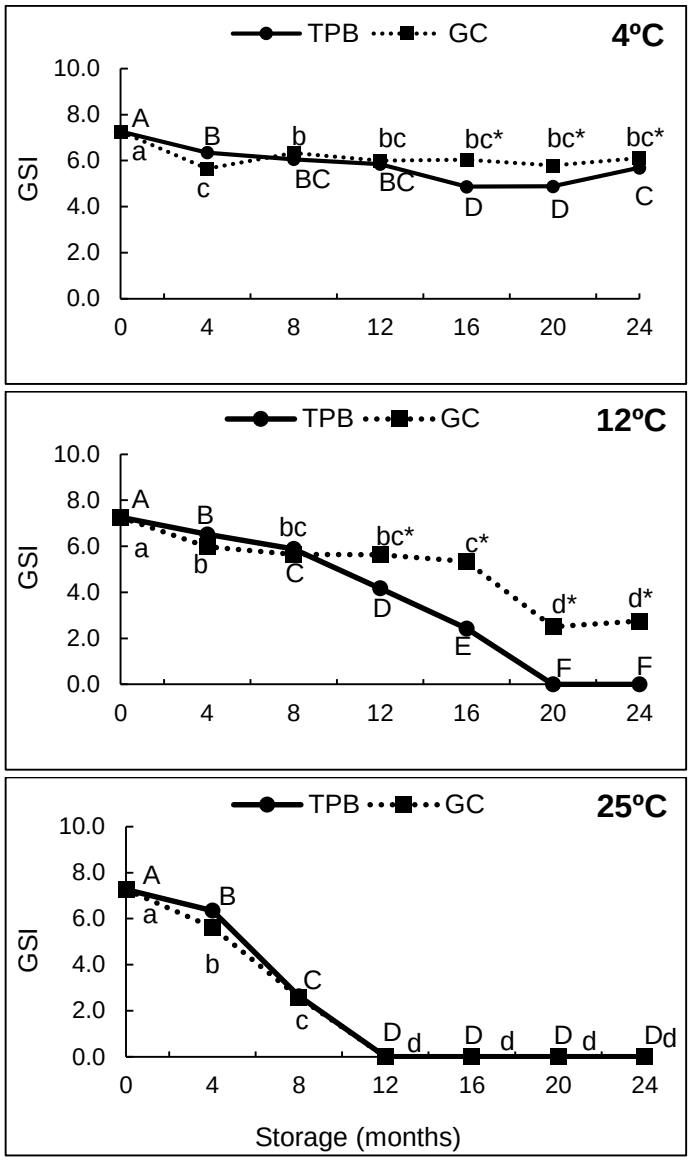

Figure 2. Germination speed index (GSI, %) of *C. fissilis* seeds stored in trifoliate paper bags (TPB) and glass container (GC) packages at 4, 12 and 25 ºC temperatures, before (time 0) and after 4, 8, 12, 16, 20 and 24 months of storage. *Capital letters* indicate significant differences between months of storage in paper bags. *Lowercase letters* indicate significant differences between months of storage in glass container. The asterisks (*) denote significant differences between the types of package in each month of storage. CV = Coefficient of variation (n = 4, CV of 4ºC = 4.23%; CV of 12ºC = 6.32%; CV of 25ºC = 8.88%).

The percentage of non-germinated seeds was also affected by the temperature and type of package during seed storage of *C. fissilis* (Fig. 3). Seed storage at 4ºC during 24 months showed no significant differences in the number of non-germinated seeds in both type of package (Fig. 3A).

At 12ºC, a significant increase of the non-germinated seeds was observed at 12 months of storage in trifoliate paper bags, while those maintained in glass containers presented significant increase at 20 months of storage (Fig. 3B). These

data showed that this type of package is able to maintain the seed viability for a longer period in this temperature compared to trifoliate paper bag.

A significant increase in the number of non-germinated seeds when stored at 25ºC was observed after 4 months, without significant effect of the type of package used (Fig. 3C).

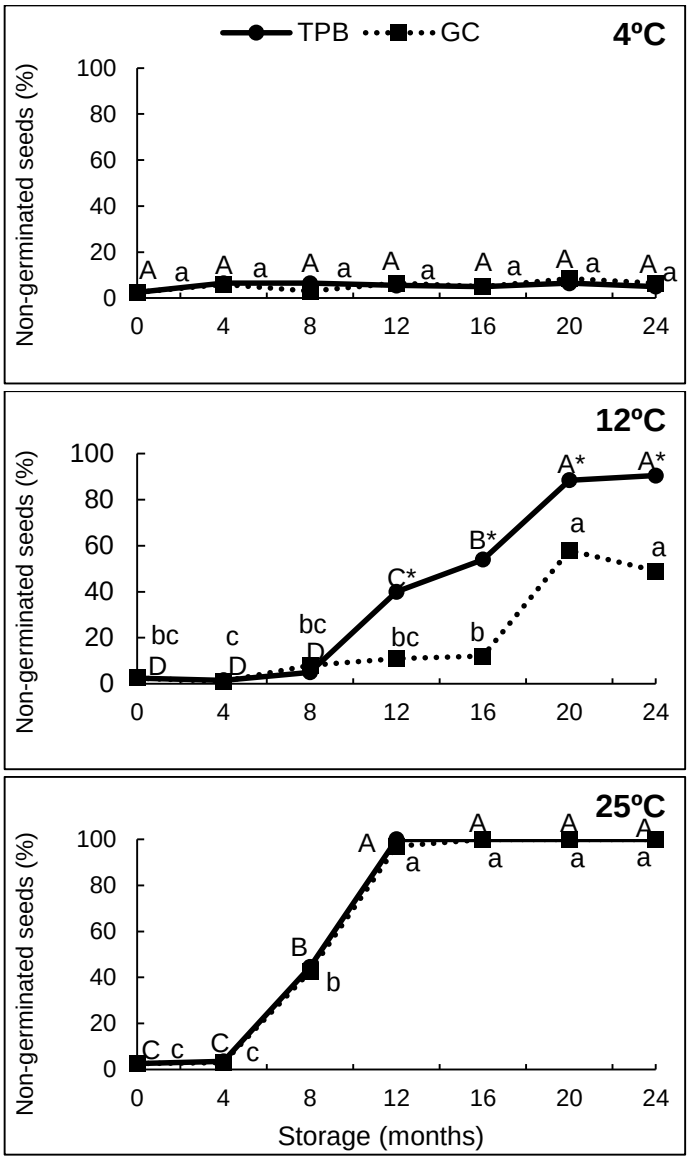

Figure 3. Non-germinated (%) seeds of *C. fissilis* stored in trifoliate paper bags (TPB) and glass container (GC) packages at 4, 12 and 25 ºC temperatures, before (time 0) and after 4, 8, 12, 16, 20 and 24 months of storage. *Capital letters* indicate significant differences between months of storage in trifoliate paper bags. *Lowercase letters* indicate significant differences between months in glass containers. The asterisks (*) denote significant differences between the types of package in each month of storage. CV = Coefficient of variation (n = 4, CV of 4ºC = 43.86%; CV of 12ºC = 15.73%; CV of 25ºC = 5.53%).

The percentage of seedlings with abnormal development was also affected by the temperature and type of package and increased significantly with the time of storage (Fig. 4).

Storage at 4ºC did not affect significantly the percentage of abnormal seedlings (Fig. 4A).

At 12ºC, there was an increase in the percentage of seedlings with abnormal development from 8th month of seeds kept in trifoliate paper bags, while those stored on glass container, the increase in the abnormal sees occurred only at 24 months of storage (Fig. 4B).

However, an increase in the percentage of seedlings with abnormal development was observed in seeds from 8 months of storage at 25ºC, in both type of package, besides no significant difference between them (Fig. 4C).

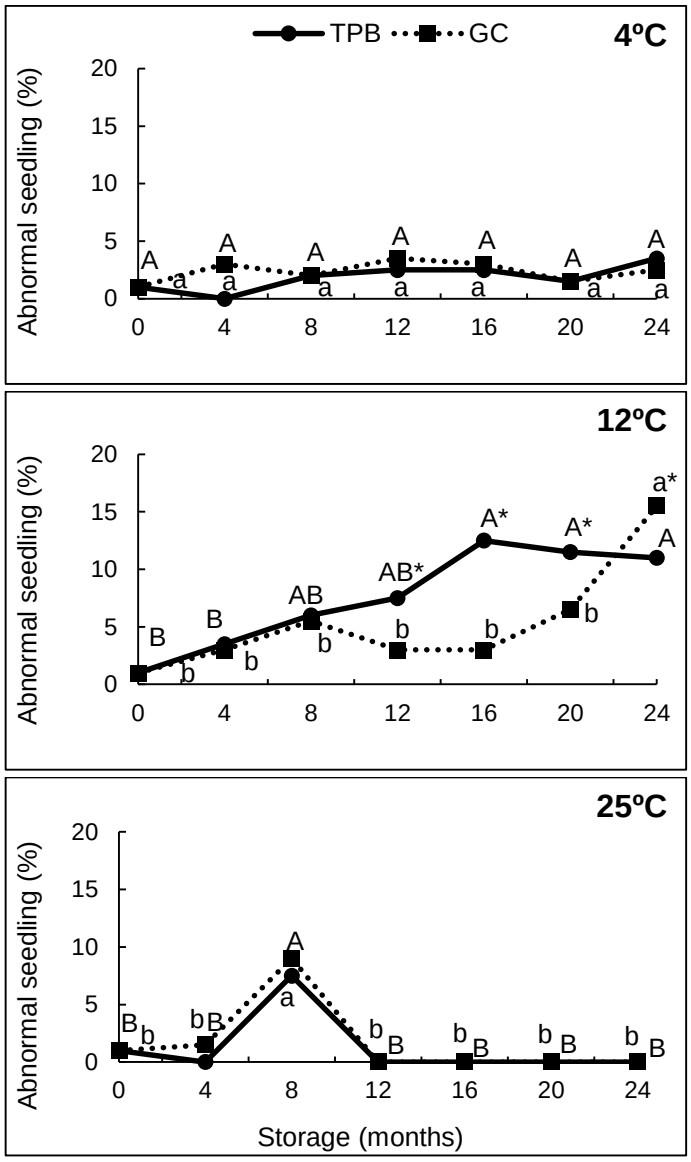

Figure 4. Percentage of seedling with abnormal development from *C. fissilis* seeds stored in trifoliate paper bags (TPB) and glass container (GC) packages at 4, 12 and 25 ºC temperatures, before (time 0) and after 4, 8, 12, 16, 20 and 24 months of storage. *Capital letters* indicate significant differences between the months of storage in trifoliate paper bags. *Lowercase letters* indicate significant differences between the months of storage in glass containers. The asterisks (*) denote significant differences between the types of package in each month of storage. CV = Coefficient of variation (n = 4, CV of 4ºC = 76.45%; CV of 12ºC = 54.51%; CV of 25ºC = 69.51%).

The seed moisture content was affected by the temperature and type of package during the periods of storage (Fig. 5).

The percentage of moisture content decreased significantly at 4 months in seeds stored at 4ºC, following by constant values up to 20 months with reduction on 4th month of storage, in both type of package used, without significant differences between them (Fig.5A). The seeds stored at 12ºC in trifoliate paper bags decreased significantly at 4 months and a significant higher moisture content at 20 and 24

months of storage, while those in the glass container showed a decrease from 4 month of storage, and increased at 20 and 24 months of storage (Fig. 5B). Comparing the two types of package, it was observed a significant increase in the moisture content in seeds stored in trifoliate paper bags compared to glass container at 12°C (Fig 5B).

Seeds stored at 25°C in trifoliate paper bags showed a higher moisture contents at 24 months, however, those stored in the glass container decreased the moisture content from 4th month, remaining constant up to 24 months (Fig. 5C). In this temperature, the moisture contents of seeds stored in trifoliate paper bags was significantly higher from 16 to 24 months of storage compared to those stored in glass container (Fig. 5C).

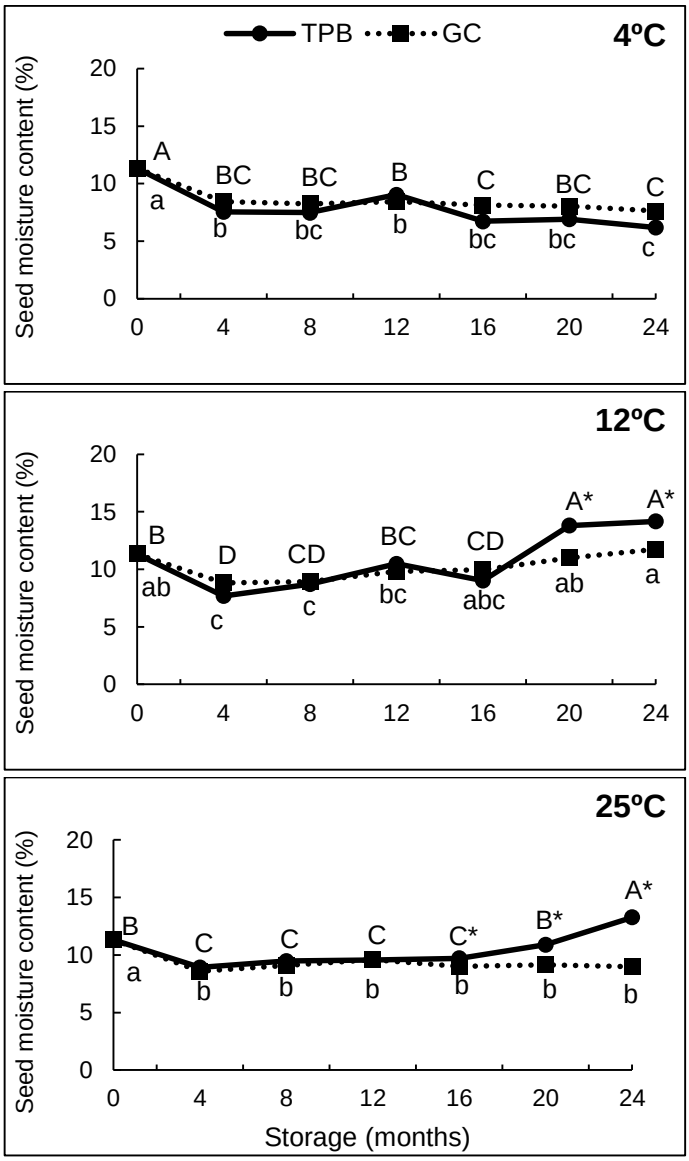

Figure 5. Moisture content (%) in *C. fissilis* seeds stored in trifoliate paper bags (TPB) and glass container (GC) packages at 4, 12 and 25 ºC temperatures, before (time 0) and after 4, 8, 12, 16, 20 and 24 months. *Capital letters* indicate significant differences between the months of storage in trifoliate paper bags. *Lowercase letters* indicate significant differences between the months of storage in glass containers. The asterisks (*) denote significant differences between the types of package in each month of storage. CV = coeficient of variation (n = 4, CV of 4ºC = 9.00%; CV of 12ºC = 8.14%; CV of 25ºC = 4.78%).

3.2. Effects of temperature and package of seed storage on endogenous free-PAs content

The endogenous free-PAs contents were determined in seeds stored at all temperatures (4, 12 and 25°C) and time of storage in trifoliate paper bags. As we did not see significant differences for the type of package (trifoliate paper bag or glass container) for germination at 4°C (the better temperature for storage) and 25°C

(the temperature not suitable for storage), the trifoliate paper bag was chosen for analysis of PAs.

Storage of seeds at 4 and 12ºC induced significant changes in the endogenous content of free-PAs Put, Spd and Spm during 24 months in *C. fissilis*, while that storage at 25ºC did not show significant differences (Fig. 6).

Seeds stored at 4ºC showed significantly higher contents of endogenous free-Put from12 to 24 months and lower contents were observed at time 0 and 8 months (Fig. 6A). At 12ºC, the content of endogenous free-Put oscillated during the time of storage, being significantly higher at 12 months, however not being significant different from time 0, 8 and 20 months, and significant lower contents were observed at 4, 16 and 24 months of storage (Fig. 6A). Comparing the three temperatures, the content of free-Put was significantly higher in the seeds stored at 4ºC, at 16 and 24 months.

The endogenous content of free Spd was significant higher at 24 months seed of storage at 4ºC compared to seeds before storage (time 0), which was not significantly different from the 8, 16 and 20 months of storage. The lower contents of Spd at 4ºC was observed in seeds before storage (time 0), being not statistically different from seeds of 4, 8, 12 and 16 months of storage (Fig. 6B). At 12ºC, the content of Spd reduced at 24 months of storage compared to 4 and 12 months. The highest Spd content was observed in seeds at 16, 20 and 24 months of storage at 4ºC compared to the other temperatures (12 and 25°C) (Fig. 6B).

A significant increase in free Spm content was also observed for seeds stored at 4°C from 4 to 24 months (Fig. 6C). At 12ºC, the content of free Spm reduced significantly in seeds at 24 months of storage compared to 8 and 12 months. The content of Spm was not significantly affected in seeds stored at 25°C (Fig. 6C). Comparing the three temperatures, the highest content of free Spm was also observed in seeds stored at 4°C. This temperature allowed the better condition to maintain seed germination probably by the regulation of the contents of these PAs, with a higher content of free Put, Spd and Spm at 24 months, in the end of storage (Fig. 6).

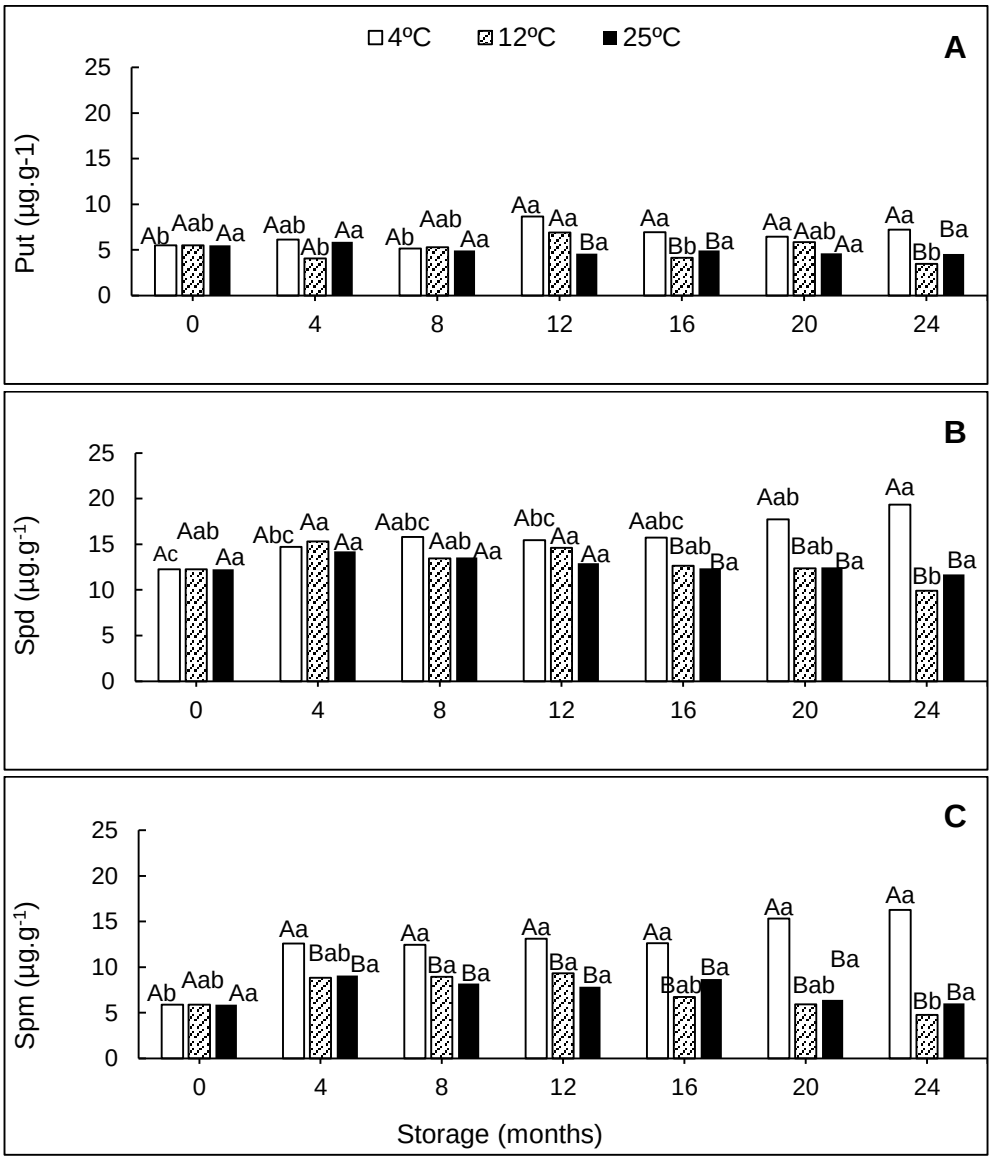

Figure 6. Endogenous contents (µg g^{-1} FM) of free Put (A), Spd (B) and Spm (C) in *C. fissilis* seeds before (time 0) and after 4, 8, 12, 16, 20 and 24 months of storage in trifoliate paper bags at 4, 12 and 25°C. *Capital letters* indicate significant differences between temperatures in each month of storage. *Lowercase letters* indicate significant differences between months of storage in the same temperature. CV = Coeficient of variation (n = 3, CV of Put = 19.07%; CV of Spd = 10.91%; CV of Spm = 18.06%).

The contents of total free-PAs were significantly higher in the seeds stored at 4ºC, increasing from 4 to 24 months of storage, whereas no significant differences was observed in the seeds stored at 25°C (Fig. 7). Comparing the three temperatures, the contents of endogenous total free-PA were higher in the seeds stored at 4ºC from the 8 to 24 months of storage, while no significant differences between seeds stored at 12 and 25ºC was observed (Fig. 7).

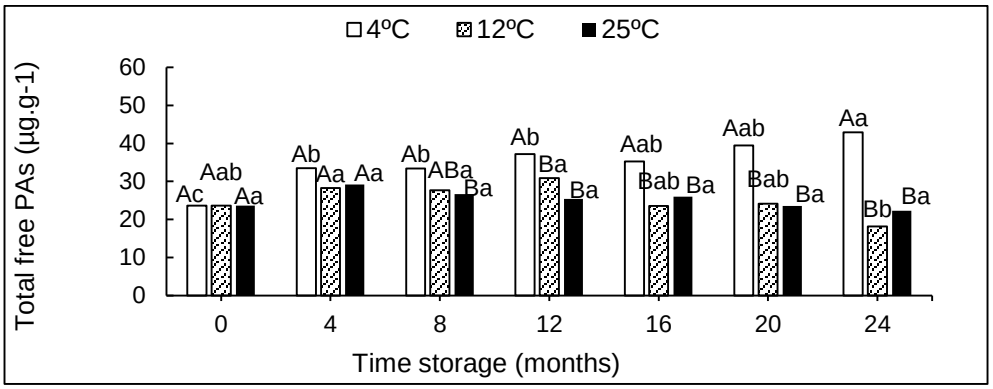

Figure 7. Endogenous contents (µg g^{-1} FM) of total free PAs in *C. fissilis* seeds before (time 0) and after 4, 8, 12, 16, 20 and 24 months of storage in trifoliate paper bags at 4, 12 and 25°C. *Capital letters* indicate significant differences between temperatures on each month of storage. *Lowercase letters* indicate significant differences between months of storage in the same temperature. CV = Coeficient of variation (n = 3, CV of total free-PAs = 11.07%).

4. DISCUSSION

The storage conditions influences directly the longevity of seeds, being the deterioration, an unavoidable process that occurs in seeds, an important factor that affects the germination during its storage for short or long periods (Barbedo et al., 2013). In this work, it was possible to verify that the conditions of storage, as temperature and packages, showed a significant effect on seed germination, GSI, seed moisture, and in the contents of endogenous free PAs during 24 months of seed storage in *C. fissilis*.

A progressive reduction on germinative capacity of *C. fissilis* seeds was observed when stored at 12 and 25ºC during 24 months, being dependent of temperature and type of package used (Fig. 1). The temperature of 25°C was used to simulate a non-adequate condition of storage, and this condition induced the loss of seed viability more quickly, with no germination observed at 12 months, compared to 4°C. These results suggests that temperature is an important factor for the maintenance of seed viability in *C. fissilis* Corvello et al. (1999) also reported a total reduction of germination in seeds of *C. fissilis* stored during 12 months in a room with non-controlled conditions, besides no biochemical analysis performed. Moreover, *C. fissilis* seeds stored in plastic bags at 4°C showed no significant reduction in the seedling emergence after 12 months of storage, besides no analysis performed until 24 months (Sousa et al., 2016). We could show that, a higher

percentage of germination can be maintained when seeds were stored at 4ºC during 24 months, compared to 12 and 25 °C, in both type of package (Fig. 1), being the first time to show a study with a longer time of storage (24 months) with seeds in *C. fissilis*.

Moreover, the temperature of storage has a greater influence in reducing the speed of deterioration processes compared to the type of package, once non-germinated seeds (Fig. 3B-C) and seedlings with abnormal development (Fig. 4B-C) were higher at 25°C compared to 4°C, without significant differences between the packages used. The manifestation of initial aging in seeds is the decreased in the vigor of viable seeds followed by a decrease in the size of seedlings, and an increase of seedlings with abnormal development (Marcos Filho, 2015). Strenske et al. (2017) observed that a not adequate control of temperature (uncontrolled temperature conditions) during the storage of *Chenopodium quinoa* seeds may explain the decline in the seed germination, and the increase in the number of abnormal seedling formed due to seed deterioration.

In addition to temperature, the type of package also is an important factor for seed storage (Abreu et al., 2013; Gupta et al., 2017). In the present work, the type of package influenced the physiological parameters, as germination and GSI, only in seeds of *C. fissilis* stored at 12ºC (Fig. 1-2). In this temperature of storage, the glass container was better than trifoliate paper bag, maintaining 86 and 36% of germination at 12 and 24 months, respectively (Fig. 1B). On the other hand, seeds of *Foeniculum vulgare* stored in glass container maintained at 25ºC and 15.4ºC don't loss their initial physiological quality during 12 months, keeping up 80% of germination (Rubim et al., 2013). In our conditions, the storage at 25°C induced the loss of seed viability in both type of package used, trifoliate paper bag and glass container (Fig. 1C)

The moisture content of seeds was affected by the temperature and type of package during storage, wherein the trifoliate paper bag allowed the significant increase in this parameter in seeds stored at 12 and 25ºC compared to glass container (Fig. 5). Similar results were observed with other species using trifoliate paper bag. In seeds of *F. vulgare*, an increase in the moisture content occurs in seeds stored in trifoliate paper bag during 12 months at 15.6 and 25ºC (Rubim et al. (2013). In addition, Corvello et al. (1999) showed an increase in the moisture content of *C. fissilis* seeds maintained in cotton bags and in a room place with non-

controlled temperature at 12 months of storage. An increase in the moisture content in seeds of *Talisia esculenta* stored in polyethylene bag at 28°C during 100 days was associated to seed deterioration (Sena et al., 2016). Thus, the increase in the moisture content in seeds of *C. fissilis* stored in trifoliate paper bags at 12 and 25ºC may be related to deterioration processes, resulting in the loss of seed viability. It has been known that an increase in the water can activated the metabolic activities necessary to seed germination (Bewley, 1997). However, in stored seeds, the seed moisture content and the non-adequate temperature can lead to deterioration, reducing seed viability (Jyoti and Malik, 2013), as observed in *C. fissilis* seeds.

A significant increase in the endogenous contents of free PAs, specially Spd and Spm was observed in seeds at 24 months compared to seeds before storage (time 0) at the lowest temperature (4°C) tested (Fig. 6B-C). In a previous work, it was not verified significant differences on PAs contents in *C. fissilis* seeds during 12 months of storage (Sousa et al., 2016). These results suggests that thee alteration in PAs contents during storage of seed in this species, with orthodox behavior of seed desiccation, occurs in a longer period of storage, as observed in the present work.

Moreover, an increase in the contents of free Spd and Spm (Fig. 6B-C) in seeds stored at 4°C could be related to the maintenance of viability in the prolonged time of storage in this species (Fig.1). It has been known that Spd and Spm are molecules with longer chain and with greater number of positive charges, and can contribute to the more pronounced protective effects of these PAs in the stabilization of molecules with negatives charges, as DNA, RNA, proteins (Kusano et al., 2008; Minocha et al., 2014) and membranes (Velikova et al., 2000). This sense, the higher contents of this PAs can be related to the protection of *C. fissilis* seeds from deterioration during storage. In addition, it has been showed the action of PAs as free radical scavengers (Ha et al., 1998; Amooaghaie, 2011; Cai et al., 2015). In seedlings of *Glycine max* the application of exogenous Put, Spd and Spm as a pre-treatment, enhanced the growth recovery roots and hypocotyls and decreased the electrolyte leakage and lipid peroxidation when exposed to 45°C, suggesting a protection of membrane integrity induced by the PAs (Amooaghaie and Moghym, 2011). In *Cucumis sativus* roots, the application of exogenous Spd elevated the activities of antioxidant enzymes, suppressed free radical production and membrane damage, and thereby mitigated the oxidative stress (Duan et al., 2008).

Thus, the higher content of endogenous Spd and Spm, may be related to the maintenance of viability of the seeds stored at 4ºC during 24 months, suggesting the protective role of these PAs in *C. fissilis* seed storage (Figs. 1A). This suggestions is in agreement with Basra et al. (1994), whose showed that seeds of *Allium cepa* stored for one-year showed a decrease in the endogenous contents of Spd and Spm. Interestingly in these aged seeds, the exogenous application of Put, Spd e Spm increased endogenous content of these PAs and was related to improved seed vigor. In addition, it was observed in seeds of *Triticum durum* stored during eight years under laboratory conditions, in glass containers, an increase in the PAs, especially Spd, during first six years of aging, followed by a sharply decline in its contents, with progressive loss of their vigor and germination capacity (Anguillesi et al., 1990). Additionally, a correlation between the highest PA content and the higher vigor of *Zea mays* seeds stored for 60 days was observed, and suggests the contents of PAs as an index for storage performance of seeds in this species (Lozano et al., 1989).

5. CONCLUSIONS

Seeds of *C. fissilis* was able to maintain the capacity of germination when stored at 4°C during 24 months, in both type of package used. When stored at 12ºC, the glass container was the best package for seed storage of *C. fissilis*, besides the decrease in the germination capacity. Storage at 25ºC, in both type of packages, is not suitable to conserve seeds during long periods. The increase on the contents of free PAs Spd and Spm in the seeds stored at 4ºC can be related to the maintenance of the germinative capacity, and the increase of these compounds can constitute a biochemical marker of viability in *C. fissilis* seeds.

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7. RESUMO E CONCLUSÕES

No presente trabalho foram obtidas informações inéditas e de relevância teórica e aplicada para conservação e armazenamento *ex situ* de sementes de *C. fissilis*. Vários experimentos foram realizados visando identificar o efeito do envelhecimento, seja durante longo tempo de armazenamento de sementes ou pelo envelhecimento artificial em curto período, sobre a germinação e viabilidade das sementes, assim como na abundância de proteínas diferencialmente abundantes e na alteração do conteúdo de PAs.

No primeiro capítulo foi mostrado que as temperaturas de indução do envelhecimento testadas afetam significativamente a germinação e viabilidade das sementes, verificando-se redução significativa nas maiores temperaturas, especialmente, a 50°C.

Desta forma, no capítulo dois, foram apresentadas alterações no perfil proteômico e no conteúdo de PAs comparando-se as duas temperaturas, uma que não afeta significativamente a germinação ao longo da incubação (41°C) com a que afeta significativamente (50°C). As sementes envelhecidas a 50ºC apresentaram acúmulo de Put, diminuição progressiva de Spd e Spm no decorrer e diminuição da abundância de proteínas importantes relacionadas com estresse e germinação e ao próprio catabolismo de Put, o que pode ter sido prejudicial às células culminando na deterioração de sementes. Estes dados foram mostrados pela primeira vez para esta espécie e são importantes para entender a relação das PAs e proteínas específicas associadas com a redução da viabilidade de sementes durante o envelhecimento.

No capítulo três foram apresentados os resultados do efeito da temperatura (4, 12 e 25ºC) e tipos de embalagem (papel multifoliado e vidro) no processo de redução da viabilidade das sementes. Observou-se que as sementes mantidas a 4ºC permaneceram viáveis e sem alteração do seu potencial germinativo por 24 meses em comparação com as mantidas a 25ºC, as quais apresentaram ausência de germinação e vigor aos 12 meses. O conteúdo de PAs foi modulado durante o armazenamento, verificando-se aumento no decorrer de 24 meses para as sementes armazenadas a 4ºC, principalmente no conteúdo de Spd e Spm, sendo conhecida na literatura a relação de proteção de membrana acionada por estas PAs devido ao seu maior número de cargas positivas quando comparado a Put. Esse comportamento foi diretamente influenciado pela menor temperatura (4°C) e pode ser associado à maior viabilidade das sementes quando comparado com aquelas armazenadas a 25ºC, com germinação comprometida a partir do 8º mês de armazenamento. Neste sentido, a alteração na concentração dessas aminas livres pode ter relação direta com a manutenção e/ou perda da viabilidade em sementes de *C. fissilis.*

A partir destas informações obtidas pode-se sugerir novos estudos a fim de compreender o complexo processo e velocidade de deterioração, que é dependente de cada espécie.

Esse é o primeiro trabalho a descrever o envolvimento de poliaminas e proteínas durante o envelhecimento de sementes de *C. fissilis* obtendo dados inéditos para maior compreensão desses compostos na semente quiescente e as suas alterações relacionadas ao processo de deterioração acelerado ou não pela temperatura. Assim, estudos futuros com abordagem proteômica comparativa nas amostras obtidas durante o armazenamento por 24 meses são relevante ser realizado, visando não somente identificar proteínas que possam ser relacionadas com a redução da viabilidade, mas também comparar com os resultados obtidos nos estudos do envelhecimento artificial. Adicionalmente, estudos associados com alterações histológicas e ultraestruturais são fundamentais para entender como o envelhecimento afeta a organização estrutural das células durante o envelhecimento das sementes.

Por fim, esse trabalho também resultou em ações práticas para o produtor viveirista ao mostrar a manutenção do potencial germinativo de sementes quando armazenadas a 4ºC, em ambas as embalagens. Estes precisam dispor de boas

condições de armazenamento condição/temperatura para a maior conservação das sementes coletadas para produção de mudas, tendo em vista que essa espécie pode ser utilizada para recuperação de áreas degradadas. Estudos futuros testando temperaturas mais baixas, como -20°C podem ser uma opção para melhor condição de armazenamento das sementes desta espécie.

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