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Daniela Goeten

Germinação e criopreservação de embriões de Arecaceae: *Butia eriospatha* **Mart. ex Drude Becc,** *Butia catarinensis* **Noblick & Lorenzi e** *Euterpe edulis* **Mart**

Florianópolis - SC
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Tese apresentada ao Programa de Pós-Graduação em Recursos Genéticos Vegetais, Departamento de Fitotecnia, do Centro de Ciências Agrárias da Universidade Federal de Santa Catarina, como requisito para obtenção do Título de Doutora em Ciências.

Orientador: Profª. Drª. Neusa Steiner

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O presente trabalho em nível de doutorado foi avaliado e aprovado por banca examinadora composta pelos seguintes membros

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Prof.ª Neusa Steiner, Dr.ª
Orientadora

Florianópolis, março de 2023

Sucesso não é o final, falhar não é fatal,
O que conta é a coragem para retornar e seguir em frente!

(Winston Churchill, 1874 – 1965)

A minha querida família, pelo apoio incondicional ao longo desta caminhada

Dedico e ofereço!

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RESUMO

A Mata Atlântica abriga uma série de espécies de palmeiras (Arecaceae) ameaçadas, devido à perda e fragmentação de seus habitats, bem como por sua superexploração. Três delas, *Butia eriospatha* Mart. ex Drude Becc*, Butia catarinensis* Noblick & Lorenzi e *Euterpe edulis* Mart., nativas da Mata Atlântica do sul do Brasil, são amplamente conhecidas por sua importância ecológica e econômica. Estratégias de conservação *ex situ* fornecem um reservatório de diversidade genética protegido de ameaças antrópicas e ambientais. As sementes são uma das fontes de germoplasma preferidas e mais utilizadas para a conservação *ex situ*. No entanto, a sensibilidade à redução do conteúdo de água aliada a germinação baixa e lenta inviabiliza o armazenamento das sementes dessas espécies através de métodos convencionais. Técnicas de cultivo *in vitro* associadas à criopreservação possibilitam a preservação a longo prazo de germoplasma vegetal ameaçado. Buscou-se, no presente estudo, desenvolver métodos de germinação *in vitro* e de criopreservação para embriões excisados dessas três espécies de palmeiras ameaçadas. Embriões de sementes maduras foram dessecados em fluxo de ar laminar e passaram por testes de germinação *in vitro*. Definindo o limiar de dessecação, dois protocolos de criopreservação foram desenhados: embriões dessecados e imersos em nitrogênio líquido (NL); e embriões previamente dessecados, incubados em soluções de vitrificação de plantas (PVS2/PVS3) pelo método de vitrificação de gotículas. O estado bioquímico específico que tornou os embriões responsivos ao protocolo de criopreservação foi analisado para *B. eriospatha*. Alterações estruturais durante o processo de criopreservação foram descritas para *B. catarinensis.* A combinação de hormônios, antioxidantes e nutrientes permitiu que os embriões frescos se desenvolvessem em plântulas normais e foi eficaz na recuperação após a criopreservação. A redução do conteúdo de água até 0,14 $gH_2O\ gMS^{-1}$ e 0,13 $gH_2O\ gMS^{-1}$ não alterou significativamente a germinação de *B. eriospatha* (93,33%) e *B. catarinensis* (82,50%), porém ambas as espécies sofreram uma redução no índice de velocidade de germinação (IVG) de aproximadamente 60%. Em contraste, embriões de *E. edulis* mostraram-se mais sensíveis à dessecação, o limite crítico de conteúdo de água (CA) foi de 0,20 $gH_2O\ gMS^{-1}$ com germinação de 42,27% e redução do IVG em 32,23%, a perda total da viabilidade ocorreu em CA de 0,11 $gH_2O\ gMS^{-1}$. Durante a dessecação dos embriões de *B. eriospatha* houve um aumento significativo da poliamina PUT, o que resultou na redução da razão [(SPD+SPM) /PUT^{-1}] MS). A intensa atividade de GPX e APX sugere que elas são as principais enzimas envolvidas na proteção celular durante a dessecação. O incremento no conteúdo de ácido glutâmico (Glu), leucina (Leu), lisina (Lys), glutamina (Gln), sugere suas ações como osmoprotetores. Em células meristemáticas de *B. catarinensis* a dessecação ocasionou invaginação das paredes celulares, retração da membrana plasmática e núcleos compactados. Ambas espécies sobreviveram a crioexposição, com taxas acima de 76% de formação de plântulas normais. Características de células viáveis, foram associadas à presença de mitocôndrias, núcleos intactos e paredes celulares preservadas. Por outro lado, 37,67% dos embriões de *E. edulis* dessecados até 0,20 $gH_2O\ gMS^{-1}$ e criopreservados sobreviveram, mas apenas 14,76% deles desenvolveram plântulas normais. O uso de PVS2 e PVS3 não incrementou os índices de sobrevivência para *B. eriospata* se mostrando ineficaz para embriões de *E. edulis.* Conclui-se que embriões de *B. eriospatha* e *B. catarinensis* tendem a se comportar de forma ortodoxa, tolerando baixa CC e congelamento e valida-se o método de dessecação parcial combinada com congelamento rápido, como estratégia de armazenamento a longo prazo para essas espécies. Para embriões de *E. edulis* o armazenamento através da criopreservação precisa ser adaptado e pesquisas adicionais das propriedades da água para uma melhor compreensão da vitrificação em altos CA ajudarão a otimizar os protocolos criopreservação para esta espécie.

Palavras-chave: Conservação; Criobiologia; Palmeira Jussara, Recalcitrância e Vitrificação em gota.

RESUMO EXPANDIDO

Introdução

O armazenamento *ex situ* a longo prazo de germoplasma vegetal é importante tanto para a conservaçao da diversidade genética de plantas. Sementes do tipo ortodoxas (tolerantes à dessecação) podem sobreviver no estado seco por períodos consideráveis e, portanto, podem ser armazenadas com sucesso em baixo conteúdo de água e temperaturas abaixo de zero. Em contraste, as sementes recalcitrantes (sensíveis à dessecação) não toleram a perda de água e, portanto, não podem ser armazenadas em condições convencionais. A criopreservação (nitrogênio líquido, -196°C) representa uma opção segura e eficiente para a conservação *ex situ* a longo prazo dessas espécies que não podem serem armazenadas em sistemas convencionais.

A nível global aproximadamente 8% das plantas possuem sementes sensíveis à dessecação, e em ecossistemas tropicais essa proporção chega a 50%. Arecaceae (Palmeiras) estão amplamente distribuídos nas zonas tropicais americana e contribuem para a diversidade de espécies e cadeias alimentares. Estima-se que 60% das espécies da ordem Arecales possuem sementes sensíveis a dessecação e a associação de recalcitrância e dormência em sementes, embora pouco compreendida, é relatada para muitas espécies de palmeiras e implica em dificuldades para conservação *ex situ*. A técnica de cultivo *in vitro* de embriões excisados, associada à criopreservação, pode contornar os longos tratamentos de estratificação necessários para a germinação da semente inteira e permite a conservaçao *ex situ*. Os mecanismos pelos quais sementes toleram a dessecação e ao congelamento são bem compreendidos, porém não há relatos sobre possíveis alterações estruturais e a relação entre poliaminas, aminoácidos e enzimas antioxidantes durante a dessecação e criopreservação de embriões de Arecaceae.

Cerca de 38,4% das espécies de Arecaceae da América do Sul encontram-se catalogadas na Red List (IUCN 2021), entre as categorias de criticamente em perigo (CR), em perigo (EN) e vulnerável (VU). Dentre essas espécies está *Butia eriospatha* (Mart. ex Drude) Becc, com status de vulnerável (VU). *Butia catarinensis* Noblick & Lorenzi é encontrado na Lista da Flora Ameaçadas de Extinção no Estado de Santa Catarina na categoria de risco muito elevado de extinção (EN), e o *Euterpe edulis* (Mart.), na lista da Secretaria do Estado do Meio Ambiente como vulnerável (VU). Neste sentido, diante ao quadro de ameaça, a perda e fragmentação de habitats e a exploração predatória dessas espécies, elaborar estratégias de conservação compatíveis são urgentes e necessárias.

Objetivos

Estudar aspectos fisiológicos, estruturais e bioquímicos do limite de tolerância a dessecação e da criopreservação de embriões de *B. eriospatha*, *B. catarinensis,* e *E. edulis* visando estabelecer protocolos para estratégias de conservação *ex situ* de sementes de Arecaceae.

Metodologia

Frutos maduros de *B. eriospatha*, *B. catarinensis,* e *E. edulis* foram coletadas em populações naturais no estado de Santa Catarina, Brasil. As sementes foram extraídas após a quebra do endocarpo com o uso de um martelo e desinfestadas. Embriões foram excisados individualmente usando bisturi e foram

assentados diretamente em placa de Petri com 2,5 g de ácido ascórbico (0,2 g L^{-1}) e ácido cítrico (0,2 g L^{-1}), para evitar a oxidação do tecido.

O conteúdo de água das sementes foi estimado após secagem em estufa (105ºC, 24 h). Os potenciais hídricos foram determinados por imersão de embriões isolados, por 24h em soluções de polietilenoglicol 6000 (PEG 6000) a 25º C em concentrações que forneceram diferentes Ψw. Embriões foram parcialmente dessecados por 0, 15, 30, 60, 120, 180, 240 e 300 min em cabine de fluxo de ar laminar a 25 ± 2 °C, 55 ± 5% de umidade relativa [UR]. Em cada tempo de dessecação os mesmos foram submetidos ao teste de germinação em MS, suplementado com 30 g L^{-1} de sacarose, 8 µM GA_3, 3 g L^{-1} carvão ativado e 2,5 g L^{-1} de fitagel. Os embriões foram mantidos, por 30 dias, em sala de crescimento e obteve-se a taxa de germinação, índice de velocidade de germinação e comprimento plântula.

Definido os limiares de dessecação, dois métodos de criopreservação foram testados i) embriões parcialmente dessecados foram envoltos em papel alumínio esterilizado e imersos diretamente em nitrogênio líquido (LN) a -196 °C; ii) embriões parcialmente dessecados foram condicionados em PVS2 e/ou PVS3 durante 60 min, em seguida foram colocados em uma tira estéril de papel alumínio com gotículas (10 - 15 µL) de solução fria de PVS2 e/ou PVS3 e depois imediatamente mergulhadas em LN. O descongelamento ocorreu em solução de sacarose (1,2 M, pH 5,8) mantida em banho-maria a 45°C por 15 min.

Analises de qualificação e quantificação de aminoácidos e poliaminas foram estimados em HPLC, enquanto que a atividade enzimática em espectrofotômetro e a descrição anatômica realizada através de microscopia de óptica e de eletrônica de transmissão. Em seguida, os embriões foram transferidos para recuperação em meio de cultura. Os dados foram submetidos à análise de variância em delineamento inteiramente casualizado e as médias foram comparadas pelo teste SNK ($p <= 0,05$).

Resultados e Discussão

A conservação *ex situ* em de bancos de sementes é considerada uma das estratégias mais importantes para preservar a variabilidade genética de espécie com reprodução sexuada. No presente estudo as sementes de *B. eriospatha*, *B. catarinensis,* e *E. edulis* estavam aderidas a um endocarpo rígido e espesso. Embora o endocarpo proteja as estruturas internas das sementes e proporcione resistência à perda de água, essa estrutura também é responsável pela baixa taxa e longa germinação, atribuída à dormência que limita o desenvolvimento do embrião, e inviabiliza do uso de estratégias convencionais de armazenamento. A remoção do endocarpo lenhoso e do endosperma seguido de cultivo *in vitro* de embriões tem sido usado para acelerar e aumentar as taxas de germinação em outras espécies de palmeiras. No presente estudo, a germinação de embriões de *E. edulis* iniciou-se após 12 dias e 100% de germinação foi observada em no máximo 16 dias, para *B. eriospatha* iniciou-se ao sétimo dia e atingiu o máximo de germinação (100%) ao 10° dia, enquanto para *B. catarinensis* iniciou-se ao sexto dia e levou 21 dias para atingir a germinação máxima (90%).

Embriões frescos, das três espécies, apresentaram altos CA e demonstraram taxas de dessecação elevadas, onde aproximadamente 90% do CA inicial foi perdido durante os 300 min de secagem. Alta CA no momento do desprendimento da planta mãe tem sido proposto como um indicador útil para prever

sementes recalcitrantes. As sementes são classificadas com base na capacidade de armazenamento e no limiar de conteúdo de água, as ortodoxas (<0,05 $gH_2O\ gMS^{-1}$) podem sobreviver à dessecação e armazenamento em baixa temperatura (< -18ºC), enquanto as recalcitrantes (<0,25 $gH_2O\ gMS^{-1}$) não. A maioria dos estudos sobre sementes de espécies de palmeiras ainda não são precisas sobre a tolerância à dessecação das sementes e, como consequência, um conhecimento limitado sobre o tempo de vida útil de armazenamento em banco de sementes. A redução do conteúdo de água até 0,14 $gH_2O\ gMS^{-1}$ e 0,13 $gH_2O\ gMS^{-1}$ não alterou significativamente a germinação de *B. eriospatha* (93,33%) e *B. catarinensis* (82,50%), porém ambas as espécies sofreram uma redução no índice de velocidade de germinação (IVG) de aproximadamente 60%.

Embriões parcialmente desidratados em cabine de fluxo de ar laminar a $gH_2O\ gMS^{-1}$, incubados em PVS2 ou PVS3 (60 min) e em seguida, criopreservadas pelo método de vitrificação de gotas, apresentou alta germinação >80% e formação normal de plântulas (66,67 e 49,64%, respectivamente). No entanto, melhores resultados para a formação normal de plântulas (85,30%) foram obtidos com o mesmo CA seguido da imersão direta em NL. O melhor método de criopreservação para embriões de *B. eriosptha,* foi testado em *B. catarinensis* e resultou em 78,75% de germinação e 76,55% de plântulas normais. Com base nesses resultados, ambas as espécies tendem a um comportamento próximo a embriões de espécies ortodoxas, uma vez que toleraram baixos conteúdos e água e o congelamento. Embriões de *B. eriospatha* e *B. catarinensis* podem ser criopreservadas com sucesso, sem a necessidade de soluções crioprotetoras, o que facilita a excussão do protocolo de crioprervação e o torna menos oneroso. No entanto, nós acreditamos que esta técnica só foi possível através da combinação entre o estado seco e algumas altercações bioquímicas, reesposáveis pela viscosidade das células, impedindo a formação de cristais de gelo. Durante a dessecação dos embriões de *B. eriospatha* houve um aumento significativo na PUT, o que resultou na redução da razão [(SPD+SPM) /PUT^{-1}] MS). A intensa atividade de GPX e APX nos levou a sugerir que elas são as principais enzimas envolvidas na proteção celular durante a dessecação. E por fim, o incremento no conteúdo de ácido glutâmico (Glu), leucina (Leu), lisina (Lys), glutamina (Gln), poderiam estar atuando como osmoprotetores. Características de células viáveis, associadas à presença de mitocôndrias, núcleos intactos e paredes celulares preservadas, após a imersão em NL, validou a dessecação parcial (0,13 $gH_2O\ gMS^{-1}$) em conjunto com o congelamento rápido para *B. catarinensis*.

Em contraste, embriões de *E. edulis* mostraram-se mais sensíveis à dessecação, o limite crítico de conteúdo de água (CA) foi de 0,20 $gH_2O\ gMS^{-1}$ com germinação de 42,27% e redução do IVG em 32,23%, a perda total da viabilidade ocorreu em CA de 0,11 $gH_2O\ gMS^{-1}$. 37,67% dos embriões de *E. edulis* dessecados até 0,20 $gH_2O\ gMS^{-1}$ e criopreservados sobreviveram, mas apenas 14,76% deles se desenvolveram plântulas normais. O uso de PVS2 e PVS3 não aumentou os índices de sobrevivência para *B. eriospata* e foi ineficaz para embriões de *E. edulis.* Para embriões de *E. edulis* o armazenamento através da criopreservação não se mostrou eficiente, provavelmente devido à sua sensibilidade a dessecação, então pesquisas adicionais das propriedades da água e uma melhor compreensão da vitrificação em altos CA ajudarão a otimizar os protocolos criopreservação para esta espécie

Considerações Finais

A dormência morfofisiológica das sementes de *B. eriospatha*, *B. catarinensis,* e *E. edulis* que impõe dificuldades na germinação e produção de mudas pode ser superada por meio do cultivo *in vitro*. Pela primeira vez, uma estratégia para conservação a longo prazo foi estabelecida com sucesso para embriões de *B. eriospatha* e *B. catarinensis*, através da combinação de dessecação e congelamento rápido. Pesquisas adicionais das propriedades da água e uma melhor compreensão da vitrificação em altos CA devem ser desenvolvidas, afim de aprimorar os protocolos de criopreservação e aumentar as taxas de sobrevivência e de formação de plântulas normais de *E. edulis.*

Palavras-chave: Conservação; Bioquimicas; Morfo-estutural, Ortodoxia e Recalcitrância.

ABSTRACT

The Atlantic Forest is home to a number of endangered palm tree species (Arecaceae), due to the loss and fragmentation of their habitats, as well as their overexploitation. Three of them, *Butia eriospatha* (Mart. ex Drude) Becc, *Butia catarinensis* (Noblick & Lorenzi) and *Euterpe edulis* (Mart.), native to the Atlantic Forest of southern Brazil, widely known for their ecological and economic importance. *Ex situ* conservation strategies provide a reservoir of genetic diversity protected from anthropic and environmental threats. Seeds are one of the preferred and most used sources of germplasm for ex situ conservation. However, the sensitivity to the reduction of water content, combined with low and slow germination, prevents the storage of seeds from these species through conventional methods. The in vitro culture technique associated with cryopreservation provides a means for the long-term preservation of endangered plant material. Therefore, the aim of this study was to develop in vitro germination and cryopreservation methods for excised embryos from these three endangered palm species. For this purpose, mature seed embryos were desiccated in laminar airflow and underwent in vitro germination tests. Defining the desiccation threshold, two cryopreservation protocols were designed: embryos desiccated and immersed in liquid nitrogen (NL); and previously dried embryos, incubated in plant vitrification solutions (PVS2/PVS3) by the droplet vitrification method. The specific biochemical state that made the embryos responsive to the cryopreservation protocol was analyzed for *B. eriospatha*. Structural changes during the cryopreservation process have been described for *B. catarinensis*. The combination of hormones, antioxidants and nutrients allowed fresh embryos to develop into normal seedlings and was effective in recovering after cryopreservation. Reducing the water content to 0.14 $gH_2O\ gMS^{-1}$ and 0.13 $gH_2O\ gMS^{-1}$ did not significantly alter the germination of *B. eriospatha* (93.33%) and *B. catarinensis* (82.50%), but both the species suffered a reduction in the germination speed index (GVI) of approximately 60%. In contrast, *E. edulis* embryos were more sensitive to desiccation, the critical limit of water content (WC) was 0.20 $gH_2O\ gMS^{-1}$ with germination of 42.27% and reduction of IVG in 32.23 %, total loss of viability occurred at CA of 0.11 $gH_2O\ gMS^{-1}$. During desiccation of *B. eriospatha* embryos, there was a significant increase in PUT, which resulted in a reduction in the ratio [(SPD+SPM) /PUT^{-1}] MS). The intense activity of GPX and APX led us to suggest that they are the main enzymes involved in cell protection during desiccation. And finally, the increase in the content of glutamic acid (Glu), leucine (Leu), lysine (Lys), glutamine (Gln), could be acting as osmoprotectors. In meristematic cells of *B. catarinensis*, desiccation caused invagination of the cell walls, retraction of the plasmatic membrane and compacted nuclei. Both species survived, survived cryoexposure, with rates above 76% of normal seedling formation. Characteristics of viable cells were associated with the presence of mitochondria, intact nuclei and preserved cell walls. On the other hand, 37.67% of *E. edulis* embryos desiccated to 0.20 $gH_2O\ gMS^{-1}$ and cryopreserved survived, but only 14.76% of them developed normal seedlings. The use of PVS2 and PVS3 did not increase survival rates for *B. eriospata* and were ineffective for *E. edulis* embryos. Therefore, it is concluded that *B. eriospatha* and *B. catarinensis* embryos tend to behave in an orthodox way, tolerating low WC and freezing, and the method of partial desiccation combined with rapid freezing is validated as a long-term storage strategy for these species. For *E. edulis* embryos storage via cryopreservation needs to be adapted, further research into the properties of water for a better understanding of vitrification at high WC will help to optimize cryopreservation protocols for this species.

Keywords: Conservation; Cryobitechnology; Jussara Palm, Recalcitrance and Drop Vitrification.

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SUMÁRIO

1. INTRODUÇÃO E JUSTIFICATIVA

A conservação *in situ* prevê a conservação de ecossistemas e hábitats naturais, mantendo e recuperando populações viáveis (CDB 1992), além de permitir a continuação de processos evolutivos (Maxted et al. 2000). Porém, se constitui como um desafio o desenvolvimento de estratégias para sua implementação considerando as especificidades de espécies ameaçadas ou em perigo de extinção (Oldfield 2009). Visando mitigar a perda da biodiversidade, a conservação *ex situ* foi implementada para conservar os componentes da diversidade biológica fora de seus hábitats naturais (CDB 1992). No ano de 2002 a Conferência das Partes (COP), o órgão supremo da Convenção da Diversidade Biológica (CDB), adotou algumas Estratégias Globais para a Conservação de Plantas (GSPC), entre as quais, foi proposto que até o ano de 2020, 75% das espécies de plantas ameaçadas deveriam estar disponíveis em coleções *ex situ,* de preferência em seu país de origem (CDB 2010). Além disso, o desenvolvimento de protocolos para a conservação e o uso sustentável de plantas, com base em pesquisas, é prioridade entre as GSPC. No entanto, nem todas as plantas podem serem conservadas facilmente, o que exige a compreensão do comportamento individual para cada espécie visando a adaptação de protocolos eficientes. Dentre as estratégias de conservação *ex situ* destacam-se os bancos de germoplasma, os quais incluem amostras de sementes, embriões e propágulos. Contudo, os bancos de germoplasma convencionais (baixa umidade e temperatura) armazenam somente sementes tolerantes à dessecação, as quais mantém viabilidade em um limiar crítico de conteúdo de água de 0.05 $gH_2O.gMS^{-1}$ (Roberts 1972; Hong and Ellis 1996), enquanto plantas com sementes sensíveis à dessecação não são capazes de manter a viabilidade a longo prazo nestas condições, pois apresentam um limite de viabilidade entre 0.20 – 0.30 $gH_2O.gMS^{-1}$ (Walters 2015).

O Brasil é um país megadiverso contemplando de 15 a 20% das espécies do planeta, apresentando a maior riqueza de espécies da flora, além dos maiores remanescentes de ecossistemas tropicais (Myers et al. 2000; Lewinsohn 2005; Forzza et al. 2012). Espécies de palmeiras, tais como *Butia catarinenses* Noblick & Lorenzi*, Butia eriospatha* (Mart. ex Drude) Becc. e *Euterpe Edulis* Mart. estão dispersas na Mata Atlântica Brasileira (Leitman et al. 2015), qual como um dos *hotspots* de biodiversidade é área prioritária global para conservação (Myers et al. 2000; Myers 2003; Mittermeier et al. 2005). No entanto, este bioma é altamente ameaçado pelo desmatamento, exploração madeireira e pela conversão das áreas florestais em áreas de agricultura e pecuária (Myers et al. 2000; dos Reis et al. 2002; Sampaio and Guarino 2007; Ribeiro et al. 2009; Vibrans et al. 2011). Além da perda de seus habitats espécies do gênero *Butia* vêm sofrendo com a pressão da extração e venda ilegal, tanto dos frutos quanto das plantas, as quais são muito usadas de forma ornamental, comprometendo a conservação da espécie (Barbieri et al. 2014; Elias et al. 2016a). Atualmente *B. eriospatha* encontra-se catalogado na *Red list* da IUCN com status de "vulnerável" (VU – vulnerable) (Noblick 1998; IUCN 2021), enquanto o *Butia catarinenses* é encontrado na Lista da Flora Ameaçadas de Extinção no Estado de Santa Catarina na categoria "EN", ou seja, em risco muito elevado de extinção. *Euterpe edulis,* assim como as espécies do gênero *Butia* aqui abordadas, vem sofrendo com os altos níveis de exploração predatória para a comercialização do palmito, comprometendo populações inteiras (dos Reis et al. 2000; Rodrigues e Durigan 2007). Tais ações aliadas ao crescimento lento e dependência de florestas bem preservadas resultaram na inclusão desta espécie na lista da Secretaria do Estado do Meio Ambiente como "vulnerável" (VU – vulnerable), (CNCFlora 2017). Diante do quadro de

ameaça dessas espécies e do cenário de declínio da Mata Atlântica, estudos que auxiliem na conservação dessas e demais espécies que compõem este bioma são de extrema importância.

Estudos apontam que aproximadamente 50% das espécies de florestas tropicais, bem como a grande parte das espécies da família Arecaceae apresentam sementes sensíveis a dessecação (Souza et al. 2015; Wyse e Dickie 2018). Para estabelecer técnicas complementares e diferenciadas de conservação *ex situ*, dentre as quais inclui a criopreservação, a caracterização das sementes e embriões quanto a sua sensibilidade ou tolerância a dessecação é crucial e determinante no sucesso de um protocolo de germinação (Walters et al. 2008, 2013; Engelmann 2011; Kaczmarczyk et al. 2012). Protocolos de cultivo *in vitro* de embriões isolados já foram desenvolvidos e vêm sendo estudados exaustivamente nos últimos 40 anos, não somente com o objetivo de conservar, mas também de cultivar essas espécies (Guerra and Handro 1988, 1998; Saldanha et al. 2006; Ribeiro et al. 2011; Saldanha and Martins-Corder 2012; Ree and Guerra 2015). No entanto, estudos para a conservação *ex situ* de sementes e ou embriões zigóticos, os quais carregam a diversidade genética, são escassos ou até inexistentes dentro do contexto abordado pela CDB e pelo GSPC.

Um protocolo bem-sucedido de criopreservação exige a determinação do conteúdo de água crítica e de mecanismos bioquímicos que permitam a sobrevivência das células e tecidos após exposição ao nitrogênio líquido (-196°C) (Pritchard 2007; Walters et al. 2008; Kaczmarczyk et al. 2012). Em sementes sensíveis à dessecação nas primeiras etapas da criopreservação, que consiste na redução do conteúdo de água intracelular, (afim de evitar a formação de cristais de gelo) podem resultar danos que levam a perda da viabilidade (Benson 2008; Day et al. 2008). Os três principais danos são: (i) mecânico: associado à redução volumétrica da célula e ao colapso de vacúolos, assim como a baixa capacidade de reestruturação das membranas durante a embebição; (ii) metabólico: associado ao acumulo de EROs (Espécies Reativas ao Oxigenio), devido à atividade metabólica, alta taxa de respiração e falha do sistema antioxidante o que leva a peroxidação lipídica da membrana e (iii) desnaturação macromolecular: associado à não de-diferenciação macromolecular (Umarani et al. 2015). Contudo, os danos continuam durante as demais etapas da criopreservação, após a exposição ao nitrogênio, estudos relatam ruptura de estruturas lipídicas, desnaturação das proteínas e a precipitação de solutos responsáveis pela permeabilidade da membrana (Steponkus et al. 1998; Engelmann 2011; Kaczmarczyk et al. 2012). Além disso, a peroxidação de ácidos graxos em fosfolipídios ocasiona danos extensos à membrana celular, o que também resulta em alterações na semi-permeabilidade da membrana (Walters et al. 2008, 2013; Bewley et al. 2013b). Estudos mostram que uma alternativa para sementes que não toleram baixos níveis de dessecação é o uso de crioprotetores (Engelmann 2011; Popova et al. 2013), porém os protocolos de criopreservação devem ser adaptados para cada espécie, em função de seu limite de tolerância à dessecação, resistência natural ao congelamento, metabolismo da planta e resposta ao agente crioprotetor (Panis and Swennen 2001; Beardmore and Whittle 2005b; Volk and Walters 2006; Chmielarz 2009; Engelmann 2011; Berjak and Pammenter 2014). Sendo assim, estudos iniciais de caracterização e comportamento de sementes se tornam chave para avançar no estabelecimento de um protocolo eficiente de criopreservação. Apesar da complexidade e dificuldade durante as etapas de criopreservação de sementes sensíveis à dessecação, diferentes protocolos foram adaptados a inúmeras espécies, mostrando que a secagem das sementes a um conteúdo de água apropriado

ou então o uso de crioprotetores permitem uma criopreservação bem sucedida (Walters et al. 2008; Chmielarz 2009; Popova et al. 2013; Michalak et al. 2015; Coelho et al. 2018; Endoh et al. 2018).

Está bem descrito na literatura que a tolerância de uma semente ao estresse, seja ele pela secagem ou pelo frio, se dá pela ativação de alguns mecanismos como a condensação da cromatina, a síntese de carboidratos (sacarose e oligossacarídeos da série da rafinose), das proteínas protetoras tais como LEAs e HSPs, das enzimas antioxidantes (CAT, SOD, APX, GSH e POD) e dos hormônios (ABA e GAs) (Farrant et al. 1996; Steadman et al. 1996; Berjak et al. 2007; Berjak and Pammenter 2013; Bewley et al. 2013a; Walters 2015; González-Morales et al. 2016; Leprince et al. 2017). Porém, até o momento, poucos estudos foram realizados visando elucidar o papel dos aminoácidos e das poliaminas associados as enzimas antioxidantes diante a condição de estresse gerada no processo de dessecação (Beardmore and Whittle 2005a, b; Panis and Lambardi 2005; Alcázar et al. 2006; Liu et al. 2011; Sagor et al. 2013; Chen et al. 2019). Esses aspectos conferem pioneirismo em nosso estudo, no sentido de estudar a relação entre esses metabólitos em resposta aos limites de sensibilidade à dessecação, uma vez que conhecer e caracterizar os limiares da sensibilidade à dessecação são princípios fundamentais para o sucesso de programas de conservação *ex situ*. Com base nisso, este trabalho tem por objetivo estudar a relação bioquímica entre o limite de dessecação e a capacidade de resposta para criopreservação para embriões zigóticos de *B. catarinensis, B. eriospatha* e *E. edulis,* que além de ameaçadas de extinção apresentam um elevado potencial de uso, devido as suas propriedades organolépticas, sensoriais e nutricionais.

Mediante o exposto, foi elaborado uma representação esquemática para ilustrar as técnicas de criopreservação, bem como os pontos das análises fisiológicas, bioquímicas e estruturais (Fig. 1).

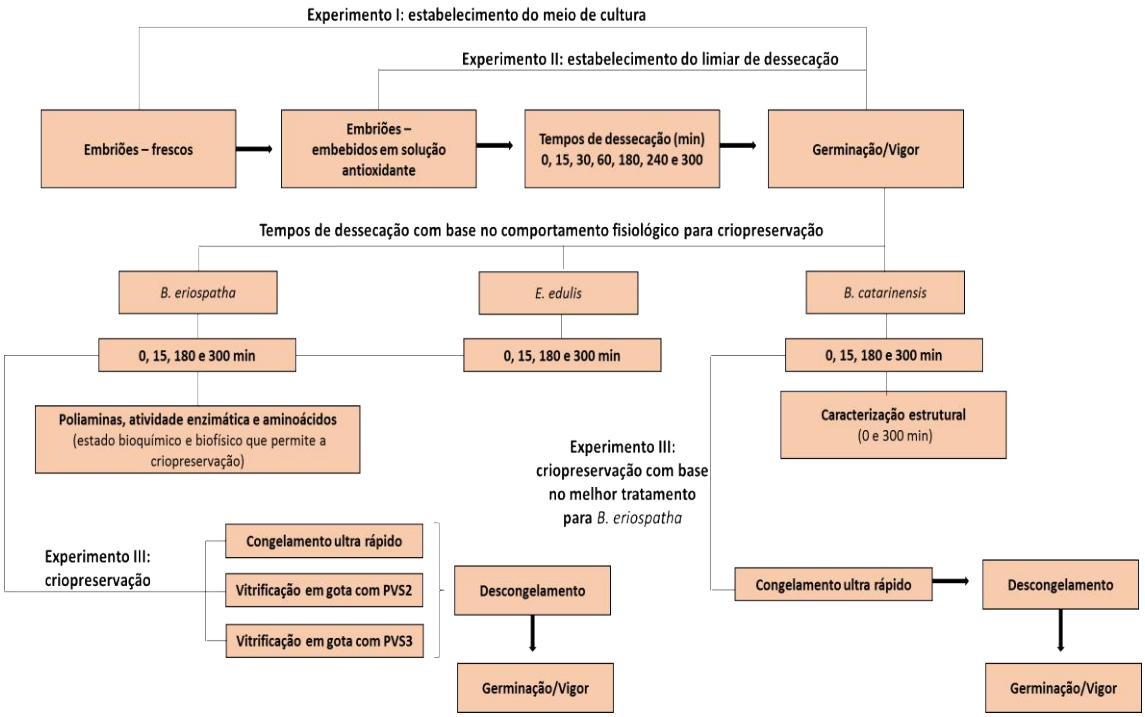

Figure 1: Ensaio dos experimentos para definição do limiar de dessecação e protocolos de criopreservação em embriões de *B. catarinensis, B. eriospatha* e *E. edulis*

2. OBJETIVO GERAL

Estudar aspectos fisiológicos, estruturais e bioquímicos do limite de tolerância a dessecação e da criopreservação de embriões de *B. eriospatha*, *B. catarinensis,* e *E. edulis* visando estabelecer protocolos para estratégias de conservação *ex situ* de sementes de Arecaceae tropicais.

2.1. Objetivos específicos e questões norteadoras da pesquisa

Questão norteadora

Qual o limiar de dessecação dos embriões de B. eriospatha, *B. catarinensis,* e *E. edulis?*

i. Avaliar e determinar o limite de tolerância a dessecação de embriões de *B. catarinensis, B. eriospatha* e *E. edulis.*

Questão norteadora

Qual a relação entre a atividade das enzimas antioxidantes, das poliaminas e dos aminoácidos em embriões de B. eriospatha e o limite de tolerância a dessecação?

ii. Caracterizar e avaliar a atividade das enzimas antioxidantes (catalase, ascorbato peroxidase, guaicol peroxidase e superóxido dismutase) o perfil de poliaminas e aminoácidos em embriões de *B. eriospatha* e durante a dessecação.

Questão norteadora

Embriões de B. catarinensis apresentam dormência morfofisiológica?

iii. Testar o efeito de GA3 na germinação de embriões de *B. catarinesis*.

Questão norteadora

Quais as alterações citoquímicas e ultraestruturas em embriões de B. catarinenses durante o processo de criopreservação?

iv. Caracterizar alterações morfo-estruturais (microscopia de luz e microscopia eletrônica de transmissão) em embriões de *B. catarinenses*, durante a dessecação e criopreservação.

Questão norteadora

É possível criopreservar embriões de B. catarinensis, B. eriospatha e *E. edulis?*

v. Desenvolver protocolos de criopreservação para embriões de *B. catarinensis, B. eriospatha* e *E. edulis.*

Questão norteadora

O uso de crioprotetores (PVS2 e PVS3) em embriões de B. eriospatha e *E. edulis permite ou melhoram a taxa de sobrevivência após o processo de criopreservação?*

vi. Avaliar a taxa de sobrevivência e geminação e as alterações estruturais nas células dos embriões de *B. eriospatha* e *E. edulis* submetidos ao processo de criopreservação.

3. REFERENCIAL BIBLIOGRÁFICO

3.1. A família Arecaceae

A família Arecaceae engloba aproximadamente 181 gêneros e 2.600 espécies (Uhl e Dransfield 1987; Baker e Dransfield 2016). A alta diversidade, assim como, a distribuição dessas espécies, ocorrem principalmente nas zonas tropicais e subtropicais, regiões essas, em que as palmeiras têm uma grande importância ecológica e econômica (Balick e Beck 1990; Lorenzi et al. 1996; Bourscheid et al. 2011). No continente americano encontram-se 87 gêneros e aproximadamente 1.440 espécies, das quais cerca de 37 gêneros e 386 espécies estão dispersos na flora brasileira (Flora do Brasil, 2023) . As Arecaceae são reconhecidas como uma família ameaçada de extinção, com um total de 670 espécies catalogadas na *Red List - International Union for Conservation of Nature e Natural Resources* (IUCN 2020). Na Lista de espécies da Flora do Brasil ameaçadas de extinção, a família Arecaceae contribui com 49 espécies, sendo que, o *E. edulis*, o e o *B. eriospatha*, estão enquadrados na categoria de "vulnerável" (VU - *vunerable*) (Heiden et al. 2020). Apesar de não listado na Lista de Espécies da Flora do Brasil Ameaçadas o *B. catarinensis* encontra-se na categoria "em perigo" (EN) da Lista Oficial das Espécies da Flora de Santa Catarina Ameaçadas de Extinção.

As espécies *B. catarinensis, B. eriospatha* e *E. edulis* estão dispersas na Mata Atlântica Brasileira (Leitman et al. 2015), um dos *hotspots* de biodiversidade e um dos biomas mundiais mais biodiversos, ameaçados e com alto nível de endemismo (Myers et al. 2000; Myers 2003). Devido a essas características, a Mata Atlântica Brasileira é considerada como área prioritária global para conservação (Myers et al. 2000; Mittermeier et al. 2005). Esse bioma é altamente ameaçado pelo desmatamento, exploração madeireira e pela conversão das áreas florestais em áreas de agricultura e pecuária (Myers et al. 2000; dos Reis et al. 2002; Sampaio e Guarino 2007; Ribeiro et al. 2009; Vibrans et al. 2011). Diante deste cenário de declínio da Mata Atlântica, estudos que auxiliem na conservação de espécies que compõem este bioma são de extrema importância.

Em geral as espécies de Aracaceae têm como principal e mais eficiente forma de propagação as suas sementes, no entanto, a sensibilidade à dessecação dificulta seu armazenamento em sistemas convencionais (Bovi e Cardoso 19778; Zona et al. 2007; Wen 2019). Logo, suas sementes são cultivadas com frequência em jardins botânicos, os quais são os maiores responsáveis pela conservação *ex situ* a longo prazo das palmeiras (Johnson 1996; Maunder et al. 2001). Assim, estratégias que visam a redução do metabolismo aliadas à criopreservação são alternativas para a conservação das sementes de palmeiras, minimizando custos da implantação e manutenção das coleções a campo, ou até mesmo como cópia de segurança desses materiais, os quais estão expostos a catástrofes ambientais.

3.1.1. O estado da arte das espécies alvo

Pertencente à subtribo Butiinae, o gênero *Butia* está distribuído nas regiões Sul, Centro-Oeste e Sudeste do Brasil, no Paraguai, Argentina e Uruguai (Noblick 2010). Vários são os usos potenciais e tradicionais atribuídos às espécies do gênero *Butia*, como o consumo *in natura* de seus frutos, ou a utilização desses no preparo de licor, cachaça, suco, geleia, bolo, bombom, recheio de doces e sobremesas como sorvete (Büttow et al. 2009). Estudos vêm incentivando a inserção da amêndoa do butiá no cardápio alimentar, devido a

seus valores nutricionais, fornecendo fibras, pró-vitamina A, vitamina C e potássio (Faria et al. 2008). Além disso, o uso ornamental, medicinal e em rituais de cunho religioso lhes agregam um maior valor cultural (Macía et al. 2011).

O Brasil possui a maioria das espécies do gênero *Butia*, 18 das 20, e dentre essas espécies o *B. eriospatha* (Fig. 2a, d) popularmente conhecido como "butiá-da-serra", é endêmico da Floresta Ombrófila Mista, distribuído exclusivamente em Campos Naturais do Paraná, Santa Catarina e Rio Grande do Sul, em regiões de alta altitude (Reitz 1974; Noblick 2010; Elias et al. 2016b; Heiden et al. 2020). No entanto, nas últimas décadas os Campos Naturais da região Sul do Brasil ocorreu uma redução de 25% na área total, principalmente devido à expansão agropecuária (Overbeck et al. 2007; Hoffmann et al. 2014). Além da perda de habitat, o *B. eriospatha* vem sofrendo com a pressão da extração e venda ilegal, tanto dos frutos quanto das plantas, as quais são muito usadas ornamentalmente, essas ações comprometem a conservação da espécie (Barbieri et al. 2014; Elias et al. 2016a). Já *B. catarinensis* (Fig. 2b, e) ainda não foi avaliado quanto à ameaça de extinção no Brasil, embora Elias et al. (2019) alertam para a necessidade de atualizações desta lista, sugerindo que a alocação do *B. catarinensis* na categoria "em perigo" (EN), uma vez que sua distribuição geográfica é restrita a uma área de ocupação inferior a 2.000 km². Além disso, a área de Restinga, vegetação nativa onde a espécie ocorre, está sob constante pressão urbana e fragmentação (Scherer et al. 2005; Ribeiro e Melo Junior 2016). Dentre os fatores de degradação, incluem a introdução de espécies exóticas que se tornaram invasoras, a remoção da vegetação para a construção de rodovias, a extração de areia e o extrativismo seletivo de plantas de importância econômica, poluição do solo e da água (Marques et al. 2015). Segundo Coradin et al. (2011), dentre as 149 espécies com alto valor comercial atual ou potencial, o *B. catarinensis* é uma das cinco espécies prioritárias nos grupos das alimentícias e fibrosas.

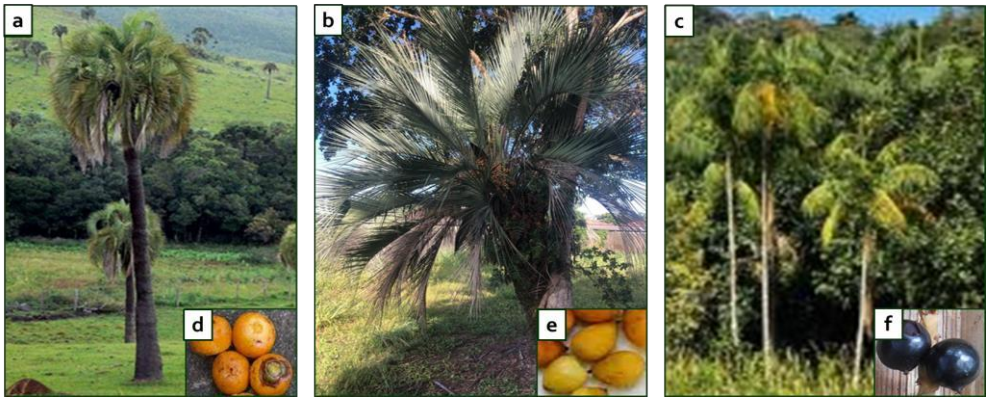

Figure 1: Espécies da família Arecaceae. *Butia catharinenses* **(a)**; *Butia eriospatha* **(b)** e *Euterpe edulis* **(c)**; em detalhe seus respectivos frutos maturos **(d, e** e **f).**

Euterpe edulis (Fig. 2c, f) conhecido como "palmito juçara" tem ampla distribuição, com ocorrência em toda a Floresta Ombrófila Densa (CNCFlora 2012; Elias et al. 2016a) é amplamente utilizada na alimentação humana (palmito, botões florais e os frutos, incluir bibliografias sobre o uso recente da polpa), na construção civil, confecção de artesanatos, apicultura, , paisagismo e produção de celulose (Carvalho 2003; Bourscheid et al. 2011). Devido aos diversos usos é considerado como a principal espécie fonte de produtos florestais não-madeireiros (PNMs) da Mata Atlântica, e também está entre as mais ameaçadas (Reis et al. 2000). Apesar de uma extensa área de ocupação, desde o final da década de 1970, a espécie sofreu com os altos níveis de exploração predatória do palmito, comprometendo populações inteiras (Reis

et al. 2000; Rodrigues e Durigan 2007). Por lei, a atividade de corte *E. edulis* no estado de Santa Catarina, só é permitida quando atende os pré-requisitos estabelecidos pela Resolução CONAMA Nº 294, de 12 de dezembro de 2001. Uma vez que a espécie está classificada na lista da Secretaria do Estado do Meio Ambiente como "vulnerável" (VU – *vulnerable*), devido ao crescimento lento e a dependência de florestas bem preservadas (CNCFlora 2017). Segundo Bovi e Cardoso (1978) sementes de *E. edulis* demoram cerca de 60 a 90 dias para germinarem, o que pode estar relacionado a espessura do endocarpo (Panza et al. 2002). No entanto, dormência fisiológica também pode ser relatada para essa espécie, pois estudos mostram que ácido giberélico (GA_3) exógeno na concentrações de 40 a 60 mgL^{-1} aumentou o desempenho germinativo em sementes de *E. edulis* (Roberto e Habermann 2010).

A degradação das florestas associada à exploração indiscriminada ainda está avançando, comprometendo a continuidade e a sobrevivência dessa espécie. Além da importância social, econômica e ecológica das espécies *B. eriospatha*, *B. catarinensis* e do *E. edulis*, entende-se que é necessário o desenvolvimento de pesquisas que visem elucidar aspectos fisiológicos e bioquímicos de suas sementes em busca de alternativas para a conservação a longo prazo dessas espécies, uma vez que as sementes é a melhor forma de propagação.

3.2. Germinação *in vitro* para a superação da dormência

A propagação de *Butia* spp. e *E. edulis* ocorrem exclusivamente por meio da dispersão do diásporo, o qual é composto por um endocarpo rígido envolvendo a semente (Ecología 2003; Ribeiro et al. 2011; Magalhães et al. 2013; Oliveira et al. 2013). Por décadas acreditava-se que resistência mecânica imposta pelo endocarpo, era o principal motivo da dormência, que resultava em baixos índices de germinação (menor que 20%), a qual era lenta e desuniforme, devido a impermeabilidade do oxigênio, água e a limitação do crescimento do embrião (Broschat 1998). Por outro lado, a remoção dessa estrutura teve pouca influência nos índices de germinação (Fior et al., 2004). Atualmente, uma reclassificação da dormência foi proposta, sugerindo que o diásporo dormente é devido a embriões subdesenvolvidos (10% do tamanho total do diásporo), portanto, dormência morfológica, ou então por restrição fisiológica, devido a um desequilíbrio hormonal ou incapacidade do embrião de empurrar o endocarpo duro (Fig. 3) (Baskin e Baskin 2004; Jaganathan 2020).

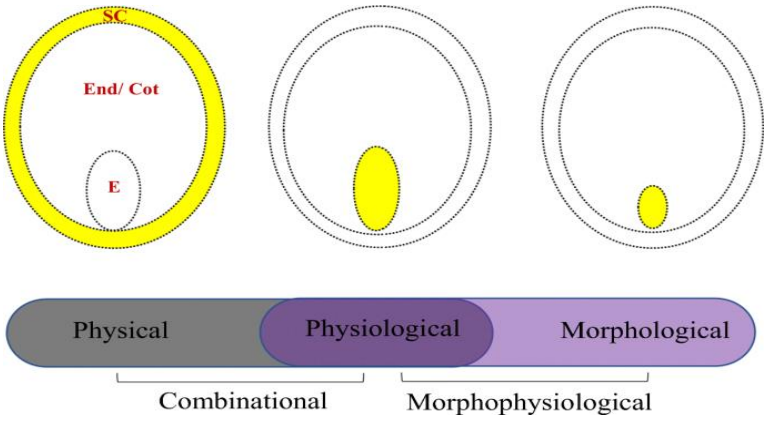

Figure 2: Representação de classes de dormência de sementes de Aerecaceae. Tipo de dormência: em amarelo. E: embrião; End/coat: endosperma/cotilédone; SC: revestimento da semente (Baskin e Baskin 2004; Jaganathan 2020)

A germinação via técnicas de cultura de tecidos *in vitro* permite o isolamento de embriões zigóticos, sendo uma técnica promissora para superação das limitações impostas pelo endocarpo (Ecología 2003; Ribeiro et al. 2011; Magalhães et al. 2013; Oliveira et al. 2013). Além disso, o cultivo do embrião em meio nutritivo, suplementado com fitorreguladores (2,4 D e GA_3), tem garantindo a produção de propágulos para produção direta de mudas ou como fornecedores de explantes para micropropagação (Waldow et al. 2013; Ferreita et al. 2017). Dessa forma, o cultivo *in vitro* de embriões isolados permite conservação *ex situ* e a produção de mudas em larga escala para seu uso no controle da erosão genética e conservação de populações naturais.,

3.3. Tolerância à dessecação e seus limiares

A água constitui a maior parte do volume celular das plantas, sendo um componente essencial para as reações químicas, além de contribuir para a estabilidade de outras moléculas como as proteínas, o DNA, os lipídios e as membranas celulares (Vertucci e Farrant 1995; Ballesteros e Walters 2011; Dekkers et al. 2015). Devido à importância e as funções da água nas células vegetais, a compressão de como cada espécie adquire ou não capacidade de sobreviverem sob quantidades limitadas de água envolve muita pesquisa, as quais são limitadas para cada espécie de forma particular.

Sementes de angiospermas apresentam três estágios de desenvolvimento, a fase histodiferenciação: - caracterizada por intensa atividade metabólica e sensibilidade acentuada à dessecação; a fase de acúmulo de reservas (maturação): - em sementes ortodoxas é caracterizada por um platô em massa seca e declínio significativo do teor de água a níveis muito baixos, sendo considerado um dos pré-requisitos para aquisição de tolerância à dessecação; e a fase de dessecação: - característica das sementes tolerantes à dessecação (Bewley et al. 2013a). Uma semente tolerante à dessecação tem a habilidade de se manter em equilíbrio entre o conteúdo de água de 0,05 a 0,15 g H_2O gMS^{-1} e a umidade relativa do ar, e assim, sobreviver por longos períodos sem acumulo de dano letal (Vertucci e Farrant 1995; Alpert 2005).

Cinco níveis de hidratação nos tecidos das sementes foram descritos, e em cada um desses níveis a água se apresenta com diferentes propriedades físicas e como consequência as sementes exibem diferentes estados metabólicos (Vertucci 1990). Nível (v): teores de água > 0,75 gH_2O gMS^{-1}, as propriedades da água no tecido são semelhantes às de uma solução diluída a chamada "água livre"; a taxa metabólica é alta, proporcionando a germinação das sementes. Nível (iv): teores de água entre 0,75 – 0,45 gH_2O gMS^{-1}, as propriedades da água se semelham a de uma solução concentrada e a interação entre água e solutos se torna mais forte, este conteúdo de água é inadequado para o crescimento e germinação, mas ocorre respiração e a síntese de proteínas e ácidos nucléicos. Nível (iii): teores de água entre 0,45 – 0,25 gH_2O gMS^{-1}, a síntese de proteína e ácido nucleico não é significativa, mas ainda possibilita a respiração celular. Nível (ii): teores de água entre 0,25 – 0,08 gH_2O gMF^{-1} a solução torna-se concentrada e viscosa, possuindo propriedades de vidro, onde apenas eventos catabólicos de baixo nível são produzidos. Nível (i): teores de água <0,08 gH_2O gMS^{-1} a água está fortemente associada às superfícies macromoleculares e sua mobilidade é reduzida a chamada de "água ligada", como consequência não há atividade metabólica (Walters 2015; Zaritzky 2015).

Nas duas últimas décadas, pesquisas trouxeram à tona a importância de conhecer os limiares da tolerância à dessecação, afim de classificar o comportamento dessas sementes e estabelecer estratégias de conservação (Walters 2015; Walters e Pence 2020). Em um extremo estão as sementes tolerantes à dessecação – chamadas de ortodoxas, como por exemplo as culturas base da alimentação humana, como *Oriza sativa*, *Triticum* spp.; *Zea mays*; *Glycine max*; *Phaseolus vulgaris* e *Helianthus annuus*, já no outro extremo as sementes sensíveis à dessecação – chamadas de recalcitrantes, como por exemplos de *Camellia sinensis*, *Castanea sativa; Castano- spermum australe; Quercus robur* e *E. edulis* (Radwan et al. 2014).

Sementes tolerantes à dessecação dispersam em baixo teor de água (abaixo de 0,15 $gH_2O\ gMS^{-1}$), se tornam quiescentes após a dispersão e podem sobreviver à dessecação (0,05 $gH_2O\ gMS^{-1}$), sem perda do vigor, essas características permitem seu armazenamento a baixa temperatura por longos períodos (Berjak e Pammenter 2008). Além disso, o baixo teor de água permite a essas sementes o armazenamento em temperaturas congelantes, sem causar danos, pois, a água presente nas células entra em um estado vítreo evitando a formação de cristais de gelo letais (Ballesteros et al. 2019). Em contraste, as sementes sensíveis à dessecação são dispersas com elevados teores de água e metabolismo altamente ativo, como consequência essas sementes não podem ser conservadas usando abordagens de banco de germoplasma padrão (Walters 2015). Devido ao metabolismo ativo no estado hidratado, a desidratação de tecidos de sementes sensíveis à dessecação produz a deterioração da membrana (plasmalema e mitocôndria) e desnaturação de proteínas (Walters et al. 2010, 2013). Evidências sugerem que tais sementes são programadas para iniciar a germinação durante ou logo após a dispersão (Farrant et al. 1993; Berjak e Pammenter 2013). Uma vez que, as espécies de plantas que produzem esse tipo de sementes evoluíram e se adaptaram a ambientes nos quais as condições são favoráveis à germinação imediata, ou seja, na mesma estação quando as sementes são dispersar, por ex. florestas tropicais com uma estação chuvosa constante e abundante (Tweddle et al. 2003; Wyse e Dickie 2017). Adicionalmente, as sementes sensíveis à dessecação carecem ou não expressam os vários processos e mecanismos que caracterizam a aquisição de tolerância à dessecação, como ocorre nas sementes tolerantes à dessecação.

A distinção ortodoxo-recalcitrante foi uma dicotomia importante para empreendimentos de bancos de sementes (FAO 2013). No entanto, um grande grupo de sementes, especialmente as de espécies tropicais, não fazem parte desta dicotomia e foram denominadas de sementes intermediarias (Ellis et al. 1990; Hong e Ellis 1996; De Vitis et al. 2020). Esta categoria foi proposta por Ellis et al. (1990), estudando a dessecação e o armazenamento de quatro variedades de *Coffea arabica* L. e concluiu que as sementes que apresentam este comportamento sobrevivem à dessecação, mas não sobrevivem aos efeitos combinados de dessecação e baixa temperatura. Estudos buscando elucidar os danos deletérios desse tipo de sementes, quando submetidas à baixas temperaturas, nos levam a crer que as interações de água e triacilgliceróis cristalizados são os grandes responsáveis pela morte de suas células (Crane et al. 2003, 2006; Walters et al. 2005; Volk et al. 2006, 2007). A classificação intermediária também tem sido usada para descrever sementes que sobrevivem aos estresses iniciais de dessecação e baixa temperatura, mas envelhecem rapidamente (De Vitis et al. 2020).

3.4. Respostas estruturais e bioquímicas envolvidas na sensibilidade à dessecação

A maioria dos tecidos hidratados, tais como as sementes sensíveis à dessecação, não suportam a desidratação necessária para que as suas células entrem em um estado de vitrificação, ou seja, um conteúdo de água de no máximo 20%, o qual permite o seu armazenado em sistemas convencionais (Panis and Lambardi 2005; Walters e Pence 2020). Estudos demonstram que a viabilidade de sementes de *E. edulis* começa a decrescer com aproximadamente 39% do teor de água da semente e é definitivamente perdida com 21% do conteúdo de água (Reis et al. 1999; Andrade 2001; Panza et al. 2007). Na literatura não se encontram relatos sobre limiar de dessecação para as espécies *B. eriospatha* e *B. catarinensis,* porém em sementes de *Butia capitata* (Mart.) Becc. a redução do conteúdo de água a 10 e 5% não afetou a viabilidade das sementes (Dias et al. 2015).

Em sementes tolerantes à dessecação, pelo menos quatro tipos de mecanismos agem sinergicamente para mitigar os efeitos deletérios da dessecação: (i) prevenção de danos oxidativos: a partir do acúmulo de compostos antioxidantes; (ii) evitar danos estrutural: a partir da modificação da parede celular, reorganização das membranas intracelulares e do citoesqueleto, e condensação da cromatina; (iii) estabilização de membranas e proteínas: por meio de açúcares não redutores, proteínas abundantes em embriogênese tardia (LEA) e proteínas de choque térmico (HSP); e (iv) sistemas eficientes de desintoxicação e reparação do DNA e de proteínas danificados durante a reidratação (Hoekstra et al. 2001; Leprince e Buitink 2010; Gaff e Oliver 2013; Dussert et al. 2018).

Nas sementes sensíveis à dessecação três tipos de danos levam a perda da viabilidade: (i) dano mecânico, associado à redução volumétrica da célula e ao colapso de vacúolos, assim como a baixa capacidade de reestruturação das membranas durante a embebição; (ii) dano induzido pelo metabolismo, associado ao acúmulo de EROs (Espécies Reativas ao Oxigênio), devido à atividade metabólica, alta taxa de respiração e falha do sistema antioxidante o que leva a peroxidação lipídica da membrana e (iii) desnaturação macromolecular, associado à não de-diferenciação macromolecular na maturação (Pammenter e Berjak 2000; Umarani et al. 2015).

3.5.1. Respostas estruturais

Entre os primeiros desafios enfrentados durante o processo de dessecação, em especial sob estresse mais severo (< 2.5 MPa) estão as perturbações mecânicas em resposta a retração e expansão do conteúdo celular (Walters 2015). Células com baixo conteúdo de matéria seca são altamente vacuoladas e retraem mais quando comparadas as células com acúmulo de reservas (Vertucci e Farrant 1995; Farrant et al. 1997). Walters e Koster (2007) descreveram que cerca de 85% do volume de uma célula moderadamente vacuolada é ocupado por água, quando exposta a -5 MPa uma redução de 75% no volume ocorre, por outro lado, uma célula carregada com corpos proteicos e grãos de amido, a água ocupa cerca de 40% do seu volume e quando exposta ao mesmo nível de dessecação, o volume da célula diminui em cerca de 22% (Fig. 4). Como consequência da retração brusca da célula, o plasma pode se desintegrar da parede celular, interrompendo as comunicações intercelulares e os sistemas de membrana podem ficar muito próximos e alterar a capacidade de partição dentro da célula (Berjak e Pammenter 2008). Com o desmantelamento das

membranas celulares, as respostas de defesa e as reações necessárias para o funcionamento do metabolismo integrado são comprometidos (Hyman e Simons 2012).

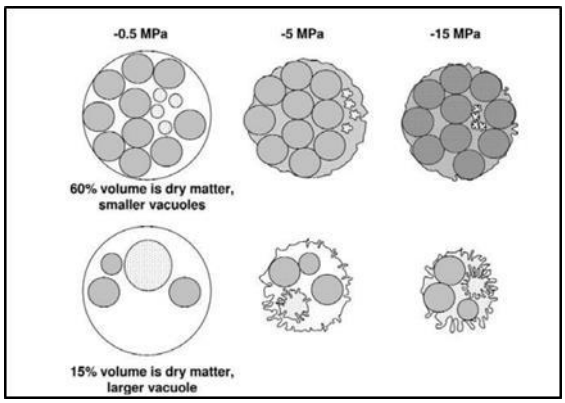

Figure 3: Representação das células contendo diferentes quantidades de matéria secas a medida em que a água é reduzida (Walters 2015).

Além disso, no processo embriogênico, em especial durante a fase de maturação, as células de semente tolerantes à dessecação passam por um platô em massa seca e um declínio significativo do teor de água a níveis muito baixos (Bewley et al. 2013a). Este evento possibilita as células de sementes tolerantes à dessecação resistirem as perturbações mecânicas durante a perda de água (Vertucci e Farrant 1995; Farrant et al. 1997; Walters e Koster 2007). A condensação da cromatina também está associada à aquisição de tolerância à dessecação, uma vez que cessa eventos de replicação e transcrição, reduzindo os processos de desdiferenciação das organelas e dos elementos do sistema endomembrana, os quais são alvos geração de EROs (Van Zanten et al. 2011).

3.5.2. O papel das enzimas no sistema antioxidante

Durante o processo de dessecação ocorre o acúmulo das chamadas espécies reativas de oxigênio (EROs), sendo elas: oxigênio singlete ($^1O_2$), radicais superóxido (O_2^{-1}), peróxido de hidrogênio (H_2O_2) e radicais hidroxila (OH^{-1}), essas moléculas são altamente reativas e tóxicas, com potencial de danificar membranas celulares, ácidos nucléicos, proteínas, carboidratos e lipídios, causando danos irreversíveis aos sistemas celulares (Gill e Tuteja 2010; Sharma et al. 2012). A peroxidação lipídica mediada por radicais livres e o rompimento das membranas celulares foram relatados como os principais danos durante a dessecação e armazenamento de sementes (Fu et al. 2015).

À medida que as espécies reativas de oxigênio aumentam, os mecanismos de defesa antioxidante enzimáticos e não enzimáticos são ativados, as principais enzimas envolvidas nesse mecanismo são superóxido dismutase (SOD), catalase (CAT), ascorbato peroxidase (APX), glutationa peroxidase (GPX), peroxidase (POD) peroxirredoxina (Prx), monodesidroascorbato redutase (MDHAR), desidroascorbato redutase (DHAR), e glutationa redutase (GR) (Gill e Tuteja 2010; Mittler 2017). A carência do sistema antioxidante enzimático foi o responsável pelo acúmulo de EROs, levando à perda do vigor e a viabilidade das sementes de *Azadirachta indica* (Sahu et al. 2017), *Vicia faba* (Arrigoni et al. 1992), *Triticum durum* (De Gara et al. 2003), *Hordeum vulgare* (Yin et al. 2014), *Avena sativa* (Kong et al. 2015). Sementes tolerantes à dessecação apresentam alta atividades de CAT e GR e baixa atividades de SOD e APX, enquanto o reverso é observado em sementes sensíveis à dessecação (Bailly et al. 2001).

Superóxido dismutase (SOD) é a primeira enzima a exercer atividade antioxidante, realizando a dismutação do O_2^{1-} em H_2O_2, embora H_2O_2 ainda seja tóxico para a semente, as demais enzimas como CAT e APX são responsáveis em reduzir o H_2O_2 em água (H_2O) (Fig. 4) (Das e Roychoudhury 2014). O ciclo ascorbato-glutationa também participa na redução do H_2O_2 e este além das enzimas chaves envolvem: monodeidroascorbato redutase (MDAR), desidroascorbato redutase (DHAR) (Fig. 5). Além de eliminar de forma direta os radicais livres, essas enzimas participam da regeneração dos poderosos antioxidantes-não enzimáticos, tais como o ácido ascórbico (vitamina C), glutationa reduzida e α-tocoferol (vitamina E) (Fig. 5) (Bailly et al. 2001).

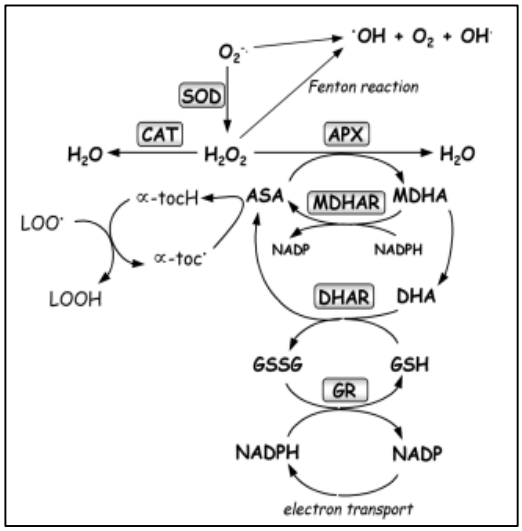

Figure 4: Principais sistemas de desintoxicação em plantas. Superóxido dismutase (SOD) dismuta o O_2^{1-} em H_2O_2. Catalase (CAT) e ascorbato peroxidase (APX) reduz o H_2O_2 em água H_2O. Ciclo ascorbato-glutationa: ascorbato peroxidase reduz o H_2O_2 em H_2O usando o ascorbato (ASA), o ascorbato oxidado (monodeidroascorbato – MDHA) é regenerado pela ação da monodeidroascorbato redutase (MDHA), quando a regeneração não o MDHA é dismutado a desidroascorbato (DHA) o qual é reduzido a ASA pela ação da desidroascorbato redutase (DHAR) usando a glutationa reduzida (GSH) e produzindo a produzindo glutationa oxidada (GSSG), finalmente, o GSSG é reduzido pela ação da glutationa redutase (GR) usando o NADPH como doador de elétrons. α-tocH, α-tocoferol; α-toc·, α-tocoferil; LOOH, peróxido de lipídio; LOO·, radical lipídico (Bailly et al., 2001).

3.3.3. O papel das poliaminas (PAs)

As poliaminas (PAs) fazem parte de um grupo de substâncias, os quais incluem compostos nitrogenados alifáticos carregados positivamente, e podem ocorrer na forma livre ou conjugada com ácidos fenólicos e moléculas de baixo peso molecular (Bouchereau et al. 1999; Wallace et al. 2003). As PAs mais abundantes nas plantas são a putrescina (PUT), a espermidina (SPD) e a espermina (SPM), aos quais são encontradas em quantidades relativamente altas quando comparadas aos hormônios (Bano et al. 2020). Elas atuam em processos que ocorrem durante o crescimento e desenvolvimento das plantas, como por exemplo, divisão e diferenciação celular (Bouchereau et al. 1999; Davies 2004), alongamento radicular (Su et al. 2006), desenvolvimento e amadurecimento de frutos (Davies 2004), apoptose (morte celular programada) (Taylor et al. 2007), síntese de DNA, transcrição gênica, tradução proteica e organização da cromatina (Feng et al. 2011). Mas, nos últimos anos, os trabalhos vem mostrando uma forte relação das PAs com à resistência a estresses abióticos (hídrico, térmico e salino) em plantas (Alcázar et al. 2006, 2010; Yamaguchi et al. 2007; Yang et al. 2007; Liu et al. 2011; Sagor et al. 2013; Chen et al. 2019; Bano et al. 2020). A interação entre PAs e estresse abiótico em plantas e seus papéis aparentemente contraditórios no processo foram documentados ao longo dos anos (Hussain et al. 2011; Shi e Chan 2014; Bano et al. 2020).

Um dos mecanismos pelo qual as PAs auxiliam na tolerância ao estresse abiótico se deve as suas características de molécula policatiônica, logo, estabiliza por meio de ligações os compostos celulares poliânions, tais como DNA, RNA, fosfolipídios, resíduos de proteínas ácidas e componentes da parede celular (Bouchereau et al. 1999; Wallace et al. 2003). Outro mecanismo de proteção é desencadeado pelo

catabolismo das PAs, uma vez que resulta em uma maior produção de H_2O_2 levando à ativação de respostas ao estresse, no entanto o excesso desta EROs pode resultar em morte celular programada, assim, as plantas devem coordenar fortemente as vias anabólicas e catabólicas dos PAs para garantir a homeostase celular sob estresse (Moschou et al. 2008; Saha et al. 2015). Sob estresse moderado, as plantas podem superar o estouro de EROs por meio do complexo de enzimas antioxidantes, as quais são induzidos pelo próprio estresse e/ou pelo acúmulo de PAs e catabolismo (Moschou et al. 2008; Yu et al. 2019). Apesar do número crescente de estudos visando elucidar a relação de PAs e sistema antioxidantes, muitos autores descrevem o mecanismo de atuação de forma equivocada, onde defendem a hipótese de que as PAs atuam simultaneamente as enzimas antioxidantes, como sequestradores de radicais, estabilizadores de membrana e inibidores da peroxidação lipídica (Gill e Tuteja 2010; Gupta et al. 2013; Sánchez-Rodríguez et al. 2016; Yu et al. 2019). Conforme revisado por Minocha (2014), os pontos exatos de interação entre PAs e EROs estão longe de ser totalmente compreendidos e permanecem como um dos fenômenos bioquímicos mais curiosos e complexos que ocorrem nas células vegetais. No entanto, seus efeitos de melhoria na superacumulação de EROs estão muito provavelmente relacionados às suas características químicas, combinando suas propriedades de ligação aniônica/catiônica neutralizantes de ácido (Gupta et al. 2013). Outro exemplo que elucida o papel de sinalização dos PAs é o seu envolvimento na regulação epigenética da expressão gênica, uma vez que elas demonstraram modular a estrutura da cromatina (Paul et al. 2018). Sua ligação à cromatina pode interromper o acesso das polimerases a certas regiões genômicas e levar a mudanças na expressão gênica. Além disso, as PAs são capazes de interagir com agentes modificadores e remodeladores da cromatina e também são capazes de promover ou inibir a atividade enzimática, levando a modificações pós-tradução da histona como acetilação, desacetilação, metilação, desmetilação e assim por diante (revisado por Paul et al. (2018) e Tiburcio et al. (2014)).

Espécies de plantas sensíveis ao estresse biótico, como a seca, tiveram seus teores de PUT geralmente aumentado, o que se reflete em uma diminuição da relação (SPM+SPD)/PUT, sendo essas mudanças geralmente acompanhadas pela geração de EROs (Wang et al. 2007; Groppa e Benavides 2008; Paul et al. 2018). Em contraste, espécies e cultivares tolerantes ao estresse são geralmente capazes de manter níveis mais elevados de SPD e SPM sob estresse, enquanto os níveis de PUT permanecem relativamente baixos, o que pode implicar em sua maior resiliência (Sánchez-Rodríguez et al. 2016). O acúmulo excessivo de PUT nas células sob estresse pode causar graves efeitos negativos, como a despolarização das membranas, levando ao extravasamento de potássio, necrose tecidual e perda de proteínas, principalmente nos tecidos foliares. Por outro lado, SPD e SPM têm efeitos anti-senescência sob estresse, sendo cruciais para preservar a integridade das membranas tilacóides (Zhao et al. 2008).

Encontramos na literatura um apanhado de trabalhos da relação de PAs e o estresse abiótico em plantas, porém estudos que investigam as funções das PAs especificamente em sementes submetidas a estresse ainda são escassos. Em sementes de *Medicago sativa* germinadas em diferentes concentrações de PEG - 4000 (polietileno glicol) e tratadas com PUT apresentaram uma melhora no índice de germinação, no crescimento e um aumento na atividade das enzimas hidrolíticas (Zeid e Shedeed 2006). Huang et al. (2017) descobriram um aumento significativo no vigor, quando embebidas em SPD. Enquanto sementes de arroz submetidas ao estresse de alta temperatura a SPD exógena proporcionou um aumentou na germinação

e no vigor (Fu et al. 2019). Estes autores acreditam que a SPD exógena esteja intimamente relacionada ao metabolismo hormonal, e com o aumento de H_2O_2. A SPD pode desempenhar funções duplas nos fenômenos de tolerância ao estresse nas plantas, uma como um composto direto de proteção ao estresse e a outra como um regulador de sinalização do estresse (Kasukabe et al. 2004). Para o estabelecimento de um protocolo de criopreservação o material requer um grau de tolerância às mudanças bruscas na temperatura, estudos mostram que a SPM exógena pode proteger a planta dos danos causados pelo choque térmico, através da ativação de diferentes fatores de transcrição do choque térmico (Sagor et al. 2013). Segundo Kasukabe et al. (2004) as plantas tolerantes ao estresse geralmente têm uma grande capacidade de melhorar a biossíntese de PAs, produzindo 2 a 3 vezes mais, comparado com as plantas não estressadas. Viera (2019) observou uma correlação positiva moderada entre mudanças nos níveis de SPD e atividade de CAT e APX como resposta a dessecação (dados não publicados). O que nos leva a acreditar que as PAs, possam ter um papel fundamental na resposta a tolerância a dessecação em sementes.

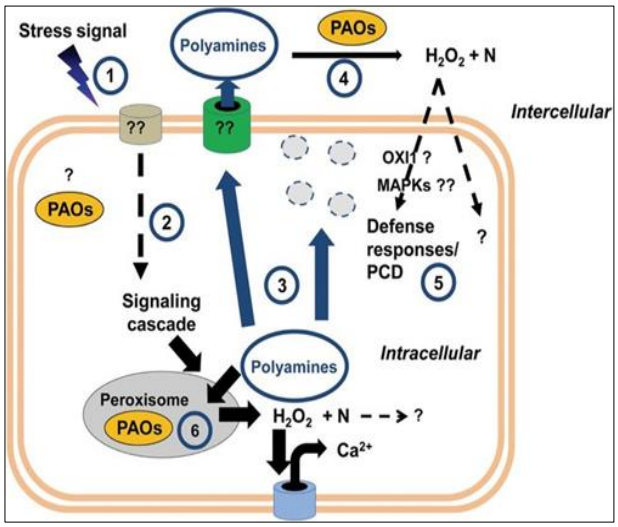

Figure 5: Modelo para as possíveis funções de oxidação de PAs em plantas. (1) Um sinal de estresse é percebido por um receptor ainda desconhecido. (2) O sinal é transduzido por várias vias celulares. (3) A via de transdução leva ao aumento dos níveis intracelulares de PAs, as quais são secretadas. (4) As PAs são oxidadas por PAOs apoplásticos para produzir H_2O_2 + nitrogênio (N). (5) H_2O_2 e N podem direcionar respostas adicionais, através de diferentes cascatas (proteínas quinases ativadas por MAPKs ou Oxidative Signal Inducible 1 (OXI1). Dependendo dos níveis de H_2O_2 produzidos: PCD é induzido ou H_2O_2 é eliminado de forma eficiente, pela ascorbato peroxidase (APX). (6) Nos peroxissomos, as PAs são convertidas de volta com a produção simultânea de H_2O_2 e outras moléculas nitrogenadas. - H_2O_2 produzido por essa via pode ativar canais permeáveis ao Ca_2^+. Por outro lado, o papel das moléculas N produzidas adicionalmente pela via de conversão reversa do PA ainda não foi elucidado (Moschou et al. 2012).

3.3.4. O papel dos aminoácidos (AAs)

Os aminoácidos (AAs) são biomoléculas base para a síntese de proteínas, além de servir como fonte de nitrogênio orgânico para a síntese de uma grande variedade de compostos, como, nucleotídeos, clorofila, hormônios e metabólitos secundários (Tegeder 2012). Os AAs são precursores de muitos metabólitos primários e secundários envolvidos nas funções metabólicas centrais, bem como, na proteção das plantas contra estresses bióticos e abióticos (Fait et al. 2006; Angelovici et al. 2011; Ali et al. 2019). Os níveis e a composição dos AAs nos tecidos vegetativos mudam consideravelmente em resposta ao estresse, geralmente tem seus níveis aumentado atuando como osmoprotetores (Hildebrandt et al. 2015; Hildebrandt e Crosby 2018; Ali et al. 2019). Os AAs também afetam a síntese e a atividade de algumas enzimas, a expressão gênica e a homeostase redox (Hasegawa et al. 2000; Meloni et al. 2001). Segundo Ali et al. (2019) os AAs desempenham seu papel na tolerância ao estresse abiótico por meio três mecanismos: (i) atuando como osmólitos compatíveis, (ii) reguladores de pH, (iii) agindo como uma reserva de nitrogênio ou carbono.

Resíduos de lisina (Lys) são a base em uma sequências altamente conservadas de 15 aminoácidos (EKKGIMDKIKEKLPG) que dão origem as deidrinas (família D-11 das LEAs), proteínas chave no processo de tolerância a dessecação (Close e Lammers 1993; Close 1996; Campbell et al. 1997; Banerjee e Roychoudhury 2016). Além disso, Yuan et al. (2013) descobriram que a Lys está envolvida diretamente com as modificações de histonas e, portanto, está associada com epigenoma e biologia de estresse em plantas. Aminoácidos como arginina (Arg) e ornitina (Orn) são precursores das rotas metabólicas para a síntese de PAs, essas rotas são denominadas arginina descarboxilasa (ADC) e ornitina descarboxilasa (ODC) e alguns estudos nos sugerem que a rota ADC responde muito mais fortemente ao estresse abiótico do que a via ODC (Do et al. 2013; Berberich et al. 2015).

Em sementes de *Medicago* L. e *Arabidopsis* o restabelecimento da tolerância à dessecação depende do ABA, uma vez que o tratamento com fluridone (inibidor da biossíntese de ABA) e o mutante *Arabidopsis aba2*-1 (insensível ao ABA) tiveram o reestabelecimento da tolerância à dessecação comprometida, e em contrapartidas nas sementes que apresentaram maior sensibilidade ao ABA a tolerância à dessecação pode ser reestabelecida (Terrasson et al. 2013; Verdier et al. 2013; Maia et al. 2014). Há evidências de que o *ABI5* forma um complexo com o *ABI3* para regular a expressão de genes que codificam proteínas LEAs, proteínas HPSs, genes envolvidos no acúmulo de proteínas de reservas, como oleosina cuja abundância está relacionada com a aquisição da tolerância à dessecação, além da biossíntese de glutationa (GSH) - enzima que confere proteção contra estresse oxidativo (Chatelain et al. 2012; Terrasson et al. 2013; Verdier et al. 2013). Batool et al. (2018) estudou funções de aminoácidos como a cisteína (Cys) e a serina (Ser) e propõe que a Cys é o fator limitante para a biossíntese de ABA nos estágios iniciais de condições de seca, e que a Ser é utilizado em várias vias biossintéticas como na síntese de nucleotídeos, nas reações de metilação e produção do poder redutor. A Ser atua como um precursor para a síntese de glicina betaína, um importante agente osmótico e eliminador de EROs, em *Arabidopsis* que não produzem glicina betaína a glutationa (derivado da γglu-cys-gly) elimina as EROs geradas durante o estresse abiótico (Rosa-téllez et al. 2020).

O citoplasma celular de plantas sob estresse ambiental pode ter seus conteúdo de prolina (Pro) aumentados em até 100 vezes (Verbruggen e Hermans 2008). Estudos sugerem que a Pro atua como um importante osmoprotetor, e desempenha um papel significativo no alívio dos efeitos adversos do estresse, como à seca (Heuer 1999; Trovato et al. 2008; Verbruggen e Hermans 2008) e à baixa temperaturas (Draper 1972; Chu et al. 1978; Naidu et al. 1991). Recentemente os trabalhos tem discutido demais funções da Pro, como na estabilização e estrutura de membranas e proteínas, eliminação de EROs e reguladora o pH citoplasmático (Rodriguez e Redman 2005; Kaul et al. 2008; Guo et al. 2010).

Em sementes de soja o uso de glutamato (Glu), cisteína (Cys), fenilalanina (Fen) e glicina (Gli) tiveram efeito positivo no metabolismo antioxidante, aumentando a atividade das enzimas antioxidantes (CAT, POD e SOD) e das enzimas de resistência (PAL e PPO) (Teixeira et al. 2017)

3.4. Métodos de criopreservação

A criopreservação consiste em manter o material biológico em temperaturas ultrabaixas, usualmente em nitrogênio líquido (NL) a -196°C, ou em sua fase de vapor a -150°C, assim, o material é submetido a

condições de baixa energia e difusão extremamente lenta, de forma que as reações químicas sejam praticamente interrompidas (Santos 2000; Pritchard 2007). Para o sucesso da técnica de criopreservação, após a exposição das sementes ao NL, estas devem ser germinadas e gerarem plântulas normais (Rica 1992; Walters et al. 2008; Engelmann 2011). Porém, a criopreservação é uma técnica complexa e existe uma série de fatores que limitam seu uso em sementes sensíveis à dessecação, como a formação de cristais de gelo letais, os quais são formados em temperaturas abaixo de 0 °C (Santos 2000; Engelmann 2011; Walters et al. 2013; Walters 2015). A formação de cristais de gelo depende da taxa de resfriamento (Fig. 7), o resfriamento lento resulta na formação de gelo extracelular, enquanto o resfriamento rápido ocasiona a formação de gelo intracelular (Gonzalez-Arnao et al. 2008). Neste contexto, várias técnicas e protocolos têm sido sugeridos, divididos em três linhas gerais: i) desidratação; ii) vitrificação; iii) congelamento (Santos 2000; Engelmann 2011; Kaviani 2011; Kaczmarczyk et al. 2012).

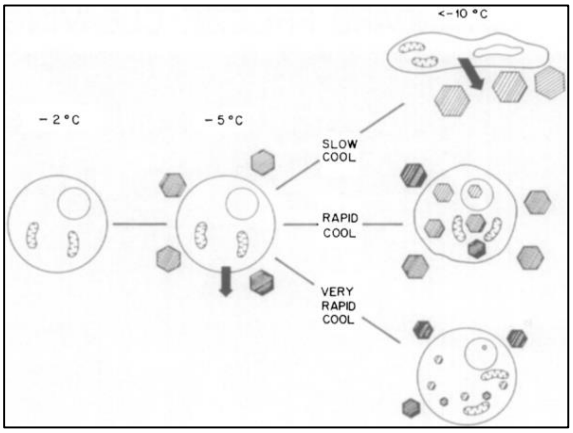

Figure 6: Ilustração esquemática dos eventos físicos nas células durante diferentes taxas de congelamento. No processo de congelamento lento (slow cool), o gelo extracelular começa a se formar abaixo do ponto de congelamento de equilíbrio. Posteriormente, a água é expelida de dentro da célula por pressão osmótica, eventualmente eliminando a formação de gelo intracelular. O processo de congelamento rápido (rapid cool), no entanto, causa a formação de gelo intracelular, pois a água não pode sair da célula rapidamente. No resfriamento ultra-rápido (very rapid cool), como no processo de vitrificação, teoricamente, nenhum gelo se formará (Mazur 1977).

3.4.1. Resfriamento ultra-rápido

Um protocolo de criopreservação bem-sucedido reside primeiramente na redução do conteúdo de água do material, seguida pelo congelamento. As células quando desidratadas não contém água que congela rapidamente e as danifica irreversivelmente. Assim, sementes que toleram à dessecação podem ser armazenadas a qualquer temperatura abaixo de zero. Porém, quando se trata das sementes sensíveis à dessecação, a dificuldade está na redução do conteúdo de água no limiar de 0.25 – 0.08 $gH_2O.gMS^{-1}$, para que a água intracelular torne-se concentrada e viscosa (vítrea) (Vertucci 1990; Walters et al. 2008; Engelmann 2011). O estado vítreo das células impede o colapso, pois minimiza reações químicas que requerem difusão, garantindo a estabilidade durante o período de armazenamento e permite a saída de solutos caotrópicos (Bruni e Leopold 1991; Leopold et al. 1994; Buitink e Leprince 2004; Moelbert et al. 2004). No entanto, protocolos de criopreservação para sementes recalcitrantes fazem uso do potencial crítico da água que limita os danos à dessecação e o congelamento da água seguido da imersão direta em nitrogênio líquido (Vertucci 1990; Pritchard 2007; Walters et al. 2008, 2013).

3.4.2. Vitrificação – agentes crioprotetores

As células de sementes sensíveis à dessecação, não suportam os limiares de dessecação necessários para a atingir um estado vítreo (conteúdo de umidade de 20 a 30% [com base no peso fresco]), devido aos efeitos

mecânicos da dessecação (Walters et al. 2008). Assim, a chave para o sucesso da criopreservação é, portanto, atrelada da tolerância ao congelamento para a tolerância à dessecação. A tolerância a dessecação e ao congelamento pode ser induzida por meio de agentes crioprotetores (ACPs), seguido da imersão em nitrogênio líquido (Panis e Lambardi 2005; Engelmann 2011; Kaviani 2011). Uma vez que, os mecanismos de proteção dos ACPs se baseiam na desidratação das células das plantas, restringindo parcialmente a mobilidade molecular, ou então desorganizando a estrutura do cristal de gelo (Volk e Walters 2006). Como alternativa, eles podem estabilizar as estruturas celulares durante a dessecação e o resfriamento (Crowe et al. 1998; Bryant et al. 2001). Os ACPs alteram as propriedades de congelamento da água e a relação entre o teor de água e a taxa de resfriamento necessária (Volk e Walters 2006). Os ACPs são classificados em dois tipos principais: não penetrantes, geralmente monossacarídeos ou dissacarídeos (sorbitol, trealose, sacarose e o polietilenoglicol), e os penetrantes, na maioria das vezes moléculas de baixo peso molecular (dimetilsulfóxido - DMSO e glicerol) (Grout 2007; Day et al. 2008; Engelmann 2011; Berjak e Pammenter 2014). Os ACPs não penetrantes permanecem nos espaços extracelulares, fazendo com que a água potencialmente congelada seja osmoticamente removida das células, aumentando a concentração de soluto intracelular. Além disso, auxilia na conformação das membranas celulares durante as etapas de desidratação, resfriamento e reidratação (Panis e Swennen 2001; Panis e Lambardi 2005; Berjak e Pammenter 2014; Streczynski et al. 2019). Já os ACPs penetrantes inibem a formação e o crescimento do gelo intracelular, atuando como solutos, aumentando o número de partículas dissolvidas e promovendo do estado vítreo (Engelmann 2011; Berjak e Pammenter 2014; Streczynski et al. 2019). Algumas misturas padrões são utilizadas como ACPs, os quais são chamados de PVS (*plant vitrification solution*). A mistura mais usada é PVS2, consistindo de sacarose, glicerol, etilenoglicol e DMSO (Grout 2007; Sakai e Engelmann 2007; Engelmann 2011). Como certos componentes do PVS podem ser tóxicos, como o DMSO, utiliza-se uma mistura crioprotetora conhecida como PVS3, que é composta somente por sacarose e glicerol (Sakai e Engelmann 2007). Adicionalmente, outras técnicas foram testadas em embriões zigóticos de *Bactris gasipaes* (pupunha), também da família Arecaceae. Os embriões foram submetidos à técnica de encapsulamento-desidratação, estes foram isolados e encapsulados em alginato com glicerol 2 M e sacarose 0,4 M e, em seguida, pré-tratados por 24 h em meio com sacarose 1,0 M e dessecada a 20% de conteúdo de água, antes do congelamento rápido. Nessas condições, 29% dos embriões congelados resistiram à preservação e desenvolveram plântulas (Steinmacher et al. 2007).

O sucesso da criopreservação é avaliado após a capacidade de regeneração de meristemas e plantas, por meio de alterações morfológicas e do reestabelecimento da produção de metabólitos (Santos 2000; Engelmann 2004, 2011; Walters et al. 2013). O ABA, as PAs, as proteínas, compostos enzimáticos e não enzimáticos da atividade antioxidante são reconhecidamente importantes na tolerância ao estresse, tanto na desidratação quanto no resfriamento. No entanto, o modo de ação da maioria dessas moléculas e enzimas durante as etapas da criopreservação de sementes recalcitrantes é pouco conhecido. Propomos com esse projeto desenvolver protocolos de criopreservação para sementes de *B. eriospatha, B. catarinensis e E. edulis* em busca de responder lacunas das condições celulares que levam a sensibilidade a dessecação a partir de avaliações bioquímicas e fisiológicas das sementes e dos protocolos estudados gerando subsídios para conservação *ex situ*.

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CAPÍTULO I

Este artigo foi aceito para a publicação na revista Plant Cell, Tissue and Organ Culture (PCTOC)

Effect of water content and biochemical cell state on the germination rate of cryopreserved *Butia eriospatha* embryos (Arecaceae)

Abstract

Rain forest in Brazil is home of around 50% of the world's plant biodiversity and many species have a high economical potential. *Butia eriospatha* is an endemic species of the Brazilian Atlantic Forest and due to the anthropic intervention, it is in a vulnerable conservation status. In this species, plants are only propagated sexually by seeds which show dormancy and have a low germination rate, combined with slow seedling emergence. To manage the limitation of the seedling establishment and conservation strategies of this species, the viability, antioxidant activity, and profiles of polyamines and amino acids of desiccated *B. eriospatha* embryos were assessed. Mature and desiccated embryos were germinated under *in vitro* conditions on Murashige and Skoog culture medium, which was supplemented with hormones and antioxidants. Embryo desiccation tolerance threshold was 0.14 g H_2O g DW^{-1} with a germination rate of 93.3%. During embryos desiccation was observed a significant increase in putrecine (PUT). Increase in guaiacol peroxidase (GPX) and ascorbate peroxidase (APX) activity led us to suggest that they are the main enzymes involved in cellular protection during desiccation. An increase of amino acids content, especially glutamic acid (Glu), leucine (Leu), lysine (Lys), glutamine (Gln), which are known as osmoprotectors also was observed. Embryos previously desiccated to 0.14 g H_2O g DW^{-1} were successfully cryopreserved, and showed a post-thawing recovery rate of 85%. The physiological and biochemistry approach of this study associated to a cryopreservation protocol can be used for plant genetic resources conservation of other Arecaceae species in the world.

Additional keywords: Orthodox. Recalcitrant. Seeds. Palm species. *Ex situ* conservation. Polyamines.

1. Introduction

Palm species (Arecaceae) are worldwide popular for their fruits with high nutritional value and organoleptic characteristics, as well as an ornamental plant species (Hoffmann et al. 2014; Souza et al. 2020). However, it is not well popularly known that about 26% of Arecaceae species are on the Red List of the International Union for Conservation of Nature (IUCN) (IUCN, 2021). Furthermore, some of the palm species, such as *Corypha taliera* Roxb. and *Cryosophila williamsii* P.H. Allen are already "extinct in the wild" (EW) (IUCN 2021). Arecaceae in Brazil are represented by 37 genera and 299 species, of which about 41% are listed as endemic (Leitman et al. 2015). *Butia eriospatha* (Martius Ex Drude) Beccari, a native Arecaceae from the South Brazilian Atlantic Forest, is among the threatened palm plants (MMA - Ministério do Meio Ambiente 2008; IUCN 2021). This species has suffered severe anthropic intervention, including both local and international illegal trade, overexploitation of fruits and habitat replacement by exotic tree and cattle farming (Nazareno and Reis 2012, 2014b; Elias et al. 2019). Consequently, the decrease of the *B. eriospatha* plant population has resulted in low levels of genetic diversity (Nazareno and Reis 2012).

B. eriospatha plants are only propagated sexually by seeds and, due to long dormancy, the germination can take up to two years and only around 50% of seedlings grow (Fior et al. 2011; Lopes et al. 2011; Schlindwein et al. 2013). All these characteristic directly influence the seedlings establishment and conservation of *B. eriospatha* and several have reported about the worrying genetic situation of the *B. eriospatha* species (Nazareno and Reis 2014a, b). *In vitro* culture protocols have been studied as an alternative to on-farm production and for preserving its genetic diversity (Waldow et al. 2013). Nonetheless, the knowledge regarding its reproductive biology is still limited, especially related to seed germination and desiccation tolerance physiology, which is essential for species preservation and restoration.

The ability of a seed to survive or not to desiccation has a direct influence on *in situ* and *ex situ* plant conservation methods (Walters and Pence 2020). Around 50% of the tropical forest tree species, including Arecaceae, produce recalcitrant seeds (Wyse and Dickie 2017; Royal Botanic Gardens Kew 2021). Recalcitrant seeds are dispersed with high water content (> 4.0 g H_2O gDW^{-1}) and metabolically active, and do not tolerate further desiccation beyond 0.25 g H_2O gDW^{-1} (Walters 2015). Seeds get lost if they are not germinated under adequate conditions, and for most recalcitrant plant species no seed banks are available (Obroucheva et al. 2016; Gonçalves et al. 2020). In this sense, in tropical ecosystems, *ex situ* conservation programs have increased due to the threats of climate change and forest reduction. Biotechnology tools like cryopreservation have largely been used to preserve tissues, seeds and embryos of those plant species that cannot be stored in conventional seed banks, are from rare collection or have natural issues to germinate (Walters and Pence 2020).

Cryopreservation conserves biological material at ultra-low temperature (-196 °C) in liquid nitrogen (LN) (Streczynski et al. 2019). Zygotic embryos are source for genetic diversity and are a suitable target for cryopreservation protocols (Steiner et al. 2020; Walters and Pence 2020). However, embryos from recalcitrant seeds usually have a low tolerance to desiccation and reduction of cell water to prevent intracellular ice formation which is the primary challenge of any cryopreservation protocol (Ballesteros et al. 2021). The prevention of mechanical cell collapse is achieved by chromatin compaction, late embryogenesis abundant proteins (LEAs) and heat shock proteins (HSPs) synthesis, reactive species

oxygen (ROS) equilibration, and the presence of lipids, polyamines (PAs), and specific amino acids (AA) which participate in the stabilization of cell membranes and the deposition of non-reducing sugars (Leprince et al. 2017; Dussert et al. 2018). These protective mechanisms are determinant to maintain plant cell viability during the desiccation process. The role of AAs and PAs has been studied for abiotic stress tolerance in *Oryza sativa* L. (Yang et al. 2007), *Glycine max* L. (Ramos et al. 2005), *Sporobolus stapfianus* (Stapf) Stent. (Martinelli et al. 2007) and *Campomanesia xanthocarpa* Berg. (Vieira et al. 2021). Nonetheless, these studies were mainly focused on plant tissues, without relating AAs and PAs to desiccation tolerance or sensibility in embryos or seeds. To cope the lack of mechanisms that prevent cell damage during desiccation and freezing, plant vitrification solutions (PVS2 and PVS3) were applied in the cryopreservation protocols (Nakkanong and Nualsri 2018). Some cryopreservation procedures include an encapsulation or a flash drying-step, followed by ultra-rapid freezing in LN (Streczynski et al. 2019). New techniques based on fast cooling and warming rates, such as droplet-vitrification and V- and D-cryoplates were developed to conserve plant cell tissue that was more challenging to cryopreserve (Orjuela-Palacio et al. 2019; Berjak and Pammenter 2014; Sinumporn et al. 2019).

Particularly during the last 40 years, many efforts have been made to establish cryopreservation protocols for Arecaceae species. So far *Elaeis guineensis.* Jacq. (Grout et al. 1983), *Phoenix dactylifera* L. (Bagnio and Engelmann 1991), *Cocos nucifera* L. (Assy-Bah and Engelmann 1992), *Sabal* spp. (Wen and Wang 2010), *Butia capitate* (Mart.) Becc. (Dias et al. 2015) and *Butia yatay* (Mart.) Becc. (Vargas et al. 2020) have been successfully cryopreserved. However, it is well-known that not all plant species can be cryopreserved with the same protocol. It is important to highlight that only some of the previously mentioned cryopreservation studies on Arecaceaes species used zygotic embryos as explants. On top of that, *B. eriospatha* is facing an alarming conservation status. Our hypothesis is that embryos of *B. eriospatha* respond well to *in vitro* germination and cryopreservation, at a specific biochemical status and desiccation level. This study assesses and discusses the role and interaction of PAs, amino acids and antioxidants on the viability of *B. eriospatha* embryos during desiccation. To the best of the authors' knowledge, this is the first publication of a cryopreservation and *in vitro* germination protocol for *B. eriospatha* embryos.

2. Material and method

2.1. Plant material

Mature fruits of *B. eriospatha* were collected from 20 plants in the Serrano Plateau of Santa Catarina State (27º 12’ 44’’ S, 50º 36’ 29’’ W), from January to March 2021. Fruits free of signs of insect or microorganism infection were manually processed to obtain the seed. Isolated seeds enveloped by the woody endocarp (pyrenes) were kept at 25ºC for three days and then stored at 8° C for maximum 15 days.

2.2. Embryo excision

The seed’s endocarp was removed manually using a hammer. Seeds were surface-sterilized in ethyl alcohol (70% v/v) for 1 min followed by 15 min in sodium hypochlorite (2.5% v/v) and then rinsed three times with sterilized distilled water. Using a scalpel, the embryos were excised from the seeds (under sterile conditions in a laminar flow hood). Embryos were placed in petri dishes onto germitest® paper (sheets 28

X 38 cm, neutral pH; CINELAB-FILM), moistened with 2.5 g of solution per gram of paper. The solution contained 0.2 g L^{-1} g ascorbic acid and 0.2 g L^{-1} citric acid, to prevent tissue oxidation.

2.3. Seed and embryo morphology and morphometry

Diameter, length and width of seeds and embryos (fresh) was measured with a caliper. A set of 100 seeds and embryos was measured digitally under the stereoscope (Olympus SZH10 equipped with image capture system DP71).

2.3. Water relations

2.3.1. Water content (WC) and water potential (Ψ_w)

Five replicates of 10 embryos were used to determine WC and Ψ_w. Water content was measured gravimetrically by difference in weight before and after drying for 24 h in an oven at 103 ± 1°C (ISTA 2004). Water content value was expressed on a dry weight basis (g H_2O gDW^{-1}).

Water potential was determined by immersing embryos in solutions of polyethylene glycol 6000 (PEG 6000) with Ψ_w of 0, −0.5, −1, −2, −3 and −4 MPa at 25ºC (Michel and Kaufmann 1973). The fresh weight of the embryos was measured gravimetrically immediately after excision and after 24h- immersion in PEG 6000 solutions.

2.4. Drying of embryos

After excision, five replicates of 10 embryos were placed on dry filter papers in a laminar airflow cabinet at 25 ± 2°C, 55 ± 5% relative humidity [RH] and an airflow of 0.46 m s^{-1}. Embryos were dried for 0 (control); 15; 30; 60; 120; 180; 240 and 300 min and the desiccation curve was calculated (Hong and Ellis 1996). The embryos' drying rate index (*k*) was calculated from the amount of water loss in dry basis (g H_2O g DW^{-1}) divided by the corresponding drying period (in minutes). Viability and vigor of embryos was assessed for all desiccation times.

2.5. Viability and vigor

2.5.1. Germination of embryos under *in vitro* conditions

Four replicates of 25 embryos of each desiccation treatment (different drying time intervals) were excised as described in the "Embryos excision" section. Embryos were immersed in a commercial sodium hypochlorite solution (0.5% v/v) for 10 min and then rinsed three times in sterile distillate water (Magalhães et al. 2013). Embryos were germinated in culture medium according to Waldow et al (2013), with modifications. Embryos were inoculated in petri dish with 25 mL of MS culture medium (Murashige and Skoog 1962) supplemented with 30 g L^{-1} sucrose, 1 g L^{-1} glutamina, 0.5 g L^{-1} inositol, 3 g L^{-1} activated charcoal, 2.77 mg L^{-1} GA_3 and 2.5 g L^{-1} phytagel at pH 5.7. A GA_3 stock solution was filter-sterilized and added to the culture medium after autoclaving at 121 °C, 1.5 atm for 15 min. The germination was performed in a growth room (25 ± 2°C; 55% RH) with white LED light (Green Power TLED W; Philips TM; 77 µmol m^{-2} s^{-1}) and 16/8 h photoperiod. An embryo was considered germinated when the radicle protrusion occurred. Assessments were recorded daily for 30 days. Germination percentage was calculated by the cumulative number of daily germinated embryos with respect of the total number of embryos

evaluated (Ranal and Santana 2006). The germination speed index (GSI) was calculated according to the Maguire's index (Mangure 1962) as counting the total number of embryos germinated per day between sowing and germination divided by the number of days of the test. Based on the results of WC, viability and vigour of the embryos, it was decided to perform the biochemical analyzes and cryopreservation experiments at desiccation times of 0, 15, 180 and 300 min.

2.5.2. Tetrazolium tests (TTC)

Four replicate of 25 embryos per treatment were soaked for 12 h in Petri dish with germitest® paper, moistened with 2.5 g of water per gram of paper. Samples were incubated in darkness at 20 ± 2 °C. Embryos were immersed for 4 h in 2,3,5-triphenyl tetrazolium chloride (TTC) [0.5% w/v; in darkness, at 35 °C], and viability was assessed by staining intensity (Ribeiro; et al. 2010). Results were expressed in percentage of embryo showing positive reaction to TTC.

2.5.3. Seedling morphometry

Length of primary root, cotyledonary petiole and leaf sheath of four replicate of 10 seedlings were measured digitally under the stereoscope (Olympus SZH10 equipped with image capture system DP71), after 30 days on germination medium.

2.6. Cryopreservation methods

2.6.1. Method A

For the dehydration method four replicate of 25 embryos were partially desiccated for 0; 15; 180 and 300 min. Then, the embryos were wrapped in sterilized aluminum foil (~20 μm thickness) and directly immersed in liquid nitrogen (LN) at -196 °C, where they were kept for 24 h (Orjuela-Palacio et al. 2019).

2.6.2. Method B

For the droplet-vitrification method two sets of 100 embryos each, were partially desiccated for 0; 15; 180 and 300 min. Desiccated embryos from the first set were incubated in Plant Vitrification Solution (PVS2) (30 % glycerol w/v, 15 % w/v dimlethylsulfoxide, 15 % w/v ethylene glycol and 13.7 % w/v sucrose) (Sakai et al. 1990), while the second set was treated with Plant Vitrification Solution 3 (PVS3) (50 % w/v sucrose and 50 % w/v glycerol) (Nishizawa et al. 1993). The PVSs (Plant Vitrification Solution) were previously autoclaved for 10 min at 15 atm and 121 °C. Both PVS treatments were performed on an orbital shaker (at 90 rpm) at 0°C for 60 min (Berjak and Pammenter 2014; Steiner et al., 2020). Five droplets (15 μl) of PVS containing one embryos each were placed on aluminum strips (~ 150 μm thickness) and submerged directly into LN for one min. Samples were transferred to cryotubes (TTP®, Trasadingen, Switzerland) and stored in a dewar containing LN. Water content was measured gravimetrically before and after the PVS treatment as described in the "Water content" section.

2.6.3. Thawing of embryos and recovery

After 24 h in LN the foil envelopes (method A) and aluminum strips (method B) with embryos were thawed in a sucrose solution (1.2 M, pH 5.8) kept in water bath at 45°C for 5 min (Walters et al. 2008).

Embryos were placed on germination medium, contained in petri dishes with culture medium (*In vitro* embryo germination section) and incubated in darkness at 25 ± 2 °C (55% RH) for three days. After three days, the embryos were exposed to white LED light (Green Power TLED W; Philips TM; 77 μmol m^{-2} s^{-1}) and 16/8 h photoperiod. Germination percentage and GSI were daily evaluated while the seedling measurements were performed at the end of experiment (60 days). Embryos without exposure to LN were used as control treatment.

2.7. Antioxidant enzyme extraction and assays

Superoxide dismutase (SOD), Catalase (CAT), Ascorbate Peroxidase (APX) and Guaiacol peroxidase (GPX) activities were measured using a spectrophotometer Spectra-Max® 190 Microplate Reader. Three samples (300 mg FW, ≅ 300 embryos) desiccated for 0, 15, 180 and 300 min were grounded using LN, followed by treatment with 1 mL of potassium phosphate buffer (50 mM, pH 7.0), 1 mM ethylenediaminetetra acetic acid (EDTA) and 1% (p/v) polyvinylpyrrolidone (PVP) according to Bailly and Kranner (2011). The homogenate was centrifuged at 15.000 × g for 20 min at 4°C, and the supernatant was collected and placed on ice. Superoxide dismutase activity was determined in a reaction mixture composed of 10 μL of enzyme extract and 290 μL of potassium phosphate buffer (50 mM, pH 7.8) containing 13 mM methionine, 75 μM nitroblue tetrazolium, 2 mM of riboflavin and 100 nM EDTA. Then, reaction mixtures were illuminated by a fluorescent lamp (30 W at 12 cm above the reaction mixtures), attached to a dark box, for 15 min. Duplicate samples kept in the dark for same time were used as controls. Activity was estimated by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium (NBT) at 560 nm (Giannopolitis and Ries 1977). Ascorbate peroxidase activity was determined in a reaction mixture composed of 10 μL of enzyme extract and 290 μL of potassium phosphate buffer (50 mM, pH 7.0) containing 4.75 mM H_2O_2, 5 mM EDTA and 50 mM ascorbic acid. The absorbance decrease at 290 nm (molar extinction coefficient 2.8 mM^{-1} cm^{-1}) was recorded for 10 min at 25°C (Koshiba 1993). Catalase activity was determined in a reaction mixture composed of 10 μL of enzyme extract and 290 μL of potassium phosphate buffer (50 mM, pH 7.0) containing 13 mM H_2O_2. The absorbance decrease at 240 nm (molar extinction coefficient 39.4 mM^{-1} cm^{-1}) was recorded for 3 min at 25°C (Peixoto et al. 1999). Guaiacol peroxidase activity was determined in a reaction mixture composed of 7 μL of enzyme extract and 293 μL of sodium phosphate buffer (10mM, pH 6.0) containing 12.6 mM hydrogen peroxide, and 0.31 M guaiacol. The absorbance increase at 470 nm (molar extinction coefficient 26.6 M^{-1} cm^{-1}) was recorded for 3 min at 32°C (Freitas and Stadnik 2015). The protein contents of the extracts were determined according to Bradford (1976), using bo24vine serum albumin (BSA) as standard.

2.8. Free polyamines (PAs) determination

For PAs determination, three samples (200 mg FW, ≅ 200 embryos) desiccated for 0, 15, 180 and 300 min were grounded using 1.6 mL of 5 % (v/v) perchloric acid. Free PAs were dansylated and quantified according to Steiner et al. (2007). Free PAs were derivatized with dansyl chloride (Sigma-Aldrich, St. Louis, MO, USA) and quantified by high-performance liquid chromatography (HPLC) (Shimadzu, Nakagyo-Ku, Kyoto, Japan) using a 5-μm C_{18} reverse-phase column (Shimadzu Shin-pack CLC ODS). Two mobile phases were utilized: (A) 10% acetonitrile in distilled water (pH 3.5 adjusted with HCl 1 N);

(B) 100% acetonitrile. The gradient used was 65% of B (100% acetonitrile) in the first 11 min, 65% to 100% from 11 to 25 min, 100% from 25 to 35 min, and then adjusted to 65% from 35 to 44 min, and the flow rate was 1.0 mL min^{-1} at 40 °C. PAs concentration was determined using a fluorescence detector with wavelengths of 340 nm (excitation) and 510 nm (emission). Peak areas and retention times were measured by comparison with standard PAs: putrescine (PUT), spermidine (SPD) and spermine (SPM). The PAs ratio was calculated using the formula (SPD+SPM)/PUT. The final concentration of PAs was expressed in mmol mg^{-1} DW. The 1,7-diaminoheptane (DAH) was used as internal standard.

2.9. Free amino acids (AA) determination

Free amino acids determination was performed using three samples (100 mg FW, ≅ 100 embryos) desiccated for 0, 15, 180 and 300 min. Embryos were ground using 1.5 mL of 80% (v/v^1) ethanol. Derivatization and quantification of amino acids was performed following the procedures described by Astarita et al. (2003). Amino acids were derivatizated using o-phtaldialdehyde (OPA) (Sigma-Aldrich, St. Louis, MO, USA), in a borate buffer (400 mM pH 9.5 adjusted with NaOH 5 N), and then filtered through a 0.45 µm nylon membrane and then injected for quantification. Quantification was obtained using HPLC (Shimadzu, Nakagyo-Ku, Kyoto, Japan) with fluorescent detector set at 250 nm (excitation) and 480 nm (emission). For the reverse phase, Luna 5 µm C18 (250 × 4.6 mm, Phenomenex, Torrance, CA, USA) was used as a stationary phase. The gradient was developed by mixing increasing proportions of 65% methanol to a buffer solution (50 mM sodium a cetate, 50 mM sodium phosphate, 20 mL L^{-1} methanol, 20 mL L^{-1} tetrahydrofuran and pH 8.1 adjusted with acetic acid). The gradient of 65% methanol was programmed to 20% over the first 32 min, increased from 20 to 100% from minute 32 to 71, and then maintained at 100% until minute 80 min, at 1 mL min^{-1} flow at 40°C. Fluorescence excitation and emission wavelengths of 250 nm and 480 nm, respectively, were used for amino acid detection. The identification of the amino acids was based on the retention time and peak area of standards of each amino acid: aspartic acid (Asp), glutamic acid (Glu), asparagine (Asn), glutamine (Gln), serine (Ser), histidine (His), arginine (Arg), glycine (Gly), cysteine (Cys), threonine (Thr), γ-Aminobutyric acid (Gaba), alanine (Ala), tyrosine (Tyr), tryptophan (Trp), methionine (Met), valine (Val), phenylalanine (Phe), isoleucine (Ile), leucine (Leu), ornithine (Orn) and lysine (Lys) (Sigma-Aldrich, St. Louis, MO, USA). The total amino acids content was determined by the sum of all amino acids and the amino acids ratio was obtained by the division of Arg value by total free amino acids content value (Arg/Total Free Amino $Acids^{-1}$) DW and Orn value by total free amino acids content value (Arg/Total Free Amino $Acids^{-1}$) DW. Final concentration of amino acids was expressed in µg mg^{-1} DW.

2.10. Statistical Analyses

Seed and embryo morphometric data were measured in four replicates of 25 seeds each (total = 100). Data analysis of water relations (WC and Ψw) was performed with five replicates of 10 embryos each (total = 50 per analyses). For viability and vigor analysis of embryos desiccated (0; 15; 60; 120; 180; 240 and 300 min) was performed on a sample size of 100 embryos per treatment (four replicates of 25 embryos each). Statistical analysis of the cryopreservation experiments was performed on a sample size of 100 embryos per treatment (four replicates of 25 embryos each). The data normality was evaluated using the

Shapiro – Wilk test, and then submitted to analysis of variance in a completely randomized design. In the case of significance of the F values, means were compared using SNK test ($p < 0.05$) (Sokal and Rohlf 1995). Statistical analyses and hierarchical cluster analysis (HCA) (package pheatmap) were performed using the R Core Team (2016).

3. Result

3.1. Morphological characterization of seeds and embryos

B. eriospatha seeds have elliptic irregular shapes, a brown seed coat (sc) and an operculum (os) forming a small protuberance at its proximal extremity (Fig. 1a). Average seed diameter (d) and length (h) were 10.29 ± 0.84 mm and 8.56 ± 0.92 mm, respectively (Fig. 1a). The white opaque endosperm (en) was covered by a thin seed coat (sc). The embryo is contained in a cavity, located immediately below the operculum (Fig. 1b). In the embryos proximal portion (pp) a cotyledonary petiole (cp) envelops a rudimentary embryonic axis while in the distal portion (dp) it is the haustorium (ha) (Fig. 1c). Embryos had an average length and width of 3.1 ± 0.04 mm and 1.2 ± 0.08 mm, respectively.

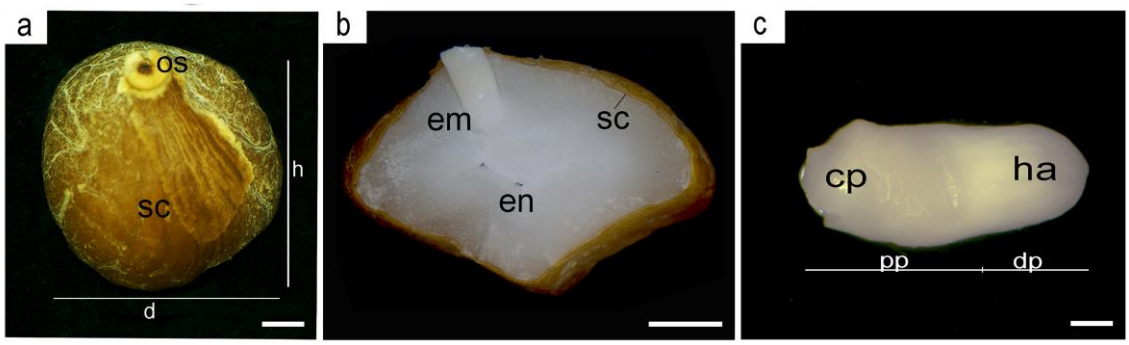

Figure 1: Morphology of seed and embryo of *B. eriospatha.* **(a)** External morphology of whole seed: operculum (os), seed coat (sc), diameter (d) and length of seed (l). **(b)** Longitudinally sectioned seed with embryo (em), endosperm (en) and seed coat (sc). **(c)** Mature embryo with cotyledonary petiole (cp) in the proximal portion (pp) and haustorium (ha) in the distal portion (dp). Bars: a, b = 2.0 mm; c = 0.5 mm

3.2. Water relations

Mature seeds (Fig. 1a) and fresh embryos (Fig. 1c) of *B. eriospatha* showed a water content (WC) of 0.24 g H_2O g DW^{-1} (19.87%) and 0.94 g H_2O g DW^{-1} (48.52%), respectively. Fresh embryos excised and immersed in antioxidant solution (control - without desiccation) had increased its WC to 2.56 g H_2O g DW^{-1}, as a consequence of the initial imbibition, and after 15 min of dehydration (0.91 g H_2O g DW^{-1}) they showed similar WC than the fresh embryos (Fig. 2a). Embryos reached a nearly stable WC (0.17 g H_2O g DW^{-1}) at 180 min of desiccation (Fig. 2a). After 300 min of desiccation, the embryos have lost 94.53% of their initial WC (0.94 g H_2O g DW^{-1}) reaching 0.14 g H_2O g DW^{-1}. The drying rate (K) of the *B. eriospatha* embryos could be subdivided into three phases. During the first 15 min the drying rate was higher (0.110 g H_2O^{-1} min^{-1}), from minute 15 to 120 it decreased (0.006 gH_2O^{-1} min^{-1}) and almost stabilized from 120 min to 300 min (0.004 g H_2O^{-1} min^{-1}) (Fig. 2a).

The water potential (Ψ_w) of fresh embryos was related to their ability to absorb or retain water (Fig. 2b). Fresh embryos (Ψ_w = 0.94 g H_2O g DW^{-1}) that were immersed in pure distilled water (Ψ_w = 0.00 MPa), for 24 hours, showed a three-fold increase in their WC (2.70 g H_2O g DW^{-1}). An increase in embryo WC was

noted when they were immerged in the Ψ_w = - 0.5 MPa solution (1.71 g H_2O g DW^{-1}) and a reduction of WC was observed at Ψ_w = - 1.0 MPa (0.51 g H_2O g DW^{-1}). At Ψ_w - 4.0 MPa a WC of 0.03 g H_2O g DW^{-1} was reached (Fig. 2b).

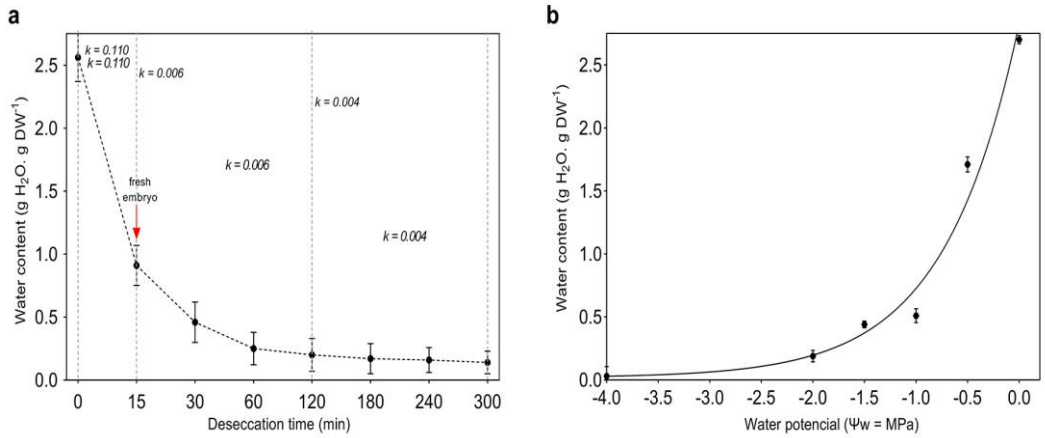

Figure 2: (a) Variation of water content (g H_2O g DW^{-1}) in embryos of *B. eriospatha* dried for 0, 15, 30, 60, 120, 180, 240 and 300 minutes. k: drying rate. Arrow at 15 minutes: water content slightly below to fresh embryo. **(b)** Relationship between water content and water potential at Ψw levels of 0, − 0.5, − 1, − 2, − 3 and − 4 MPa. Data points: mean ± standard error. n = 5 (five independent replicates, each being a pool of 10 embryos). Linear regression of water content in function to water potential: $r^2 > 0.98$

3.3. Viability and vigor

Desiccation time and WC did not have a significant effect on the germination of *B. eriospatha* embryos under *in vitro* conditions. Both fresh and dehydrated embryos (300 min) showed germination rates of about 93% (Fig. 3a). The tetrazolium test was validated by the germination experiment, indicating a suitable protocol for embryo viability in this species (Fig. 3a). However, germination dynamic was affected by desiccation time. Control treatment (without dehydration) and embryos dried for up to 180 min germinated earlier and faster (maximum 13 days) compared to the embryos that were desiccates for 240 and 300 min (17 and 18 days, respectively) (Fig. 3b). These contrasting dynamics could also be observed by the decrease in germination speed index (GSI). The lowest value of GSI occurred in embryos dehydrated for 240 and 300 min (GSI = 0.44 ± 0.04 and GSI = 0.43 ± 0.03, respectively) compared to the control treatment and embryos desiccated for up to 180 min (GSI = 0.71 ± 0.05 and GSI = 0.59 ± 0.04, respectively) (Fig. 3c). Embryos dehydrated for 300 min showed a significant decrease in sheath and primary root length, compared to the 15-minute treatment (13 ± 2.22 mm) (Fig. 3d). Desiccation times greater than 120 min resulted in shorter primary root lengths (rp) (10.8 ± 3.30 mm) compared to embryos desiccated up to 15 min (19.7 ± 7.76 mm) (Fig. 3d).

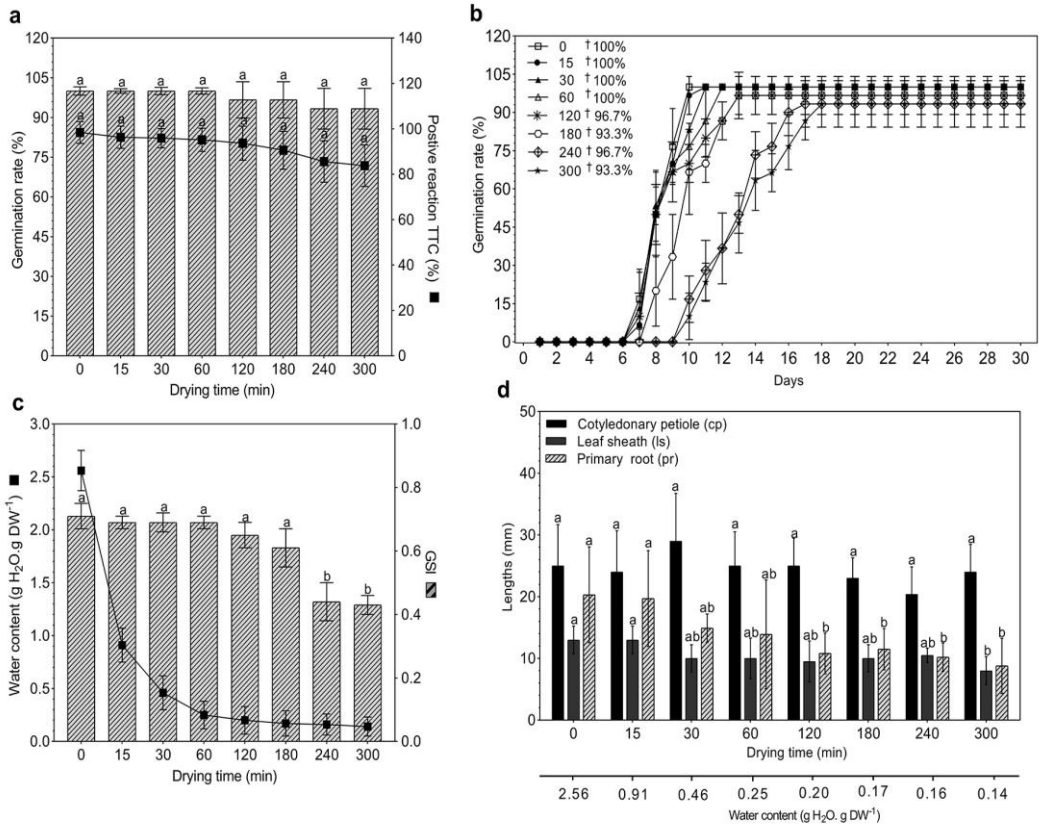

Figure 3: (a) *In vitro* germination rate (%) and reaction to 2,3,5-triphenyltetrazolium chloride (TTC) of *B. eriospatha* embryos desiccated for 0, 15, 30, 60, 120, 180, 240 and 300 minutes. **(b)** In vitro germination rate (%) versus inoculation time (days). † Embryo germination rate after four weeks. **(c)** Water content (WC = g H_2O g DW^{-1}) and germination speed index (GSI) versus drying time. **(d)** Length of primary root (rp), cotyledonary petiole (cp) and leaf sheath (ls). Bars: mean ± standard error. n = 100 (four replicates, each being a pool of 25 embryos. Different letters indicate significant differences ($p < 0.05$) between treatments based on the SNK test.

3.4. Cryopreservation

Non-dehydrated embryos (control) and embryos dehydrated for 15 min, regardless of having been incubated in PVS2 or PVS3 solution, did not germinate after being cryopreserved with method A or B (Table 1). These embryos remained unchanged in size for more than 60 days, without elongation or callus formation. Embryos dehydrated for 180 and 300 min, treated or not with PVS2 or PVS3, showed germination rates of 83.33% or higher and no differences in the GSI values (Table 1). However, 60 days after thawing, it was observed that embryos dehydrated for 180 min and cryopreserved with method A showed a significantly lower rate of abnormal seedlings (12.50 ± 0.75), compared to embryos cryoprotected with Method B, both with PVS2 (29.17 ± 0.88) and PVS3 (14.81 ± 0.80) [Table 1; Fig 4a].

Table 1. Water content, germination rate, germination speed index and seedling morphological of cryopreserved *Butia eriospatha* embryos.

Methods	Drying time (min)	Water content (g H_2O g DW^{-1})	Germination rate (%)	GSI	Seedlings morphometry				
					Normal (%)	Abnormal (%)	Cotyledonary petiole (cm)	Leaf sheath (cm)	Root (cm)
Dehydration (Method A)	0	2.56 ± 1.15	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{f}	0.00 ± 0.00^{f}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}
	15	0.94 ± 0.15	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{f}	0.00 ± 0.00^{f}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}
	180	0.17 ± 0.07	91.67 ± 6.66^{a}	0.34 ± 0.04^{a}	79.17 ± 0.75^{b}	12.50 ± 0.75^{d}	2.00 ± 0.22^{a}	0.90 ± 0.19^{a}	1.30 ± 0.47^{a}
	300	0.14 ± 0.10	92.44 ± 2.41^{a}	0.30 ± 0.02^{a}	85.3 ± 0.31^{a}	7.14 ± 0.31^{e}	1.90 ± 0.24^{a}	1.00 ± 0.16^{a}	1.30 ± 0.32^{a}
Droplet vitrification/PVS2 (Method B)	0	1.30 ± 0.05	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{f}	0.00 ± 0.00^{f}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}
	15	0.43 ± 0.09	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{f}	0.00 ± 0.00^{f}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}
	180	0.36 ± 0.07	86.11 ± 5.31^{a}	0.34 ± 0.02^{a}	56.94 ± 0.88^{d}	29.17 ± 0.88^{b}	2.00 ± 0.30^{a}	1.20 ± 0.38^{a}	1.30 ± 0.44^{a}
	300	0.22 ± 0.10	86.67 ± 7.24^{a}	0.33 ± 0.02^{a}	49.64 ± 1.05^{e}	37.03 ± 1.05^{a}	1.80 ± 0.45^{a}	1.50 ± 0.43^{a}	1.00 ± 0.48^{a}
Droplet vitrification/PVS3 (Method B)	0	0.25 ± 0.10	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{f}	0.00 ± 0.00^{f}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}
	15	0.24 ± 0.02	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{f}	0.00 ± 0.00^{f}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}
	180	0.46 ± 0.10	86.11 ± 2.85^{a}	0.32 ± 0.01^{a}	71.30 ± 0.80^{c}	14.81 ± 0.80^{c}	1.70 ± 0.58^{a}	0.90 ± 0.43^{a}	0.70 ± 0.43^{a}
	300	0.35 ± 0.08	83.33 ± 7.95^{a}	0.32 ± 0.06^{a}	66.67 ± 0.81^{c}	16.66 ± 0.81^{c}	2.40 ± 0.48^{a}	0.80 ± 0.28^{a}	0.90 ± 0.47^{a}

Data mean ± SD (n = 4).
Different letters indicate significant differences at $P < 0.05$, according to the SNK test

Embryos with primary root (pr) and leaf sheath formation (ls) and elongation of the cotyledonary petiole (cp), were considered as normal. Abnormal embryos did not develop a primary root (arrow) [Fig. 4b] nor a leaf sheath (Fig. 4c) but showed a swelled cotyledonary petiole (cp) and callus proliferation in the basal region of the embryo (Fig. 4d).

Figure 4: Development of zygotic embryos of *B. eriospatha* after cryopreservation. **(a)** Droplet-vitrification method (PVS3): normal seedling with primary root (pr), cotyledonary petiole (cp) and leaf sheath (ls), 60 days after thawing. **(b)** Droplet-vitrification method (PVS2): abnormal seedling with missing primary root (asterisc). **(c)** Droplet-vitrification method (PVS2): abnormal seedling without leaf sheath. **(d)** Abnormal embryo. Bars: a, b = 2.0 mm; c, d = 0.5 mm

3.5. Antioxidant system

Control treatment (without dehydration) and *B. eriosptha* embryos dehydrated for 15 min showed a higher superoxide dismutase (SOD) activity (96.23 $Umin^{-1}$ mg^{-1} protein) compared to embryos dehydrated for 180 and 300 min (68.05 and 51.43 $Umin^{-1}$ mg^{-1} protein, respectively) [Fig. 5a; Table S1]. In contrast to SOD enzyme behavior, ascorbate peroxidase (APX) activity was lower after 15 min of desiccation (0.69 µmol min^{-1} mg^{-1} protein), and showed an increase after 300 min (1.09 µmol min^{-1} mg^{-1} protein) [Fig. 5b; Table S1]. Guaiacol peroxidase (GPX) activity values in embryos desiccated for 300 min were 34 times

higher (0.90 umol min^{-1} mg^{-1} protein) than for the control treatment (without dehydration) and embryos dehydrated for 15 min (increase of 0.03 umol min^{-1} mg^{-1} protein) [Fig 5c; Table S1]. Catalase activity (CAT) increased during the first 180 min, reaching its maximum activity at 180 min (0.88 µmol min^{-1} mg^{-1} protein) and decreased drastically after 300 min of drying (0.19 µmol min^{-1} mg^{-1} protein) [Fig. 5d; Table S1].

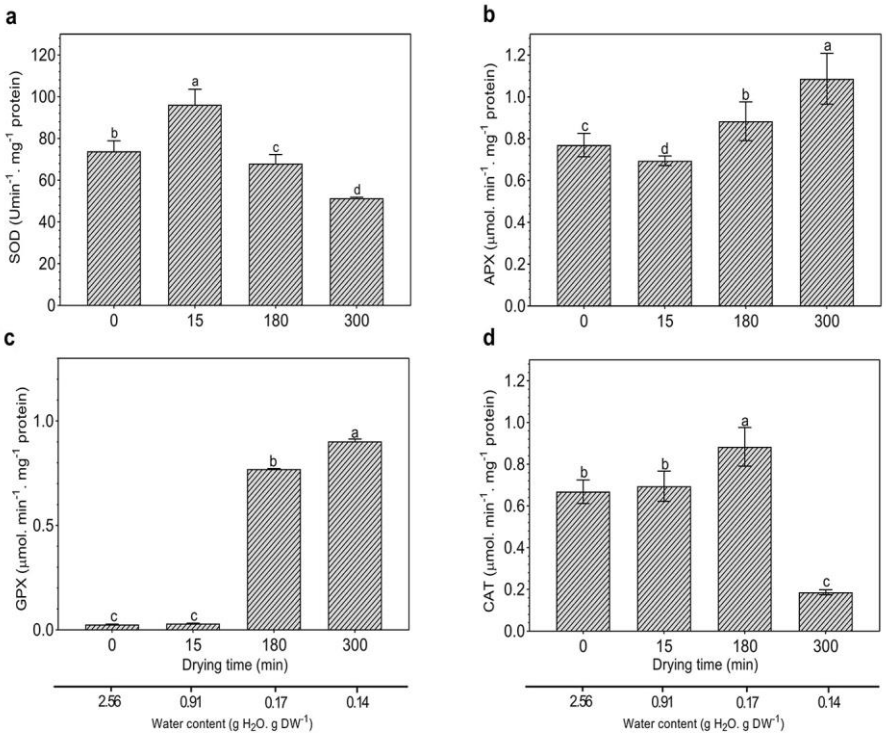

Figure 5: Antioxidant enzyme activities in *B. eriospatha* embryos in response to drying time (min) 0; 15; 180; and 300 minutes. **(a)** SOD. **(b)** APX. **(c)** GPX. **(d)** CAT. Data mean ± standard error (n = 3) independent replicates, each being a pool of ≅ 300 embryos. Means followed by different letters are significantly different ($P < 0.05$) according to the SNK test. Absolute values Supplementary Data – Table S1

3.6. Polyamines (PAs) content

Control treatment (without dehydration) embryos had a significantly higher content of total free polyamines (PAs) (689.21 mmol mg DW^{-1}) than the desiccated ($P< 0.05$) [Fig. 6a]. The lowest contents of putrescine (PUT), spermidine (SPD) and spermine (SPM) were observed in embryos desiccated for 15 min (Fig. 6b – d). Putrescine (PUT) content increased in embryos desiccated for 300 min (39.30 mmol mg DW^{-1}) compared to the control (without dehydration) (30.66 mmol mg DW^{-1}) [Fig. 6b; Table S2]. The opposite behavior was observed for SPD and SPM, where the control treatment embryos (without dehydration) (SPD = 455.34 and SPM = 203.21 mmol mg DW^{-1}) showed a higher content than in embryos desiccated for 300 min (SPD = 292.80 and SPM = 174.77 mmol mg DW^{-1}) [Fig. 6c-d; Table S2]. When embryos were desiccated for 300 min, increased PUT and reduced SPD and SPM contents were observed. Consequently, also the PA ratio [(SPD + SPM)/PUT] decreased.

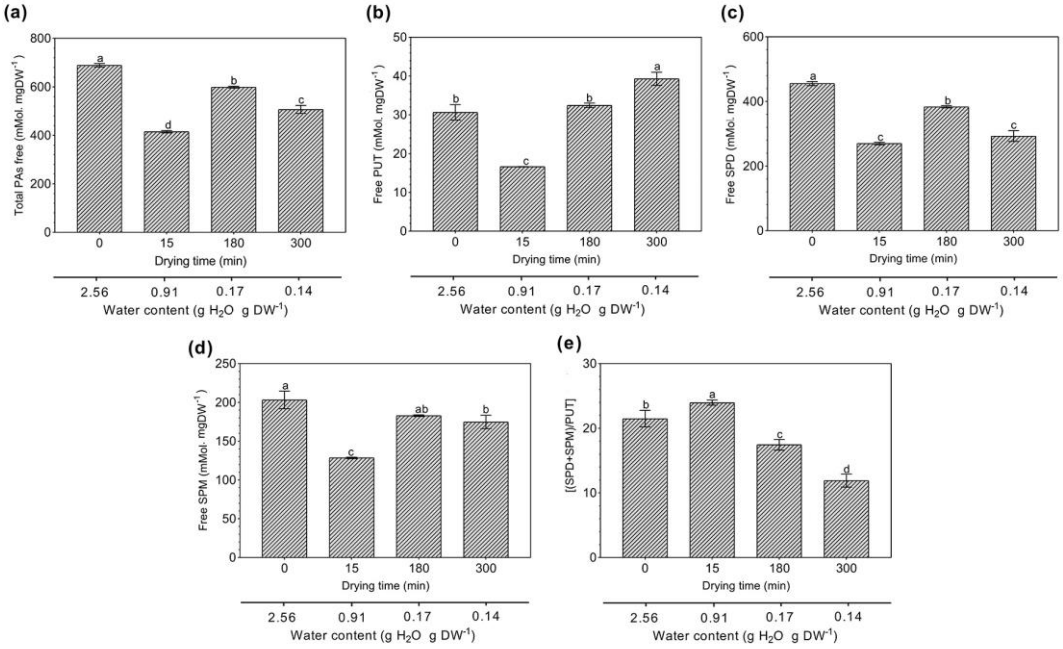

Figure 6: Free polyamines contents (mMol mg DW^{-1}) in *B. eriospatha* embryos in response to drying time (min) for 0; 15; 180; and 300 minutes. **(a)** Putrescine (PUT). **(b)** Spermine (SPD). **(c)** Spermidine (SPM). **(d)** PAs [(SPD + SPM)/PUT] ratio. Data mean ± standard error (n = 3) independent replicates, each being a pool of ≅ 200 embryos. Means followed by different letters are significantly different ($P < 0.05$) according to the SNK test. Abs olute values Supplementary Data – Table S2

3.7. Free amino acid content

Embryos dehydrated for 300 min had a significantly higher content of total free amino acids (23565.61 µg mg DW^{-1}) compared to the control treatment (3849.47 µg mg DW^{-1}) and dehydration times of 15 min (9035.52 µg mg DW^{-1}) and 180 min (10372.56 µg mg DW^{-1}) [Fig. 7a]. Lysine (Lys) was the most abundant amino acid for all assessed desiccation times and represented 42% of the total free amino acid profile (Fig. 7b; Table S3 The content of gamma-aminobutyric acid (Gaba) and arginine (Arg) did not change significantly during drying. Lysine (Lys), leucine (Leu), isoleucine (Ile) glutamine (Gln) and glutamic acid (Glu) practically doubled their contents when the embryo was desiccated for 300 min (Fig. 7b; Table S1). After a desiccation time of 300 min, these five amino acids represented approximately 97.85% of the total amino acid content. The ratio of ornithine (Orn) and arginine (Arg) to total amino acids indicate that Orn increase preferentially to Arg with dehydration (Fig. 7c). Control treatment (without dehydration) had higher Arg content (0.06 µg mg DW^{-1}), which decreased at 15 min of desiccation (0.02 µg mg DW^{-1}) and showed no statistical differences until 300 min of desiccation (0.01 µg mg DW^{-1}) (Fig. 7c). The Orn content was higher in the 15 min of desiccation (0.93 µg mg DW^{-1}) compared to the control treatmen (without dehydration) (0.27 µg mg DW^{-1}), but did not differ from 180 min (0.47 µg mg DW^{-1}) and 300 min (0.48 µg mg DW^{-1}) of desiccation (Fig. 7c).

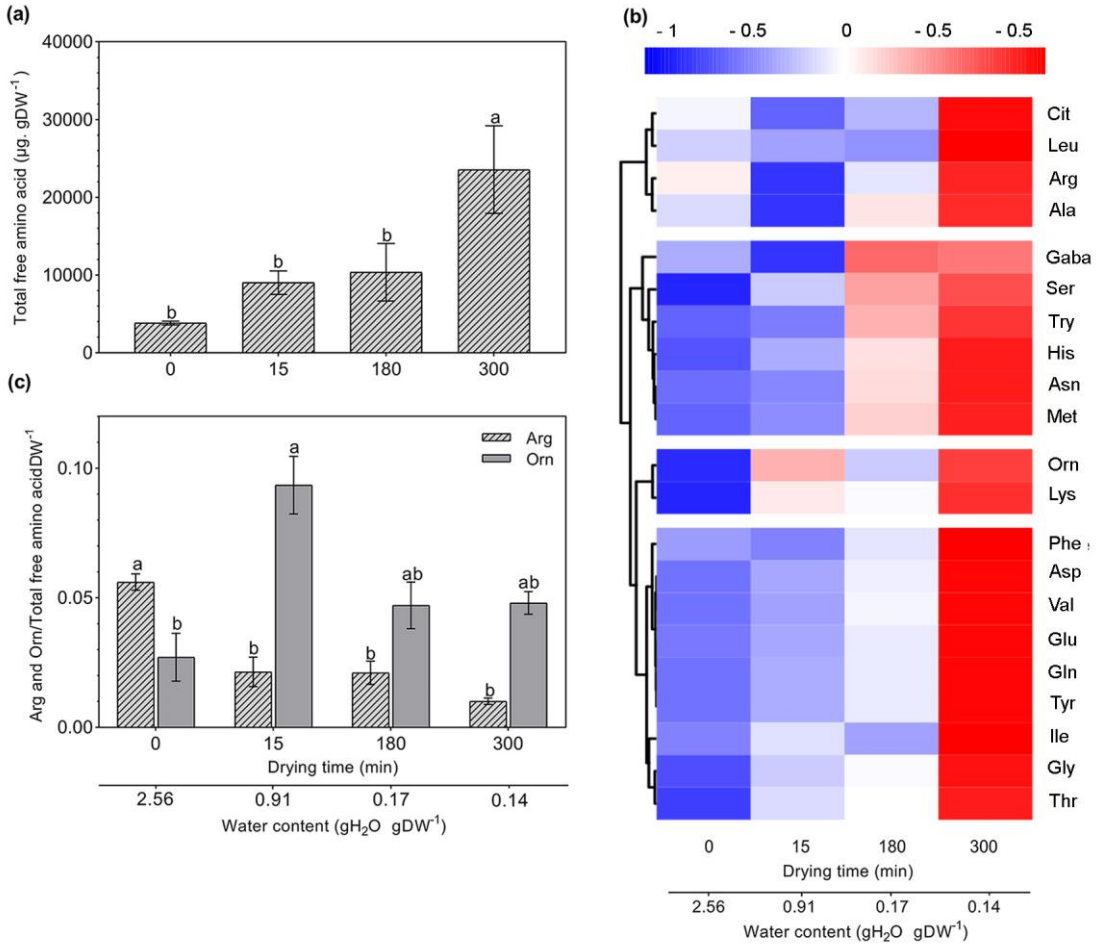

Figure 7: (a) Total free amino acid contents (µg g DW^{-1}) in *B. eriospatha* embryos in response to drying time 0; 15; 180 and 300 minutes. **(b)** Amino acids heatmap cluster according to embryos drying time and water content. Color gradients represent the differences value of amino acid contents. **(d)** The ratio of arginine (Arg) and ornithine (Orn) to total amino acids. Data mean ± standard error (n = 3) independent replicates, each being a pool of ≅ 100 embryos. Means followed by different letters are significantly different ($P < 0.05$) according to the SNK test. For absolute values and statistical analysis see Supplementary Data – Table S3

4. Discussion

4.1. Water relations and cryopreservation of *B. eriospatha* embryos

Fresh *B. eriospatha* embryos had a water content of 0.94 g H_2O g DW^{-1} and an *in vitro* germination rate of 100%. As the embryos' viability was still high (90%) at a water content of 0.14 g H_2O g DW^{-1}, it is postulated that embryos of *B. eriospatha* tend to have an orthodox behavior. Seeds are classified based on its WC threshold and capacity to be stored at low temperatures. Orthodox seeds can survive at low WC (<0.05 g H_2O g DW^{-1}) and temperature (< -18ºC), while recalcitrant seeds will not survive under these conditions (Walters 2015). However, in between of these two categories are intermediate seeds, that can survive to the WC threshold of orthodox seeds, but may not be compatible with sub-zero temperatures (Ballesteros et al. 2021). Studies on palm species seeds still not precise about seed desiccation tolerance threshold and this is a bottleneck about life time storage of tropical species in seed banks (Pritchard et al. 2004; Royal Botanic Gardens Kew 2021). In the present study we assessed embryos, in contrast to many other studies that used seeds. This approach can give us valuable information on how to monitor and

maintain long-term viability of seeds in seed banks. Fresh seeds of *B. eriospatha* had a similar WC (19.87%) than *B. odorata* (Barb. Rodr.) Noblick (17.48%) (Schlindwein et al. 2019) and *B. capitata* (Mart.) Becc. (20.93%) (Neves et al. 2010). The seed desiccation threshold of *B. odorata* was 6.14% (WC), and seeds maintained a minimum viability rate of 90% when stored at -20 °C, which is a typical orthodox seed behavior (Fior et al. 2020). Seeds of *B. capitata* (same genus) could be dehydrated to 5-10% of WC but did lose their viability and vigor at low temperature, which corresponds to the behavior of intermediate seeds; but interestingly isolated embryos were successful cryopreserved (Dias et al. 2015). Seeds of many Arecaceae species as *Attalea crassispatha* (Mart.) Burret Haiti, *Acoelorraphe wrightii* (Griseb. & H. Wendl.) H. Wendl. ex Becc., *Desmoncus orthacanthos* Mart., Schippia *concolor* Burret., *Zombia antillarum* (Desc.) L.H. Bailey, *E. guineensis* and *Adonidia merrilli* (Becc.) Becc., are tolerant to dehydration but sensitive to low temperatures (Dickie et al. 1992; Orozco-Segovia et al. 2003). As seed conservation scientists, we should consider that whole seeds and isolated embryos can have different viability rates when they are exposed to identical desiccation and storage conditions. Our results indicate that embryos of *B. eriospatha* are orthodox, but this might not be true for whole seeds at the same conditions.

Seeds are the most preferable source of plant material for *ex situ* conservation, as they conserve the genetic background of the species. Additionally, seed collections are space- and cost-efficient (Rajasekharan 2015). However, seed size, dormancy and low and uneven germination rates were limiting factors for establishing successful conservation strategies in *Butia* spp. (Fior et al. 2011). Our results showed that germinating excised embryos under *in vitro* conditions is a suitable technique for the conservation of palm species. Desiccated embryos showed a high rate of seedling formation (Fig. 3d). Excised embryos (<1 mg) can be uniformly desiccated and cooled at a rate of 1000 °C/s, which make them easier to cryopreserve compared to whole seeds, as the formation of intracellular ice crystals is avoided (Walters et al. 2008; Walt et al. 2022). *B. eriospatha* embryos were small (Fig. 1c) our results showed that embryos dehydrated for 15 minutes did not establish an adequate WC (2.56 and 0.91 g H_2O g DW^{-1}) for successful cryopreservation. However, *B. eriosphata* embryos desiccated for 300 minutes (0.17 to 0.14 g H_2O g DW^{-1}) did show a high post-thawing germination rate of 91% when they were cryopreserved with the dehydration method (method A) [Table 1]. Ours results are similar to the successful embryos cryopreservation protocols reported for others species of this genus as *Butia paraguayensis* (Barb.Rodr.) Bailey.; *B. capitata*; *B. odorata* and *B. yatay* (Taniguchi et al. 2020). The vitrification-droplet method (PVS2 and PVS3), was only successful when the *B. eriosphata* embryos were dehydrated at 0.17 to 0.14 g H_2O gDW^{-1}. Although, desiccated embryos cryopreserved with PVS2/PVS3 solution showed a germination rate of 83% or higher, more embryos showed abnormal seedling formation (PVS2: 30%; PVS3: 15%) [Table 1]. The adverse effects of PVS2/PVS3 are as relevant as their protective function (Zamecnik et al. 2021). PVS3 is a non-permeable cryoprotectant agent (sucrose and glycerol), while PVS2 contains dimethyl sulfoxide (DMSO) and ethylene glycol (EG) which penetrate the plant tissues, hence it is more toxic than PVS3 (Fahy et al. 2004; Zamecnik et al. 2021). Certain plant vitrification agents may cause damage to the membrane system due to an unbalanced redox state and the excessive generation of ROS (Uchendu et al. 2010; Ren et al. 2013). In *Syzygium maire* (A.Cunn.) embryos, the droplet vitrification and droplet vacuum infiltration methods (using PVS2) resulted in cell death after cryopreservation (Walt et al.

2021). In contrast, *Hevea brasiliensis* L. embryos had a high tolerance to PVS2 exposition, while PVS3 was critical for growth of embryos after cryopreservation (Nakkanong and Nualsri 2018). In the present study, it was shown that embryos of *B. eriospatha* previously dehydrated to a WC of 0.14 g H_2O g DW^{-1} can successfully be cryopreserved with a dehydration protocol. About 85% of the cryopreserved embryos developed into normal seedlings.

4.2. Biochemical characterization revealed increment of antioxidant enzymes specific and differences content of PAs and amino acids during the desiccation of *B. eriospatha* embryos

In present study, as *B. eriospatha* embryo desiccate, the SOD and CAT (Fig. 5a, d) activity significant decrease while the APX and GPX (Fig. 5b, c) increase suggesting a control window of oxidative stress by eliminating ROS which may have kept the embryos viability after desiccation and cryopreservation protocol. The ROS scavenging by the antioxidant enzymes activities is the main regulators of cell homeostasis under stress condition (Hasanuzzaman et al. 2020). In desiccation sensitive seeds, protective antioxidant enzymes are not able to remove ROS quickly enough as they are produced culminating in metabolism collapse under desiccation (Leprince and Buitink 2015). On the other hand, in desiccation tolerant seeds the ROS production decrease, not only because of antioxidant enzymes activities, but as a consequence of mitochondrial respiration reduction (Vertucci and Farrant 1995; Leprince et al. 2000). For the successful scavenging of ROS, antioxidant enzymes work together, SOD catalyze O_2^- in H_2O_2 and O_2, and then H_2O_2 produced is effectively converted to H_2O and O_2 by APX, GPX e CAT (Bailly 2004; Gill and Tuteja 2010). In *Antiaris toxicaria* L. embryos and *Pongamia pinnata* L. seeds the loss of viability was associated with an H_2O_2 increase and a decrease in CAT and APX activity during desiccation (Cheng and Song 2008; Sahu et al. 2017). In contrast, for *B. capitata* embryos desiccation tolerance was related to SOD activities high, while CAT kept stable and APX was no detected (Gonçalves et al. 2020). According by Bailly (2004) reduction of SOD and CAT activity, can be compensated by the increase in APX and GPX. This leads us to think that, for B. *eriopstha*, APX and GPX are the main enzymes involved in the embryo viability during desiccation.

In *B. eriospatha* embryos, total free PAs content decreased during desiccation (Fig. 6a). In general, water stress tolerance in plant tissues and seeds were increased by biosynthesis and accumulation of PAs (Liu et al. 2015; Vieira et al. 2021). PAs act on osmotic adjustment, maintain cation-anion balance, and bind to phospholipids, stabilizing the cell membrane (Pál et al. 2015; Saha et al. 2015). Another suggested role for PAs is the scavenging of ROS by stimulating the activity of antioxidant enzymes (Liu et al. 2015). In *C. xanthocarpa* seeds under desiccation a correlation between endogenous SPD and the activity of CAT and APX, indicating its complementary role in the antioxidant defense system also was found by (Vieira et al. 2021). In the same way, exogenous SPD was able to stimulate SOD, CAT, APX and GPX enzymes actives that moderates the oxidative stress in *Trifolium repens* L. seed germination under drought stress (Li et al. 2014). However, the exact points of interaction between PAs and ROS are far from being fully understood and remain a complex biochemical phenomena that occur in plant cell stress (Minocha 2014). In desiccated *B. eriospatha* embryos the SPD and SPM content decreased while PUT increased (Fig. 6c, d). Polyamines can interconvert from PUT to SPM and SPM to SPD and back (Pál et al. 2015). Thus, the increase in PUT and a decrease in SPD content, can be related to the absense of PUT converstion into other

PAs. Increase in PUT concumitant to decrease in SPD and SPM resulted in a decrease in the ratio [(SPD + SPM)/PUT]). Pál et al. (2015) reported that the greater accumulation of PUT, leading to a low ratio [(SPM + SPD)/PUT] may even injure plants. In aquatic species as *Nymphoides peltatum* L. (Wang et al. 2007) and *Potamogeton crispus* L. (Yang et al. 2010) sensitive to drought, also had their PUT content increased, as well as a decrease of (SPM+SPD)/PUT] ratio. Compared to this report, our results showed that in parallel with the increase in PUT (Fig. 6b) and the decrease in (SPM+SPD)/PUT] ratio (Fig. 6e), the embryos showed a reduction in GSI (Fig. 3c). Some authors argued that PUT content increases is generally accompanied by the generation of ROS, caused injure plants (Groppa and Benavides 2008; Paul et al. 2018). Despite the reduction in vigor, desiccated *B. eriospatha* embryos maintained high germination, revealing that PUT content increases had no negative effects on embryo viability.

Desiccation tolerance of *B. eriospatha* embryos observed in this study might be strongly related to the amino acids metabolism. Desiccated embryos showed approximately six-fold increase in total free amino acid levels (Fig. 7a) and maintained a germination rate of 93.33% (Fig. 3a). Amino acids represent an important form of storage and transport of organic nitrogen and carbon, for the synthesis of enzymes and proteins, and are precursors of several secondary metabolites (Hildebrandt et al. 2015). In plants under abiotic stress, amino acids act in osmotic adjustment, pH regulation and ROS detoxification (Deivanai et al. 2011; Ali et al. 2019). Nonetheless, there are literally no mentions considering endogenous amino acids behavior and its possible relationship with PAs and antioxidant enzymes during seed or embryos drying. In the present study, Lys was the most abundant amino acid and its content increased about 33-fold when the embryos were dehydrated, showing its fundamental role in maintaining the viability of desiccated *B. eriosptha* embryos (Fig. 7b; Table S3). In fact, previous studies have already reported an increase of Lys content in *Solanum tuberosum* L. (Muttucumaru et al. 2015) grown under water-deficient conditions. In *Raphanus sativus* seeds L., exogenous Lys was used to overcoming the adverse effects of drought stress (Noman et al. 2018). Though all these studies reveal a potential role of Lys in desiccation tolerance through different modes, its effective concentration as well as the mechanisms are still to be explored (Ali et al. 2019). We suggest that a possible role of Lys in desiccation tolerance may be related to the dehydrins synthesis, which are identified by their highly conserved domain composed of 15 Lys-rich amino acids -EKKGIMDKIKEKLPG-, called the K segment (Galau and Close 1992). We also observed that desiccation increased the Glu and Gly content by 30 and 6-fold, respectively (Fig. 7b; Table S3). Glu is a precursor of Pro and their high concentration was associated to wild *Cicer arietinum* L. drought-tolerant seeds (Behboudian et al. 2001; Rontein et al. 2002). A putative role of Glu in the oxidative defense mechanism was proposed in *G. max* seeds, since its increase provided an increase in the antioxidant activity of CAT, GPX and SOD enzymes (Teixeira et al. 2017). Gly is also appears to be indirectly indirectly related to desiccation tolerance, since one of the glycinebetaine (GB) synthesis pathways is through glycine N-methylation (Chen and Murata 2002, 2011).

In present study, the ratio between Orn or Arg in relation to the total free amino acid revealed a decrease in the proportion of Arg and an increase in the proportion of Orn, during desiccation (Fig. 7c). Interestingly, the amino acid Arg is the precursor of arginine decarboxylase (ADC) and Orn is the precursor of ornithine decarboxylase (ODC), both responsible for PUT biosynthesis (Chen et al. 2019). By this way, our results suggest that Arg was being used by the ADC pathway for the biosynthesis of PUT, which had its contents

increased during seed desiccation (Fig. 6b). Indeed, gene expression analysis showed that the biosynthesis of PAs via ADC responds much more strongly to abiotic stress than the ODC pathway (Do et al. 2013; Berberich et al. 2015). Our results showed a significant increase in Met content in embryos dehydrated (Fig. 7b; Table S3). Met is the precursor of S-adenosylmethionine (SAM) which are involved with the generation of SPD and SPM by the addition of one or two aminopropyl groups, respectively (Mustafavi et al. 2018). The increase in the Met content and the reduction of SPD and SPM content during desiccation, may be because this biosynthesis pathway is not highly active, also confirmed by the decrease in the ratio [(SPM + SPD)/PUT] (Fig. 6e).

4.3. Integration between the desiccation threshold and the biochemical status of *B. eriospatha* embryos for *ex situ* conservation strategies

This study designs a schematic integration of the *in vitro* germination and cryopreservation protocols of *B. eriospatha* embryos and its biochemical status associated to plant population remnants as well to an *ex situ* conservation (germplasm bank) strategy (Fig. 8). Our study points out for the first time that *B. eriospatha* embryos can have more than 90% of *in vitro* germination in a water content range of 2.56 to 0.14 g H_2O g DW^{-1}. This germination protocol using culture medium (MS) supplemented with hormones (2.77 mg L^{-1} GA_3) and antioxidants (3 g L^{-1} activated charcoal) ensures the large-scale seed germination of remnant plant population to guarantee the genetic diversity preservation (Fig. 8a). In addition to advances in understanding the physiological behavior of desiccation tolerance, we provide relationships between amino acids, antioxidant activity, and PAs during desiccation stress. At a specific embryo desiccation threshold (0.14 g H_2O g DW^{-1}) compare to the previous embryos water content, the increase in the activity of APX and GPX suggest they were highly efficient against the oxidative stress caused during desiccation (Fig. 8). Also, the increase in the endogenous contents of PUT and the decrease in the biosynthesis of SPD and SPM, in relation to the control treatmen (2.56 g H_2O g DW^{-1}), resulted in a reduction in the ratio [(SPD + SPM)/PUT] (Fig. 8b). This biochemical event combined with the increase in the content of Gln, Glu, Lys, Leu, Orn and Met seems to play a key role in the desiccation tolerance and maintenance of embryo viability. The combination of this embryo stage and dehydration with subsequent direct immersion in LN, allowed us successfully established a cryopreservation protocol with *in vitro* germination and normal seedling development above 85%.

5. Conclusion

In this study it was showed that the precise information about the cell state related to water content and its biochemical composition were essential for the establishment of cryopreservation protocols with the reduction of metabolism without cell damage. Known the loss of habitats of *B. eriospatha* combined with the seed dormancy and low germination is essential to develop accessible technologies that ensure propagation and conservation of this species. In this way we recommend for long term storage partial desiccation of the embryos (0.14 g H_2O g DW^{-1}) followed by direct immersion in LN and thawing in sucrose solution (1.2 M) for 5 min, finally *in vitro* germination in MS medium supplemented with GA_3 (2.77 mg L^{-1}) and activated charcoal (3g L^{-1}). Thus, both *in vitro* germination and the cryopreservation protocol here established are powerful tools directly applied and useful to preserve a vulnerable tropical species from

Brazilian Atlantic tropical forest, which is one of the most important hotspots of biodiversity in the world. The results indicate that *B. eriospatha* embryos tending to an orthodox behavior, once are tolerate desiccation and low storage temperature.

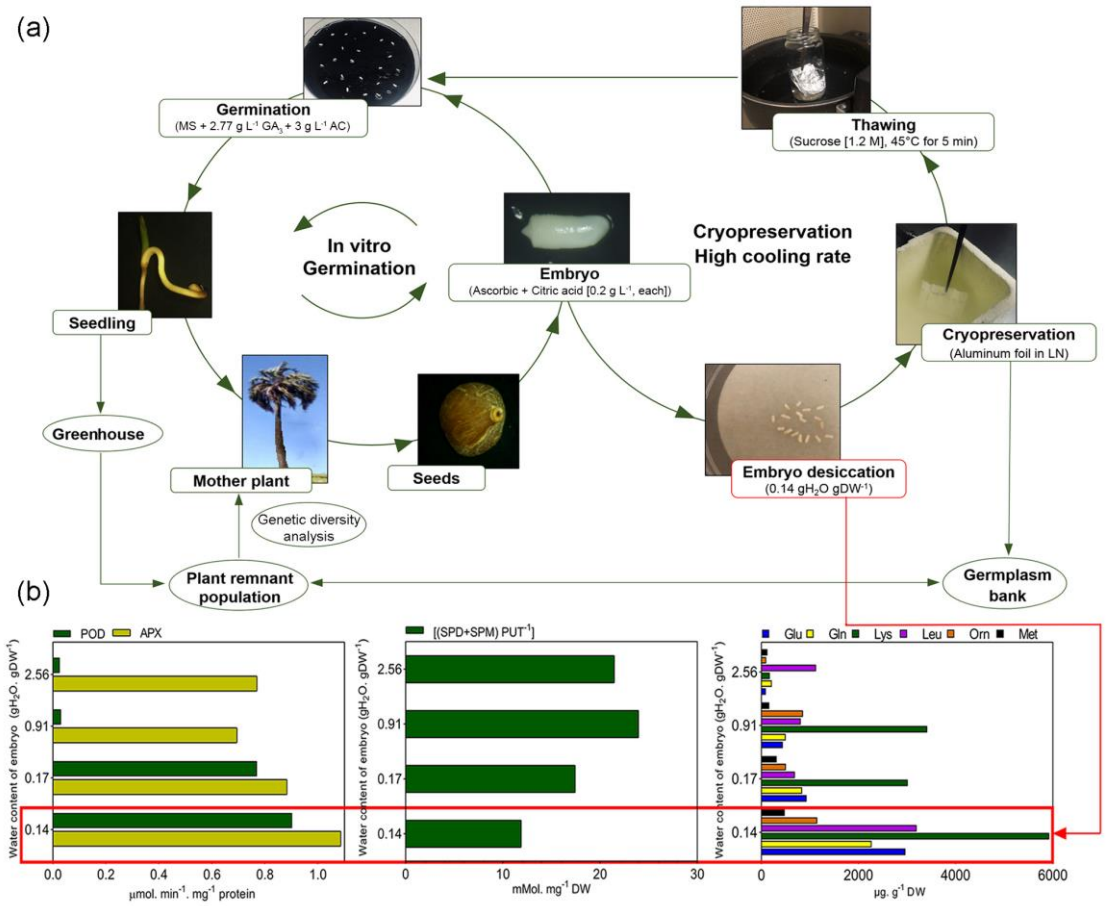

Figure 8: (a) Schematic integration of the in vitro germination and cryopreservation protocol; **(b)** and main biochemical changes of *B. eriospatha* embryos associated to plant population remnants as well to an ex situ conservation strategy. Excised fresh embryos immersed in antioxidant solution (ascorbic and citric acid 0.2 g L^{-1}, each) and the dried by 300 min (0.14 g H_2O gDW^{-1}) were in vitro germinate (60 days). These embryos showed a significant increase in the enzymatic activity of GPX and APX, in the endogenous content of amino acids (Glu, Gln, Lys, Leu, Orn, Met) and reduction in PAs [(SPD + SPM)/PUT] ratio. Specific embryo stage (0.14 g H_2O g DW^{-1}) and its biochemical state were used to establish a highly efficient cryopreservation protocol (germination > 90%). Dehydrated embryos were immersed in liquid nitrogen (LN) for 24h and thawed in sucrose solution (1.2 M, pH 5.8) at 45°C for 5 min and then in vitro germinated using MS medium with GA_3 (2.77 mg L^{-1}) and activated charcoal (3g L^{-1}). Cryopreserved embryos can be long term storage in a germplasm bank and seedlings can be transferred to a greenhouse and after to the field

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CAPÍTULO II

High-efficiency in propagation and cryopreservation of *Butia catarinensis* zygotic embryos: physiological and ultrastructural characterization

Abstract

Cryobiotechnology has guaranteed the conservation of endangered plants that cannot germinate or be long time stored conventional seed banks, such as *Butia catarinensis*, an endemic Arecaceae species from southern coast of Brazil. We investigated physiological and cytological aspects of *in vitro* germination and sensitivity to desiccation and freezing of *B. catarinensis* embryos, with the aim of designing an *ex situ* conservation strategy. The underdeveloped embryo and addition of gibberellin (GA_3) in culture medium associated with the germination high rates (90%), leads us to infer that of *B. catarinensis* seeds have morphophysiological dormancy. The meristematic cells of fresh embryos (1.42 $gH_2O\ gDW^{-1}$) showed characteristics associated with desiccation tolerant described in other palms species, such as the presence of small vacuoles and lipid and protein reserves, but also had many mitochondria and endoplasmic reticulum - found in embryos of palms desiccation sensitive. Although dehydration (0.13 $gH_2O\ gDW^{-1}$) had caused invagination of the cell walls, retraction of the plasma membrane and nuclei condensation not significantly alter the germination rates (82.5%). For the first time guidelines for cryopreservation of *B. catarinensis* embryos were described combining partial desiccation and direct immersion in liquid nitrogen (LN), with 79% of seedling normal. Cytoplasmic content filling the cellular spaces caused during desiccation, intact nuclei, mitochondria and preserved cell walls were indicative of viability. *B. catarinensis* embryos tend to behave orthodox, tolerating low WC and freezing. Validation of the use of cryobiotechnology as a long-term storage strategy, allows us to protect genetic diversity this species from environmental and anthrophonic threats.

Additional Keywords: Cell regrowth, dormancy, germination rate, zygotic embryo, *Ex situ* conservation.

1. Introduction

Palms (Arecaceae) are fundamental components of tropical and subtropical forests (Eiserhardt et al. 2011; Baker and Dransfield 2016). Brazilian palm flora has around 299 species but due to human disturbances of this ecosystem about 15% are listed on the Red List – International Union for Conservation of Nature (IUCN) (Leitman et al. 2015; IUCN 2021). The ability of a seed to survive or not to desiccation has a direct influence in the choice conservation methods (Walters and Pence 2020). Estimates that around 61% of species of Arecales produce desiccation-sensitive (DS) seeds and cannot be storage in conventional seed bank (Wyse and Dickie 2017). In addition, the seeds present low germination associated to morphophysiological dormancy, precluding seedlings produces on a large scale (Baskin and Baskin 2014). The coexistence of dormancy and desiccation-sensitivity has been reported in a very few temperate species as *Asesculus hippocastanum* (Obroucheva and Lityagina 2007) and species of Quercus (Sun et al. 2021). Although, has been considerable interest in identifying the seed storage behavior of Arecaceae seeds, available data represent only c. 7–10% of the species (SID 2020; Jaganathan 2021).

Butia catarinensis Noblick and Lorenzi is a palm species which is endemic of southern coast of Brazil (Leitman et al. 2015). It is an ornamental tree and leaves are used in utilitarian and decorative objects. Fruits are consumed by humans and local fauna and oil have anti-inflammatory potential (Kumagai and Hanazaki 2013). Urban pressure and ecosystem fragmentation result in the inclusion of *B. catarinensis* as critically endangered species on the official List of Endangered Flora Species for the State of Santa Catarina (CONSEMA 2014; Elias et al. 2019). Propagated exclusively by seeds a low germination rate (17.5%) was achieved only after removal of the operculum (Marques et al. 2014). These findings suggest that, in addition to the difficulties encountered in overcoming the growth/germination restrictions imposed by adjacent tissues, the embryo may present morphophysiological dormancy.

In vitro germination of isolated zygotic embryos is a promising alternative for propagation of *Butia* spp. (Magalhães et al. 2013; Waldow et al. 2013; Fior et al. 2018) circumventing the low rates and longtime of germination reported in whole seeds. GAs increase the growth potential of the embryo during germination by stimulating cell division and elongation (Schwech-heimer 2008). The supplementation of gibberellic acid (GA_3) in culture medium was necessary for promote higher germination rates in embryos of *Butia eriospatha* (Martius Ex Drude) (Waldow et al. 2013) and improves the conversion into seedlings and *ex vitro* survival in *Cocos nucifera* L. (Pech et al. 2007). Until present moment there are literally no studies concerning *in vitro* culture for *B catarinensis* embryos, therefore, it is necessary to find out an efficient propagation method for this species.

In the 1980s, Grout et al. (1983) first reported the successful storage of Palm zygotic embryos (*Elaeis guineensis* Jacq.) in liquid nitrogen (LN). Since the grout study, cryobiotechnology has been used as an effective and long-term *ex situ* conservation tool for many other Arecaceae species (Bagnio and Engelmann 1991; Assy-Bah and Engelmann 1992; Hemanthakumar et al. 2013; Han et al. 2021). This technical involves storage biological material at ultra-low temperatures in LN (−196 °C) together with *in vitro* technologies (Walters and Pence 2020). The principle of cryopreservation is to stabilize cellular structure and limit metabolic processes without loss of physiological quality (Engelmann 2011). It is crucial to establish a successful cryopreservation protocol to prevent lethal ice formation within cells and this is possible through solidification of the cytoplasm, combining drying and rapid cooling (Walters and Pence

2020). Previous searches with *Butia capitata* (Mart.) Becc (Dias et al. 2015); *Butia yatay* (Vargas et al. 2020); *Butia lallemantii* (Deble & Marchiori); *Butia paraguayensis* (Barb.Rodr.) Bailey; *Butia odorata* (Barb. Rodr.) Noblick (Taniguchi et al. 2020) and *Butia eriospatha* (Mart. ex Drude) Becc. (Goeten et al. 20220 have shown the successful partial air desiccation of embryos followed by direct immersion in LN.

Steps in the cryopreservation process expose embryonic tissues to numerous physical and metabolic stresses which are associated with cytological factors (Raju et al. 2021). Disruption of the lipid structure and denaturation of proteins alter membrane semi-permeability and are major contributors to cell death during cryopreservation (Kaczmarczyk et al. 2012; Wesley-Smith et al. 2014; Umarani et al. 2015). In that context, ultrastructural and cytochemical evaluations are important tool for the comprehension of cellular and morphological events underlying cryopreservation. Both analyzes have been employed in studies of seed behavior during desiccation (Panza et al. 2007; Vieira et al. 2021) and cooling (Alla-N'nan et al. 2014; Ballesteros et al. 2014; Wesley-Smith et al. 2015) contributing to physiological knowledge and assisting in *ex situ* conservation (Vitis et al. 2020).

In this sense, this article describes for the first time the use of *in vitro* culture as a method of rescuing *B. catarinensis* embryos to overcome the unviability and dormancy of its seeds. Furthermore, we studied the physiological behavior of *B. catarinensis* isolate embryos during the desiccation process and their response to cryostorage. We have determined the WC in which embryos can be safely cryopreserved. Survival and seedling emergence after thawing was assessed *in vitro* while cell integrity was assessed based on ultrastructural analysis.

2. Material and method

2.1. Plant material

Mature fruits of *B. catarinensis* were collected from 10 plants in Santa Catarina State (28º 20'34" S; 48º 67'80" W) Brazil, in March 2022. Fruits without insect attack and microorganism infection were manually processed to obtain the pyrenes. The seeds were extracted after breaking the pyrenes by using a hamber. Seeds were surface-sterilized in ethyl alcohol (70% v/v) for 1 min followed by 15 min in sodium hypochlorite (2.5% v/v) and then rinsed three times with sterilized distilled water. At a laminar flow hood, embryos were excised from the seeds, using scalpel and then placed in petri dishes with 25 mL of ascorbic acid (0.2 g L^{-1}) and citric acid (0.2 g L^{-1}) solution, to prevent tissue oxidation.

2.2. Seed and embryo morphology and morphometry

Length and diameter of 100 seeds and embryos (fresh) were measurements, using caliper. Seeds and embryos were pictured with a stereomicroscope (Olympus® - SZH10) equipped with image capture system (Olympus® - DP71).

2.3. *In vitro* germination tests

Four replicates of 25 excised embryos of were disinfected with commercial sodium hypochlorite (0.5% v/v) for 10 min, followed by a triple-washed with autoclaved distilled water (Magalhães et al. 2013). Embryos were germinated in culture medium according to Waldow et al (2013), with adaptations. Embryos

were germinated in petri dish with 25 mL of MS culture medium (Murashige and Skoog 1962) supplemented with sucrose (30 g L^{-1}), activated charcoal (3 g L^{-1}), and phytagel (2.5 g L^{-1}). The pH of the culture mediums was adjusted to 5.8 and autoclaved at 121 ºC, 1.5 atm for 15 min.

The effect of GA_3 in germination in excised embryos was tested with the best concentration described for Waldow et al. (2013) for *B. eriospatha*. The GA_3 (8 mg L^{-1}) solution was filter-sterilized (PES membrane 0.22 µm) and added to the culture medium, previously described, after autoclaving. Both experiments (with and without GA_3) embryos were maintained in a germination chamber type BOD (25 ± 2 °C, 60% RH, 25.5 µmol m^2 s^{-1} intensity light and 12/12 h photoperiod). Embryo was scored germinated when the radicle protrusion occurred. Assessments were recorded daily for 30 days. The germination percentage and germination speed index (GSI) were calculated according to the Ranal and Santana (2006) and Mangure (1962) respectively.

2.4. Water content (WC) and drying of embryos

The initial WC of was determined by the oven method (ISTA 2004), using five replicates of 10 seeds and embryos. WC was measured gravimetrically by difference in weight before and after drying for 24 h in an oven at 103 ± 1°C. WC value was expressed on a dry weight basis (g H_2O gDW^{-1}).

Five replicates of 10 embryos were dried for 0; 15; 30; 60; 120; 180; 240 and 300 min in a laminar airflow cabinet at 25 ± 2°C and an airflow of 0.46 m s^{-1}. Embryos WC were measured gravimetrically and then desiccation curve was calculated accordingly Hong and Ellis (1996). Four replicates of 25 embryos of each desiccation treatment (different drying time intervals) were *in vitro* germinated of according to the description of *In vitro germination tests* - section, in medium supplemented with GA_3. Germination rate and GSI were assessed for all desiccation times. Based on these analyzes and on the WC, the drying times for the cryopreservation experiments were determined.

2.5. Cryopreservation, thawing and recovery

Four replicate of 25 embryos were partially dried for 0; 60; 180 and 300 min. Then, these embryos were wrapped in sterilized aluminum foil (~20 µm thickness) and directly immersed in liquid nitrogen (LN) at -196 °C. After 24h, the thawing was done by removing foil envelopes from the cryotank and directly immersing them a sucrose solution (1.2 M, pH 5.8), kept in water bath at 45°C for 5 min (Goeten et al. 2022).

Embryos were germinated of according to the description of *In vitro germination tests* section, in medium supplemented with GA_3 and kept in in a germination chamber type BOD (25 ± 2 °C, 60% RH) in the dark for three days. After three days, the embryos were exposed to light (i*ntensity* of 25.5 µmol m^2 s^{-1}) and 12/12 h photoperiod. After 30 days in germination medium, germination rate, GSI and normal seedling formation were assessments. A seedling was scored as normal those presenting cotyledonary petiole, primary root, and first leaf sheath as described by Magalhães et al. (2013).

At 40 days individual seedlings were transferred for glass test tubes (150 x 925 mm) containing 15 mL of MS culture medium (Murashige and Skoog 1962) with sucrose (30 g L^{-1}) and phytagel (2.5 g L^{-1}). The pH was adjusted to 5.8 and autoclaved at 121 ºC, 1.5 atm for 15 min. The seedlings were maintained in growth room (25 ± 2°C; 55% RH) with white LED light (Green Power TLED W; Philips TM; 77 µmol m^{-2} s^{-1}) and 16/8 h photoperiod.

2.6. Seedlings morphometry

Primary root and the first eophyll were measurements in four replicate of 10 seedlings, after 75 days of cryopreservation. Seedlings were pictured in stereomicroscope (Olympus® - SZH10) equipped with image capture system (Olympus® - DP71) and Controller software.

2.7. Light microscopy

Shoot apical meristem (sam) of fresh embryos, without desiccation (control) and desiccated for 300 min cryopreserved (+LN) or not (-LN) were used for histological analysis. Light microscopy was carried out according to Steiner et al. (2015). Embryos were fixed in phosphate buffer 0.1 M (pH 7.2) containing 2.5 % formaldehyde for 24h, followed by dehydration in increasing series of ethanol aqueous solutions. Then, the embryos were infiltrated with HistoResin (Leica® HistoResin, Heidelberg, Germany). Semi-thin sections (3 µm) were submitted to periodic acid-schiff (PAS) and coomassie brilliant blue (CBB) 0.4% in Clarke's solution double staining (Schmidt et al. 2012). PAS was used to identify the presence of neutral polysaccharides (starch grains and cellulose), and CBB to detect total protein. Relevant aspects have been captured using a microscope (Olympus® BX-40) equipped with image capture system (Olympus® DP 71) and Controller software.

2.8. Electron microscopy analysis

Shoot apical meristem (sam) of fresh embryos, without desiccation (control) and desiccated for 300 min cryopreserved (+LN) or not (-LN) were used for electron microscopy analysis. Ultrastructure analysis was performed according to Steiner et al. (2015). Embryos were fixed were initially fixed for 24 h in fixation buffer composed by 2.5% glutaraldehyde, 0.1 M sodium cacodylate buffer (pH 7.2), and 18 mM sucrose. After that, the embryos were subjected to washing with 0.1 M sodium cacodylate buffer (pH 7.2) and pos-fixed for 4 h in a buffer composed of 1% osmium tetroxide in 0.1 M sodium cacodylate buffer. Then, the embryos were dehydrated by acetone series (30°, 50°, 70°, 80°, 90° and 100°), followed by a series of pre-infiltration with 100% acetone and Spurr's resin (3:1; 2:1; 1:1; 1:2; 1:3, pure, pure, 96 h each at 4 °C) until embedded in Spurr's resin (EMS Diasum, Hatfield, PA, USA) according to manufacturer's instructions. Ultrathin sections were stained with uranyl acetate and lead citrate (Reynolds 1963), and the images were acquired using TEM JEM 1011 (JEOL Ltd., Akishima, Tokyo, Japan) at 80 kV.

2.9. Statistical Analyses

The data normality was evaluated using the Shapiro – Wilk test, and then submitted to analysis of variance (ANOVA) in a completely randomized design. For cryopreservation was performed two crossed

fixed factors (drying time × exposition or not to LN). In the case of significance of the F values, means were compared using SNK test ($p<0.05$) (Sokal and Rohlf 1995). Statistical analyses were performed using the R Core Team (2016).

3. Result

3.1. Morphometry and morpho-anatomy of seeds and embryos

B. catarinensis pyrenes are oval and are composed of a woody endocarp (end) [Fig. 1a]. Seeds had an oval shape, were covered by a thin brown seed coat (sc) and an operculum (os) forming a small protuberance at its proximal extremity (Fig. 1b). Average seed diameter and length were 9.5 ± 0.04 mm and 5.9 ± 0.02 mm, respectively and weighed 22.8 ± 4.12 mg. An embryo (em) white opaque was located under the operculum (op) surrounded by a transduced endosperm (en) [Fig. 1c]. In the embryos proximal portion (pp) a cotyledonary petiole (cp) envelops a rudimentary embryonic axis while in the distal portion (dp) it is the haustorium (ha) [Fig. 1d]. At an oblique angle to the cotyledon axis; the plumule and the hypocotyl–radicle axis can be distinguished (Fig. 1e). The plumule (pl) has two leaf primordia (s1 and s2) and a shoot apical meristem (sam) [Fig. 1f]. The hypocotyl–radicle axis consists of root apical meristem (ram) formed by just a few cells, a ground meristem (mz), and procambium (pc) [Fig. 1f]. Procambial (pc) strands appears originating from the embryonic axis that are located centrally in the cotyledonary petiole (cp) and peripherally in the haustorium (ha) [Fig. 1e]. The embryo was typically cylindrical average 4.2 ± 0.02 mm of length and 2.0 ± 0.01 mm of diameter and weighing 1.2 ± 0.05 mg. and with two regions: cotyledonary petiole (cp) and haustorium (ha) [Fig. 1d-e].

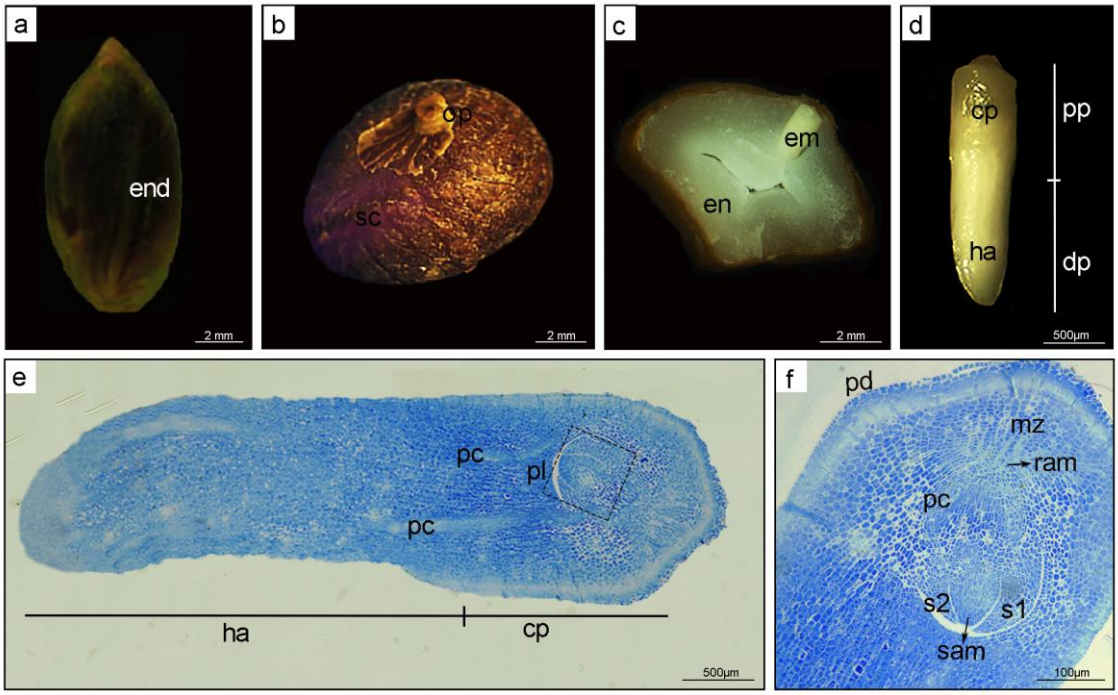

Figure 1: Morphology characterization and light microscopy of seed **(a-b)** and mature embryo **(c-e)** of *B. catarinensis*. Seeds external morphology showing seed coat (sc) and operculum (op) regions **(a)**. Seed longitudinally sectioned showing embryo (em) embedded in the endosperm (en) **(b)**. Mature embryo with cotyledonary petiole (cp) in the proximal portion (pp) and haustorium (ha) in the distal portion (dp) **(c).** Mature embryo stained with TB-O showing procambial (pc) and plumule (pl) **(d).** Detail of the cotyledonary petiole (cp) demonstrating the M zone (mz) between the protoderm (pd) and the root apical meristem (ram), first (s1), second leaf (s2) and shoot apical meristem (sam).

3.2. Effect of GA_3 *in vitro* germination

Effect of GA_3 on embryos germination and vigour was assessed for 30 days. Embryos cultivated in medium supplemented with GA_3 germinated faster (6 days) and reached earlier (21 days) its maximum value of 90%, resulting in a GSI of 1.63 (Fig. 2). While control embryos (without GA_3) started germination later (12th day) tending to stabilize after 26 days and showed lower germination values (25.77%) and GSI (0.14) [Fig. 2].

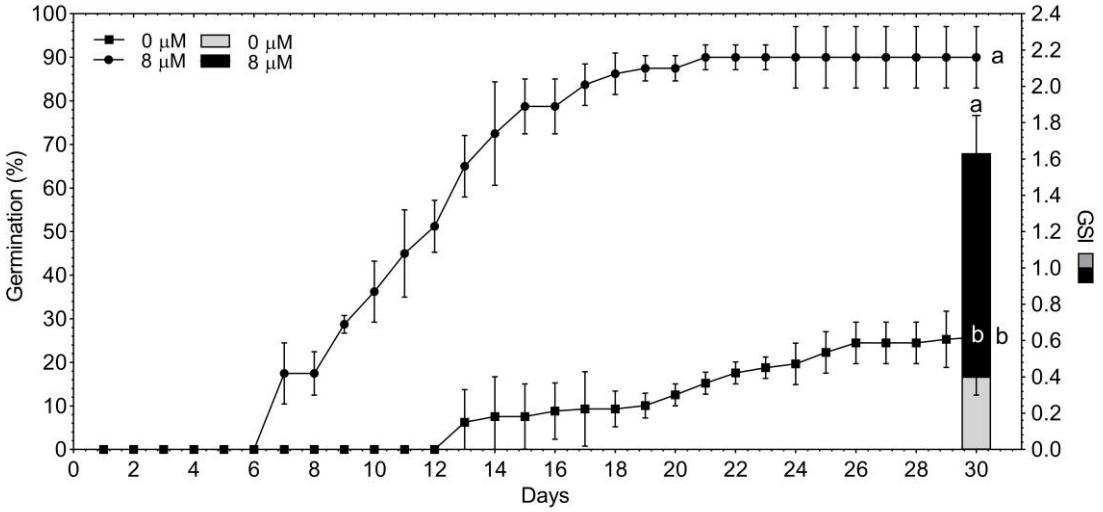

Figure 2: Germination dynamic and germination speed index (GSI) of *B. catarinensis* embryo inoculated or not in medium supplemented with GA_3. Data mean ± standard error (n = 4). Means followed by different letters are significantly different ($p < 0.05$) according to the SNK test.

3.3. Effect of desiccation in *B. catarinensis* embryos in viability and vigor

B. catarinensis mature seeds (Fig. 1a) and fresh embryos (Fig. 1c) presented a WC of 0.68 gH_2O gDW^{-1} and 1.42 gH_2O gDW^{-1} respectively. Fresh embryos excised (1.42 gH_2O gDW^{-1}) and immerse in antioxidant solution (control treatment; WC = 3.22 gH_2O gDW^{-1}) had their WC increased by 1.8 times (Fig. 3). The half of the initial WC was lost within at 45 min of drying (1.54 gH_2O gDW^{-1}). At 60 min (1.49 gH_2O gDW^{-1}) WC was slightly higher than that found in the initial conditions (1.42 gH_2O gDW^{-1}), probably the time needed for the exit of the antioxidant solution (Fig. 3). After 180 min (0.29 gH_2O gDW^{-1}) WC slowly reduced tending to stability. At 300 min embryos final water loss were 95.96% and WC was of 0.13 gH_2O gDW^{-1} (Fig. 3).

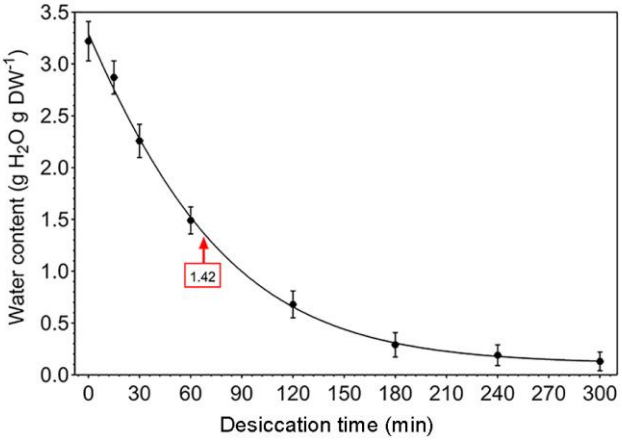

Figure 3: Water contents (gH2O gDW^{-1}) of *B. catarinensis* embryos in response to desiccation for 0; 15; 30; 60; 120; 180; 240 and 300 min. Red arrow: time (~60 min; WC = 1.49 gH2O gDW^{-1}) indicating WC slightly longer than fresh embryo (1.42 gH2O gDW^{-1}). Data mean ± standard error (n = 5).

The reduction of WC did not have a significant effect on *B. catarinensis* embryos viability (Fig. 4a). Embryos without dehydration (control treatment; WC = 3.22 gH_2O gDW^{-1}) had a germination of 90%, while embryos with WC of 0.13 gH_2O gDW^{-1} had 82.5% (Fig. 4a).

Embryos with WC until 1.49 gH_2O gDW^{-1} did not showed significant effects in the GSI values (Fig. 4b), they germinated earlier and faster (maximum 17 days) [Fig. S1]. Reduction of WC at 0.13 gH_2O gDW^{-1} resulted in a decrease of 35% in the GSI values (1.07) compared to control treatment (1.63) [Fig. 4b]. These embryos germinated later taking approximately 25 days to complete it (Fig. S1).

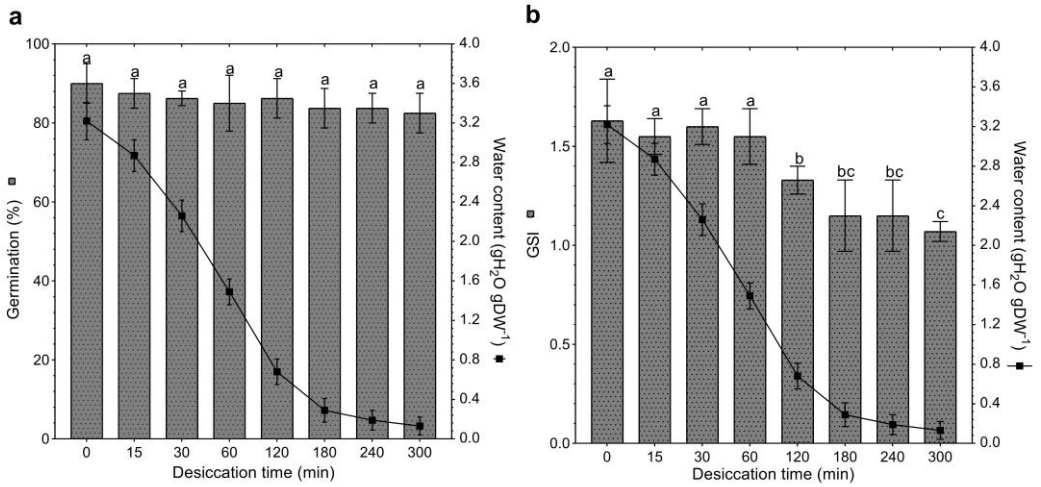

Figure 4: Germination and germination speed index (GSI) of *B. catarinensis* embryos in response to desiccation for 0; 15; 30; 60; 120; 180; 240 and 300 min. Water contents (axis right) and final germination (%) - (axis left) **(b)**. Water contents (axis right) germination speed index (GSI) - (axis left) **(c)**. Data mean ± standard error (n = 4). Means followed by different letters are significantly different ($p < 0.05$) according to the SNK test.

3.4. Effect of cryopreservation on *B. catarinensis* embryos in viability and vigor

B. catarinensis embryos fully hydrated (control treatment; WC = 3.22 gH_2O gDW^{-1}) immersed in LN lost viability completely and did not germinate (Fig. 5a). Low survival rate (3.75%) was observed in embryos with WC of 1.49 gH_2O gDW^{-1}. Reduction of WC at 0.29 gH_2O gDW^{-1} allowed the cryopreservation of embryos (germination = 66.25%). However, the germination rate was increased to

78.75 % when the embryos were dried at a WC of 0.13 $gH_2O\ gDW^{-1}$ (Fig. 5a). Cryopreservation in embryos with WC of 0.29 and 0.13 $gH_2O\ gDW^{-1}$ had no significant effect on GSI values, compared to control (no exposure to LN) [Fig. 5b]. These embryos beginning germination 10th day and took 24 and 25 days, respectively, to reach maximum values (Fig. S2).

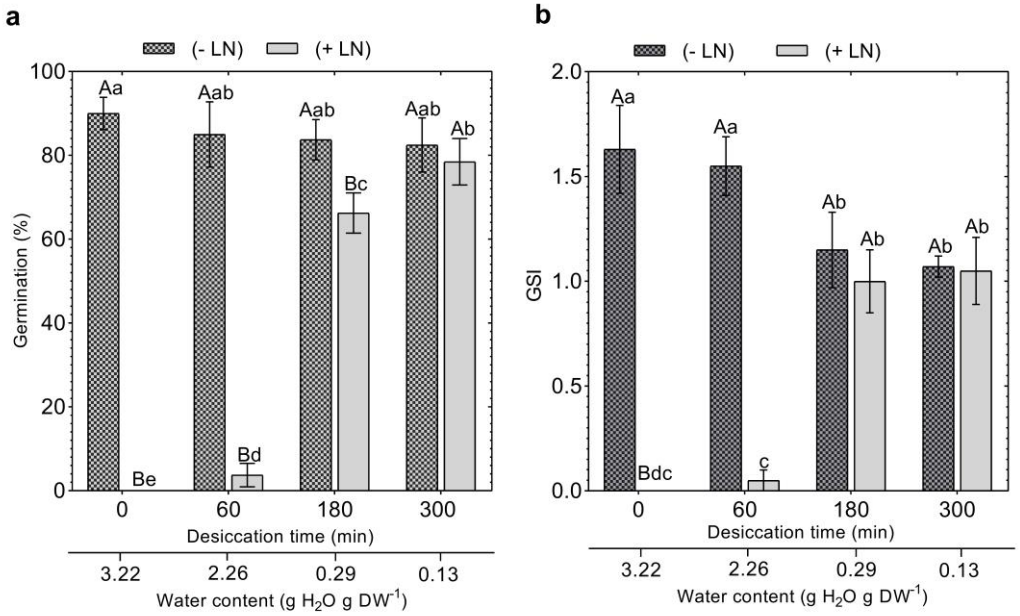

Figure 0-5: Germination and germination speed index GSI of *B. catarinensis* embryos to desiccation for 0; 60; 180 and 300 min and cryopreserved **(a-b)**. Final germination (%) **(a)** GSI values **(b)**. Data mean ± standard error (n = 4). Means followed by different letters are significantly different ($p < 0.05$) according to the SNK test.

3.5. Effect structural of cryopreservation in *B. catarinensis* embryos

Shoot apical meristem (sam) zone of fresh embryo (WC = 1.42 $gH_2O\ gDW^{-1}$) showed cells with high nucleus-cytoplasm ratio, small vacuoles (v) and preserved cell walls (cw) (Fig. 6a-b). Intense reaction to CBB and PAS staining allowed us to identify a dense cytoplasm, abundant in proteins and the presence of neutral polysaccharides in cell wall (cw) [Fig 6a]. Mitochondria (m), endoplasmic reticulum (er), lipid body (lb) and vacuoles containing protein residues (pvs) were abundant and positioned in peripherally of cell (Fig. 6c).

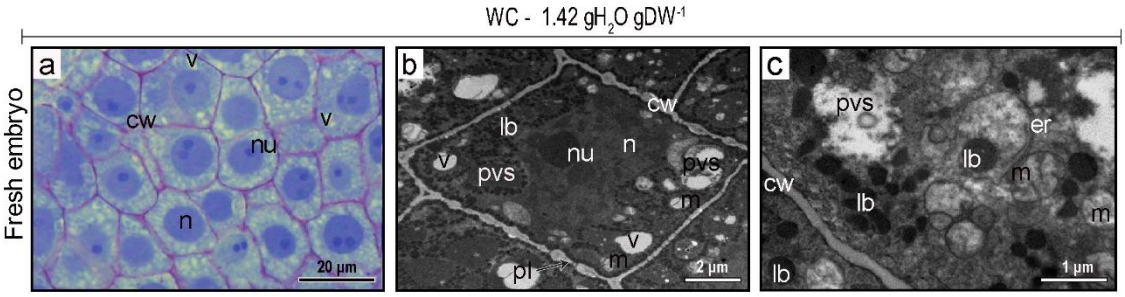

Figure 6: Shoot apical meristem (sam) zone of *B. catarinensis* fresh embryos (WC = 1.42 gH2O gDW^{-1}) **(a-c)**. Light microscopy of cells stained with (PAS + CBB) showing organized nuclei (n) and with nuclei (nu) in the central region, small vacuole (v) and cell wall (cw) **(a)**. Transmission electron microscopy of cells showing large central nuclei (n) with prominent nucleoli (nu), vacuoles (v) containing protein residues and preserved cell wall (cw) **(b)**. In detail, mitochondria (m), endoplasmic reticulum (er), protein storage vacuoles (pvs) and lipid body (lb) in cellular peripherally **(c)**.

Cells of the control treatment (without desiccation; WC = 3.22 $gH_2O\ gDW^{-1}$) were turgid with evident protein storage vacuoles (pvs) and compacted nucleus (n) in the peripheral region close to the cell wall (cw) (Fig. 7 a-b). Protein reserves were abundant inside the vacuoles (pvs) compared to the fresh embryo, indicating mobilization (Fig. 7b). Nuclear envelope (ne) preserved, mitochondria (m) and numerous lipid bodies (lb) organized around the protein storage vacuoles (pvs) were observed. Cryopreservation theses embryos promoted cellular reduction of cytoplasm, coalescence of lipids body (lb) and some cells showed leakage cell contents (*asterisks*) [Fig. 7d-e]. Consequently, due to the extensive lysis of some cells no intracellular structure could be identified (*asterisks*) [Fig. 7e]. Cytoplasm was disorganized with nuclei (n) and nucleoli (nu) showing signs of degradation, losing its rounded shape and elongating (Fig. 7f).

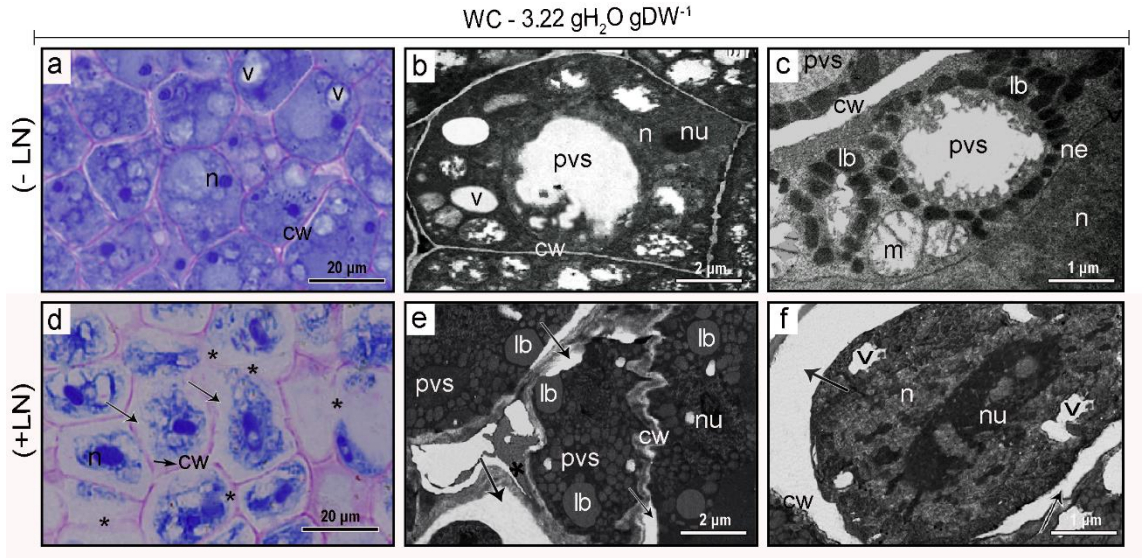

Figure 7: Shoot apical meristem (sam) of *B. catarinensis* embryos with content water of 3.22 $gH_2O\ gDW^{-1}$ (without esiccation) before (-LN) and after (+LN) cryopreservation, double staining (PAS + CBB) **(a; d)** and transmission electron microscopy **(b-c; d-e)**. Turgid cells with compacted nucleus (n) in the peripheral region close to the cell wall (cw) and evident vacuole (v) **(a)**. Cells with many vacuoles filled with protein (pvs), nuclei (n) with nucleoli (nu) and cell wall (cw) preserved **(b)**. In detail, mitochondria (m) and lipid bodies (lb) organized around the protein storage vacuoles (pvs), nuclear envelope (ne) and nuclei (n) preserved **(c)**. Cells with plasmalemma detached from their cell walls (cw) [arrow] **(d-e)**. Cell wall (cw) [asterisks] disruption **(d)**. Coalescence of lipid body (lb) **(e)**. In detail, cytoplasm disorganized with nuclei (n) and nucleoli (nu) degraded **(f)**.

In cells of embryos desiccated (0.13 $gH_2O\ gDW^{-1}$) nuclei (n) with nucleoli (nu) were compacted and located centrally within the cell compartment (Fig. 8a-b). The cells showed cytoplasmic shrinkage characterized by the separation of the plasmalemma from their cell walls (cw - arrow) [Fig. 8a-c]. Consequently, it is observed loss of communication between cells through the of plasmodesmata (pl) [Fig. 8d]. These cells were marked by small infolds of cell wall (cw), compared to fresh embryo and control treatment (Fig. 8c). Post cryo, the cells were turgid, the spaces formed between the cell wall (cw) and the plasmalemma during desiccation reduced, being filled by cytoplasmic content (arrow) [Fig. 8d-e]. The cell wall (cw) was structurally preserved and without folds, lipid bodies (lb) adjacent to the cell wall (cw) and mitochondria were observed (Fig. 8f). The mobilization of protein stores is evidenced by the lower number

of protein storage vacuoles (psv) and a less dense cytoplasm observed by CBB staining compared to fresh and control embryos (Fig. 8d-e).

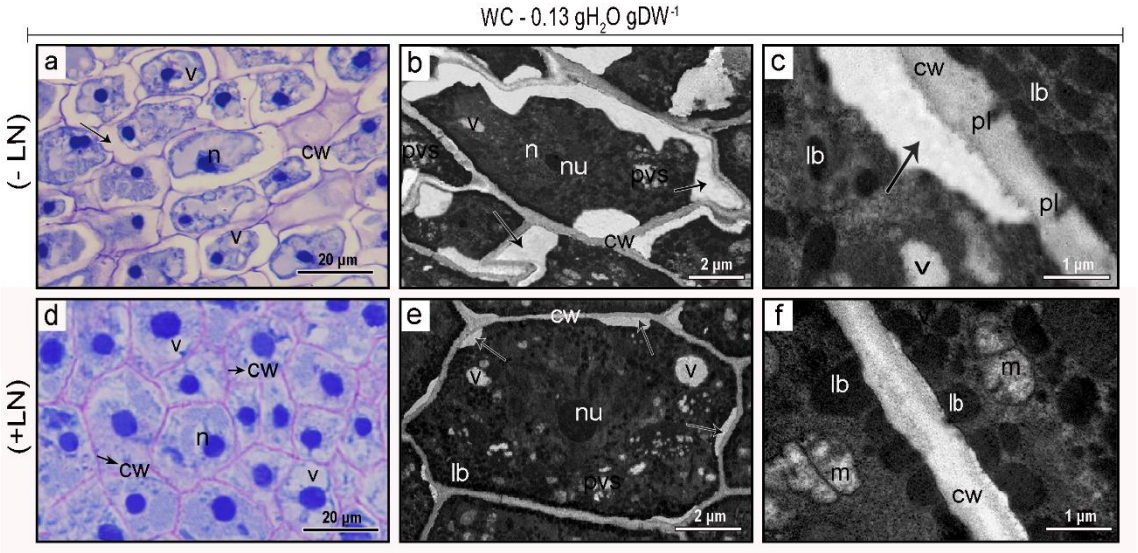

Figure 8: Shoot apical meristem (sam) of *B. catarinensis* embryos with content water of 3.22 gH_2O gDW^{-1} (without desiccation) before (-LN) and after (+LN) cryopreservation, double staining (PAS + CBB) **(a; d)** and transmission electron microscopy **(b-c; d-e)**. Cells showing separation of the plasmalemma from their cell walls (cw) [arrow] organized nuclei (n) and small vacuole (v) **(a-b)**. Cells wall (cw) with small infolds (**b**). In detail, detachment plasmalemma from their cell walls (cw) [arrow] interrupting communication between cells through the of plasmodesmata (pl) **(c)**. Turgid cells with evident nucleus (n) small vacuole (v) and preserved cell wall (cw) **(d-e)**. In detail, lipid bodies (lb) adjacent to the cell wall (cw) and mitochondria (**f**).

3.6. Seedling recovery after cryopreservation of *B. catarinensis* embryos

After three days of *in vitro* culture cryopreserved embryos were completely white and intumescent. At the 10th day the protrusion of the primary root and cotyledonary petiole elongates occurred, indicating the beginning of germination (Fig. 9a). At 45 days of *in vitro* culture the first leaf sheath will emerged from the opposite side of the primary root (Fig. 9b-c). All desiccate embryos that germinate produced normal seedling (Table 1). Reduced WC favored the normal seedling development and highest rate was obtained in embryos with WC of 0.13 gH_2O gDW^{-1} with 76.55% (Table 1). At 75 days the second sheath, followed by the eophyll and lateral roots were observed (Fig. 9d-e). The loss of water had no significant effect on the length of the eophyll but led to a reduction in the length of the roots, consequence of delayed germination (Table 1; Fig S2). Embryos desiccated at 0.13 gH_2O gDW^{-1} followed by immersion in LN had shorter leaf lengths when compared to desiccated embryos not exposed to LN, but with no significant difference in primary root length (Table 1).

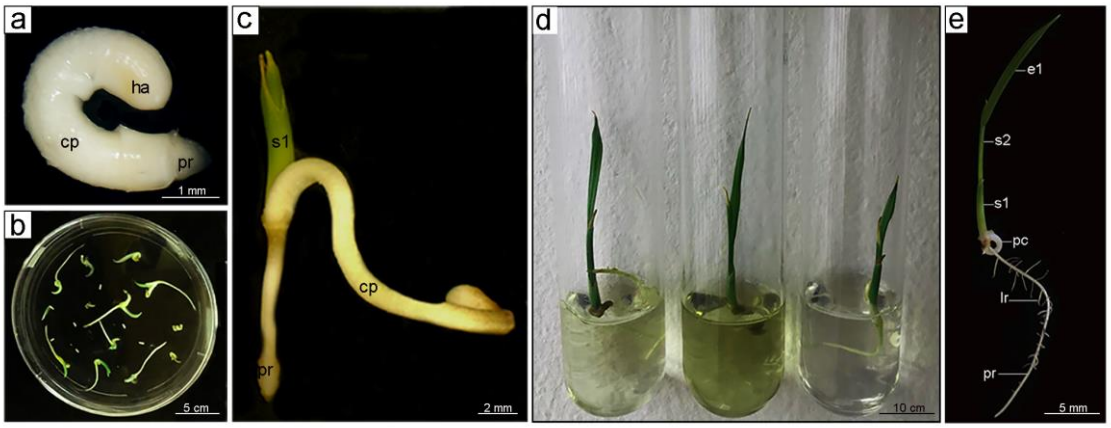

Figure 9: *B. catarinensis* embryo and seedling development after desiccation (WC = 0.13 $gH_2O\ gDW^{-1}$) and cryopreserved for high cooling technique **(a-d)**. Embryo germinated, 10 days of *in vitro* culture, showing haustorium (ha), cotyledonary petiole (cp) elongation and radicle protrusion (pr) **(a)**. Embryos regenerated after 45 days of *in vitro* culture **(b)**. In detail normal seedling, showing formation of leaf sheath (s1), cotyledonary petiole (cp) and primary root (pr) elongation **(c)**. Seedlings after 75 days of *in vitro* culture with first (s1) and second leaf sheaths (s2) the emission of the first eophyll (e1) and lateral roots (lr) **(d-e)**.

Table 1: *B. catarinensis* seedling growth from cryopreserved embryos

Desiccation time (min)	Water content ($gH_2O\ gDW^{-1}$)	Water loss (%)	Normal seedlings (%)		Seedlings morphometry (cm)			
					Primary root		Eophyll	
			(-LN)	(+LN)	(-LN)	(+LN)	(-LN)	(+LN)
0	3.22±0.19	------	90.00±5.01^{aA}	0.00±0.00^{dB}	4.92±0.03^{aA}	0.00±0.00^{dB}	4.22±0.02^{aA}	0.00±0.00^{bB}
60	1.49±0.13	53.73	85.00±7.07^{aA}	1.17±0.14^{cB}	4.44±0.01^{bA}	2.83±0.04^{cB}	4.15±0.05^{aA}	3.46±0.15^{aB}
180	0.29±0.12	90.99	83.75±5.12^{abA}	45.87±5.12^{bB}	3.62±0.34^{cA}	3.02±0.01^{bB}	4.04±0.12^{aA}	3.34±0.12^{aB}
300	0.13±0.10	95.96	82.50±5.03^{abA}	76.55±7.16^{aA}	3.08±0.04^{dA}	3.29±0.02^{aA}	4.01±0.04^{aA}	3.19±0.03^{aB}

Data mean ± standard error (n = 4). Small letters compare each drying time and capital letters compare embryos no exposed to liquid nitrogen (-LN) and exposed to liquid nitrogen (+LN). Means followed by the same letter are not significantly different by the SNK test ($p < 0.05$).

4. Discussion

4.1. The use of GA_3 is essential for the germination of *B. catarinensis* embryos

The use of gibberellic acid (GA_3) in culture medium accelerated in six days and increased about 3.5 times the germination of *B. catarinensis* embryos (Fig. 2). The onset of germination is closely regulated by the endogenous balance of gibberellin (GA) and abscisic acid (ABA) (Lando et al. 2020). Embryos in contact with GA_3 produces a specific signal activating ABA catabolism and increasing the synthesis of GAs, activating the germinal processes (Liu and Hou 2018). Failures in the endogenous regulation of GA_3 inhibit the activity of enzymes responsible for loosening the cell wall of the tissues surrounding the radicle, preventing germination (Bewley et al. 2013; Lando et al. 2020). Similar to our results embryos of *B. eriospatha in vitro* had a high germination and speed index when added GA_3 in culture medium (Waldow et al. 2013). In the present study, embryos were excised from seeds from mature fruits (yellow-reddish color), but embryo anatomy showed that the hypocotyl-radicle axis was less differentiated than the plumule (Fig. 1f). Anatomical immaturity is common in the embryo of palms during dispersion of the fruit, and has been attributed to morphophysiological dormancy (Baskin and Baskin 2014; Jaganathan 2020). In *B. capitata* embryos the differentiation of the radicle pole of occurred very slowly and only became recognizable when the first plumule primordium developed (Magalhães et al. 2013). Until now, studies have linked the delay and even the absence of germination of *B. catarinensis* to mechanical dormancy,

preventing gas exchange and water absorption (Marques et al. 2014). Considering the underdeveloped embryos and that even after the removal of the endocarp the germination was smaller and slower in relation to the treatment with GA_3, we believe that the embryos of *B. catarinensis* present germination inhibitors and fit the classification of morphophysiological dormancy, proposed by Baskin and Baskin (2014) and Jaganathan (2020).

4.2. *B. catarinensis* **embryos are tolerant of low water contents**

Initial WC of *B. catarinensis* embryo was shed highly hydrated (1.42 gH_2O gDW^{-1}, respectively). This characteristic combined with high nucleus-cytoplasm ratio and the large number of mitochondria, observed in apical meristem of embryo [Fig. 6a-c], suggest a state of high metabolic activity. Highly WC at the time of shedding from mother plant has even been proposed as a useful indicator for predicting to recalcitrant seeds (Marques et al. 2018). Once pre-shedding development of orthodox seeds is characterized by a third and final phase, maturation drying, during which the acquisition of dry mass is maximum and a large part of the water is lost, this phase seems to be absent in recalcitrant types (Radwan et al. 2014). The cytochemical and ultrastructural analyzes allowed us to compare cellular characteristics found in previous studies with orthodox and recalcitrant palm seeds. Meristematic cells of *B. catarinensis* embryo were similar to *Euterpe edulis* Mart. and *Mauritia flexuosa* L. (recalcitrant embryos), in the large number of mitochondria and endoplasmic reticulum, but they differed in that they had small vacuoles, lipid reserves and a dense cytoplasm spread throughout the cell compartment (Panza et al. 2004; Veloso et al. 2016). On the other hand, embryo cells of *E. edulis* (Panza et al. 2004), show an abundance of rough starch grains, features absent in *B. catarinensis* and in the palm embryos orthodox as *Phoenix dactylifera* L., *Acrocomia aculeata* (Jacq.) Lodd. ex Mart and *Washingtonia filifera* (Lindl.) Wendl (Demason and Thomson 1981; Demason 1988; Mazzottini-dos-Santos et al. 2020).

In the present work, the reduction in WC to 0.13 gH_2O gDW^{-1} did not significantly affect viability (germination = 82.5%) [Fig. 3a]. Storage studies report that for some palm species whole seeds do not tolerate WC less than 10–15%, while in excised embryos less than this content is not lethal (Dias et al. 2015; Jacob et al. 2017). Jaganathan (2021) proposed that excessive drying of pyrenes leads to endosperm cell damage and elongated embryo cannot penetrate the endocarp as the requires exceptional strength supplied by the nutrition from the endosperm, resulting in germination failure. In addition, the rapid dehydration inhibits the accumulation of reactive oxygen species (ROS), which react with proteins, lipids and nucleic acids causing oxidative damage to cells (Ballesteros et al. 2014; Vieira et al. 2021). In most palms, the endocarp and other covering structures prevents water loss embryo, so dry pyrenes take several days to decrease the WC (von Fintel et al. 2004; Jaganathan 2021), which can lead to loss of viability.

Interestingly, the anatomical features of rudimentary embryos (hypocotyl-radicle axis less differentiated than the plumule) [Fig. 1f] was also described in other palm embryos that desiccation tolerant as *B. capitata* end *S. romanzoffiana* (Magalhães et al. 2013; Suhana Zakaria et al. 2016). On the other hand, in mature embryos of seeds recalcitrant such *Euterpe* spp., the hypocotyl–radicle axis was more developed with complete cap and very evident radicle (Aguiar and Mendonça 2002; Panza et al. 2007). According to Golovina et al. (2001) desiccation tolerance can be acquired by the meristematic cells in the developing embryo and is independent of seed morphological development stage. Some evidence that poorly developed

embryos may have survived dehydration came from researches on *Zea mays* L. (Sheridan and Clark 1993) and *Arabidopsis thaliana* L. (Devic and Sylvie Albert 1996). A study with *Livistona chinensis* (Jacq.) R. Br. ex Mart., a recalcitrant seed palm, showed that there is a transient stage exhibiting partial intracellular dedifferentiation and accumulation of protective sugars and proteins, this stage corresponds to the peak of desiccation and cryotolerance tolerance and occurs in the intermediate stages of seed development (Wen 2011). Different, Barbedo et al. (2013) proposed a model to explain the recalcitrant behavior of seeds based on the degree of maturation in which the seeds are when spilled from the mother plant. In this model, the further away from maturity, the more recalcitrant the seed would be. The authors explain that early stages of development are marked by high metabolism, indicated by increased synthesis of adenosine triphosphate (ATP), essential for the production of reserve compounds in subsequent development (Barbedo et al. 2013). However, ATP synthesis is correlated indirectly with the formation of ROS, which can be eliminated by antioxidant enzymes synthesized in the later stages of development (Barbedo et al. 2013).

Cells of shoot apical meristem from desiccated embryos of *B. catarinensis* showed compact nuclei, protein presence (reacting positively to CBB) and small infolds of cell wall (Fig. 8a-b). Previous studies have shown that the ability to resist drying coincides with chromatin compaction, as well as regular patterns of wall folding (Webb and Arnott 1982; Zanten et al. 2011). It is known that cells with more dry matter reserves are more tolerant of water loss because they are able to minimize volume changes (Walters and Koster 2007). Thus, protein reserves may be contributing to minimize the retraction of the cell volume. In addition, seed storage proteins have been described as a powerful ROS scavenging system, as they are primary targets for oxidation and thus protect cellular components that are important for embryo survival (Davies 2005; Sano et al. 2015). Protein storage vacuoles were attributed to the high viability of the post-desiccation embryos of *B. capitata* (Gonçalves et al. 2020). It is important to note that although we do not have a qualitative analysis of proteins, some of them are already well known in the literature associated with desiccation tolerance, such as those abundant in late embryogenesis (LEA) and thermostable heat shock (HSPs) (reviewed by Matilla 2021).

4.3. *B. catarinensis* embryos may cryopreserved successfully through partial desiccation and fast cooling

B. catarinensis embryos fully hydrated not survive exposure to LN (Fig. 4a). Disorganized and detachment cytoplasm from their cell walls, deformed nuclei and leakage of cell contents were associated with loss of viability (Fig. 7d-f). During cooling and thawing steps nucleation centers may form, resulting in an arrangement which defines the crystal structure of the solid (Wowk 2010). Thus, cellular damage shown here is a consequence of the high WC present in the cells, which upon cooling forms ice crystals resulting in a series of mechanical disturbances. Similar, viability reduction in cryopreserved fresh embryos of *Fortunella polyandra* (Ridl.) Tanaka *L. chinensis* was closely correlated to rupture of walls and membranes cell, caused loss of intercellular connections through plasmodesmata (Wen et al. 2012; Al Zoubi and Normah 2015). Physical damage to the cell membrane results in the loss of its semipermeability dissolving the cytoplasm, thus rendering it functionless and lacking in organelle activity (Uemura et al. 2006; Kaviani 2011; Streczynski et al. 2019). In addition, here, lipid bodies retained their integrity in hydrated *B. catarinensis* embryos and only coalesced post-cryo (Fig. 7g). Studies of the relationships

between thermal transitions in oil-rich seeds have concluded that triacylglycerols interact with water to cause damage (Hor et al. 2005; Crane et al. 2006; Hamilton et al. 2009). Possibly because lipids crystallize and destabilize the glassy matrix (Ballesteros et al. 2020). Desiccated *B. catarinensis* embryos showed high survival rate after cryo-exposure (78.75%) producing root and shoot (Fig. 6b-e). The reduction of WC increase cytoplasmic viscosity, reducing the intracellular mobility of water and the heat to be dissipated during cooling; preventing ice-crystal growth (Walters et al. 2008; Yashaswi and Mona 2022). In present study number of protein storage vacuoles was lower in desiccated embryos (Fig. 8e).

Since there is no crystallization event, vitrification outruns the processes of ice nucleation and growth and thus their potential adverse effects (Yashaswi and Mona 2022). Therefore, WC is one of the main factors affecting *B. catarinensis* embryos survival after storage in LN. *B. catarinesis* embryo tend to an orthodox response with 82.5% germination after desiccation to 0.13 $gH_2O\ gDW^{-1}$ and store success under low temperatures (−196). The ultrastructure analysis and the high indices of normal seedlings validated the method of partial desiccation followed by direct immersion in LN to cryopreserve excised embryos of *B. catarinenises.*

5. Conclusion

The morphophysiological dormancy of *B. catarinensis* seeds that impose difficulties in the germination and production of seedling was overcome with *in vitro* culture of embryos, in medium supplemented with GA_3 (Fig. 10). For the first time an *in vitro* conservation strategy was described for *B. catarinensis* embryos; the water state of the tissue being the limiting factor for embryos cryopreservation... Thus, the reduction in WC about 0.13 $gH_2O\ gDW^{-1}$, followed by direct immersion in LN and subsequent thawing in sucrose at 45°C followed by recovery *in vitro* (Fig. 7) is the suggested protocol. Advantages of the method are its simplicity and low cost, as compared to others, which require the use of cryoprotectant agents. The protocol described here can be used for long-term conservation of *B catarinensis* serving as a reservoir of genetic diversity, protected from environmental and anthropic threats.

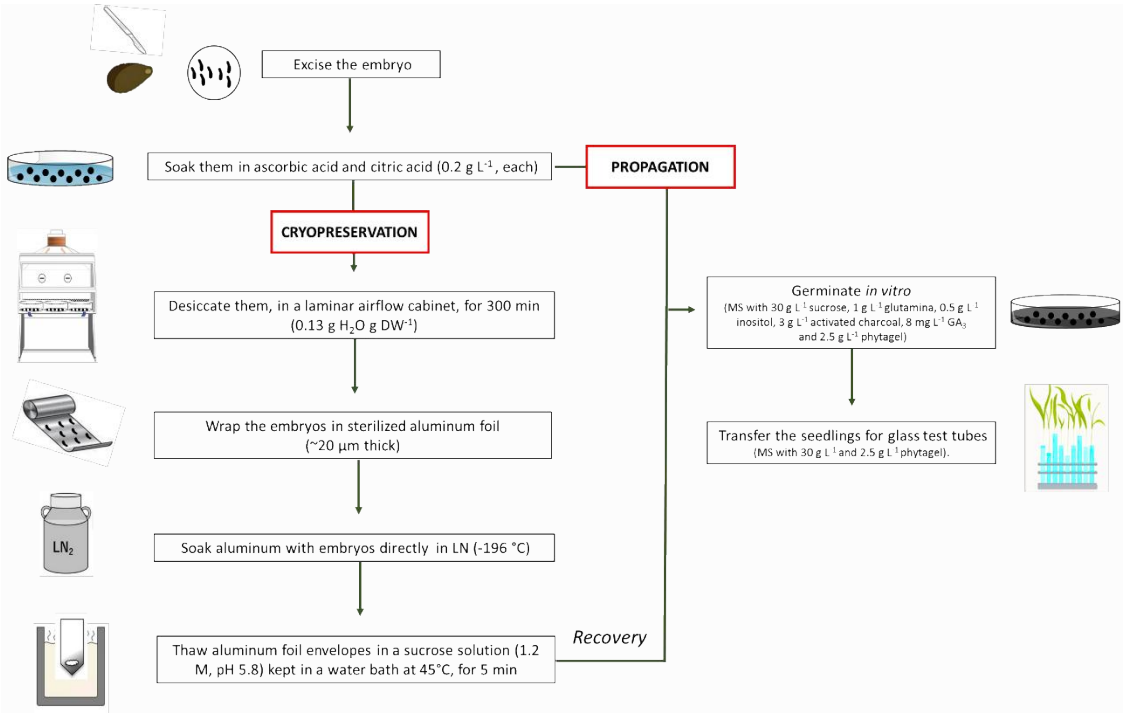

Figure 10. Flowchart depicting the activities involved the conditions of *in vitro* propagation, as well as in embryo cryopreservation of *B. catarinensis*

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CAPÍTULO III

Desiccation sensitivity and cryopreservation of *Euterpe edulis* excised embryos

Abstract

Euterpe edulis is a key species in the Atlantic Forest and predatory exploitation for the harvest of palm heart and the reduction of its habitat have put it in endangered status. Recalcitrance and low germination are major challenges for long-term storage of their seeds. Cryogenic storage in liquid nitrogen (LN) and *in vitro* technologies may be an alternative promissory in *ex situ* conservation this species that cannot be stored in conventional seed banks. Here, the physiology of desiccation sensitivity and the possibility of cryopreservation of *Euterpe edulis* isolated embryos were investigated. Once set the desiccation threshold, two cryopreservation protocols were tested: embryos air-dried wrapped in aluminum envelope and immersed in liquid nitrogen (LN); embryos air-dried in combination with solutions cryoprotective agents (PVS2/PVS3), followed by droplet vitrification. A combination of hormones, antioxidants and nutrients allowed 100% of fresh embryos develop in normal seedling, also being effective in the recovery of embryos after cryopreservation. Viability and vigor gradually decreased as the water content (WC) was reduced. The minimum germination rate was of 42.2% in embryos dried at 0.20 $gH_2O\ gDW^{-1}$ and loss of viability occurred in embryos with WC of 0.11$gH_2O\ gDW^{-1}$. The use of PVS2 and PVS3 was toxic, reducing up to 28.7% germination rate. 37.7% of embryos desiccated at 0.20 $gH_2O\ gDW^{-1}$ germinate post-cryo, but only 14.8% of them developed into normal seedlings. The low normal seedlings development obtained here requires attention, and a thermal analysis of desiccate embryo will help ensure the glassy state. In addition, a study of the reduction of sensitivity to desiccation of the embryo could help to improve its recovery.

Additional Keywords: Jussara Palm. Desiccation Sensitive Seeds. Zygotic Embryo. Droplet Vitrification.

1. Introduction

Euterpe edulis Mart (palm tree juçara) is native to the Brazilian Atlantic Forest, and its disappearance represents a danger to the local fauna and, indirectly, to the flora, since these animals play important roles in mutualistic processes, such as pollination and seed dispersal (Carvalho et al. 2016). Palm tree juçara is known for the production of palm hearts, ranking second among the most exploited non-timber forest product species in the Atlantic Forest (Muler et al. 2014; Carvalho et al. 2022). Given that the apical meristem is extracted to and that the *E. edulis* is a single-stemmed palm that does not re-sprout after harvesting, thus palm heart extraction leads to implies the death of individual (Reis et al. 2002). As an aggravating factor, palm juçara heart come of extractivism and since the late 1970s is suffering with high levels of exploitation, compromising populations and leading to local extinction in several areas (Reis et al. 2002; Rodrigues and Durigan 2007). This activity culminated in the its inclusion in the Red List of Brazilian Flora (annex 1), as endangered (EN) (MMA - Ministério do Meio Ambiente 2008). Currently the predatory exploitation of palm tree juçara is prohibited by law, constituting an environmental crime and its cutting is only allowed when meets the prerequisites established by CONAMA Resolution nº. 294, of 12/01/2001. In a sustainable approach the fruit of juçara has been exploited increasing the income of small local farmers (Maier et al. 2018). Despite efforts to maintain the species in its natural habitat, the natural regeneration has not been sufficient, thus the propagation of palm tree juçara is fundamental to maintain and increase the remaining populations (Reis et al. 2000).

Reproduction of *E. eduli* occur exclusively by seeds, but the seedling production is limited due low germination rates, usually linked to dormancy (Henzel et al. 2020; Jaganathan 2021) representing a major challenge for producers, seed companies and for the conservation of germplasm. Until now somatic embryogenesis has been considered the best form way to *E. edulis* regeneration (Guerra and Handro 1988, 1998; Saldanha et al. 2006; Saldanha and Martins-Corder 2012). However, in terms of diversity conservation, the use of this tool is limiting, because although it allows obtaining a large number of individuals, these are genetically identical, being preferably applied to propagating of elite genotypes (Martínez et al. 2019). *In vitro* germination of zygotic embryo have been studied as an alternative to plant production and to preserve its genetic diversity of palm with dormant seeds (Minardi et al. 2011; Magalhães et al. 2013; Waldow et al. 2013). Nonetheless, in addition to a limited number of studies with *in vitro* zygotic embryos of *E. edulis*, these have focused on their competence in inducing somatic embryos (Saldanha et al. 2006; Saldanha and Martins-Corder 2012).

There is even interest in the development of effective protocols for germplasm preservation of *E. edulis* (Nodari et al. 1998; Andrade 2001; Martins et al. 2009). However, without the possibility of drying, due to the recalcitrant nature of its seeds, storage is restricted to less than one year (Andrade 2001; Vitis et al. 2020). Long-term storage of recalcitrant seeds is sometimes possible by isolating the embryo and subjecting them to cryopreservation technique, where they are rapidly cooled to ultra-low temperatures (-196 °C) using liquid nitrogen (LN) (Walters and Pence 2020). Cryopreservation methods based on vitrification procedures, in which the formation of ice crystals is inhibited or minimized, has been used in many zygotic embryos of palms species in which seeds cannot be maintained through seedbanks (Wen 2011; Dias et al. 2015; Santos and Salomão 2017; Vargas et al. 2020). Generally, in seeds this state is obtained at water contents below 0.25 $gH_2O\ gDW^{-1}$ followed by ultra-rapid cooling at temperatures below

− 190°C (Walters and Pence 2020). In *E. edulis*, desiccation thresholds between 1.2 at 2.3 $gH_2O\ gDW^{-1}$ was established, based on different drying methods of whole seeds (Panza et al. 2007). In these circumstances, the vitrification technique by exposure to solutions with high osmotic potential is recommended (Volk and Walters 2006; Berjak and Pammenter 2014). Protectants solution, have the capacity to induce glassy matrices within cytoplasm without inducing major structural changes to the cell (Volk and Walters 2006). These solutions are known as PVS formulations (Plant Vitrification Solution) composed of substances cryoprotectant such as sucrose, glycerol, polyethylene glycol and DMSO (Sakai et al. 1990; Nishizawa et al. 1993). High viability rates of zygotic embryos cryopreserved using cryoprotective solutions have been reported for some palm species such as *Butia yatay* Mart. (Vargas et al. 2020) and *Cocos nucifera* L. (Sajini et al. 2011).

This work sought to understand the physiological behavior of isolated *E. edulis* embryos under desiccation stress, generating useful information for the development of reliable protocols for cryogenic storage. Two cryopreservation methods were investigated: i) partial desiccation followed by immersion in LN and; ii) partial desiccation in combination with cryoprotective agents (PVS2 and PVS3) followed by the technique of droplet-vitrification. This is the first relate of cryobiotechnology use for *E. edulis* embryos.

2. Material and method

2.1. Plant material

Mature fruits of *E. edulis* were collected in March 2021 from a natural plant population in Santa Catarina state (27º 02'05'' S; 49º 27'36'' W), Brazil. Fruits free of insects or microorganisms attack were manually processed to obtain the seeds. The seeds were immersed in ethanol 70% (v/v) for 5 min and disinfested in sodium hypochlorite (1% v/v) for 15 min and then washed three time with sterile distilled water.

In a laminar flow chamber, embryos were excised from seeds using scalpel and a hamber. Then, embryos were placed immersed in an ascorbic acid (0.2 $g\ L^{-1}$) and citric acid (0.2 $g\ L^{-1}$) solution, to prevent oxidation.

2.2. Seeds and embryo morphology and morphometry

Diameter and length of 50 seeds and embryos (fresh) were measurements using a caliper. Images were acquired in Stereo Microscope (Olympus® - SZH10, Shinjuku, Tokyo, Japan) coupled with a digital camera (Olympus® - DP71, Shinjuku, Tokyo, Japan) using the software Cell Sens Dimension (Olympus®, Shinjuku, Tokyo, Japan)

2.3. Water content (WC)

Five replicates of 15 seeds and embryos (fresh) measured gravimetrically by difference in weight before and after drying at 103 ± 1° C for 24h (ISTA 2004). Water content value was expressed on a dry weight basis ($g\ H_2O\ g\ DW^{-1}$).

2.4. Water potential (Ψ_w)

Five replicates of 15 embryos (fresh) were immersed in different concentrations of polyethylene glycol 6000 (PEG 6000) at 25º C, for 24h. The concentrations provided Ψ_w of 0, −0.5, −1, −1.5 −2, −2.5 and −3 MPa (Michel and Kaufmann 1973). The fresh weight of the embryos was measured gravimetrically immediately after excision and after 24h- immersion in PEG 6000 solutions. Then a curve of WC (g H_2O g DW^{-1}) against Ψw was constructed.

2.5. Desiccation of embryos

Four replicates of 25 embryos (immersed in antioxidant solution) were desiccated by 0; 15; 30; 60; 120; 180; 240 and 300 min, in a laminar airflow cabinet at 25 ± 2 °C and an airflow of 0.46 m s^{-1}. WC was determined gravimetrically and desiccation curve calculated (Hong and Ellis, 1996). Embryos drying rate index (*k*) was calculated from the amount of water loss in dry basis (gH_2O gDW^{-1}) divided by correspondent elapsed time in minutes (Xia et al. 2012).

2.6. *In vitro* germination and germination speed index (GSI)

Four replicates of 25 embryos, in each time intervals of desiccation, were inoculated in petri dish with 25 ml of MS culture medium (Murashige and Skoog 1962), supplemented with 30 g L^{-1} sucrose, 2.77 mg L^{-1} GA_3, 3 g L^{-1} activated charcoal and 2.5 g L^{-1} phytagel. The pH was adjusted to 5.8 and autoclaved at 121 ºC, 1.5 atm for 15 min. The germination was performed in a growth room (25 ± 2° C; 55% RH) with white LED light (Green Power TLED W; Philips TM; 77 μmol m^{-2} s^{-1}) and 16/8 h photoperiod. Germinated embryos were recorded every day considering radicle protrusion. Germination rate was calculated by the cumulative number of daily germinated seeds with respect of the total number of seeds evaluated (Ranal and Santana 2006). Total number of embryos germinated per day between sowing and germination divided by the number of days of the test was used to calculate GSI (Mangure 1962).

2.7. Seedlings morphometry

Length of radicle and aerial part of four replicates of 15 seedlings were measurements, after 60 days of sowing. Images were acquired in Stereo Microscope (Olympus® - SZH10, Shinjuku, Tokyo, Japan) coupled with a digital camera (Olympus® - DP71, Shinjuku, Tokyo, Japan) using the software Cell Sens Dimension (Olympus®, Shinjuku, Tokyo, Japan).

2.8. Cryopreservation methods

For both methods described below, the embryos were previously desiccated at 0; 30; 120 and 240 min, laminar airflow cabinet at 25 ± 2 °C, and an airflow of 0.46 m s^{-1}. The times were estimated based on the previous results of WC, germination rate and vigour (GSI and seedling morphometry).

2.8.1. Method I: desiccation

Four replicates of 25 embryos previously desiccated were wrapped in sterilized aluminum foil and directly immersed in liquid nitrogen (LN) at -196 °C, where they were kept for 48 h. Embryos not exposed to LN were used as controls.

2.8.2. Method II: desiccation and droplet-vitrification

Two sets of four replicates of 25 embryos previously desiccated were used. In first set embryos previously desiccated were incubated in Plant Vitrification Solution 2 (PVS2) (30 % glycerol w/v, 15 % w/v dimlethylsulfoxide, 15 % w/v ethylene glycol and 13.7 % w/v sucrose) (Sakai et al. 1990), while the second set was treated with Plant Vitrification Solution 3 (PVS3) (50 % w/v sucrose and 50 % w/v glycerol) (Nishizawa et al. 1993). Both solutions of PV's were previously autoclaved for 10 minutes at 15 atm and 120°C. Embryos were exposed to cryoprotectant solutions (PVS2 e/or PVS3) for 60 min, and kept on an orbital shaker at 90 rpm at 0°C. After, five droplets (15 µL) of PVS were placed onto a sterile strip of aluminum foil (3.0 cm long x 0.5 cm wide x 0.05 mm deep), each drop contained an embryo then strip was plunged immediately into LN. Embryos desiccated and immersed in PVS2 or PVS3 not exposed to LN were used to assess the toxicity of the respective solutions.

2.9. Thawing of embryos and recovery

Foil envelopes (method I) and aluminum strips (method II) with embryos were rapidly thawed in a sucrose solution (1.2 M, pH 5.8) kept in water bath at 45°C for 15 min (Walters et al. 2008). Then, embryos were transferred for recovery in culture medium of germination (described *In vitro germination* - section) and kept in growth room (25 ± 2°C; 55% RH) in the dark for three days. After the embryos were exposed to white LED light (Green Power TLED W; Philips TM; 77 µmol m^{-2} s^{-1}) and 16/8 h photoperiod. Germination rate and GSI were daily evaluated, during 30 days. Seedling morphometry was performed at the end of experiment at 60 days.

2.10. Statistical Analyses

The data normality was evaluated using the Shapiro – Wilk test, and then submitted to analysis of variance in a completely randomized design. In the case of significance of the F values, means were compared using SNK test ($p <= 0.05$) (Sokal and Rohlf 1995). Second-order polynomial regression analysis was used to adjust the desiccation and water potential curves ($p <= 0.05$). Statistical analyses were performed using the R Core Team (2016).

3. Results

3.1. Morphological characterization of seeds and embryos

Mature fruit of *E. edulis* was globose, with a purple-black color (Fig. 1a). Seeds was spherical with brown seed coat (sc), hilum (hi) and an operculum (os) and surrounded by mesocarp fibers (fb) [Fig. 1b-c]. Average seed diameter was 12.56 ± 1.62 mm. The embryo was located immediately below the operculum (os) embedded in the endosperm (en) [Fig. 1d]. Embryo was conical with two distinct regions, in the proximal portion (pp) the cotyledonary petiole (cp) and in the distal portion (dp) the haustorium (ha) [Fig. 1e]. Embryos had an average length and width 3.2 ± 2. 70 mm and 1.2 ± 1.15 mm, respectively.

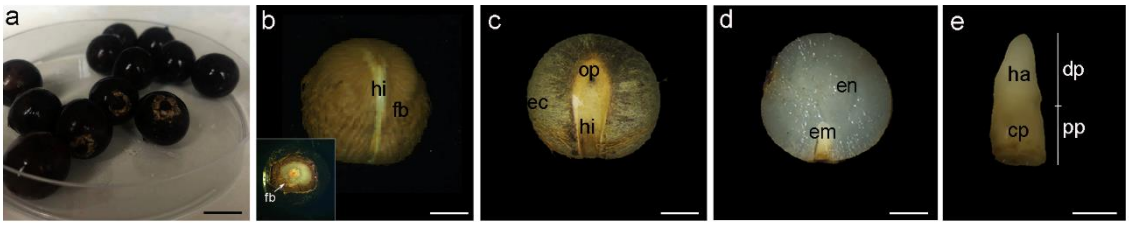

Figure 1: Morphology of *E. edulis* seed **(a-d)** and embryo **(e)**. Mature fruit (**a**). Seed adhered to fibrous mesocarp (fb) and with hilum (hi) visible **(b)**. Seeds showing seed coat (sc), hilum (hi) and operculum (op) region **(c)**. Longitudinally sectioned seed with embryo (em) distal portion (pd) embedded in the endosperm (en) and proximal portion adjacent to the operculum (op) **(d)**. Mature embryo with cotyledonary petiole (cp) in the proximal portion (pp) and haustorium (ha) in the distal portion (dp) **(e)**. Bars: a = 5.0 mm b, c, d = 2.0 mm; e = 500 µm

3.2. Desiccation and osmotic potential

Mature seeds (Fig. 1c) and fresh embryos (Fig. 1e) of *E. edulis* had WCs of 2.17 $gH_2O\ gDW^{-1}$ and 4.22 $gH_2O\ gDW^{-1}$ respectively. Embryos immersed in antioxidant solution (control treatment) had a WC of 7.35 $gH_2O\ gDW^{-1}$ (Fig. 2a). At 30 min of dehydration (4.39 g H_2O g DW^{-1}) embryos showed WC slightly higher than the fresh embryos (4.22 $gH_2O\ gDW^{-1}$). Dry embryos up to 180 min had a high drying rate (k = 0.0389 $gH_2O^{-1}\ min^{-1}$) and lost 95.10% (WC = 0.36 $gH_2O\ gDW^{-1}$) of their initial WC. From 180 to 240 min the water loss occurred slowly (k = 0.0028 $gH_2O^{-1}\ min^{-1}$) and embryos reached WC of 0.20 g H_2O g DW^{-1}. After 240 min of desiccation, the water loss was minimal tending to stabilize (k = 0.0013 $gH_2O^{-1}\ min^{-1}$). At 300 min of desiccation, embryos had WC of 0.11 g H_2O g DW^{-1} and lost 98,09% of their initial WC (Fig. 2a).

Fresh embryos (WC = 4.22 $gH_2O\ gDW^{-1}$) in a Ψw = 0 MPa had an approximately three-fold increase in their WC (WC = 7.50 $gH_2O\ gDW^{-1}$). At Ψw equal to or greater than -0.5 MPa (WC = 3.71 $gH_2O\ gDW^{-1}$) embryos reductions in their WC. In potentials more negative (> Ψw = -1.5 MPa) the loss of water was slower, tending to establishment. At Ψ w – 3.0 MPa water loss from the embryos was 90%, reaching 0.78 $gH_2O\ gDW^{-1}$.

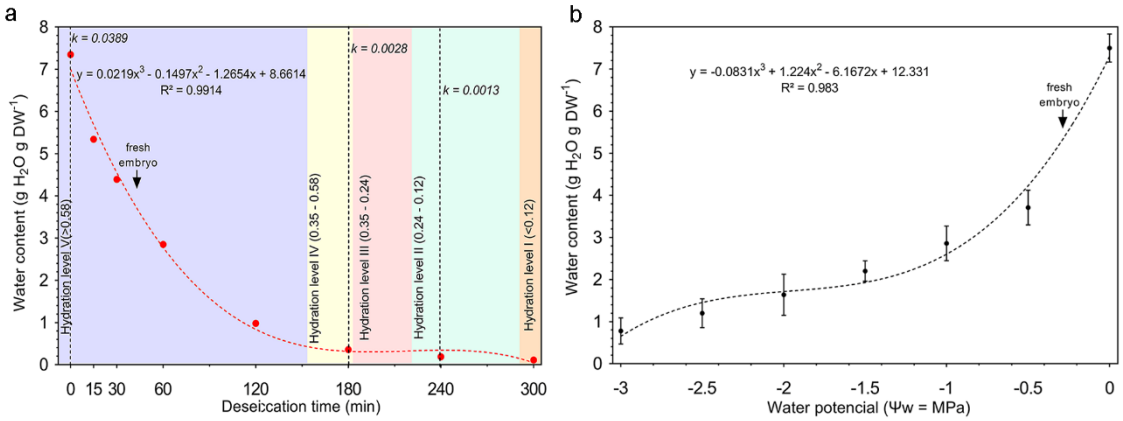

Figure 2: Variation of WC ($gH_2O\ gDW^{-1}$) of *E. edulis* embryo in response to drying for 0; 15; 30; 60; 120; 180; 240 and 300 min: drying rate (k); WC slightly higher than the fresh embryos (arrow). Hydration levels was adapted for water content of E. edulis isolate embryo using Vertucci (1990). **(a).** WC in response to levels Ψw of 0; − 0.5; − 1; − 1.5; − 2; − 2.5 and − 3 MPa **(b)**. Data: means ± standard error (n = 4). Second-order polynomial regression analysis of water content in function to desiccation and water potential was used to adjust curves ($p <= 0.05$).

3.4. Germination test and vigor

E. edulis embryos desiccated up to 4.39 $gH_2O\ gDW^{-1}$ (30 min) showed no significantly reduction in final germination rate with percentages ranging from 98.66 at 100% (Fig. 3a). The germination rate of

embryos with WC below 2.19 $gH_2O\ gDW^{-1}$ gradually decreases. Germination minimum (42.27%) in embryos with WC of 0.20 $gH_2O\ gDW^{-1}$ (240 min) and loss of viability in WC of 0.11 $gH_2O\ gDW^{-1}$ were observed (Fig. 3a).

The reduction of WC affected the germination dynamics of *E. edulis* embryos. Desiccated embryos up to 2.85 $gH_2O\ gDW^{-1}$ embryos germinated faster between 7 and 8 days and reached earlier its maximum value (15 days after sowing) [Fig. 3b]. While, desiccated embryos for WC equal to or less than 0.98 $gH_2O\ gDW^{-1}$ the protrusion of the radicle started at 12th day and took 16 to 17 days to complete germination (Fig. 3b).

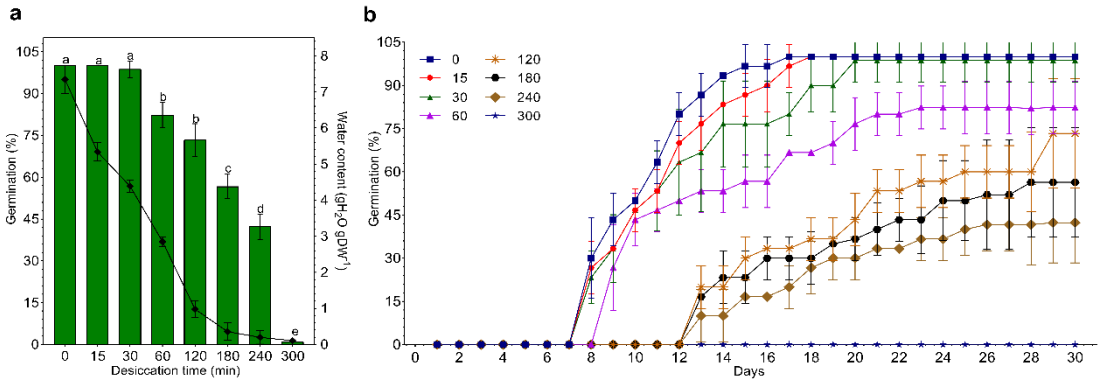

Figure 3: Final germination (%) (axis right) and water content (WC = $gH_2O\ gDW^{-1}$) (axis left) of *E. edulis* embryos in response to desiccation time (0; 15; 30; 60; 120; 180; 240 and 300 min) **(a)**. Germination dynamics during 30 days after sowing **(b)**. Data mean ± standard error (n = 5). Means followed by different letters are significantly different ($p < 0.05$) according to the SNK test.

The delay in germination directly reflected in the germination speed index (GSI) (Fig. 3b; Table 1). Embryos with WC equal to or less than 2.19 $gH_2O\ gDW^{-1}$ showed a gradual reduction in GSI values (Table 1). Embryos with WC of 0.20 $gH_2O\ gDW^{-1}$ (240 min) had the lowest GSI value (0. 62), a reduction of approximately 75% compared with embryos of control treatment (without desiccation) (GSI = 2.47) (Table 1).

Embryos dehydrated at 0.20 $gH_2O\ gDW^{-1}$ (300 min) had a significant decrease in radicle (1.50 ± 0.63) and aerial part (0.24 ± 0.09) length, compared to the control embryos (without desiccation) [3.44 ± 0.32 and 0.62 ± 0.06, respectively] and desiccated embryos at 5.34 $gH_2O\ gDW^{-1}$ (15 min) [2.96 ± 0.39 and 0.62 ± 0.04, respectively] {Table 1}.

Table 1: Physiological behavior of *E. edulis* embryos under desiccation and their relating its water content, water loss, germination speed index and seedlings morphometry

Drying time (min)	Content water ($gH_2O\ gDW^{-1}$)	Water loss (%)	Germination speed index (GSI)	Seedlings morphometry (cm)	
				Radicle	Aerial part
0	7.35 ± 0.19	------	2.47 ± 0.06^{a}	3.44 ± 0.32^{a}	0.62 ± 0.06^{a}
15	5.34 ± 0.16	27.35	2.37 ± 0.07^{a}	2.96 ± 0.39^{ab}	0.62 ± 0.04^{a}
30	4.39 ± 0.16	40.27	2.19 ± 0.07^{b}	2.76 ± 0.44^{abc}	0.54 ± 0.06^{a}
60	2.85 ± 0.13	61.22	1.77 ± 0.13^{c}	2.26 ± 0.44^{abc}	0.36 ± 0.09^{bc}
120	0.98 ± 0.13	86.67	1.03 ± 0.19^{d}	2.14 ± 0.29^{abc}	0.46 ± 0.10^{ab}
180	0.36 ± 0.12	95.10	0.83 ± 0.09^{e}	1.84 ± 1.00^{bc}	0.32 ± 0.06^{bc}
240	0.20 ± 0.10	97.27	0.62 ± 0.05^{f}	1.50 ± 0.63^{c}	0.24 ± 0.09^{c}
300	0.11 ± 0.13	98.09	0.00 ± 0.00^{g}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}

Data mean ± standard error (n = 5)
Means followed by different letters are significantly different ($p < 0.05$) according to the SNK test

3.5. Cryopreservation

Embryos immersed in PVS2 and PVS3 had significant reductions in germination rate and vigor (Fig. 4; Table 2). Embryos with WC greater than 4.39 gH_2O gDW^{-1} and immersed in PVS's had their WC reduced but did not survive after exposure to LN (method II) [Fig. 4; Table 2]. Embryos with WC of 0.98 gH_2O gDW^{-1} and cryopreserved by droplet-vitrification technique (method II) had germination rates of 4.83% (PVS2) and 12.88% (PVS3). However, these embryos, only 1.56% (PVS2) and 3.69% (PVS3) formed normal seedling (Table. 2) The results bests of survival pos-cryo (germination = 37.67%), vigour (GSI = 0.57) and formation of normal seedlings (14.76%) were obtained in embryos desiccated at 0.20 gH_2O gDW^{-1} and immersed directly in LN (method I) [Fig. 4; Table 2].

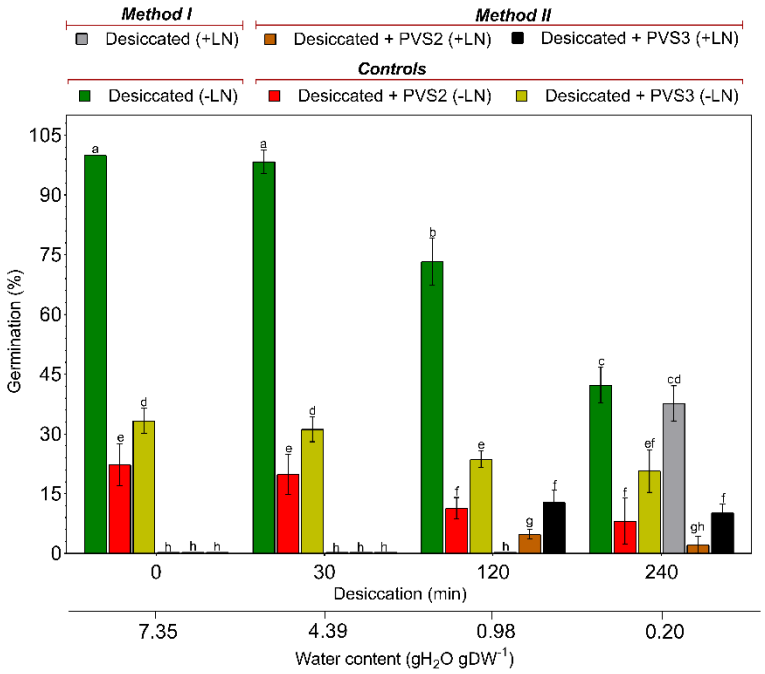

Figure 4: Final germination (%) of *E . edulis* embryos desiccated for 0; 30;120 and 240 min and incubated or not in solution cryoprotectant (PVS2 or PVS3 – for 60 min) (-LN). Embryos desiccated and immerse directly in LN (Method I) and embryos desiccated incubated in solution cryoprotectant followed by droplet-vitrification (Method II) (+LN). Data mean ± standard error (n = 5). Means followed by different letters are significantly different ($p < 0.05$) according to the SNK test

Table 2: Physiological behavior of *E. edulis* embryos under vitrification and cryopreservation and their relating its water content, water loss, germination speed index an d seedlings morphometry

Desiccation time (min)	Vitrivicant Agents	Water content ($gH_2O\ gDW^{-1}$)	Germination speed index (GSI)		Normal seedlings (%)	Normal seedlings (%)	Seedlings morphometry (cm)			
							Radicle	Radicle	Aerial part	Aerial part
			(-LN)	(+LN)	(-LN)	(+LN)	(-LN)	(+LN)	(-LN)	(+LN)
0	Desiccation (Method I)	7.35 ± 0.19	2.47 ± 0.06^{aA}	0.00 ± 0.00^{bB}	100.00 ± 0.00^{aA}	0.00 ± 0.00^{dB}	3.44 ± 0.32^{aA}	0.00 ± 0.00^{dB}	0.62 ± 0.06^{aA}	0.00 ± 0.00^{cB}
30		4.39 ± 0.16	2.19 ± 0.07^{bA}	0.00 ± 0.00^{bB}	98.33 ± 2.30^{aA}	0.00 ± 0.00^{dB}	2.76 ± 1.44^{abA}	0.00 ± 0.00^{dB}	0.54 ± 0.06^{abA}	0.00 ± 0.00^{cB}
120		0.83 ± 0.13	1.03 ± 0.19^{cA}	0.00 ± 0.00^{bB}	13.33 ± 3.89^{bA}	0.00 ± 0.00^{dB}	1.84 ± 1.00^{bA}	0.00 ± 0.00^{dB}	0.32 ± 0.06^{bcA}	0.00 ± 0.00^{cB}
240		0.20 ± 0.10	0.62 ± 0.03^{bA}	0.57 ± 0.06^{aA}	22.27 ± 2.10^{cA}	14.76 ± 0.35^{aB}	1.44 ± 0.35^{bA}	1.13 ± 0.10^{abA}	0.24 ± 0.09^{cA}	0.22 ± 0.03^{aA}
0	PVS2 (Method II)	5.08 ± 3.19	0.52 ± 0.24^{bA}	0.00 ± 0.00^{bB}	11.87 ± 3.25^{dA}	0.00 ± 0.00^{dB}	2.11 ± 1.22^{abA}	0.00 ± 0.00^{dB}	0.42 ± 0.07^{bA}	0.00 ± 0.00^{cB}
30		2.79 ± 0.08	0.36 ± 0.16^{bcA}	0.00 ± 0.00^{bB}	10.12 ± 5.98^{dA}	0.00 ± 0.00^{dB}	1.17 ± 0.78^{bA}	0.00 ± 0.00^{d}	0.40 ± 0.06^{bA}	0.00 ± 0.00^{cB}
120		2.71 ± 0.76	0.22 ± 0.09^{bcA}	0.08 ± 0.04^{cB}	8.28 ± 3.07^{eA}	1.56 ± 1.09^{cB}	1.02 ± 0.58^{bA}	0.54 ± 0.12^{cB}	0.22 ± 0.07^{cA}	0.15 ± 0.12^{bA}
240		2.40 ± 1.03	0.17 ± 0.10^{cA}	0.04 ± 0.03^{cB}	8.43 ± 1.07^{eA}	1.89 ± 1.14^{cB}	1.06± 0.44^{bA}	0.38 ± 0.10^{cA}	0.21 ± 0.05^{cA}	0.11 ± 0.10^{bA}
0	PVS3 (Method II)	3.91 ± 2.52	0.52 ± 0.12^{bA}	0.00 ± 0.00^{bB}	15.13 ± 3.15^{dA}	0.00 ± 0.00^{dB}	2.23 ± 1.14^{ab}	0.00 ± 0.00^{dB}	0.52 ± 0.06^{abA}	0.00 ± 0.00^{cB}
30		2.31 ± 0.79	0.54 ± 0.10^{bA}	0.00 ± 0.00^{bB}	11.36 ± 4.22^{dA}	0.00 ± 0.00^{dB}	2.14 ± 0.32^{bA}	0.00 ± 0.00^{dB}	0.55 ± 0.05^{aA}	0.00 ± 0.00^{cB}
120		2.24 ± 0.77	0.36 ± 0.07^{bA}	0.21 ± 0.04^{bB}	5.92 ± 3.87^{dA}	3.69 ± 2.24^{bcB}	1.88 ± 0.78^{bA}	0.98 ± 0.12^{bA}	0.30 ± 0.03^{cA}	0.10 ± 0.09^{bA}
240		2.21 ± 1.10	0.33 ± 0.04^{bA}	0.19 ± 0.04^{bB}	8.76 ± 3.80^{dA}	5.34 ± 1.10^{bB}	1.34 ± 1.10^{bA}	1.01 ± 0.10^{bA}	0.23 ± 0.04^{cA}	0.08 ± 0.03^{bA}

Data mean ± standard error (n = 5)

Small letters compare each vitrification technical and capital letters compare embryos no exposed to liquid nitrogen (-LN) and exposed to liquid nitrogen (+LN). Means followed by the same letter are not significantly different by the SNK test ($p < 0.05$)

A seedling was considered normal when it presented ligule (li), first cataphyll (ct), primary (pr) adventitious (ar) roots and haustorium (ha) (Fig. 5b; c). The combination of reducing WC to 0.20 gH_2O gDW^{-1} and immersed directly in LN (method I) generated seedlings with longer radicle (1.13 cm) and aerial part (0.22 cm) compared to method II (Fig. 5a; Table 2). Both methods (I and II) the embryos that did not germinate remain unchanged in size and with cotyledonary petiole (cp) highly oxidized, indicated by dark brown coloration (Fig. 5a; d). Abnormal embryos showed oxidized ligule (li), deformed roots (pr) and the cataphyll (ct) development were not visualized (Fig. 5e - f).

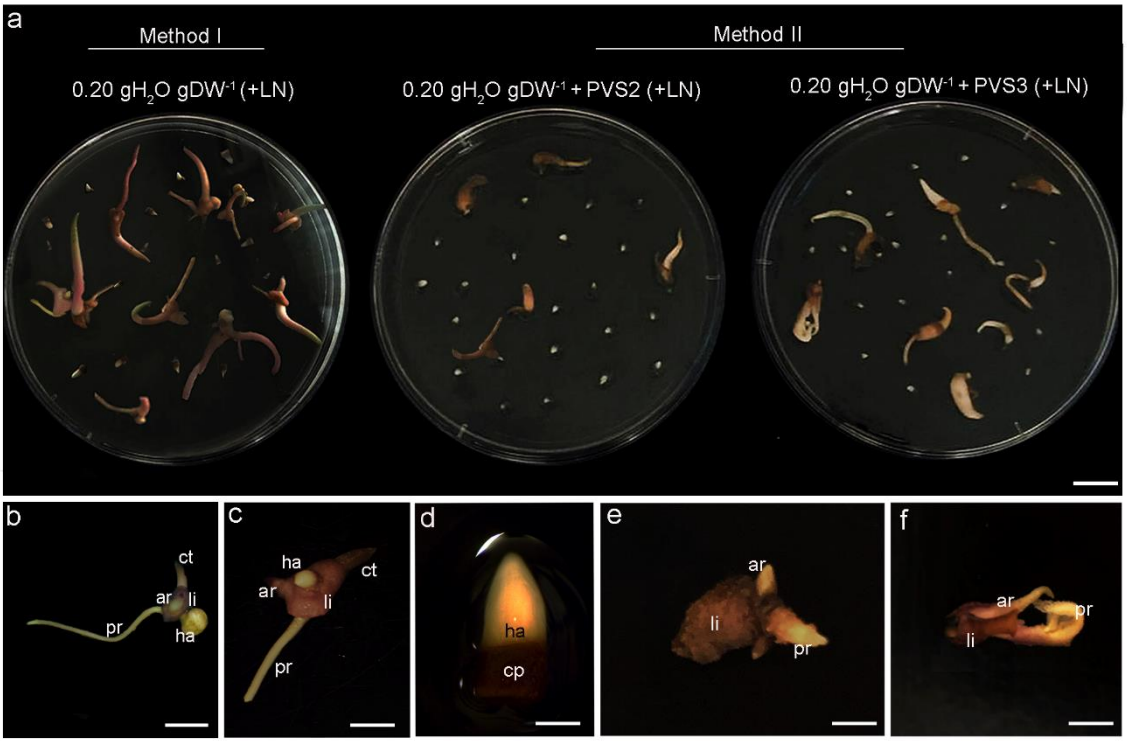

Figure 5: *E. edulis* embryos development after 60 days of cryopreservation. Control **(b)** and embryo desiccated at 0.20 gH_2O gDW^{-1} (240 min) and immersed directly in LN (Method I) **(c)** both showing normal seedling with ligule (li), haustorium (ha), primary root (pr), adventitious root (ar) and cataphyll (ct) **(b-c)**. Embryos desiccated 0.20 gH_2O gDW^{-1} (240 min) incubated in PVS2 followed by droplet-vitrification (Method II) showing stagnant embryo, with cotyledonary petiole (cp) oxidated **(d).** Embryos desiccated 0.20 gH_2O gDW^{-1} (240 min) incubated in PVS3 followed by droplet-vitrification (Method II) showing development abnormal of root (pr), oxidation of the ligule (li) structure and absence of cataphyll (ct) **(d-e)**. Bars: a = 2.0 mm; b, c, d, e and f = 500 µm

4. Discussion

E. edulis embryos were embedded in a rigid endosperm, surrounded by a woody endocarp (Fig. 1a, b). Although the endocarp protects the internal structures of the seeds and provide resistance for water loss, this structure also is responsible for low and over a long time germination, attributed to mechanical dormancy (Baskin and Baskin 2014; Jaganathan 2021). Since the 70s to the present day, many efforts have been made to establish an efficient dormancy breaking method for *E. edulis* seeds, but germination rates ranged from 16 to 76% taking up to 150 days to start germinating (Bovi and Cardoso 1975; Roberto and Habermann 2010; Guimarães et al. 2018; Henzel et al. 2020). In the present study, in vitro germinated excised *E. edulis* embryos showed radicle protrusion in 12 days and 100% germination was observed in a maximum of 16 days (Fig. 3a, b). *In vitro* culture of isolates embryos has been used in other palm species for circumvent long stratification treatments needed in germination of whole seed (Pritchard et al. 2000;

Wen and Wang 2010; Magalhães et al. 2013; Dias et al. 2015). In our study, after 30 days in germination medium, embryos gave rise to normal seedlings, developing cataphyll, primary and adventitious roots, haustorium and ligule (Table 1; Fig. 4d). *In vitro* culture technique was a rapid and efficient for obtain *E. edulis* normal seedlings, which can be used by companies and for the restoration of remaining areas of the species.

Despite the economic and ecological importance of *E. edulis*, little progress has been made beyond the short-term storage of its seeds (Nodari et al. 1998; Andrade 2001; Martins et al. 2009). Seed banks is considered one of the most important strategies to preserve the genetic variability of species (Rajasekharan 2015; Walters and Pence 2020). However, the size, low and uneven germination and sensitivity to water loss of *E. edulis* seeds limits the use of the conventional conservation strategy (Munhoz et al. 2005; Panza et al. 2007; Martins et al. 2009). *In vitro* embryo banks have allowed the conservation of numerous endangered plant species, including those with limitations in germination and storage through whole seeds. (Pence 2011; Chauhan et al. 2019). Different *in vitro* conservation methods are employed, depending on the storage duration requested. For short and medium term storage, growth is reduced and intervals between subcultures are increased (Engelmann 2011). For long-term storage the only current method is the use of cryopreservation (Walters and Pence 2020). To date, there are no reports in the literature on the use of *E. edulis* embryos in cryopreservation studies. The first step to for the development of cryopreservation protocol it is necessary to understand the behavior of water, and set a desiccation threshold, especially for seeds/embryos that are unable to survive low WC drying (Day et al. 2008; Walters 2015). In the present study, the shape of the desiccation curves and water potential (Ψ) indicated that the WC decreased markedly during the initial drying times and at higher Ψ and then slowly until it stabilized (Fig. 2a, b). In addition, viability and vigor reduced as embryos were dried (Table 1; Fig. 3a, b). The outflow of water from tissues during desiccation and the reduction in viability are related to water functions within the cells and have been discussed Vertucci (1990). Five levels of hydration are described and generally the dehydration process and physiological damage are associated with the first three. However, our results showed a reduction of 17.67% in viability and 0.16 in GSI in WC of 2.85 $gH_2O\ gDW^{-1}$ i.e. when type V water was removed (Table 1; Fig 3a). Levels V (>0.58 $gH_2O\ gDW^{-1}$) and IV (0.35 - >0.58 $gH_2O\ gDW^{-1}$) corresponding free water, while III (0.35 - 0.24 $gH_2O\ gDW^{-1}$) form bridges over hydrophobic portions of macromolecules and this three levels thus as easily lost (Vertucci 1990), hence the faster rate of water loss at the start of drying. On the other hand, type II water (0.12 - >0.24 $gH_2O\ gDW^{-1}$) interacts with the polar surfaces of macromolecules and is difficult to remove by drying (Vertucci 1990), so water loss is slow. In the present study the type II water removal reduced the germination rate to 42.27% (Fig 3a). In recalcitrant embryos this water removal is lethal, due to the phase change of the membranes (Walters 2015). A previous study in whole mature *E. edulis* seeds desiccated, in silica gel and drying chamber, the loss viability of embryos occurred in WC of 0.81 and 0.53 $gH_2O\ gDW^{-1}$ respectively (Panza et al. 2007). Often, embryos tolerated lower water contents than whole seeds, however, of such comparison researches are a few. In palm species as *Elaeis guineensis, Butia capitate* (Mart.) Becc. and *Borassus flabellifer* L. a greater tolerance to the dehydration embryo in comparison to whole seed has been reported (Grout 1986; Dias et al. 2015; Han et al. 2021). This is because isolated embryos allows for faster drying, reducing exposure time to potentially harmful intermediate WC (Pukacka et al. 2011; Varghese et al. 2011; Vieira et al. 2021).

In our study, the subsequent desiccation of embryos to WC of 0.11 $gH_2O\ gDW^{-1}$ caused removal of type I water that took in loss of viability total. Water type I (<0.12 $gH_2O\ gDW^{-1}$) is one that binds as a structural component, very difficult to remove (Vertucci 1990), this explains water loss to be so slow it tends to stabilize.

Cryostorage of *E. edulis* embryos with high WC (> 0.20 $gH_2O\ gDW^{-1}$) indicated that the excess of water in the tissue caused freeze injury, probably due to the formation of ice crystals. A survival rate of 37.63% in embryos desiccated WC at 0.20 $gH_2O\ gDW^{-1}$ followed by direct immersion in LN was obtained, showing possible cryopreservation of *E. edulis* (Fig. 4). The reduction of WC resulted in a decrease in freezing temperature and in cytoplasm vitrification, which prevented ice nucleation in the cells (Fahy and Wowk 2021). Conventionally, for species with recalcitrant seeds/embryos the cryopreservation successful is possible with solidified cytoplasm obtained WC less than 0.20 $gH_2O\ gDW^{-1}$ that corresponds to the value at which no more frozen water (Walters and Pence 2020). In our study all embryos with WC of 0.20 $gH_2O\ gDW^{-1}$ that germinated gave rise to normal seedlings (42.27%), but after LN exposure (method I) this number was restricted to 14.77% (Table 1, 2). Ultrastructural studies of embryos isolated during cryopreservation show that an adequate number of meristematic cells with subcellular organization is necessary for the development of normal seedlings (Sershen et al. 2012; Walt et al. 2022). Thus, it can be inferred that failed to seedling develop *E. edulis* is due to the sensitivity to freezing of the meristematic root and stem tissues of the embryos. In the present study, the selected of embryo for cryopreservation was based WC, obtained from mass-weighted averages. The standard error observed in the size of the embryos (3.2 ± 2. 70 mm in length and 1.2 ± 1.15 mm in diameter) combined with the anatomical heterogeneity, due to the degree of tissue differentiation (Panza et al. 2004), may have influenced the homogeneity of desiccation by making some regions of embryos susceptible to the formation of ice crystals. Embryo anatomy and tissue topography have been implicated in low rates of seedling recovery or abnormal growth following cryopreservation (Wesley-Smith et al. 2015). Recalcitrant embryos of *Livistona chinensis* (Jacq.) R. Br. (Arecaceae) have an acquisition of cryotolerance at the early developmental stage, exhibiting partial dedifferentiation of central cells at the epicotyl end root of the embryo (Wen 2011).

The principle for cryopreserving recalcitrant embryos is based on solidification of the cytoplasm, exhibiting a glassy state, which can be achieved through a combination of drying and rapid cooling (Walters and Pence 2020). Some seeds/embryos cannot vitrify themselves, due to high WC or because they do not have glass-inducing substances (Zamecnik et al. 2021). In these cases, are use of cryoprotectant solutions which dehydrate living cells and help vitrify them during cooling (Engelmann 2011; Zamecnik et al. 2021). In order to increase germination and normal seedling rates, we tested the effects of PVS2 and PVS3 thr ough the droplet vitrification technique (Method II) In embryo with WC >4.39 $gH_2O\ gDW^{-1}$ the cryoprotectant (PVS2 and PVS3) solutions were able to trigger dehydration due to exposure to hypertonic environment. Nonetheless, embryos no desiccated and conditioned in PVS2 or PVS3 had their viability reduced in 77.73% and 66.86%, respectively, and when cryopreserved they did not survive (Fig. 4; Table 2). Differently from the results obtained for other recalcitrant embryos from *Cocos nucifera* L.(Sajini et al. 2011) and *Hevea brasiliensis* L. (Nakkanong and Nualsri 2018). *Quercus robur* L. produce recalcitrant seeds and since the 1990s attempts at cryostorage of their embryonic axes have been carried out, but generally with little success (Poulsen 1992; Chmielarz 1997; Berjak et al. 1999). Dehydrating the axes to

WC 0.43 $gH_2O\ gDW^{-1}$ plus PVS3 has brought advances in cryopreservation protocols for this species (Nuc et al. 2016). However, for *E. edulis* embryos, air drying to WC less than 0.98 $gH_2O\ gDW^{-1}$ plus PVS2 e/or PVS3 negatively impacted germination and normal seedling formation compared with desiccation and immersion directly in LN (Method I) (Fig. 4, Table 2). Therefore, cryoprotectants (PVS2 and PVS3), used to induce vitrification, had toxic effects on *E. edulis* embryos. Responses to cryoprotectant solutions vary between species, although *Q. robur* had good results with PVS3, toxic effect has been reported for PVS2 (Nuc et al. 2016). While for *H. brasiliensis* embryos, the effects were the opposite (Nakkanong and Nualsri 2018).

This was study first the investigate the physiological responses during desiccation of *E. edulis* embryos to various WC levels, in order to define a desiccation threshold that would prevent the intracellular ice formation lethal during freezing. Although 42.27% of embryos desiccated at WC of 0.20 $gH_2O\ gDW^{-1}$ germinated, this level of desiccation combined with direct immersion in LN promoted a survival rate of 37.67% and of these 14.76% formed normal seedlings. Nevertheless, these results were not enough to achieve a high survival and normal seedling. The use of PVS2 or PVS3 proved to be toxic to *E. edulis* embryos. As cryopreservation is the only viable option for the long-term conservation this species, future perspectives for successful cryopreservation of should investigate exogenous inducers of desiccation tolerance to increase embryo survival following drying. Gaps in knowledge during embryo desiccation will need to be filled, eg thermal analysis will help ensure that the glassy state is reached after rapid desiccation. Cryopreservation of embryo plumule may also be another potential technique for the conservation of this species.

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5. CONSIDERAÇÕES FINAIS E PERSPECTIVAS

No presente trabalho contornamos os baixos, longos e desuniformes índices de germinação relatados para *Butia* spp. e *E. edulis*, através da germinação *in vitro* de embriões isolados. Elucidamos o comportamento fisiológico dos embriões de *B. eriospatha*; *B. catarinenses* e *E. edulis* baseando-se nas mudanças bioquímicas e estruturais relacionadas à sensibilidade à dessecação. Definimos os limiares de dessecação para embriões isolados de ambas espécies, e testamos a eficiência de dois protocolos de criopreservação; (i) dessecação rápida em câmera de fluxo vertical, seguido congelamento rápido e; (ii) vitrificação com PVS2 e PVS3, seguido da técnica de vitrificação em gota. Os avanços alcançados neste trabalho nos permitiram estabelecer estratégias de conservação a longo prazo para três Arecaceae, até então não relatadas na literatura. De modo geral, a dessecação parcial seguida a imersão direta em nitrogênio líquido é o melhor método para criopreservar embriões das três espécies aqui estudas.

Estudos adicionais de aclimatização precisam ser realizados para ambas as espécies. Novas hipóteses foram levantadas, na perspectiva de melhorar os índices de geminação e formação de plântulas normais de *E. edulis.* Entre elas, estão o incremento da tolerância dessecação, a temperaturas de descongelamento, o estágio de desenvolvimento. A elucidação do comportamento da água, através da determinação de temperaturas de cristalização durante a dessecação, estudos de composição lipídica, e de parede celular são fundamentais para melhorar esses índices.