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A relação entre ácido abscísico, poliaminas e enzimas antioxidantes exerce função primordial na tolerância à dessecação de sementes de *Campomanesia xanthocarpa*

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A relação entre ácido abscísico, poliaminas e enzimas antioxidantes exerce função primordial na tolerância à dessecação de sementes de *Campomanesia xanthocarpa*

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RESUMO

A sensibilidade à dessecação das sementes impõe restrições à conservação em bancos de germoplasma convencionais e desenvolvimento de protocolos diferenciados faz-se necessário para a conservação *ex situ* de espécies sensíveis. Atualmente, existe a possibilidade de indução da tolerância à dessecação (TD) em sementes sensíveis por meio da alteração do conteúdo de dois reguladores do crescimento vegetal (ácido abscísico-ABA e giberelina-GA) considerados decisivos na aquisição de TD. Nesse estudo foi avaliado o efeito da aplicação de sete soluções (H_2O, ácido abscísico (ABA), diniconazole (DE - inibidor do catabolismo do ABA), fluridone (FLU - inibidor da biossíntese do ABA), ABA/DE, FLU/ DE e paclobutrazol (PAC – inibidor da biossíntese de giberelina) para avaliar a tolerância à dessecação (TD) de sementes de *Campomanesia xanthocarpa*. As sementes foram embebidas por 36 horas nas respectivas soluções e parte delas foi colocada para germinar, e outra parte foi dessecada em sílica gel até atingir 0,07 $gH_2O.gMS^{-1}$ sendo em seguida submetidas ao teste de germinação, quantificação de poliaminas, atividade de enzimas antioxidantes e peroxidação lipídica. Observou-se que a aplicação das soluções não influenciou a germinabilidade e índice de velocidade de germinação (IVG) das sementes que não foram dessecadas, enquanto que as sementes submetidas à dessecação apresentaram diferença na germinabilidade e IVG na maioria dos tratamentos. Foi observada maior germinabilidade quando as sementes foram embebidas em H_2O e ABA (H_2O: 37,5% ± 6,4; ABA: 43,7% ± 8,5) quando comparadas com as sementes que foram apenas dessecadas (20,2% ± 5,2). Foi observada redução da germinabilidade e IVG no tratamento onde foi aplicado o fluridone (7,5 ± 5), indicando que o ABA é necessário para aumento da TD. Foi observado aumento do conteúdo de PAs depois da dessecação em todos os tratamentos, especialmente naqueles com menor taxa de germinação, como controle, FLU e FLU/DE. A atividade das enzimas antioxidantes e níveis de peroxidação lipídica também foram afetados pela dessecação. Foi observado aumento expressivo da atividade de Superóxido Dismutase (SOD) e Ascorbato Peroxidase (APX) no tratamento ABA depois da dessecação e redução da atividade de SOD nas sementes que foram apenas dessecadas. Além disso, observamos redução dos níveis de MDA nesse tratamento. Os resultados indicaram que o ABA exerceu uma função primordial no aumento da TD, principalmente por participar da ativação do sistema antioxidante, reduzindo o estresse causado pela dessecação. Apesar dos resultados promissores deste trabalho, mais estudos ainda precisam ser realizados para que o armazenamento dessas sementes seja uma opção viável para conservação, permitindo assim maior exploração do potencial de uso da espécie.

Palavras-chave: Ácido abscísico. Myrtaceae. Conservação

RESUMO EXPANDIDO

Introdução

A Mata Atlântica é considerada um dos principais hotspots para a conservação da biodiversidade (Myers et al. 2000, Rezende et al. 2018). Nesse ecossistema, cerca de 50% das plantas produzem sementes que não toleram a dessecação (Tweddle et al. 2003). A tolerância à dessecação (TD) refere-se à capacidade de um organismo de desidratar abaixo de 10% de água em uma base de massa seca (ou 0,1 $gH_2O.g^{-1}$ peso seco) sem acúmulo de dano letal (Alpert 2005; Ballesteros et al. 2020). Como esse ecossistema é caracterizado por estações bem definidas e regimes hídricos abundantes e constantes, observa-se menos pressão seletiva para aquisição de TD e longevidade de suas sementes (Tweddle et al. 2003, Wyse e Dickie 2017). Essas sementes germinam assim que são dispersas (Marques et al. 2018, Pammenter e Berjak 2013, Tweddle et al. 2003) promovendo o rápido estabelecimento da muda (Pammenter e Berjak 2014, Tweddle et al. 2003). No entanto, a perda de TD como característica adaptativa torna essas espécies altamente dependentes de habitats tropicais úmidos e, como consequência da redução e fragmentação desses ecossistemas, podem enfrentar riscos de extinção iminente (Achard et al. 2002).

Uma vez que a aquisição de TD em sementes TD ou a falta dela em sementes sensíveis à dessecação (SD) é o resultado de uma série de processos celulares e moleculares mediados principalmente pelo equilíbrio no conteúdo de reguladores de crescimento vegetal (principalmente ácido abscísico -ABA e giberelina - GA) (Bewley et al. 2013, Marques et al. 2018, 2019), alguns estudos têm destacado a possibilidade de induzir TD por modulação dos conteúdos de PGRs (Beardmore e Whittle 2005, Marques et al. 2019).

Os mecanismos ativados durante o aumento ou indução de TD nessas sementes estão associados à resposta ao estresse abiótico. Esses mecanismos incluem síntese de proteínas, lipídios, carboidratos e ativação de enzimas antioxidantes (Berjak e Pammenter 2014; Walters 2015). As principais enzimas antioxidantes envolvidas na desintoxicação celular são superóxido dismutase (SOD), catalase (CAT), glutationa redutase (GR) e ascorbato peroxidase (APX). Geralmente as sementes SD mostram alta atividade de SOD e APX, enquanto as sementes TD mostram alta atividade de CAT e GR (Bailly 2004). Outros reguladores conhecidos como moduladores importantes do crescimento, germinação e

ativação da resposta da planta ao estresse abiótico são as poliaminas (PAs) (Huang et al. 2017). As principais PAs presentes em plantas superiores são putrescina (Put), espermidina (Spd) e espermina (Spm) (Bouchereau et al. 1999), eles são pequenos compostos nitrogenados policatiônicos alifáticos (Huang et al. 2017) e, portanto, podem se ligar a várias macromoléculas celulares (Kusano e Suzuki 2015).

Existem evidências de que os níveis de ABA, a atividade do sistema antioxidante e os níveis de poliaminas são alterados em algumas plantas sob estresse. Estudos recentes apontam que o ABA tem diferentes efeitos no acúmulo de poliaminas em diferentes condições de estresse (Liu et al. 2005; Lando et al. 2019) e também afeta a expressão de genes que codificam enzimas antioxidantes (Hu et al. 2016). No entanto, há poucos estudos explorando como o sistema antioxidante e o conteúdo de PAs são afetados pela aplicação exógena de alguns reguladores do crescimento em sementes SD. Levando em consideração que a dessecação é um tipo de estresse abiótico, esperamos esclarecer a relação entre ABA, PAs e sistema antioxidante durante a indução ou aumento da TD nas sementes, ampliando o escasso conhecimento disponível nessa área.

Objetivo

Estudar a influência de reguladores de crescimento vegetal e seus inibidores no aumento da tolerância à dessecação em sementes de *Campomanesia xanthocarpa***.**

Metodologia

Frutos de *C. xanthocarpa* foram coletados em uma população natural na região central do estado de Santa Catarina, Brasil. Os frutos foram armazenados sob refrigeração e as sementes foram extraídas manualmente com auxílio de peneira, sendo imediatamente submetidas aos experimentos. Sementes frescas foram colocadas em placas de Petri de 10 cm contendo 10 mL de uma das seguintes sete combinações de H_2O, ABA, diniconazol (DE), fluridona (FLU) e placobutrazol (PAC): (1) H_2O; (2) ABA 200 μM; (3) DE 200 μM; (4) FLU 200 μM; (5) ABA 200 μM + DE 200 μM; (6) DE 200 μM + FLU200 μM e (7) PAC200 μM. As placas de Petri foram mantidas em câmara incubadora tipo B.O.D (Biochemical Oxigen Demand), com fotoperíodo de 12 horas e temperatura de 25 ºC por 36 horas. O tratamento controle corresponde aquele cujas sementes não passaram pelas 36 horas de incubação.

Após o processo de aplicação dos reguladores de crescimento de plantas, as sementes de cada tratamento foram colocadas diretamente para germinar em um teor de água de 0,85

gH_2O^{-1}. gMS^{-1} (Tratamento 1) ou foram dessecados em sílica gel até atingir 0,07 gH_2O^{-1}. gMS^{-1} (Tratamento 2). A partir disso cada tratamento foi submetido ao teste de germinação em B.O.D (25ºC, 12/12h fotoperíodo), onde após 30 dias foi calculada a taxa de germinação e índice de velocidade de germinação (IVG). O conteúdo de poliaminas de cada tratamento foi estimado por HPLC, e a atividade das enzimas antioxidantes (superóxido dismutase (SOD), catalase (CAT) e ascorbato peroxidase (APX), assim como a peroxidação lipídica foram estimados por espectrofotômetro.

Todos os dados obtidos nos experimentos foram sujeitos à ANOVA e as diferenças entre médias comparadas pelo teste Tukey ($p < 0,05$).

Resultados e discussão

A aplicação dos reguladores de crescimento vegetal e seus inibidores não influenciou a germinabilidade quando comparada às sementes controle nos tratamentos onde o teor de água foi superior a 0,10 gH_2O^{-1}. gMS^{-1} (0,85). No entanto, quando as sementes foram secas a 0,07 gH_2O^{-1}. gMS^{-1} foi observada uma diferença significativa na germinabilidade e IVG nos tratamentos H_2O e ABA (H2O: 37,5% ± 6,4; ABA: 43,7% ± 8,5) em comparação com sementes que não foram expostas a nenhum pré-tratamento antes da dessecação (Controle: 20,2% ± 5,2). Esse resultado sugere que a redução do teor de água para 0,07 gH_2O^{-1}. gMS^{-1} após a aplicação de ABA e H_2O suprimiu a atividade metabólica e contribuiu para induzir a resposta ao estresse, o que permitiu a ativação do mecanismo de proteção e aumento da tolerância à dessecação (Kleinwächter et al. 2014). Outros estudos também relataram a eficiência na indução ou aumento de TD em sementes SD quando ABA sozinho (Meurs et al. 1992) ou combinado com seu inibidor de catabolismo (Beardome e Whittle 2005) foram aplicados antes do tratamento de dessecação.

Observamos também redução significativa da germinabilidade e do IVG em alguns tratamentos quando comparados às sementes controle, indicando redução da viabilidade e presença de danos por dessecação. Essas reduções foram maiores no tratamento onde foi aplicado o inibidor da biossíntese de ABA (FLU) (7,5 ± 5), o que indica que algumas quantidades de ABA são necessárias para conferir TD. O tratamento que combinou ABA e seu inibidor de catabolismo (DE) não aumentou a TD, isto indica que a quantidade de ABA endógeno pode ter excedido a concentração ótima para *C. xanthocarpa*.

Assim, nossos resultados sugerem que o ABA tem um papel no aumento da tolerância à dessecação das sementes de *C. xanthocarpa*, provavelmente ativando os mecanismos de

proteção devido ao estresse causado pela dessecação.

Em relação às poliaminas (PAs) observou-se aumento dos teores de PAs totais após a dessecação no controle e nos tratamentos com H_20, PAC, FLU, FLU + DE quando comparadas às sementes submetidas aos mesmos tratamentos, mas que não foram dessecadas (0,85 gH_2O^{-1}. gMS^{-1}).

Ao comparar a diferença entre os tratamentos em sementes com 0,85 gH_2O^{-1}. gMS^{-1}, observou-se diferença nos conteúdos de Spd e Spm, principalmente entre o tratamento controle e PAC, com maior teor de PAs no tratamento controle. Sementes com teor de água 0,07 também apresentaram diferença nos teores de PAs, de modo geral, as PAs foram maiores nos tratamentos onde nem ABA nem seu inibidor de catabolismo (DE) foram usados. A espermidina foi a poliamina mais abundante, seguida por Spm e Put em todos os tratamentos.

Nossos resultados mostraram que o conteúdo de PAs foi maior nos tratamentos em que ABA não foi utilizado (Controle, PAC, FLU, FLU + DE). Esses tratamentos apresentaram menores taxas de germinação provavelmente porque os danos causados pela dessecação foram elevados e essas sementes não conseguiram germinar, mesmo com o aumento do teor de PAs. Na maioria desses tratamentos, inibidores da biossíntese de ABA foram usados, o que poderia ter neutralizado os mecanismos de proteção que poderiam ser ativados pelo ABA (Liu et al. 2005). Outros estudos também relataram um aumento significativo no conteúdo de PAs quando as sementes foram tratadas com H_20 e FLU (Lando et al. 2019).

O sistema antioxidante das sementes de *C. xanthocarpa* foi afetado pela dessecação. Observamos maior atividade da superóxido dismutase (SOD) nos tratamentos H_2O, ABA, PAC, DE, FLU, ABA + DE e FLU + DE após a dessecação quando comparado às sementes que não foram dessecadas dos mesmos tratamentos. O maior aumento da atividade da SOD foi observado em H_2O (295%), ABA (652%), e o menor aumento foi em FLU (218,6%). O tratamento controle foi o único em que a atividade da SOD foi reduzida após a dessecação. Em contraste com o comportamento da SOD, não foi observada diferença na atividade da catalase (CAT) após a dessecação, todos os outros tratamentos não mostraram diferença antes e depois da dessecação, sugerindo que a catalase provavelmente não está envolvida na tolerância à dessecação de *C. xanthocarpa*. A única alteração que observamos na atividade da ascorbato peroxidase (APX) após a dessecação foi no tratamento com ABA, onde sua atividade aumentou quando comparada ao tratamento com ABA a 0.85 gH_2O^{-1}. gMS^{-1} de conteúdo de água. Esses resultados sugerem que em sementes de *C. xanthocarpa*, SOD

juntamente com APX são as principais enzimas protetoras que atuam contra os danos causados durante a dessecação. Em relação ao níveis de peroxidação lipídica, o único tratamento em que os níveis não aumentaram após a dessecação foi o ABA. É importante notar que seu nível também foi o mais baixo no tratamento ABA após a dessecação (2,92 nM $g^{-1}W$), indicando que o tratamento ABA foi o único onde os efeitos negativos causados por ERO na peroxidação lipídica foram contidos.

Em resumo nossos resultados indicaram que o ABA desempenhou um papel fundamental no aumento da TD, principalmente porque participou da ativação do sistema antioxidante, reduzindo o estresse causado pela dessecação. No entanto, apesar dos resultados promissores deste trabalho, mais estudos ainda são necessários de forma a tornar o armazenamento dessas sementes uma opção viável para a conservação, permitindo assim uma maior exploração do potencial de uso da espécie.

ABSTRACT

Seed desiccation sensitivity imposes restrictions to conservation on conventional germplasm banks and the development of differentiated protocols is necessary for the *ex situ* conservation. Currently, is possibly to induce desiccation tolerance (DT) in sensitive seeds by altering the content of two plant growth regulators (abscisic acid-ABA and gibberellin-GA) considered decisive in the acquisition of DT. We studied the effect of seven solutions (H_2O, abscisic acid (ABA), diniconazole (DE - ABA catabolism inhibitor), fluridone (FLU - ABA biosynthesis inhibitor), ABA/DE, FLU/DE and paclobutrazol (PAC – gibberellin biosynthesis inhibitor) on desiccation tolerance (DT) of *Campomanesia xanthocarpa* seeds. Seeds were soaked for 36 hours in the respective solutions and part of them was placed to germinate, and another part was desiccated in silica gel until reaching 0.07 gH_2O^{-1}. $gDW^{-1}$and then was submitted to germination test, polyamine quantification, antioxidant enzyme activity and lipid peroxidation. It was observed that the treatments did not influence the germination and germination speed index (GSI) of seeds that were not desiccated, while the seeds submitted to desiccation showed difference in germinability and GSI in most treatments. Greater germinability was observed when seeds were soaked in H_2O and ABA (H_2O: 37.5% ± 6.4; ABA: 43.7% ± 8.5) when compared to seeds that were only desiccated (20.2% ± 5.2). A reduction in germinability and IVG was also observed in the treatment where fluridone was applied (7.5 ± 5), indicating that ABA is necessary to increase DT. An increase in PAs content was observed after desiccation in all treatments, especially those with lower germination rate, such as control, FLU and FLU/DE. Antioxidant enzyme activity and lipid peroxidation levels were also affected by desiccation. A significant increase of SOD and APX activity was observed in the ABA treatment after desiccation and a reduction in SOD activity in seeds that were only desiccated were also observed. In addition, we observed a reduction in MDA levels in this treatment although the majority of the treatments showed increase of MDA levels. Those results indicated that ABA played a key role in increasing DT, mainly because it participated in the activation of the antioxidant system, reducing the stress caused by desiccation. Despite the promising results of this work, more studies are still needed to be carried out to make storage of these seeds a viable option for conservation, thus allowing greater exploration of the potential use of the species.

Keywords: Abscisic acid. Myrtaceae. Conservation.

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Figura 1 – . Germination (%) (**a)** and Germination Speed Index (GSI) (**b**) of *Campomanesia xanthocarpa* seeds submitted to different pre-treatments solutions without (0.85 gH_2O^{-1}. gDW^{-1}) and prior (0.07 gH_2O^{-1}. gDW^{-1}) to desiccation. Control is seeds without pre-treatment solutions in both water content. Data mean ± standard error (n = 3). Uppercase letters compare the same treatment at different water content and lowercase letter compares different treatment at the same water content. Means followed by the same letters are not significantly different according to Tukey test ($p < 0.05$)……..…….35

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LISTA DE ABREVIATURAS E SIGLAS

ABA Ácido abscísico

APX Ascorbato Peroxidase

CAT Catalase

DE Diniconazole

DT Desiccation Tolerant

DS Desiccation Sensitive

FLU Fluridone

GA Giberelina

HSP Heat Shock Protein

LEAs Late Embryogenesis Abundant

MDA Complexo malondialdeído-ácido tiobarbitúrico

PAC Paclobutrazol

PAs Poliaminas

PGR Plant Growth Regulators

Put Putrecina

SOD Superóxido dismutase

Spd Espermidina

Spm Espermina

ROS Reactive Oxygen Species

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1 FUNDAMENTAÇÃO TEÓRICA

1.1 Campomanesia xanthocarpa Berg

A família Myrtaceae compreende 142 gêneros e ca. 5.800 espécies, distribuídas nas zonas tropicais e subtropicais do Hemisfério Sul (Wilson 2011). É uma das famílias mais importantes dos neotrópicos. O Brasil é considerado um centro de diversidade da família, com 24 gêneros, cerca de 1034 espécies, das quais 744 são endêmicas (Sobral et al. 2015). A família é excepcionalmente rica em espécies na Mata Atlântica e no Cerrado do Sudeste e Sul do Brasil, onde pode compor 10% a 15% das angiospermas arbóreas (Mori et al.1983, Oliveira-Filho et al.1983, Fontes 2000).

No Brasil, apresenta as seguintes características: árvores ou arbustos com glândulas oleíferas; folhas inteiras, simples, geralmente opostas, normalmente com nervura marginal; flores hermafroditas geralmente brancas, 4–5 sépalas e pétalas livres, ovário ínfero; bagas ou drupas com uma a numerosas sementes (Sobral et al. 2015). Muitas espécies têm importância econômica, destacando-se as do gênero *Eucalyptus* L'Hérit. com potencial madeireiro, gênero *Pimenta* Lindl. de interesse medicinal e as dos gêneros *Eugenia L., Psidium* L. e *Campomanesia*. com interesse alimentício, principalmente devido a produção de frutos comestíveis (Govaerts et al. 2008, Wilson 2011, Sobral et al. 2015).

O gênero *Campomanesia* compreende ca. 45 táxons distribuídos predominantemente em áreas de florestas tropicais e vegetação de savana (Landrum 1986, Govaerts et al. 2008). Segundo Sobral et al. (2015), 39 espécies de *Campomanesia* são conhecidas do Brasil, sendo que 29 delas são endêmicas. O gênero faz parte da tribo Myrteae, que inclui mais espécies do que qualquer outra nas Myrtaceae (Wilson 2011). Pode ser separado de outros gêneros de Myrtaceae pelas flores pentâmeras, geralmente alto número de lóculos (3–18), a presença de uma parede locular glandular que cobre a semente e serve como um tegumento falso, um embrião com um grande hipocótilo e pequenos cotilédones (Landrum 1986).

Dentre as 29 espécies do gênero endêmicas do Brasil, pode-se destacar a *Campomanesia xanthocarpa*, uma espécie arbórea de ampla distribuição, sendo encontrada no planalto meridional dos estados de Santa Catarina, Paraná e Rio Grande do Sul (CNCFlora 2019). A espécie está incluída no livro "Plantas para o Futuro" do Ministério do Meio Ambiente (Lisboa et al 2011), onde é ressaltado o seu potencial econômico para a região Sul do Brasil. Seus frutos possuem elevado valor nutricional, sendo promissores como

complemento à dieta humana (Vallilo et al. 2008) e principalmente como recurso alimentar para a fauna nativa. A espécie tem potencial para uso na recuperação de áreas degradadas, em sistemas agroflorestais, bem como no paisagismo (Lisboa et al 2011).

O hábitat de ocorrência da *C. xanthocarpa* é principalmente florestas tropicais úmidas (CNCFlora 2019). Nesses hábitats cerca de 50% das plantas, assim como a *C.xanthocarpa*, produzem sementes sensíveis à dessecação (SD) (Tweddle et al. 2003), mais conhecidas como sementes recalcitrantes (Roberts 1972, 1973), que diferente das sementes ortodoxas não passam pela fase da dessecação, durante o desenvolvimento da semente e por isso são dispersas com elevado conteúdo de água, metabolismo ativo e possuem baixa longevidade (Farrant e Oliver 2004, Pammenter e Berjak 2000, 2013, Walters 2015).

1.2 Tolerância à dessecação

As sementes das angiospermas apresentam três estágios de desenvolvimento, a histodiferenciação caracterizada por intensa atividade metabólica e sensibilidade acentuada à dessecação, a fase de acúmulo de reservas (maturação) - que, em sementes tolerantes à dessecação 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 TD e a fase de dessecação, característica das sementes tolerantes à dessecação (Bewley et al. 2013).

A tolerância à dessecação se refere à habilidade de uma planta ou parte dela de manter equilíbrio entre o conteúdo de água de aproximadamente 0,05 a 0,15 gH_2O^{-1}. gMS^{-1}e a umidade relativa do ar e sobreviver por longos períodos sem acúmulo de dano letal (Alpert 2005, Leprince e Buitink 2010). A aquisição da TD consiste de uma fase programada do desenvolvimento embrionário que ocorre durante a fase de maturação, de modo que, na maturação tardia, os tecidos das sementes estão predispostos a suportar as tensões impostas pela dessecação (Berjak et al. 2007). Em *Arabidopsis* Heynh., a maturação das sementes é controlada por reguladores mestres, que interagem de uma maneira complexa e incluem o fator de ligação CCAAT-box, LEAFY COTYLEDON (LEC1) e três proteínas ABSCISIC ACID INSENSITIVE (ABI3), FUSCA (FUS3) e LEC2. Coletivamente, eles são conhecidos como a rede LAFL. Esta rede controla os processos de desenvolvimento e a maturação normais das sementes, incluindo a aquisição de TD, afetando a expressão de fatores de transcrição (FT), vias hormonais e síntese e acumulação de proteínas LEAs (do inglês: Late Embryogenesis Abundant) (Maia et al. 2014).

As principais características físicas intracelulares que se modificam com a aquisição de TD incluem deposição de proteínas insolúveis dentro de vacúolos, proporcionando assim aumento da resistência mecânica contra o colapso celular, acúmulo de sacarose e oligossacarídeos da família rafinose, o que aumenta a capacidade tampão do volume intracelular e associado às proteínas LEAs contribuem para formação do estado vítreo, condensação da cromatina, que atua aumentando a tolerância contra estresse oxidativo e ruptura de membrana e coincide com a interrupção dos processos de replicação e transcrição (Pammenter e Berjak 1999, Berjak et al. 2014, Leprince et al. 2017).

Além das mudanças físicas há um aumento da síntese de proteínas LEAs, das proteínas de choque térmico (HPS do inglês Heat Shock Protein), alteração no conteúdo das poliaminas, ativação de um sistema de proteção contra estresse oxidativo pelo aumento da síntese de enzimas antioxidantes (Berjak et al. 2007, Berjak et al. 2014, Dekkers et al. 2015, Leprince e Buitink 2015). As proteínas LEAs são estáveis ao calor, apresentam flexibilidade estrutural e propriedades de retenção de água e podem formar uma barreira de hidratação ao redor de estruturas intracelulares, incluindo macromoléculas e membranas, protegendo-as contra desnaturação durante a dessecação (Berjak et al. 2007, Sano et al. 2016). As HPSs também estão associadas com proteção intracelular durante a dessecação, mantendo as proteínas citoplasmáticas solúveis em estado vítreo. Sua expressão ocorre intracelularmente em todos os tecidos do embrião durante a fase de dessecação. Há registros de que a aplicação exógena de ABA em sementes pré-germinadas (sensíveis à dessecação) pode induzir a expressão de HPS e consequentemente a indução de TD (Pammenter e Berjak 1999, Maia et al. 2014, Dekkers et al. 2015, Leprince et al. 2017).

Em condições de estresse, como a dessecação de sementes SD a produção de Espécies Reativas de Oxigênio (ERO) pode aumentar ou a eficácia dos sistemas antioxidantes pode diminuir, de tal forma que a atividade oxidativa das ERO sobrecarrega a capacidade antioxidante, e os processos oxidativos degradativos predominam (Pammenter e Berjak 2013). Além disso, Varghese et al. 2011 demonstraram que há uma relação clara entre declínio da viabilidade e redução da atividade antioxidante durante a dessecação de eixos embrionários de *Trichilia dregeana* Sond., uma espécie que produz sementes SD. Similarmente, o aumento da produção de EROs e a diminuição da atividade das enzimas antioxidantes também foram associados a sensibilidade à dessecação das sementes de *Antiaris toxicaria* Lesch. (Cheng e Song 2008).

Todas as mudanças citadas anteriormente contribuem para redução da atividade

metabólica e aumento da longevidade. A incapacidade de redução da atividade metabólica das sementes recalcitrantes é provavelmente o principal fator responsável pela sensibilidade à dessecação destas. A manutenção do metabolismo ativo do desenvolvimento até a germinação restringe drasticamente o período que as sementes podem ser armazenadas (Berjak e Pammenter 2014).

A tolerância à dessecação pode ser considerada um mecanismo complexo construído por múltiplos componentes genéticos, onde cada componente contribui aditivamente para o estabelecimento da TD e a falta de cada um deles resultará na redução da longevidade e, em última análise, na perda da TD (Marques et al. 2018). A ativação desses mecanismos envolve a participação de vários fatores de transcrição, proteínas protetoras, tais como LEAs e HSPs, enzimas antioxidantes, poliaminas e principalmente hormônios (ABA e GAs) (Pammenter e Berjak 2000, Bewley et al. 2013, González-Morales et al. 2016).

1.3 Influência dos hormônios e poliaminas na tolerância e sensibilidade à dessecação

O balanço endógeno entre ABA e as GAs durante a maturação das sementes está associado com a aquisição de TD ou falta dela, nas sementes sensíveis (Bewley et al. 2013). Interrupção na síntese ou sinalização de ABA durante a maturação resulta em perda de TD ou falha na indução de TD bem como no reestabelecimento de TD em sementes germinadas (Maia et al. 2011, 2014, Dekkers et al. 2015). As GAs regulam o crescimento e vários processos de desenvolvimento, incluindo o alongamento do caule, a quebra da dormência e a indução da germinação e seus picos de concentração ocorrem durante a embriogênese, mas são reduzidos para níveis muito baixos no início da maturação em sementes TD, enquanto que nas sementes SD seu nível permanece elevado (Kucera et al. 2005, Maia et al. 2014, Marques et al. 2019). Assim, a aquisição de TD em sementes tolerantes ou a falta dela em sementes SD é considerada uma consequência da alteração do balanço endógeno entre estes fitohormônios (Bewley et al. 2013, Marques et al. 2018, 2019).

Como na maioria dos hormônios vegetais, os níveis locais de ABA ativo é controlado por um balanço entre biossíntese e inativação do ABA por catabolismos ou conjugação (Kucera et al. 2005). Acredita-se que o catabolismo desempenha um papel tão importante quanto a síntese, uma vez que os mutantes *cyp707a* (incapazes de catabolizar ABA) acumulam muito mais ABA do que os mutantes que superexpressam as enzimas de biossíntese do ABA

(Finkelstein 2013). Em sementes germinadas de *Medicago* L. e *Arabidopsis* o restabelecimento de TD depende do ABA, uma vez que o tratamento com fluridone (um inibidor da biossíntese de ABA) e o mutante *Arabidopsis aba2*-1 (insensível ao ABA) tiveram o reestabelecimento de TD comprometida, e em contrapartidas nas sementes que apresentaram maior sensibilidade ao ABA a TD pode ser reestabelecida (Terrasson et al. 2013, Verdier et al. 2013, Maia et al. 2014). As sementes que apresentaram maior sensibilidade ao ABA apresentaram superexpressão de genes de biossíntese do ABA (*NCED3*, *NCED5*, *NCED9*, *ABA1*, *ABA2* e *ABA3*) e subexpressão do gene catabólico do ABA (*CYP707A2*), o que confirma a importância do acúmulo, sinalização e sensibilidade do ABA na aquisição de TD (Maia et al. 2014).

A regulação dos mecanismos de aquisição de TD pelo ABA envolve a participação de fatores de transcrição responsivos ao ABA, como *ABI3*, *ABI4* e *ABI5*. Esses fatores de transcrição controlam a expressão de genes responsivos ao ABA associados à aquisição de TD, como os genes das LEAs (Buitink e Leprince 2018, Finkelstein 2013). Além disso, o *ABI4* aumenta a biossíntese de ABA enquanto diminui a biossíntese de GA, interagindo diretamente com as regiões promotoras do *NCED6*, um gene da biossíntese do ABA, e do *GA2OX7*, um gene inativador de GA (Shu et al. 2016). O *ABI5* é considerado um fator de transcrição crucial na sinalização do ABA. Há evidências de que o *ABI5* forma um complexo com o *ABI3* para regular a expressão de genes que codificam proteínas LEAs, proteínas HPSs e também genes envolvidos no acúmulo de proteínas de reservas, como oleosina cuja abundância está relacionada com a aquisição de TD no desenvolvimento de sementes de *Medicago* (Chatelain et al. 2012, Verdier et al. 2013). Além disso, em sementes de *Medicago*, *ABI3* atuam na biossíntese de glutationa (GSH), uma enzima que confere proteção contra estresse oxidativo nas sementes maturas e também atuam na superexpressão de genes de sensibilidade ao ABA, como *RD26* e *PROTEIN-L-ISOASPARTATE METHYLTRANSFERASE 1* (*PIMT1*), ambos associados ao reparo de proteínas depois da dessecação (Terrasson et al. 2013). Os principais processos celulares suprimidos por esses fatores de transcrição responsivos ao ABA durante a fase de aquisição de TD incluem divisão celular, que tem associação com redução do metabolismo e produção de metabólitos secundários, associados à defesa contra estresses bióticos, como ataque de patógenos.

Em sementes de um mutante *abi3* (insensível ao ABA) foi observado acúmulo de proteínas relacionadas à germinação nos estágios posteriores do desenvolvimento (Meurs et al. 1992). A ausência de proteínas relacionadas à maturação em sementes *abi3* concorda com

a visão geral de que o ABA estimula a síntese de proteínas que estão envolvidas na proteção contra danos causados pela dessecação (Black et al. 1999), como observado em sementes SD de *A. saccharinum*, onde a aplicação exógena de ABA e de um inibidor do seu catabolismo promoveu aumento do conteúdo endógeno de ABA e aumento da síntese de proteínas dehidrinas, cuja função está associada com estabilização de membranas durante a dessecação, e também aumento da síntese de proteínas de armazenamento (Beardmore e Whittle 2005).

Alguns estudos tem demostrado que outras moléculas têm seu conteúdo endógeno alterado em condições de estresses abióticos, a exemplo das poliaminas (PAs), que não apenas regulam o crescimento e o desenvolvimento das plantas (Chen et al. 2019), mas também desempenham papéis importantes na minimização dos efeitos deletérios do estresse (Liu et al. 2005, 2015; Tiburcio et al. 2014). O acúmulo de PAs nos tecidos vegetais sinaliza estresse e também pode ter papel de proteção ao estimular a atividade do sistema antioxidante (Liu et al. 2015). As três principais PAs encontradas nas plantas vasculares são espermidina (Spd), espermina (Spm) e putrescina (Put) (Alcazar et al. 2006; Kusano et al. 2007). As PAs são compostos nitrogenados alifáticos de baixo peso molecular. É importante ressaltar que eles também são policatiônicos, e por essa razão pode ligar-se a várias macromoléculas celulares (Kusano e Suzuki 2015).

O papel crítico das poliaminas na tolerância ao estresse pode ser exercido sob diferentes mecanismos: em primeiro lugar, quando os níveis de transcrição de genes biossintéticos de PAs, e a atividade das enzimas, são induzidas pelo estresse; em segundo lugar, quando há elevação dos níveis endógenos de PAs proveniente da aplicação exógena, ou a superexpressão de genes biossintéticos de PAs, resultando em maior tolerância ao estresse; e em terceiro lugar, quando a redução do conteúdo endógeno de PAs é acompanhada pela diminuição da tolerância ao estresse (Liu et al. 2015).

Uma série de estudos demonstrou que as PAs atuam na tolerância ao estresse, em grande parte modulando a homeostase de espécies reativas de oxigênio (ROS), devido aos seus papéis diretos ou indiretos na regulação dos sistemas antioxidantes ou na supressão da produção de ROS (Chen et al. 2019; Liu et al. 2015; Lando et al. 2019). Além disso, há evidências de que os níveis de ABA e de PAs são alterados em algumas plantas sob estresse, e a relação entre eles vale a pena ser investigada. Resultados anteriores sugerem que o ABA tem diferentes efeitos no acúmulo de PAs sob diferentes condições (Liu et al. 2005; Liu et al. 2016; Lando et al. 2019). Contudo ainda é desconhecido se a relação entre ABA e PAs pode

conferir proteção às sementes sensíveis à dessecação, a exemplo da *C. xanthocarpa*, quando submetidas a tratamentos de dessecação (Vieira et al. 2021).

O uso de inibidores da biossíntese e catabolismo do ABA e GA possibilita o estudo e compreensão de como essas duas rotas do metabolismo do ABA interferem no conteúdo e sinalização do ABA e em última análise na ativação dos mecanismos de indução de TD. A aplicação de um inibidor da biossíntese de GA (PAC) em sementes SD de *C. limon* promoveu indução de TD por meio da superexpressão de genes de resposta ao estresse abiótico, como aqueles relacionados à síntese de proteínas HPSs (*HSC70-1, HSP60, HSP101*) e também superexpressão de genes de biossíntese do ABA (*NCED6*) indicando aumento do conteúdo endógeno. Além disso, os genes responsivos ao ABA que atuam na regulação da indução de TD também foram superexpressos (*GBF3*, *GPCR-type G protein 1*, *MFT* e MARD). Em contrapartida esse mesmo inibidor desencadeou subexpressão de genes associadas à biossíntese de ácido salicílico (*TGA10*) e ácido jasmônico (*ATJAZ1*, *JAZ2* e *JAZ3*) que são relacionados à proteção ao estresse biótico como ataque o de patógeno (Marques et al. 2019). Em suma vários estudos tem destacado a importância do acúmulo e sinalização de ABA na ativação dos mecanismos de indução de TD em sementes sensíveis (Beardmore e Whittle 2005, Vieira et al. 2010, Maia et al. 2011, 2014, Dekkers et al. 2015, Marques et al. 2019) e como mencionado anteriormente é justamente a alteração do balanço hormonal entre o ABA e a GA que confere acúmulo do ABA e tem sido relacionado em última análise a aquisição de TD.

1.4 OBJETIVOS

1.4.1 **Objetivo Geral**

Estudar a influência de reguladores de crescimento vegetal e seus inibidores no aumento da tolerância à dessecação em sementes de *Campomanesia xanthocarpa*.

1.4.2 **Objetivos Específicos**

Estudar o efeito dos pré-tratamentos com H_2O, ABA, paclobutrazol (PAC), diniconazole (DE), fluridone (FLU), ABA/DE e DE/FLU na germinação das sementes de *C. xanthocarpa* antes e após a dessecação.

Estudar o efeito dos pré-tratamentos com H_2O, ABA, PAC, DE, FLU, ABA/DE e DE/FLU no conteúdo endógeno de poliaminas em sementes de *C. xanthocarpa* antes e após a dessecação.

Determinar a atividade das enzimas antioxidantes (superóxido dismutase, catalase, ascorbato peroxidade), nas sementes de *C. xanthocarpa* em resposta aos pré-tratamentos com H_2O, ABA, PAC, DE, FLU, ABA/DE e DE/FLU antes e após a dessecação.

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3 CAPÍTULO ÚNICO

The relationship between abscisic acid, polyamine and antioxidant enzymes play pivotal role on desiccation tolerance of *Campomanesia xanthocarpa* seeds

3.1 ABSTRACT

Seed desiccation sensitivity imposes restrictions to conservation on conventional germplasm banks and the development of differentiated protocols is necessary for the *ex situ* conservation. Currently, is possibly to induce desiccation tolerance (DT) in sensitive seeds by altering the content of two plant growth regulators (abscisic acid-ABA and gibberellin-GA) considered decisive in the acquisition of DT. We studied the effect of seven solutions (H_2O, abscisic acid (ABA), diniconazole (DE - ABA catabolism inhibitor), fluridone (FLU - ABA biosynthesis inhibitor), ABA/DE, FLU/DE and paclobutrazol (PAC – gibberellin biosynthesis inhibitor) on desiccation tolerance (DT) of *Campomanesia xanthocarpa* seeds. Seeds were soaked for 36 hours in the respective solutions and part of them was placed to germinate, and another part was desiccated in silica gel until reaching 0.07 gH_2O^{-1}. $gDW^{-1}$and then were submitted to germination test, polyamine quantification, antioxidant enzyme activity and lipid peroxidation. It was observed that the treatments did not influence the germination and germination speed index (GSI) of seeds that were not desiccated, while the seeds submitted to desiccation showed difference in germinability and GSI in most treatments. Greater germinability was observed when seeds were soaked in H_2O and ABA (H_2O: 37.5% ± 6.4; ABA: 43.7% ± 8.5) when compared to seeds that were only desiccated (20.2% ± 5.2). A reduction in germinability and IVG was also observed in the treatment where fluridone was applied (7.5 ± 5), indicating that ABA is necessary to increase DT. An increase in PAs content was observed after desiccation in all treatments, especially those with lower germination rate, such as control, FLU and FLU/DE. Antioxidant enzyme activity and lipid peroxidation levels were also affected by desiccation. A significant increase of SOD and APX activity was observed in the ABA treatment after desiccation and a reduction in SOD activity in seeds that were only desiccated were also observed. In addition, we observed a reduction in MDA levels in this treatment although the majority of the treatments showed increase of MDA levels. These results indicated that ABA played a key role in increasing DT, mainly because it participated in the activation of the antioxidant system, reducing the stress caused by desiccation. Despite the promising results of this work, more studies are still needed to be carried out to make storage of these seeds a viable option for conservation, thus allowing greater exploration of the potential use of the species.

Keywords: Abscisic acid, Myrtaceae, Desiccation

3.2 INTRODUCTION

The Atlantic Forest is considered one of the main hotspots for biodiversity conservation (Myers et al. 2000, Rezende et al. 2018). In this ecosystem about 50% of the plant produces seeds that do not tolerate desiccation (Tweddle et al. 2003). Desiccation tolerance (DT) refers to the capacity of an organism to dehydrate below 10% water on a fresh weight basis (or 0.1 g $H_2O.g^{-1}$ dry weight) without accumulation of lethal damage (Alpert 2005; Ballesteros et al. 2020). Since this ecosystem is characterized by well-defined seasons and abundant and constant water regimes, less selective pressure for the acquisition of DT and for the longevity of its seeds is observed (Wyse and Dickie 2017). Those seeds germinate as soon as they are dispersed promoting the rapid establishment of the seedling (Pammenter and Berjak 2014). However, the loss of DT as an adaptive characteristic makes these species highly dependent on humid tropical habitats and, as a consequence of the reduction and fragmentation of these ecosystems they may face risks of imminent extinction (Achard et al. 2002).

Since the acquisition of DT in DT seeds or the lack of it in desiccation sensitive (DS) seeds is the result of a series of cellular and molecular processes mediated mainly by the balance in the content of plant growth regulators (PGRs) (mainly abscisic acid -ABA and giberelins – GA) (Bewley et al. 2013, Marques et al. 2018, 2019), some studies have highlighted the possibility of inducing DT by modulation of PGRs contents (Beardmore and Whittle 2005, Marques et al. 2019). Desiccation sensitive seeds of *Acer sacharinum* L. incubated in ABA solution with tetcyclacis, an inhibitor of GA biosynthesis and ABA catabolism, (Beardmore and Whittle 2005) and *Citrus limon* L. seeds incubated in paclobutrazol solution, an inhibitor of GA biosynthesis (Marques et al. 2019) had the induction of DT. In both studies, prolonged incubation in these solutions altered the balance of ABA and GA, potentiating the accumulation of ABA, which triggered the activation of mechanisms associated with the acquisition of DT of these seeds, previously sensitive to desiccation (Marques et al. 2019).

The mechanisms activated during the enhancement and induction of DT in those seeds are associated with response to abiotic stress. These mechanisms include synthesis of protein, lipids, carbohydrates and activation of antioxidant enzymes (Berjak and Pammenter 2014; Walters 2015). The main antioxidant enzymes involved in cell detoxification are superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), and ascorbate

peroxidase (APX). Generally DS seeds show high activity of SOD and APX whereas DT seeds show high activity of CAT and GR (Bailly 2004).

Other PRGs know as important modulators involved in plant growth, germination and activation of plant response to abiotic stress are polyamines (PAs) (Huang et al. 2017). The main PAs present in higher plants are putrescine (Put), spermidine (Spd) and spermine (Spm) (Bouchereau et al. 1999), they are small aliphatic polycationic nitrogenous compounds (Huang et al. 2017), and can thus bind to several cellular macromolecules (Kusano and Suzuki 2015). Polyamines participates in tissues development processes via interactions with other plant hormones such as auxins, GA, ABA and ethylene (ET) (Mattoo et al. 2008, Pieruzzi et al. 2011, Huang et al. 2017). There are evidences that accumulation of PAs can induce its catabolism and generate ROS (reactive oxygen species), which can elicits stress-responsive genes and stimulates the antioxidant system (Tiburcio et al. 2014; Lando et al. 2019). Recent studies indicate that polyamines may act as cellular signals in the crosstalk with hormonal pathways, including ABA especially on the regulation of abiotic stress responses, where they play a protective rule (Alcázar et al. 2010, 2011). Some of them have demonstrated that the up-regulation of PA-biosynthetic genes and accumulation of Put under abiotic stress are mainly ABA-dependent responses. Since stress-responsive and ABA-responsive elements (ABRE and/or ABRE-related motifs) are present in the promoters of the polyamine biosynthetic genes (Alcázar et al. 2006). This reinforces the view that in response to abiotic stress, the expression of some of the genes involved in polyamine biosynthesis is regulated by ABA (Alcázar et al. 2010).

There are evidences that ABA levels, activity of the antioxidant system and polyamine levels are both altered in some plants under stress (Steiner et al. 2007). Previous results suggest that ABA has effects on polyamine accumulation (Liu et al. 2005; Lando et al. 2019), and also affects the expression of genes encoding antioxidant enzymes (Hu et al. 2006, Lando et al. 2020). However, studies exploring how the antioxidant system and PAs content are affected by exogenous application of some PGRs in DS seeds are still lacking. Taking in account that desiccation is a kind of abiotic stress we hope to clarify the relationship between ABA, PAs and antioxidant system during the induction or enhancement of seed DT, expanding the scarce knowledge available in that area. Thus, the possibility of inducing DT in those seeds offers a viable option for the long-term storage of *C. xanthocarpa* seeds and other

species that produce sensitive seeds, promoting strategies for *ex situ* conservation of vulnerable and or endangered species.

3.3 MATERIAL AND METHODS

Plant Material

Mature fruits of *C. xanthocarpa* were collected from natural populations located in the central region of southern Brazil, at the Santa Catarina state (S 27º36'74", W 50º56'87"). After collecting, fruits were transferred to the Plant Physiology Laboratory at the Federal University of Santa Catarina (UFSC), Florianópolis/Brazil, and stored under refrigeration for less than one month until the start of the experiments. During the seed processing, the fruits were opened, funicular pulp was manually removed and seeds were washed in distilled water prior to the pre-treatments. Due the nature of their desiccation behaviour, seeds had to be immediately submitted through the planned experiments after being extracted from the fruits.

Treatments

Plant Growth Regulators Application

Fresh seeds were placed in 10 cm Petri dishes containing 10 mL of one of the following seven combinations of H_2O, ABA, diniconazole (DE), fluridone (FLU) and placobutrazol (PAC): (1) H_2O; (2) 200 µM ABA; (3) 200 µM DE; (4) 200 µM of FLU; (5) 200 µM ABA + 200 µM DE; (6) 200 µM DE + 200 µM FLU and (7) 200 µM PAC. The Petri dishes were kept in a B.O.D. chamber (Biochemical Oxigen Demand), with a photoperiod of 12 hours and a temperature of 25 ºC for 36 hours. The control treatment was seeds without 36 hours of incubation. Placobutrazol, DE and FLU solutions were prepared by dissolving the compounds in acetone (0.1% v/v), followed by dilution with water (Kim et al. 2008, Hu et al. 2012, Lando et al. 2019). Abscisic acid was dissolved in NaOH (0.01% v/v), according to manufacturer's instructions. The concentrations of solvents were tested in previous experiments and shown to have no effect on germination (unpublished data).

Water content

After the plant growth regulators application process seeds of each treatment were either put straight to germinate in a water content of 0.85 gH_2O^{-1}. gDW^{-1} (Factor 1) or were desiccated in silica gel until reach 0.07 gH_2O^{-1}. gDW^{-1} water content (Factor 2).

Seed Germination

Four replicates of 25 seeds of each treatment were disinfested for 30 seconds in ethylic alcohol 70º GL, one minute in commercial sodium hypochlorite (0.5% v/v) and rinsed three times in sterile distilled water. Seeds were then placed in 10 cm Petri dishes containing one sheet of Germitest® moisturised with 7 ml of distilled water and incubated in a B.O.D (25 ± 2ºC, 12/12h photoperiod). Seeds were monitored daily for one month and considered germinated with the protrusion of the radicle.

Germination Parameters

Germination Speed Index (GSI) and Germination Percentage

The germination speed index (GSI) was calculated according to the Maguire's index, where GSI = (G1/N1) + (G2/N2) +… + (Gn/Nn); G1, G2,…, Gn: number of germinated seeds in first, second, to the last count; and N1, N2,…, Nn: number of days from sowing to the first, second and last count (Maguire 1962; Brown and Mayer 1988). Germination percentage was calculated by the cumulative number of daily germinated seeds with respect of the total number of seeds evaluated (Ranal and Santana 2006).

Biochemical analyses

For all biochemical analysis the embryos of seeds were collected after 36 hours of incubation in the different solutions for seeds at 0.85 gH_2O^{-1}. gDW^{-1} and for seeds at 0.07 gH_2O^{-1}. gDW^{-1} after they are incubated, dried and reached this water content.

PAs Determination

For PAs determination, three samples (300 mg fresh mass - FM) of embryos from each treatment were ground in 1.6 mL of 5% (v/v) perchloric acid. Free and conjugated PAs were extracted, dansylated and quantified according to Steiner et al. (2007), with modifications. Free PAs were directly determined from the supernatant. Free PAs were derivatized with dansyl chloride and quantified by HPLC using a 5-µm C18 reverse-phase column (Shimadzu Shin-pack CLC ODS). The gradient of absolute acetonitrile was programmed to 65% over the

first 10 min, from 65 to 100% for 10 to 13 min, and 100% for 13–21 min, using 1 mL min−1 flow rate at 40 °C. PAs concentration was determined using a fluorescence detector with a wavelength of 340 nm (excitation) and 510 nm (emission). Peak areas and retention times were measured by comparison with standard PAs: Put, Spd, and Spm. The compound 1,7-diaminoheptane (DAH) was used as internal standard.

Antioxidant Enzyme Extraction and Assays

For the antioxidant enzyme assays, embryos (300 mg FM) were homogenized on ice with 1 mL of a 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM ethylenediaminetetra acetic acid (EDTA) and 1% polyvinylpyrrolidone (PVP) using an Ultra-Turrax Homogenizer, according to Bailly and Kranner (2011), with modifications. The homogenate was centrifuged at 15,000×g for 20 min at 4 °C and the supernatant was used for the determination of enzyme activities. All steps in extracting the enzymes were carried out at 4 °C. Catalase (CAT; EC 1.11.1.6) activity was estimated by the decrease in absorbance of H_2O_2 (extinction coefficient 39.4 mM−1 cm−1) at 240 nm for 7 min (Peixoto et al. 1999). The 300 µl reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 12.5 mM H_2O_2, and 10 µl of enzyme extract. Catalase activity was expressed as µmol min−1mg−1 protein. Ascorbate peroxidase (APX; EC 1.11.1.11) activity was estimated following the decrease in absorbance at 290 nm for 10 min (extinction coefficient 2.8 mM−1 cm−1) (Koshiba 1993). The 300 µl reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 50 mM ascorbic acid, 4.75 mM H_2O_2, 5 mM EDTA and 10 µl of the enzyme extract. APX activity was expressed as µmol min−1 mg−1 protein. Superoxide dismutase (SOD; EC 1.15.1.1) activity was assayed by monitoring the 50% inhibition of photochemical reduction of NBT at 560 nm, according to the method of Giannopolitis and Ries (1977). The 300 µl reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8), 13 mM methionine, 75 µM NBT, 2 mM riboflavin, 100 nM EDTA and 10 µl of enzyme extract. The reaction mixtures were illuminated for 15 min. Protein contents in the enzyme extracts were determined by spectrophotometry according to the method of Bradford (1976) at 595 nm, with bovine serum albumin (BSA) as standard. The activity of enzymes and protein content were performed with the use of a spectrophotometer Spectra-Max® 190 Microplate Reader.

Lipid Peroxidation

The level of lipid peroxidation was estimated according to Hodges et al. (1999) with modifications. Embryos (300 mg FM) of were homogenized with 3 ml of 0.1% (w/v) trichloroacetic acid (TCA) and centrifuged at 12.000×g for 15 min. An aliquot of 0.5 ml of the supernatant was added to 1.5 ml of 0.5% (w/v) 2-thiobarbituric acid and 20% (w/v) TCA, incubated in pre-heated water (100ºC) for 30 min and then transferred to an ice bath for another 30 min. Thereafter, the samples were centrifuged at 10.000×g for 15 min. Content of malondialdehyde-thiobarbituric acid complex (MDA) was measured using Spectra-Max® 190 Microplate Reader at 532 nm and corrected by subtracting the absorbance at 600 nm and 440 nm. Lipid peroxidation was calculated using the extinction coefficient of 157 mM-1 cm-1 and expressed as nmol of MDA per gram of fresh weight.

Statistical Analyses

Germination data was measured using four replicates of 25 seeds per treatments. Data normality was evaluated using the Shapiro–Wilk test. Analysis of variance was performed on all data set using variance analysis with two crossed fixed factors (degree of desiccation × pre-treatments—plant growth regulator followed by the Tukey test ($p < 0.05$) (Sokal and Rohlf 1995) using the "R" statistical program (Team 2014). Germination data were represented by the mean germination percentage (n = 4 repetitions) and standard error.

3.4 RESULTS

Desiccation tolerance of *C. xanthocarpa* seeds is enhanced by ABA, and inhibited by FLU

The application of the plant growth regulators and their inhibitors in the pre-treatment did not influence germinability when compared to control seeds in treatments where the water content was 0.85 gH_2O^{-1}. gDW^{-1}. Seeds treated with ABA did not show reduction on germinability when compared with control treatment (Fig. 1a). On germination speed index we observed a different response. At water content of 0.85, the only treatments with significantly difference on GSI when compared to the control (1.62±0.04) were DE (1.04±0.22) and DE + FLU (1.12±0.15). In these both treatments, an ABA catabolism inhibitor (DE) was present, which indicates that possibly the catabolism route increased the sensitivity to ABA and consequently delayed germination (Fig. 1b). However, when seeds

were dried to 0.07 gH_2O^{-1}. gDW^{-1} we observed an increase on germinability and GSI in H_2O and ABA (H_2O: 37.5% ± 6.4; ABA: 43.7% ± 8.5) when compared with seeds that were not exposed to any pre-treatment prior to desiccation (Control: 20.2% ± 5.2) (Fig. 1a,b). We also observed a significant reduction on germinability and GSI in some treatments when compared with control seeds, indicating reduction of viability and presence of desiccation damage. These reductions were higher in the treatment were the ABA biosynthesis inhibitor (FLU) was applied (7.5 ± 5).

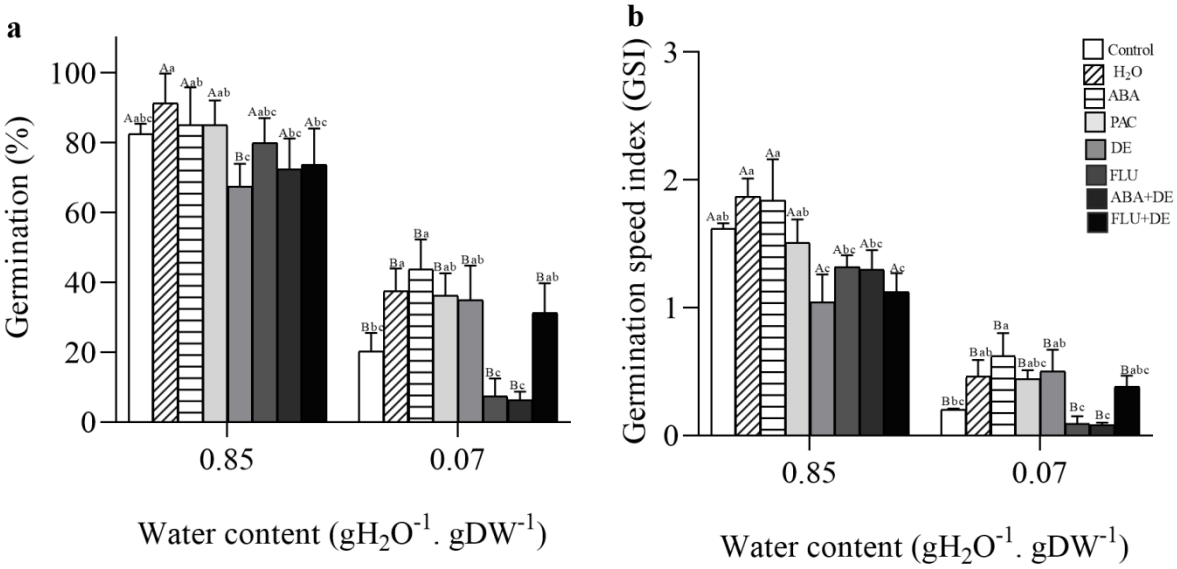

Fig. 1 Germination (%) (**a)** and Germination Speed Index (GSI) (**b**) of *Campomanesia xanthocarpa* seeds submitted to different pre-treatments solutions without (0.85 gH_2O^{-1}. gDW^{-1}) and prior (0.07 gH_2O^{-1}. gDW^{-1}) to desiccation. Control is seeds without pre-treatment solutions in both water content. Data mean ± standard error (n = 3). Uppercase letters compare the same treatment at different water content and lowercase letter compares different treatment at the same water content. Means followed by the same letters are not significantly different according to Tukey test ($p < 0.05$).

Endogenous content of polyamine is affected by exogenous PGRs and their inhibitors after desiccation of *C. xanthocarpa* seeds

We observed an increase of Total PAs contents after desiccation (0.07 gH_2O^{-1}. gDW^{-1}) in control and pre-treatments with H_20, PAC, FLU, FLU+DE when compared to seeds submitted to the same pre-treatments but that were not desiccated (0.85 gH_2O^{-1}. gDW^{-1}) (Fig. 2a). The level of all PAs increased in those treatments after desiccation (Fig. 2b,c,d), however we observed no difference in the (Spd + Spm):Put ratio, and this could be due to the low content of Put in relation to Spd and Spm (Fig. 2e). When comparing the difference between

the pre-treatments in seed with 0.85 gH_2O^{-1}. gDW^{-1} we observed slight difference in Spd and Spm contents, especially between control and PAC pre-treatment, where PAs content was higher in control (Fig. 2 b,c). Seeds with 0.07 water content also showed difference in PAs contents, in general, PAs were higher in treatments where neither ABA or their catabolism inhibitor (DE) were used. Spermidine was the most abundant polyamine, followed by Spm and Put in all pre-treatments. The majority of pre-treatments where Total PAs contents increased when compared the same pre-treatments at different water content, where those with the most significant reduction on the germinability (Control, FLU, FLU+DE). These results could indicate a possible role of PAs in the stress signalization in order to cope with the damages caused by desiccation.

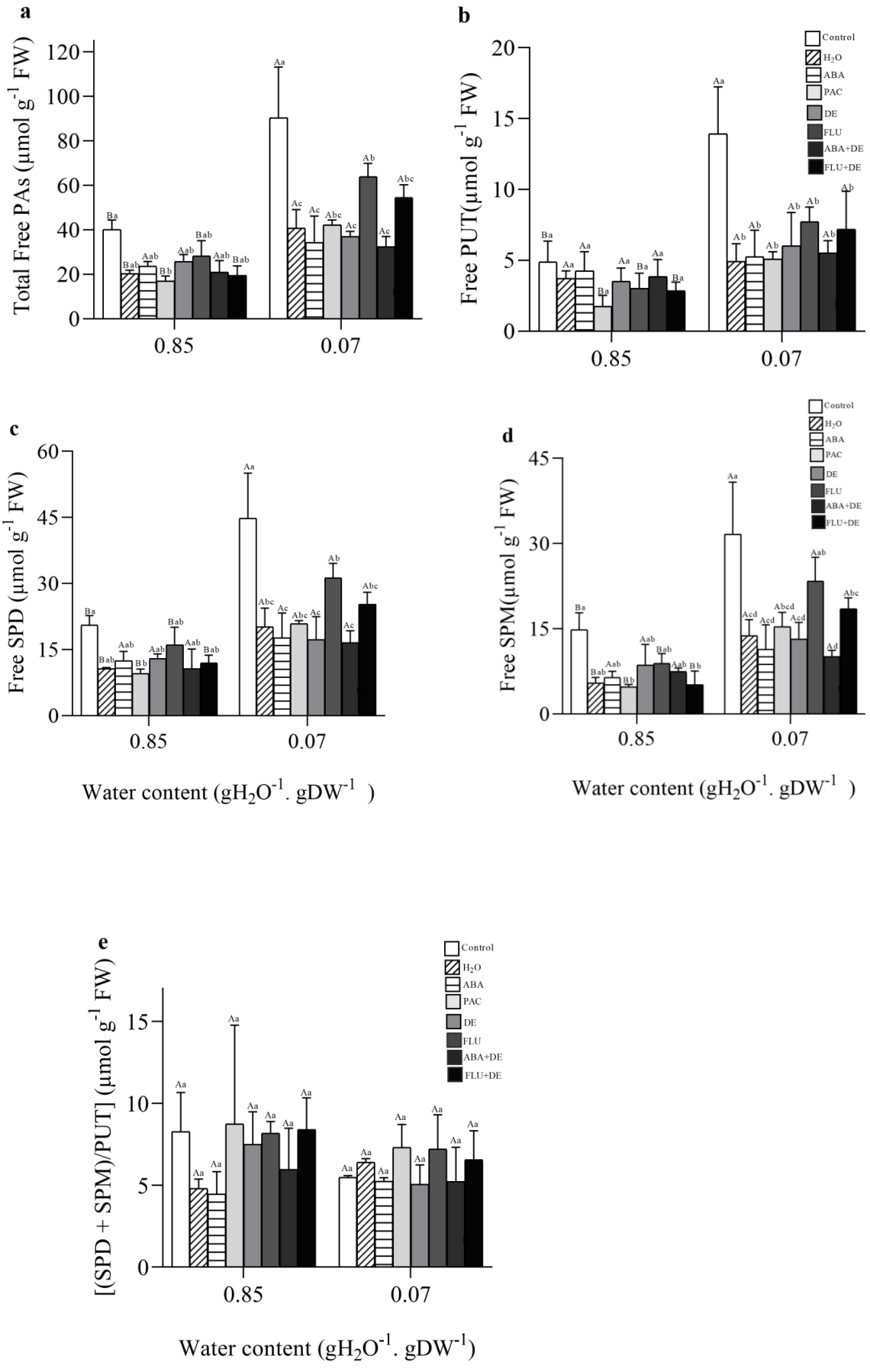
a
Total Free PAs (µmol g-1 FW)
b
Free PUT(µmol g-1 FW)
Control
H2O
ABA
PAC
DE
FLU
ABA+DE
FLU+DE
c
Free SPD (µmol g-1 FW)
d
Free SPM(µmol g-1 FW)
0.85
0.07
Water content (gH2O-1. gDW-1)
e
[(SPD + SPM)/PUT] (µmol g-1 FW)
Water content (gH2O-1. gDW-1)

Fig. 2 Endogenous contents (μmol g−1 FW) of polyamines: total free PAs (**a**), Put (**b**), Spd (**c**), Spm (**d**) and PAs ratio (Spd+Spm)/Put (**e**) of *Campomanesia xanthocarpa* seeds submitted to different pre-treatments without (0.85 gH_2O^{-1}. gDW^{-1}) and prior (0.07 gH_2O^{-1}. gDW^{-1}) to desiccation. Control is seeds without pre-treatment in both water content. Data mean ± standard error (n = 3). Uppercase letters compare the same treatment at different water content and lowercase letter compares different treatment at the same water content. Means followed by the same letters are not significantly different according to Tukey test ($p < 0.05$).

Antioxidant system is affected by exogenous PGRs and their inhibitors after desiccation of *C. xanthocarpa* seeds

The antioxidant system of *C. xanthocarpa* seeds was affected by desiccation (Fig. 3). We observed higher superoxide dismutase (SOD) activity in H_2O, ABA, PAC, DE, FLU, ABA+DE and FLU+DE pre-treatments after desiccation (129.15, 180.43, 116.1, 114.49, 85.49, 123.07, 80.9 $Umin^{-1}$. mg^{-1} protein, respectively) when compared to seeds that were not desiccated of the same pre-treatments (43.69, 27.64, 45.58, 58.64, 39.28, 62.8 and 64.07 $Umin^{-1}$. mg protein-1, respectively). The major increase of SOD activity was observed in H_2O (295%), ABA (652%), and the lower increase was in FLU (218.6%). Control treatment was the only one where SOD activity reduced after desiccation (0.85 gH_2O^{-1}. gDW^{-1}: 139.85 and 0.07 gH_2O^{-1}. gDW^{-1}: 68.93 $Umin^{-1}$. mg^{-1} protein, respectively) (Fig. 3a). When comparing different pre-treatments at 0.07 gH_2O^{-1}. gDW^{-1} control, as FLU and FLU+DC, they had no significantly difference between them (Fig. 3a). In contrast to SOD behaviour, we observed no difference in catalase (CAT) activity after desiccation (Fig. 3b), all others pre-treatments showed no difference before and after desiccation. The only change in ascorbate peroxidase activity (APX) was observed after desiccation in ABA pre-treatment, were its activity increased when comparing to ABA pre-treatment at 0.85 gH_2O^{-1}. gDW^{-1} (Fig. 3c). These results suggest that in *C. xanthocarpa* seeds, SOD together with APX are the main protective enzymes acting against the damages caused by ROS during desiccation. We also studied the effects of desiccation and the influence of pre-treatments used on lipid peroxidation. The levels of MDA (Malondialdehyde-thiobarbituric acid complex) product had a significantly increased at almost all pre-treatments after desiccation. The only pre-treatment were the levels of MDA did not increase after desiccation were ABA, all other treatments showed substantial increase of MDA level at 0.07 gH_2O^{-1}. gDW^{-1} (Fig. 3d). It worth noting that MDA level was also the lowest at ABA pre-treatment after desiccation (2.92 nM g^{-1} W), indicating that this

treatment was the only one where the negative effects caused by ROS on lipid peroxidation were restrained (Fig. 3d). Our results showed that the enzyme activities had a close relationship with the MDA content and exogenous ABA pre-treatment and, the higher the activities of SOD was the lower was the oxidative lipid peroxidation.

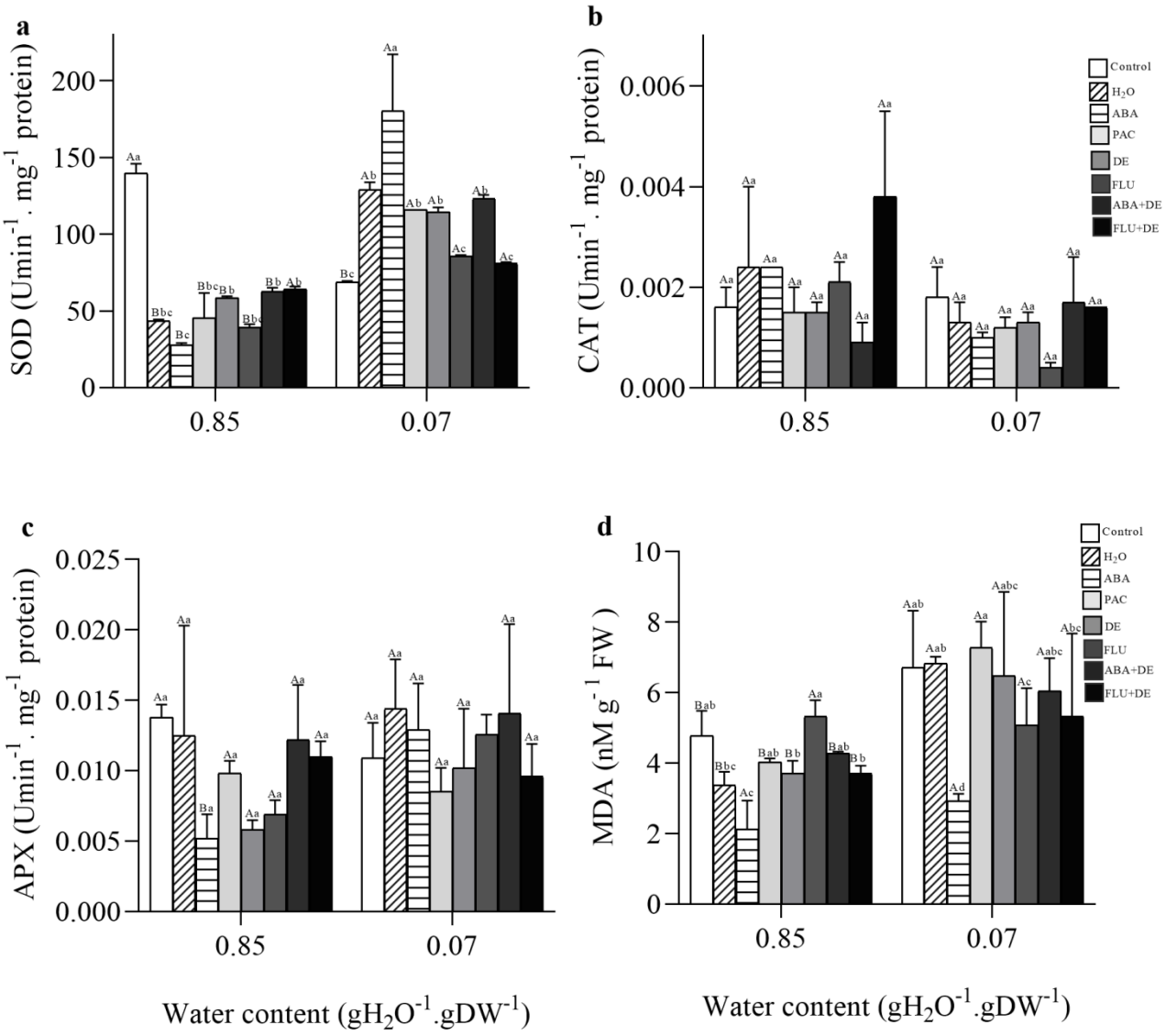

Fig. 3 Activities of antioxidant enzyme and reaction of MDA product of *Campomanesia xanthocarpa* seeds submitted to different pre-treatments without (0.85 gH_2O^{-1}. gDW^{-1}) and prior (0.07 gH_2O^{-1}. gDW^{-1}) to desiccation (**a-d**). SOD activity (**a**). APX activity (**b**). CAT activity (**c**). MDA content (**d**). Control is seeds without pre-treatment in both water content. Data mean ± standard error (n = 3). Uppercase letters compare the

same treatment at different water content and lowercase letter compares different treatment at the same water content. Means followed by the same letters are not significantly different according to Tukey test ($p < 0.05$).

3.5 DISCUSSION

Although all recalcitrant seeds are considered as desiccation sensitive, the degree of water loss tolerance shows a wide spectrum between the species (Marques et al. 2019). Seeds of different *Campomanesia* species vary widely in their level of DT (Dresch et al. 2014, 2015, Nunes et al. 2015, Emer et al. 2019, Vieira et al. 2021). *Campomanesia xanthocarpa* seeds have been considered intermediate or recalcitrant by different studies (Nunes *et al.* 2015; Vieira et al. 2021). This interspecific dehydration tolerance variability may be the result of different mother plant environments where seeds were collected (Tweddle et *al.* 2003, Marques et al. 2018, 2019). In this study, *C. xanthocarpa* seeds seem to display an intermediate behaviour as they showed severe loss of viability only when dried to water content below 0.10 on a dry weight basis (gH_2O^{-1}. gDW^{-1}) (Fig. 1).Our results suggest that the reduction water content to 0.07 gH_2O^{-1}. gDW^{-1} after the application of ABA and H_2O suppressed metabolic activity and contributed to induce the stress response, which allowed activation of protective mechanism and enhancement of desiccation tolerance (Kleinwächter et al. 2014)**.** Previous studies have also reported the efficiency in induction or enhancement of DT in DS seeds when ABA alone (Meurs et al. 1992) or combined with its catabolism inhibitor (Beardome and Whittle 2005) were applied prior to the desiccation treatment. The endogenous balance between ABA and GAs during seed maturation is associated with the acquisition of DT or lack of it in DS seeds (Bewley et al. 2013). Interruption in ABA synthesis or signalling during maturation results in loss of DT or failure to induce DT as well as reestablishment of DT in germinated seeds (Dekkers et al. 2015, Maia et al. 2011, 2014). Giberelins regulate growth and various development processes, including stem elongation, dormancy breaking and germination induction and their concentration peak occurs during embryogenesis, but drops to very low levels at the beginning of seed maturation in DT seeds, while in DS seeds its level remains high (Kucera et al. 2005, Maia et al. 2014, Marques et al. 2019). Thus, the acquisition of DT in DT seeds or the lack of it in DS seeds is considered a consequence of the alteration of the endogenous balance between these phytohormones (Bewley et al. 2013, Marques et al. 2018, 2019).

Some studies have pointed out that high GA and low ABA levels or low sensitivity to ABA in DS seeds are responsible for the maintenance of active metabolism and the skipping of maturation drying in DS seeds (Leprince and Buitink 2015, Marques et al. 2018, 2019). When maturation drying is lacking, the corresponding stress responses are not induced and the protective mechanisms are missing. In consequence those seeds lose their viability when desiccated beyond their critical water content. In contrast, seeds which have undergone a maturation drying are desiccation tolerant and are enabled to remain viable (Dekkers et al. 2015, Kleinwächter et al. 2014). The pre-treatment ABA and H_2O enhanced DT, but there was no difference between them. This indicate that enhance of desiccation tolerance is not a direct consequence of increased ABA content, suggesting that another mechanism, such as enhanced of ABA sensitivity or signalling, is probably involved (Maia et al. 2014). It is possible that ABA induced DT in *C. xanthocarpa* seeds by the regulation of a few essential protective elements whereas most of these were already present in mature seeds, and were just activated during the pre-treatment with H_2O and ABA (Meurs et al. 1992, Maia et al. 2014, Dussert et al. 2018).

In the pre-treatments were the ABA biosynthesis inhibitor (FLU) was applied, DT could no longer be enhanced or induced. This indicates that protective mechanisms were not activated; probably because the endogenous content of ABA was lower than optimum. Similar results were observed in *Medicago* seeds treated with FLU, where the re-establishment of DT could no longer be activated in consequence of the inability to repair desiccation-induced damage upon subsequent rehydration (Buitink et al. 2003). On the other hand, the pre-treatment ABA+DE did not enhance DT probably because the amount of ABA endogenous exceeded the optimum concentration for *C. xanthocarpa* seeds. Other studies have reported negative or null effects on DT when ABA content was higher than optimum, as reported by Beardmore and Whittle (2005) in *Acer saccharinum*. Therefore, further studies are needed to determine the optimum concentration of ABA to confer higher DT in *C. xanthocarpa* seeds. Nevertheless, the results presented here indicate that the overall concentration and sensitivity of ABA, regardless of whether it is externally applied or endogenously produced, is probably one of the most important factors to increase DT in *C. xanthocarpa* seeds. Our results suggests that ABA sensitivity plays a pivotal role in the enhancement of DT in *C. xanthocarpa* seeds, and that ABA accumulation is not always necessary to elicit stress, since modulation of ABA perception and signalling can be sufficient to induce an appropriate stress response (Maia et al. 2014).

Since ABA, antioxidant activity and polyamine levels are both altered in some plants under stress, the relationship between them is worth investigating (Liu et al. 2005). Our results showed that PAs content was higher in the pre-treatments where ABA was not used (Control, PAC, FLU, FLU+DE). These treatments showed lower germination rates probably because the damage caused by desiccation were high and those seeds could not germinate, even with the increase of PAs content. In the majority of those treatment ABA biosynthesis inhibitor were used, which could have counteracted the protective mechanisms that could be activated by ABA (Liu et al. 2005). Others studies have also found a significant increase in PAs contents when seeds were treated with H_20 and FLU (Lando et al. 2019). There are evidences suggesting that PAs accumulation may function as a general plant response to abiotic stresses (Liu et al. 2015), and this is supported by our results since polyamine level showed significant increase under severe stress conditions. However, it is still unclear how the cause-effect relationship between PAs accumulation and protection works, since the increase of PAs did not improve DT of *C. xanthocarpa* seeds. Our data showed that Spd and Spm was the most commons PAs in *C. xanthocarpa*. This result corroborates with the idea that in most cases, only one or two type of the three PAs shows a significant increase (Liu et al. 2015). For example, sweet orange callus was reported to show increases in Spd content when exposed to abiotic stress conditions (Wang and Liu 2009), and grape (*Vitis vinifera*) plants showed accumulation of Spd and Spm following salt stress (Ikbal et al. 2014). These observations agree with previous reports demonstrating that increases in the cellular levels of Spd and Spm are homeostatically regulated more tightly than those of Put (Alcázar et al. 2006). Several studies highlighted the importance of the Spd and Spm during abiotic stress, especially because they contain one and two additional primary amino groups ($-NH_2$), respectively, compared to Put, allowing them to be more efficient on protective functions (Liu et al. 2015, 2016, Huang et al. 2017). Furthermore, Spd is known to participate in stress tolerance via interactions with other plant hormones such as auxins, GA, ABA and ET (Alcazar et al. 2006, 2011, Steiner et al. 2007, Pieruzzi et al. 2011). Since exogenous ABA can increase polyamine levels (Pesci and Reggiani 1992, Aurisano et al. 1993, Lando et al. 2019), it can also cause a decrease (Chen and Wang 2002), our results suggest that ABA may mediate polyamine accumulation under stress, since PAs content increased in treatments were ABA was not applied, however it is not clear yet how it is done.

As mentioned before ABA can also affects the activity of the antioxidant system which is directly relate to the ability of *C. xanthocarpa* seeds to tolerate desiccation, and to remain viable. An efficient scavenging system that maintains low levels of ROS is a prerequisite for seed survival during the desiccation and prolonged storage since elevated levels of ROS might inhibits many repair processes and can also disturb the metabolic balance of ROS generation, which means that protective antioxidant reactions are not able to remove ROS quickly enough (Sahu et al. 2017). Many works have reported elevated rates of ROS production during drying of embryo as *Castanea sativa* Mill. (Roach et al. 2008, 2010), *Mimusops elengi* L. *(*Luo et al. 2012), *Araucaria bidwillii* (Francini et al. 2006) and *Norway maple* (Pukacka and Ratajczaka 2007). The protective roles of superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) to neutralize the damage caused by ROS have been widely discussed during desiccation (Bailly 2004; Kibinza et al. 2006; Sahu et al. 2017). The first line of defense against ROS is provided by SOD (Gill and Tuteja 2010), which catalyzes superoxide radicals (O_2^-) producing hydrogen peroxide (H_2O_2) and oxygen (O_2). In our study SOD was the main enzyme that increased its activity after desiccation especially in H_2O and ABA treatments. Previous studies reported that *C. xanthocarpa* seeds had an increase of SOD activity when fresh seeds were submitted through 6 and 12 h of drying, which could mean that SOD was responding to the initial intracellular production of O_2^- (Vieira et al. 2021). Our result may be associated with ABA's role in promoting the expression of genes encoding SOD, leading to an increased synthesis of this enzyme (Hu et al. 2006). Furthermore, there is evidence that ABA can causes an increased generation of ROS which could act as a signalling of abiotic stress (Arve et al., 2014) since when ROS accumulation increased in a certain limit it could have trigged the antioxidant system to counteract the damaged caused by desiccation ((Jiang and Zhang 2002). Similar results were observed during re-establishment of DT in *Medicago* seeds, where genes for enzymes involved in protection against oxidative stress were up-regulated (Buitink et al. 2006). On the other hand, when seeds were desiccated without any prior treatment (control 0.07 $gH_2O^{-1}.gDW^{-1}$) SOD activity sharply declined. This could corroborate our hypothesis that ABA was helping alleviate the damage caused by desiccation increasing antioxidant activity. Another result that could corroborate our hypothesis was the increase activity of APX in ABA treatment. Ascorbato peroxidase is known as H_2O_2-scaveging enzyme and is considered to have a central role in maintaining steady cellular contents of O_2- and H_2O_2 and considering

that SOD levels increased along desiccation, it could be assumed that its product, H_2O_2, may have also increased, justifying the increase of APX activity (Pukacka and Ratajczak, 2007).

Reactive Oxygen Species can also mediate lipid peroxidation, a mechanism of cellular injury in dry seeds (Bailly 2004; Parkhey et al. 2012) and MDA is one of the main biomarkers of oxidative damage because it is one of the last products released during the lipid peroxidation process (Min et al. 2017). High levels of MDA is frequently suggested to cause loss of viability in orthodox seeds of *Helianthus annuus* (Balešević-Tubić et al. 2007) intermediate seeds of *Pongamia pinnata* (Sahu et al. 2017) and recalcitrant seeds of *Azadirachta indica*, *Shorea robusta* (Varghese and Naithani 2008; Parkhey et al. 2012). *Campomanesia xanthocarpa* seeds showed significant increase of lipid peroxidation after desiccation in all treatment, except the one where ABA were applied exogenous. Again, we see the ABA may be directly related to the activation of antioxidant system during desiccation. Our results indicated that ABA played a key role in increasing DT, mainly because it participated in the activation of the antioxidant system, reducing the stress caused by desiccation. However, despite the promising results of this work, more studies are still needed to be carried out to make storage of these seeds a viable option for conservation, thus allowing greater exploration of the potential use of the species.

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4 CONCLUSÃO

Os resultados apresentados no presente trabalho, aprimoram o conhecimento fisiológico sobre a tolerância à dessecação em sementes de *C. xanthocarpa*, são promissores especialmente pela possibilidade de aumentar a tolerância à dessecação de espécies que possuem sementes sensíveis, além de aprofundar o conhecimento da relação poliaminas, enzimas antioxidantes e reguladores de crescimento na tolerância à dessecação. Os resultados indicaram que o ABA exerceu uma função primordial no aumento da TD, principalmente por participar da ativação do sistema antioxidante, reduzindo o estresse causado pela dessecação e promovendo aumento da germinação.

Espera-se que os resultados deste trabalho sirvam de base biológica para futuros estudos da fisiologia e conservação de sementes sensíveis à dessecação nativas com valor ecológico e econômico.