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GUILHERME DE ALMEIDA GARCIA RODRIGUES

FISIOLOGIA DE SEMENTES DE *Eugenia* (Myrtaceae) SUBMETIDAS À DESSECAÇÃO

Florianópolis

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Orientadora: Profª. Neusa Steiner, Drª

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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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Profª. Neusa Steiner, Drª.
Orientadora

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Aos que veem a luz das estrelas e têm sonhos impossíveis. Que vocês jamais desistam de fazê-los se tornar realidade.

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Muito obrigado! *Thank you so much! Merci beaucoup!*

Quanto mais conhecemos sobre as leis fixas da natureza, mais incríveis os milagres se tornam (Autobiografia de Charles Darwin, 1809–82)

RESUMO

Eugenia L. (Myrtaceae) é um importante componente florístico da Mata Atlântica, onde possui mais de 250 espécies produtoras de frutos carnosos apreciados pela fauna e pela população local. Apesar da importância ecológica, econômica e potencial farmacológico, *Eugenia* ainda é subutilizada, especialmente pelas dificuldades de manejo das suas sementes, consideradas sensíveis à dessecação. A utilização das sementes para cultivo em larga escala, estudos científicos e proteção efetiva das espécies que produzem este tipo de semente ainda é um desafio. Desta forma, este trabalho buscou aprofundar o conhecimento fisiológico e bioquímico de sementes de *Eugenia*, visando a ampliação do uso e facilitar a conservação de espécies sensíveis à dessecação. Nossas hipóteses são de que sementes de diferentes espécies possuem níveis de tolerância à dessecação distintos, os quais estão relacionados com suas respectivas atividades antioxidantes e perfis metabólicos e o local de origem das sementes. Além disso, acreditamos que é possível diminuir a sensibilidade a dessecação de sementes de *Eugenia* através da aplicação de inibidores de ácido fosfatídico. Frutos maduros de *E. uniflora, E. involucrata*, *E. pyriformis*, *E. brasiliensis* e *E. astringens* foram coletados e despolpados, sendo as sementes lavadas em água corrente. Em seguida, as sementes foram dessecadas até diferentes conteúdos de água para investigarmos a tolerância a dessecação e sua relação com a origem das sementes e distribuição geográfica das espécies. Em seguida, analisamos o conteúdo de poliaminas (PAs) endógenas e atividade antioxidante de sementes de *E. involucrata*, *E. pyriformis*, *E. brasiliensis* e *E. astringens* submetidas a dessecação. Ainda, avaliamos o conteúdo e o comportamento do perfil metabólico de sementes de *E. uniflora* e *E. astringens* frente à dessecação. Finalmente, aplicamos 1-butanol (1-BUT) e N-aciletanolamina (NAE), inibidores de ácido fosfatídico, em sementes de *E. astringens* para tentar diminuir a sensibilidade à dessecação e verificar o efeito destes compostos no comportamento fisiológico e bioquímico das sementes. Observamos que as sementes das espécies apresentaram diferentes tolerâncias à dessecação, sendo que *E. uniflora* e *E. astringens* foram consideradas a mais e a menos sensível, respectivamente, e esta sensibilidade pode estar relacionada não só aos fatores intrínsecos das sementes, mas também com fatores abióticos ambientais, como índice pluviométrico e temperatura, e com a amplitude em que estas espécies habitam. Em relação ao conteúdo de PAs, notamos que sementes de *E. involucrata* e *E. pyriformis* acumularam putrescina, enquanto sementes de *E. brasiliensis* acumularam espermidina e sementes de *E. astringens* não apresentaram modificações no conteúdo de poliaminas durante a dessecação. Ainda, verificamos que não houve acúmulo de espermina em nenhuma das espécies estudadas. Quanto ao perfil metabólico de sementes de *E. uniflora* e *E. astringens*, verificamos um alto índice de sacarose em *E. uniflora*, enquanto frutose e glicose foram os principais carboidratos em sementes de *E. astringens*, os quais aumentaram durante a dessecação. Também notamos uma maior concentração de aminoácidos em sementes de *E. uniflora* do que de *E. astringens*, os quais foram degradados em ambas as espécies durante a dessecação. Por fim, foi possível diminuir a sensibilidade à dessecação em sementes de *E. astringens* através de uma embebição prévia em 1-BUT ou em água, que diminuíram o tempo de dessecação e aumentaram a atividade de compostos antioxidantes. Nossos resultados evidenciam as particularidades das espécies de *Eugenia*, cujas sementes possuem diferentes tolerâncias à dessecação, comportamento fisiológico e conteúdo metabólico. Ainda, comprovamos que é possível diminuir a sensibilidade à dessecação de sementes. Este trabalho amplia o conhecimento sobre sementes de *Eugenia* e traz novos métodos para a conservação de espécies sensíveis à dessecação da Mata Atlântica.

Palavras-chave: Conservação. Estresse oxidativo. Germinação de Sementes. Metabolismo. Poliaminas. Sensibilidade à dessecação.

RESUMO EXPANDIDO

Introdução

Eugenia é um importante componente florístico da Mata Atlântica, onde apresenta mais de 250 espécies. Espécies deste gênero produzem flores brancas e frutos carnosos que servem de alimento para a fauna local e transitória, além de também serem apreciadas pelo ser humano na alimentação e ornamentação, e possuírem potencial na indústria farmacêutica. Sementes de *Eugenia* têm a capacidade de gerar novos indivíduos mesmo após serem altamente predadas. Além disso, não existe uma distinção visível entre o embrião e os cotilédones fusionados, e células cotiledonares podem dar origem à novas plântulas caso a semente seja fracionada ou a primeira plântula seja perdida. Ainda, uma característica das sementes de *Eugenia* é de serem sensíveis à dessecação, o que significa que elas são dispersas com elevados conteúdos de água e metabolismo ativo. Ao serem expostas à perda d'água, sementes sensíveis à dessecação podem perder sua viabilidade, dependendo do nível de desidratação e do tempo de exposição ao estresse.

A perda da viabilidade está ligada ao acúmulo de danos mecânicos e metabólicos causados pela dessecação, como desbalanço oxidativo devido ao acúmulo de espécies reativas de oxigênio (ROS), desestruturação das membranas celulares, extravasamento do citoplasma e desnaturação de macromoléculas. Isto acontece porque sementes sensíveis à dessecação não são equipadas ou não expressam de maneira ideal os mecanismos protetores necessários para sobreviver ao estresse. Dentre estes mecanismos, podemos citar a diminuição da respiração celular para evitar a superprodução de ROS, a deposição de matéria seca para estabilizar o citoplasma, o acúmulo de carboidratos, amino ácidos e outras moléculas protetoras para manter a estrutura de membranas e o turgor das células, e a ativação de moléculas antioxidantes, como poliaminas e enzimas antioxidantes.

Poliaminas (PAs) são moléculas alifáticas presentes em todas as células vivas. Em sementes, as PAs mais abundantes são a putrescina (PUT), a espermidina (SPD) e a espermina (SPM). Apesar do conteúdo de PAs variar dependendo da espécie ou etapa do desenvolvimento, elas possuem diversas funções, incluindo sinalização e combate direto e indireto contra estresses bióticos e abióticos, estabilização de membranas, remoção de ROS e indução de enzimas antioxidantes. Algumas das enzimas antioxidantes mais conhecidas são a superóxido dismutase (SOD), a catalase (CAT), o ascorbato peroxidase (APX) e a glutationa redutase (GR).

Cada enzima tem potencial de combater o estresse oxidativo por uma via específica, e a partir da ação das mesmas, o equilíbrio do metabolismo oxidante pode ser restabelecido.

Atualmente existem poucos estudos que exploram o comportamento de *Eugenia* frente à estresses abióticos, especialmente no que diz respeito ao comportamento fisiológico e bioquímico. Este trabalho foi desenvolvido visando compreender o efeito da dessecação na fisiologia de espécies sensíveis do gênero *Eugenia*, especialmente relacionando as características ambientais e o metabolismo oxidativo com a tolerância das espécies. Ainda, este trabalho buscou caracterizar metabolicamente as sementes e explorar técnicas de indução à tolerância à dessecação, com a finalidade de ampliar e preencher lacunas de conhecimento na área de fisiologia de sementes sensíveis.

Objetivo

O objetivo principal foi avaliar o efeito da dessecação no comportamento ecofisiológico e metabólico de sementes sensíveis de *Eugenia*, para aumentar o conhecimento acerca da fisiologia de sementes e facilitar o manejo e uso das espécies. Além disso, cada capítulo possui seus objetivos específicos. No primeiro, verificamos a relação entre a procedência e a tolerância à dessecação de cinco espécies de *Eugenia*: *E. uniflora*, *E. pyriformis*, *E. involucrata*, *E. brasiliensis* e *E. astringens*. No segundo, avaliamos o comportamento das poliaminas endógenas e das enzimas antioxidantes em sementes de *E. pyriformis*, *E. involucrata*, *E. brasiliensis* e *E. astringens* durante a dessecação. No terceiro, comparamos o perfil metabólico de sementes de *E. uniflora* e *E. astringens* durante a dessecação. No quarto, analisamos o efeito de inibidores de ácido fosfatídico no comportamento fisiológico de sementes de *E. astringens* antes e após a dessecação.

Material e Métodos

Frutos maduros de *E. uniflora*, *E. brasiliensis* e *E. astringens* foram coletados de populações de cerca de dez plantas em Florianópolis-SC (27°36’14.0”S 48°31’17.9”O), enquanto frutos maduros de *E. involucrata* e *E. pyriformis* foram coletados em São Paulo-SP (23°38’30.7”S 46°37’14.2”O) e Urupema-SC (28°01’26.9”S 49°52’16.0”O), respectivamente. Os frutos foram despolpados e as sementes foram lavadas em água corrente e secadas em papel toalha. O teor de água inicial das sementes foi avaliado através da estufa à 105ºC por 24h. A dessecação das sementes foi realizada em sílica gel até que atingissem os teores de água desejados para cada experimento. A sílica gel foi substituída duas vezes por dia nos dois

primeiros dias, e uma vez por dia nos dias posteriores. Para verificar o teor de água, as sementes foram pesadas a cada 3h no primeiro dia, a cada 6h no segundo e terceiro dias, e a cada 12h nos dias seguintes. Nos testes de germinação, sementes foram dispostas em papel Germitest umedecido com água destilada. Para o primeiro capítulo, sementes foram dessecadas até que atingissem 0,44, 0,33, 0,25, 0,17 e 0,12 g H_2O g MS^{-1}. Em seguida, avaliamos a germinação, a condutividade elétrica (CE) e o crescimento de plântulas. Também relacionamos com a distribuição das espécies no Brasil e os domínios morfoclimáticos e habitats destas. No segundo capítulo, foram coletadas 300 mg provenientes de dez sementes dessecadas até 0,44, 0,33 ou 0,25, e 0,12 g H_2O g MS^{-1} para verificar a dinâmica de poliaminas (PAs) endógenas (putrescina, espermidina e espermina) através do HPLC, enquanto enzimas antioxidantes (superóxido dismutase, catalase, ascorbato peroxidase e glutationa reductase) e acúmulo de peroxidação lipídica (malondialdeído, MDA) foram estimados a partir de espectrofotômetro. No terceiro capítulo, o perfil de metabólitos de sementes de *E. uniflora* e *E. astringens* frescas e dessecadas até a viabilidade atingir 50% e <10% foi estimado por GC-MS. No quarto capítulo, inibidores de ácido fosfatídico (1-butanol e N-aciletanolamina) foram aplicados em sementes frescas de *E. astringens* antes de serem dessecadas até 0,25 g H_2O g MS^{-1}. Em seguida, a capacidade germinativa, a condutividade elétrica (CE), a atividade antioxidante, o conteúdo de PAs endógenas e o acúmulo de MDA foram aferidos.

Resultados e discussão

As espécies de *Eugenia* estudadas neste trabalho apresentaram diferentes ocorrências geográficas, sendo *E. uniflora* a espécie mais dispersa, ocorrendo em 4 domínios morfoclimáticos e 8 ambientes, e *E. astringens* a mais restrita, ocorrendo apenas na Mata Atlântica e em dois ambientes: floresta ombrófila mista e restinga. Além disso, as sementes de *E. uniflora*, *E. involucrata*, *E. pyriformis*, *E. brasiliensis* e *E. astringens* apresentaram diferentes níveis de sensibilidade à dessecação, o que pode estar relacionado não só com o fato de as sementes serem de espécies distintas, mas também com as características dos ambientes nos quais as sementes foram coletadas, como pluviosidade e temperatura. As sementes de *E. uniflora* foram dispersas durante altos índices pluviométricos e temperaturas mais quentes e apresentaram alta sensibilidade à dessecação. Já as sementes de *E. astringens*, que foram dispersas durante o inverno e com índices pluviométricos extremamente baixos, foram menos sensíveis à dessecação. Cada espécie possui uma estratégia diferente para prosperar em

determinado ambiente. Neste caso, espécies mais restritas, consideradas especialistas em comparação com as espécies amplamente distribuídas (generalistas), podem apresentar características de tolerância à estresses que mais vantajosas em relação as generalistas. Sugerimos que existe uma relação entre a tolerância à dessecação de sementes e sua origem geográfica. Portanto, para estas cinco espécies, a combinação de fatores intrínsecos das sementes e condições ambientais agiram sinergicamente para promover o comportamento fisiológico das sementes e diferentes sensibilidades à dessecação.

A partir da análise de PAs, observamos que PUT se elevou durante a dessecação nas sementes de *E. pyriformis* e *E. involucrata*, enquanto SPD aumentou em sementes de *E. brasiliensis*. Por outro lado, SPM não foi regulado positivamente durante a dessecação para nenhuma das espécies de *Eugenia* estudadas. A ativação da expressão de genes relacionados à sinalização de ABA é um importante passo para tolerância à dessecação e pode estar relacionada com PAs, especialmente SPM. Portanto, acreditamos que as PAs podem estar relacionadas à sensibilidade e manutenção da viabilidade parcial das sementes de *Eugenia* durante a dessecação, e que possivelmente a falta de regulação de SPM pode ter relação com uma falta de sinalização de ácido abscísico (ABA), vista em sementes tolerantes. Com relação as enzimas antioxidantes, percebeu-se que a atividade das enzimas foi mais intensa em sementes de *E. involucrata* e *E. astringens*, sugerindo que a perda da viabilidade pode estar relacionada a um aumento do estresse oxidativo causado pelo acúmulo de radicais livres. Cada enzima possui maneiras especificas de combater os efeitos adversos da dessecação e manter a homeostase celular. A SOD é a primeira a atuar, combatendo superóxido e convertendo-o em peroxido de hidrogênio, que em seguida será convertida em água e oxigênio pela CAT, APX e GR. Por outro lado, as enzimas antioxidantes tiveram um papel menos expressivo em sementes de *E. pyriformis* e *E. brasiliensis*, o que pode indicar que a perda da viabilidade nestas sementes se deu primariamente por estresse mecânico, visto o aumento de peroxidação lipídica observado durante a dessecação destas espécies.

Com relação ao perfil metabólico de sementes de *E. uniflora* e *E. astringens*, as sementes mais e menos sensíveis à dessecação deste trabalho, respectivamente, percebemos que ambas possuem um elevado nível de carboidratos. Porém, o principal carboidrato de sementes de *E. uniflora* foi a sacarose, enquanto em sementes de *E. astringens*, a frutose e a glicose foram os carboidratos presentes em maior quantidade. Além disso, observamos que diversos carboidratos importantes para a tolerância à dessecação apresentaram uma regulação positiva durante a dessecação de sementes de *E. astringens*, como polióis, enquanto nas

sementes de *E. uniflora* a maioria destes compostos não foram regulados positivamente ou só foram regulados positivamente quando as sementes já haviam perdido a maior parte de sua viabilidade. No caso dos aminoácidos, verificamos uma maior presença em sementes de *E. uniflora*, mas apesar da maior presença, aminoácidos importantes para a tolerância à estresses não foram regulados positivamente, com exceção de ácido piroglutâmico. O mesmo ocorreu para sementes de *E. astringens*, indicando que a falta de regulação positiva dos aminoácidos pode ter relação com a perda da viabilidade de sementes de *Eugenia*. Quanto aos ácidos orgânicos, grande parte deste grupo que atua no ciclo de Krebs estava presente em maiores quantidades em sementes de *E. uniflora* durante a dessecação em comparação com sementes de *E. astringens*. Isto pode indicar que as sementes continuaram com maiores taxas respiratórias durante a dessecação e consequentemente acumularam maiores quantidades de ROS e radicais livres, fator prejudicial para estas sementes. O perfil de ácidos graxos foi similar entre as duas espécies e não sofreu grandes alterações, mas observamos um acúmulo de ácidos graxos insaturados durante a dessecação, indicando que houve um aumento da peroxidação lipídica e desestabilização de membranas nas sementes de ambas as espécies após a perda da viabilidade. Observamos ainda que sementes de *E. uniflora* apresentaram pequenas quantidades de alantoína, e a mesma não foi regulada positivamente durante a dessecação. Juntamente com a ausência em sementes de *E. astringens*, a não regulação de alantoína pode ser mais um indicativo de que o ABA não foi ativado durante a dessecação destas sementes, o que possivelmente resultou no comportamento sensível à dessecação de sementes de *Eugenia*.

Com relação à indução da tolerância à dessecação de sementes de *E. astringens*, observamos que todos os tratamentos apresentaram melhorias significativas na germinação, porém a embebição em H_2O ou 1-BUT foi capaz de reduzir o tempo de dessecação e promover germinações acima de 50%, valores muito altos em comparação com as sementes dessecadas e não embebidas, as quais germinaram apenas 13% e levaram mais tempo para dessecar. Enzimas antioxidantes como SOD e CAT foram reguladas positivamente nas sementes embebidas e dessecadas de 1-BUT, enquanto SPD e SPM aumentaram em sementes embebidas e dessecadas de H_2O. Portanto, sugere-se que os mecanismos envolvidos no combate ao estresse oxidativo foram ativados durante a dessecação de sementes de *E. astringens* que foram embebidas previamente nestes tratamentos. Como visto pela regulação positiva de SPM, H_2O pode estar promovendo uma sinalização em cascata de ABA, levando ao aumento da tolerância à dessecação nestas sementes.

Este trabalho traz diversas novidades acerca da fisiologia de sementes de *Eugenia* sensíveis à dessecação. Os dados apresentados demonstraram características fisiológicas e relações entre diferentes fatores e compostos que ainda não foram descritos para espécies de *Eugenia*, além de mostrar novas perspectivas sobre o comportamento de sementes florestais nativas do Brasil. Resultados promissores foram apresentados com relação à diminuição da sensibilidade à dessecação em sementes de *E. astringens*, e estes dados podem servir de alicerce para futuros trabalhos relacionados à preservação de germoplasma de espécies sensíveis.

ABSTRACT

Eugenia L. (Myrtaceae) is an important floristic component of the Atlantic Forest, where it holds more than 250 species. These species produce fleshy fruits consumed by the fauna and local human populations. Moreover, *Eugenia* presents great ecological and economic importance and pharmacological potential. However, this genus is understated and understudied due to their desiccation-sensitive seeds, which hampers seed management. Therefore, to use *Eugenia* desiccation-sensitive seeds for large scale production, scientific studies and to effectively protect these species is still a challenge. Our study aimed to increase the physiological and biochemical knowledge of *Eugenia* seeds, in order to broaden the use and ease the conservation of desiccation-sensitive species. We hypothesized that seeds of each *Eugenia* species have different degrees of desiccation tolerance, which are related to their individual antioxidant activities and metabolic profiles. Moreover, we also hypothesized that it is possible to attenuate their desiccation sensitivity through the inhibition of phosphatidic acid (PhA). Mature fruits of *E. uniflora, E. involucrata, E. pyriformis, E. brasiliensis*, and *E. astringens* were collected and pulped, and seeds were rinsed thoroughly in tap water. Next, seeds were desiccated in silica gel until they reached different water contents to verify their desiccation tolerance threshold and its relation to seed provenance and species geographical distribution. Moreover, seeds of *E. involucrata, E. pyriformis, E. brasiliensis* and *E. astringens* in several water contents were used in endogenous polyamines (PAs) and antioxidant enzymes analysis. We also analyzed the metabolic profile of *E. uniflora* and *E. astringens* seeds upon desiccation. We used seeds of *E. astringens* to study two PhA inhibitors, 1-butanol (1-BUT) and N-acylethanolamine (NAE), with the aim of attenuating the desiccation sensitivity of these seeds and to analyze the effects of these compounds on seed physiological and biochemical behavior. Seeds displayed different desiccation tolerance thresholds, with *E. uniflora* being the most sensitive and *E. astringens* the most tolerant among the five species. The desiccation tolerance seems to be influenced by abiotic factors, such as rainfall and temperature of the collection sites, and with the species range. Regarding the endogenous content of PAs in the seeds, we noticed that *E. involucrata* and *E. pyriformis* accumulated putrescine upon desiccation, while of *E. brasiliensis* increased spermidine and *E. astringens* did not present any differences in PA content. Also, spermine did not enhance upon desiccation in none of the species, suggesting that there is no modulation of abscisic acid upon desiccation. The metabolic profile of the seeds revealed a high sucrose rate in *E. uniflora*, while fructose and glucose were the main carbohydrates in *E. Astringens*, increasing upon desiccation. A higher amino acid rate was detected from *E. uniflora* seeds than from *E. astringens*, but for both species this metabolite class was degraded upon desiccation. Finally, we were able to attenuate desiccation sensitivity of *E. astringens* seeds through previous imbibition in water and 1-BUT, which decreased desiccation time and increased the activity of antioxidant compounds upon desiccation. Our results evidence the particularities of *Eugenia*, which showed different desiccation tolerances, physiological behavior and metabolic content. Moreover, we proved that it is possible to decrease the desiccation sensitivity of *Eugenia* seeds. This work broadens the knowledge about *Eugenia* seeds and present new conservation methods to DS seeds from the Atlantic Forest.

Keywords: Conservation. Desiccation sensitivity. Metabolism. Oxidative Stress. Polyamines. Seed germination.

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LISTA DE ABREVIATURAS E SIGLAS

DS Desiccation-sensitive
DT Desiccation-tolerant
WC Water Content
DW Dry Weight
UFSC Federal University of Santa Catarina
INMET Brazilian National Institute of Meteorology
GSI Germination Speed Index
MGT Mean Germination Time
EL Electrolytic Leakage
SOD Superoxide Dismutase
CAT Catalase
APX Ascorbate Peroxidase
GR Glutathione Reductase
ROS Reactive Oxygen Species
MDA Malondialdehyde, Lipid Peroxidation
PA Polyamines
PUT Putrescine
SPD Spermidine
SPM Spermine
HPLC High-performance Liquid Chromatography
ABA Abscisic Acid
GABA γ-Amino Butyrate
TCA Tricarboxylic Acid
GC-MS Chromatography-Mass Spectrometry
FS Fresh Seeds
US Unviable Seeds
G50 Half Viability
ANOVA Analysis of Variance
PCA Principal Component Analysis
HCA Hierarchical Cluster Analysis
PhA Phosphatidic Acid
PLD Phospholipase D
NAE N-acylethanolamine
1-BUT 1-butanol
H_2O Water
CNT Control
HD Hydration-dehydration Cycles

SUMÁRIO

1 INTRODUÇÃO E JUSTIFICATIVA

O Brasil possui dois dos 25 *hotspots* de biodiversidade do mundo: o Cerrado e a Mata Atlântica (Lambais et al. 2006). A Mata Atlântica é considerada pela UNESCO um dos cinco hotspots de biodiversidade prioritários para conservação, pois é um dos ambientes mais ricos do planeta e possui um alto nível de endemismo (Burnham & Graham, 1999; Conservation International, 2015). Uma das hipóteses para o alto nível de endemismo da Mata Atlântica é sua ampla extensão latitudinal e topográfica, que resulta em uma diversidade de variações climáticas, principalmente em termos de pluviosidade (Amorim & Alves, 2012). Apesar de sua grande importância ecológica, devido ao alto índice de desmatamento, expansão da agricultura e pecuária (Mayrinck et al. 2019), atualmente restam apenas 12% de toda a cobertura original da Mata Atlântica, distribuídos em pequenos fragmentos, sendo cerca de 9% destes protegidos por lei (Tabarelli & Peres, 2002; Castilho et al. 2018). Embora a Mata Atlântica seja considerada um dos ambientes mais ameaçados, os investimentos para assegurar a conservação de sua biodiversidade continuam escassos e práticas ilegais continuam impactando negativamente o remanescente florestal, mesmo em áreas protegidas (Castilho et al. 2018; Mayrinck et al. 2019).

Nos ambientes de Mata Atlântica, a família Myrtaceae é a sexta família mais representativa de angiospermas (Amorim & Alves, 2012; Lucas & Bünger, 2015). Myrtaceae compreende 142 gêneros e 5500 espécies e é um importante componente florístico nas áreas em que ocorre em maior abundância, especialmente nas florestas da América do Sul (Vasconcelos et al., 2017). É predominante no hemisfério sul e possui gêneros de alta importância econômica, como *Eucalyptus*, *Melaleuca*, *Psidium* e *Eugenia* (Thornhill et al., 2015). No Brasil, é considerada a quarta maior família de angiospermas, com 23 gêneros e c. 1030 espécies. *Eugenia* L. é o maior gênero de Myrtaceae e um dos maiores gêneros de angiospermas, com mais de 1000 espécies descritas, sendo o Brasil considerado centro de diversidade do gênero, possuindo cerca de 378 espécies das quais 82% são endêmicas (Mazine et al., 2016; Vasconcelos et al., 2018). A maior riqueza de espécies é encontrada na Mata Atlântica, com aproximadamente 250 espécies, que são reconhecidas pela importância ecológica, cultural e econômica que possuem. Produzem frutos carnosos que alimentam aves e mamíferos, e flores brancas generalistas que fornecem pólen a uma grande variedade de espécies de abelhas (Berger et al., 2016; Mazine et al., 2016; Vasconcelos et al., 2017).

Espécies do gênero *Eugenia* são reconhecidas graças às suas flores, geralmente tetrâmeras, de cálice com os lobos livres e separados no botão floral, com hipanto não

prolongado e nem tubular, e ovários biloculares e multi-ovulados (Giaretta et al., 2019). Todavia, devido à alta variedade de espécies e da homogeneidade morfológica, *Eugenia* é considerado um grupo complexo no aspecto taxonômico (Mazine et al. 2014). Quanto às suas sementes, o embrião possui cotilédones fusionados numa massa sólida e homogênea que não apresenta diferença visível entre o eixo embrionário e os cotilédones, com alto potencial meristemático em toda sua extensão e com capacidade totipotente de germinar mesmo quando cortada em mais de uma parte (Calvi et al., 2017). Diversas espécies nativas com frutos comestíveis estão amplamente distribuídas pelo Brasil, mas apesar do alto potencial de produção para consumo *in natura* ou indústria, produção de medicamentos, arborização urbana e especialmente restauração de áreas degradadas, poucas são largamente cultivadas (Gomes et al., 2016).

Eugenia uniflora, conhecida como pitanga, é uma das espécies domesticadas do gênero que possui distribuição expressiva no Brasil. Além de possuir compostos com potencial farmacológico, é altamente apreciada pelo sabor de seus frutos, sendo comercializada no mercado nacional de diversas formas (Sobeh et al., 2019). *E. pyriformis*, conhecida popularmente por uvaia, é uma espécie arbórea nativa da Mata Atlântica, que ocorre naturalmente nas regiões Sul e Sudeste do país (Silva et al., 2018). A uvaieira mede de 5 a 15m de altura e possui grande potencial alimentício, industrial e medicinal. A espécie é utilizada em sistemas agroflorestais, produz frutos amarelados e carnosos de sabor adocicado e acidulado comestíveis, que são utilizados na produção de bebidas, geleias, vinagre, entre outros (Delgado; Barbedo, 2007; Lamarca et al., 2013; Lamarca et al., 2016). *E. involucrata*, chamada popularmente de cereja-do-mato, apesar de menos conhecida, tem recebido atenção nos últimos anos graças ao alto potencial antioxidante e anti-inflamatório que possui em seus frutos, folhas e sementes (Giardelo et al. 2020). *E. astringens*, conhecida como guapê ou guamirim, é uma espécie arbustiva ou arbórea não domesticada, endêmica da restinga e pouco estudada, que produz frutos muito apreciados por aves (Delgado & Barbedo, 2007; Côrtes et al., 2009). *E. brasiliensis*, por sua vez, conhecida como grumixama, é nativa da Mata Atlântica e ocorre desde Santa Catarina até a Bahia e também apresenta uso alimentício e medicinal. Ainda, os frutos dessas cinco espécies são atrativos para a fauna e as mesmas são recomendadas para implantação em agroflorestas bem como recuperação de áreas degradadas heterogêneas (Kohama et al., 2006, Magina et al., 2009). Algumas espécies de *Eugenia* também possuem a capacidade de sobreviver submersas temporariamente ou ainda de germinar mesmo quando altamente predadas e de gerar diversas plântulas de uma mesma semente, além de diversos

mecanismos para evitar a perda de água (Barbedo, 2018; Alonso & Barbedo, 2020; Amorim et al., 2020).

Considerando a importância ecológica e econômica de *E. uniflora*, *E. pyriformis*, *E. involucrata*, *E. brasiliensis* e *E. astringens*, e o fato da maioria das cinco espécies serem negligenciadas e subutilizadas, é evidente a demanda de estudos das mesmas. Neste sentido, a compreensão dos fatores que controlam a sensibilidade à dessecação das sementes permite contribuir para estratégias de uso destas espécies, e principalmente para a conservação e compreensão dos aspectos relacionados a regeneração delas em seus habitats, especialmente em um domínio morfoclimático tão ameaçado como a Mata Atlântica. Sementes de pitanga, uvaia, cereja-do-mato, guapê e grumixama, assim como outras espécies do gênero *Eugenia*, são dispersas com teores de água acima de 0,4 g H_2O g MS^{-1} e consideradas sensíveis a dessecação (Cripa et al., 2014). Ao estudar sementes de *E. pyriformis* após a dessecação, Andrade e Ferreira (2000) perceberam perda da viabilidade quando o grau de umidade atingiu cerca de 0,16 g H_2O g MS^{-1}. Da mesma maneira, Delgado e Barbedo (2007) observaram que a germinação de *E. uniflora*, *E. pyriformis*, *E. astringens* e *E. brasiliensis* era inviabilizada em teores entre 0,16 e 0,25 g H_2O g MS^{-1}. Hossel et al. (2017) notaram que após 30 dias de armazenamento a 5ºC e em temperatura ambiente, a emergência de plântulas de *E. pyriformis* era menor que 10%. Além disso, sementes podem sofrer com a pressão de seleção do ambiente, podendo se adaptar as exigências germinativas às condições de seu local de origem, especialmente do clima. Isto significa que as respostas que espécies vegetais exibem diferem dependendo das adversidades impostas pelo seu habitat (Lamarca et al., 2011). Portanto, a utilização limitada destas espécies deve-se à falta de informação sobre a fisiologia de suas sementes, pois apesar da sensibilidade à dessecação apontada, ainda não se sabe quais os mecanismos fisiológicos e influências ambientais que as levam a perder a viabilidade e como esta perda poderia ser evitada.

Estima-se que mais de 44% de todas as espécies arbóreas de regiões neotropicais produzam sementes sensíveis à dessecação, as quais não passam pela última fase de maturação na planta-mãe – a dessecação (Kettle et al., 2012; Umarani et al., 2015). Estas sementes são dispersas com o metabolismo completamente ativo e elevados conteúdos de água (acima de 0,4 g H_2O g MS^{-1}), o que caracteriza as sementes como totalmente hidratadas, dispondo de água do tipo 5 – livre e não ligada a macromoléculas (Vertucci, 1990). Este nível de hidratação é suficiente para que a germinação das sementes ocorra logo após sua dispersão (Delgado & Barbedo, 2007). O nível de sensibilidade está ligado a perda d'água livre do tipo 3, responsável pelo umedecimento da superfície matricial e ligação aos sítios hidrofóbicos de macromoléculas

(Vertucci, 1990; Chen et al. 2017), e esta perda geralmente ocorre ao atingir cerca de 0,2 g H_2O g MS^{-1} de conteúdo hídrico. Este limite de água é considerado o mais baixo a manter sementes sensíveis à dessecação viáveis, porém o mesmo pode ser maior dependendo do tecido, maturidade, ecotipo ou espécie (Walters, 2015).

Quando a desidratação ocorre, o citoplasma das células é gradativamente condensado e os componentes intracelulares ficam cada vez mais próximos. Estas condições são propícias para que inúmeros processos indesejáveis aconteçam (Kalemba & Ratajczak, 2018), e estes processos podem levar à perda da viabilidade de sementes. Acredita-se que existam três tipos de danos causados por tais processos, que levam à morte de sementes: o dano mecânico, que atinge estruturas celulares (vacuolização, falha na recomposição do citoesqueleto e dano permanente às membranas celulares); o induzido pelo metabolismo (aumento exacerbado de radicais livres e declínio na atividade de enzimas antioxidantes); e a desnaturação de macromoléculas (funcionamento irregular de organelas celulares) (Umarani et al. 2015). A estrutura da membrana, por exemplo, que é determinada basicamente por sua constituição lipídica, é extremamente sensível às mudanças no conteúdo de água (Chen et al., 2017). Quando hidratadas, as membranas permanecem em fase lamelar. Porém, quando a semente é dessecada, as membranas podem sofrer alterações em seu formato, tornando-se hexagonais do tipo I ou II, levando à lixiviação do conteúdo celular (Yu et al., 2015). A partir da dessecação de sementes sensíveis, enzimas fosfolipases – especialmente da família D, aumentam sua atividade e hidrolisam os fosfolipídios estruturais da membrana e os convertem em ácido fosfatídico (Yao & Xue, 1018). Em pequenas quantidades, este fosfolipídio atua como molécula sinalizadora de diversos processos, mas em excesso, causa desestabilização da membrana celular (Ruelland et al., 2015).

Além disso, a dessecação desregula o metabolismo da semente, levando à produção descontrolada de espécies reativas de oxigênio (ROS). Por sua vez, as ROS são altamente reativas e oxidam lipídeos e proteínas, e consequentemente alteram a estrutura e funcionalidades da membrana celular (Bailly, 2004; Chandra & Keshavkant, 2018; Bailly, 2019). Dentre as ROS conhecidas, o ânion superóxido (O_2^-), radical hidroxila (-OH) e o peróxido de hidrogênio (H_2O_2) são os mais potentes em causar toxicidade às células (Pammenter & Berjak, 2014). Ainda, o acúmulo de malondialdeído (MDA), produto final da peroxidação lipídica, está altamente associado com o dano na integridade da membrana, gerando deformidades e vazamentos (Parkhey et al., 2012). Assim, percebemos que existe uma sucessão de fatores que levam sementes sensíveis à dessecação a perderem sua viabilidade.

Grande parte dos dados sofridos por sementes sensíveis a dessecação é proveniente da falta de mecanismos protetores, que por sua vez estão presentes em sementes tolerantes, como a redução do metabolismo, a estabilização estrutural, o acúmulo de moléculas protetoras e a remoção de ROS (Wang et al., 2015). Sementes precisam diminuir suas taxas respiratórias como forma de preparo para o período de baixo conteúdo de água e armazenar reservas para poder germinar. A estabilização estrutural está relacionada com a deposição de proteínas insolúveis nos vacúolos e o acúmulo de reservas de amido e lipídios, além da condensação da cromatina e da interrupção da replicação e transcrição de DNA e RNA. Ainda, mudanças na organização do citoesqueleto também fará parte da proteção das células contra o colapso, juntamente com o acúmulo de proteínas *late embryogenesis abundant* (LEA) e proteínas *heatshock* (HSP) e a vitrificação do citoplasma (Berjak e Pammenter, 2013). No geral, essa preparação para tolerar a dessecação não é observada em sementes sensíveis à dessecação. Já na perspectiva oxidante, antigamente ROS era conhecido apenas pelo potencial de causar dano a moléculas, mas atualmente sua essencialidade para o processo de germinação começa a ser reconhecida. A homeostase de ROS é crítica, seguindo o conceito da "janela oxidante da germinação", que restringe a ocorrência dos eventos associados a germinação à um limite critico de ROS, onde são estabelecidos limites mínimos e máximos de ROS para que a mesma ocorra (Bailly, 2019). Assim, durante estágios iniciais da dessecação, compostos que combatem o excesso de ROS são essenciais para manter a viabilidade das sementes, visto que ROS deve ser estritamente controlado nas sementes. O ciclo glutationa-ascorbato tem papel crucial na desintoxicação de ROS usando metabólitos antioxidantes, como ascorbato, glutationa e NADPH, assim como enzimas como superóxido dismutase (SOD), ascorbato peroxidase (APX), catalase (CAT), glutationa reductase (GR), entre outras moléculas com potencial antioxidante, como poliaminas, tocoferóis e aminoácidos (Moothoo-Padayachie et al., 2018). Os mecanismos de combate ao estresse oxidativo, por sua vez, estão presentes em sementes sensíveis à dessecação, já que estes auxiliam no combate à diversos estresses abióticos.

As poliaminas (PAs), por exemplo, são moléculas policatiônicas presentes em todas as células vivas e são essenciais para o desenvolvimento, crescimento e sobrevivência destas, sendo consideradas como uma nova classe de reguladores do crescimento vegetal e mensageiros secundários hormonais da proliferação e diferenciação celular em diversos processos (Steiner et al., 2007). As principais poliaminas são a putrescina (PUT), a espermidina (SPD) e a espermina (SPM), sendo a primeira precursora das demais. As PAs participam de processos celulares bioquímicos e fisiológicos, como regulação dos canais iônicos, manutenção da

estrutura da cromatina e das membranas celulares e modulação de enzimas (Seo et al., 2019). Ainda, diversos autores demonstraram que as PAs têm a capacidade de atenuar os danos causados por diversos estresses abióticos, como seca (Liu et al., 2016), salinidade (Parvin et al., 2014), frio (Sheteiwy et al., 2017) e dessecação (Shi et al., 2010). PAs podem agir de forma direta contra o estresse ou ainda modular outros compostos.

É certo que a sensibilidade à dessecação de sementes traz desvantagens ecológicas à diversas espécies, e elas podem ser menos resilientes às mudanças ambientais advindas das mudanças climáticas (Wyse and Dickie, 2018), e, portanto, presume-se que o risco de extinção de espécies sensíveis a dessecação seja maior (Marques et al., 2018). A tolerância à dessecação está geralmente associada a plantas que crescem em ambientes mais secos, enquanto a sensibilidade é mais comum em ambientes úmidos (Angelovici et al., 2010). Espécies que produzem sementes sensíveis habitam regiões onde o índice de pluviosidade é elevado e chuvas são regulares, e consequentemente não são necessários mecanismos de tolerância à dessecação (Marques et al., 2018). Por outro lado, estimativas apontam que até 2100 as áreas áridas do mundo aumentem em 10% (Mayrinck et al., 2019), o que poderia levar espécies não tolerantes ou mais resistentes a este tipo de habitat a serem extintas.

Devido à importância ecológica e econômica da família Myrtaceae, além do conhecimento limitado sobre a qualidade fisiológica, armazenamento e germinação de sementes de *Eugenia* em relação aos limites de sensibilidade à dessecação, este trabalho foi desenvolvido visando contribuir com o manejo e conservação de espécies deste gênero. Nossas hipóteses são de que sementes de *E. uniflora, E. involucrata, E. pyriformis, E. brasiliensis* e *E. astringens* (Figura 1) possuem níveis de tolerância à dessecação distintos, os quais estão relacionados com as condições climáticas, o local de origem das sementes e a distribuição das espécies, à atividade antioxidante e à assinatura metabólica. Além disso, acreditamos que é possível diminuir a sensibilidade a dessecação de sementes de *Eugenia* através da aplicação de inibidores de ácido fosfatídico. As informações contidas nesta tese são de extrema importância para o conhecimento sobre espécies sensíveis à dessecação de *Eugenia* e poderão servir de base para estudos futuros dentro do tema da conservação de sementes.

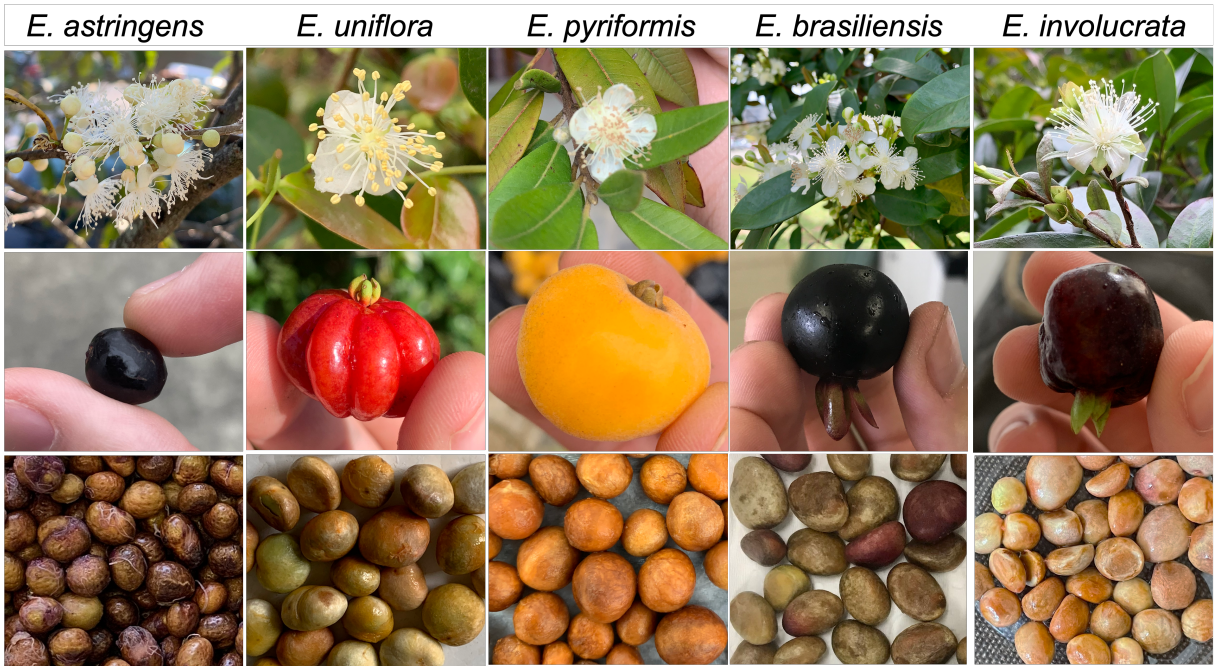

Figura 1. Morfologia das flores, frutos e sementes do gênero *Eugenia* estudadas.

2 QUESTÕES NORTEADORAS

Cada um dos quatro capítulos desta tese possui uma questão científica norteadora alinhada aos objetivos específicos. Aqui, estas questões são apresentadas em ordem crescente com relação ao número de espécies estudadas:

- A distribuição das espécies e as condições meteorológicas do ambiente no qual as sementes de *Eugenia* são coletadas influenciam os limiares de tolerância à dessecação das sementes?

- Existe relação entre o limite de tolerância à dessecação das sementes de *Eugenia* e o perfil de poliaminas endógenas e atividade de enzimas antioxidantes?

- Como é caracterizado e qual o comportamento do perfil metabólico de sementes de *E. uniflora* e *E. astringens* quando são submetidas à dessecação?

- É possível atenuar a sensibilidade à dessecação de sementes de *E. astringens* a partir da aplicação de inibidores de ácido fosfatídico?

3 OBJETIVOS

OBJETIVO GERAL

Avaliar o comportamento fisiológico e metabólico de sementes de espécies do gênero *Eugenia* submetidas a dessecação, visando ampliar o conhecimento sobre as espécies e estabelecer protocolos que possam servir de base para futuros estudos de conservação e o manejo de espécies tropicais nativas.

OBJETIVOS ESPECÍFICOS

- Avaliar a germinação e vigor de sementes de *E. uniflora*, *E. pyriformis*, *E. involucrata*, *E. brasiliensis* e *E. astringens* submetidas à dessecação e testar se o limite de tolerância e o conteúdo de água estão relacionadas às condições climáticas do local de coleta das sementes;

- Determinar o perfil e a dinâmica de PAs endógenas e da atividade antioxidante de sementes de *E. pyriformis*, *E. involucrata*, *E. brasiliensis* e *E. astringens* durante a dessecação e associá-los com a viabilidade e vigor das sementes;

- Avaliar comparativamente o perfil metabólico de sementes de *E. uniflora* e *E. astringens* e associar as mudanças metabólicas aos limiares de tolerância à dessecação de cada espécie;

- Estudar o efeito de inibidores de ácido fosfatídico (1-butanol e N-aciletanolamina) no comportamento germinativo, na qualidade fisiológica, na atividade antioxidante e no conteúdo endógeno de PAs de sementes de *E. astringens* submetidas à dessecação.

HIPÓTESES DE TRABALHO

Desenvolvemos hipóteses baseadas no que poderia ser esperado dentro de cada um dos capítulos, atreladas aos objetivos específicos dos mesmos:

- Sementes de *E. uniflora, E. pyriformis, E. involucrata, E. brasiliensis* e *E. astringens* apresentam diferentes níveis de sensibilidade à dessecação, os quais estão relacionados com as condições meteorológicas dos ambientes de origem das sementes;

- A sensibilidade à dessecação das sementes de *E. involucrata, E. pyriformis, E. brasiliensis* e *E. astringens* estão relacionadas ao comportamento de poliaminas e enzimas antioxidantes, que aumentam durante a dessecação para manter a viabilidade parcial das sementes;

- Perfis de metabólitos distintos levam a diferenças no comportamento das sementes de *E. astringens* e *E. uniflora* durante a dessecação em sementes, fazendo com que *E. astringens* seja menos sensível do que *E. uniflora*;

- É possível atenuar os efeitos da dessecação em sementes de *E. astringens* a partir da aplicação de inibidores de ácido fosfatídico.

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5 CAPÍTULO I

Este capítulo foi publicado no periódico “Journal of Plant Research”.

What affects the desiccation tolerance threshold of Brazilian *Eugenia* (Myrtaceae) seeds?

What affects the desiccation tolerance threshold of Brazilian *Eugenia* (Myrtaceae) seeds?

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ABSTRACT

Desiccation sensitive (DS) seeds are shed at high water contents (WC) and metabolically active, but WC thresholds vary broadly among species even in the same genus. *Eugenia* is an important ecological genus that has high occurrence in several Brazilian morphoclimatic domains. In this study, we assessed seed desiccation tolerance of five *Eugenia* species collected in specific meteorological conditions. We reported the species geographical ranges and verified the rainfall and temperature of species sites in the year prior to seed collection. We also assessed initial WC, seed germination and vigor and seedling growth upon desiccation. *Eugenia uniflora* was the widest spread among the five species, while *E. astringens* was the most restricted. In this specific study, widespread species showed a higher WC threshold than restricted species. In the same way, the WC of fresh seeds was not correlated to the desiccation tolerance threshold. Seed desiccation tolerance was species dependent and correlated with the environmental status of seed collection sites. Wetter and warmer conditions were correlated to the *E. uniflora* higher DS threshold. Low rainfall and temperature corresponded to a lower desiccation sensitivity of *E. astringens* seeds. Seeds of the five species lost half viability between 0.44 to 0.25 g H_2O g DW^{-1} and after 65 to 270 h of desiccation. Our results indicate that abiotic factors impact plant populations during the seed production season and can drive seed desiccation tolerance threshold and physiological behavior. These results should be taken into account in ex-situ plant conservation programs and tropical species management.

Keywords: Climate change, Germination, Provenance, Recalcitrant, Seed conservation, Wild species

INTRODUCTION

One of the main evolutionary steps in plant life was the ability to survive on dry land, which includes seed development (Smolikova et al. 2020). However, seeds by themselves still have a wide water content (WC) threshold window and can be classified as desiccation tolerant (DT) or desiccation sensitive (DS) seeds (Walters et al. 2013). It is estimated that ca. 50% of the studied neotropical species produce DS seeds (Tweddle et al. 2003; Wyse and Dickie 2017). Moreover, 36% of the critically endangered plant species worldwide produce DS seeds (Wyse et al. 2018). These seeds are shed at high WCs (> 0.4 g H_2O g DW^{-1}) and remain metabolically active after dispersal (Umarani et al. 2015). Consequently, DS seeds are short-lived, do not form persistent soil seed banks and tend to germinate soon after shedding (Angelovici et al. 2010; Subbiah et al. 2019). WC threshold for DS seeds is about 0.2 g H_2O g DW^{-1}, but there is a broad variation above this value depending on the species, population or ecotype (Walters 2015). The DS trait evolved multiple times in different lineages from a DT ancestral state, and the modification of just one of the involved genes could cause a reversal to the DS state (Chen et al. 2018; Marques et al. 2018).

Even though the DS trait represents a survival strategy at specific habitats and biogeographical contexts (Subbiah et al. 2019), desiccation sensitivity represents a major constraint for growers, seed companies, and germplasm repositories of tropical species. Seeds of many major tropical crops are DS (e.g., cocoa, coconut, coffee, rubber tree), and cannot be stored in conventional seed banks (Dussert et al. 2018). Besides, species producing DS seeds may be less resilient to the water shortage predicted under climate change scenarios (Wyse and Dickie 2018). Therefore, one of the biggest challenges currently faced is to understand the response of plant species to land dryness and climate change and to establish suitable conservation strategies (Vincent et al. 2020).

Brazil is home of one of the main biodiversity hotspots, the Atlantic Rainforest (Mittermeier et al. 1997; Myers et al. 2000). It is also the diversity center of the tropical Myrtaceae genus *Eugenia* (Lucas and Bünger 2015), with 31% of endemic species (Thornhill et al. 2015). Due to the white generalist flowers and fleshy berries produced, *Eugenia* species supply pollen and serve as food to a variety of animal species, with ecological, cultural and economic value (Mazine et al. 2016; Vasconcelos et al. 2017). Some *Eugenia* species share the interesting ability of producing new seedlings through successive germinations (Alonso and Barbedo 2020). If one root is developed under undesired conditions and ends up wilting, a new germination begins (Amorim et al. 2020 and authors therein).

Despite of the relevance of the genus *Eugenia* in tropical forests, it is still unclear how species distributions are associated with seed physiology and desiccation tolerance, as well as germination and seedling establishment. Wyse and Dickie (2018) developed a methodological approach to predict the probability of a species to produce DS seeds, using statistical models based on species' traits and habitat such as rainfall and temperature. Previously, using taxonomy-based models these authors predicted that the order Myrtales holds more than 25% of species with DS seeds (Wyse and Dickie 2017). In Myrtaceae, around 85% were found to be DS-seeded species (Mayrinck et al. 2019). Lamarca et al. (2011) compared the thermal requirements for the seed germination of four *Eugenia* species (*E. brasiliensis*, *E. involucrata*, *E. pyriformis*, and *E. uniflora*) and found out that the environment of the mother plant might strongly impact seed physiological strategy. Lamarca et al. (2013) also reported a relationship between rainfall and temperature in *E. pyriformis* seed maturation and vigor. Despite that, it remains to be elucidated how deep provenance relates with the seed WC threshold window and geographical distribution of tropical species, including *Eugenia*. In this context, our study reported the geographical ranges and habitats of *E. uniflora*, *E. involucrata*, *E. pyriformis*, *E. brasiliensis* and *E. astringens* in Brazil, as well as the meteorological conditions experienced

by the plant populations from which we collected seeds, throughout the year prior to seed collection. Then, we verified the desiccation tolerance of the seeds, to identify the WC thresholds that permit seeds to remain viable and to connect them to the meteorological conditions of each species collection site. We specifically aim to verify if the rainfall and temperature at the seed collection site affect the seed WC and desiccation tolerance. Our hypothesis is that the provenance will shape the seed desiccation tolerance degree of a species. Ultimately, we seek to increase information on seed physiology, which can be useful for the conservation and management of genetic resources of tropical plants.

MATERIAL AND METHODS

Distribution and morphoclimatic domains

We investigated species distribution, the morphoclimatic domains and ecosystem vegetations of the sites where each species occurs in Brazil through the data provided online by Flora do Brasil (2020) and the speciesLink network (https://specieslink.net), which combines the data of several herbariums. We extracted the occurrence points available in the speciesLink database for each species and plotted them into maps designed using the software QGis (2022). We separated the species into generalist and specialist based on the criteria: the species distribution range and the number of morphoclimatic domains covered by each, in order to objectively evaluate the predictions concerning their seed WC thresholds according to description suggested by Denelle et al. (2020).

Plant material

The experiments were carried out from November 2018 to May 2021 in the Plant Physiology Lab at the Federal University of Santa Catarina (UFSC). Ripe fruits of *E.*

brasiliensis, *E. astringens* (synonym *E. umbelliflora*) and *E. uniflora* were harvested from populations of 10 individuals at Florianopolis-SC (27°36'14.0"S 48°31'17.9"W) at sea level in 2018, 2019 and 2021, respectively, while *E. pyriformis* and *E. involucrata* were harvested in 2019 at Urupema-SC at elevation 1330 m above sea level (28°01'26.9"S 49°52'16.0"W) and Sao Paulo-SP at elevation 760 m above sea level (23°38'30.7"S 46°37'14.2"O), respectively. Fruits of each species were collected directly from the ground in one day, carried to the laboratory subsequently and stored at 5-8 ºC for a maximum of seven days before the beginning of the experiments (Barbedo et al. 1998).

Meteorological data

The meteorological data for the collection sites of each species in this study were obtained from the Brazilian National Institute of Meteorology (INMET 2021). We focused on five variables: accumulated annual rainfall (mm), monthly rainfall (mm), mean annual temperature (ºC), minimum monthly temperature (ºC), and maximum monthly temperature (ºC).

Seed initial WC and desiccation

Seeds from mature fruits were freshly removed from the pulp. After, they were rinsed carefully with distilled water until all the seed residue were removed and then dried superficially with paper, before conducting desiccation procedures. Initial WC was determined by the oven method at 105 ± 2 ºC for 24 h using 4 replicates of 25 freshly harvested seeds (Brazil 2013). WC was expressed in dry basis (g H_2O g DW^{-1}) using the formula (WC) = [initial fresh weight (FW) - final dry weight (DW)]/DW) (Hong and Ellis 1996)". For seed desiccation dynamics, seeds were immediately seat on grids in hermetically sealed plastic boxes containing silica gel at room temperature (24 ± 3 ºC). To evaluate the desiccation on silica, seed samples were

weighted every 3 h in the first 24 h; then every 6 h for the next 48 h and every 12 h afterwards. The selected WC for germination and electrolytical leakage tests were 0.44, 0.33, 0.25, 0.17 and 0.12 g H_2O g DW^{-1}. Silica gel was replaced every 12 h during the first two days and every 24 h subsequently.

Germination tests

Germination tests were carried out according to seed availability. Four replicates of 30 seeds for *E. involucrata*, four replicates of 25 seeds for *E. brasiliensis*, three replicates of 20 seeds for *E. astringens* and E. *pyriformis*, and three replicates of 15 seeds for *E. uniflora* were used in each of the 6 WC (five desiccation levels and control with no desiccation) treatments. Seeds were sterilized for 10 min in 1% bleach and rinsed three times in distilled water. Seeds at 0.12 g H_2O g DW^{-1} were set over a thin water blade in a plastic box for 12 h without direct contact to gain seed moisture from the humid atmosphere, before seeds were set in direct contact with liquid water. This is recommended to avoid imbibition injury since 0.12 g H_2O g DW^{-1} seeds were extremely dried (Hong and Ellis 1996). Seeds were placed in Germitest roll papers moistened with distilled water and incubated in germination chambers at 25 ± 1 ºC and 12h photoperiod (Barbedo et al. 1998). Roll papers were moistened as required. Germination tests were carried out for 12 weeks and seeds were assessed every two days. Seeds were considered as germinated when they presented 2 mm of radicle protrusion. At the end of the test, germination rate (%) and vigor, based on the germination speed index (GSI) (Maguire 1962) and germination mean time (MGT) (Edmond and Drapala 1958), were analyzed. GSI was calculated as the sum of the ratio between the number of germinated seeds per day and the respective day of observation: $GSI = G_1/N_1 + G_2/N_2 + G_3/N_3 + \ldots + G_n/N_n$, where G_1, G_2, G_3, …, G_n = number of germinated seeds on the day of observation, and N_1, N_2, N_3, …, N_n = the number of days elapsed from sowing (Maguire 1962). MGT was calculated as the sum of the

multiplication between the number of germinated seeds per day and the respective day of observation, divided by the total number of germinated seeds: MGT = $(N_1G_1+N_2G_2+\ldots+N_nG_n)$ / $(G_1+G_2+\ldots+G_n)$, where G_1, G_2, G_3, …, G_n = number of germinated seeds on the day of observation, and N_1, N_2, N_3, …, N_n = the number of days elapsed from sowing (Edmond and Drapala 1958). At the end of the germination test, we picked 20 seedlings (when available) from each treatment to measure seedling growth. Seedlings were randomly taken from different replicates without looking to impede seedling selection (Nakagawa 1999).

Electrolytic leakage (EL)

Three replicates of 15 seeds of each desiccation treatment were submerged in 50 ml of distilled water for 12 h (Marcos-Filho et al. 1987). Afterwards, the electrolytic leakage was measured using a conductometer mCA-150P MS Tecnopon. Final leakage was expressed in fold change. The fold changes were calculated using the average amount of EL ($\mu S\ cm^{-1}\ g^{-1}$) with the formula (A2 – A1) / A1, where A2 is the total amount of EL at the indicated WC point and A1 is the total amount of EL in fresh seeds.

Statistical analysis

Experiments were carried out in a completely randomized design with six WC levels. Germination, GSI and EL data were subjected to homoscedasticity (Bartlett test) and normality (Shapiro-Wilk test) assessments. One-way ANOVA was performed and the means were compared by Tukey's test ($p \leq 0.05$). Data were expressed as the means ± SD of the replicates. Statistical analysis was performed in R core team (2021).

RESULTS

Species distribution

The five species of *Eugenia* differ in their geographical ranges (Fig. 1), occurring in up to four morphoclimatic and phytogeographic domains and up to 12 ecosystem vegetations (Table 1). *E. uniflora* covers the largest area in Brazil, mainly concentrated in the South, Southeast and Northeast, and is essentially present in three morphoclimatic domains (Atlantic Rainforest, Central Brazilian Savanna and Pampa), with a few individuals in the Amazon Forest. The species occurs in eight ecosystem vegetations (Ombrophyllous Forest, Mixed Ombrophyllous Forest, Coastal Sand Forest, Cerrado, Seasonally Semideciduous Forest, Seasonal Evergreen Forest, Gallery Forest and Anthropic area), covering the entire Brazilian coastal zone (Fig. 1). Hence, we considered *E. uniflora* as generalist. The second generalist and most widely distributed species is *E. involucrata*, which is mostly found in the Central Brazilian Savanna, in the Pampa, and in the Atlantic Rainforest, in Southern Brazil. Moreover, *E. involucrata* is present in the widest variation of ecosystem vegetations, totaling nine (Ombrophyllous Forest, Mixed Ombrophyllous Forest, Cerrado, Seasonally Deciduous Forest, Seasonally Semideciduous Forest, High Altitude Grassland, Highland Rocky Field and Rock Outcrop Vegetation). *E. pyriformis* is abundantly present in Southern and Southeastern Brazil in the Atlantic Rainforest and at lesser frequencies in the Central Brazilian Savanna, which also gives the species the generalist status. *E. pyriformis* occurs in three different ecosystem vegetations (Mixed Ombrophyllous Forest, Cerrado and Seasonally Semideciduous Forest). Conversely, we considered *E. brasiliensis* a specialist species, since it only occurs in the Atlantic Rainforest, in the South and Southeast and in small fractions of Northeastern of Brazil, in four different ecosystem vegetations (Ombrophyllous Forest, Mixed Ombrophyllous Forest, Coastal Sand Forest and Seasonally Semideciduous Forest). *E. astringens* is also a specialist and the most limited of the five species: although it can be found from the South to Northeast, it is predominantly restricted to the Atlantic Rainforest in the Coastal area of Brazil, and is

exclusive of two different ecosystem vegetations (Ombrophyllous Forest and Coastal Sand Forest).

Table 1 Habitats where the *Eugenia* species studied in this work can be found. Morphoclimatic domains: AM. Amazon Forest; AR. Atlantic Rainforest; CBS. Central Brazilian Savanna; P. Pampa. Ecosystem Vegetations: 1. Ombrophyllous Forest (Tropical Rain Forest); 2. Mixed Ombrophyllous Forest; 3. Coastal Sand Forest (Restinga); 4. Cerrado; 5. Seasonally Deciduous Forest; 6. Seasonally Semideciduous Forest; 7. Seasonal Evergreen Forest; 8. Riverine and/or Gallery Forest; 9. High Altitude Grassland; 10. Highland Rocky Field; 11. Rock Outcrop Vegetation; 12. Anthropic area. (Data source: Flora do Brasil 2020).

Species	*E. uniflora*	*E. involucrata*	*E. pyriformis*	*E. brasiliensis*	*E. astringens*
Morphoclimatic domains	AM, AR, CBS, P	AR, CBS, P	AR, CBS	AR	AR
Ecosystem vegetations	1,2,3,4, 6,7,8,12	1,2,4,5, 6,9,10,11	2,4,6	1,2,3,6	1,3

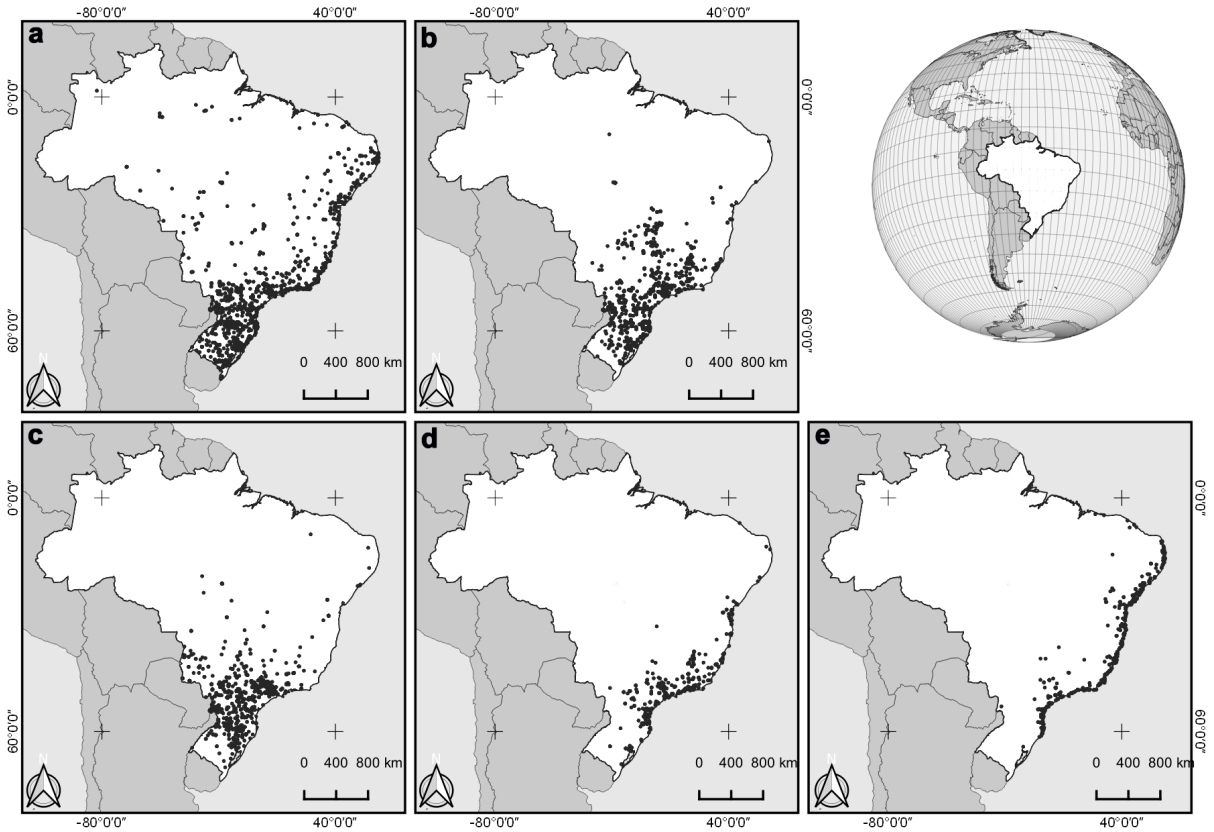

Fig. 1 Geographical distribution of the five *Eugenia* species in Brazil. Maps were designed using the data points available in the speciesLink database. a. *E. uniflora*. b. *E. involucrata*. c. *E. pyriformis*. d. *E. brasiliensis*. e. *E. astringens*

Meteorological status during seed maturation up to dispersal

Seeds of the five *Eugenia* species were dispersed at distinct seasons of the year and specific meteorological conditions (Fig. 2). *E. uniflora* seeds shed in the beginning of the Brazilian Summer, when monthly temperatures ranged from 22 to 28 ºC and the accumulated monthly rainfall was 391 mm at the collection site (Fig. 2a). *E. involucrata* seeds shed in the beginning of the Brazilian Spring, when temperatures ranged from 18 to 27 ºC and accumulated monthly rainfall was approximately 111 mm (Fig. 2b). *E. pyriformis* seeds shed in the beginning of the Brazilian Autumn, at the collection site with the lowest temperatures, ranging from 13 to 19 ºC and accumulated monthly rainfall of 147 mm (Fig. 2c). *E. brasiliensis* seeds shed in the end of the Brazilian Spring, at the collection site with the highest temperatures and second highest accumulated monthly rainfall at dispersal season, ranging from 24 to 32 ºC and 236 mm, respectively (Fig. 2d). *E. astringens* was the only species that dispersed seeds in the beginning of the Brazilian Winter when it was mostly dry at the collection site, with only 5 mm of monthly accumulated rainfall and temperatures ranging from 14 to 23 ºC (Fig. 2e). Annual data is shown in Fig. 2f, where we observe that the *E. pyriformis* plant population experienced the highest overall precipitation (1773 mm) and the lowest mean temperature (15 ºC), followed by *E. uniflora* (1687 mm and 21 ºC) and *E. involucrata* (1578 mm and 22 ºC). *E. brasiliensis* presented the lowest precipitation (1360 mm), and mean temperature of 22 ºC. *E. astringens* faced the second lowest precipitation (1390 mm) and the highest mean temperature (23 ºC).

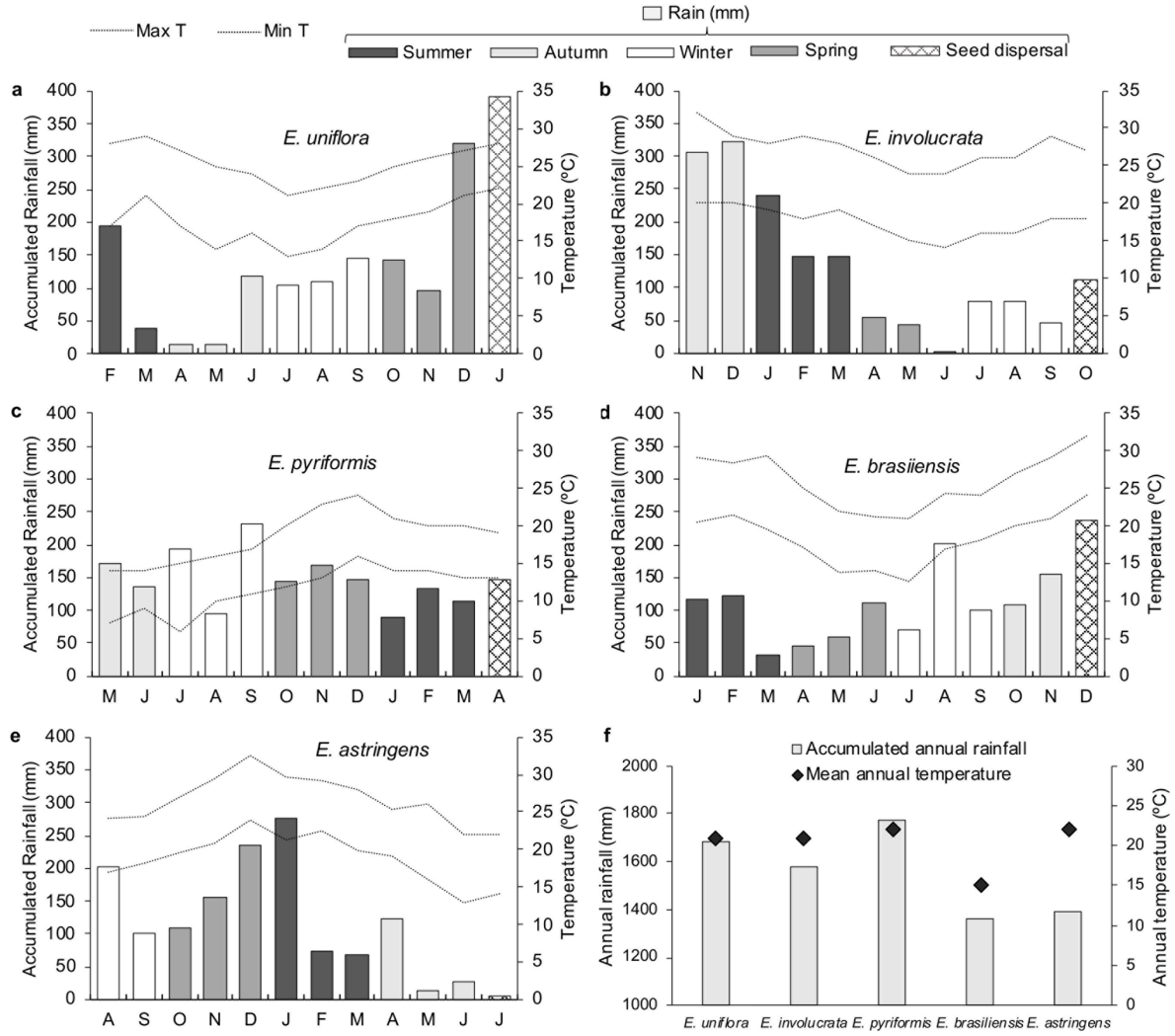

Fig. 2 Abiotic conditions of the sites where seeds of each *Eugenia* species were produced. **a-e** Accumulated monthly rainfall (mm) and monthly minimum and maximum temperatures (ºC) of a year before seed shedding. Different bar colors indicate different seasons of the year, while the plaid bar shows the month that seeds were harvested. Winter: white; Autumn: light grey; Spring: dark grey; Summer: black. **f.** Comparison of the accumulated annual rainfall (mm) and mean annual temperature (ºC)

Seed physiological behavior upon desiccation

Seeds of all five species were shed with similar WCs (ca. 1 g H_2O g DW^{-1}), except for *E. involucrata* seeds, which presented higher WCs (1.4 g H_2O g DW^{-1}). Despite of the similar initial seed WCs among the species, we observed a variation in the number of hours of desiccation for each species to reach 0.12 g H_2O g DW^{-1}. *E. pyriformis*, *E. brasiliensis*, *E.*

involucrata took around 140, 145 and 170 h, respectively, followed by *E. astringens* (275 h) and *E. uniflora*, taking nearly 700 hours (Fig. 3a).

Fresh seeds of all five species exhibited high seed germination rates, but different WC thresholds in relation to seed viability (Fig. 3c). Based on our results, *E. uniflora* seems to be the most DS of the five species. Although 67% of seeds germinated at 0.44 g H_2O g DW^{-1} (>170 h of desiccation), below this point the maximum germination achieved was 22% at 0.33 g H_2O g DW^{-1}, after 240 h of desiccation. Thus, we considered 0.44 g H_2O g DW^{-1} the WC threshold for *E. uniflora*. We observed ~50% germination for *E. involucrata* and *E. pyriformis* with a WC threshold of 0.33 g H_2O g DW^{-1}, after 72 and 65 h of desiccation, respectively. Only 50% of *E. brasiliensis* seeds were able to germinate after 65 h of desiccation, with 0.25 g H_2O g DW^{-1}. *Eugenia astringens* also maintained half of the germination rate at 0.25 g H_2O g DW^{-1}, but took 95 h of desiccation. This species still presented 35% germination at 0.17 g H_2O g DW^{-1}, after 135 h of desiccation.

The fall of 50% in the germination rates was accompanied by a drop in seed vigor, expressed as GSI (Fig. 3b, Table S1). Fresh seeds of *E. uniflora* germinated faster (1.05) in comparison to seeds desiccated to the WC threshold (0.44 g H_2O g DW^{-1}), which after 170 h presented a reduction of 80% in the GSI (0.23). *Eugenia involucrata* and *E. pyriformis* presented the same WC threshold (0.33 g H_2O g DW^{-1}), with distinct GSI. *Eugenia involucrata* fresh seeds presented the highest GSI, which dropped from 1.57 to 0.34 at the WC threshold (72 h of desiccation). *Eugenia pyriformis* fresh seeds presented the lowest GSI (0.4) and it dropped by half (0.21) after 65 h of desiccation. *Eugenia brasiliensis* and *E. astringens* also shared the same WC threshold (0.25 g H_2O g DW^{-1}) and were closely related in seed vigor. As desiccation was implied over *E. brasiliensis* seeds, GSI decreased from 0.78 in fresh seeds to 0.35 after 65 h of desiccation. After 95 h of desiccation, we observed a GSI decrease from 0.83 in *E. astringens* fresh seeds to 0.31 at the WC threshold (Fig. 3b).

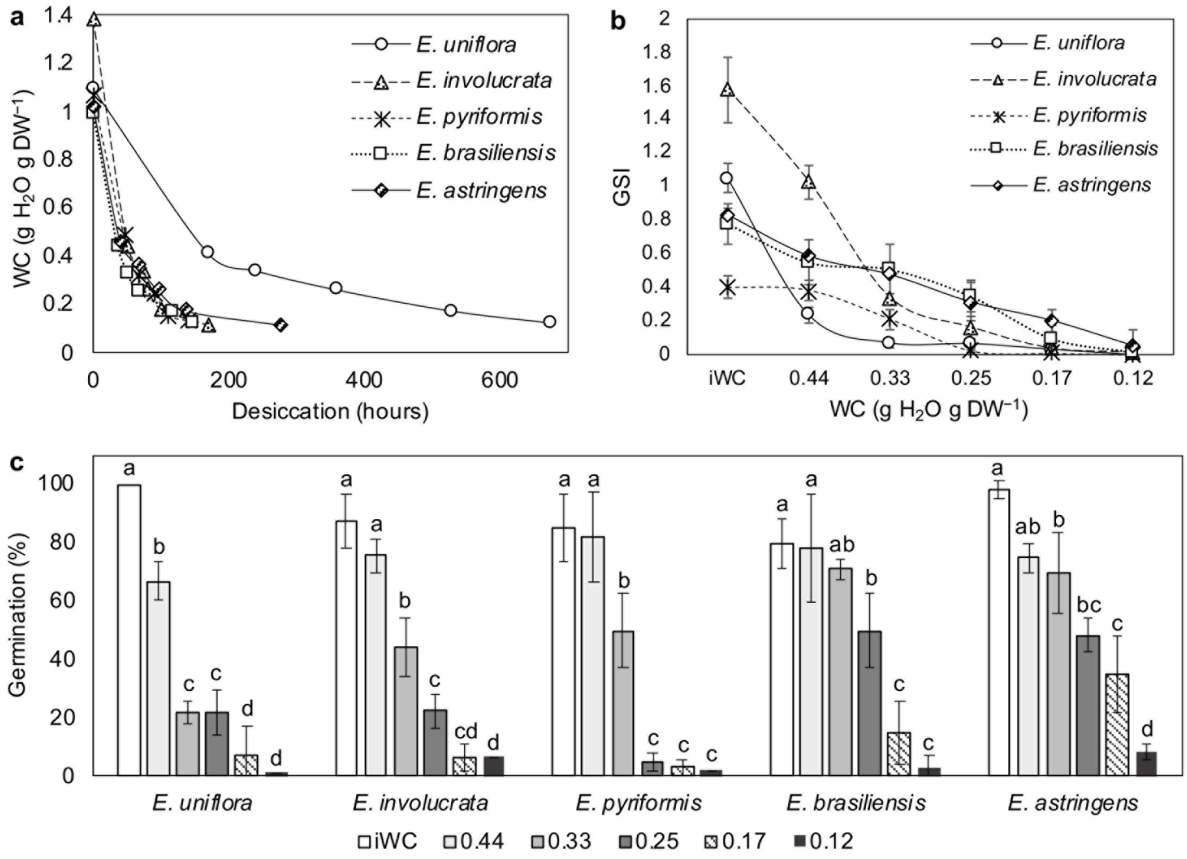

Fig. 3 Desiccation effects on seeds of *Eugenia* species. **a** Water content (WC) (g H_2O g DW^{-1}) and seed desiccation speed. **b** Germination speed index (GSI). **c** Germination rate (%). Seeds were desiccated to 0.44, 0.33, 0.25, 0.17 and 0.12 g H_2O g DW^{-1}. Germination was assessed for 12 weeks. iWC: initial WC. Values are mean of replicates and vertical bars represent ± SD. Columns within the same species that have different letters are significantly different by the Tukey test at 5% probability

In order to identify the possible injuries caused by desiccation, we examined the EL and observed significant increases for the five species, but not necessarily at the WC threshold (Fig. 4). In comparison to fresh seeds, *E. uniflora*, *E. pyriformis* and *E. brasiliensis* presented significant rises of 1.63, 2.16 and 1.9-fold at the WC threshold and continued rising progressively. *Eugenia involucrata* seeds showed significant increases of 1.54 and 2.49-fold only at 0.44 and 0.12 g H_2O g DW^{-1}, respectively. *Eugenia astringens* rose by 1.41-fold at 0.44 g H_2O g DW^{-1} and remained stable until its WC threshold, increasing progressively thereafter (Fig. 4).

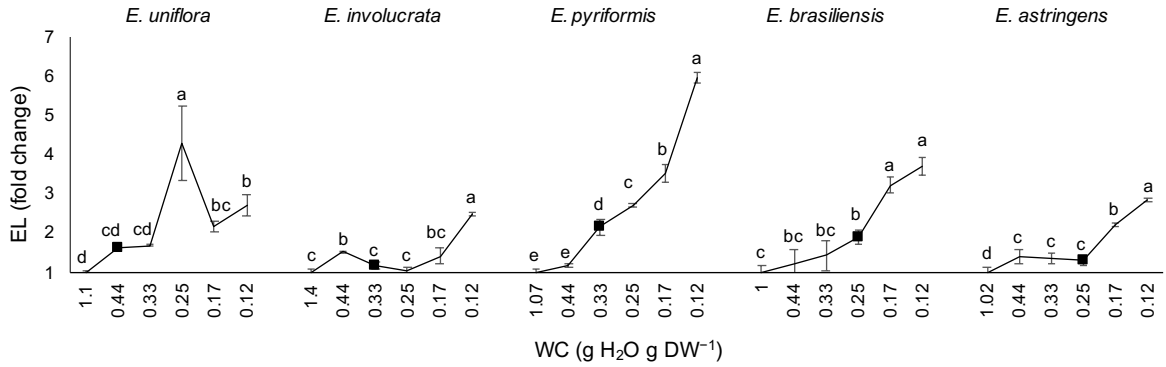

Fig. 4 Changes in electrolytical leakage (EL) of *Eugenia* species throughout desiccation. EL was measured after 12 h of seed imbibition in distilled water. Black squares represent the water content (WC g H_2O g DW^{-1}) at which half or more seeds germinated. The fold changes were calculated using the average amount of EL with the formula (A2 – A1) / A1, where A2 is the total amount of indicated EL at the indicated WC point and A1 is the total amount of EL in fresh seeds. Values are mean of replicates and vertical bars represent ± SD. Points within the same species that have different letters are significantly different by the Tukey test at 5% probability

After the end of germination tests, we measured the seedlings originated from the germinated seeds and verified the MGT (Fig. 5, Table S2). Root size was primarily disturbed by desiccation, but it was always longer than shoots for all species, especially in desiccated seeds. When no desiccation was applied, seedling total size of *E. uniflora* and *E. involucrata* were the longest, reaching c. 18 cm (Fig. 5a, b) and a MGT of 14 and 17 days (Fig. 5f), respectively. Next, *E. brasiliensis* and *E. astringens* seedlings presented 9.5 and 8.5 cm (Fig. 5d, e), and a MGT of 25 and 33 days (Fig. 5f), respectively. *Eugenia pyriformis* presented the shortest seedlings (6 cm), as observed in Fig. 5c, and the highest MGT, 47 days (Fig. 5f). Seeds desiccated to the respective WC thresholds gave rise to shorter seedlings for all species, except *E. involucrata*. *Eugenia uniflora* seedling size decreased to 14 cm when desiccated to the WC threshold (0.44 g H_2O g DW^{-1}), a 22% decrease (Fig. 5a), and MGT increased to 51 days, more than three times longer (Fig. 5f). Next, *E. involucrata* seedling size presented 15 cm at the threshold (0.33 g H_2O g DW^{-1}) (Fig. 5b), and the MGT doubled (Fig. 5f). *Eugenia pyriformis* seedling size was slightly favored by desiccation to 0.44 g H_2O g DW^{-1}. On the other hand, it

was extremely affected by desiccation to the WC threshold (0.33 g H_2O g DW^{-1}), at which shoots were barely present and root size decreased by more than a half (~2.5 cm) (Fig. 5c). However, MGT remained statistically unchanged (Fig. 5f). *Eugenia brasiliensis* and *E. astringens* shared the same WC threshold (0.25 g H_2O g DW^{-1}) and also reduced seedling total size proportionally at the WC threshold to 6 and 5.5 cm, respectively, a decrease of ~35% (Fig. 5d, e). MGT also increased for both species, with *E. brasiliensis* taking 5 days longer (38 days), and *E. astringens* taking twice the number of days (50 days) (Fig. 5f) at the WC threshold.

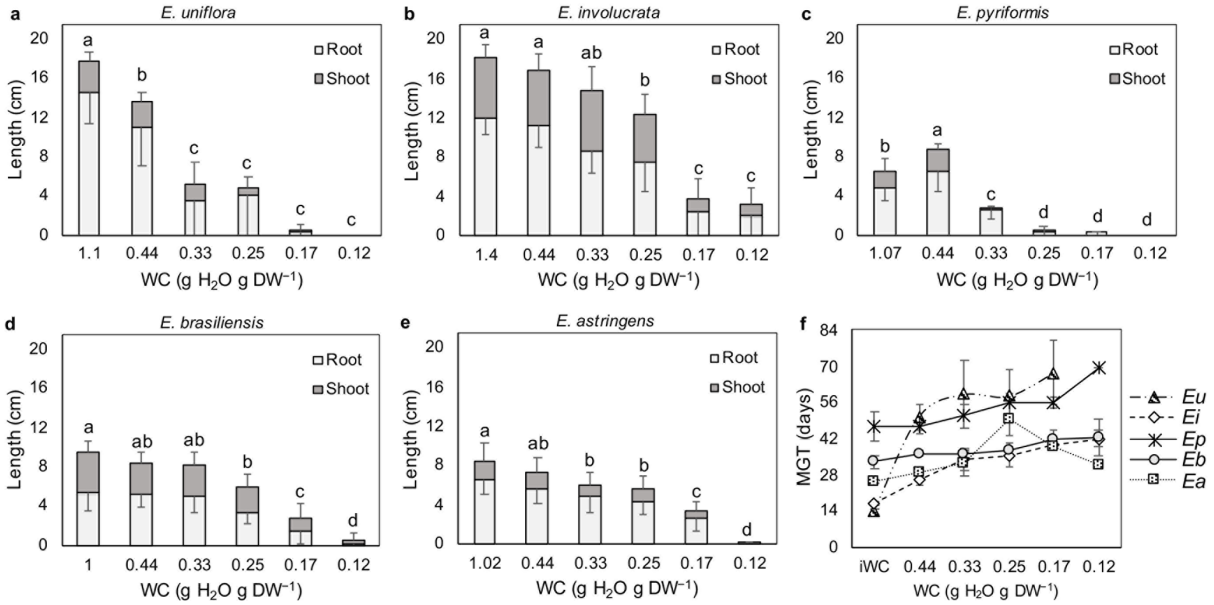

Fig. 5 a-e Seedling size (cm) of five native Brazilian *Eugenia* spp. after 12 weeks of seed sowing. Light bars represent the root length and dark bars represent the shoot length. Columns having different letters are significantly different by the Tukey test at 5% probability. **f** Mean germination time (MGT, days) of *Eugenia* seeds. Seeds were subjected to desiccation at different WC levels. Values are mean of replicates and vertical bars represent ± SD

DISCUSSION

The five *Eugenia* species occurred in the Atlantic Rainforest, but some were also present in the Amazon Forest, Brazilian Central Savannah and Pampa (Table 1). In general, the number of DS-seeded species decrease with a drying climate (Tweddle et al. 2003). On the other hand,

stable ecosystems with predictable rainy seasons have a high presence of DS-seeded species, and they eventually became specialists in this type of habitat (Garwood 1989; Vázquez-Yanes and Orozco-Segovia 1993). Specialist species are more restricted in their distribution to a narrow range of habitat conditions, while generalist species can thrive in diverse environments (Denelle et al. 2020; Marinsek et al. 2015). Therefore, *E. uniflora*, *E. involucrata* and *E. pyriformis* fit as generalists, since they were present in four, three and two morphoclimatic domains, respectively, while *E. brasiliensis* and *E. astringens* occupied only one and were then considered as specialists. It is important to highlight that the generalist and specialist dichotomy is usually used in environmental contexts, which could also fluctuate, especially in the face of climate change (Wallis De Vries 2014). Our study demonstrated that specialist species displayed lower WC thresholds than generalist species (Fig. 3). Specialists might show consistent stress-tolerance characteristics and outperform generalists in their habitats (Denelle et al. 2020). However, this division may not be maintained under different meteorological conditions, since temperature and rainfall may have a great impact on the desiccation tolerance degree of seeds (Lamarca et al. 2016). Moreover, as environmental factors will affect both generalists and specialists, each species needs a different strategy to prosper in a determined environment (Büchi and Vuilleumier 2014). Therefore, we evaluated environmental factors such as rainfall and temperature, in order to connect at least three aspects in each *Eugenia* species: geographical occurrence (Fig. 1), meteorological status (Fig. 2) and seed desiccation tolerance (Fig. 3).

Eugenia uniflora is the most widespread generalist species (Fig. 1) and disperses seeds at warm temperatures and the highest rainfall period (Fig. 2a). *F*resh seeds of *E. uniflora* presented the second highest GSI (Fig. 3b), and in lab conditions, seeds took the longest time to lose water (Fig. 3a). In this case, the dispersers would be able carry seeds through longer distances without losing viability in virtue of desiccation. The strategy of *E. uniflora* seeds to

germinate fast (Figs. 3b, 5f) and retain water for longer periods (Fig. 3a) than other *Eugenia* species in this study might be associated with the larger geographical occurrence conquered by the species (Fig. 1). This slow desiccation might be caused by the high sucrose content, which is the main reserve carbohydrate in *E. uniflora* seeds and is known to accumulate upon desiccation and stabilize membranes and the cytoplasm (Mello et al. 2010). *Eugenia uniflora* occurs in several environments and plays a role as food source for the fauna (Salgueiro et al. 2004). The species also participates in the maintenance of the shrubby coastal vegetation due to the ability to survive disturbed sites (Almeida et al. 2012; Eguiluz et al. 2017), which endorses the generalist status of *E. uniflora*. On the other hand, the desiccation speed of *E. uniflora* seeds might be associated with desiccation tolerance reduction (Fig. 4c), since after 170 h the seed WC threshold (0.44 g H_2O g DW^{-1}) allowed only 65% germination. Different results were obtained by Delgado and Barbedo (2007), which found *E. uniflora* seeds to take in average the same drying time to reach ~0.44 g H_2O g DW^{-1} as *E. involucrata*, *E. pyriformis*, *E. brasiliensis* and *E. astringens* (288 h). However, the authors collected seeds at a different location, which should explain the difference with our results. Under desiccation, it is already known that the DS seed metabolic system begins to break down, and slow development or even germination failure may be expected (Umarani et al. 2015). As *E. uniflora* seeds desiccated slowly and the aqueous-based metabolism of DS seeds continues to be active upon desiccation, it may have induced a failure of the antioxidant system and uncontrolled activity of free radicals (Viana et al. 2020). These also make sense with our results, since seeds of *E. uniflora* show a remarkable EL rise (Fig. 4), drop in germination and GSI (Fig. 3c, b) as well as an arrested seedling development (Fig. 5a) and increased MGT (Fig. 5f) under desiccation, which was also previously reported for damaged seeds (Vitis et al. 2020).

Eugenia involucrata seeds were dispersed at similar maximum temperatures as *E. uniflora*, but at lower minimum temperatures and rainfall regimes (Fig. 2 b), and was also

included in the generalist category. However, *E. involucrata* seeds showed a higher desiccation tolerance than *E. uniflora* seeds, but took only 72 h to reach its WC threshold of 0.33 g H_2O g DW^{-1} and germinate 50% (Fig. 3a). Seeds that present faster desiccation might show higher tolerance due to a lower accumulation of toxic compounds, generated by the degenerative metabolism (Walters 2015). Also, in a previous report Delgado and Barbedo (2007) found similar WC thresholds (c. 0.33 g H_2O g DW^{-1}) for *E. involucrata* seeds. Interestingly, fresh seeds of *E. involucrata* presented the highest GSI (1.57) in comparison to the other *Eugenia* species in this work. On the other hand, in *E. involucrata* seeds, GSI decreased by 80% (0.32) (Fig. 3b) and MGT doubled (34 days) (Fig. 5f) in seeds desiccated to 0.33 g H_2O g DW^{-1}. Also, smaller seedlings were also observed below the WC threshold for this species (Fig. 5b). According to Silvertown and Charlesworth (2009), species with low vigor can be less competitive and reduce the rates of population growth in stable ecosystems. Considering these and the vigor reduction of *E. involucrata* seeds under desiccation observed in our work, changes in rainfall due to climate change might become a concern for the regeneration of this species. Despite of the seed vigor reduction, the EL rates of *E. involucrata* seeds were low in comparison to the other species, but with significant increase after seeds were desiccated to 0.12 g H_2O g DW^{-1} (Fig. 4). Therefore, it is reasonable to consider that in *E. involucrata* seeds, the solute leakage may not be the main cause of seed death. This was also mentioned by Chappell et al. (2015) which stated that EL amplification may represent a reflex of viability loss or a post-mortem event rather than the main cause of DS seed death.

Even though desiccation behavior of *E. pyriformis* seeds (65 h of desiccation and ~50% of germination at 0.33 g H_2O g DW^{-1}) was closely related to seeds of *E. involucrata*, the GSI of 0.4 (Fig. 3b), the MGT of 47 days and seedling development were inferior than the other four species (Fig. 5f, c). These results were consistently related to the colder meteorological conditions at the collection site of *E. pyriformis* seeds (Fig. 2c). Lamarca et al. (2013) collected

seeds of *E. pyriformis* shed from the end of the Brazilian Spring to beginning of the Brazilian Summer from several distinct sites, where maximum temperatures reached ca. 30 ºC and compared the seed vigor. These authors observed that the GSI were up to three times higher in comparison to the seeds of our study, which were shed at the end of Autumn when temperatures ranged from 13 to 19 ºC (Fig. 2c). In nature, seeds dispersed in Autumn experience short days and cool conditions soon after dispersal, while seeds dispersed in Spring experience the contrary (Donohue 2005). The low GSI, high MGT and slow seedling development of *E. pyriformis* may be used by the species as a strategy to prevent overwinter seedling mortality. From an evolutionary perspective, plants have developed a number of mechanisms to interpret signals from their surrounding dynamic environment and use them to adjust their life processes (Vaishak et al. 2019), including seed development, maturation and shed. Our study and the results of Lamarca et al. (2013) for *E. pyriformis* seeds can be correlated to the ability of generalist plant species to use seed physiological strategies to cope with adverse conditions and thus occur in distinct morphoclimatic domains. It was already reported that seed physiological ability could be used to gradually populate neighboring habitats and establish in larger areas (Büchi and Vuilleumier 2014). This would explain the wide range of this generalist species (Fig. 1). Desiccation of *E. pyriformis* seeds led to the highest EL increases (>2.16-fold) (Fig. 4), which may originate from severe ultrastructural damage suffered by these seeds upon desiccation and storage, as reported by Justo et al. (2007). Desiccation also impacted the development of *E. pyriformis* seedlings, and initially led to a slight increase of the roots at 0.44 g H_2O g DW^{-1} (Fig. 5c). Increased root length might be strategic to overcome desiccation stress by investing energy in root development. Selective pressures may favor plasticity in root growth, increasing surface to improve water uptake and promote seedling survival (Padilla et al. 2007). On the other hand, at the subsequent WC of 0.33 g H_2O g DW^{-1} the root length decreased by 50% and led to absence of shoots in *E. pyriformis* seedlings (Fig. 5c).

The three generalist species (*E. uniflora*, *E. involucrata* and *E. pyriformis*) produced seeds considered more prone to DS than the two specialists (*E. brasiliensis* and *E. astringens*) (Fig. 3c). Although generalist species can occupy several habitats, specialist species are closely related to a determined environment (Denelle et al. 2020). *E. brasiliensis* seeds resisted further desiccation and presented 50% germination at 0.25 g H_2O g DW^{-1} after 65 h of desiccation, suggesting that desiccation speed is not the only factor affecting the tolerance of DS seeds. According to Lavergne et al. (2004), in comparison with widespread species, restricted endemic plant species may exhibit more stress-tolerance traits, such as the observed higher seed desiccation tolerance of *E. brasiliensis*. Lamarca et al. (2011) verified that seeds of *Eugenia* from different species collected at the same site presented a closer performance than populations of the same species from distinct sites. This report supports the hypothesis that the environmental status strongly impacts seed physiological behavior. Interestingly, meteorological conditions at seed dispersal also differed between *E. brasiliensis* and the other species, with the second highest rainfall (236 mm) and highest range of temperatures (24-32 ºC) (Fig. 2d). This suggests that temperature and rainfall may act synergically at seed development and shed, and can influence the desiccation behavior (Barbedo, 2018). *Eugenia brasiliensis* desiccated seeds (0.25 g H_2O g DW^{-1}) presented EL rates almost two times higher than fresh seeds (1.9-fold), increased MGT by 5 days, and 35% smaller seedlings. These may be a result of mechanical damage during desiccation, which destabilizes cell membranes and cytoskeleton, and leads to high solute leakage and poor seedling development (Oliver et al. 2020).

Eugenia astringens seeds were shed in Winter, at low temperatures (14-23 ºC) and rainfall (5 mm precipitation) (Fig. 2e). The WC threshold of 0.25 g H_2O g DW^{-1} was reached after 95 h of desiccation, with ~50% of germination (Fig. 3a, c), 30 h longer in comparison to *E. brasiliensis*. The strategy of *E. astringens* to disperse seeds in Winter was also described by

Pammenter and Berjak (2000) to temperate species, which avoid the abrupt seed desiccation. *Eugenia* seeds are mainly dispersed by birds (Biffin et al. 2010), but in *E. astringens* only 30% of berries are removed from the tree, the majority are just pecked (Côrtes et al. 2009). Thus, seeds are exposed to the environment and consequently to desiccation even before dispersal. Seedlings of *E. astringens* developed slower and reached 8.5 cm, such as observed for *E. pyriformis*, which also dispersed seeds in the cool season. On the other hand, *E. astringens* seedlings decreased by 35% after desiccation (Fig. 5e, f). These two seed species showed an arrested root development under desiccation as reported before for others species (Buitink et al. 2003).

The collection sites of all *Eugenia* seeds presented annual accumulated rainfall between 1300 and 1800 mm (Fig. 2f). According to Wyse and Dickie (2018), seed desiccation sensitivity can be more strongly related to precipitation at seed development and dispersal season than annual precipitation. The contrasting seed desiccation behavior of *E. uniflora* and *E. astringens* can be easily associated with the warmer and wetter vs. cooler and drier conditions, respectively, during seed maturation and shed of these species. Also, our results shows that closely related species presented different drying times and seed desiccation tolerances, which interestingly indicates that phylogenetics does not play an exclusive role either. For instance, *E. pyriformis* and *E. brasiliensis* took 65 h to reach their distinct respective WC thresholds of 0.33 and 0.25 g H_2O g DW^{-1}. However, *E. uniflora*, a species from the same phylogenetic section as *E. brasiliensis* (Mazine et al. 2018), required almost three times longer (170h) to reach its WC threshold (0.44 g H_2O g DW^{-1}). Our study shows that all five species present WC thresholds above 0.25 g H_2O g DW^{-1}, losing mostly of their germination capacity before reaching 0.17 g H_2O g DW^{-1} (Fig. 3c). To these five *Eugenia* species, the combination of intrinsic seed features and environmental conditions synergically drove the seed desiccation tolerance and physiological behavior (Table 2). According to Barbedo (2018), it is possible that

the environmental conditions interfere on which characteristics will be displayed by seeds to enhance the chances of survivability and germination success. So, the crosstalk between environmental abiotic factors and seed physiology behavior presented in this study might indicate that specific habitat conditions may influence how seeds of different species will behave. This is alarming considering the actual climate change scenario, as well the tropical forest conservation status. From this perceptive, it is important to take into account the abiotic factors inflicted over plant populations during the seed production season to draw DS seed conservation and species management conclusions. We also suggest that future studies on genotypical and morphological features on seeds of *Eugenia* species can help to understand the WC threshold and the phenotypic behavior of DS seeds in the short and long run.

Table 2 Summary view of the main abiotic trends and physiological features regarding desiccation tolerance of the five *Eugenia* species from each specific collection. Arrows indicate higher or lower values when each parameter is compared among species.

Dispersal Season	Summer	Spring	Autumn	Summer	Winter
Species	***E. uniflora***	***E. involucrata***	***E. pyriformis***	***E. brasiliensis***	***E. astringens***
Occurrence	↑↑ Very Wide	↑ Wide	↑ Wide	↓ Restrict	↓↓ Very restrict
Temperature	↑↑ Very high	↑ High	↓↓ Very low	↑↑ Very high	↓ Low
Rainfall at Dispersal	↑↑ Very high	↓ Low	↓ Low	↑ High	↓↓ Very low
Annual precipitation	↑↑ Very high	↑↑ Very high	↑↑ Very high	↑ High	↑ High
Germination speed	↑↑ Very fast	↑↑ Very fast	↓↓ Very slow	↓ Slow	↑ Fast
WC at dispersal	↑ High	↑↑ Very high	↑ High	↑ High	↑ High
WC at half germination	↑↑ Very high	↑ High	↑ High	↓ Low	↓ Low
Desiccation speed	↓↓ Very slow	↑↑ Very fast	↑↑ Very fast	↑↑ Very fast	↑ Fast
Desiccation sensitivity	↑↑ Very high	↑ High	↑ High	↓ Low	↓↓ Very low

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Author contributions

N.S. supervised the research; G.A.G.R. performed experiments and analyzed data; D.S. assisted the experiments; G.A.G.R, D.S. and M.I.R. collected seeds; O.A.L. assisted the experiments; G.A.G.R. and N.S. wrote the article with contributions of S.A., C.J.B. and R.A.K.

Statement and Declarations

Conflict of interest Authors have no competing interests.

Ethical approval Not applicable.

Consent to participate Not applicable.

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6 CAPÍTULO II

Are polyamines and antioxidant enzymes related to the desiccation sensitivity of *Eugenia* (Myrtaceae) seeds?

Are polyamines and antioxidant enzymes related to the desiccation sensitivity of *Eugenia* (Myrtaceae) seeds?

Rodrigues G.A.G., Goeten D., Silva D., Ribeiro M.I., Barbedo C.J., Steiner N.

ABSTRACT

Eugenia is the largest genus within Myrtaceae and an important floristic component of the Atlantic Rainforest. *Eugenia* species commonly produce desiccation-sensitive (DS) seeds, however, studies about the seed physiology in this genus are still scarce. Desiccation leads to the accumulation of toxic molecules, such as reactive oxygen species (ROS), which will further cause the loss of seed viability in sensitive species. Therefore, investigating the antioxidant system response to desiccation is crucial to manage and preserve *Eugenia* DS seeds. We hypothesized that the metabolism of PAs and antioxidant enzymes are enhanced before their point of viability loss, which is related individually to each WC threshold. Our study analyzed the dynamics of polyamines, antioxidant enzymes and the accumulation of lipid peroxidation (MDA) upon seed desiccation of *E. pyriformis, E. involucrata, E. brasiliensis and E. astringens* to verify the relationship between desiccation tolerance threshold and the antioxidant constituent. We observed that the response of PAs and antioxidant enzymes was species-dependent. Overall, putrescine (PUT) content increased in *E. pyriformis* and *E. involucrata* seeds at the WC threshold, while spermidine (SPD) increased for *E. brasiliensis* seeds. Conversely, spermine (SPM) was downregulated or remained constant in all species studied. In the case of antioxidant enzymes, ascorbate peroxidase (APX) was the only enzyme to increase activity for the four species at the WC threshold, while superoxide dismutase (SOD) and catalase (CAT) only increased activity upon desiccation in *E. involucrata* and *E. astringens* seeds, and glutathione reductive (GR) was downregulated or unresponsive. MDA increased progressively upon desiccation of *E. pyriformis* and *E. brasiliensis* seeds, while in *E. involucrata* and *E. astringens* seeds the increase was only significant after viability was completely lost. We observed that although PAs and antioxidant enzymes responded to seed desiccation, no specific pattern could be determined among the species. This indicates that the loss of viability upon seed desiccation is caused by different mechanisms across different *Eugenia* species, and may be not exclusively derived from metabolism-damage accumulation. Our work highlights the relevance of studying individual wild species and brings new insights on the metabolic behavior of the four *Eugenia* species upon desiccation.

Key words: putrescine, spermidine, spermine, seed conservation, wild species, recalcitrant

INTRODUCTION

With the advance of climate change, studying seed desiccation appears as a promising avenue to understand and manage the water content (WC) threshold and drought tolerance towards the preservation of tropical plants (Priyanka et al. 2021). Seeds can survive extreme protoplasmic dehydration and still flourish upon rehydration because of their desiccation tolerance (Oliver et al. 2020). However, not all of them are able to survive dehydration, with ca. 47% of plant species from tropical and subtropical regions producing seeds that lack this ability (Wyse and Dickie 2017). *Eugenia* is a pantropical Myrtaceae genus that produces desiccation sensitive (DS) seeds and is considered one of the largest among angiosperms, comprising ca. 11000 species (Delgado and Barbedo, 2007; Vasconcelos et al., 2017). Besides the ecological importance, several *Eugenia* species possess antibacterial (*E. astringens*), antifungal (*E. brasiliensis*), antioxidant (*E. pyriformis*, *E. brasiliensis* and *E. involucrata*) and anti-inflammatory properties (Lazarini et al., 2018). Fruits are appreciated by people and consumed *in natura* or utilized in food industries (Araújo et al., 2019). *Eugenia* seeds are resistant to physical damage, and one seed can generate more than one seedling even if is fractioned (Amorim et al. 2020). On the other hand, *Eugenia* seeds are shed at high WC (>0.7 g H_2O g DW^{-1}) and the majority cannot survive desiccation below 0.17 g H_2O g DW^{-1} (Rodrigues et al. 2022). High respiratory rates, the fully active and disarranged oxidative metabolism, and low protection against reactive oxygen species (ROS) are the main events that can accelerate seed deterioration and death (Umarani et al. 2015, Moothoo-Padayachie et al., 2018). Therefore, the cultivation as well as the preservation of *Eugenia* seeds require expertise on the physiological behavior upon seed desiccation.

Polyamines (PAs) are low molecular organic cations with stimulatory roles in diverse plant metabolic processes, including seed development, germination and adaptation to environmental stresses (Mustafavi et al. 2018). The most common PAs in higher plants are the

diamine putrescine (PUT), the triamine spermidine (SPD), and the tetraamine spermine (SPM) (Liu et al., 2017). PUT is the obligate precursor of SPD and SPM, but each PA is able to be converted back to their previous form, if necessary, by the removal of one amine group (Minocha et al. 2014, Singh et al. 2018). PAs have a positive charge that allows them to bind at DNA, RNA, chromatin and proteins, by hydrogen and ionic binding, electrostatic and hydrophobic interactions, which can stabilize these molecules (Lechowska et al. 2022). PAs are also able to induce the activity of antioxidant enzymes to scavenge reactive oxygen species (ROS), directly or indirectly (Juzón et al, 2017). Understanding the effects of desiccation on the metabolism of *Eugenia* seeds is essential for maintaining seed viability and conserving seeds. Though PAs participate on the general seed metabolism, including development and germination (Lando et al. 2019, Zandoná et al. 2021), there is a lack of studies on the possible relationship between PAs and antioxidant enzymes during DS seed desiccation (Viana et al. 2020, Vieira et al. 2022). Therefore, investigating this phenomenon may generate important insights for future DS seed management, specially of *Eugenia*.

The set of antioxidant enzymes includes superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR). These enzymes have specific pathways to neutralize ROS excess and to maintain cellular homeostasis (Aguilera et al., 2015; Chandrakar et al, 2016). This is crucially important because the uncontrolled generation of ROS due to the unbalanced metabolism is responsible for the oxidation of proteins and nucleic acids, damage to cellular structures, and severe cellular disorder (Roach et al., 2008; Walters, 2015; Chandra and Keshavkant, 2018). ROS also initiate reactions with polyunsaturated fatty acids that leads to the destruction of cellular membranes, lipid peroxidation and accumulation of malondialdehyde (MDA), the final product of the lipid peroxidation (Parkhey et al. 2012; Wang et al., 2015). All of this metabolic damage accumulation is specially concerning for desiccation-sensitive (DS) seeds. With the advance of climate change, dryland areas on Earth are expected

to rise by 10% by 2100 (Mayrinck et al. 2019). Therefore, DS-seeded species may face desiccation even in their natural habitats in the near future, which could put them at challenging conditions to survive.

In this study, we investigated the dynamics of endogenous PAs and antioxidant enzymes upon desiccation of four *Eugenia* DS seeded-species: *E. pyriformis*, *E. involucrata*, *E. brasiliensis* and *E. astringens.* By comparing seeds from *Eugenia* with different WC thresholds (Rodrigues et al. 2022), we hypothesize that the metabolism of PAs and antioxidant enzymes are enhanced before their point of viability loss, independently of what their WC threshold is. Our study was developed aiming to serve as base for future studies that focus on improving seed management in the field and in seed collection through antioxidant compounds. Moreover, exploring the desiccation effects on the metabolism of *Eugenia* seeds may assist in finding techniques to attenuate their sensitivity. The results of this work bring new insights on the desiccation effects over *Eugenia* seed physiological behavior. Ultimately, we seek to increase information on the physiology of DS-seeded species, which can be useful for genetic resource conservation of tropical plants.

MATERIAL AND METHODS

Plant material

The experiments were carried out from November 2018 to May 2021. Ripe fruits of *E. brasiliensis* and *E. astringens* (synonym *E. umbelliflora*) were harvested from populations of 10 plants at Florianopolis-SC, Brazil (27°36’14.0”S 48°31’17.9”W), while *E. pyriformis* ripe fruits were harvested in Urupema-SC, Brazil (28°01’26.9”S 49°52’16.0”W) and *E. involucrata* in Sao Paulo-SP, Brazil (23°38'30.7"S 46°37'14.2"O). Fruits of each species were collected

directly from the ground in one day, carried to the laboratory subsequently and stored at 5-8 ºC for a maximum of seven days before the beginning of the experiments (Barbedo et al. 1998).

Seed desiccation and germination assays

Seeds from mature fruits were freshly removed from the pulp. After, seeds were rinsed thoroughly with distilled water until all pulp residue was removed and then dried superficially with paper before conducting the desiccation procedures. Initial WC was determined by the oven method at 105 ± 2 ºC for 24 h using 4 replicates of 25 freshly harvested seeds (Brazil 2013) and WC was expressed in dry basis (g H_2O g DW^{-1}). For seed desiccation dynamics, seeds were immediately seat on grids in hermetically sealed plastic boxes containing silica gel at room temperature (24 ± 3 ºC). Seeds were desiccated until they reached 0.44, 0.33, 0.25 and 0.12 g H_2O g DW^{-1}. To evaluate the desiccation on silica, seed samples were weighted every 3 h in the first 24 h; then every 6 h for the next 48 h and every 12 h afterwards. Silica gel was replaced every 12 h during the first two days and every 24 h subsequently.

Germination tests were carried out according to seed availability. Four replicates of 30 seeds were used for *E. involucrata*, four replicates of 25 seeds for *E. brasiliensis*, and three replicates of 20 seeds were used for *E. astringens* and E. *pyriformis* at each of the 5 WC (initial, 0.44, 0.33, 0.25 and 0.12 g H_2O g DW^{-1}). Seeds were disinfested in sodium hypochlorite (1% v/v) for 10 min and rinsed three times in distilled water. Seeds at 0.12 g H_2O g DW^{-1} were set over a thin water blade in a plastic box for 12 h without direct contact to gain seed moisture, before seeds were set in direct contact with liquid water. This is recommended to avoid imbibition injury since 0.12 g H_2O g DW^{-1} seeds were extremely dried (Hong and Ellis 1996). Seeds were placed in Germitest roll papers moistened with distilled water and incubated in germination chambers at 25±2ºC and 12h photoperiod (Barbedo et al. 1998). Roll papers were moistened as required. Germination tests were carried out for 12 weeks and seeds were assessed

every two days. Seeds were considered as germinated when they presented 2 mm of radicle protrusion. At the end of the test, germination rates (%) were recorded.

Biochemical assays

We selected four WC based on the seed viability upon desiccation to verify the dynamics of antioxidant enzymes, MDA and PAs of each species. The four WC were: initial WC (maximum viability, max), 0.44 g H_2O g DW^{-1} (viability >75%), 0.33 or 0.25 g H_2O g DW^{-1} (viability 50%), and 0.12 g H_2O g DW^{-1} (viability <10%). For the antioxidant enzymes, MDA and PAs assays, three samples (300 mg FM) originated from a mix of ten seeds of *E. involucrata*, *E. pyriformis*, *E. brasiliensis* and *E. astringens* at each of the four WC were used.

For the antioxidant enzymes assays, samples were ground with 1 mL of 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 few modifications. The homogenate was centrifuged at 15000 g for twenty minutes at 4ºC. The resulting supernatant was filtered and used for the enzyme assays. Superoxide dismutase (SOD; EC 1.15.1.1) activity was measured by monitoring the inhibition of NBT photochemical reduction at 560 nm, according to Giannopolitis and Ries (1977). Ascorbate peroxidase (APX; EC 1.11.1.11) activity was determined by following the decrease at 290 nm (extinction coefficient 2.8 mM^{-1} cm^{-1}) (Koshiba, 1993). Catalase (CAT; EC 1.11.1.6) activity was determined by following the consumption of H_2O_2 (extinction coefficient 39.4 mM^{-1} cm^{-1}) at 240 nm (Peixoto et al., 1999). Glutathione reductase (GR; EC 1.6.4.2) activity was determined by following the oxidation of NADPH at 340 nm (extinction coefficient 6.22 mM^{-1} cm^{-1}) (Bailly and Kranner, 2011). Protein content was determined according to Bradford (1976) at 595 nm, with bovine serum albumin (BSA) as standard. SOD, CAT, APX and GR activities of each extract were measured three

times, and the results correspond to the means ± SD of the values obtained with three different extracts and three measurements per extract (i.e., nine measurements). The enzyme activities and protein content were performed using a spectrophotometer Spectra-Max® 190 Microplate Reader.

MDA measurements were estimated according to Hodges et al. (1999), with few modifications. Samples were homogenized with 3 ml of 0.1% (w/v) trichloroacetic acid (TCA) and centrifuged at 12.000 rpm 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 rpm for 15 min. Content of malondialdehyde-thiobarbituric acid complex (MDA) was measured using spectrophotometer (Spectra-Max® 190 Microplate Reader) at 532 nm and corrected by subtracting the absorbance at 600 nm. Lipid peroxidation was calculated using the extinction coefficient of 157 mM^{-1} cm^{-1}.

For PAs assays, free PAs were extracted, dansylated and quantified according to Steiner et al. (2007), with few modifications. Samples were ground in 1.6 mL of 5% (v/v) perchloric acid. 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. PA contents were expressed in dry basis (µmol. g^{-1} DW).

Statistical analysis

Experiments were carried out in a completely randomized design with five WC levels. Data were subjected to homoscedasticity (Bartlett test) and normality (Shapiro-Wilk test) assessments. Germination, antioxidant enzymes, PAs and MDA data were subjected to one-way ANOVA and the means were compared by Tukey's test ($p \leq 0.05$). We also carried out a Pearson's correlation analysis to verify the possible strong relationships between WC, antioxidant enzymes, PAs and MDA. Data were expressed as the means of the replicates ± SD. Statistical analysis was performed in R core team (2021).

RESULTS

Eugenia seeds reached WC threshold between 0.33 and 0.25 g H_2O g DW^{-1}

Seeds of *E. pyriformis* were dispersed with 1.06 g H_2O g DW^{-1} and took 45, 65, 90 and 140 h to reach 0.44, 0.33, 0.25 and 0.12 g H_2O g DW^{-1}, respectively (fig. 1A). Fresh seeds of *E. pyriformis* germinated 85%, and remained over 80% at 0.44 g H_2O g DW^{-1}. At 0.33 g H_2O g DW^{-1}, germination decreased to 50%, and no germination was displayed below this WC (fig. 1A). *Eugenia involucrata* seeds presented the highest WC at dispersal (1.38 g H_2O g^{-1} DW), and took 50, 72, 81 and 168 h to reach each of the respective analyzed WC (fig. 1B). The germination rates followed the same pattern as *E. pyriformis*, with fresh seeds germinating 87%, 76% germination at 0.44 g H_2O g DW^{-1} and half germination (44%) at 0.33 g H_2O g DW^{-1}, and low germination at 0.25 and 0.12 g H_2O g DW^{-1} (22 and 6%, respectively) (fig. 1B). Seeds of *E. brasiliensis* possessed 0.99 g H_2O g DW^{-1} at dispersal and were the fastest to desiccate to 0.44, 0.33 and 0.25 g H_2O g DW^{-1}, taking 35, 50 and 65 h, respectively, and 145 h to reach 0.12 g H_2O g DW^{-1} (fig. 1C). Fresh seeds of *E. brasiliensis* presented 80% of germination, and this rate was maintained until 0.33 g H_2O g DW^{-1}. At 0.25 g H_2O g DW^{-1}, germination of *E. brasiliensis* seeds decreased to a half, and no germination was displayed by seeds at 0.12 g H_2O

g DW^{-1} (fig. 1C). Seeds of *E. astringens* were dispersed with 1.02 g H_2O g DW^{-1}, and took 40, 65 and 95 h to desiccate to 0.44, 0.33 and 0.25 g H_2O g DW^{-1}, respectively, and were the slowest to reach 0.12 g H_2O g DW^{-1}, taking more than 100 h in comparison to the other species (275 h) (fig. 1D). Seed germination of *E. astringens* reached 100% at the initial WC, and decreased to 75% at 0.44 g H_2O g DW^{-1}. Germination decreased to 48% at 0.25 g H_2O g DW^{-1}, and only 8% of *E. astringens* seeds germinated at 0.12 g H_2O g DW^{-1} (fig. 1D). Overall, seed germination was higher than 75% at 0.44 g H_2O g^{-1} DW and lower than 10% at 0.12 g H_2O g^{-1} DW for the four species (fig. 1). Based on the germination results upon desiccation, *E. pyriformis* and *E. involucrata* seeds at initial WC, 0.44, 0.33 and 0.12 g H_2O g DW^{-1}, and *E. brasiliensis* and *E. astringens* seeds at initial WC, 0.44, 0.25 and 0.12 g H_2O g DW^{-1} were utilized in the PAs, antioxidant enzymes and MDA analysis and discussion (fig. 2 and 3).

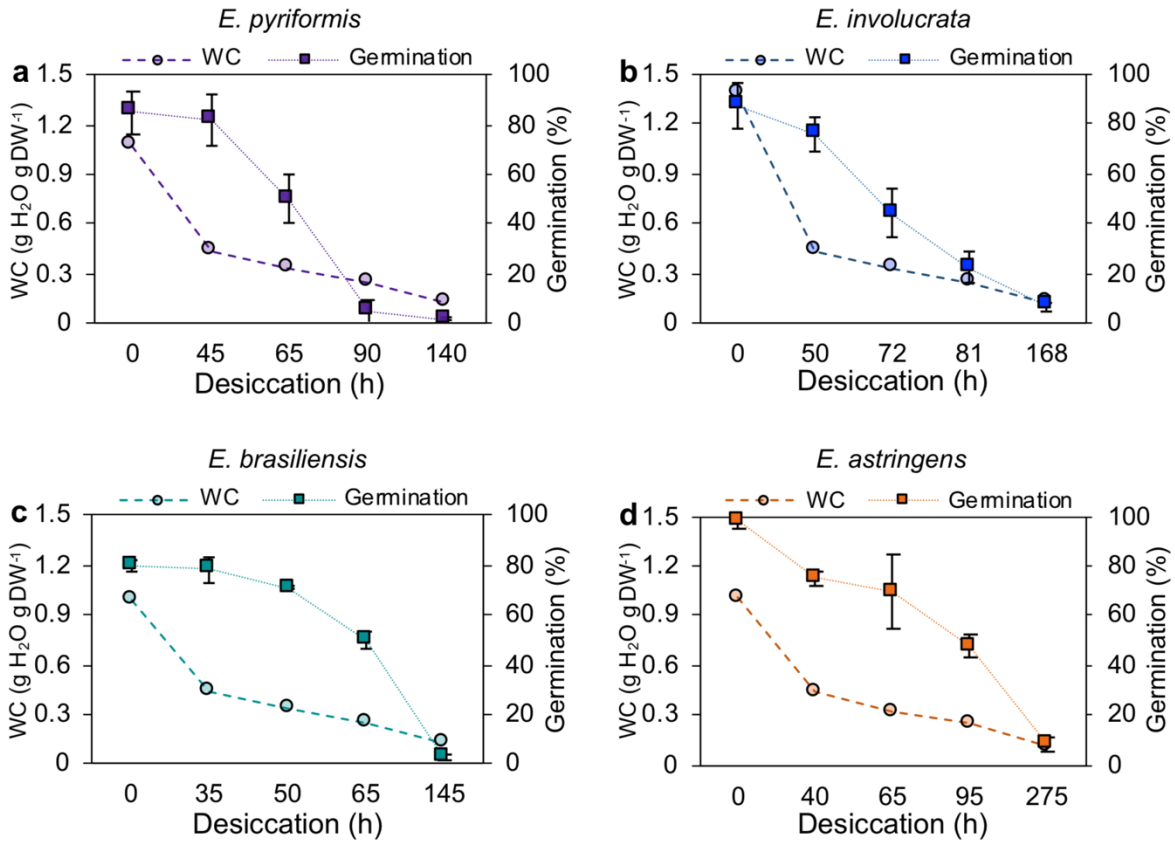

Figure 1. Water content (WC) and germination (%) of **a.** *E. pyriformis*, **b.** *E. involucrata*, **c.** *E. brasiliensis* and **d.** *E. astringens* seeds upon desiccation. WC was expressed in dry weight (g H_2O g DW^{-1}). Values are mean of replicates and vertical bars represent ± SD.

PAs exhibited distinct patterns for each species upon desiccation

The initial content and dynamics of PAs during desiccation was species dependent (fig. 2, Table S1). The greatest amounts of PAs were found in *E. pyriformis* seeds (fig. 2A). Total PAs were reduced at 0.44 g H_2O g^{-1} DW (viability >75%) but went back up at the WC threshold (0.33 g H_2O g^{-1} DW, viability 50%) (fig. 2A). In seeds of *E. astringens*, total PAs declined at 0.44 g H_2O g^{-1} DW and stabilized (fig. 2A). No significant changes were observed in the total PA content in *E. involucrata* and *E. brasiliensis* seeds during desiccation (fig. 2A).

PUT was the lowest free PA present in *E. pyriformis* fresh seeds (max viability), but it presented the highest increase (fig. 2B). As desiccation reached the WC threshold (0.33 g H_2O g^{-1} DW, viability 50%), free PUT content increased by 4.5-fold (fig. 2B). *Eugenia involucrata* seeds presented the lowest initial PUT content, but it also increased by 4.6-fold at 0.33 g H_2O g^{-1} DW (viability 50%) (fig. 2B). In *E. brasiliensis* seeds, PUT was reduced at 0.12 g H_2O g^{-1} DW, when viability dropped <10% (fig. 2B). Seeds of *E. astringens* presented a significant decrease in PUT content at 0.44 g H_2O g DW^{-1} (viability >75%) and a slightly increased at the WC threshold (0.25 g H_2O g DW^{-1}, viability 50%) (fig. 2B).

SPD was the most abundant PA in *E. pyriformis* fresh seeds, at maximum viability (fig. 2C). This PA decreased by ca. 30% at 0.44 g H_2O g^{-1} DW and returned to initial contents at the subsequent WC (fig. 2C). Fresh seeds of *E. involucrata* presented the lowest SPD content and no significant changes were observed upon desiccation (fig. 2C). *Eugenia brasiliensis* SPD content increased 2-fold in seeds desiccated to the WC threshold (0.25 g H_2O g DW^{-1}, viability 50%) and remained elevated at the subsequent WC (fig. 2C). SPD contents of *E. astringens* seeds remained constant along desiccation (fig. 2C).

SPM content of *E. involucrata* seeds significantly decreased at 0.12 g H_2O g DW^{-1} (viability <10%) in comparison to 0.44 g H_2O g DW^{-1} (viability >75%) (fig. 2D). In *E. astringens* seeds, SPM content dropped at the WC threshold (0. 25 g H_2O g DW^{-1}, viability 50%) in comparison to 0.44 g H_2O g DW^{-1} (fig. 2D). SPM content remained constant throughout desiccation in *E. pyriformis* and *E. brasiliensis* seeds (fig. 2D).

Eugenia pyriformis fresh seeds displayed the lowest PA ratio [PUT/(SPD+SPM)] among the species studied. However, PA ratio increased by 5-fold when seeds reached the WC threshold (0.33 g H_2O g^{-1} DW, viability 50%) (fig. 2E). PA ratio increased gradually in *E. involucrata* seeds during desiccation (fig. 2E). In *E. brasiliensis*, PA ratio significantly decreased at 0.12 g H_2O g DW^{-1} (viability <10%) (fig. 2E). The PA ratio of *E. astringens* was reduced when seeds were desiccated to 0.44 g H_2O g DW^{-1}, but increased again at the next WC (fig. 2E).

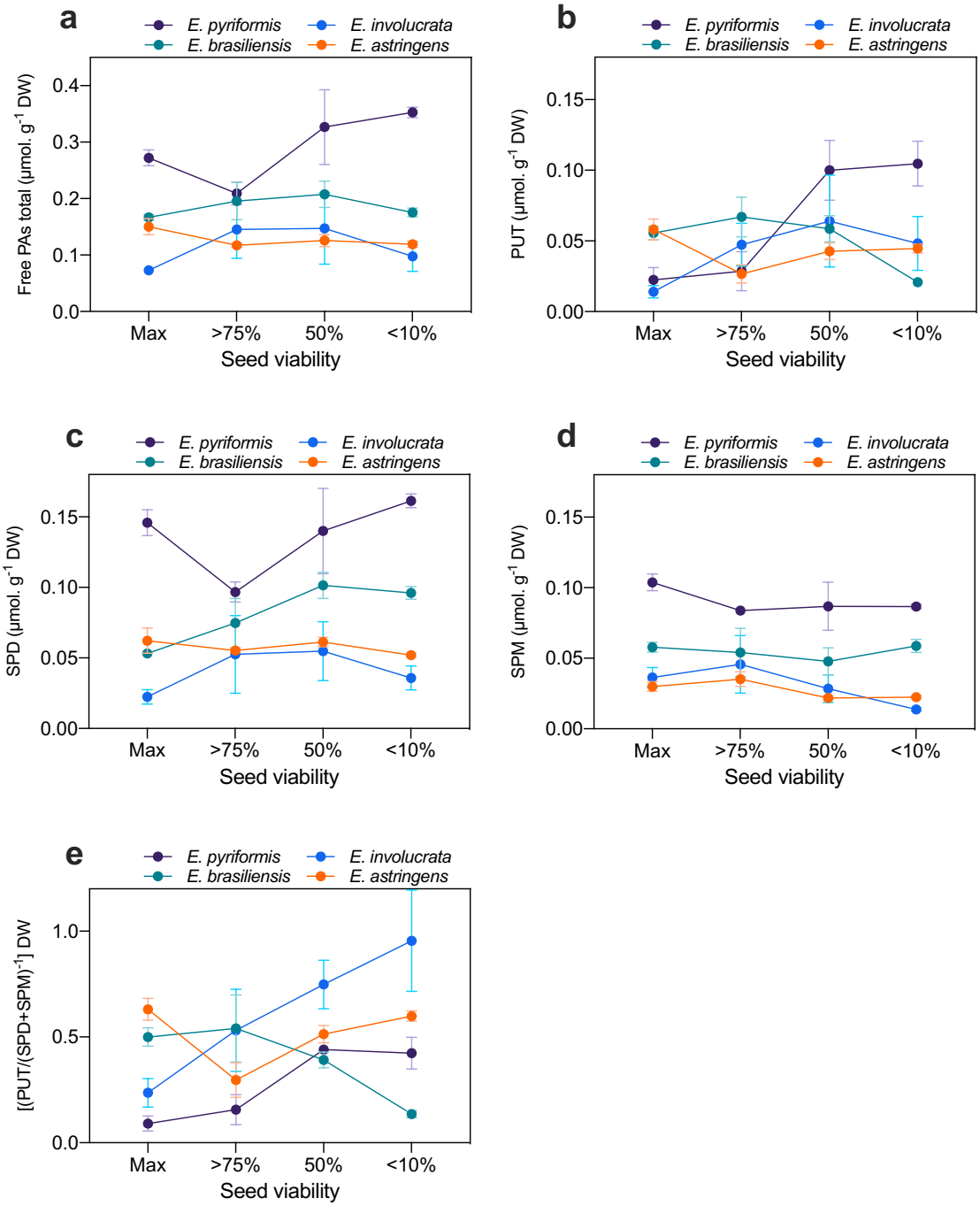

Figure 2. Polyamines (PAs) dynamics in *Eugenia* seeds during desiccation, in relation to seed viability and water content (WC). **a.** free PAs total, **b.** Putrescine (PUT), **c.** Spermidine (SPD), **d.** Spermine (SPM) and **e.** PA ratio (PUT/SPD+SPM). Maximum (Max) viability: initial WC; viability >75%: 0.44 g H_2O g DW^{-1}; viability 50%: 0.33 g H_2O g DW^{-1} (*E. pyriformis* and *E. involucrata*) or 0.25 g H_2O g DW^{-1} (*E. brasiliensis* and *E. astringens*); viability <10%: 0.12 g H_2O g DW^{-1}. Values are mean of replicates and vertical bars represent ± SD.

Eugenia seeds differ on the activation of antioxidant enzymes upon desiccation to maintain viability

The activity of antioxidant enzymes and the accumulation of MDA were species dependent as well (fig. 3, Table S2). In *E. pyriformis* seeds, SOD activity remained low until WC threshold was reached (0.33 g H_2O g DW^{-1}, 50% viability) and more than triplicated at 0.12 g H_2O g^{-1} DW (viability <10%) (fig. 3A). In *E. involucrata* seeds, SOD activity doubled at 0.44 g H_2O g DW^{-1} (viability >75%) and decreased gradually at the next WC (fig. 3A). Seeds of *E. astringens* presented the highest SOD activity among the four species. It slightly increased at the WC threshold (0.25 g H_2O g^{-1} DW, 50% viability) and increased by 5.6-fold at 0.12 g H_2O g DW^{-1} (viability <10%) in comparison to fresh seeds (fig. 3A). SOD activity fluctuated in seeds of *E. brasiliensis*, but the changes were not significantly expressive (fig. 3A).

Seeds of *E. involucrata* presented the highest activities of CAT among the studied species, which doubled at 0.44 g H_2O g DW^{-1} (viability >75%) and decreased progressively afterwards (fig. 3B). CAT activity of *E. brasiliensis* seeds significantly decreased at 0.25 g H_2O g DW^{-1} (viability 50%) and doubled at 0.12 g H_2O g DW^{-1} (viability <10%) in comparison to fresh seeds (fig. 4 B). Seeds of *E. astringens* presented a significant increase in CAT activity when seeds were desiccated to the WC threshold (0.25 g H_2O g DW^{-1}, 50% viability), and decreased to initial values at 0.12 g H_2O g DW^{-1} (viability <10%) (fig. 3B). In seeds of *E. pyriformis*, CAT activity remained low and constant in general (fig. 3B).

APX activity was higher in *E. pyriformis* and *E. involucrata* seeds in comparison with *E. brasiliensis* and *E. astringens* (fig. 3C). *Eugenia pyriformis* APX reached maximum activity at the WC threshold (g H_2O g DW^{-1}, viability 50%), and decreased at 0.12 g H_2O g DW^{-1} (viability <10%) (fig. 3C). APX increased progressively in *E. involucrata* seeds up to 7.4-fold at the WC threshold (0.33 g H_2O g DW^{-1}, viability 50%) and moderately decreased at 0.12 g H_2O g DW^{-1} (fig. 3C). In *E. brasiliensis* and *E. astringens* seeds, APX behaved similarly (fig.

3C), though the activity was more prominent in *E. astringens*. As desiccation hit the seeds, the enzyme activity primarily decreased at 0.44 g H_2O g DW^{-1} (viability >75%) but progressively increased afterwards, with the highest activity at 0.12 g H_2O g DW^{-1} (viability <10%) (fig. 3C).

GR activity decreased in *E. pyriformis* seeds desiccated to 0.44 g H_2O g DW^{-1} (viability >75%), and returned to initial values at 0.12 g H_2O g DW^{-1} (viability <10%) (fig. 3D). In *E. brasiliensis* seeds, GR activity decreased progressively and was significantly reduced at 0.12 g H_2O g DW^{-1} (fig. 3D). In *E. astringens*, GR activity increased by 7-fold at 0.12 g H_2O g DW^{-1} (viability <10%) (fig. 3D). GR remained generally constant in *E. involucrata* seeds upon desiccation (fig. 3D).

MDA content in *E. pyriformis* increased when seeds reached the WC threshold (0.33 g H_2O g DW^{-1}, viability 50%) (fig. 3E). In *E. involucrata* seeds, MDA increased up to 2.5-fold at 0.12 g H_2O g DW^{-1} (viability <10%) (fig. 3E). *Eugenia brasiliensis* seed MDA content increased progressively and exhibited the first significant rise at the WC threshold (0.25 g H_2O g DW^{-1}, viability 50%) and the highest peak at the lowest WC (0.12 g H_2O g DW^{-1}, viability <10%). MDA content in seeds of *E. astringens* doubled at 0.12 g H_2O g DW^{-1} (viability <10%) (fig. 3E).

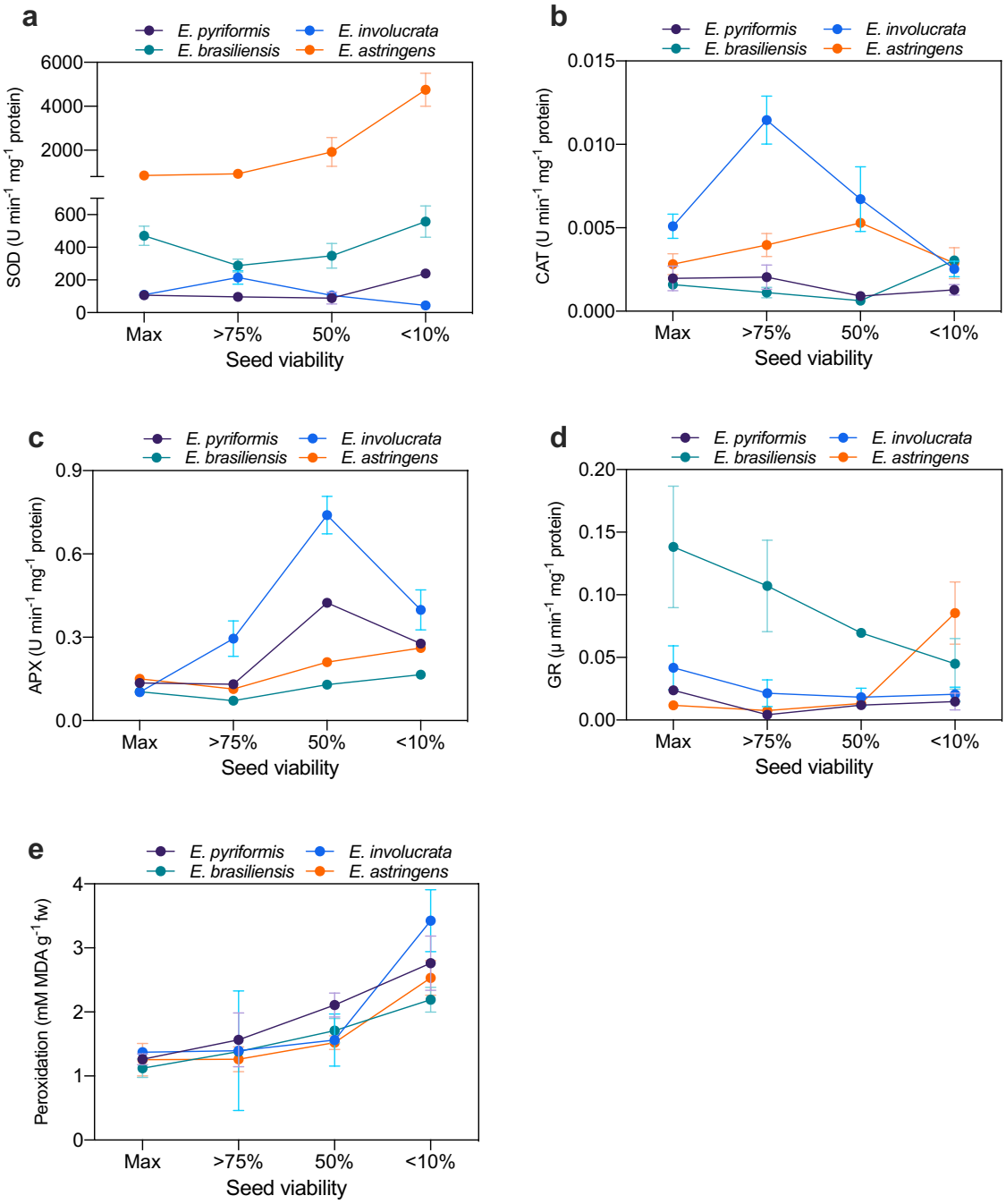

Figure 3. Enzyme activity of **a.** superoxide dismutase (SOD), **b.** catalase (CAT), **c.** ascorbate peroxidase (APX) and **d.** glutathione reductase (GR), and **e.** lipid peroxidation (MDA) of *Eugenia* seeds during desiccation in relation to seed viability and water content (WC). Max viability: initial WC; viability >75%: 0.44 g H_2O g DW^{-1}; viability 50%: 0.33 g H_2O g DW^{-1} (*E. pyriformis* and *E. involucrata*) or 0.25 g H_2O g DW^{-1} (*E. brasiliensis* and *E. astringens*); viability <10%: 0.12 g H_2O g DW^{-1}. Values are mean of replicates and vertical bars represent ± SD.

Comparison of WC, PAs and antioxidant enzymes by Pearson's correlation analysis in Eugenia seeds showed individual associations

The values of Pearson's pairwise correlation for each pair of the analyzed components were calculated and the significant relationships ($p < 0.05$) were shown in a correlation matrix (fig. 4). In *E. involucrata* seeds, we observed a positive correlation between WC and GR, and SOD and CAT, and a negative correlation between PUT and GR (fig. 4A). In the case of *E. pyriformis* seeds, one significant negative correlation was displayed between CAT and APX (fig. 4B). In seeds of *E. brasiliensis*, a negative correlation was shown GR and MDA (fig. 4C). In *E. astringens* seeds, positive correlations were observed between SOD, GR and MDA (fig. 4D).

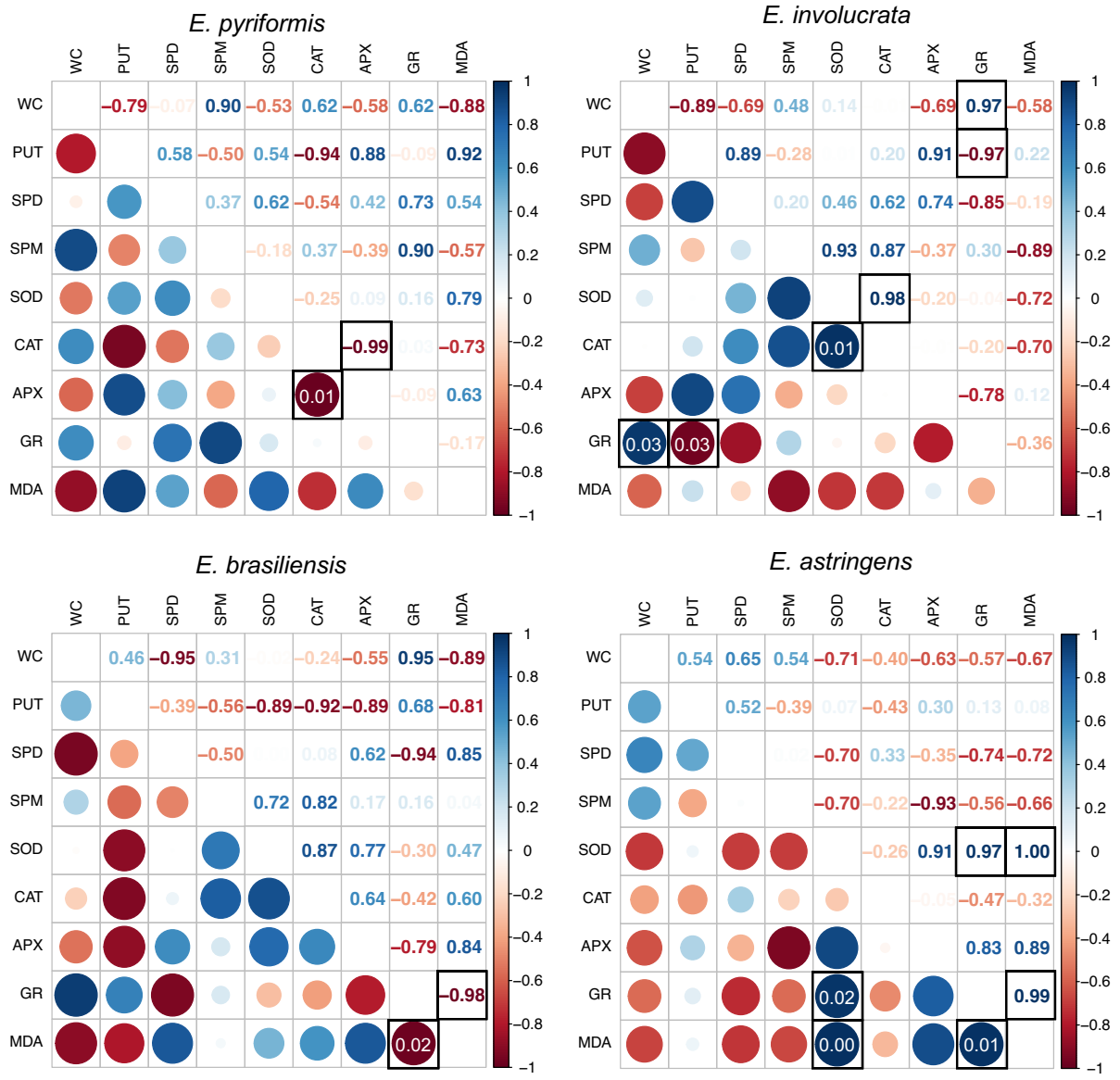

Figure 4. Correlation matrix of polyamines (Putrescine: PUT, Spermidine: SPD and Spermine: SPM), antioxidant enzymes (Superoxide dismutase: SOD, Catalase: CAT, Ascorbate peroxidase: APX and Glutathione reductase: GR) and lipid peroxidation (MDA) in relation to water content (WC) in *Eugenia* seeds. Each square indicates the Pearson's correlation coefficient of a pair of compounds. Correlation coefficients are represented by the intensity of blue or red colors in the lower triangular matrix as indicated on the color scale and by the values in the upper triangular matrix. Black squares show the significant correlations ($p<0.05$), while the white values inside show the p-value.

DISCUSSION

This is the first study to analyze the PAs content and antioxidant enzymes activity during desiccation of *Eugenia* seeds and how it correlates with seed viability. Originally, fresh seeds

of the four *Eugenia* species presented high germination rates (fig. 1). However, upon desiccation seeds of *E. pyriformis* and *E. involucrata* were slightly more sensitive (0.33 g H_2O g DW^{-1}) than *E. brasiliensis* and *E. astringens* seeds (0.25 g H_2O g DW^{-1}) (fig. 1). Since accumulation of oxidative stress is known to take place during desiccation of DS seeds (Umarani et al. 2015), we suggest that the differences in desiccation sensitivity were related to the behavior of antioxidant compounds, such as PAs (fig. 2) and antioxidant enzymes (fig. 3). Therefore, we examined the dynamics of these compounds to shed light to the possible causes of *E. involucrata*, *E. pyriformis*, *E. brasiliensis* and *E. astringens* seed desiccation intolerance and understand their physiological behavior.

The highest and lowest content of PAs were found in *E. pyriformis* and *E. involucrata* seeds, the most sensitive species in this study, respectively, while *E. brasiliensis* and *E. astringens* presented similar intermediary contents (fig. 2A). PAs are considered as a class of new plant growth regulators that take a crucial role in diverse physiological processes, cellular metabolism and stress tolerance (Arun et al., 2016; El-Tarabily et al., 2020). Endogenous PAs may vary remarkably depending on the species or developmental stage, and their dynamics during DS seed desiccation is not fully understood (Pál et al., 2015). For instance, PUT displayed a ~4.5-fold increase in the two most sensitive species (*E. pyriformis* and *E. involucrata*) at the WC threshold (0.33 g H_2O g DW^{-1}, viability 50%) (fig. 2B), suggesting a protective role for PUT in these species. Alet et al. (2011) also reported increased endogenous PUT in response to dehydration in transgenic Arabidopsis seedlings that led to an improved desiccation tolerance. PUT can rapidly translocate to bind in cell membranes and cell walls, and possibly prevent proteins from ROS oxidation (Rady and Hemida 2015). The beneficial impact of PUT might be either to PUT itself or due to its conversion to SPD and SPM (Yiu et al. 2009). In fact, a gradual rise in SPD was observed in *E. brasiliensis* seeds while PUT content remained stable, and when it dropped, SPD stopped rising (fig. 2B,C). This might be due to the

fact that SPD indirectly controls the PUT and SPM contents during abiotic stresses, since it can be converted into both PAs (Hussain et al., 2011; Li et al., 2015). Also, an exogenous application of SPD was reported to improve *Oryza sativa* seed germination under stress (Sheteiwy et al., 2017). SPD modulates surface charge, regulates membrane permeability and stability, and can bind to antioxidant enzymes to scavenge ROS (Parvin et al. 2014), which thus may be responsible for maintaining *E. brasiliensis* seeds viable at 0.25 g H_2O g DW^{-1} (viability 50%). In *E. pyriformis* seeds, SPD decreased at 0.44 g H_2O g DW^{-1}, but increased back to initial contents at the threshold (0.33 g H_2O g DW^{-1}) (fig. 2C). A similar SPD trend was observed by Vieira et al (2021) in Myrtaceae DS seeds of *Campomanesia xanthocarpa* upon desiccation, which suggests that seeds were sensing the stress and trying to respond. In the case of *E. involucrata* and *E. astringens* seeds, no differences were observed in SPD (fig. 2C), but distinct PAs are stimulated depending on the species and source of stress, so usually not all the three types are significantly involved (Liu et al., 2015). Similarly, *E. astringens* and *E. involucrata* showed a decrease in SPM, while no changes were displayed by *E. pyriformis* and *E. brasiliensis* seeds upon desiccation (fig. 2D). SPM is synthesized from SPD and it is suggested to be related to defense response against abiotic stresses and to induce the expression of ABA-responsive signaling pathway (Seifi and Shelp, 2019). Therefore, the low SPM response of *Eugenia* seeds upon desiccation might indicate a lack of ABA modulation, which is already known to quickly accumulate in desiccation-tolerant seeds (Marco et al., 2019).

In relation to antioxidant enzymes, SOD, CAT, APX and GR have specific pathways to counteract the desiccation adverse effects and keep the seed cell homeostasis (Aguilera et al., 2015). SOD is the first antioxidant enzyme to act against ROS, due to its ability to catalyze O_2^- into H_2O_2 (Mitler 2017). A boost in SOD was observed in *E. involucrata* and *E. astringens* while seeds kept viability >75% and 50%, respectively, which possibly helped to mitigate the O_2^- burst, as observed in desiccated axes of *Castanea sativa* seeds (Roach et al. 2008). The lack

of SOD response in *E. brasiliensis* and *E. pyriformis* seeds might suggest that no O_2^- accumulation took place while seeds were partially viable, as reported for *Azadirachta indica* seeds, in which a surge in O_2^- was accompanied by increased SOD (Varghese and Naithani, 2002). As SOD activity leads to rises in H_2O_2, other antioxidant enzymes take action to diminish the oxidative stress.

CAT is one of the responsible for converting H_2O_2 into water and oxygen (Bailly et al. 2004), and increased for *E. involucrata* and *E. astringens* before seed viability dropped below 50% (0.12 g H_2O g DW^{-1}, viability <10%) (fig. 3B). This is also an indication that an oxidative damage accumulation was taking place in these species during desiccation. CAT is essential for the removal and avoidance of oxidative stress-related damage and is directly proportional to the oxidative state of cells (Puac et al. 2018). The functioning of CAT also suggests an efficient antioxidant defense system in *E. involucrata* and *E. astringens*; this may be sufficient to control ROS accumulation during mild desiccation and allow partial germination (Bailly et al. 2008).

We observed APX increases for the four species at the WC threshold (fig. 3C), which suggests that this enzyme has an important role during the desiccation of *Eugenia* species. The activation of APX is one of the most effective H_2O_2 scavengers in plant cells, reducing it to water through the use of ascorbic acid as electron donor (Pukacka and Ratajczak, 2010; Shigeoka et al 2002). APX rise was also associated to increases in PUT in *E. pyriformis* and *E. involucrata* (fig. 3C), which was also observed during *Trichocline catharinensis* seed germination (Lando et al. 2019). Therefore, it is reasonable to assume that desiccation induced PUT accumulation, which then participated in the activation of APX. In the case of *E. brasiliensis* and *E. astringens* seeds, the rises in APX were smaller. Since APX depends on ascorbic acid as source of reducing power (Pandey et al., 2017), this might mean that *E. brasiliensis* and *E. astringens* seeds have lower ascorbic acid contents. GR also plays a role in the control of endogenous H_2O_2, through an oxide-reduction cycle involving glutathione and

ascorbate (Rejeb et al., 2015). However, this enzyme was reduced for all species before viability <10% (0.12 g H_2O g DW^{-1}), as shown in fig. 3D. Reduction in GR activity suggests a decrease in the levels of reduced glutathione, known to be an important component to prevent oxidative injuries (Bailly et al., 1996). MDA content of *E. pyriformis* and *E. brasiliensis* seeds increased by 67 and 50% at their WC threshold (0.33 and 0.25 g H_2O g DW^{-1}, respectively) (fig. 3E). Promotion in MDA levels is closely related to leaky or distorted integrity of membranes, and can derive from mechanical or metabolic damage (Parkhey et al., 2012). This indicates that membrane disruption took part in the viability loss of these species during desiccation. On the other hand, in seeds of *E. involucrata* and *E. astringens*, significant rises in MDA content were only observed after viability dropped below 10% (0.12 g H_2O g DW^{-1}), suggesting that MDA accumulation was not the main cause of seed death, but a consequence of antioxidant system failure. Chandra and Keshavkant (2018) reported a ROS accumulation in DS seeds of *Madhuca latifolia* upon desiccation, which further led to increases in MDA.

Overall, the generally high PUT and SPD, and low antioxidant enzymes response of *E. pyriformis* seeds and *E. brasiliensis* seeds may indicate that the viability loss may be mainly related to an accumulation of lipid peroxidation, which led to a gradual membrane destabilization upon desiccation. In a previous report (Rodrigues et al., 2022), we showed a progressive increase in electrolytic leakage for *E. pyriformis* and *E. brasiliensis*, which may be a reflex of membrane injury that was confirmed by the MDA increases observed in this study (fig. 3E). Moreover, Justo et al. (2007) showed that *E. pyriformis* seeds presented cell cytoplasmatic disruption upon desiccation and storage, which is another proof of cellular destabilization. On the other hand, in both *E. involucrata* and *E. astringens* the activity of antioxidant enzymes, especially SOD, CAT and APX rose at the WC threshold (viability 50%). This may suggest that a metabolic disturbance took place in these seeds during desiccation and led to ROS accumulation, which was mainly responsible for seed viability loss. Taken together,

our results evidence that the relationship between antioxidant enzymes and PAs during desiccation of *Eugenia* DS seeds is species-dependent. However, despite of the different degrees of seed desiccation sensitivity and PAs response, these molecules somehow responded at the WC threshold (viability 50%) in the four species, especially PUT and SPD. Furthermore, our results indicate that though desiccation was lethal for the four *Eugenia* species, *E. involucrata* and *E. astringens* possibly lost viability through antioxidant systems failure due ROS accumulation, and *E. pyriformis* and *E. brasiliensis* through membrane disruption due lipid peroxidation accumulation. Finally, the WC threshold at which only half viability of *E. pyriformis* and *E. involucrata* seeds was maintained was 0.33 g H_2O g DW^{-1}, while this threshold occurred at 0.25 g H_2O g DW^{-1} for *E. brasiliensis* and *E. astringens* seeds. The WC thresholds need to be taken into consideration during seedling production and seed conservation in seed banks.

From this perspective, finding solutions for low desiccation tolerance is imperative if we want to conserve these species. The exogenous application of PAs has been used to improve the tolerance of seeds and seedlings to dehydration (Shi et al. 2010), drought (Liu et al. 2016), and salinity (Verma et al. 2005). Perhaps, the application of PAs may be an alternative method to enhance desiccation tolerance of these seeds, but this needs further investigation. Our work brings new insights into the metabolic response of *Eugenia* DS seeds to desiccation, unveiling the antioxidant enzymes and PA dynamics upon the stress. Moreover, changes may also be related to PA signaling pathways instead of the actual endogenous content, which also needs evaluation. Therefore, future studies should focus on the application of specific PAs to verify their possible effects on desiccation sensitivity attenuation, and on their endogenous molecular signaling.

Table S1. Polyamines (PAs) dynamics in *Eugenia* seeds during desiccation, in relation to seed viability and water content (WC). **a.** free PAs total, **b.** Putrescine (PUT), **c.** Spermidine (SPD), **d.** Spermine (SPM) and **e.** PA ratio (PUT/SPD+SPM). Maximum (Max) viability: initial WC;

viability >75%: 0.44 g H_2O g DW^{-1}; viability 50%: 0.33 g H_2O g DW^{-1} (*E. pyriformis* and *E. involucrata*) or 0.25 g H_2O g DW^{-1} (*E. brasiliensis* and *E. astringens*); viability <10%: 0.12 g H_2O g DW^{-1}.

Free Polyamines (PAs) Total				
	E. involucrata	*E. pyriformis*	*E. brasiliensis*	*E. astringens*
Max	0.0728 ns	0.2721 ab	0.1665 ns	0.1502 a
>75%	0.1454 ns	0.2090 b	0.1956 ns	0.1172 b
50%	0.1472 ns	0.3267 a	0.2077 ns	0.1257 b
<10%	0.0977 ns	0.3526 a	0.1755 ns	0.119 b
Putrescine (PUT)				
	E. involucrata	*E. pyriformis*	*E. brasiliensis*	*E. astringens*
Max	0.014 b	0.0224 b	0.0555 a	0.0581 a
>75%	0.0473 ab	0.0285 b	0.0669 a	0.0266 c
50%	0.064 a	0.0999 a	0.0585 a	0.0427 b
<10%	0.0482 ab	0.1046 a	0.0208 b	0.0446 ab
Spermidine (SPD)				
	E. involucrata	*E. pyriformis*	*E. brasiliensis*	*E. astringens*
Max	0.0224 ns	0.1459 a	0.0532 c	0.0622 ns
>75%	0.0525 ns	0.0967 b	0.0747 bc	0.0553 ns
50%	0.0548 ns	0.1399 a	0.1014 a	0.0612 ns
<10%	0.0358 ns	0.1613 a	0.096 ab	0.052 ns
Spermine (SPM)				
	E. involucrata	*E. pyriformis*	*E. brasiliensis*	*E. astringens*
Max	0.0364 ab	0.1037 ns	0.0578 ns	0.0299 ab
>75%	0.0456 a	0.0837 ns	0.054 ns	0.0352 a
50%	0.0284 ab	0.0868 ns	0.0478 ns	0.0218 b
<10%	0.0137 b	0.0866 ns	0.0587 ns	0.0224 b
Polyamine Ratio (PUT/SPD+SPM)				
	E. involucrata	*E. pyriformis*	*E. brasiliensis*	*E. astringens*
Max	0.2363 c	0.0906 b	0.4999 a	0.6308 a
>75%	0.5315 bc	0.1564 b	0.54 a	0.2962 b
50%	0.7481 ab	0.4405 a	0.3913 a	0.5134 a
<10%	0.9547 a	0.4231 a	0.135 b	0.5988 a

Values are mean of replicates. Columns within the same species that have different small letters are significantly different by the Tukey test at 5% probability.

Table S2. Enzyme activity of **a.** superoxide dismutase (SOD), **b.** catalase (CAT), **c.** ascorbate peroxidase (APX) and **d.** glutathione reductase (GR), and **e.** lipid peroxidation (MDA) of *Eugenia* seeds during desiccation in relation to seed viability and water content (WC). Max viability: initial WC; viability >75%: 0.44 g H_2O g DW^{-1}; viability 50%: 0.33 g H_2O g DW^{-1} (*E. pyriformis* and *E. involucrata*) or 0.25 g H_2O g DW^{-1} (*E. brasiliensis* and *E. astringens*); viability <10%: 0.12 g H_2O g DW^{-1}.

Superoxide Dismutase (SOD)				
	E. involucrata	*E. pyriformis*	*E. brasiliensis*	*E. astringens*
Max	108.782 b	106.259 b	446.381 ^{ns}	849.024 c
>75%	215.853 a	95.93 b	267.493 ^{ns}	926.487 c
50%	105.647 b	88.72 b	434.129 ^{ns}	1610.707 b
<10%	44.344 c	240.02 a	510.890 ^{ns}	5189.945 a
Catalase (CAT)				
	E. involucrata	*E. pyriformis*	*E. brasiliensis*	*E. astringens*
Max	0.00508 bc	0.001972 ^{ns}	0.00158 b	0.00217 b
>75%	0.01145 a	0.002047 ^{ns}	0.001120 bc	0.003705 ab
50%	0.00672 b	0.000902 ^{ns}	0.00062 c	0.00553 a
<10%	0.00252 c	0.001282 ^{ns}	0.00304 a	0.00288 b
Ascorbate Peroxidase (APX)				
	E. involucrata	*E. pyriformis*	*E. brasiliensis*	*E. astringens*
Max	0.1025 c	0.1356 c	0.1036881 c	0.1505789 c
>75%	0.2952 b	0.13108 c	0.07145948 d	0.1138324 d
50%	0.7401 a	0.4238 a	0.129047 b	0.2106555 b
<10%	0.3986 b	0.22 b	0.1653351 a	0.2617223 a
Glutathione Reductase (GR)				
	E. involucrata	*E. pyriformis*	*E. brasiliensis*	*E. astringens*
Max	0.04177 ^{ns}	0.02544 a	0.06045 a	0.0068 b
>75%	0.02146 ^{ns}	0.004241 c	0.04682 ab	0.0068 b
50%	0.01831 ^{ns}	0.011883 bc	0.03041 ab	0.0058 b
<10%	0.02061 ^{ns}	0.018585 ab	0.0196 b	0.0280 a
Malondialdehyde (MDA)				
	E. involucrata	*E. pyriformis*	*E. brasiliensis*	*E. astringens*
Max	1.37 b	1.26 b	1.122 c	1.254 b
>75%	1.395 b	1.564 b	1.38 bc	1.26 b
50%	1.562 b	2.653 a	1.707 b	1.32 b
<10%	3.425 a	2.762 a	2.19 a	2.53 a

Values are mean of replicates. Columns within the same species that have different small letters are significantly different by the Tukey test at 5% probability.

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Author contributions

N.S. supervised the research; G.A.G.R. performed experiments and analyzed data; D.G. ran polyamine analysis; D.S. assisted the experiments; G.A.G.R, D.S., M.I.R. and C.J.B. collected seeds; G.A.G.R. and N.S. wrote the article.

Statement and Declarations

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7 CAPÍTULO III

Uncovering the desiccation effects on the metabolic profile of *Eugenia astringens* and *E. uniflora* (Myrtaceae) seeds

Uncovering the desiccation effects on the metabolic profile of *Eugenia astringens* **and** *E. uniflora* **(Myrtaceae) seeds**

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ABSTRACT

The desiccation tolerance trait requires an orchestration of complex processes that are in general not or insufficiently expressed in desiccation-sensitive (DS) seeds. *Eugenia astringens* and *E. uniflora* are Brazilian tropical species with poorly understood seed biology. We test the hypothesis that the WC threshold is dependent of the seed metabolic profile, with *E. astringens* activating more stress-related metabolites upon desiccation than *E. uniflora*. We evaluated the global metabolite profile of their mature seeds and the metabolic changes that took place upon desiccation. Seeds of *E. astringens* lost half viability at 0.17 g H_2O g DW^{-1}, while this occurred at 0.41 g H_2O g DW^{-1} for *E. uniflora* seeds. We identified 103 annotated metabolites, and through principal component analysis the species were separated into two groups based on the differences in their metabolic profile. Seed desiccation altered the abundance of 70 metabolites in *E. astringens* and 77 metabolites in *E. uniflora*. Upon desiccation, 66% of the 70 metabolites were downregulated in *E. astringens*, while 64% of the 77 metabolites were upregulated in *E. uniflora* seeds. We identified twenty-nine stress-related metabolites in *Eugenia* seeds. However, most of these metabolites was not ideally upregulated upon desiccation in neither of the species. Our study describes the profile of carbohydrates, amino acids, organic acids, fatty acids and others and unveils the metabolic behavior of each metabolite in *Eugenia* seeds upon desiccation. The loss of viability in *Eugenia* seeds seems to be caused by a failure in enhancing the metabolism to deal with desiccation. This study brings new insights into the fundamental processes of seed viability under desiccation, which can be useful to understand the bottlenecks of the ex-situ and in-situ seed conservation of wild tropical species.

Keywords: allantoin, amino acids, carbohydrates, fatty acids, organic acids, recalcitrant, seed bank

INTRODUCTION

Seed desiccation has been a concerning scientific subject in tropical forests in the century of climate change and forest restauration. Global plants produce seeds with a wide range of water content (WC) threshold between desiccation tolerant (DT) and desiccation sensitive (DS) seeds (Walters, 2015). However, around 47% of evergreen rainforest plant species produce DS seeds (Wyse and Dickie, 2017), and their habitats have been significantly reduced (Mayrinck et al., 2019). Brazil is home of two biodiversity hotspots and is the center of diversity of *Eugenia* (Myrtaceae), from which 82% of species are endemic (Mazine et al., 2018). This genus has been cited in the "Plants of the Future" list due to the ecological importance and economical potential, especially because of the edible fruits and the cosmetic and medicinal use (Delgado and Barbedo, 2007; Vieira et al., 2018). However, there is a lack of studies about the physiology of *Eugenia* seeds, especially due to their DS behavior. How desiccation affects seeds is a crucial topic towards food security, climate change and plant conservation (Leprince et al., 2017).

DS seeds are shed highly hydrated and remain metabolically active after dispersal (Umarani et al., 2015). In these seeds, desiccation causes a disruption of the overall metabolic system, interrupting the functioning of numerous physiological processes and causing structural destabilization (Vertucci,1990; Parkhey et al., 2012). Even the loss of small amounts of water can cause severe damage, and if WC threshold is reached (usually c. 0.2 g H_2O g DW^{-1}), viability loss is expected (Walters, 2015; Moothoo-Padayachie et al., 2018). On the other hand, DT seeds are able to resist intense water loss to levels below 0.1 g H_2O g DW^{-1} and subsequent rehydration, without accumulating lethal damage (Leprince et al., 2017). At the end of DT seed development during embryo maturation, cells are loaded with dry matter to stabilize the cellular cytoplasm (Walters, 2015). In these seeds, carbohydrates and amphiphilic compounds replace water molecules in the hydration shell of the membranes (Leprince et al., 2017; Marques et al.,

2018). Amino acids, such as proline and γ-amino butyrate (GABA), together with sugars, act as osmolytes to slow down water and maintain cell turgor (Oliver et al., 2020). In a different way, in DS seeds the amount and types of carbohydrates, organic acids, amino acids and lipids can be diverse and specific for each species or family, and most of them has not been studied yet (Aguirre et al., 2018). Moreover, in DS seeds a failure in the orchestration of the protective mechanisms occurs and the specific sugars and amino acids is generally insufficient or absent (Dekkers et al., 2015; Marques et al., 2018; Oliver et al., 2020). Some DS seeds have only small traces of lipids that might be oxidized under unfavorable conditions and lead to seed deterioration (Guimarães et al., 2020). During desiccation, the respiratory rate of DS seeds remains elevated until seed death, while in DT seeds a decrease in the tricarboxylic acid (TCA) cycle intermediates would decrease respiration and avoid oxidative damage (Caccere et al., 2013). Despite of the advances previously made, the physiological behavior of tropical seeds is distant from being fully comprehended, and the seed metabolism upon desiccation still remains as a gap to be elucidated (Angelovici et al, 2010).

Metabolomics is a powerful technique to measure the abundance of metabolites at a global level (Das et al., 2017), and the study of seed metabolism can help explaining the complex relationship between seed viability and WC threshold of tropical species. Global metabolites have been studied with the goals of understanding the changes during germination, desiccation and storage of numerous wild and cultivated species (Han et al., 2017; Yan et al., 2018; Hell et al., 2019). Metabolomics of tropical mature seeds and upon desiccation allow us to identify chemical markers that may regulate seed viability or help to predict seed desiccation sensitivity. In this study, we evaluated the global metabolite profile of mature seeds and then upon desiccation of two *Eugenia* species. These species have an interesting geographical occurrence, once *E. astringens* is restricted to the coastal Restinga vegetation, while *E. uniflora* is widespread in several Brazilian regions (Flora do Brasil, 2020; Rodrigues et al. 2022). So far,

we lack details regarding on the metabolic profile of *E. uniflora* and *E. astringes* seeds and how they are modified under desiccation conditions to keep viability. Previous studies demonstrated that both species produce DS seeds (Delgado and Barbedo 2007). In addition, Rodrigues et al. (2022) reported that the seed desiccation tolerance of *E. uniflora* is lower than *E. astringens*, which may be related to seed provenance. Our hypothesis is that the WC threshold also relies on the seed metabolic profile, with *E. astringens* possessing more stress-related metabolites which are upregulated upon desiccation in comparison to *E. uniflora*. In this sense, this study evaluated the global metabolite profile of *E. uniflora* and *E. astringens* mature seeds and their behavior upon desiccation using chromatography-mass spectrometry (GC–MS). We compared the metabolic profile of the *Eugenia* species and highlighted metabolites known by their involvement in plant stress-related responses, which might also be related to the WC threshold window between DS and DT seeds. We focus on understanding the behavior of tropical seeds and link it with their associated metabolism during desiccation.

MATERIAL AND METHODS

Plant material

The experiments were carried out from June 2019 to February 2021. Ripe fruits of *E. astringens* and *E. uniflora* were harvested from wild populations of 10 plants of each species at Florianopolis, Santa Catarina state, Brazil (27°36’14.0” S 48°31’17.9” W). After harvest, fruits were stored at 5ºC for up to three days until the beginning of the experiments.

Initial WC and desiccation

Seeds from mature fruits of *E. astringens* and *E. uniflora* were removed from the pulp, rinsed with distilled water and dried superficially with paper. To verify the WC of fresh seeds (FS), four replicates of 15 seeds were inserted in the oven at 105±2ºC for 24 h (Brazil, 2013).

WC was expressed in dry basis (g H_2O g DW^{-1}). For seed desiccation dynamics, seeds were immediately seat on grids in hermetically sealed plastic boxes containing silica gel at room temperature (24±3ºC). To evaluate the desiccation on silica, seed samples were weighted every 3 h in the first 24 h; then every 6 h for the next 48 h and every 12 h afterwards. Silica gel was replaced every 12 h at the first two days and every 24 h subsequently.

Germination assay

Three replicates of 20 seeds were used for *E. astringens* and three replicates of 15 seeds for *E. uniflora* at each of the 4 WC (fresh, 0.41, 0.17 and 0.12 g H_2O g DW^{-1}) treatments. Seeds were disinfested in sodium hypochlorite (1% v/v) for 10 min and rinsed three times in distilled water. For 0.12 g H_2O g DW^{-1}treatments, seeds were left 12 h to humidify in a moist atmosphere to raise seed moisture before seeds were set to germinate in direct contact with liquid water to avoid imbibition injury (Hong & Ellis, 1996). Seeds were placed in Germitest roll papers moistened with distilled water and incubated in germination chambers at 25±1ºC and 12h photoperiod. Roll papers were moistened as required. Germination tests were conducted for 80 days and seeds were assessed every two days. Seeds were considered as germinated when they presented 2 mm of radicle protrusion. At the end of the test, germination percentage (%) was assessed.

Tetrazolium assay

Three replicates of 10 seeds of *E. astringens* and *E. uniflora* at each of the four WC (fresh, 0.41, 0.17 and 0.12 g H_2O g DW^{-1}) were cut longitudinally, and immersed in 1% 2,3,5-triphenyltetrazolium chloride solution at 30 ºC for two hours in the dark (Brasil, 2009). This test distinguishes live and dead tissues based on the relative respiration rate (Hartmann and Kester, 1959). A positive reaction to tetrazolium is indicated by a red staining on the seed tissue.

Results were shown as images along the WC treatments and germination curves, with the most common reaction among seeds in each treatment.

Metabolite extraction

Based on the seed germination and viability upon desiccation, three independent seed samples of 300 mg at three different WC of *E. astringens* (fresh, 0.17 and 0.12 g H_2O g DW^{-1}) and *E. uniflora* (fresh, 0.41 and 0.12 g H_2O g DW^{-1}) were collected and utilized to run a metabolic profiling using the GC-MS (i.e., 18 samples in total). Ground dried samples of 5 mg were resuspended in 1 ml of frozen (-20°C) water:acetonitrile:isopropanol (2:3:3) containing Ribitol at 4 µg.ml^{-1} and extracted for 10 min at 4°C with shaking at 1500 rpm in an Eppendorf Thermomixer. Insoluble material was removed by centrifugation at 16 300 g for 10 min. In total, 100 µl were collected and 10 µl of myristic acid d27 at 30 µg ml^{-1} were added as an internal standard for retention time locking. Extracts were dried for 4 h at 35°C in a Speed-Vac and stored at -80°C.

Metabolomics by Gas Chromatography−Mass Spectrometry (GC-MS)

After the extraction, the relative metabolite contents of the 18 samples (2 spp. x 3 WC x 3 replicates) were determined by GC-MS. All steps for GC-MS analyses were carried out as previously described (Ponnaiah et al., 2019). Samples were taken out of -80°C, warmed to 15 min before opening and dried again in a Speed-Vac evaporator for 1.5 h at 35°C before adding 10 µL of 20 mg.ml^{-1} methoxyamine in pyridine to the samples. The reaction was performed for 90 min at 30°C under continuous shaking in an Eppendorf thermomixer. Here, 90 µL N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) (Regis Technologies, Morton Grove, IL, USA) were then added and the reaction continued for 30 min at 37°C. After cooling, all the samples were transferred to an Agilent vial for injection. At 4 h after derivatization, 1 µl of sample was

injected in splitless mode on an Agilent 7890B gas chromatograph coupled to an Agilent 5977A mass spectrometer. The column was an Rxi-5SilMS from Restek (30 m with 10 m Integra-Guard column). An injection in split mode with a ratio of 1:30 was systematically performed for saturated compounds quantification. Oven temperature ramp was 60°C for 1 min then 10°C min^{-1} to 325°C for 10 min. Helium constant flow was 1.1 $ml.min^{-1}$. Temperatures were the following: injector: 250°C, transfer line: 290°C, source: 230°C and quadrupole 150°C. The quadrupole mass spectrometer was switched on after a 5.90 min solvent delay time, scanning from 50 to 600 m/z. Absolute retention times were locked to the internal standard d27-myristic acid using the RTL system provided in Agilent's Masshunter software. Retention time locking reduces run-to-run retention time variation. Samples were randomized. A fatty acid methyl esters mix (C8, C9, C10, C12, C14, C16, C18, C20, C22, C24, C26, C28, C30) was injected at the beginning of analysis for external RI calibration. The Agilent Fiehn GC/MS Metabolomics RTL Library (version June 2008) was employed for metabolite identifications. Peak areas determined with the Masshunter Quantitative Analysis (Agilent Technologies, Santa Clara, CA, USA) in splitless and split 30 modes. Resulting areas were compiled into one single MS Excel file for comparison. Peak areas were normalized to Ribitol and Dry Weight. 103 annotated metabolites were identified in *E. astringens* and *E. uniflora* seeds. We classified the metabolites according to their functional class into carbohydrates, organic acids, amino acids, fatty acids and other metabolites. Metabolite contents were expressed in arbitrary units (AU g^{-1} DW) (semi-quantitative determination).

Data analysis

Statistical differences of the metabolite quantification of *E. astringens* and *E. uniflora* seeds at the three WC (fresh, 0.41 or 0.17, and 0.12 g H_2O g DW^{-1}) were calculated by two-way ANOVA. Data were expressed as the means ± SD of the replicates. Principal component

analysis (PCA) models were generated using Prism software (version 9.0.4) and RDA analysis was generated using R core team (2021) to verify the organization of samples in relation to the several studied metabolites. Hierarchical cluster analysis (HCA) was performed using the R core team (2021) package pheatmap to analyze the behavior of metabolites when seeds were desiccated.

RESULTS

Desiccation effects on seed germination

Fresh seeds of *E. astringens* and *E. uniflora* were dispersed with 1.02 and 1.12 g H_2O g DW^{-1} respectively (fig. 1A). *Eugenia astringens* seeds reached 0.41, 0.17 and 0.12 g H_2O g DW^{-1}after 52, 180 and 275 h, respectively. Seeds of *E. uniflora* took around three times more to reach 0.41 (170 h) and 0.17 g H_2O g DW^{-1} (528 h), and more than twice the time to reach 0.12 g H_2O g DW^{-1} (674 h) (fig. 1A). Fresh seeds of both species presented full germination (100%), but as seeds were desiccated to 0.41 g H_2O g DW^{-1}, *E. uniflora* seed germination decreased to 65%, while *E. astringens* maintained 75% of germination (fig. 1B). Upon further desiccation, seeds of *E. astringens* lost approximately half of its germination capacity at 0.17 g H_2O g DW^{-1} (48%), and seeds of *E. uniflora* were barely germinating (ca. 5%). At 0.12 g H_2O g DW^{-1}, only 8% of *E. astringens* seeds were able to germinate and no germination was observed in *E. uniflora* seeds (fig. 1B). In *E. astringens* treatments using fresh seeds and seeds desiccated to 0.41 and 0.17 g H_2O g DW^{-1}, most of them displayed a positive reaction to Tetrazolium (fig. 1C). In *E. uniflora* seeds, the Tetrazolium test was only positive in fresh seeds and seeds desiccated to 0.41 g H_2O g DW^{-1} (fig. 1C).

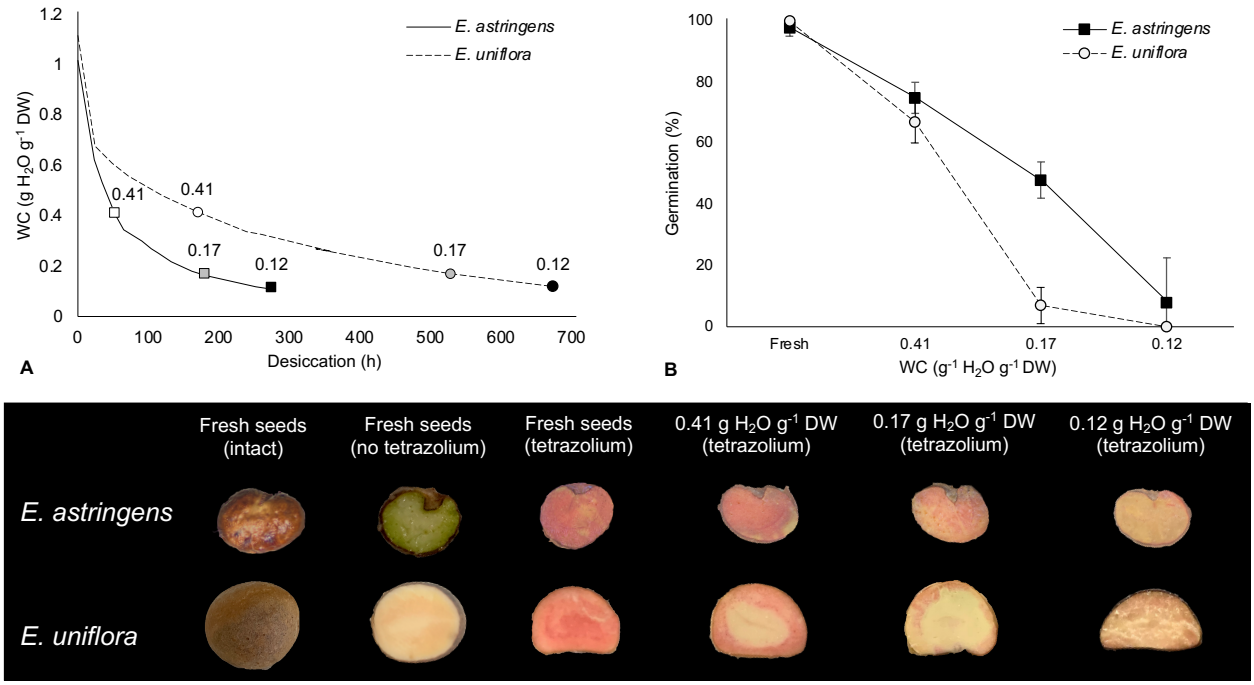


Figure 1. *Eugenia astringens* and *E. uniflora* seed desiccation dynamics and germination upon desiccation. **A.** Desiccation dynamics (hours). **B.** Germination percentage (%). **C.** Seed reaction to 2,3,5-triphenyltetrazolium chloride (Tetrazolium) solution. Positive reaction is indicated by the red staining on the tissue. WC of fresh seeds: *E. uniflora*, 1.12 g H_2O g DW^{-1}, and *E. astringens*, 1.02 g H_2O g DW^{-1}.

We compared the metabolic profile at the same seed viability rates for both species, which derived from different WC for each species (Table 1). *Eugenia astringens* samples were collected at 1.02 (fresh seeds, FS), 0.17 (half germination, G50) and 0.12 g H_2O g DW^{-1} (unviable seeds, US), while *E. uniflora* samples were collected at 1.12 (FS), 0.41 (G50) and 0.12 g H_2O g DW^{-1} (US) (Table 1).

Table 1. Water content (WC), desiccation hours and germination percentage of *Eugenia uniflora* and *E. astringens* seeds used in the GC-MS metabolite analysis. FS: fresh seeds. G50: half viability. US: unviable seeds.

	FS	G50	US
E. astringens			
WC (g H_2O g DW^{-1})	1.02	0.17	0.12
Desiccation (h)	-	180	275
Germination (%)	98	48	8
E. uniflora			
WC (g H_2O g DW^{-1})	1.12	0.41	0.12
Desiccation (h)	-	170	674
Germination (%)	100	65	0

Principal Component Analysis of metabolites

Through PCA, the metabolites of *E. astringens* and *E. uniflora* seeds at the three different WC (FS, G50 and US) were separated into three distinct groups (fig. 3A). In this case, PCA (PC1+PC2) explained 59.52% of total variation of the metabolic behavior and split samples of *E. astringens* (FS, G50 and US) from *E. uniflora* (FS and G50), and *E. uniflora* (US) (fig. 3A). This suggested that the metabolite profile involved in the physiological behavior of *E. astringens* and *E. uniflora* seeds were distinct. Also, it was possible to identify that the metabolite behavior of FS and G50 in *E. uniflora* diverged from US. Next, we analyzed the seed metabolites of each species separately (fig. 2B and C). In *E. astringes* seeds, PCA (PC1+PC2) explained 72.04% of seed germination and WC based on the behavior of metabolites upon desiccation. G50 and US metabolites were more similar to each other than to FS in *E. astringens* (fig. 3B). Conversely, in *E. uniflora* seeds, PCA (PC1+PC2) explained 79.56% of the metabolite variation upon desiccation and suggested that FS and G50 seeds were more similar to each other than to US (fig. 3C). In relation to the RDA, the analysis explained 28.68% of total data variation (fig. 3D). Samples were separated along WC, whereas the different metabolites organized each species separately. In general, *E. uniflora* samples were separated by sucrose and quinic acid, and *E. astringens* samples were separated by fructose and glucose.

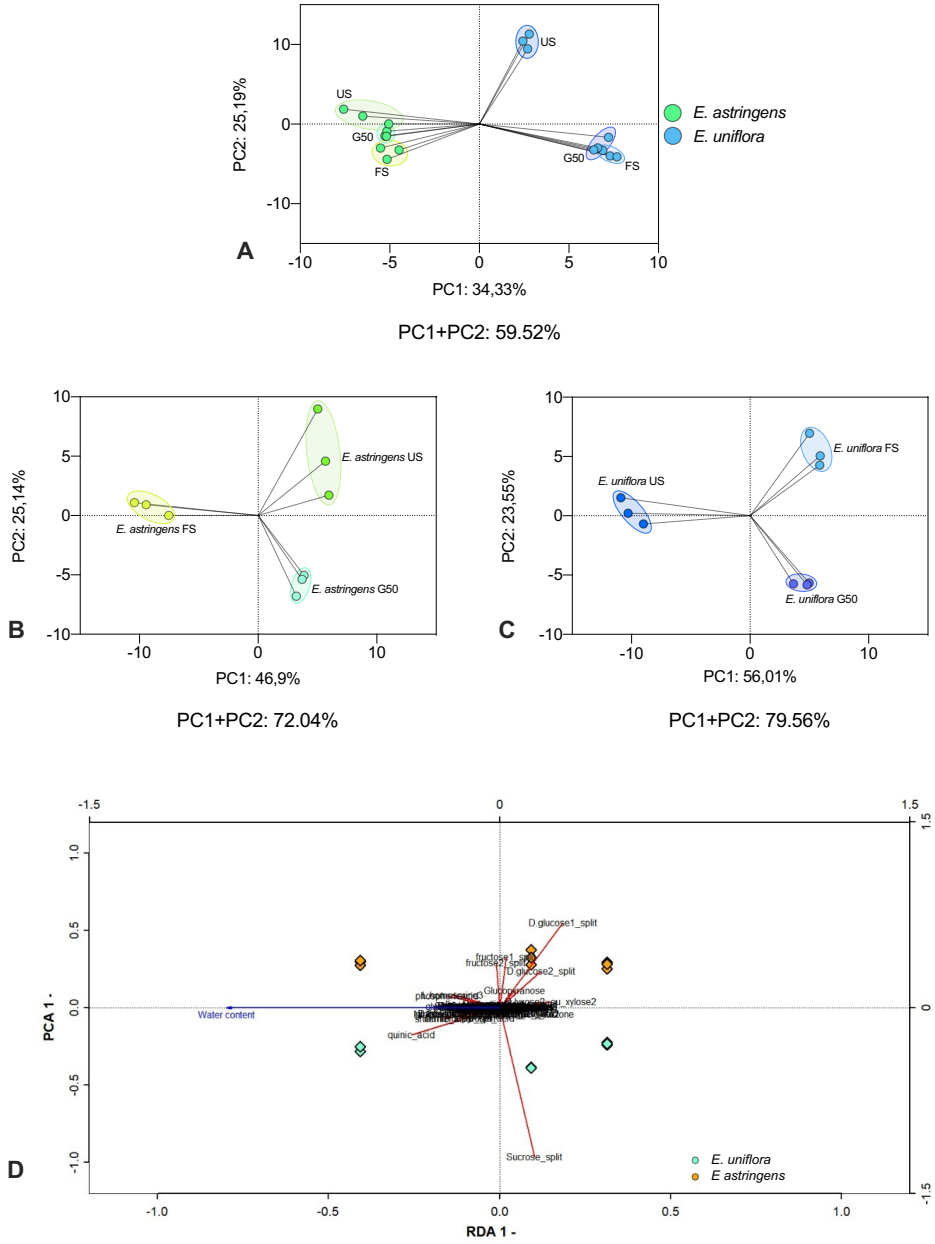


Figure 2. Score plots of the principal component analysis (PCA) of the identified metabolites by GC-MS in *Eugenia astringens* and *E. uniflora* seeds according to the germination and water content (WC). **A.** PCA comparing *E. astringens* and *E. uniflora* seed metabolite profile variation. **B.** PCA comparing the metabolite profile variation at distinct WC in *E. astringens* seeds. **C.** PCA comparing the metabolite profile variation at distinct WC in *E. uniflora* seeds. WC include: fresh seeds (FS), half viability (G50) and unviable seeds (US). PC1, the first principal component; PC2, the second principal component. **D.** Biplot of the constrained Redundancy analysis (RDA) of the identified metabolites.

Metabolic profile of seeds during desiccation

In *E. astringens* FS, carbohydrates represented the majority of metabolite content (79.1%), followed by organic acids (18.7%), amino acids (1.35%), fatty acids (0.5%) and others (0.35%) (fig. 3). At 0.17 g H_2O g DW^{-1} (G50) seeds of *E. astringens*, carbohydrates slightly increased to 85.75%, while we observed a small decrease in organic acids (12.4%), amino acids (1.2%), fatty acids (0.4%) and others (0.25%). Upon further desiccation, the amino acids of *E. astringens* seeds at 0.12 g H_2O g DW^{-1} (US) decreased in more than 70% and 60% in comparison to *E. astringens* FS and G50, respectively, representing 0.4% of the metabolite content (fig. 3).

Carbohydrates also represented most of the metabolite content of *E. uniflora* FS (72.2%), followed by organic acids (24.5%), amino acids (2.57%), fatty acids (0.43%) and others (0.3%) (fig. 3). Upon desiccation to 0.41 g H_2O g DW^{-1} (G50), seeds of *E. uniflora* presented a small increase in carbohydrate content (77.4%), while a decreased was observed in organic acids (19.9%), amino acids (2.1%), fatty acids (0.34%) and others (0.24%). In *E. uniflora* 0.12 g H_2O g DW^{-1} (US), carbohydrate content reached 87.4% of total metabolite content, while organic acids (11.15%), amino acids (1.16%), fatty acids (0.2%) and others (0.12%) decreased in more than 55% and 41% in comparison to *E. uniflora* FS and G50, respectively (fig. 3). Comparatively, we observed a slight increase in carbohydrates, a slight decrease in organic acids, fatty acids and other metabolites, and a considerable reduction in amino acids upon desiccation for both species (fig. 3).

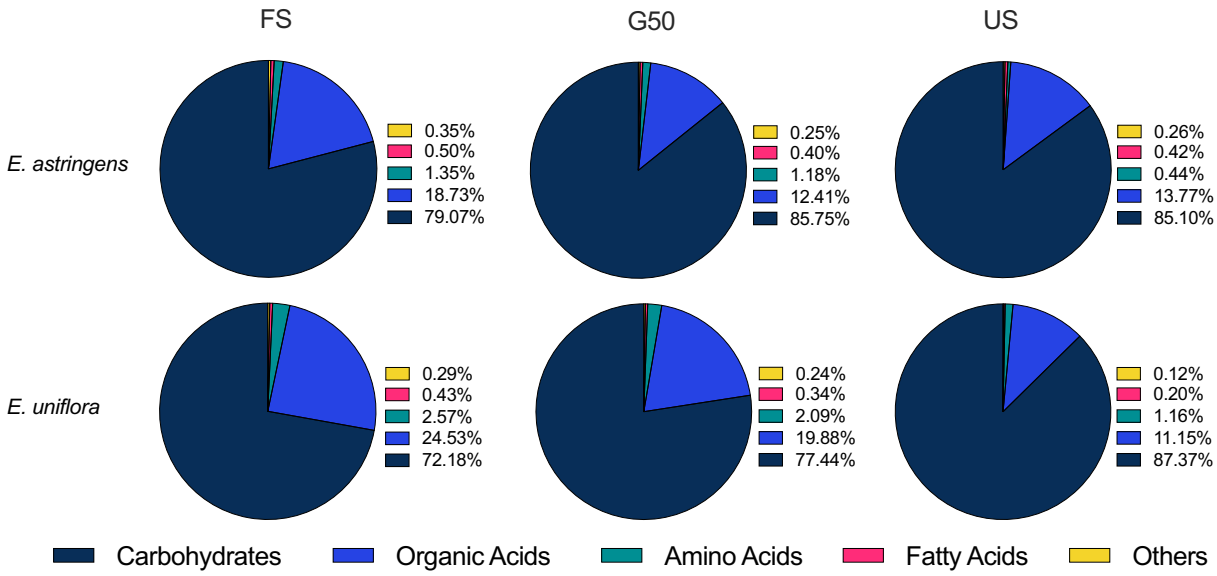


Figure 3. Functional classification (%) of the identified metabolites in *E. uniflora* and *E. astringens* fresh seeds (FS), half viability (G50) and unviable seeds (US).

We also evaluated the metabolites inside each functional class separately (fig. 4). In *E. astringens* FS, 95.5% of carbohydrates were composed of hexoses, mainly by fructose (45.17%) and D-glucose (48.83%). The proportion of carbohydrates in *E. astringens* remained nearly constant after desiccation to 0.17 g H_2O g DW^{-1} (G50) and 0.12 g H_2O g DW^{-1} DW (US). On the other hand, the carbohydrate portion of *E. uniflora* FS was mainly comprised of sucrose (67.4%), followed by fructose (18.51%) and D-glucose (11.12%). In *E. uniflora* seeds desiccated to 0.41 g H_2O g DW^{-1} (G50), the sucrose proportion increased to 80.6%, while fructose and D-glucose decreased to 13.2 and 4.6%, respectively. In seeds of *E. uniflora* at 0.12 g H_2O g DW^{-1} (US), the proportion of sucrose decreased to 61.85%, while fructose increased to 17.38% and D-glucose increased to 19.38% (fig. 4A).

The organic acids of *E. astringens* FS were mainly constituted of phosphoric acid (37.17%), TCA cycle intermediates (30.9%) – especially citric (17.45%) and malic (12.76%) acids, and quinic acid (23.13%) (fig. 4B). In G50 seeds of *E. astringens*, phosphoric acid and TCA cycle intermediates slightly increased (47.25 and 36%, respectively), while quinic acid

decreased (9.1%) (fig. 4B). In *E. astringens* US, TCA cycle intermediates returned to FS proportions (32%) while quinic acid increased to 16% and phosphoric acid maintained G50 proportions (45%). In a similar way, the largest portion of organic acids of *E. uniflora* FS was comprised of TCA cycle intermediates (32.9%) – mainly citric (19.8%) and malic (12.6%) acids, quinic acid (32.1%), and phosphoric acid (25.3%). In *E. uniflora* G50 seeds, TCA cycle intermediates and quinic acid slightly increased (35.6% and 36.6%, respectively), and phosphoric acid decreased (19.3%). In *E. uniflora* US, quinic acid (30%) and phosphoric acid (26%) returned to FS proportions, and TCA cycle intermediates (37.9%) remained at G50 proportions (fig. 4B).

Amino acids of *E. astringens* FS were mainly composed of L-homoserine (62%) and L-pyroglutamic acid (22%) (fig. 4C). In seeds of *E. astringens* at 0.17 g H_2O g DW^{-1} (G50), L-pyroglutamic acid decreased to 15.8% and aspartic acid rose up to 16.5%, while L-homoserine remained 62%. In *E. astringens* US, L-homoserine was reduced to 45.4%, while L-pyroglutamic acid returned to FS proportions (25.4%) and aspartic acid remained as G50 (15.8%) (fig. 4C). In FS of *E. uniflora*, amino acids were composed of N-methyl glutamic acid (31.6%), L-pyroglutamic acid (23.6%), L-homoserine (13.6%), GABA (8.4%), aspartic acid (6%) and L-glutamic acid (5.6%). In *E. uniflora* seeds at 0.41 g H_2O g DW^{-1} (G50), increases were observed for L-pyroglutamic acid (61.4%), L-glutamic acid (13.4%) and aspartic acid (10%), while N-methyl glutamic acid strongly decreased (0.23%) and GABA decreased by half (4.4%). In *E. uniflora* US, aspartic acid increased (52.4%), while L-pyroglutamic and L-glutamic acids returned to FS proportions (27% and 4.3%, respetively), and GABA decreased to 1.53%.

Fatty acid composition of *E. astringens* and *E. uniflora* FS were similar and mainly composed of saturated fatty acids (98.2 and 97.6%, respectively) (fig. 4D), especially palmitic (50.4% and 47.63%, respectively) and stearic (36.87% and 38.53%, respectively) acids.

Eugenia astringens unsaturated fatty acids increased to 3.14% in G50 seeds and to 4.78% in US. In seeds of *E. uniflora*, unsaturated fatty acids represented 2.17% in G50 and 2.93% in US (fig. 4D).

Other metabolites covered similar slices in FS of both species and included ethanolamine (61%), 2-hydroxypiridine (10%), 4-hydroxypiridine (5%) and porphine (5%) (fig. 4E). In *E. astringens* FS, we also observed phendimetrazine (12.6%). In seeds of *E. astringens* at 0.17 g H_2O g DW^{-1} (G50), ethanolamine increased to 67.7% and phendimetrazine decreased to 3.2% (fig. 4E). In US seeds of *E. astringens*, phendimetrazine increased to 6%. On the other hand, in FS of *E. uniflora*, phendimetrazine represented 6.7% and urea 5.8% of other metabolites. In *E. uniflora* G50 seeds, phendimetrazine increased to 9.41% and urea decreased to 3.9%. In *E. uniflora* US, phendimetrazine went back to FS levels (6.7%) and urea decreased to 2% (fig. 4E).

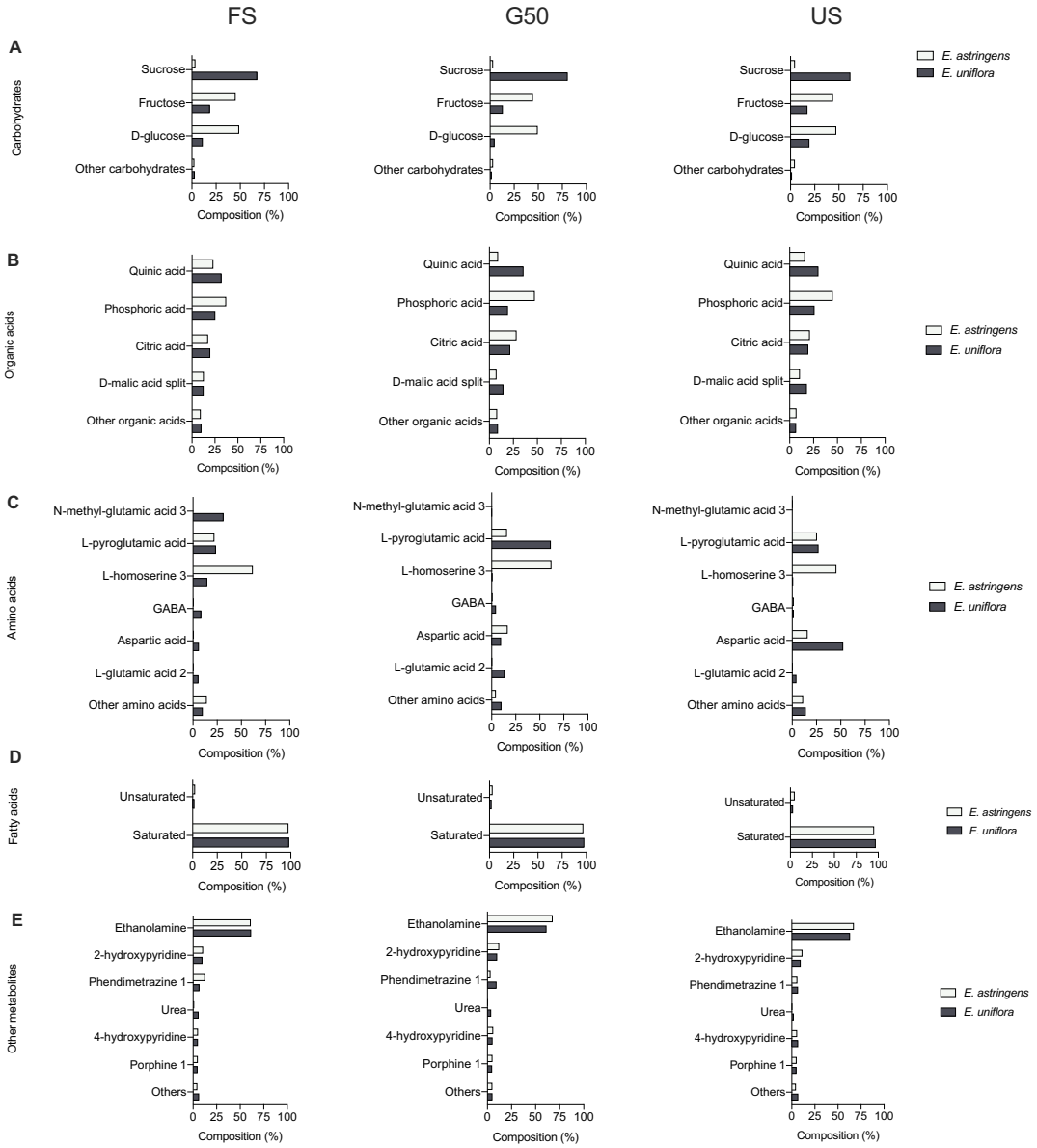
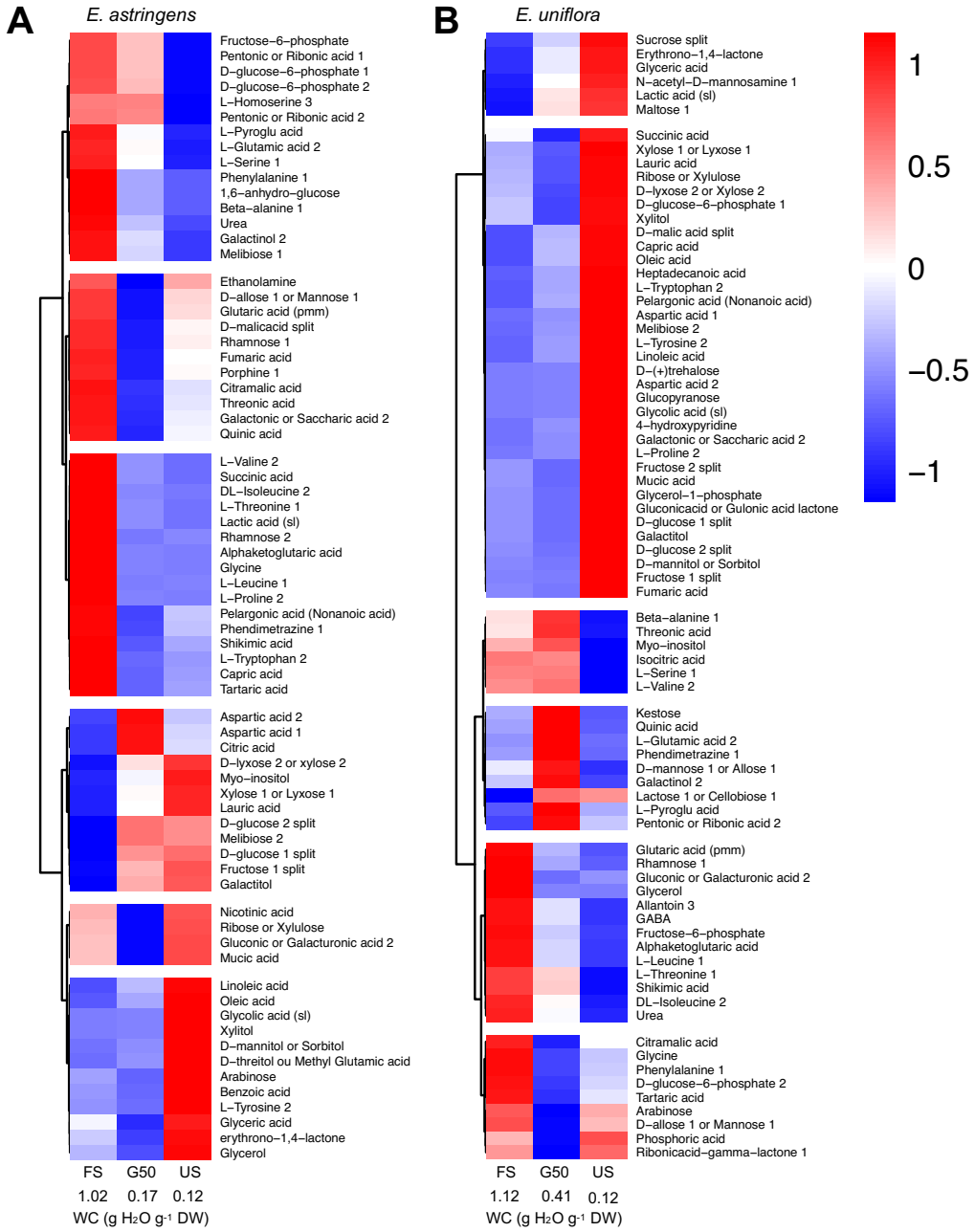


Fig. 4. Relative composition of metabolites (%) inside each functional class upon desiccation of *E. astringens* and *E. uniflora* seeds. **A.** carbohydrates **B.** organic acids **C.** amino acids **D.** fatty acids **E.** other metabolites. FS: Fresh seeds. G50: half viability. US: unviable seeds.

Metabolic changes upon seed desiccation of E. astringens and E. uniflora

The metabolic HCA shows that 70 metabolites of *E. astringens* seeds and 77 metabolites of *E. uniflora* seeds were significantly affected by desiccation and viability loss (ANOVA, $P<0.05$) (fig. 5). From the 70 metabolites of *E. astringens* seeds, 66% were downregulated upon desiccation, including galactinol, malic acid, succinic acid, pyroglutamic acid, phenylalanine, glycine and proline (fig. 5A). Metabolites such as *myo*-inositol, xylose, glucose, fructose,



galactitol, citric acid, aspartic acid and lauric acid were upregulated in *E. astringens* seeds at 0.17 g H_2O g DW^{-1} (G50). In *E. uniflora* seeds, 64% of metabolites were upregulated upon desiccation, including sucrose, maltose, galactinol and pyroglutamic acid that were upregulated at 0.41 g H_2O g DW^{-1} (G50) (fig. 5B). On the other side, allantoin, GABA, glycine, phenylalanine, arabinose and galacturonic acid were downregulated in G50 (fig. 5B).

Figure 5. Hierarchical cluster analysis (HCA) showing the coordinated changes in metabolite content upon seed desiccation. **A.** *E. astringens* coordinated changes in metabolites from 1.02

(FS) to 0.17 (G50) and 0.12 (US) g H_2O g DW^{-1} **B.** *E. uniflora* coordinated changes in metabolites from 1.12 (FS) to 0.41 (G50) and 0.12 (US) g H_2O g DW^{-1}. FS: fresh seeds. G50: half viability. US: unviable seeds. The deeper the red color, the higher the metabolite content; similarly, the deeper the blue color, the lower the metabolite content.

Comparison of metabolites involved in seed desiccation

We compared the relative content (fig. 6) and described the role (table S1) of 12 carbohydrates, six amino acids, five organic acids, four fatty acids and two other metabolites that were previously reported by several authors (see table S1) to be involved in stress-related responses of plants. The majority of carbohydrates, specifically fructose, galactinol, galactitol, glucose, maltose, mannitol, trehalose presented higher contents in *E. astringens* FS and remained higher than in *E. uniflora* seeds upon desiccation (fig. 6A). On the other hand, the six stress-related amino acids (aspartic acid, GABA, glycine, phenylalanine, proline and pyroglutamic acid) were higher in *E. uniflora* FS, with pyroglutamic acid increasing in G50 seeds and aspartic acid and proline increasing in US. In *E. astringens* seeds, only aspartic acid slightly increased in G50 seeds (fig. 6B). In relation to organic acids, metabolites were slightly higher in *E. uniflora* seeds (fig. 6C). In relation to fatty acids, relative contents were similar, except for lauric acid which started to accumulate earlier in *E. astringens* seeds (fig. 6D). In relation to other metabolites, allantoin content was higher in *E. uniflora* in all WC, while ethanolamine content was similar for both species (fig. 6E).

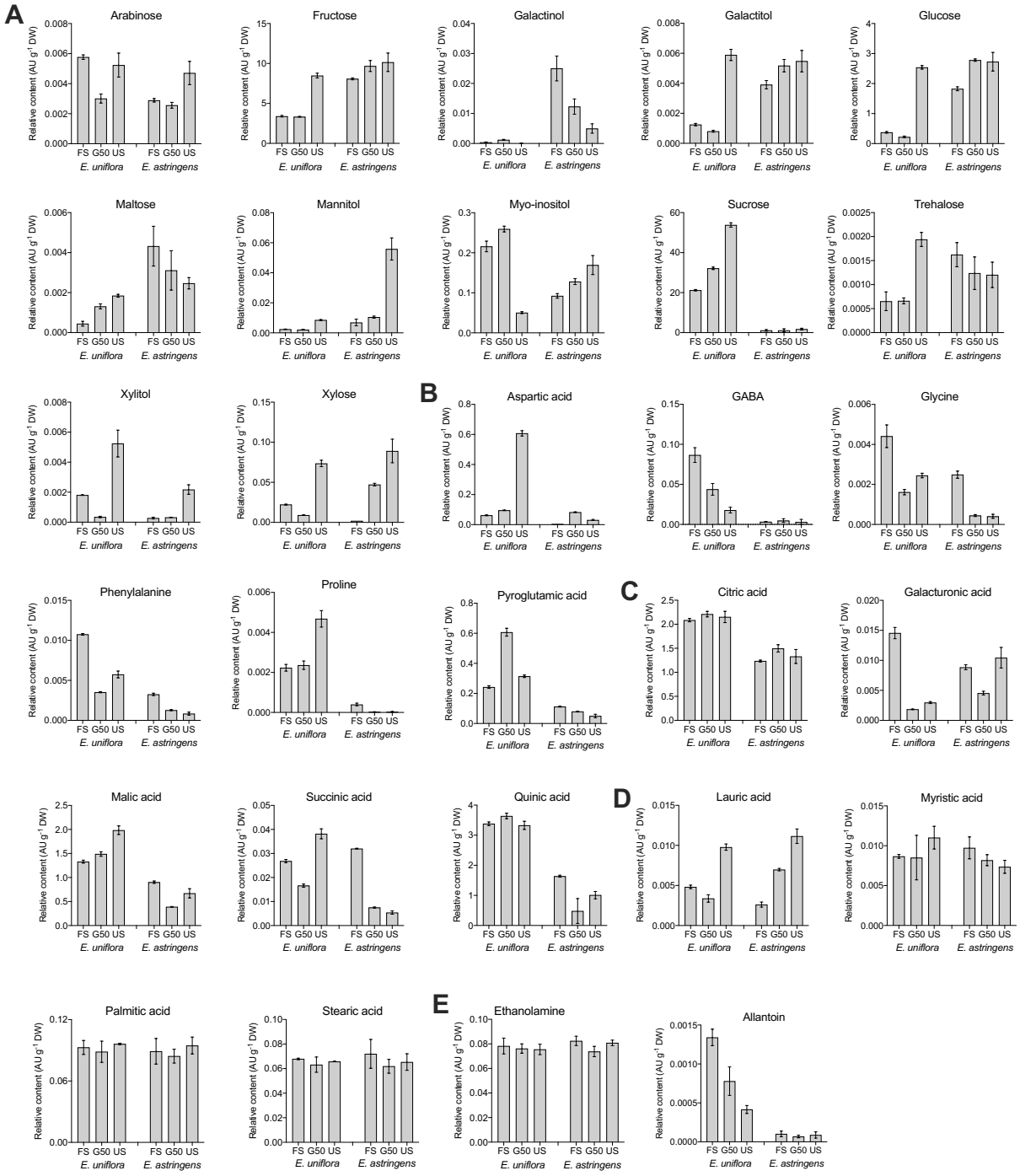


Fig. 6. Comparison of the relative content of metabolites identified in *E. uniflora* and *E. astringens* seeds involved in plants stress-related responses. **A.** Carbohydrates. **B.** Amino acids. **C.** Organic acids. **D.** Fatty acids. **E.** other compounds. Values were expressed as average contents in arbitrary units (AU) relative to the internal standard and normalized to dry weight (DW) and are mean of replicates. Vertical bars represent ± SD. FS: fresh seeds. G50: half viability. US: unviable seeds.

DISCUSSION

Seed metabolite profile goes through specific changes to tolerate partial desiccation

Our study is the first to show the global metabolite changes of DS *Eugenia* seeds and opens a wide range of possibilities to uncover the underline information about tropical DS-seeded species by exploring the physiological behavior of several metabolites upon desiccation. Fresh seeds from wild plant populations of *Eugenia astringens* and *E. uniflora* showed high viability (>98% germination). Upon desiccation, *E. uniflora* seeds presented a WC threshold (G50) of 0.41 g H_2O g DW^{-1} and *E. astringens* 0.17 g H_2O g DW^{-1}, which showed a significant difference in desiccation sensitivity (fig. 1B). Interestingly, WC thresholds (G50) were reached after similar desiccation times, with 170 h for *E. uniflora*, the widespread species, and 180 h for *E. astringens*, which is restricted to the coastal Restinga vegetation (fig. 1A). Restinga is an ecosystem known by high solar incidence, salinity, and sandy soils with low nutrient availability (Weidlich et al., 2020). The harsh and inhospitable environment of Restinga may be a constraint for DS seeded-species, once they would require stress-tolerance traits (Lavergne et al., 2004). Our data suggest that *E. astringens* seeds activate mechanisms to tolerate further desiccation (0.17 g H_2O g DW^{-1}) comparatively to *E. uniflora* seeds (0.41 g H_2O g DW^{-1}). It is known that seed responses under stress conditions involve complex shifts in metabolic pathways for physiological adaptation (Saito and Mastuda, 2010). Therefore, it might be reasonable to think that *E. astringens* seed metabolite profile goes through specific changes to tolerate desiccation to 0.17 g H_2O g DW^{-1}, while specific metabolic switches may slow down water loss of *E. uniflora* seeds. The metabolite composition was distinct between *E. astringens* and *E. uniflora* seeds, with distinct changes occurring upon desiccation in carbohydrates, organic acids and amino acids, as we further discuss.

Carbohydrates are the main components of E. uniflora and E. astringens seeds, but composition was distinct

In seeds of *E. uniflora*, carbohydrates increased up to 77.4% at 0.41 g H_2O g DW^{-1} (G50) and 87.4% at 0.12 g H_2O g DW^{-1} (US) of total metabolite content (fig. 3). Carbohydrates are recognized by their involvement in the desiccation tolerance and high storability of DT seeds (Chabrillange et al., 2000). Galactinol, maltose, *myo*-inositol and sucrose were upregulated in *E. uniflora* G50 seeds. Sucrose represented 80% of carbohydrates in G50 (fig. 4A) and is known to be involved in the protection and stabilization of lipid bilayers under desiccation, where it primarily aggregates in the center of the interbilayer space (Stachura et al., 2019). Mello et al. (2010) also reported sucrose as the main carbohydrate of *E. uniflora* seeds. The high proportion of sucrose may have contributed to slow down seed desiccation and preserve, at least in part, the viability of *E. uniflora* seeds at 0.41 g H_2O g DW^{-1} (G50). Maltose has the ability to protect proteins and membranes from drying damage, but only at physiologically relevant amounts (Lu and Sharkey, 2006). It might not be the case in *E. uniflora* G50 seeds, since maltose comprised only 0.003% of the carbohydrates, with 0.0013 AU g^{-1} DW (fig. 6A). Fructose, galactitol, glucose, mannitol, trehalose, xylitol and xylose were significantly upregulated in *E. uniflora* at 0.12 g H_2O g DW^{-1} (US) (fig 5A, 6A). *Myo*-inositol, galactitol, mannitol, sorbitol and xylitol are polyols (sugar alcohols) that control the osmotic balance, enabling water adjustment in cytoplasm and providing the stabilization of membranes in abiotic stress conditions (Tari et al., 2010). Trehalose is a highly stable non-reducing sugar that holds a water replacement mechanism by forming hydrogen bonds with a membrane or macromolecules during dehydration (Macovei et al., 2019). Therefore, the delay in accumulating polyols and trehalose may help explaining the viability loss of *E. uniflora*.

In the case of *E. astringens* seeds, carbohydrates comprised 86% of metabolites at 0.17 g H_2O g DW^{-1} (G50) (fig. 3). Fructose, glucose, galactitol, *myo*-inositol and xylose were

upregulated in *E. astringens* G50 seeds (fig. 5B, 6B). Fructose and glucose composed 95% of total carbohydrates in *E. astringens* seeds (fig. 4A), and are known to accumulate earlier than other metabolites in response to stress (Fàbregas and Fernie, 2019). On the other hand, fructose and glucose are monosaccharides that act as promoters of Maillard reaction products, which stimulate respiration and promote free radical formation, therefore contributing to the DS state (Rangel-Fajardo et al., 2011). Arabinose, mannitol and xylitol were upregulated in *E. astringens* seeds at 0.12 g H_2O g DW^{-1} (US) (fig. 5B, 6A). An accumulation of arabinose is found in the cell wall of DT seeds and it acts as plasticizer agent by increasing cell wall flexibility and diminishing strong interactions among acidic pectic polysaccharides (Caccere et al., 2013). This may suggest that the death of *E. astringens* US evaluated in our study might also be related to a deficiency in cell wall flexibility upon desiccation, either due to an insufficient or to a late arabinose accumulation. Galactinol content was progressively reduced in *E. astringens* during seed desiccation (fig. 5B, 6A). Galactinol is produced during the first step of the raffinose family oligosaccharides (RFOs) biosynthesis pathway, and in *Brassica oleracea* seeds the decrease in galactinol was correlated with a reduction in seed viability over time (Vidigal et al., 2016). This also makes sense with the gradual viability loss of *E. astringens* seeds upon desiccation.

It seems like seeds of *E. astringens* responded to desiccation by upregulating several stress-related carbohydrates in G50 seeds, while the majority of carbohydrates in *E. uniflora* seeds accumulated only after seed death (US). Notwithstanding, the sole presence of specific sugars in *E. astringens* and *E. uniflora* was not sufficient to keep seeds viable at the lowest WC (0.12 g H_2O g DW^{-1}). Though the stress-related sugars might have demonstrated potent protective mechanisms up to a certain point, they form “fragile” glasses that may not handle appropriately environmental fluctuations in moisture (Walters, 2015), especially the ones observed in these tropical seeds.

Organic acids were upregulated in E. uniflora and downregulated in E. astringens seeds under desiccation

In relation to organic acids, half of these compounds remained unchanged at 0.41 g H_2O g DW^{-1} (G50) in seeds of *E. uniflora* (fig. 5A) and were upregulated at 0.12 g H_2O g DW^{-1} (US). Organic acids may have important roles in cell metabolism, including energy production, amino acid biosynthesis and plant adaptation to the environment (Lòpez-Bucio et al., 2000). Seeds of *E. uniflora* desiccated to 0.41 g H_2O g DW^{-1} (G50) presented an increase in quinic acid, which covered 36% of total organic acids (fig. 4C, 5A, 6C). Derivates of quinic acid were observed to act against potential oxidative damage and deleterious effects of reactive oxygen species (ROS), and could also help in the protection of cell membranes against damage caused by desiccation (Moore et al., 2005). Therefore, this compound might have been broken down to produce derivatives that relief desiccation stress in G50 seeds of *E. uniflora*. In the case of *E. astringens*, 80% of the organic acids were downregulated in G50 seeds, including all TCA cycle intermediates, except citric acid (fig. 5B, 6C). The TCA cycle is a vital respiratory pathway that is crucial for energy delivery to different organelles and for the maintenance of several physiological processes (Das et al., 2017). However, during acquisition of desiccation tolerance of *Erythrina speciosa* DT seeds there is a metabolic shutdown and the TCA cycle intermediates citric acid, malic acid and succinic acid were downregulated (Hell et al., 2019). The downregulation of TCA cycle intermediates might suggest that *E. astringens* seeds also decreased the respiratory rates at 0.17 g H_2O g DW^{-1} (G50). Conversely, citric acid was the only organic acid upregulated in *E. astringens* G50 seeds (fig. 4B, 5B, 6C). Citric acid may have supplied more substrate for mitochondrial citric dehydrogenase to alleviate the oxidative damage and to regulate the mitochondrial oxidative balance caused by desiccation (Zhong et al., 2016). Galacturonic acid is involved in stress-related responses as one of the precursors of

ascorbic acid, a molecule that acts as an antioxidant and cell signaling modulator (Hemavathi et al., 2009). Therefore, a decrease in the production of antioxidant compounds may have taken place, since this compound was downregulated in G50 seeds of both species (fig. 5, 6C).

The participation of amino acids upon desiccation was species-specific

Amino acid content represented 2.57% of total metabolite content in FS in *E. uniflora* seeds (fig. 3). Many amino acids play roles as osmolytes by acting as precursors for most of the osmoprotectants and also help preventing membrane damage (Suprasanna et al., 2016). Here, 40% of the amino acids were downregulated in *E. uniflora* seeds upon desiccation (fig. 5B) and GABA decreased progressively upon desiccation (fig. 6B). During desiccation of DT seeds, there is an accumulation of GABA (Fait et al., 2008; Angelovici et al., 2010), which is supposed to facilitate early metabolic reorganization during germination and to regulate plant growth and development (Du et al., 2020; Li et al., 2021). GABA also plays a role in oxidative stress mitigation by preventing ROS accumulation (Bouché and Fromm, 2004). Therefore, the degradation of GABA during *E. uniflora* seed desiccation may also have predisposed seeds to aqueous-based metabolism oxidative stress. Pyroglutamic acid was upregulated and accounted for 61.4% of total amino acids in G50 of *E. uniflora* seeds (fig. 4C). Pyroglutamic acid can be easily obtained from glutamic acid by intramolecular dehydration (Kumar and Bachhawat, 2012). It is a constrained analogue of GABA and was found to accumulate in response to salt stress and to function as an osmoprotectant (Kumar and Bachhawat, 2012; Jiménez-Arias et al., 2019). Therefore, N-methyl-glutamic acid of *E. uniflora* FS may have been converted into pyroglutamic acid in G50 seeds to favor water adjustment and oxidative stress alleviation. Glycine and phenylalanine decreased in more than 50% and represented less than 1% of the amino acid content each in *E. uniflora* G50 seeds (fig. 6B). Glycine and phenylalanine may act directly or indirectly in the attenuation of oxidative stresses. Phenylalanine synthesizes

antioxidative compounds that can balance the redox equilibrium and minimize oxidative damage (Teixeira et al., 2017). Glycine regulates the cell osmotic pressure and stabilizes macromolecular activities and membrane integrity (Quan et al, 2004; Yang et al., 2018). Aspartic acid and proline were upregulated in *E. uniflora* US (fig. 6B). Proline accumulation is an intracellular signal connected with water loss prevention mechanisms (Lehmann et al., 2010). It can also promote the protection of the integrity of cell membranes and proteins, reduction of lipid peroxidation, ROS scavenge and antioxidant enzyme activation (Abrantes et al., 2019; Kijowska-Oberc et al., 2020). The late accumulation of proline in *E. uniflora* seeds may suggest a failure in stress sensing and signaling, which then would lead to cellular damage accumulation due to the no activation of protective mechanisms. In the case of *E. astringens*, aspartic acid was the only amino acid upregulated in G50 seeds (fig. 5A, 6B), taking 16.5% of total amino acid content. Aspartic acid is a precursor of multiple biomolecules for plant growth and defense, and may be mobilized into other amino acids such as methionine (Han et al., 2017; Han et al., 2021). In order to be converted into methionine, the aspartic acid has to be first converted into homoserine, by the homoserine dehydrogenase enzyme (Azevedo et al., 2006). This amino acid is considered to be a major limiting factor of the methionine pathway, besides also having a stimulative role in the accumulation of methionine (Lee et al., 2005). Perhaps, homoserine was not being converted in methionine in a satisfactory rate, leading to a low methionine production. Other amino acids, such as glycine and phenylalanine decreased in more than 80 and 60% in G50 and US *E. astringens* seeds, respectively, while GABA and proline were barely detected (fig. 6B). Based on our results, it seems that amino acids might have played a higher role in *E. uniflora* seeds in comparison to *E. astringens*, by regulating water balance and preventing oxidative damage. Amino acids can play an important role in maintaining the osmotic potential during seed desiccation and serve as precursors of many secondary metabolites in response to abiotic stresses (Yan et al., 2018). It was also suggested

that amino acid accumulation under stress may be used in protein biosynthesis and accelerate the post-stress recovery (Zandalinas et al., 2017). However, the metabolism of stress-related amino acids in both species might have not been desirably enhanced upon desiccation, which may also have contributed to the loss of viability of *E. astringens* and *E. uniflora* seeds.

Fatty acids followed similar patterns in E. uniflora and E. astringens upon desiccation

Fatty acid composition was similar between *E. uniflora* and *E. astringens*, with higher amounts of saturated than unsaturated fatty acids (fig. 4D). Their behavior upon desiccation was also similar between the two species, with decreased saturated and increased unsaturated fatty acid content (fig. 5, 6D). Lauric, palmitic, myristic and stearic acids are saturated fatty acids and may contribute to the preservation of membrane functionality after desiccation (Guimarães et al., 2020). On the other hand, unsaturated fatty acids reached up to 3% and 4.7% of total fatty acid contents in US seeds of *E. uniflora* and *E. astringens*, respectively. Unsaturated fatty acids are less stable than saturated fatty acids, and their oxidation leads to an increase in malondialdehyde levels, which is cytotoxic and in high levels cause seed deterioration (Parkhey et al., 2012). Even though they were present in small relatively amounts, the increase in unsaturated fatty acids may have reflected in a rise in lipid peroxidation, membrane destabilization and consequently viability loss of *E. uniflora* and *E. astringens* US seeds.

The role of other compounds in E. uniflora and E. astringens seed desiccation

A couple of other compounds were identified in *Eugenia* seeds and might also play a role in seed desiccation, such as ethanolamine and allantoin (fig. 6E). Ethanolamine, which increased in Arabidopsis under drought stress (Rizhsky et al., 2004), is considered essential for membrane biogenesis and is precursor of some important osmoprotectants such as glycine

betaine (Du et al., 2012). In our study, no changes occurred in ethanolamine for either species, which may represent a response failure and justify the decrease in glycine levels upon desiccation. Allantoin is synthesized from purine nucleotides and is known to be upregulated in response to various abiotic stresses in DT species, including drought and osmotic stress, and to stimulate ABA biosynthesis as a protective mechanism (Kaur et al., 2021 and references therein). Allantoin gradually declined upon desiccation of *E. uniflora*, and was not significantly present in *E. astringens* seeds (fig. 6E). This suggests that the step of DT induction where ABA is the main signaling molecule was not activated during seed maturation. ABA signaling is an essential step for the acquisition of DT of seeds, and low levels of ABA or a disruption of the ABA signaling pathway possibly results in a DS behavior even in DT seeds (Gutierrez et al., 2007).

Final thoughts and perspectives

We observed distinct metabolite profiles in *E. uniflora* and *E. astringens* seeds, specifically carbohydrates, organic acids, amino acids and others (fig. 4, 5 and 6). Moreover, our results demonstrated that distinct mechanisms were activated in *E. astringens* and *E. uniflora* seeds in order to survive partial desiccation (G50). In *E. astringens* G50 seeds, fructose, glucose, galactitol, myo-inositol and xylose were upregulated (fig. 5A, 6A) and may have stabilized the cytoplasm. TCA cycle intermediates declined, which might indicate a respiratory rate reduction (fig. 5A, 6C). On the other hand, antioxidant amino acids were mostly degraded or not representative, with only aspartic acid being upregulated (fig. 6B). Unsaturated fatty acids increased and might have led to membrane destabilization and enhanced lipid peroxidation (fig. 4D, 5A). Therefore, the viability loss of *E. astringens* seeds at 0.12 g H_2O g DW^{-1} (US) may be due to a failure in the balance of the redox status and to a ROS accumulation. In the case of *E. uniflora* seeds, sucrose was the only relevant carbohydrate that was enhanced

in G50 seeds (fig. 5B, 6A), and possibly slowed down water loss. Respiratory rates remained stable and probably increased in *E. uniflora* US, as displayed by the rise in organic acid levels (fig. 5B). Pyroglutamic acid represented a significant part of amino acid composition in *E. uniflora* G50 seeds (fig. 4C, 6B), possibly dealing with the oxidative stress and maintaining partial viability of G50 seeds. Nonetheless, it was the only amino acid upregulated in *E. uniflora* G50 seeds, while the rest was either downregulated upon desiccation (GABA, glycine and phenylalanine), or only upregulated in *E. uniflora* US (aspartic acid and proline) (fig. 5B, 6B). Unsaturated fatty acid proportions also increased in *E. uniflora* seeds upon desiccation (fig. 3D). Allantoin was progressively downregulated, which might be related to a possible ABA decrease such as reported for other species in the literature (fig. 6E). Therefore, death of *E. uniflora* US might be related to a delay in stress-sensing signaling and late accumulation of osmotic and antioxidant compounds. Table 2 summarizes the list of metabolites with stress-related functions identified in *Eugenia* seeds and briefly describes their function. We confirm that *E. uniflora* and *E. astringes* are placed in the DS category by also linking their metabolite profiles with their seed viability and WC threshold. This study brings new insights into the fundamental processes of seed viability under desiccation, which can be useful to understand the bottlenecks of the ex-situ and in-situ seed conservation of wild tropical species. Further studies targeting specific compounds should be conducted and might select compounds that could serve as ways to induce desiccation tolerance in DS seeds.

Table 2. List of metabolites present in *Eugenia astringens* and *E. uniflora* seeds known to be involved in plant stress responses. The table displays their abundance in fresh seeds (FS) (high/low in comparison between species), how they are regulated (up/down) in half viable (G50) and unviable seeds (US), and main functions.

Compounds	*E. astringens*			*E. uniflora*			Remark	Ref.
	FS	G50	US	FS	G50	US		
Carbohydrates	**Water replacement, structural polymers, glass formation**							
Arabinose	Low	-	↑	High	↓	↑	Plasticizer; increases cell wall flexibility	1
Fructose	High	↑	-	Low	-	↑	Osmoprotectant; overall structure stability	2
Galactinol	High	↓	↓	Low	↑	↓	Protective role; biosynthesis of RFOs and seed longevity marker	3
Galactitol	High	↑	-	Low	-	↑	Osmoprotectant	5
Glucose	High	↑	-	Low	-	↑	Osmoprotectant; overall structure stability	2
Maltose	High	-	-	Low	↑	↑	Protective role; membrane stability and biosynthesis of other carbohydrates and proline	4
Mannitol/Sorbitol	Low	-	↑	Low	-	↑	Osmoprotectant	5
Myo-inositol	Low	↑	-	High	-	↓	Stress response, cell wall formation, osmotic adjustment and membrane transport; biosynthesis of RFOs	8
Sucrose	Low	-	-	High	↑	↑	Osmoprotectant; overall structure stability	2
Trehalose	High	-	-	Low	-	↑	Osmoprotectant; seed vitrification	6, 20
Xylitol	Low	-	↑	High	-	↑	Osmoprotectant; stabilizing agent	7
Xylose	High	↑	-	Low	-	↑	Osmoprotectant; major precursor in hemicellulose of the cell wall	17
Amino acids	**Protein synthesis, N_2 metabolism, stress response, osmolytes**							
Aspartic acid	Low	↑	↑	High	↑	↓	Biosynthesis of essential amino acids; N_2 transport and storage compound	22
GABA	Low	-	-	High	↓	↓	Protective role; oxidative stress response, signaling molecule, cytosolic regulation of pH and N_2 metabolism	12
Glycine	Low	↓	-	High	-	-	Protective role; membrane integrity, enzyme and protein structural stabilization	10
Phenylalanine	Low	↓	↓	High	↓	↑	Plant adaptation; precursor of phenolic compounds directly involved in plant stress resistance	11
Proline	Low	-	-	High	-	↑	Protective role; osmoregulation, protein activation, ROS removal, lipoperoxidation reduction, and membrane stabilization	13

continuação da tabela 1.

Pyroglutamic acid	Low	↑	↓	High	↓	↓	ROS scavenging enhancement; improves osmotic and water balance; glutathione cycle involvement	21
Organic acids	**Energy metabolism (TCA cycle), plant modulation upon stress**							
Citric acid	High	↑	↓	High	-	-	Reduction of ROS production and regulation of mitochondrial oxidative balance during stress	18
Galacturonic acid	High	↓	↑	High	↓	-	Intermediate in the ascorbic acid pathway, an important antioxidant against oxidative stress	16
Malic acid	Low	↓	↑	High	-	↑	Maintenance of osmotic pressure	17
Quinic acid	Low	↓	↑	High	↑	↓	Precursor of antioxidant and stabilizing compounds	9
Succinic acid	High	↓	-	High	-	↑	Stress tolerance through respiration enhancement	18
Fatty acids	**Structure role in membranes, secondary metabolite intermediates**							
Lauric acid	Low	↑	↑	Low	-	↑	Preservation of membrane functionality after desiccation	14, 15
Myristic acid	High	-	-	High	-	-	Preservation of membrane functionality after desiccation	14, 15
Palmitic acid	High	-	-	High	-	-	Preservation of membrane functionality after desiccation	14, 15
Stearic acid	High	-	-	High	-	-	Preservation of membrane functionality after desiccation	14, 15
Other compounds								
Allantoin	Low	-	-	High	↓	↓	Long-term stress tolerance mechanisms; ABA biosynthesis stimulation and N_2 mobilization	19
Ethanolamine	High	↓	↑	High	-	-	Membrane biogenesis and precursor of osmoprotectants such as glycine betaine	17

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8 CAPÍTULO IV

Is it possible to attenuate the desiccation sensitivity of *Eugenia astringens* (Myrtaceae) seeds?

Is it possible to attenuate the desiccation sensitivity of *Eugenia astringens* (Myrtaceae) seeds?

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ABSTRACT

Eugenia is a promising and highly representative Myrtaceae genus of the Atlantic rainforest that produces desiccation-sensitive (DS) seeds. Due to their seed morphology, at which seeds are large and there is no visible distinction between the embryonic axis and the fused cotyledons, the conservation of *Eugenia* species may demand specific and innovative methods. In this study, we test the hypothesis that N-acylethanolamine (NAE) and 1-butanol (1-BUT), two phosphatidic acid (PhA) inhibitors, are capable of attenuating the desiccation sensitivity of *E. astringens* seeds. We imbibed seeds in NAE, 1- BUT or distilled water (H_2O) for 24 h in the dark and verified their effects on desiccation and germination. We also analyzed the oxidative metabolism through antioxidant enzymes, lipid peroxidation (MDA), electrolytic leakage (EL) and polyamines (PAs). Imbibed seeds desiccated faster than CNT seeds. Surprisingly, seeds imbibed in H_2O and 1-BUT presented germinations above 50%, while NAE-imbibed seeds maintained 28% germination and control (CNT) seeds displayed only 13% after desiccation to 0.25 g H_2O g DW^{-1}. H_2O, 1-BUT and NAE-desiccated seeds presented lower EL and MDA rates in comparison to CNT seeds. H_2O-desiccated seeds showed increased PA content, specifically spermidine and spermine, while 1-BUT-desiccated seeds displayed increased superoxide dismutase and catalase antioxidant activities. This work brings new insights on the influence of PhA inhibitors in the desiccation tolerance and oxidative metabolism of *E. astringens*, together with new possibilities of improving germination of desiccated seeds through H_2O imbibition.

Keywords: 1-butanol, N-acylethanolamine, oxidative metabolism, seed conservation, recalcitrant, wild species

INTRODUCTION

Brazilian biomes detain a considerable portion of the plant diversity worldwide. *Eugenia* is one of the largest Myrtaceae and angiosperm genus, and is highly representative in Brazil, with 376 spp. from which 82% are endemic (Mazine et al. 2018). This genus is mostly found in the Atlantic Rainforest and has great ecological importance and economic potential (Amorim et al. 2020). *Eugenia* is a promising source of natural antioxidants and has caught a large industrial attention in food, cosmetic and pharmaceutical areas (Araújo et al. 2019). Interestingly, *Eugenia* seeds share the ability of germinating even if a large piece of the reserve tissue is predated or removed; if seeds are fractioned into four pieces, each will give rise to a healthy seedling (Alonso and Barbedo 2020). Also, there is no visible distinction between the embryo and the fused cotyledons (Lucas et al. 2005). If a seedling dies or is detached from the seed, the seed will originate a new one due to its capacity of differentiating new embryos directly from the cotyledons (Amorim et al. 2020). The large seed size and the rare abilities mentioned above suggest that *Eugenia* seed reserves are present in amounts greater than the necessary to generate a seedling (Barbedo 2018). Another important characteristic of *Eugenia* seeds is that they are sensitive to desiccation (Delgado and Barbedo 2007).

Desiccation-sensitive (DS) seeds are dispersed highly hydrated, with water contents (WC) above 0.4 g H_2O g DW^{-1} and metabolically active, and generally lose viability if WC drops below 0.2 g H_2O g DW^{-1} (Walters 2021). Moreover, DS seeds may lack the ideal metabolic orchestration to resist water loss, such as efficiency for repairing DNA damage, effective antioxidant systems and accumulation of protective molecules (Leprince et al. 2017). During DS seed desiccation, the active metabolism generates uncontrolled amounts of reactive oxygen species (ROS), which may cause damage to cell organelles, proteins and DNA, lipid peroxidation and disruption of the membrane integrity (Umarani et al. 2015). The membrane systems become irreversibly disrupted under desiccation and unable to reorganize into the original structure upon water uptake, leading to cytoplasmic leakage and consequently seed death (Yu et al. 2015). This is caused in part by the activity of phospholipases D (PLD), which hydrolyses membrane phospholipids into phosphatidic acid (PhA) and respective head group (Ruelland et al. 2015). PhA molecules are important for signaling under several stresses, including freezing, wounding, salt and dehydration (Vadovic et al. 2019). However, high levels of PhA predispose membrane leakage and seed deterioration (Devaiah et al. 2007). Therefore, the inhibition of PLD activity and PhA formation may increase seed desiccation tolerance by

maintaining cell membranes stable and decreasing the metabolism-induced oxidative damage. Chen et al. (2017) imbibed *Castanea mollissima* seeds in 1-butanol (1-BUT) or N-acylethanolamine (NAE), which are PLD activity inhibitors, and found increased post-desiccation seed viability. The authors also found similar results by imbibing *Litchi chinensis* with NAE 12:0. Therefore, studying the effects of these compounds might help understanding the behavior of DS-seeded species upon desiccation that leads to viability loss. Moreover, 1-BUT and NAE may be a promising way to enhance the desiccation tolerance of *Eugenia* seeds as well.

Dryland areas in the globe are expected to increase by 10% by 2100 due to climate change (Mayrinck et al. 2019), thus the discovery of methods that can induce the desiccation tolerance of DS seeds is essential for biodiversity maintenance. For instance, the conservation of DS seeds may be achieved by drying and cryopreserving excised embryonic axes, which are smaller than the whole seed (Wesley-Smith et al. 2015). However, in the case of *Eugenia* species it is particularly tricky due to the seed characteristics. Therefore, finding alternative procedures that can lead to achieve the requirements to preserve the genetic material of *Eugenia* seeds may be challenging. In this study, we used *E. astringens* as a model, since it is a non-domesticated, endemic and restricted to the Atlantic Rainforest species, and has been proven to produce DS seeds (Delgado and Barbedo 2007, Rodrigues et al. 2022). We aimed to investigate the effects of 1-BUT and NAE on the seed physiology of *E. astringens* upon desiccation, especially on the seed oxidative metabolism. Our goal was to analyze the effects of 1-BUT and NAE, and their potential in decreasing the desiccation sensitivity of *E. astringens* seeds. We hypothesized that 1-BUT and NAE attenuate the sensitivity of *E. astringens* to desiccation by decreasing membrane disruption. We analyzed and linked their effects on seed germination and desiccation with electrolytic leakage (EL), lipid peroxidation, antioxidant enzymes and polyamines (PAs). Our study brings new insights into *Eugenia* seed physiology behavior associated with new conservation procedures.

MATERIAL AND METHODS

Plant material

The experiments were carried out from July 2019 to February 2021. *Eugenia astringens* ripe fruits were harvested from a population of 10 plants at Florianopolis-SC (27°36'14.0"S

48°31'17.9"W). After harvest, fruits were carried to the laboratory and stored at 4ºC for three days before the beginning of the experiments.

Application of inhibitors and seed desiccation

Seeds were removed from the fruit pulp and rinsed thoroughly in distilled water until seeds were cleaned from pulp residual, then dried superficially with paper. Initial WC was determined by the oven method at 105±2ºC for 24 h using 4 replicates of 25 seeds, and WC was expressed in dry weight basis (g H_2O g DW^{-1}) (Brasil 2013). Seed desiccation was performed by seating seeds on grids in hermetically sealed plastic boxes containing silica gel. For the application of inhibitors, seeds were desiccated for 5 h at room temperature (24±3ºC) on silica and then imbibed in distilled water (H_2O), 0.3% (v/v) 1-butanol (1-BUT) or 100 μM N-Acylethanolamine 12:0 (NAE) for 24 h in the dark. Control (CNT) seeds were not imbibed in any solution. Next, seeds of each imbibition treatment and CNT seeds were separated in two batches: one was immediately used in germination, EL, antioxidant enzymes, lipid peroxidation and PAs assays, while the other was reinserted in the boxes with silica to desiccate until they reached 0.25 g H_2O g DW^{-1} before the conduction of the same assays. To evaluate the desiccation on silica, seed samples were weighted every 3 h in the first 24 h; then every 6 h for the next 48 h and every 12 h afterwards. Silica gel was replaced every 12 h during the first two days and every 24 h subsequently. Briefly, eight treatments were used in all the assays: seeds imbibed in H_2O, 1-BUT and NAE for 24h prior and after desiccation to 0.25 g H_2O g DW^{-1}, and CNT seeds at initial WC and 0.25 g H_2O g DW^{-1}.

Germination assay

Four replicates of 30 seeds from each of the eight treatments were disinfested in sodium hypochlorite (1% v/v) for 10 min and rinsed three times in distilled water. Seeds were placed in Germitest roll papers moistened with distilled water and incubated in germination chambers at 25±2ºC and 12h photoperiod. Roll papers were moistened as required. Germination tests were carried out for 14 weeks and seeds were assessed every two days. Seeds were considered as germinated when they presented 2 mm of radicle protrusion. At the end of the test, germination rate (%) and germination speed index (GSI) (Maguire, 1962) were analyzed.

Electrolytic leakage (EL)

Three replicates of 20 seeds from each of the eight treatments were submerged in 50 ml of distilled water during 12 h. After, the electrolytic leakage was measured using a conductometer mCA-150P MS Tecnopon. Seed replicates were weighted before the test to express final leakage as $\mu S\ cm^{-1}\ g^{-1}$.

Antioxidant enzymes analysis

For the antioxidant enzyme assays, three samples (300 mg FM) originated from ten seeds of *E. astringens* from each of the eight treatments were ground with 1 mL of 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 few modifications. The homogenate was centrifuged at 15000 g for 20 min at 4ºC. The resulting supernatant was filtered and used for the enzyme assays. Superoxide dismutase (SOD; EC 1.15.1.1) activity was measured by monitoring the inhibition of NBT photochemical reduction at 560 nm, according to Giannopolitis and Ries (1977). Ascorbate peroxidase (APX; EC 1.11.1.11) activity was determined by following the decrease at 290 nm (extinction coefficient 2.8 $mM^{-1}\ cm^{-1}$) (Koshiba, 1993). Catalase (CAT; EC 1.11.1.6) activity was determined by following the consumption of H_2O_2 (extinction coefficient 39.4 $mM^{-1}\ cm^{-1}$) at 240 nm (Peixoto et al., 1999). Protein content was determined according to Bradford (1976) at 595 nm, with bovine serum albumin (BSA) as standard. SOD, CAT and APX activities of each extract were measured three times, and the results correspond to the means ± SD of the values obtained with three different extracts and three measurements per extract (i.e., nine measurements). The enzyme activities and protein content were performed using a spectrophotometer Spectra-Max® 190 Microplate Reader.

Lipid peroxidation

Malondialdehyde (MDA) measurements were estimated according to Hodges et al. (1999), with few modifications. Three samples (300 mg FM) originated from ten seeds of *E. astringens* from each of the eight treatments were homogenized with 3 ml of 0.1% (w/v) trichloroacetic acid (TCA) and centrifuged at 12.000 rpm 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 rpm for 15 min. Content of malondialdehyde-thiobarbituric acid complex (MDA) was measured using spectrophotometer

(Spectra-Max® 190 Microplate Reader) at 532 nm and corrected by subtracting the absorbance at 600 nm. Lipid peroxidation was calculated using the extinction coefficient of 157 mM^{-1} cm^{-1}.

PAs quantification

For PAs quantification, three samples (300 mg FM) originated from ten seeds of *E. astringens* from each of the eight treatments were ground in 1.6 mL of 5% (v/v) perchloric acid. Free PAs were extracted, dansylated and quantified according to Steiner et al. (2007), with few 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: putrescine (PUT), spermidine (SPD), and spermine (SPM). The compound 1,7-diaminoheptane (DAH) was used as internal standard. PA contents were expressed in dry basis (µmol. g^{-1} DW).

Statistical analysis

Experiments were carried out in a completely randomized design with 4 treatments (CNT, H_2O, 1-BUT and NAE) and 2 WC (initial and 0.25 g H_2O g DW^{-1}). Germination, EL, antioxidant enzymes, lipid peroxidation and PA data were subjected to one-way ANOVA and the mean differences were compared by Tukey ($p \leq 0.05$). Data were expressed as the mean ± SD. Statistical analyses were performed in R core team (2021).

RESULTS

Imbibition reduced desiccation time and improved germination of desiccated seeds

Seeds of *E. astringens* were shed with 1.02 g H_2O g DW^{-1} and after 5 h of desiccation WC decreased to 0.86 g H_2O g DW^{-1} (fig. 1A). Seeds desiccated for 5 h and then imbibed in the respective solutions increased WC to 1.06 g H_2O g DW^{-1} after 24 h (fig. 1A). Nonetheless, imbibition in H_2O, 1-BUT and NAE led to faster desiccations in comparison with CNT seeds, at which seeds took 170, 214 and 234 h until 0.25 g H_2O g DW^{-1}, respectively. CNT seeds were the slowest to desiccate, taking almost 300 h to reach the same WC (fig. 1A).

We also verified the effects of H_2O, 1-BUT and NAE imbibition on seed germination (fig. 1B). Seeds of *E. astringens* imbibed for 24 h in H_2O, NAE or 1-BUT and CNT seeds that were not desiccated achieved 100% of germination by 6 weeks (fig. 1B). However, desiccated seeds from all treatments presented reduced germination (fig. 1B). Surprisingly, H_2O- and 1-BUT-desiccated seeds exhibited 62 and 53% of germination, respectively, while NAE-desiccated seeds germinated 28% and CNT-desiccated seeds presented only 13% of germination at 0.25 g H_2O g DW^{-1} (fig. 1B).

GSI was also significantly affected by imbibition and desiccation (fig. 1C). Although a 100% of seeds germinated in the four treatments before desiccation, GSI was slightly higher in CNT non-desiccated (1.5) and H_2O-imbibed (1.39) seeds in comparison to NAE- and 1-BUT-imbibed seeds, with 1.27 and 1.26 of GSI, respectively (fig 1C). However, seeds desiccated to 0.25 g H_2O g DW^{-1} that were previously imbibed in H_2O and 1-BUT germinated faster (0.38 and 0.31, respectively) than NAE-desiccated seeds (0.11) and CNT-desiccated (0.04) seeds, though still very slow compared to non-desiccated seeds (fig. 1C).

The EL of *E. astringens* seeds imbibed in H_2O, NAE and 1-BUT was three times lower than the EL of CNT non-desiccated seeds, which displayed 14.16 μS cm^{-1} g^{-1} of EL (fig. 1D). At 0.25 g H_2O g DW^{-1}, H_2O, NAE and 1-BUT-desiccated seeds displayed an average of 12 μS cm^{-1} g^{-1} of EL, while CNT-desiccated seeds presented 31.37 μS cm^{-1} g^{-1} (fig. 1D).

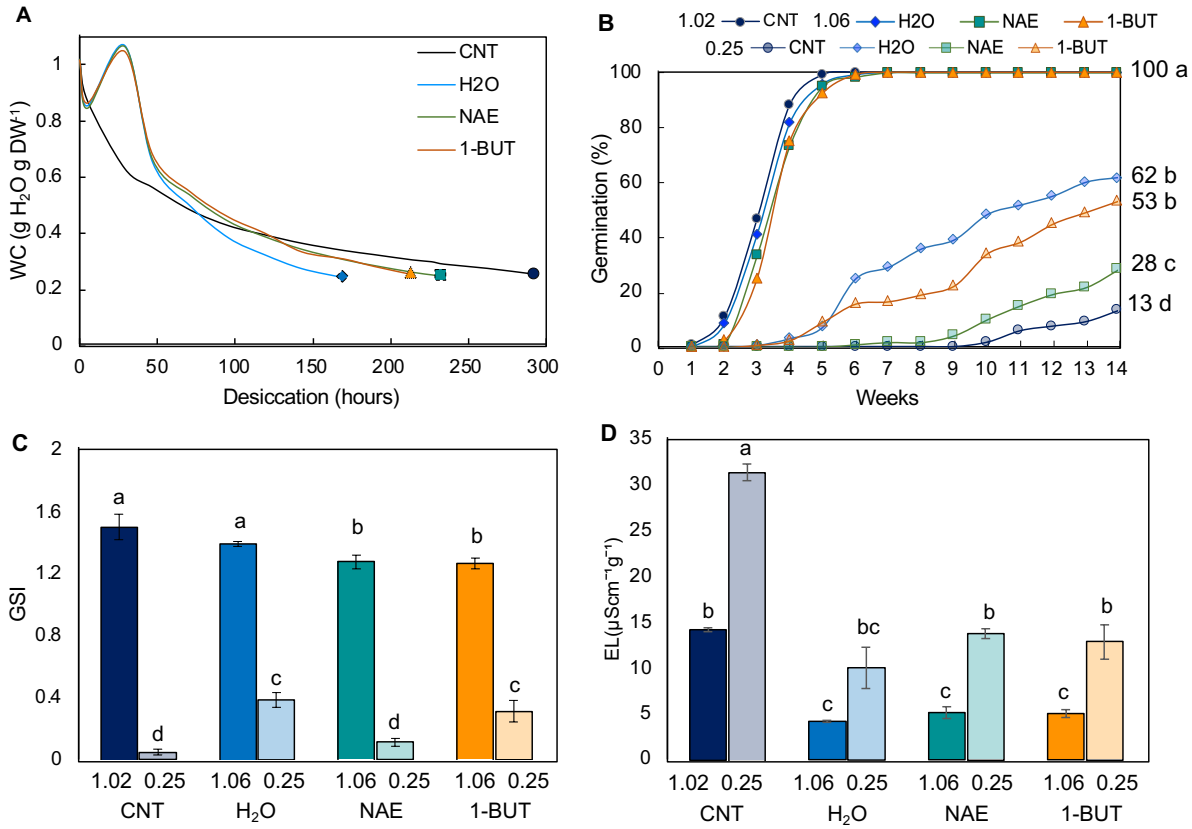

Figure 1. Seed physiological behavior of *E. astringens* upon desiccation. **(a)** desiccation speed, **(b)** germination dynamics (%), **(c)** germination speed index (GSI) and **(d)** electrolytic leakage (EL). Seeds at 1.02 g H_2O g DW^{-1} were initially desiccated in silica gel for 5 h, then imbibed in H_2O, 100 µM N-Acylethanolamine 12:0 (NAE) or 0.3% 1-Butanol (1-BUT) for 24 h, which increased WC to 1.06 g H_2O g DW^{-1}, and put back to desiccate until they reached 0.25 g H_2O g DW^{-1}. Control seeds (CNT) were not imbibed in any solution and followed direct desiccation. Data are means of 4 replicates ± SD. Columns having different letters are significantly different by the Tukey test at 5% probability.

Imbibition affected antioxidant enzymes and reduced lipid peroxidation

SOD activity increased in H_2O and NAE-imbibed seeds, while it decreased in 1-BUT-imbibed seeds when no desiccation was applied in comparison with CNT non-desiccated seeds (fig 2A). When *E. astringens* seeds were desiccated to 0.25 g H_2O g DW^{-1}, SOD activity increased by 5.5-fold in 1-BUT-desiccated seeds and by 3.7-fold in CNT-desiccated seeds. Conversely, SOD decreased by 57% in NAE-desiccated seeds (fig. 2A).

CAT activity decreased in H_2O- and NAE-imbibed seeds in comparison with CNT non-desiccated seeds (fig. 2B). At 0.25 g H_2O g DW^{-1}, CAT increased in 1-BUT-desiccated seeds, which presented the highest activity of this enzyme upon desiccation, while significantly decreased in CNT-desiccated seeds (fig. 2B).

NAE-imbibed seeds presented increased APX, while 1-BUT-imbibed seeds presented reduced APX activity in relation to CNT non-desiccated seeds (fig. 2C). When *E. astringens* seeds were desiccated to 0.25 g H_2O g DW^{-1}, APX decreased for all treatments, with a reduction of 85% in NAE- and H_2O-desiccated seeds, 52% in 1-BUT-desiccated seeds and 45% in CNT-desiccated seeds (fig. 2C).

Initial lipid peroxidation was similar between H_2O-, NAE- and 1-BUT-imbibed seeds and CNT non-desiccated seeds, with an average of 0.9 mM MDA g FW^{-1} (fig. 2D). Lipid peroxidation increased for *E. astringens* seeds in all treatments at 0.25 g H_2O g DW^{-1}. H_2O-, 1-BUT- and NAE-desiccated seeds presented the lowest increases in lipid peroxidation (~70%), while lipid peroxidation of CNT-desiccated seeds increased by 2.15-fold at 0.25 g H_2O g DW^{-1} (fig. 2D).

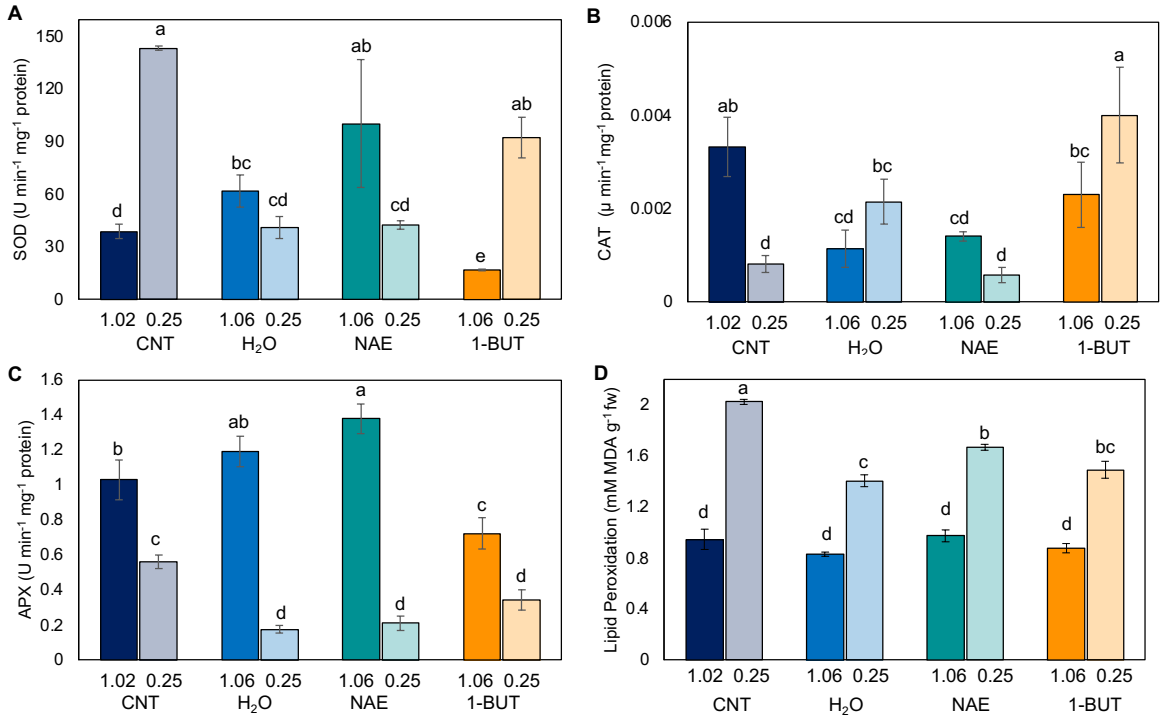

Figure 2. Enzyme activity of **(a)** superoxide dismutase (SOD), **(b)** catalase (CAT), (**c**) ascorbate peroxidase (APX), and **(d)** lipid peroxidation (MDA) of *E. astringens* seeds. Seeds at 1.02 g H_2O g DW^{-1} were initially desiccated in silica gel for 5 h, then imbibed in H_2O, 100 μM N-

Acylethanolamine 12:0 (NAE) or 0.3% 1-Butanol (1-BUT) for 24 h, which increased WC to 1.06 g H_2O g DW^{-1}, and put back to desiccate until they reached 0.25 g H_2O g DW^{-1}. Control seeds (CNT) were not imbibed in any solution and followed direct desiccation. Data are means of 3 replicates ± SD. Columns having different letters are significantly different by the Tukey test at 5% probability.

1-BUT increased PA content and ratio prior desiccation, but reduced at 0.25 g H_2O g^{-1} DW

We analyzed total free PA content, free PUT, SPD and SPM, and PA ratio [PUT/(SPD+SPM)] in *E. astringens* seeds before and after desiccation to 0.25 g H_2O g^{-1} DW in the four treatments (fig. 3). Total PA content was higher in seeds imbibed in 1-BUT in comparison to CNT non-desiccated and H_2O-imbibed seeds (fig. 3A). When *E. astringens* seeds were desiccated to 0.25 g H_2O g DW^{-1}, total PA doubled in H_2O-desiccated seeds, while no significant changes were observed in other treatments (fig. 3A).

Next, we analyzed the effects of H_2O, NAE and 1-BUT on the PAs separately (fig. 3B, C, D). Seeds of *E. astringens* imbibed in 1-BUT presented increased PUT in comparison to CNT non-desiccated and H_2O-imbibed seeds (fig. 3B). At 0.25 g H_2O g DW^{-1}, the only significant PUT change was observed in 1-BUT-desiccated seeds, at which PUT decreased by 95% (fig. 3B). In relation to SPD, when no desiccation was applied 1-BUT-imbibed seeds presented higher contents in comparison to H_2O-imbibed seeds (fig. 3C). After desiccation to 0.25 g H_2O g DW^{-1}, H_2O was the only treatment at which seeds presented significant increases in SPD (2.16-fold increase) (fig. 3C). The contents of SPM were also higher in 1-BUT-imbibed seeds in comparison to H_2O- and NAE-imbibed seeds (fig. 3D). After desiccation to 0.25 g H_2O g DW^{-1}, SPM content increased by 2.29-fold in H_2O-desiccated seeds (fig. 3D). H_2O- and 1-BUT-imbibed seeds presented increased PA ratio before desiccation (fig. 3C). PA ratio of 1-BUT-desiccated seeds strongly decreased and was lower than all other treatments at 0.25 g H_2O g DW^{-1} (fig. 3C).

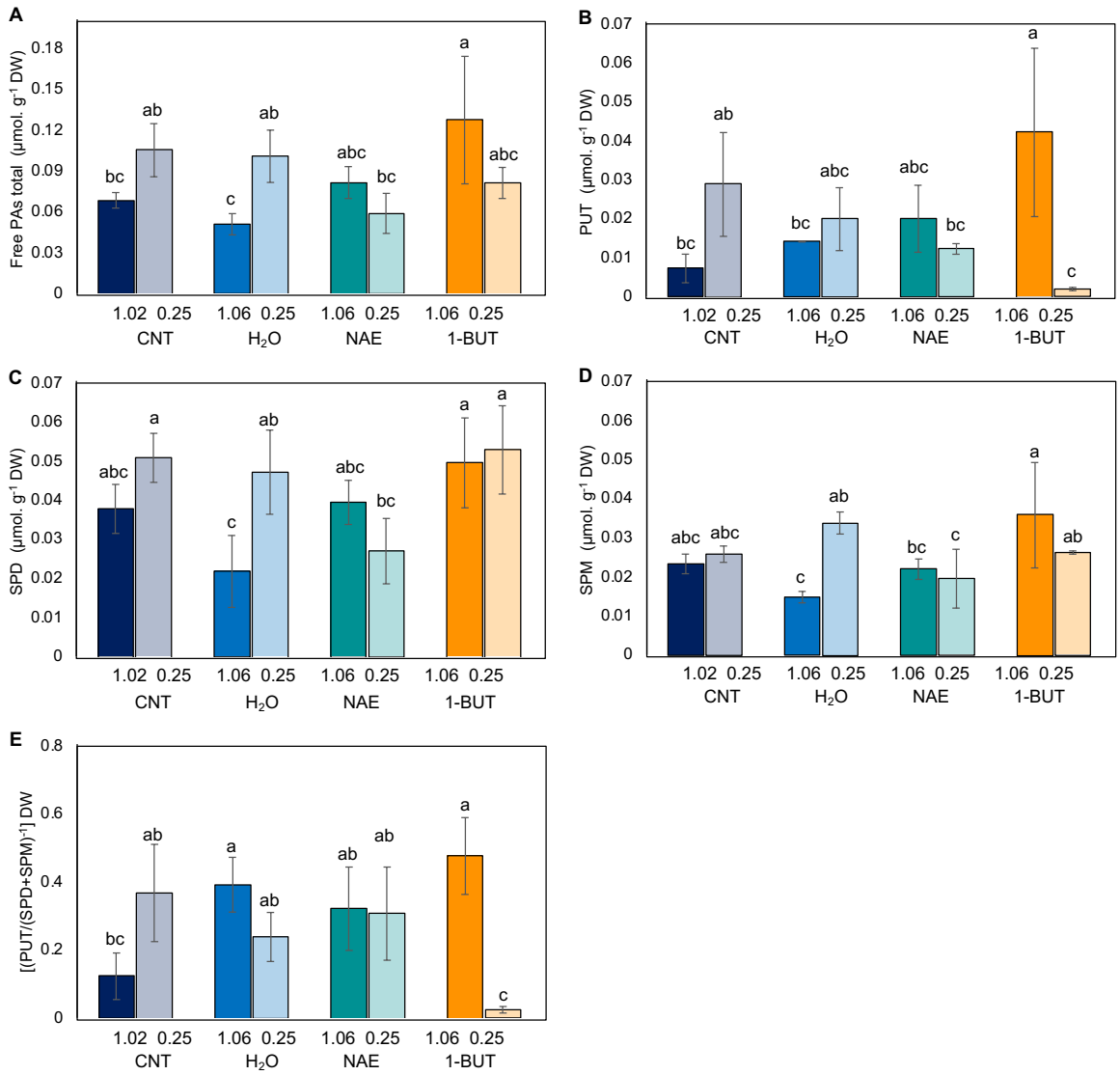

Figure 3. Polyamines (PAs) of *Eugenia astringens* seeds. **(a)** free PAs total, **(b)** Putrescine (PUT), **(c)** Spermidine (SPD), **(d)** Spermine (SPM) and **(e)** PA ratio [PUT/(SPD+SPM)]. Seeds at 1.02 g H_2O g DW^{-1} were initially desiccated in silica gel for 5 h, then imbibed in H_2O, 100 µM N-Acylethanolamine 12:0 (NAE) or 0.3% 1-Butanol (1-BUT) for 24 h, which increased WC to 1.06 g H_2O g DW^{-1}, and put back to desiccate until they reached 0.25 g H_2O g DW^{-1}. Control seeds (CNT) were not imbibed in any solution and followed direct desiccation. Data are means of 3 replicates ± SD. Columns having different letters are significantly different by the Tukey test at 5% probability.

DISCUSSION

Desiccation to 0.25 g H_2O g DW^{-1} severely affected *E. astringens* seeds and led to a reduction in germination and vigor for all treatments. In a previous report, we have shown that desiccating *E. astringens* seeds for over 275 h was lethal (Rodrigues et al. 2022). In agreement with our previous results, CNT seeds presented only 13% of germination after ~300 h of desiccation (fig. 1A, B). Interestingly, the treatments at which seeds desiccated faster presented higher viability (fig. 1B), suggesting that the speed of water removal from seeds can influence their survival. A reduction of the exposure time to desiccation might increase the limit of water removal tolerated by seeds (José et al. 2011). Thus, the decrease in desiccation time might help explaining why H_2O, 1-BUT and NAE-imbibed seeds presented enhanced germination rates when desiccated to 0.25 g H_2O g DW^{-1} in comparison to CNT-desiccated seeds (fig. 1B). The desiccation period is where most of the degenerative processes occur, from a disruption of the metabolic regulation to a failure of antioxidant systems (Berjak and Pammenter 2013). Therefore, the slower the desiccation, the higher the accumulation of toxic compounds from the degenerative metabolism in DS seeds (Walters et al. 2021). Nevertheless, 1-BUT seeds took only 20 h less than NAE seeds to reach 0.25 g H_2O g DW^{-1}, but presented the same germination capacity as H_2O seeds, which took 64 h less (fig. 1A, B). The explicit above led us to consider that drying time was not the only factor affecting desiccation tolerance, but that other processes were taking place in these seeds upon imbibition and desiccation. Also, imbibing seeds in either NAE or 1-BUT before desiccation caused a slight reduction in the GSI, while H_2O had no such effects (fig. 1D). In a similar way, 1-BUT and NAE showed inhibitory effects on Arabidopsis seed germination and seedling development, which was reversed when seeds were imbibed in H_2O (Motes et al. 2005). The germination delay showed by NAE-imbibed seeds may be related to the link between this compound and abscisic acid (ABA), since it was previously shown that an exogenous NAE application increased the expression of ABA-encoded genes to inhibit seed germination (Coulon et al. 2012).

Both NAE and 1-BUT are known to knockdown the activity of PLD enzymes, and the inhibition of these led to enhanced viability of Arabidopsis seeds during storage (Devaiah et al. 2007). NAE-desiccated seeds doubled the germination rate in comparison to CNT-desiccated seeds and this may be a consequence of the ABA signaling promoted by NAE (Coulon et al. 2012). ABA is known to be responsible for the acquisition, induction and re-establishment of desiccation tolerance in tolerant-seeded species (Maia et al. 2014, Marques et al. 2019).

However, germination of NAE-desiccated seeds was still low and perhaps this is because NAE only inhibits PLDα enzyme activity (Jia and Li 2018). This indicates that the role previously described for NAE by itself was not capable of avoiding the germination reduction of *E. astringens* seeds upon desiccation (fig. 1B). Also, perhaps the NAE concentration used in *E. astringens* seeds was insufficient to induce a satisfactory PLDα inhibition at 0.25 g H_2O g DW^{-1}, since it is dependent of a NAE dose-response mechanism (Motes et al. 2005). On the other hand, 1-BUT inhibits PhA formation by any isoform of PLD (Jia and Li 2018), thus preventing a massive PhA accumulation during unbalanced metabolism upon desiccation (Chen et al. 2017). PhA is produced via several pathways and rapidly accumulates in the membranes as a reaction to numerous stresses (Pokotylo et al. 2018). Therefore, the PhA production inhibition effect described above for 1-BUT may have been more efficient in attenuating *E. astringens* seed viability loss upon desiccation (fig. 1B). To a certain degree, PhA serves as a signaling molecule for many fundamental metabolic processes, including the response to abiotic stress, such as drought, salt and oxidative stress, and membrane degradation (Yu et al. 2015).

In relation to EL, *E. astringens* seeds imbibed in H_2O, NAE or 1-BUT displayed lower increases in the EL in comparison with CNT seeds at 0.25 g H_2O g DW^{-1}, which pointed to a more damaged set of membranes in CNT-desiccated seeds (fig. 1D). Desiccation of DS seeds causes structural and permanent damage to vacuoles, cytoskeleton and cell membranes due to the water removal, leading to a higher membrane permeability and solute leakage during rehydration (Pammenter and Berjak 2014, Umarani et al. 2015). Cell membranes are in a lamellar phase when hydrated, but can form non-lamellar phases under water shortage, which is predisposed by PhA excess (Chen et al. 2017). PhA also activates NADPH oxidase to produce superoxide (O_2^-), a ROS that can be quickly converted to hydrogen peroxide (H_2O_2) (Abreu et al. 2018).

Next, the uncontrolled production of ROS caused by extended stress promotes lipid peroxidation, which is considered a major contributor of seed deterioration (Devaiah et al. 2007). Hence, the desiccation tolerance improvement observed in *E. astringens* seeds at 0.25 g H_2O g DW^{-1} after imbibing seeds in 1-BUT might also be related to the enhanced antioxidant enzyme activity and decreased lipid peroxidation observed in fig. 2. Antioxidant enzymes are part of the complex defense network system of seeds, which acts to scavenge the excessive ROS and to protect cells under oxidative stress (Goel et al. 2003).

The set of antioxidant enzymes includes superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) (Sano et al. 2016). SOD is the very first step taken towards

detoxification of cellular ROS, converting two O_2^- radicals into H_2O_2 and oxygen (Chandrakar et al. 2016). Apparently, NAE-desiccated seeds might have been unable to deal with the accumulated O_2^- at 0.25 g H_2O g DW^{-1}, which corresponded to the decrease in SOD displayed by seeds (fig. 2A). On the other hand, the increase in SOD activity in CNT- and 1-BUT-desiccated seeds might suggest that an elevated conversion of O_2^- into H_2O_2 took place during *E. astringens* seed desiccation. Next, CAT will be responsible for the dismutation of H_2O_2 into water and oxygen (Bailly et al. 2004). CAT is a crucial enzyme, since H_2O_2 is frequently considered as the most critical ROS; H_2O_2 has stability in the cell pH, can cross membranes and cause severe cell damage due to the highly aggressive hydroxyl radical it can generate (Kibinza et al. 2011). While CAT activity increased in 1-BUT-desiccated seeds at 0.25 g H_2O g DW^{-1}, CAT dropped for NAE- and CNT-desiccated seeds at the same WC (fig. 2B). Therefore, *E. astringens* 1-BUT-desiccated seeds might be scavenging H_2O_2 and controlling the oxidative stress caused by desiccation to 0.25 g H_2O g DW^{-1}, but NAE- and CNT-desiccated seeds were possibly accumulating these toxic molecules. H_2O_2 accumulation is also controlled by the APX activity (fig. 2C). APX is important for H_2O_2 detoxification, since it reduces H_2O_2 using ascorbate as an electron donor (Goel et al. 2003). High endogenous ascorbate levels are essential to regulate APX activity because APX enzyme is labile in the absence of this compound (Caverzan et al. 2012). However, APX decreased in all treatments, which might indicate that ascorbate was degraded upon desiccation of *E. astringens* seeds, especially in NAE and H_2O-desiccated seeds at 0.25 g H_2O g DW^{-1} (fig. 2C). Another evidence of oxidative and structural damage is lipid peroxidation (fig. 2D). Lipid peroxidation, measured by the accumulation of its final product MDA, is closely linked to leaky or disrupted membrane integrity (Parkhey et al. 2012). We observed that the higher the MDA levels, the lower the germination in *E. astringens* seeds at 0.25 g H_2O g DW^{-1} (fig. 2D). Similar results were observed in *Madhuca latifolia* DS seeds, at which increased MDA was associated with EL rise, elevated ROS levels and decreased germination (Chandra and Keshavkant, 2018).

The protection of membranes and ROS scavenging may also be achieved by the action of low molecular weight non-enzymatic antioxidants, such as polyamines (PAs) (Wimalasekera et al. 2011). The increase in total PA content of 1-BUT-imbibed seeds mainly derived from the rise in PUT, which increased by ~6-fold in relation to CNT non-desiccated seeds (fig. 3B). Interestingly, the exogenous application of PUT was reported to increase the activity of PLD enzymes in response to a short period of drought in maize, which could be an early protective mechanism (An et al. 2012). Similarly, the accumulation of PUT in 1-BUT-imbibed seeds may

be an early response that was activated when seeds were previously desiccated for 5 h and then imbibed in 1-BUT which would prevent seeds from further desiccation damage. Conversely, after desiccation to 0.25 g H_2O g DW^{-1}, the PUT content of 1-BUT-desiccated seeds was strongly degraded (fig. 3 B). PUT is the central product of the common PA biosynthetic pathway and can be the precursor of SPD and SPM (Chen et al. 2019). Hence, PUT may have served as a direct substrate for the maintenance of the SPD and SPM content in *E. astringens* 1-BUT-desiccated seeds at 0.25 g H_2O g DW^{-1} (fig. 3 C,D). An accumulation of PA content has been reported under several abiotic stresses, and high cellular levels of PAs correlated with increased stress tolerance (Hussain et al. 2011). PAs are known to stabilize membrane phospholipids, directly scavenge ROS and serve as signal molecules in ABA-regulated stress response pathway (Minocha et al. 2014, Juzón et al. 2017), which are crucial steps during seed desiccation. In our study, it appears that imbibing *E. astringens* seeds in H_2O enhanced desiccation tolerance by increasing free PA contents to improve membrane stabilization, ROS scavenge and ABA signaling, more specifically SPD and SPM (fig. 3C, D). These PAs are responsible for modulating membrane permeability and stability (Parvin et al. 2014). Another protective role of SPD is to increase the transcription level of genes encoding antioxidant enzymes (Pál et al. 2015), while SPM refines the antioxidant responses of enzymes such as SOD and CAT (Seifi and Shelp 2019). Therefore, SPD and SPM might have an involvement in regulating the activities of SOD and CAT in H_2O-desiccated seeds, in comparison to the SOD drop displayed by NAE-desiccated seeds and the CAT decrease seen in CNT-desiccated seeds at 0.25 g H_2O g DW^{-1} (fig. 2 A, B).

What is interesting is our study is that in *E. astringens,* H_2O-desiccated seeds at 0.25 g H_2O g DW^{-1} maintained higher germination rates and higher GSI in comparison to CNT- and NAE-desiccated seeds (fig. 1 B, C). Previous imbibition in H_2O caused similar germination improvements in *Castanea mollissima* seeds upon desiccation (Chen et al. 2017). This may be a consequence of the hydration-dehydration (HD) cycles that seeds went through during our experiment. The discontinuity during water absorption of seeds stimulates alterations in the seeds, such as differential protein expression, which can improve survival rate during desiccation and promote faster germinations (Contreras-Quiroz et al. 2016, Lima and Meiado 2018), such as observed in our study (fig. 1 B,C). Also, Maia et al. (2014) reported that the establishment of desiccation tolerance is driven by the modulation of ABA sensitivity rather than the ABA content, which may have been promoted by the HD cycles. The authors also suggested that that the acquisition of desiccation tolerance after dispersal is genetically distinct

from the innate tolerance acquired during seed maturation. Therefore, one hypothesis in need of further investigation is that the H_2O imbibition and desiccation activated an ABA cascade signaling that induced the improved desiccation tolerance of *E. astringens* seeds by increasing their ABA sensitivity. Taken together, our results demonstrate that it is possible to enhance the desiccation tolerance of *E. astringens* through seed imbibition in H_2O and 1-BUT, which will promote reduction in MDA and EL levels, improvements in PAs and antioxidant enzymes, and decrease seed desiccation time (fig. 4). Imbibing seeds in H_2O may serve as a cheap and easy way to enhance the desiccation tolerance of DS seeds, which may be the first step to promote the preservation of *Eugenia* seed material. Our study also brings new insights on the possible relationship between PAs and desiccation tolerance induction, which might involve PA metabolism. Understanding the DS seed physiology can have a strong impact when we look towards the concerning challenges of climate change and *ex-situ* seed conservation of wild species. In this aspect, advances in this area require scientific knowledge on the maintenance of seed viability under the expected increases in dry areas worldwide. Further studies should focus on analyzing the metabolic changes promoted by H_2O and 1-BUT imbibition in *Eugenia* seeds, as well as the ABA signaling pathway. Moreover, verifying the capacity of H_2O and 1-BUT in improving the desiccation tolerance of parts of *Eugenia* seeds would also be suitable for conservation techniques.

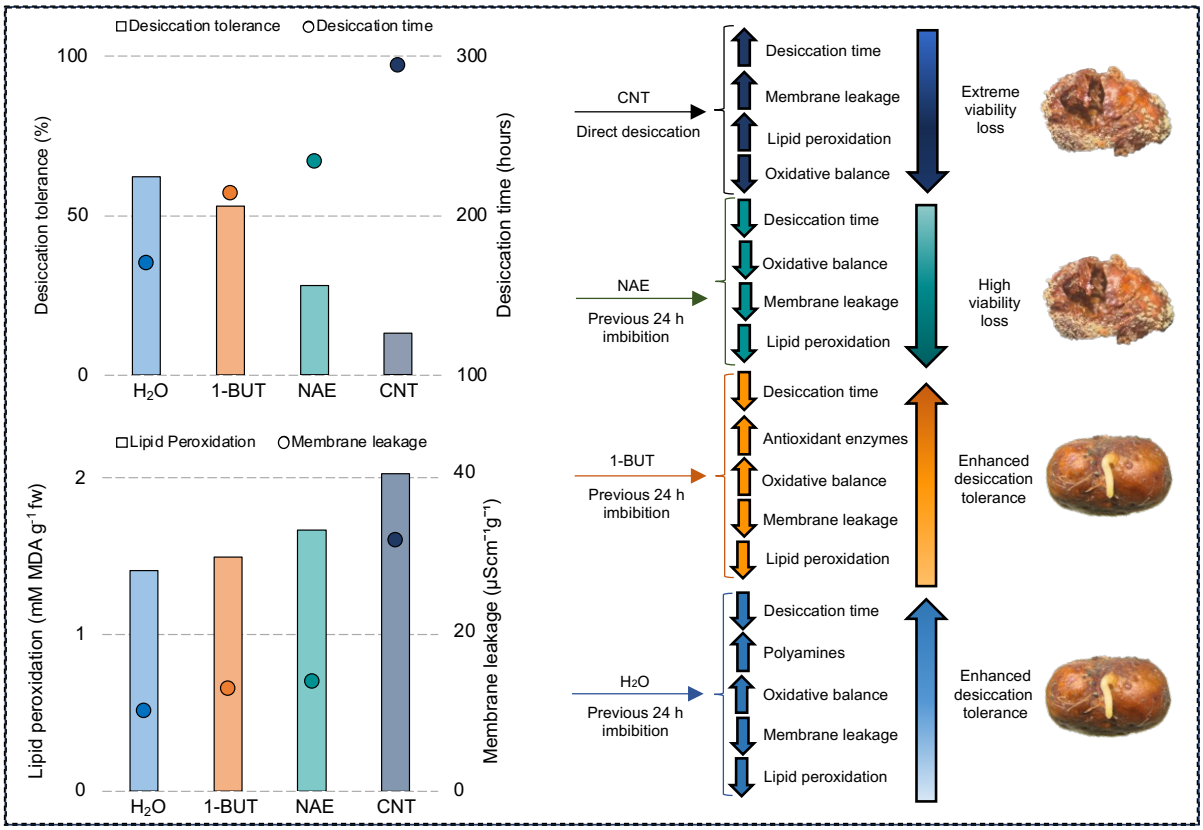

Figure 4. Graphical abstract of the impacts of imbibing *E. astringens* sensitive seeds in N-acylethanolamine (NAE), 1-butanol (1-BUT) and distilled water (H_2O) and desiccating afterwards. While germination decreased to ~10% in control (CNT) non-imbibed and desiccated seeds at 0.25 g H_2O g DW^{-1}, imbibition of seeds in either H_2O or 1-BUT before desiccating to 0.25 g H_2O g DW^{-1} improved germination to >50%. This might be related to decreased desiccation time, membrane leakage and lipid peroxidation, and increased polyamine contents and antioxidant activity brought by H_2O and 1-BUT imbibition, respectively. On the other hand, NAE presented lower MDA and EL rates, but no antioxidant action enhancement, thus only slightly improving germination (~30%). Colors bars and arrows indicate the specific physiological behavior and biochemistry results after seed imbibition and desiccation in each treatment.

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AUTHOR CONTRIBUTIONS

N.S. supervised the research; G.A.G.R. performed experiments and analyzed data; D.S. assisted experiments; D.G. ran polyamine analysis; G.A.G.R. and N.S. wrote the article.

STATEMENT AND DECLARATIONS

Conflict of interest Authors have no competing interests.

Ethical approval Not applicable.

Consent to participate Not applicable.

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9 CONCLUSÕES

As sementes de *Eugenia* estudadas neste trabalho possuem diferentes níveis de sensibilidade à dessecação, o que pode estar relacionado com os fatores ambientais presentes nos ambientes de origem das sementes, como temperatura e pluviosidade, e a amplitude de ocorrência das espécies, em que espécies mais restritas possuem menor sensibilidade do que aquelas mais amplamente distribuídas. Além disso, a atuação de enzimas antioxidantes e PAs pode ser responsável pela manutenção da viabilidade parcial de sementes sensíveis, visto o aumento observado nestes compostos em sementes durante a dessecação. Porém, não existe um padrão de comportamento das poliaminas e das enzimas antioxidantes, sendo que cada espécie respondeu particularmente de uma maneira, o que pode indicar que a viabilidade das sementes é perdida por diferentes origens, como mecânica em *E. pyriformis* e *E. brasiliensis* e metabólica em *E. involucrata* e *E. astringens*. Outro fator endógeno relacionado à viabilidade de sementes de *E. uniflora* e *E. astringens* é sua composição. Encontramos uma alta composição de carboidratos, que foram regulados positivamente durante a dessecação. Porém, *E. uniflora* possuiu uma maior presença de sacarose, enquanto *E. astringens* possuiu frutose e glicose. Ainda, aminoácidos estavam presentes apenas em pequenas quantidades e não foram regulados positivamente para nenhuma das duas espécies durante a dessecação. Ácidos orgânicos relacionados à respiração se mantiveram mais altos em sementes de *E. uniflora* durante a dessecação em comparação com sementes de *E. astringens*, que foram menos sensíveis ao estresse. Ainda, ácidos graxos saturados compuseram 98% da composição de ácidos graxos, enquanto a percentagem de ácidos graxos insaturados aumentou progressivamente durante a dessecação. Também verificamos que é possível diminuir a sensibilidade à dessecação de sementes de *E. astringens* a partir da embebição prévia em H_2O ou 1-BUT, onde germinações se mantiveram acima de 50%, mesmo após dessecação. Os resultados deste trabalho ampliam o conhecimento sobre a fisiologia das sementes de *Eugenia* sensíveis à dessecação nativas da Mata Atlântica, demonstrando a sensibilidade das sementes e a relação aos fatores exógenos (ambiente) e endógenos (metabolismo). Finalmente, as informações contidas nesta tese ampliam o conhecimento sobre espécies sensíveis à dessecação de *Eugenia* e apresenta técnicas que poderão servir de base para futuros estudos relacionados a conservação *ex-situ* de sementes sensíveis à dessecação.