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CARACTERIZAÇÃO ESTRUTURAL, FISIOLÓGICA E BIOQUÍMICA DE SEMENTES DE *Calibrachoa sellowiana* (SENDTN.) WIJSMAN

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Orientadora: Prof.ª Dr.ª Neusa Steiner

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por

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Eu mesmo, o que sou: O que foi que eu fiz?
Eu recolhi e utilizei tudo o que ouvi e observei.
Minhas obras foram alimentadas por milhares de indivíduos diversos.
Ignorantes e sábios, pessoas cultas e tolos.
A infância, a maturidade e a velhice vieram me oferecer suas ideias.
Suas faculdades, suas maneiras de ser.
Minha obra é a de um ser coletivo que leva o nome de LUAN

(GOETHE, 1832)

RESUMO

Calibrachoa sellowiana (Sendtn.) Wijsman (Solanaceae) é uma espécie endêmica nativa do Sul do Brasil e tem se tornado uma alternativa importante como planta ornamental. A germinação de sementes é chave essencial para a conservação, melhoramento genético e cultivo desta espécie. Assim, o presente estudo descreve pela primeira vez uma ampla gama de características fisiológicas de sementes de *C. sellowiana*, demonstrando as modificações morfoestruturais e bioquímicas associadas ao comportamento fisiológico na germinação de sementes. Sementes escuras e claras foram encontradas dentro de cápsulas apresentando diferenças fisiológicas entre elas. Enquanto as sementes escuras apresentaram desenvolvimento completo, as sementes pálidas apresentaram tegumento incompleto, um embrião atrofiado e um endosperma retraído, evidenciando as características de qualidade fisiológica dessas sementes. No entanto, uma germinação muito baixa foi observada em sementes escuras frescas, o que nos levou a assumir a presença de dormência fisiológica nesta espécie. O tratamento com Ácido Giberélico aumentou a germinação final e o índice de velocidade de germinação. Durante a protrusão radicular, todas as Poliaminas aumentaram consideravelmente, mostrando uma correlação entre Poliaminas e Giberelinas. Além disso, os dados mostram que as sementes de *C. sellowiana* toleraram à dessecação, até um teor muito baixo de água e armazenamento a baixas temperaturas, sem perda de vigor e viabilidade. Assim, este é o primeiro relato que mostra essas relações entre análises fisiológicas, ultraestruturais e bioquímicas em sementes de *C. sellowiana*, que podem ser úteis para futuros estudos sobre uso e conservação desta espécie bem como de sementes de outras espécies herbáceas e endêmicas.

Palavras-Chave: Solanaceae. Fisiologia da Semente. Poliaminas. Ortodoxia. Dormência.

RESUMO EXPANDIDO

Introdução

Calibrachoa Cerv. é um gênero botânico que pertence à família Solanaceae e tem se tornado um importante cultivar no paisagismo. *Calibrachoa* é endêmico do sul e sudeste brasileiro, sendo que a maioria das espécies deste gênero ocorrem nos estados do Rio Grande do Sul e Santa Catarina (STEHMANN, 2018). Com flores vistosas e muito coloridas, muitas *Calibrachoa* spp. possuem mecanismos de autoincompatibilidade polínica e são polinizadas por abelhas e vespas (síndrome de polinização melitofílica) gerando grande número de sementes (STEHMANN e SEMIR, 2001). No entanto, apesar do aumento considerável de dados de análise de sementes de espécies nativas, muitas ainda carecem de informações básicas referentes às condições ideais de germinação e armazenamento e os estudos têm sido pouco aprofundados em relação ao comportamento germinativo de algumas espécies de interesse ornamental, como os requerimentos que a semente necessita para germinar, os tipos de dormência, embebição, estoque de reservas e dinâmicas da germinação (BASKIN e BASKIN, 2004).

A dormência de sementes é considerada um bloqueio de germinação de uma semente viável intacta, mesmo que esta receba condições ótimas para germinar como água, luz e temperatura ideal (Bewley et al. 2013). A família Solanaceae inclui culturas economicamente importantes como *Solanum tuberosum* e *S. melongena* e muitas outras consideradas dormentes. Assim, esta família na qual o embrião é cercado por um endosperma rígido, apresenta modelos adequados para a compreensão da biologia da germinação e pesquisa de dormência (Koornneef et al. 2002). A dormência fisiológica é a forma mais abundante encontrada em sementes de Solanaceae, incluindo *Solanum lycopersicum* e *Nicotiana* spp. (Finch-Savage e Leubner-Metzger 2006) e seu mecanismo envolve os reguladores de crescimento vegetais, principalmente Ácido Abscísico e Giberelinas. Além disso, este balanço hormonal provoca alterações em outras biomoléculas, como as Poliaminas, que podem atuar como segundos mensageiros na via de sinalização das Giberelinas, mediando a dormência e a germinação de sementes (Palavan e Galston, 1982).

Poliaminas são pequenas aminas alifáticas de baixo peso molecular encontradas em quase todos os tipos de células (Bouchereau et al. 1999; Gupta et al. 2013). Putrescina, Cadaverina, Espermina e Espermidina constituem os principais Poliaminas (Kaur-sawhney et al. 2003) e estão

envolvidos em alguns procedimentos de replicação, transcrição, tradução (Kaur-sawhney et al. 2003), estabilização de membrana, modulação de atividade enzimática, divisão celular e expansão (Gupta et al. 2013). Devido a estas características, eles também estão envolvidos na germinação das sementes, afetando todo o metabolismo da planta. Poliaminas também estão envolvidas na defesa contra fatores abióticos estressantes, como dessecação ou frio durante o armazenamento das sementes.

O armazenamento de sementes consiste em armazenar as sementes tentando manter a máxima qualidade fisiológica, sendo um método barato de conservação *ex situ* (Souza et al. 2016). Um método que tem sido frequentemente usado para a conservação de sementes é o armazenamento à frio. Essa técnica é conhecida como opção viável para armazenamento de células vegetais, tecidos, sementes e embriões (Reed, 2008). Estudos de informações fisiológicas, estruturais e bioquímicas em relação à capacidade do armazenamento de sementes de espécies aumentaram devido à necessidade de sementes viáveis para programas de conservação. Esta informação é estritamente necessária, uma vez que é considerada decisiva para manter a viabilidade após a exposição a baixas temperaturas.

Neste contexto, a investigação de características básicas de *Calibrachoa sellowiana* (Sendtn.) Wijsman, como qualidade fisiológica, vigor e viabilidade, tolerância à dessecação e armazenamento, se tornam imprescindíveis para melhor compreensão da biologia do desenvolvimento da espécie bem como do uso destas sementes para o cultivo. Também, não há estudos específicos na literatura sobre o perfil hormonal e características estruturais de sementes de *C. sellowiana*, uma espécie que está presente nos habitats sulinos brasileiros e que ainda permanece incógnita para muitas pessoas.

Objetivos

O objetivo deste trabalho foi estudar sementes maturas e germinadas de *C. sellowiana* afim de relacionar modificações anatômicas, ultraestruturais e bioquímicas com o comportamento de dormência fisiológica nas três fases da germinação. Além disso, determinamos o limiar de tolerância à dessecação e armazenamento a baixas (8ºC, -20ºC) e ultrabaixa (-196ºC) temperaturas de sementes de *C. sellowiana*, bem como analisamos o conteúdo do perfil de Poliaminas livres e alterações ultraestruturais durante a dessecação e armazenamento dessas sementes. Este é o primeiro relato que apresenta uma ampla gama

de características de uma espécie endêmica e ruderal do Sul do Brasil, mostrando as relações entre características fisiológicas, ultraestruturais e bioquímicas em sementes de *C. sellowiana*, uma espécie ornamental promissora.

Metodologia

Frutos (cápsulas) de *C. sellowiana* foram coletadas no município de Curitibanos (987 metros de altitude; 27º16'58" latitude Sul; 50º35'04" longitude Oeste) no Estado de Santa Catarina, em campo natural aberto.

O teste de Tetrazólio foi realizado com 3 amostras de 25 sementes embebidas em água destilada por 0, 2, 4, 6, 8, 10, 12 horas, em seguida, reagidas com cloreto de 2,3,5 Trifenil Tetrazólio (1%) a 25±2ºC no escuro por 1 hora (Brasil 2009). A Eletrocondutividade foi medida com 4 amostras de 25 sementes pesadas e submersas em 75,0 ml de água destilada. As amostras foram mantidas em câmara de incubação a 25±2ºC por 12 horas. A cada hora, a Eletrocondutividade foi medida com um eletrocondutivímetro de massa e expressa $\mu S.cm^{-1}g^{-1}$.

Para o teste de Germinação, as sementes foram previamente desinfetadas em solução etanólica (70%) por 30 segundos, em solução de hipoclorito de sódio (1%) por 1 minuto e, finalmente lavadas 3 vezes em água estéril. As amostras foram inoculadas em placas de Petri com papel Germitest® umedecido com 2,0 ml de água ou diferentes concentrações de Ácido Giberélico (50, 100, 250, 500, 750, 1000 µM) a 25±2ºC à luz (120 µEm-2s-1, 12/12 horas) por 50 dias. O Índice de Velocidade de Germinação foi calculado de acordo com Maguire (1962). A Morfometria das plântulas foi medida por imagens usando o software Image J® (versão 1.52, 2018) onde foram analisadas 24 plântulas para cada tratamento. A Curva de Embebição foi realizada por 4 amostras de 50 sementes. Foi pesado a cada 30 minutos até 6 horas; então a cada 1 hora até 12 horas e, depois disso a cada 24 horas, até atingir 51% de germinação (Brasil 2009).

Análises de Microscopia de Luz, Microscopia Eletrônica de Varredura e Transmissão (Steiner et al. 2015) foram realizadas de acordo com protocolos já estabelecidos. As análises de Poliaminas foram realizadas por 3 amostras de 200 mg de sementes moídas em 1,6 ml de ácido perclórico (5%). As Poliaminas livres foram extraídas, dansiladas e identificadas por HPLC de fase reversa, de acordo com Steiner et al. (2007).

Para o armazenamento, as sementes foram dessecadas a 4,02% e 6,96% do conteúdo inicial de água (11,63%) através do método em sílica gel (Rao *et al*. 2006). As amostras provindas da dessecação foram

armazenadas em três temperaturas: 8°C, -20°C e -196°C. Então, as sementes foram submetidas aos testes de Tetrazólio, Eletrocondutividade, Germinação, Análise de Poliaminas e Microscopia Eletrônica de Transmissão conforme já descritos anteriormente.

Os dados obtidos foram submetidos à análise de variância (ANOVA) e as médias comparadas pelo teste de Tukey ou SNK através do Software R® (versão 3.4.1, 2017), considerando estatisticamente significativos os valores de $p<0,05$.

Resultados e Discussão

No presente trabalho, encontramos dois tipos de sementes de *C. sellowiana* de acordo com a coloração da testa: sementes escuras (DS) e sementes pálidas (PS). Além do maior peso fresco e do teor relativo de água, DS apresentaram uma testa completamente desenvolvida, mostrando a presença de polissacarídeos neutros, proteínas e compostos fenólicos. Seu embrião totalmente desenvolvido apresentava células com núcleos evidentes e citoplasma denso, formando os meristemas apicais vegetativo e de raiz, nas extremidades. Em torno do embrião havia o endosperma composto por grandes células com paredes espessas e proteínas como reserva de armazenamento, bem como muitos vacúolos. Por outro lado, PS apresentaram a testa da semente não bem desenvolvida, um embrião atrofiado e um endosperma retraído que implicaram em uma baixa taxa de germinação. Mesmo demonstrando viabilidade tecidual, as sementes embebidas em água mostraram uma taxa de germinação muito baixa. Assim, sugerimos a presença de dormência fisiológica, uma vez que PS tratadas com Ácido Giberélico não apresentaram germinação satisfatória, enquanto em DS esse regulador de crescimento aumentou a taxa germinativa e o índice de velocidade de germinação. Consequentemente, uma curva de embebição na Fase III foi observada apenas em DS embebidas em Ácido Giberélico. Além disso, durante a protrusão radicular, o conteúdo de Poliaminas foi muito maior do que nas outras fases de germinação. Putrescina, Cadaverina, Espermidina e Espermina foram encontrados em grandes quantidades em DS embebidas em Ácido Giberélico na Fase III, caracterizando uma associação entre Giberelinas e Poliaminas em sementes de *C. sellowiana*. A partir da análise ultraestrutural, podemos deduzir que todas as células da Fase III embebidas em Ácido Giberélico eram altamente ativas, uma vez que apresentavam alto grau de mobilização de proteínas e vacuolização.

DS maturas de *C. sellowiana* apresentaram um comportamento ortodoxo, uma vez que foi observada tolerância à dessecação até 4,02% de conteúdo de água e a germinação das sementes não diferiu do tratamento controle (11,63%). A germinação das sementes foi baixa (~ 9%) nas sementes embebidas em água em comparação às sementes embebidas em Ácido Giberélico, pois aumentou consideravelmente (77%), e isso foi associado à dormência fisiológica desta semente. Em todas as temperaturas do armazenamento, as sementes mantiveram a viabilidade uma vez que a germinação não diferiu estatisticamente do tratamento controle. Alterações ultraestruturais indicaram alterações das paredes celulares e mobilização de reservas lipídicas e proteicas durante a dessecação e tolerância ao armazenamento. A análise indireta da viabilidade pelo teste de Tetrazólio não validou os dados de germinação de sementes e isto indica que este teste deve ser melhorado em experimentos futuros. Nossos resultados indicam que o teor de Poliaminas das sementes de *C. sellowiana* não se alterou significativamente durante a tolerância à dessecação e ao armazenamento e que a Espermidina foi a Poliamina mais abundante, o que pode estar associada à manutenção da viabilidade das sementes.

Considerações finais

A partir dos detalhes deste trabalho, futuros estudos poderão ser realizados para investigar os efeitos da aplicação exógena de Poliaminas na germinação de sementes de *C. sellowiana* visando avaliar seu papel na quebra da dormência. Além disso, a quantificação de hormônios durante a germinação de sementes seria essencial para inferir sobre a qualidade fisiológica das sementes desta espécie. Além disso, estudos poderiam ser feitos com a intenção de utilizar inibidores de Poliaminas antes da criopreservação de sementes de *C. sellowiana*, com o objetivo de entender melhor como essas moléculas poderiam estar associadas à tolerância ao frio e a dessecação.

Palavras-chave: Solanaceae. Fisiologia da semente. Dormência. Giberelinas. Poliaminas. Armazenamento.

ABSTRACT

Calibrachoa sellowiana (Sendtn.) Wijsman (Solanaceae) is a native, endemic ruderal species from South Brazil and has become an important alternative to ornamental crop. Seed germination is an essential key for conservation, genetic improvement and cultivation of this species. Thus, this study describes for the first time a large range of *C. sellowiana* seed physiology characteristics demonstrating the morphostructural and biochemical seed modifications linked to the physiological behaviour during seed germination. Dark and pale seeds were found inside capsules presenting physiological differences between them. While dark seeds presented a complete development, pale seeds presented seed coat not well developed, a stunted embryo and a retracted endosperm, evidencing their physiological quality features. However, a very low germination was observed in dark fresh seeds, which led us to assume the presence of physiological dormancy on this species. The treatment with Gibberellic Acid increased final germination and the germination speed index. During the radicular protrusion all Polyamines increased considerably showing a correlation between Polyamines and Gibberellins. Moreover, these data showed that there is capacity of *C. sellowiana* seeds to tolerate desiccation and chilling stress as the seeds tolerated desiccation to a very low water content and storage at low and ultra-low temperatures without loss on its vigour and viability. Hence, this is the first report showing these relationships among physiological, ultrastructural and biochemical analysis in *C. sellowiana* seeds which may be useful for further studies on seed conservation and use of this species as well of other herbaceous and endemic species.

Keywords: Solanaceae. Seed Physiology. Polyamines. Orthodoxy. Dormancy.

LISTA DE FIGURAS

CAPÍTULO I

Fig. 1 Stereomicroscope images showing the external features of Calibrachoa sellowiana (Sendtn.) Wijsman pale (PS) (**A**) and dark (DS) (**B**) seeds. Scanning electron microscopy images of DS C. sellowiana seed coat (SC) structures showing in the lateral view (**C,D**) the ornamentations of the surface and in the hilar view (**E**), the hilum (H) and micropyle (MI). The graphs show the average number of DS and PS by fruit (**F**), weight of 1000 seeds calculated from capsules attached to branches (**G**) and Initial/Relative Water Content (IWC/RWC) (**H**) of post-harvested C. sellowiana seeds. The vertical bars indicate ±SD. Means followed by the same letters are not significantly different according to the Student-T test ($p < 0.05$). A-B, bar: 500 µm; C, bar: 250 µm; D, bar: 100 µm; E, bar: 50 µm.....54

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1. INTRODUÇÃO E JUSTIFICATIVA

Calibrachoa Cerv. é um gênero botânico que pertence à família Solanaceae e tem se tornado um importante cultivar no paisagismo, pois se afigura em muitos aspectos a um gênero muito conhecido chamado *Petunia* Jussieu, de alto valor econômico e ecológico (WATERWORTH e GRIESBACH, 2001). *Calibrachoa* é endêmico do sul e sudeste brasileiro, sendo que a maioria das espécies deste gênero ocorrem nos estados do Rio Grande do Sul e Santa Catarina (STEHMANN, 2018). Com flores vistosas e muito coloridas, muitas *Calibrachoa* spp. possuem mecanismos de autoincompatibilidade polínica e são polinizadas por abelhas e vespas (síndrome de polinização melitofílica) (STEHMANN e SEMIR, 2001). Conforme Stehmann e Semir (2001), a polinização por himenópteros maximiza a produção de sementes destas espécies. No entanto, apesar do aumento considerável de dados de análise de sementes de espécies nativas, muitas ainda carecem de informações básicas referentes às condições ideais de germinação e armazenamento e os estudos têm sido pouco aprofundados em relação ao comportamento germinativo de algumas espécies de interesse ornamental, como os requerimentos que a semente necessita para germinar, os tipos de dormência, embebição, estoque de reservas e dinâmicas da germinação (BASKIN e BASKIN, 2004). A qualidade fisiológica da semente é a soma destas propriedades e determina o potencial para uma rápida germinação, emergência uniforme e desenvolvimento de plântulas normais (CAO et al., 2010). Muitos estudos bioquímicos e fisiológicos têm sido focados em membros da família Solanaceae (BOVE; JULLIEN e GRAPPIN, 2001), porém, pouco se relata sobre esses aspectos quando se trata de sementes. Neste contexto, a investigação de características básicas de *Calibrachoa sellowiana* (Sendtn.) Wijsman, como qualidade fisiológica, vigor e viabilidade, tolerância à dessecação e armazenamento, se tornam imprescindíveis para melhor compreensão da biologia do desenvolvimento da espécie bem como do uso destas sementes para o cultivo. Também, não há estudos específicos na literatura sobre o perfil proteico, hormonal e características estruturais de sementes de *C. sellowiana*, uma espécie que está presente nos habitats sulinos brasileiros e que ainda permanece incógnita para muitas pessoas.

2. REVISÃO BIBLIOGRÁFICA

A família Solanaceae A. L. Jussieu é composta por 2.678 espécies, distribuídas entre 115 gêneros (THE PLANT LIST, 2013), incluindo plantas com característicos tubérculos e frutos, cujas plantas são consideradas medicinais e ornamentais. Essa família é uma das maiores e mais complexas dentre as Angiospermas (SILVA e AGRA, 2005) sendo o terceiro táxon de plantas mais importantes quando se trata de culturas hortícolas, desempenhando também um papel significativo nos modelos científicos (MUELLER et al., 2005). A biodiversidade brasileira é rica em espécies de Solanaceae, devido ao fato que a América do Sul é um dos principais centros de diversidade e endemismo desta família (HUNZIKER, 2001; AGRA et al., 2009).

Calibrachoa Cerv. pertencente à família Solanaceae, é um gênero americano cujo centro de diversidade ocorre na região Sul do Brasil (STEHMANN e SEMIR, 2001). É composto por 25 espécies e faz parte dos domínios fitogeográficos de savana, mata atlântica e pampa (STEHMANN et al., 2015). Historicamente, *Calibrachoa* foi incluída em *Petunia* devido às similaridades morfológicas (STEHMANN e SEMIR, 1997; JĘDRZEJUK et al., 2017), porém Wijsman (1990) desmembrou os dois grupos de acordo com características citogenéticas (JĘDRZEJUK et al., 2017), onde verificou que *Calibrachoa* possuía 2n=18 cromossomos enquanto *Petunia* possuía 2n=14 cromossomos (STEHMANN, 1999). O gênero *Calibrachoa* abriga espécies anuais ou perenes, herbáceas ou lenhosas. Suas flores possuem cálice médio-lobulado, corola afunilada esbranquiçada, amarela, púrpura ou vermelha, podendo acumular quantidades significativas de flavonoides e carotenoides nas pétalas (JĘDRZEJUK et al., 2017). Devido a estas características de floração, este gênero vem se tornando um novo e importante grupo para o paisagismo (KANAYA et al., 2010) (Figura 1).

Visando a disseminação de espécies importantes economicamente, as sementes são responsáveis pela propagação, mas também são responsáveis pela adaptação, dispersão e sobrevivência da espécie vegetal (YAN e CHEN, 2016). Como é normalmente formada através de reprodução sexuada, as sementes provêm diversidade genética e uma maneira eficaz na dispersão de novos indivíduos (BEWLEY et al., 2013). Segundo Nambara e Nonogaki (2012) as sementes são componentes fundamentais no ciclo de vida da planta, como estoque de informação genética necessária para a próxima geração, para dispersar, se estabelecer, desenvolver e eventualmente se reproduzir para manter a espécie.

Estudos baseados na morfologia geral de 42 espécies de sementes de Solanaceae, demonstraram que, externamente, as sementes podem ser classificadas em duas categorias: 1 – Tamanho moderado (>1,5 mm em comprimento), bastante circular vista de perfil e fortemente comprimida; 2 – Tamanho pequeno (<1,5 mm em comprimento), angular e não-comprimida ou oblonga (GUNN e GAFFNEY, 1974). Já a ornamentação do tegumento das sementes, pode ser utilizada como caracteres de identificação de espécies. As células epidérmicas do tegumento são fragmentadas durante a maturação persistindo somente as paredes celulares laterais, assim o tegumento torna-se semelhante a uma malha. A estruturação desta malha difere entre as espécies de Solanaceae. No quesito coloração, as sementes de Solanaceae são normalmente uniformes e monocromáticas. As cores podem variar entre preto, marrom, cinza, ocre, palha e amarelo (GUNN e GAFFNEY, 1974). Internamente, de acordo com os mesmos autores, os embriões podem ser classificados em três principais tipos: 1 – linear, fortemente curvado e com cotilédones bem desenvolvidos; 2 – entroncado, curvado e com cotilédones pouco desenvolvidos; 3 – espatulado, ereto e com cotilédones bem desenvolvidos. As propriedades morfológicas externas e internas das sementes, auxiliam na identificação de diferentes espécies.

Com o intuito de conservar a capacidade germinativa, diversidade genética e estrutural das sementes, os bancos de sementes têm surgido como uma ferramenta de conservação das espécies no âmbito da restauração e reintrodução (MERRITT e DIXON, 2011; HAY e PROBERT, 2013). Uma gama de bancos de sementes tem se estabelecido ao redor do mundo com o intuito da conservação *ex situ* da diversidade de plantas (HAY e PROBERT, 2013) e a técnica do armazenamento a frio é um dos métodos mais interessantes na preservação de tais sementes, pois interrompe o metabolismo celular e preserva o DNA, tecidos e germoplasma, devido as baixas temperaturas impostas pela técnica (WALTERS, 2015). Contudo, algumas sementes não suportam a exposição à baixas temperaturas por um longo período, então, a viabilidade e a germinação de sementes expostas ao processo de baixas temperaturas, devem ser testadas para cada espécie (ZEVALLOS et al., 2013). Faria et al. (2016) demonstraram que em sementes de *Physalis angulata* (Solanaceae) a desidratação e criogenia em nitrogênio líquido por 60 minutos, não alterou as taxas de germinação, evidenciando que as sementes desta espécie podem ser armazenadas sem a perda do potencial germinativo. Em trabalhos realizados com *P. peruviana*, as sementes ficaram armazenadas por mais de um ano a 5ºC, 25ºC e -196ºC sem a perda da capacidade de germinação (SOUZA et al., 2016). Tal técnica é

utilizável para muitas plantas, mas poderia ser uma estratégia permanente para a conservação de muitas outras espécies, se os métodos de armazenamento fossem melhor estudados (BONNER, 1990; WALTERS, 2015). É sabido que durante o período de armazenamento, que deve ser iniciado na maturidade fisiológica, a qualidade da semente não pode ser maximizada, porém pode ser preservada com o uso de técnicas específicas que mantenham também as taxas de umidade e temperatura específicas para a sobrevivência da espécie em estudo (ZUCHI et al., 2013; VILLELA e PERES, 2004). Neste sentido, aspirando à inalteração das particularidades da semente, se faz necessário um estudo dos caracteres morfológicos, bioquímicos e fisiológicos visando o mantenimento de tais particularidades. Dessa forma, a caracterização morfológica através de técnicas histológicas permite a identificação de características teciduais e celulares da semente. Tais análises, através de métodos como Microscopia Eletrônica de Transmissão (MET), Microscopia Eletrônica de Varredura (MEV) e Microscopia de Luz (ML), revelam a natureza estrutural e ultraestrutural das células (ROGGE-RENNER et al., 2013), que compreendem as partes integrantes de uma semente, como ornamentações no tegumento, o estado das membranas celulares e tipos de reservas. Também, com o auxílio destes métodos, é possível descrever a presença de estruturas que antes não haviam sido citadas na literatura (GALLETI, 2003; ROGGE-RENNER et al., 2013). Como resultado gerado através destas observações, há uma optimização da compreensão da organização dos tecidos vegetais e funções celulares (ROGGE-RENNER et al., 2013), que podem ser associadas com a qualidade das sementes e com os processos fisiológicos que ocorrem durante a conservação das sementes de espécies nativas.

Segundo Bewley et al. (2013) o sucesso com que o novo indivíduo será estabelecido – o tempo, o habitat o vigor da nova plântula – é determinado por características fisiológicas e bioquímicas da semente. Estes atributos estão também intimamente ligados a características ultraestruturais e têm sido investigados com ênfase em espécies de importância econômica, medicinal ou ornamental, sendo que, o conhecimento das etapas nos estágios iniciais do desenvolvimento das sementes é expressamente interessante nas áreas da sistemática, ecologia, fisiologia e bioquímica. Tais processos, que têm recebido atenção por parte dos pesquisadores, incluem o desenvolvimento da semente e maturação, estoque e mobilização de reservas, germinação e respostas de sementes a vários fatores ambientais. Neste mesmo sentido, o estudo da morfofisiologia permite a identificação de diferenças nos status celulares e bioquímicos entre sementes sob várias condições ambientais

(NAMBARA; NONOGAKI, 2012), associados à qualidade e longevidade das sementes.

O desenvolvimento da semente, em geral, transcorre por três fases distintas as quais incluem modificações morfológicas e fisiológicas. A primeira fase é identificada pela fecundação, estabelecimento dos domínios embrionários, meristemas e eixo embrionário. É nesta fase que a semente adquire um aumento no peso fresco e é quando a divisão mitótica se intensifica. Também, em alguns casos, há a formação de endosperma. A segunda fase, de maturação, é identificada pelo acúmulo de reservas (proteínas, lipídios e carboidratos) e expansão das células, que tendem a aumentar o tamanho da semente. É durante esta fase que os compostos de armazenamento se acumulam, o peso seco aumenta e os vacúolos diminuem de tamanho. Então, para a maioria das sementes, o desenvolvimento chega ao fim durante a terceira fase, onde ocorre algum grau de secagem devido à perda de água pela semente. Nesta fase, a semente exibe quiescência e o embrião entra em um estado mínimo de metabolismo (CASTRO et al., 2004; BEWLEY et al., 2013). Devido a este estado desidratado e com baixo metabolismo, muitas sementes adquirem capacidade de resistência ao estresse. Algumas moléculas são sintetizadas tardiamente no desenvolvimento de sementes e estão envolvidas na tolerância à dessecação (CASTRO et al., 2004). As análises destas moléculas nos diferentes estágios de desenvolvimento da semente, bem como na aquisição de dormência e no processo de germinação, têm provido informações interessantes sobre a regulação bioquímica destes processos (NAMBARA e NONOGAKI, 2012). Neste sentido, uma nova classe de reguladores de crescimento, chamada Poliaminas (PAs), tem sido sugerida como efetivas contra o estresse em ambientes extremos (HUSSAIN et al., 2011). As PAs espermidina (Spd), espermina (Spm) e putrescina (Put) estimularam o crescimento *in vitro* de culturas embriogênicas de *Araucaria angustifolia* (Bertol.) Kuntze (STEINER et al., 2007) demonstrando que as PAs regulam endogenamente funções celulares importantes nas estruturas vegetais, como crescimento e diferenciação. De acordo com Bais e Ravishankar (2002), as PAs podem atuar resultando em efeitos similares a outros fitormônios, como na divisão celular e elongação, além de influenciar no crescimento de raízes, floração, desenvolvimento de frutos e estabilização de membranas e paredes celulares. Conforme Liu et al. (2007), as PAs possuem a característica de serem mais estáveis que outras moléculas protetivas e previnem a desnaturação do sistema de membranas sob condições de estresse. Também, segundo os mesmos autores, esses reguladores de crescimento compartilham características similares àquelas de alguns

solutos, como hidrofilicidade, proteção de macromoléculas, eliminação de espécies reativas de oxigênio, e manutenção do pH celular. Sabendo que a qualidade da semente é determinada durante seu desenvolvimento, as PAs têm um papel importante no crescimento do embrião, desenvolvimento da semente e defesa contra fatores abióticos estressantes. O conteúdo e variações de PAs são influenciados pelo status fisiológico e de desenvolvimento (CAO et al., 2010) e as concentrações destas moléculas dependem da espécie de planta, do órgão e tecido (HUSSAIN et al., 2011). Porém Urano et al. (2005) relataram que alguns transcritos modulam a síntese de PAs em sementes e são altamente ativos durante o desenvolvimento do embrião e do endosperma, desempenhando assim funções importantes durante seu crescimento.

Para que a germinação ocorra, as sementes quiescentes precisam ser hidratadas sob condições que reativem o metabolismo, como requisitos adequados de temperatura, luminosidade e oxigênio (BOVE; JULLIEN e GRAPPIN, 2001; BEWLEY, 2013). Se mesmo sob condições ótimas a semente não germina, ela é dita como dormente e reguladores de crescimento podem ser necessários para a indução e/ou aceleração de processos germinativos. Os hormônios são mensageiros químicos que podem modular processos celulares, interagindo com proteínas específicas que funcionam como receptores da rota de transdução de sinais. São um grupo de substâncias orgânicas de ocorrência natural que influenciam processos fisiológicos em baixas concentrações. Tais processos consistem principalmente de crescimento, diferenciação e desenvolvimento (DAVIES, 2010). Assim, uma classe de hormônios que estão frequentemente envolvidos diretamente no controle da germinação são as Giberelinas (GAs). As GAs são um grupo de diterpenos tetracíclicos composta atualmente por 136 tipos distintos. São responsáveis pela elongação do caule, crescimento foliar, partenocarpia e estimulam a germinação de sementes (BUCHANAN; GRUISSEM e JONES, 2015). Sob condições normais de germinação como por exemplo água, luz, oxigenação e temperatura ideais, a síntese de ácido giberélico (GA) começa rapidamente após a embebição sendo indispensável para a ruptura do endosperma e da testa da semente, evidenciando os processos pós-germinativos (PISKUREWICZ et al., 2008). Em cereais as GAs transportadas à camada de aleurona pelo embrião em desenvolvimento são responsáveis pela síntese de α-amilase, uma enzima que lançada no endosperma hidrolisa amido e proteínas, suprindo nutricionalmente o embrião em desenvolvimento. Não obstante, os sinais ambientais são de extrema importância nos processos germinativos de sementes. Em sementes dormentes que necessitam de frio/calor para que ocorra a

germinação, a aplicação exógena de GAs pode ser uma alternativa para contornar a aplicação destes estímulos em muitas espécies de vegetais. Porém nestes casos maior eficácia é atingida quando há estratificação a frio/calor mais a aplicação exógena de GAs (FINCH-SAVAGE e LEUBNER-METZGER, 2006).

Assim, estudos morfológicos de ultraestrutura e biomoléculas em sementes de *C. sellowiana*, se fazem necessários para uma melhor compreensão da fisiologia do desenvolvimento de sementes desta espécie e ampliam os dados sobre os aspectos germinativos e estruturais da família Solanaceae. Além disso, tais estudos podem ser utilizados para o uso e conservação destas espécies, e assim potencializando a valoração econômica e ornamental de *C. sellowiana*, uma espécie que está presente nos habitats sulinos brasileiros e que ainda permanece desconhecida pela maioria dos moradores destas regiões.

Figura 1. Imagens de indivíduos adultos de uma população natural de plantas de *Calibrachoa sellowiana* (Sendtn.) Wijsman mostrando o hábito das plantas. A população se encontra na cidade de Curitibanos (27º16'58" Sul e 50º35'04" Oeste) no estado de Santa Catarina – Brasil, habitando ambientes de solo pedregoso com incidência luminosa direta.

3. OBJETIVOS

3.1. Objetivo Geral

Caracterizar morfoanatomicamente, ultraestruturalmente e fisiologicamente sementes maturas, dessecadas e armazenadas de *Calibrachoa sellowiana* (Sendtn.) Wijsman, bem como avaliar o conteúdo endógeno de Poliaminas livres durante a germinação e armazenamento, a fim de gerar contribuições para a utilização e conservação pelo uso desta espécie.

3.2. Objetivos Específicos

Estudar sementes frescas e germinadas de C. sellowiana para relacionar modificações anatômicas, ultraestruturais e bioquímicas com o comportamento da dormência fisiológica ao longo das três fases da germinação;

Determinar o limiar de tolerância à dessecação e armazenamento a baixas (8ºC, -20ºC) e ultrabaixa (-196ºC) temperaturas de sementes de *C. sellowiana* bem como analisar as alterações no conteúdo de Poliaminas e alterações ultraestruturais durante a dessecação e armazenamento à frio dessas sementes;

Os objetivos supracitados foram estudados e organizados em forma de dois capítulos, na modalidade de artigo científico, conforme seguem.

Capítulo I

Physiological dormancy overcoming of *Calibrachoa sellowiana* (Sendtn.) Wijsman seeds is mediated by Polyamines

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Abstract

Calibrachoa sellowiana (Sendtn.) Wijsman (Solanaceae) is an endemic and ruderal species from South of Brazil and have been considered an important ornamental plant. Seed germination is an essential key for conservation, genetic improvement and cultivation of this species. Thus, our study describes for the first time a large range of *C. sellowiana* seed physiology characteristics and demonstrate the morphostructural and biochemical seed modifications linked to the physiological behaviour over three phases of germination. Dark and pale seeds were found inside capsules presenting physiological differences between them. Dark seeds presented seed coat constituted of neutral polysaccharides, proteins and phenolic compounds; a well-developed embryo and endosperm presenting many vacuoles and protein bodies. Ultrastructure analysis demonstrated embryo cells filled with lipid bodies and proteins while endosperm cells constituted basically by protein bodies. However, pale seeds presented seed coat not well developed, a stunted embryo and a retracted endosperm. Besides its viability tissue, a very low germination was observed may be because reserves of embryo and endosperm cells were in advanced stage of mobilization by autophagic vesicles. The treatment of dark seeds with Gibberellic Acid was successful to overcoming the supposed nondeep physiological dormancy. On the radicular protrusion of Gibberellic Acid imbibed seeds, all the Polyamines content considerably increased showing us a possible correlation between Polyamines and Gibberellins.

Keywords: Solanaceae. Germination. Gibberellins. Putrescine. Spermidine. Spermine.

Abbreviations

2,3,5 TTC	2,3,5-Triphenyl tetrazolium chloride
ADC	Arginine decarboxylase
CBB	Coomassie Brilliant Blue
DAS	Days after sowing
DS	Dark seeds
FC	Ferric Chloride
FM	Fresh Mass
GA_3	Gibberellic Acid
GAs	Gibberellins
GSI	Germination speed index
H_2O	Water
IWC	Initial water content
LDC	Lysine decarboxylase
ODC	Ornithine decarboxylase
PAs	Polyamines
PAS	Periodic Acid-Schiff
PS	Pale seeds
Put	Putrescine
RWC	Relative water content
SAMDC	S-adenosylmethionine decarboxylase
Spd	Spermidine
Spm	Spermine
TB-O	Toluidine blue - O
TEM	Transmission electron microscopy

Introduction

Seed dormancy is considered an arrest of germination of an intact viable seed even under optimal conditions to germinate such water, light and temperature (Bewley et al. 2013). Solanaceae family includes economically important crops such as *Solanum tuberosum* and *S. melongena* and many others considered dormant. Thus, this family in which the embryo is surrounded by a rigid endosperm, presents suitable models in understanding germination biology and dormancy research (Koornneef et al. 2002). Physiological dormancy is the most abundant form found in Solanaceae seeds, including *Solanum lycopersicum* and *Nicotiana* spp. (Finch-Savage and Leubner-Metzger 2006) and its mechanism involve plant growth regulators, mainly Abscisic Acid and Gibberellins (GAs). The hormonal balance regulate the maintenance and overcoming of seed dormancy (Baskin and Baskin 2004): while Abscisic Acid is involved in the establishment and maintenance of dormancy, GAs play a key role in dormancy release and germination (Finch-Savage and Leubner-Metzger 2006; Gupta and Chakrabarty 2013). GAs acts in two ways as proposed by Kucera et al. (2005): first, GAs increase the growth potential of the embryo; second, it is necessary to overcome the mechanical restraint conferred by micropylar endosperm which cover the radicle. It was evaluated by Lee et al. (2012) in *N. tabacum* where they observed a deposition of saccharides in the endosperm cell walls next to the radicle limiting germination. Thus, endosperm rupture is one of the main germination-limiting process in seeds of *Solanaceae*, as well *Asteraceae*, and *Rubiaceae* families, and GAs are likely to be involved in cell wall hydrolysis by hydrolytic enzymes breaking the dormancy and promoting germination (Kucera et al. 2005). Exogenous GAs applications in economically important species such as *S. centrale*, *S. cunninghamii*, *S. diversiflorum* and *S. phlomoides* increasing their germination considerably, once the dormancy was overcome (Commander et al. 2008). GAs form a tetracyclic group of diterpenoid hormones (Buchanan et al. 2015) exerting their function as first extracellular signals which are capable of binding to the receptor GA-insensitive dwarf1 (GID1) and promote ubiquitination and degradation of DELLA proteins which, in turn, are repressors of GAs responses (Schwechheimer 2008). Moreover, hormonal balance elicit changes in other biomolecules, such as Polyamines (PAs), which may act as second messengers on the GA signaling pathway, mediating seed dormancy and germination (Palavan and Galston 1982).

PAs are small aliphatic amines of low molecular weight found in almost all cell types (Bouchereau et al. 1999; Gupta et al. 2013). Putrescine (Put), Cadaverine (Cad), Spermine (Spm) and Spermidine (Spd) constitute the major PAs (Kaur-sawhney et al. 2003) and have been regarded as a new class of plant growth regulators, interacting with other ones (Martinez-Madrid et al. 1996; Santa-Catarina et al. 2006; Steiner et al. 2007; Krawiarz and Szczotka 2008; Farias-Soares et al. 2014; Liu et al. 2016; Zhang et al. 2017). PAs are found in all plant cell compartments such as vacuoles, mitochondria, chloroplast (Ahmad et al. 2013), and nucleus that indicates its participation in various cellular processes (Bachrach 2010; Gupta et al. 2013). They are synthesised in large amounts in meristems and growing tissues (Astarita et al. 2003) interacting with negatively charged molecules such as DNA, RNA, proteins and phospholipids leading to their stabilization or structural modification (Hussain et al. 2011). The concentrations of PAs in plants are higher than the endogenous plant growth regulators due to the fact that PAs are involved in many processes like protective function against biotic and abiotic stresses (Bouchereau et al. 1999). Like plant growth regulators, PAs are involved in some processes like replication, transcription, translation (Kaur-sawhney et al. 2003), membrane stabilization, enzyme activity modulation, cell division and expansion (Gupta et al. 2013). These processes are all key steps to seed germination, leading to the hypothesis that PAs are important during the early stages of plant development. Hussain et al. (2011) have related that PAs biosynthesis pathways and their enzymes are under complex metabolic control and it is necessary for efficient regulation of cellular metabolism. In this sense, PAs synthesis has mainly two pathways: The reaction to form Put is catalysed by ornithine decarboxylase enzyme (ODC) from ornithine or by arginine decarboxylase enzyme (ADC) from arginine. In addition, Spd and Spm can be formed from Put by S-adenosylmethionine decarboxylase (SAMDC) from a complex pathway of reactions (Bachrach 2010). Cad is formed from lysine through lysine decarboxylase enzyme (LDC) which is found in plastids of Solanaceae family and leguminous plants (Jancewicz et al. 2016) and has been associated to the synthesis of compounds involved in protection against herbivores (Bagni and Tassoni 2001).

Calibrachoa sellowiana (Sendtn.) Wijsman (Solanaceae) is a ruderal species with dormant seeds, endemic of fields in South Brazil that has become an important species for ornamental purposes (Waterworth and Griesbach 2001). Cultivation as well conservation of endemic species by seeds, require physiological knowledge of seed germination

behaviour. According to Bewley et al. (2013), seed germination refers to the physiological process culminating in the emergence of the embryo form the covering layers, such endosperm and seed coat. Germination commences with the physical uptake of water by imbibition of the dry seed, followed by embryo expansion (Kucera et al. 2005) and penetration of the radicle through surrounding tissues. After imbibition, a set of physiological mechanisms are activated allowing the initiation of metabolism and the occurrence of complex subcellular changes such as reestablishment of basal cell activities, alleviation of dormancy and preparation of the embryo for radicular emergence (Bewley et al. 2013). Moreover, the initial seedling growth is supported by metabolites produced by the hydrolysis and conversion of the major stored seed reserves such as proteins and lipids, which are mediated by plant growth regulators (Zienkiewicz et al. 2014). Indeed, cellular, anatomical, ultrastructural and biochemical features define the seed physiological quality and could be related to the germinative aspects. Despite the usefulness and economic importance of *C. sellowiana*, little is known regarding the morphostructural features as well as the seed regulation and germination mechanism. Thus far, no systematic studies have been made on the PAs content during the dormancy release associated with GA_3, neither associating it to the cell characterization of this process. Considering it, this work studies the mature and germinating seed of *C. sellowiana* in order to link morphohistological and biochemical modifications with the physiological dormancy behaviour over three phases of germination. Our study presents a large range of characteristics of an endemic and ruderal species of the South Brazil demonstrating morphological, physiological and biochemical features of *C. sellowiana* seed germination.

Materials and methods

Plant material

Calibrachoa sellowiana capsules were collected in January 2018 from plants of a natural population located near Curitibanos (27º16'58" South and, 50º35'04" West), Santa Catarina State, Brazil. A hundred capsules were used to calculate the number of seeds by flower. The weight of 1000 seeds was analysed according to Brasil (2009).

Water content

The water content was measured by 3 samples of 500 seeds fresh weighed, then oven dried at 103±2ºC for 24 hours and weighed again. Moreover, fresh seeds were distributed in Germitest® paper imbibed with water for 24 hours at 5ºC (Brasil 2009), then the initial and relative water content (IWC/RWC) were calculated according to Sun (2002).

Tetrazolium tests

Tetrazolium test was realized with 3 samples of 25 seeds imbibed in distilled water for 0, 2, 4, 6, 8, 10, 12 hours, then reacted with 2,3,5 triphenyl tetrazolium chloride (2,3,5 TTC) (1%) at 25±2ºC in the dark for 1 hour (Brasil 2009).

Electroconductivity

The electroconductivity was measured with 4 samples of 25 seeds weighed and submerged in 75.0-ml of distilled water. The samples were maintained in an incubation chamber (BOD) at 25±2ºC for 12 hours. At each hour the electroconductivity was measured with a mass conductivity meter and expressed by $\mu S.cm^{-1}g^{-1}$.

Germination tests

The seeds were previously disinfected in an ethanol solution (70%) for 30 seconds, then in a sodium hypochlorite solution (1%) for 1 minute and finally, washed 3 times in sterile water. Germination test was carried out by 8 samples of 25 seeds inoculated in 6-cm Petri dishes on Germitest® paper moistened with 2.0-ml water or different concentrations of Gibberellic Acid (GA_3) (50, 100, 250, 500, 750, 1000 µM) at 25±2ºC in the light (120 $\mu Em^{-2}s^{-1}$, 12/12 hours) for 50 days. The germination speed index (GSI) was calculated according to Maguire (1962). Seedling morphometry was measured by images using the Image J® Software (version 1.52, 2018) where we analysed 24 plantlets for each treatment.

The imbibition curve was performed by 4 samples of 50 seeds. It was weighed every 30 minutes until 6 hours; then every 1 hours until 12 hours and, after that every 24 hours, until reached 51% of germination (Brasil 2009).

Light microscopy

Light microscopy analyses were carried out according to Steiner et al. (2015) with modifications. Seeds were fixed in paraformaldehyde (2.5%) containing phosphate buffer (0.1 M) at room temperature for 7 days. Subsequently, a puncture was made on the seed coat and the samples were dehydrated in an increasing ethanolic series. Then, the samples were infiltrated with Historesin® (Leica Historesin, Heidelberg, Germany) for 14 days. Semi-thin sections (4 μm) containing the samples were submitted to different histochemical techniques. Periodic Acid-Schiff (PAS) was used to identify neutral polysaccharides (O'Brien and McCully 1981), Toluidine Blue O (TB-O) to identify acid polysaccharides (O'Brien et al. 1964), Ferric Chloride (FC) to identify phenolic compounds (Johansen 1940) and Coomassie Brilliant Blue (CBB) to identify proteins (Ventrella et al. 2013). Some of the sections were double-stained (PAS + CBB). Light microscopy sections were analysed using Olympus BX 41 microscopy equipped with the Olympus DP 40 image capture system and Q-capture pro Software® (version 5.1).

Transmission electron microscopy

Endosperm and embryo ground meristem of the hypocotyl-radicle axis were separated and fixed in glutaraldehyde (7.5%) containing sodium cacodylate buffer (0.3 M) and sucrose (2%) for 7 days. The material was post-fixed with sodium cacodylate buffer (0.1 M) containing osmium tetroxide (1%) for 4 hours. The material was dehydrated in an increasing ethanolic series and then embedded in LR White Acrylic Resin® (Merck KGaA, Darmstadt, Germany) for 15 days. Ultrathin sections (70 nm) were collected on grids recovered with Parlodion than stained with aqueous uranyl acetate followed by lead citrate. Two grids for treatment were examined in the JEM 1011 transmission electron microscopy (TEM) (JEOL Ltd., Tokyo, Japan) at 100 kV.

Scanning electron microscopy

Seeds were fixed in a similar manner to that described for TEM. The samples were also dehydrated in an increasing ethanolic series, dried in the CO_2 critical point dryer (EM-CPD-030; Leica, Heidelberg, Germany), and gold sputter-coated prior to examination in the JSM 6390 LV (JEOL Ltd., Tokyo, Japan) at 20 kV (Steiner et al. 2015).

Polyamines determination

Polyamines analysis was carried out by 3 samples of 200 mg of seeds grounded in 1.6-ml of perchloric acid (5%). Free polyamines (PAs) were extracted, dansylated and identified by reverse phase HPLC, according to Steiner et al. (2007). PAs content was determined using a fluorescence detector at 340 nm (excitation) and 510 nm (emission). Peak areas and retention times were measured by comparison with standard PAs: Putrescine (Put), Cadaverine (Cad), Spermidine (Spd) and Spermine (Spm).

Statistical analysis

Data have been analysed for significance using Student-T test as well as one-way ANOVA (R® Software, version 3.4.1, 2017). Tukey and SNK (Student-Newman-Keuls) multiple range tests have been used for the determination of significant differences between germination values and Polyamines content, respectively ($P<0.05$).

Results

Ripe seeds structural and histological characteristics associated to the physiological behaviour of seed.

Morphological observations of *Calibrachoa sellowiana* seeds allowed us to classify them into two groups according to the colour of the seed coat (SC): Pale Seeds (PS) and Dark Seeds (DS) (Fig. 1A-B). In both groups, seed showed a size of 1.0 mm in length and 0.8 mm in width and a lateral side compressed (Fig. 1C). Scanning electron microscopy analysis of seed coat or testa showed concave shape and polygonal ornamentations in the entire surface of both DS and PS (Fig. 1D) as well an inconspicuous and circular hilum (H) close to the micropyle (MI) (Fig. 1E).

A total of 86 ripe seeds per single *C. sellowiana* fruit (capsule) were identified; 44 of them were PS while 42 DS (Fig. 1F). Different weight of 1000 seeds were also identified in the two morphological groups, with PS weighing 0.136g and DS 0.239g (Fig. 1G). Usually, the weight seed set is related with the fresh mass (FM) and moisture content at ripe stage, however the initial water content (IWC) in *C. sellowiana* PS and DS did not show significant differences. On the other hand, the relative water content (RWC) observed was 7.17% in PS while in DS seed was 20.31% (Fig. 1H) indicating a significative difference in the two seed groups, which was possible related to seed mass and physiological behaviour during germination.

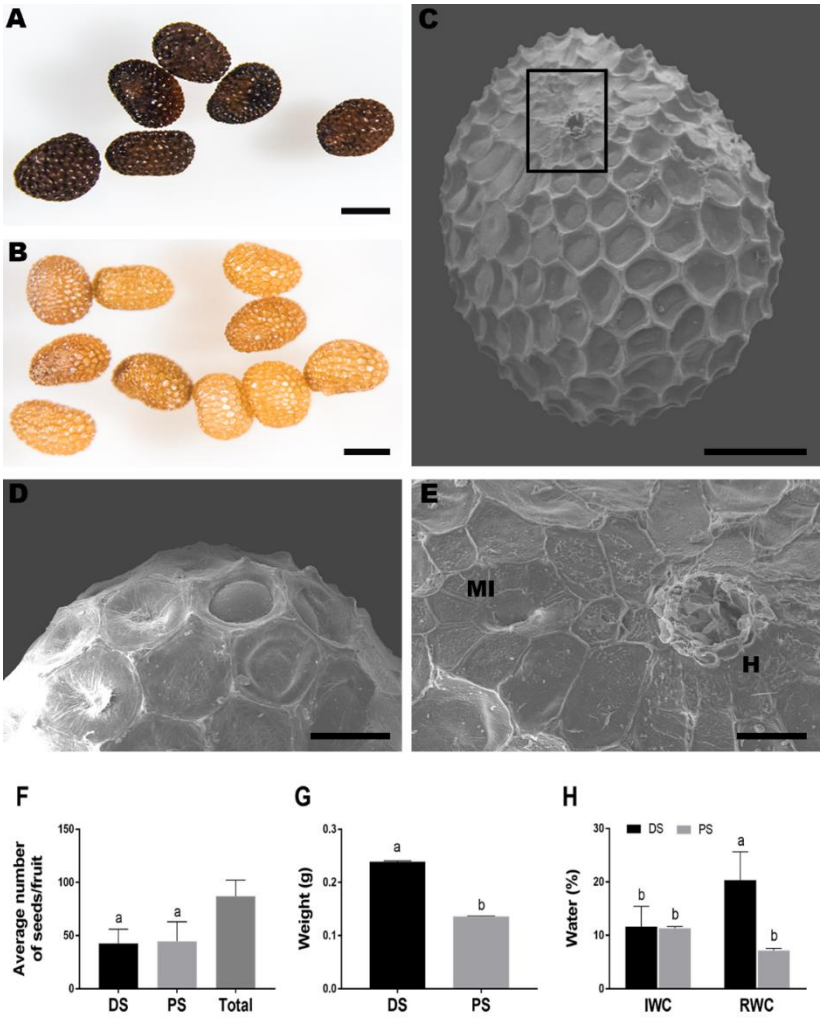

Fig. 1 Stereomicroscope images showing the external features of *Calibrachoa sellowiana* (Sendtn.) Wijsman pale (PS) (**A**) and dark (DS) (**B**) seeds. Scanning electron microscopy images of DS *C. sellowiana* seed coat (SC) structures showing in the lateral view (**C,D**) the ornamentations of the surface and in the hilar view (**E**), the hilum (H) and micropyle (MI). The graphs show the average number of DS and PS by fruit (**F**), weight of 1000 seeds calculated from capsules attached to branches (**G**) and Initial/Relative Water Content (IWC/RWC) (**H**) of post-harvested *C. sellowiana* seeds. The vertical bars indicate ±SD. Means

followed by the same letters are not significantly different according to the Student-T test ($p < 0.05$). A-B, bar: 500 µm; C, bar: 250 µm; D, bar: 100 µm; E, bar: 50 µm.

Moreover, the differences identified in the weight and RWC of the two groups of seeds could be at least in part explained by the histological analysis. Seed structure was different in the two groups. In *C. sellowiana* DS, seed coat cells showed abundant presence of acid polysaccharides and phenolic compounds identified by metachromatic reaction to the Toluidine Blue-O (TB-O) (Fig 2A). It was also observed a positively strong reaction of seed coat cells to the Periodic Acid-Schiff (PAS) indicating the presence of neutral polysaccharides. The PAS staining of seed coat cells showed mainly two polysaccharides layer cells, one most tangential external (TE) and other close to the endosperm (EN) region, the tangential internal (TI) (Fig. 2B). Over to the polysaccharides tangential internal layer it was identified a proteic thin cell layer by the Coomassie Brilliant Blue (CBB) stain, around the endosperm (Fig. 2C). Tangential external and tangential internal cell layers showed an internal region full fit of neutral polysaccharides (Fig. 2B) and phenolic compounds (Fig. 2D) once this region react positively to PAS and Ferric Chloride (FC). DS showed all these structures in all analysed seeds, while only a few percentages of PS demonstrated the same characteristics (Fig. 2E). Most of PS showed a seed coat not fully developed, separated by an interstice from a retracted endosperm and a stunted embryo (EM) (Fig. 2F). In these seeds the seed coat cells were rich in acid polysaccharides as seen in the TB-O reaction (Fig. 2G). In DS, the endosperm region was categorized in three regions according to the position and number of cell layers: chalazal endosperm (CE), micropylar endosperm (ME) and peripheral endosperm (PE) (Fig. 2A). Chalazal and micropylar endosperm are composed by 1 to 3 cell-layers and the peripheral endosperm is composed by 3 to 8 cell-layers. The parenchymatous endosperm cells are polyhedral, with thick cell walls and protein bodies (PB) in the cytoplasm as was evidenced by the positive reaction of double (PAS + CBB) staining respectively (Fig. 2H). Mature embryo is slightly curved composed by two cotyledons (CT) with shoot apical meristem (SAM) and root apical meristem (RAM) separated by hypocotyl. The outermost cell layer of the embryo is the protoderm (PD) with thin-walled cells (Fig. 2I) next to ground meristem (GM) which showed cells with many vacuoles (V) and protein bodies (Fig. 2J). In the hypocotyl-radicle axis was identified procambium (PC) with elongated and thin-walled cells containing also protein bodies as storage reserve (Fig. 2K). Shoot apical

meristem was characterized by a set of isodiametric cells with central and large nucleus and nucleoli reactive to TB-O stain (Fig. 2L) while root apical meristem (RAM) containing a set of cells with central small nuclei. In this stage, it was observed the beginning of the differentiation of the root cap (RC) with elongated cells and evident nucleus on root apical meristem (Fig. 2M).

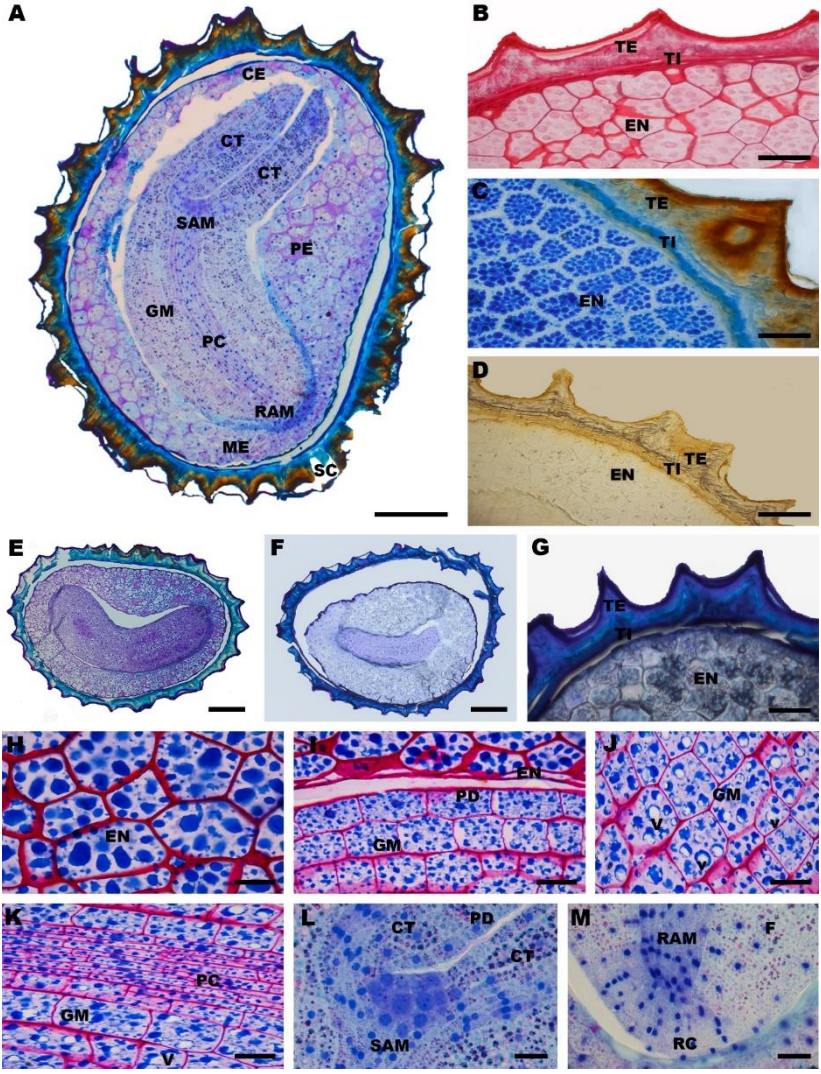

Fig. 2 Light microscopy analyses of *Calibrachoa sellowiana* (Sendtn.) Wijsman. Dark seeds (DS) stained in Toluidine Blue-O (TB-O) showing a mature embryo (EM) presenting two cotyledons (CT), shoot apical meristem (SAM), root apical meristem (RAM) ground meristem (GM) and procambium (PC); an endosperm (EN) classified in three regions: micropylar (ME), chalazal (CE) and peripheral (PE); and a seed coat (SC) (**A**). Details of DS SC with a tangential external layer (TE) and the tangential internal layer (TI) reactive to the Periodic Acid-Schiff (PAS) stain (**B**). Additional histochemical tests of seed coat in DS indicated the presence of protein bodies by Coomassie Brilliant Blue (CBB) stain (**C**) and the presence of phenolic compounds by the Ferric Chloride stain (FC) (**D**). Structures of pale seeds (PS) stained with TB-O showing differences between them (**E-F**), mainly on the seed coat (**G**). DS details of endosperm cells with a cell wall rich in neutral polysaccharides and protein bodies in the cytoplasm indicated by double (PAS+CBB) staining (**H**). Details of the embryo showing protoderm (PD) and ground meristem (GM) cells with presence of vacuoles (V) and proteins bodies (**I-J**). Details of procambium cells (PC) which are elongated with an evident nucleus (**K**). Double (PAS+CBB) staining **(H-K)**. Details of shoot apical meristem (SAM) (**l**) and root apical meristem (RAM) by TB-O stain. Close to the root apical meristem it was observed the root cap (RC) (**M**). A, bar: 100 µm; B-D, bar: 50 µm; E-F, bar: 100 µm; G, bar: 50 µm; H-K, bar: 20 µm, L-M, bar: 50 µm.

Transmission electron microscopy (TEM) analysis allowed us to obtain additional data about the subcellular organization of mature DS and PS (Fig. 3). The cytoplasm of the ground meristem cells present in the hypocotyl-radicle axis was completely filled by lipid bodies (LB) and protein bodies. Protein bodies of DS embryo cells showed a rounded shape formed by a homogenous electron-dense matrix while lipid bodies filled the cytoplasm with numerous, smaller and electron-transparent bodies of rounded shape (Fig. 3A,B). DS endosperm cells presented a cytoplasmic content constituted by large, numerous and rounded shape protein bodies formed by an electron-dense matrix (Fig. 3C,D).

On the other hand, both mature PS embryo (Fig. 3E,F) and endosperm (Fig. 3G,H) cells presented large clusters of protein bodies diffused inside structures similar to vacuoles. Close to them, we observed autophagic vesicles (AV). The lipid bodies were sinuous, less numerous and compressed in direction to the cellular periphery.

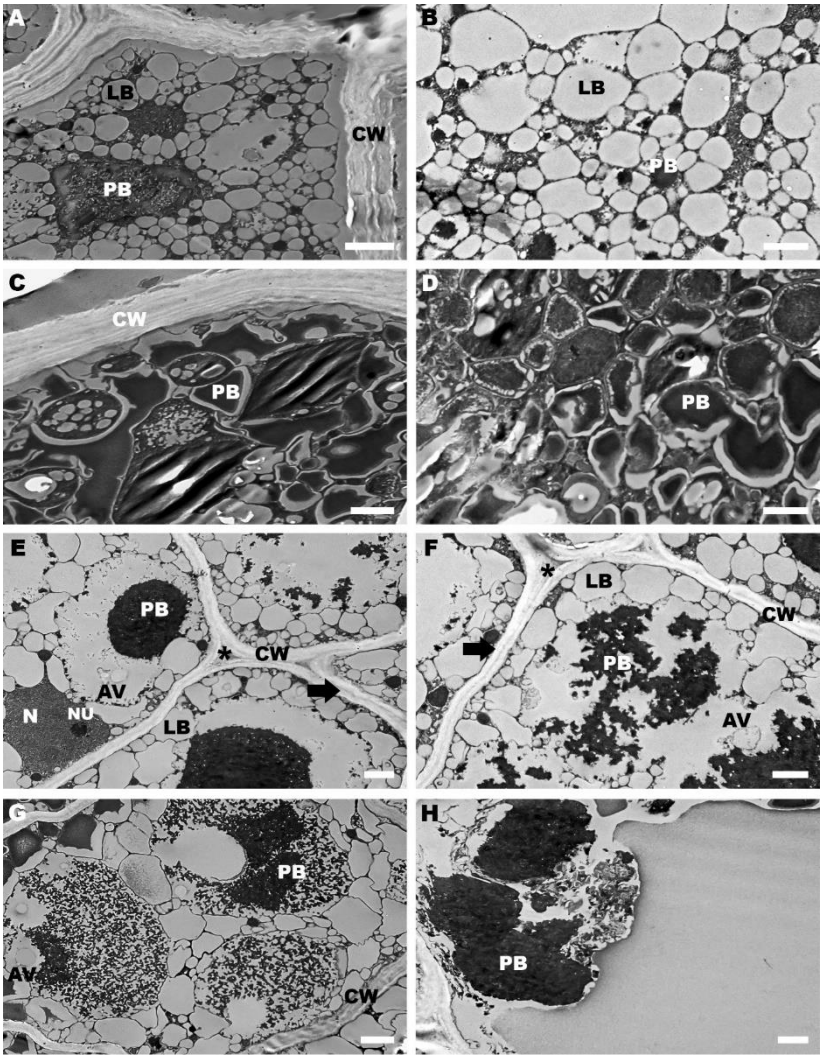

Fig. 3 Transmission electron microscopy (TEM) images of mature *Calibrachoa sellowiana* (Sendtn.) Wijsman dark (DS) (**A-D**) and pale (PS) (**E-H**) seeds. Embryo (EM) cell presenting a cell wall (CW) well developed and a cytoplasm filled with electron-dense protein bodies (PB) surrounded by densely populated of small and less electron-dense lipid bodies (LB) (**A**) and the details of its cytoplasmic content showing protein and lipid bodies organization (**B**). Endosperm (EN) cell presenting many protein bodies as storage reserve (**C**) and the details of these structures (**D**). Embryo cells of PS showing a nucleus (N)

with its nucleolus (NU), clusters of protein bodies and autophagic vesicles (AV) inside compartments similar to vacuoles (V) (**E**). Details of the mobilization of proteins clusters with different degrees of electron density and sinuous lipid bodies compressed to the cell wall. An intercellular space (*) and the middle lamella (arrow) may be perceived (**F**). PS endosperm cell in the intermediate state of mobilization of protein clusters with different degrees of electron density evidencing the presence of many autophagic vesicles (**G**) and the detail of its disintegration (**H**). A, bar: 2 µm; B, bar: 1 µm; C, bar: 2 µm; D, bar: 1 µm; E-G, bar: 2 µm; H, bar: 1 µm.

Viability and vigour of seeds associated to the morphohistological characteristics.

Tetrazolium test of DS indicated the better imbibition times over 12 hours were associated with the high value of seed positive reaction (78%) while in PS the better imbibition time was 6 hours with 67% and a continuous decrease of this value until 16 hours (Fig. 4A). The electroconductivity values of the two groups of C. sellowiana seeds were considerably different, showing stabilization after 12 hours of imbibition. At this time, PS showed a higher electrolyte value (709.71 $\mu S.cm^{-1}.g^{-1}$) comparatively to DS (255.79 $\mu S.cm^{-1}.g^{-1}$) (Fig. 4B).

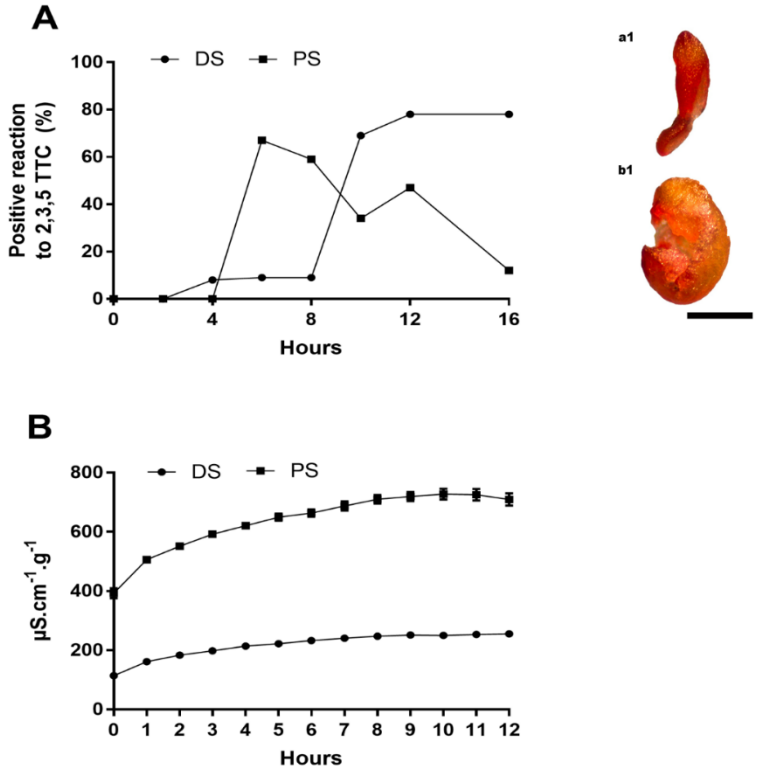

Fig. 4 Positive reaction to 2,3,5-Triphenyl Tetrazolium Chloride (2,3,5 TTC) (1%) of post-harvested *Calibrachoa sellowiana* (Sendtn.) Wijsman pale (PS) and dark (DS) seeds showing the reaction on embryo (**a1**) and endosperm (**b1**) (**A**). Electroconductivity variation ($\mu S.cm^{-1}g^{-1}$) at different imbibition times (**B**). Bar: 500 µm.

Germination dynamics of either water (H_2O) or Gibberellic Acid (GA_3) *C. sellowiana* imbibed PS and DS were very different. Water imbibed DS and PS started to germinate at 10 and 17 days after sowing (DAS), respectively (Fig. 5A), however low final germination value of PS (4%) and DS (9%) were observed (Fig. 5B). On the other hand, the exogenous application of GA_3 promoted DS germination at 6 DAS with final germination rate of 77%, while PS did not germinated (Fig. 5A,B). Considering the low germination value of *C. sellowiana* PS observed and the morphohystological analyses we decided to work only with DS. In this seed group, we identified a similar germination dynamic at all concentrations of the GA_3 imbibed DS differing only from the seed control treatment (H_2O) (Fig. 5C). The highest germination was observed in 750µM of GA_3, although it did not differ significantly from the other concentrations except to the control (9%) (Fig. 5D). In the same way, germination speed index (GSI) showed high value in all the GA_3 imbibed DS and it there was no statistical differences between them, although they were different from the control treatment (Fig. 5E). Furthermore, we evaluated the morphology of the seedlings and none of their morphological characteristic (hypocotyl (H), radicle (R), total length (TL)) showed significative differences in GA_3 imbibed DS. However, we identified that only hypocotyl showed significant value in GA_3 imbibed DS comparatively to the control treatment (Fig. 5F).

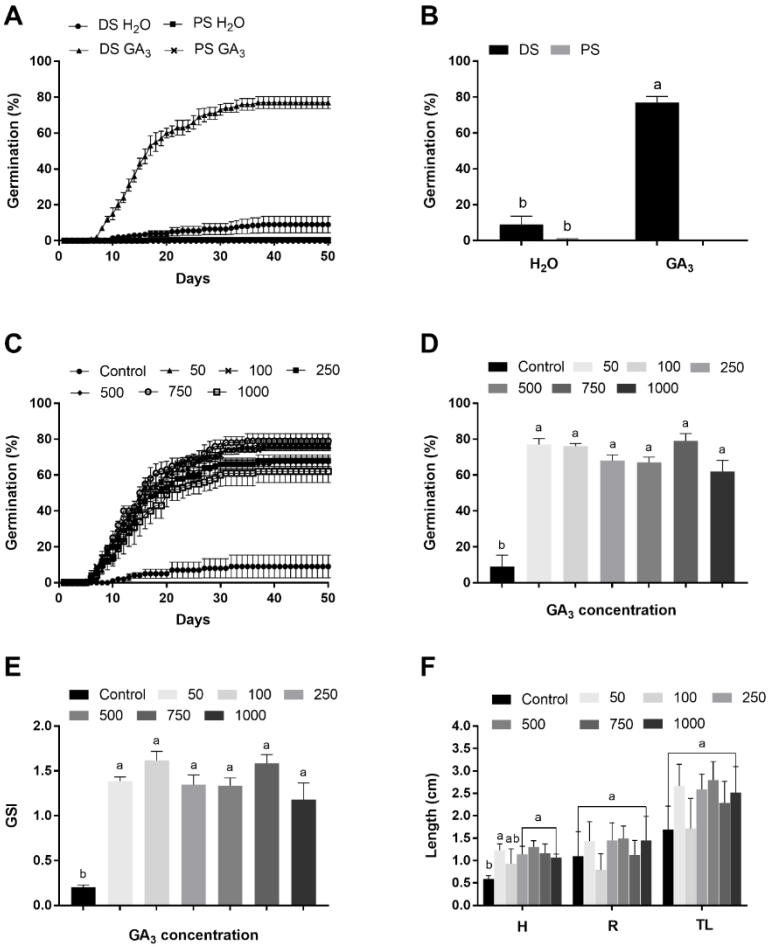

Fig. 5 Seed germination dynamics of *Calibrachoa sellowiana* (Sendtn.) Wijsman dark (DS) and pale (PS) seeds imbibed in H_2O and GA_3 (50 µM) (**A**) and their respective final germination value (%) at 50 days after sowing (DAS) (**B**); Germination dynamics of DS treated with different GA_3 concentrations (0, 50, 100, 250, 500, 750, 1000 µM) (**C**) and their respective final germination (%) at 50 DAS (**D**). Germination speed index (GSI) (**E**) and morphometry (cm) of hypocotyl (H), radicle (R) and total length (TL) from *C. sellowiana* seedlings (**F**). The vertical bars indicate ±SD. Means followed by the same letters are not significantly different according to the Tukey test ($p < 0.05$).

C. sellowiana GA_3-imbibed DS followed the triphasic imbibition curve pattern (Fig. 6A). The results demonstrated a fast imbibition on phase I that finished at 6-hours after inoculation (Fig. 6B) with a fresh mass (FM) increase of 65.91% comparatively to the mature seed. Phase II started after 6 hours and lasted up to 288 hours, with a fresh mass increase of 80.45% (Fig. 6C). Finally, the phase III started after 288 hours with a fresh mass increase of 201.59% (Fig. 6D). At this phase we observed the radicular protrusion from seed coat (Fig 7A,B) with posterior seedling establishment and development (Fig. 7C).

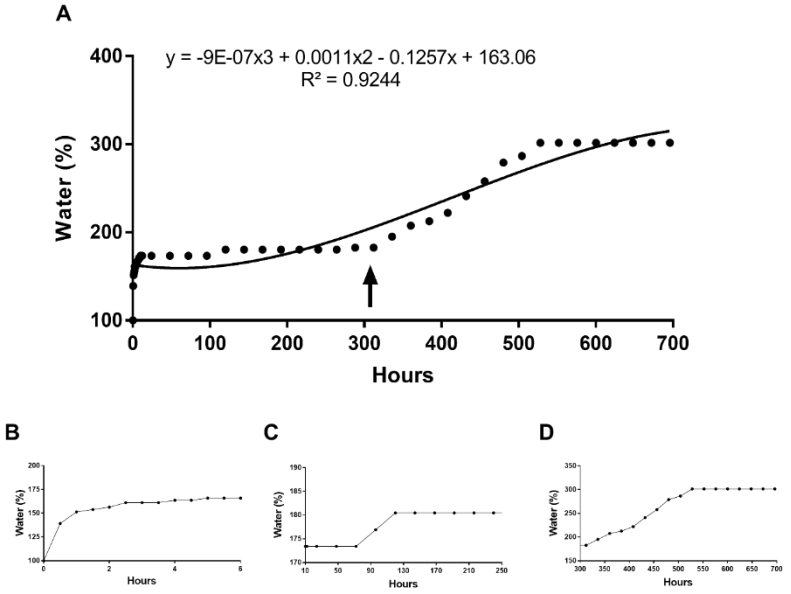

Fig. 6 Imbibition curve of *Calibrachoa sellowiana* (Sendtn.) Wijsman dark seeds (DS) treated with GA_3 (50µM), showing the triphasic pattern (**A**) and the respective and isolated phase I (**B**), phase II (**C**) and phase III (**D**) of seed germination (arrow).

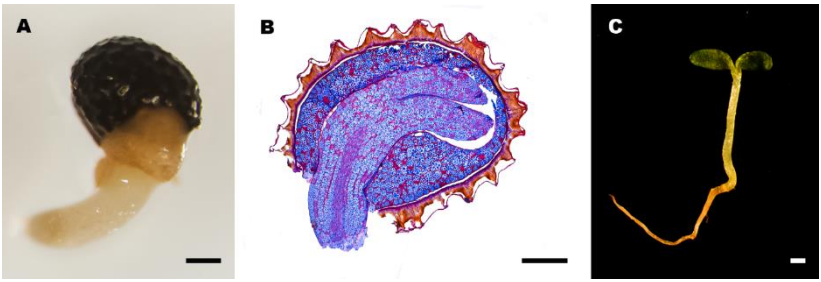

Fig. 7 Stereomicroscope (**A**) and light microscopy (**B**) analyses of *Calibrachoa sellowiana* (Sendtn.) Wijsman DS showing the radicular protrusion and seedling development at 15 days after germination (**C**). A, bar: 200 µm; B, bar: 100 µm; C, bar: 1000 µm.

Transmission electron microscopy (TEM) analysis allowed us to understand the cellular modifications between H_2O and GA_3-imbibed DS during the phase III of seed germination in endosperm cells and ground meristem of the hypocotyl-radicle axis (Fig. 8). In H_2O-imbibed seed, embryo cells presented many protein bodies clusters in process of mobilization inside compartments similar to vacuoles and also sinuous lipid bodies compressed to the cell wall (Fig. 8A). Endosperm cells presented many intact electron-dense protein bodies as storage reserve (Fig. 8B). On the other hand, GA_3-imbibed seeds embryo cells showed the formation of a single vacuole with inclusions (Fig. 8C) while endosperm cells contained a large vacuole and many less electron-dense protein bodies also compressed to the cell wall which were in way to disrupting (Fig. 8D).

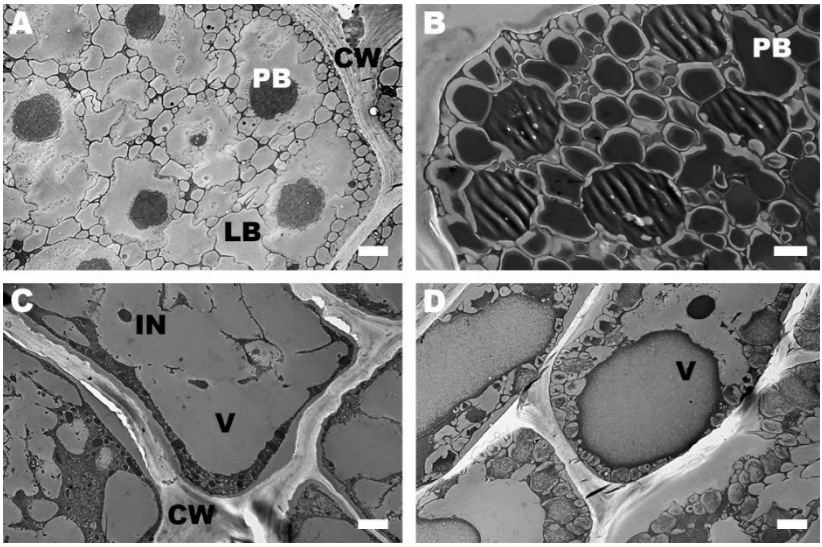

Fig. 8 Transmission electron microscopy (TEM) images of phase III *Calibrachoa sellowiana* (Sendtn.) Wijsman dark seeds (DS) imbibed in H_2O (**A-B**) or GA_3 (**C-D**). Embryo (EM) cell presenting some protein bodies (PB) clusters with different degrees of electron density surrounded by many sinuous lipid bodies (LB) compressed to the cell wall (CW) (**A**). Endosperm (EN) cell presenting many intact electron-dense protein bodies (PB) as storage reserve (**B**). Elongated embryo cells showing the union of many compartments similar to vacuoles in a large central vacuole containing inclusions (IN) in its interior (**C**). Endosperm cells evidencing the presence of large vacuoles (V) and less electron-dense protein bodies compressed to the cell wall. The disruption of cell wall may be perceived (**D**). A-C, bar: 2 µm; D, bar: 1 µm.

The relationship between Gibberellins and Polyamines content during seed germination.

Ours results indicate that all PAs, Putrescine (Put), Cadaverine (Cad), Spermidine (Spd) and Spermine (Spm) content increased at phase III of C. sellowiana GA_3 imbibed DS. We observed an increase of Put in GA_3 imbibed DS at phase III almost three-fold higher (0.10 µmol.g^{-1} FM) than in phases I and II or even in the mature seed. However, in H_2O imbibed DS at phase III it was observed a tiny increase in Put content comparatively to phases I and II (Fig. 8A). In both treatment of seed germination, Cad was found only in phase III of germination, with four-fold higher (0.04 µmol.g^{-1} FM) content in GA_3 imbibed seeds than in H_2O imbibed seeds (0.01 µmol.g^{-1} FM) (Fig. 8B). Spd was the most abundant PA found in C. sellowiana seeds comparatively to the other PAs, and the value found in mature seeds (0.11 µmol.g^{-1} FM) was almost two-fold lower than phase III (0.21 µmol.g^{-1} FM) in GA_3 imbibed seeds. However, we observed that the Spd content in mature seeds was higher than phases I or II of seed germination. In H_2O imbibed seeds it was observed higher endogenous Spd contents in mature seeds than in phases I, II or III of seed germination (Fig. 8C). The endogenous Spm content had an increase almost three-fold higher on phase III (0.05 µmol.g^{-1} FM) on GA_3 imbibed seeds comparatively to mature seeds (0.02 µmol.g^{-1} FM), but phases I and II presented similar content between them. H_2O imbibed seeds showed similar Spm content in phases I, II and III but in mature seeds Spm content was more elevated (Fig. 8D). In both H_2O and GA_3 treatment, total free PAs increased at phase III at the same time that radicular protrusion occurred. Mature seeds presented three-fold (0.137 µmol.g^{-1} FM) more total PAs than phases I and II (0.039 µmol.g^{-1} FM) (Fig. 8E). PAs ratios were not different between the phases of germination, but the values obtained for H_2O and GA_3 imbibed seeds were significantly higher than mature seeds.

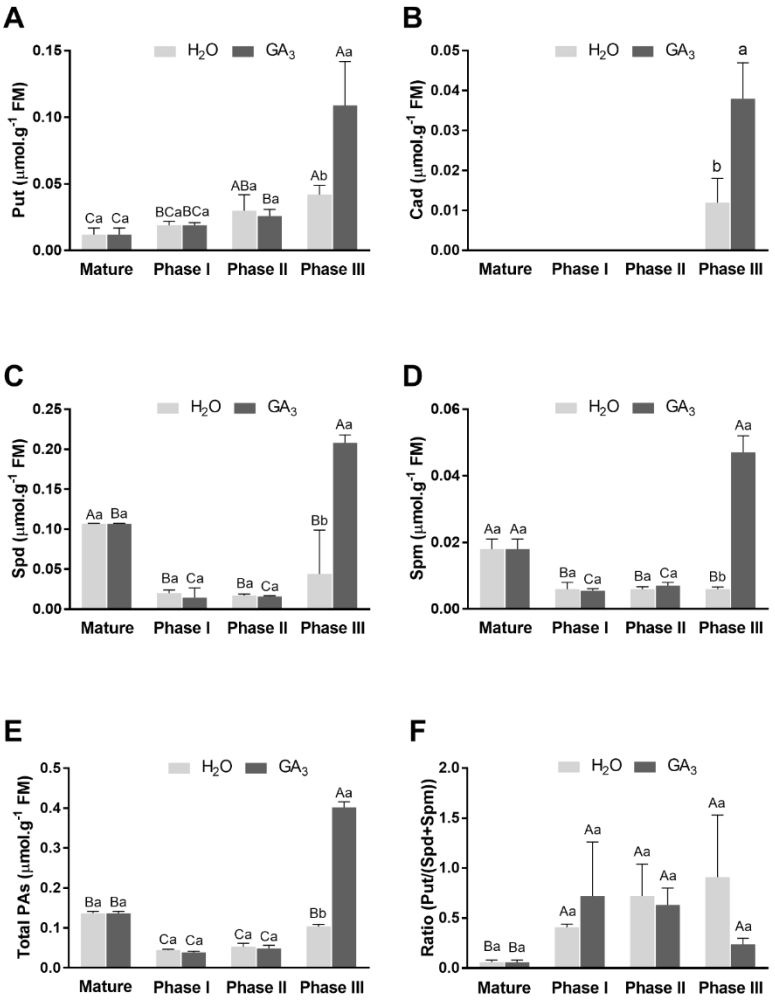

Fig. 9 Endogenous free polyamines (µmol.g^{-1} FM): putrescine (Put) (**A**), cadaverine (Cad) (**B**), spermidine (Spd) (**C**) and spermine (**D**) in mature *Calibrachoa sellowiana* (Sendtn.) Wijsman seeds and over of the three phases of H_2O and GA_3 (50µM) imbibed seed germination. Total polyamines (PAs) (**E**) and the ratio (Put/(Spd+Spm)) (**F**) (µg.g^{-1} FM). The vertical bars indicate ±SD. Means followed by same letters are not significantly different according to the SNK test ($p < 0.05$). Capital letters compare H_2O or GA_3 at distinct phases and small letters compare H_2O and GA_3 at the same phase.

Discussion

The morphohistological characterisation of mature seeds demonstrated differences between groups of seeds.

Our study describes for the first time a large range of physiological characteristics of *C. sellowiana* seed which is an endemic and ruderal species from South Brazil. We demonstrated anatomical, ultrastrucutral and biochemical seed modifications linked to the physiological behaviour over three phases of germination. Most angiosperms seeds consist in an embryo, triploid endosperm surrounded by covering layers such as the maternal tissue nominated seed coat (Weitbrecht et al. 2011). Seed coat is the first structure which comes into direct contact with the environment and faces biotic and abiotic stresses. In ruderal species usually, it is a maternal diploid tissue, mostly composed by dead cells (Finch-Savage and Leubner-Metzger 2006) and it must be rigid and lightweight allowing a successful dispersion. In such way, *C. sellowiana* presents two-layer seed coat as observed in light microscopy providing resistance against stresses. Also, it is possible to verify by histochemical tests the presence of some neutral and acid polysaccharides in the cell wall of tangential external cell layer (Fig. 2). These characteristics were also found in seeds of *Nycandra physalodes*, a Solanaceae species (Lovey et al. 2007). In *Arabidopsis thaliana* the tangential external layer of the cell wall is composed manly by pectin and cellulose, which was associated to the adhesion and osmotic control (Weitbrecht et al. 2011). In our work, phenolic compounds were also found by histochemical reaction in the cell layers of the seed coat. This compound is associated with protective capacities and correlated with the colour of this structure (Attree et al. 2015).

In this study, we found two morphological groups of seeds classified according to the seed coat colour – pale seeds (PS) and dark seeds (DS). In the PS, the seed coat colour was associated to the cell compounds once these cells did not react to the histochemical tests indicating absence mainly of cell wall polysaccharides and lignification comparatively to DS. Moreover, the seed coat colour is determined by flavonoid concentrations and has been linked to germination potential across multiple angiosperms once the more intense the pigmentation, the lower the permeability and the more dormant the seed. (Macgregor et al. 2015). This was observed in *C. sellowiana* seeds once PS and DS showed seed coat differences at cellular level. Next to the seed coat of *C.*

sellowiana seed is found the three endosperm regions surrounding the embryo similar to what was observed in *Nicotiana tabacum* (Lee et al. 2012). This tissue has been described as an important regulator of germination processes in some Solanaceae like *Solanum lycopersicum* and *N. tabacum* (Lee et al. 2012). Finch-Savage and Leubner-Metzger (2006) usually related one of these regions, the micropylar endosperm, to the germination constraint of Solanaceae seeds due to the abundance of compounds such cellulose, arabinan and xyloglucan polysaccharides (Lee et al. 2012). In our work, the parenchymatic cells of the endosperm have thick cell walls as evidenced by PAS reaction. This consists in neutral polysaccharides involved not only in the cell protection against biotic and abiotic stresses but also associated to the regulation of intercellular adhesion, ionic status and cell wall porosity (Lee et al. 2012). We also observed that the endosperm and embryo storage reserves were mostly proteins which reacted with CBB and it was corroborated with transmission electron microscopy analysis. In mature DS, protein bodies filled the entire cytoplasm although mature PS presented large protein bodies clusters formed by the coalescence of numerous proteins bodies and close to them autophagic vesicles were found digesting these reserves (Fig. 3). Seed storage proteins are usually accumulated in special organelles termed protein storage vacuoles, a kind of protein body surrounded by a membrane provided from the cellular endomembrane system (Bewley et al. 2013). Nguyen et al. (2015) postulated these protein bodies might be involved in seed longevity contributing to seed vigour and giving support to seedlings upon germination. However, PS have demonstrated an early digestion of proteins from endosperm cells since these seeds had not yet initiated germination.

Finally, we observed an embryo composed by an embryonic axis, two cotyledons and meristems. At the apex of the embryonic axis near the chalazal endosperm was found the shoot apical meristem. On the other extremity, near to the micropillar endosperm was found the root apical meristem. *C. sellowiana* showed cells of shoot and root apical meristems with very evident nuclei, dense cytoplasm and isodiametric. The shoot and root apical meristems are structures at the tip of the shoot and root, respectively, that are responsible for generating almost all tissues of the plant. They are dynamic structures composed by undifferentiated stem cells involved in the formation of new cells, tissues and organs (Shapiro et al. 2015).

Plant embryos present a tissue organization comprising tree major types: the protoderm, ground tissue and centrally localized vascular tissue (De Rybel et al. 2016). The protoderm is the most external tissue of the

embryo. As we observed in *C. sellowiana* DS, protoderm cells are elongated with small nuclei, vacuolated, perfectly juxtaposed with straight cell walls and usually store products of metabolism. It is associated with embryo covering preventing the action of mechanical shocks and the invasion of pathogens, besides restricting the loss of water (Evert 2006). Underlying the protoderm is found the ground meristem composed of parenchymatic cells characterized by thin-walled cells, presence of small nucleus as well as numerous small vacuoles. Mature DS transmission electron microscopy analysis of ground meristem evidenced the presence of small and numerous rounded lipid bodies surrounding protein bodies. This kind of architecture was also evidenced in *Helianthus annuus* where most of the volume of the cell was occupied by lipid and proteins (Walters et al. 2005). On the other side, mature PS presented large protein bodies clusters in an advanced stage of digestion by autophagic vesicles. As in *Solanum tuberosum* (Evert 2006), *C. sellowiana* embryo presents storage reserve composed basically by lipids and proteins. However, differently of mature embryo cells from DS which demonstrates an intact cellular content, embryo cells of PS showed also advanced metabolic status of digestion of reserves without the initiation of the germinative processes decreasing the physiological quality of these seeds.

The microscopy analysis of *C. sellowiana* showed elongated procambium cells with small nuclei and PB. This tissue and cellular components evidence a normal EM capable to germinate. In the DS we found all these intact structures while in PS not. The growth and maintenance of future vascular tissues occurs through cell periclinal divisions in zones with high mitotic activity such procambium (De Rybel et al. 2016). Besides histochemical difference of the tissues, most PS presented a large interstice between this structure and endosperm and a stunted embryo. All these features interfere in the weigh and water content of PS and could be the cause of the low germination. On the other hand, *C. sellowiana* DS possess a slightly curved embryo filling the entire seed cavity which Martin (1946) classified as a linear axile embryo type in his research investigating Solanaceae seeds.

The physiological data shows loss of tissue viability in pale seeds and signs of nondeep dormancy in dark seeds.

C. sellowiana mature seeds demonstrated an initial water content of 11.63%. Thus, an orthodox behaviour of its seeds is suggested once

this initial water content (~10%) constitutes a desiccation-tolerant state, according to Weitbrecht et al. (2011). The relative water content was higher in DS than PS due to the presence of an interstice, previously demonstrated in histological analysis, and in their ability for osmotic adjustment (Black and Pritchard 2002) which was corroborated with electroconductivity test (Fig. 4). In *C. sellowiana*, the nonviable seeds (PS) have the capacity to uptake more H_2O than others due to the fact that their membranes are probably not intact, and there was absence of lignification and polysaccharides in the seed coat as describe before. This was also mentioned in other angiosperms seeds and are also related to the water potential of these seeds (Weitbrecht et al. 2011). PS had almost three-fold higher electrolytes leaching than DS, indicating the destabilization of PS cell membranes during imbibition. The electroconductivity test indirectly evaluate the extent of damage caused to the cell membranes resulting from seed deterioration (Abreu et al. 2011). The rapid seed imbibition generates internal cell tensions and can result in the disruption of cell membranes and extrusion of cellular contents (Bewley et al. 2013). Based on this, the effectiveness of the electroconductivity test has been reported to evaluate differences between lots of *H. annuus* and *Arachis hypogaea* seeds (Sun et al. 2014; Szemruch et al. 2015).

In our work, the seed coat colour was an indicative of the germinative potential and this was also confirmed by the 2,3,5 TTC (Fig. 4). This biochemical compound is used as an indicator of the reduction process that happens inside the living cells. H^+ ions released during the Krebs cycle of living tissues are transferred to an enzyme group (dehydrogenase) which interacts with the 2,3,5 TTC forming the reduced red formazan (Brasil 2009). For some Solanaceae species the process begins by imbibing 2,3,5 TTC through the micropylar pore and not by the seed coat rupture, resulting in the embryo staining (Macgregor et al. 2015). Our results showed that after 6 hours of imbibition, PS tissue viability was reduced considerably. On the other hand, with 8 hours of imbibition, DS increased the metabolic activation and maintained tissue viability. Similar results were observed when different lots of *Melocactus ernestii* and *M. zehntneri* seeds were submitted to 2,3,5 TTC test (Assis et al. 2015). All these tests corroborate with the results of PS and DS seed germination. PS did not germinate even when they were imbibed in GA_3 and this should be related to the imbibition as well as the short viability time (6 hours) which probably caused a strong cell membranes destabilization. Also, the anticipated mobilization of reserves demonstrated by transmission electron microscopy analysis, would cause

the consequent loss of viability. Moreover, as we described before, PS showed retraction of endosperm and stunted embryo which corroborate with the results of viability test.

On the contrary, beside the low value of total germination (9%) of the H_2O-imbibed (control) DS a high percentage of the GA_3-imbibed DS germinated (77%) (Fig. 5). The DS germination results indicated that they were sensible to the entire concentration spectrum of GA_3. In GA_3 C. sellowiana seeds presented a tree-phase imbibition curve (Fig. 6). At phase I a fast H_2O uptake and an increase in cell turgor from their dry state was observed. The H_2O uptake on this phase is driven by the matrix potential (Weitbrecht et al. 2011) independently of seed viability or dormancy. Thus, the imbibition causes a release of some solutes such as sugars, organic acids, ions, amino acids, and proteins that were evident in the electroconductivity test (Bewley et al. 2013; Steinbrecher and Leubner-Metzger 2018). The phase I is followed by a gap phase II, where the seed metabolism is activated. In this phase the H_2O uptake is minimal, and the metabolic events which occurs either in dormant or nondormant seeds are initiation of respiration, mitochondrial and DNA repair, and preparation of radicle protrusion, known as phase III, in which there is the increment of fresh mass and H_2O content (Bewley et al. 2013). In C. sellowiana seed germination the phase III started 288 hours after imbibition (Fig. 7). At this phase occurs the cell expansion aiming the radicular protrusion which involves selective cell wall loosening and consequently cell wall creep. It is a mechanical process facilitated by cell wall loosening, stress relaxation and wall extension driven by water uptake (Steinbrecher and Leubner-Metzger 2018) and by hydrolytic enzymes or non-enzymatic proteins like α-expansins. A similar dynamics was found in *Solanum lycocarpum* by Pinto et al. (2007) which displayed the common triphasic pattern of water uptake and seed germination. However, the time course of each phase can be variable according the species and seed physiological characteristics (Liu 1996). *C. sellowiana* H_2O-imbibed seeds remained in phase II and did not reach the radicular protrusion while GA_3 exogenous application stimulates germination demonstrating the importance of this plant hormones.

GAs promote cell wall loosening, thereby facilitating endosperm weakening while Abscisic Acid inhibits this process (Steinbrecher and Leubner-Metzger 2018). Degradation of micropylar endosperm is under the control of the embryo. Diffusible signals, such GA_3, induce gene expression on the micropylar endosperm to activate a mechanism of cell wall degradation. This process is accompanied by mobilization of protein bodies in the cells near the radicle. In the same way, besides the

endosperm degradation, the embryo presents a growth potential generated which provides pressure against the endosperm. Thus, germination occurs by the radicular protrusion of the embryo through the seed coat (Nonogaki 2014). GAs molecules act as signals to activate a signal transduction cascade and trigger some cellular process. The signal transduction, an intra or extracellular effector, interact with receptors and its message is relayed through some steps within the cell, eliciting a change in the behaviour of the cell (Richards et al. 2001). Environmental factors such presence of H_2O, light and adequate temperature determine the relative levels of both GAs and Abscisic Acid. GAs synthesis occurs just after imbibition and have a role in seed coat and endosperm rupture while Abscisic Acid content drops rapidly at this time. When the GAs content are elevated, the signal transduction leads to the degradation of RGA-LIKE2 (RGL2) which has a conserved DELLA motif essential for its proteasome-mediated destruction (Weiss and Ori 2007; Piskurewicz et al. 2008). Thus, transcription and translation of genetic material can occur and produce enzymes. In cereals, for example, the GAs released in the aleurone layer induce the transcription of several genes encoding hydrolytic enzymes, including α-amylase. These enzymes degrade starch and proteins of the endosperm that are available to supply the embryo (Weiss and Ori 2007). In the same way, these authors had explained that in *Arabidopsis* GAs had a role in radicle growth. Due to this, we also evaluated some aspects of post-germinative growth of *C. sellowiana* by measuring the radicle, hypocotyl and total size of seedlings. The morphometry analysis of *C. sellowiana* seedlings showed they are normal in size and morphology. These three analysed characteristics were similar between them, except the hypocotyl in the control treatment which was the only one differing statistically. This elongation of the hypocotyl cells was probably caused by GA_3 activity and it is also observed in *Arabidopsis* (Richards et al. 2001) and *Pisum sativum* (Dai et al. 1982). Thus, the presence of GA_3 in the spectrum used in our experiment was not limiting to the seedling growth and could be used successfully to induce germination in *C. sellowiana* seeds.

Considering *C. sellowiana* seeds only germinate with exogenous GA_3 we also point out that these seeds have some kind of dormancy (Fig. 8). Besides H_2O importance, the seeds require oxygen, appropriate temperature and light. Even in these conditions if the seed does not germinate, it is called a dormant seed (Bewley et al. 2013). In our experiments C. sellowiana DS presented only 9% of germination under proposed conditions of H_2O, oxygen, light and temperature indicating some degree of dormancy. In general, dormancy may be imposed by

physiology, integument, morphology or combination of them (Finch-Savage and Leubner-Metzger 2006). The morphological dormancy in *C. sellowiana* seeds was not found in our analysis because the embryo was intact and completely developed according to the microscopy analysis. In addition, the tetrazolium test showed the tissue viability of the embryo. In the same way, the imbibition curve puts in evidence that the problem of low germination rate would not be related to seed imbibition. Along these lines, we proposed an investigation of physiological dormancy when we treated the seeds with GA_3. In this case, the germination rate increased considerably as well the germination speed index. The germination speed had the same statistical pattern than final germination and the seeds imbibed in GA_3 maintained a germination speed index much higher than those obtained when the seeds were imbibed only in H_2O, indicating the exposure to this plant growth regulator stimulated the germination speed. It was demonstrate by Bezerra et al. (2006), that *Egletes viscosa* seeds had a greater germination speed index when imbibed in different concentrations of GA_3 than those imbibed in H_2O. In the same way, Oliveira et al. (2010) observed in *Annona* spp. a germination speed index increase in seeds treated with GA_3. The physiological dormancy is the most abundant form and according to Baskin and Baskin (2004) it could be classified in three levels: deep, intermediate and nondeep. Based on these authors, the nondeep physiological dormancy can be broken submerging seeds on GA_3 or also by scarification, after-ripening in dry storage, and cold or warm stratification (Baskin and Baskin 2004; Finch-Savage and Leubner-Metzger 2006). Thus, we carried out cold stratification (data not shown) in *C. sellowiana* seeds but this treatment was not satisfactory to break dormancy. The seeds were imbibed in H_2O and GA_3 then stored at 8ºC for 30 days or 60 days. None of these treatments showed seed germination or positive 2,3,5 TTC reaction, indicating their ineffectiveness in breaking dormancy.

In this case, the nondeep physiological dormancy may be imposed by embryo or embryo-covering structures, like endosperm and seed coat (Kucera et al. 2005). The endosperm encasing the embryo acts as a mechanical barrier for radicle protrusion where the tissue resistance of the endosperm decreases before radicle emergence. In some Solanaceae species the endosperm resistance is greater than embryo growth potential, preventing germination (Finch-Savage and Leubner-Metzger 2006). The endosperm weakening and consequently seed germination is a complex process and we need to understand the hormonal and physiological aspects involved. An early signal from the embryo is usually required to induce germination processes, and this

signal can be replaced by GAs (Finch-Savage and Leubner-Metzger 2006; Steinbrecher and Leubner-Metzger 2018). In GAs-deficient tomatoes, for example, there is no natural germination unless the seed coat and endosperm are removed, showing the importance of the hormonal control in endosperm weakening. These processes are under control of many hormones, especially Abscisic Acid and GAs and could be related with other hormones, such as PAs as evidenced on this paper.

Seeds treated with Gibberellic Acid had an increase in all Polyamines during the radicular protrusion.

Upon GA_3 treatment, all PAs increased during germination (Fig. 9). Put was the second most abundant PA found in phase III of the GA_3-imbibed seeds and the less abundant in *C. sellowiana* mature seeds. During the germination the embryo needs to elongate and multiply cells of the radicle to break the endosperm and the seed coat which were weakened by GAs action. Thus, Put is related to the stimulus of cell division being a prerequisite to differentiation and cellular division in *S. melongena* according to Yadav and Rajam (1998). In embryogenic cultures of *Araucaria angustifolia* this PA was associated with high cell multiplication and with the enhancement of the Indol-3-Acetic Acid endogenous content (Steiner et al. 2007). Unlike other PAs, in *C. sellowiana* seeds Cad was only found in the phase III, indicating this PA is only synthesized during root protrusion. Gamarnik and Frydman (1991) found this molecule is involved in cell division and proliferation in germinating *Glycine max* seedlings. It could be considered an ecological mechanism once Bagni and Tassoni (2001) had postulated that in some plant families, including Solanaceae, Cad is often related to alkaloids synthesis in the radicular system, which are secondary metabolites involved on insect defence. Moreover, Cad has also been reported to contribute to plant growth and development, cell signalling and stress response to heat, drought, salt and oxidative stresses (Jancewicz et al. 2016). Spd was the most abundant PA found in *C. sellowiana* mature seeds as well in GA_3-imbibed seeds phase III. In S. *melongena* it was associated with the in vitro morphogenetic potential, indicating somatic embryogenesis competence (Yadav and Rajam 1998; Pal Bais and Ravishankar 2002). Moreover, this molecule promoted a cellular alteration in *A. angustifolia* pro-embryogenic masses forming mature somatic embryo (Dutra et al. 2013) showing an important role in the embryo development. On the other hand, Spm was found in very low concentration in *C. sellowiana* mature seed as well GA_3-imbibed seeds phase III. In *Solanum lycopersicum* it was also quantified and in low concentrations however this molecule enhanced antioxidant enzymes activity and lipid peroxidation protection providing thermotolerance (Cheng et al. 2009). Spm may be related with cell membrane stabilization and antioxidant activity which provide cellular integrity (Steiner et al. 2007). Besides that, Kusano et al. (2007) described a defensive role of Spm in salt and drought stresses in *Arabidopsis thaliana* suggesting its role as cellular defense component.

As seen previously, GA_3 showed a positive correlation with PAs in *C. sellowiana* seeds due to the PAs increment in germination, mainly in the treatments with GA_3. The higher values were found in treatments where there was radicular protrusion since the H_2O-imbibed seeds did not germinate. It was reported that GA_3 induced four-fold the ODC activity during the germination of *Hordeum vulgare* seeds (Kyriakidis 1983) consequently increasing the endogenous content of free PAs. Likewise, dormant seeds of *Fagus sylvatica* had increased ODC and ADC activity when imbibed in GA_3 (Krawiarz and Szczotka 2008). In grape berries, the application of GA_3 increased Put content about 150% above control (Shiozaki et al. 1998). The ADC was also increased by application of GA_3 in *P. sativum*. Thus, Put, Spm and Spd were significantly raised by action of this enzyme, supporting the idea that PAs are regulated by GAs. An ADC release already exists before the stimuli of growth, implicating in a cause and effect relationship between GAs and PAs biosynthesis (Dai et al. 1982). Krishnan and Merewitz (2017) have postulated a cross-talking between PAs and GAs isoforms in *Agrostis stolonifera* as well *Triticum aestivum* (Liu et al. 2016) when they analysed treatments containing Spm and Spd and verified these PAs increased the endogenous GAs, suggesting it is regulated by PAs. Thus, we also postulated an association between GAs and PAs in C. sellowiana seeds once we found a strict correlation among them in the germination process.

C. sellowiana phase III H_2O or GA_3-imbibed DS have demonstrated differences at cellular level too. Transmission electron microscopy analysis showed ground meristem of H_2O-imbibed seeds presented sinuous lipid bodies surrounding the protein bodies clusters. It is formed by the coalescence of protein bodies, as seen in the mature seed, which agglomerate and grow in size then can be mobilized by autophagic vesicles during germination. On the other hand, ground meristem of GA_3-imbibed seeds presented the complete mobilization of the protein bodies clusters forming a large vacuole containing inclusion which are the rest of proteins and the disappearance of lipid bodies. This process was also observed in *Vigna radiata* and *Olea europaea* at the germinative process (Van der Wilden et al. 1980; Zienkiewicz et al. 2011) where the breakdown of seed reserves is known to occur after radicle protrusion in order to supply the growing seedling (Walters et al. 2005) and the high vacuolation allow the elongation of embryo cells. Thus, the low germination rate of H_2O-imbibed seeds is in part explained by the fact that it had not yet mobilized its reserves not allowing embryo growth and radicular protrusion.

Endosperm cells also have shown differences between H_2O or GA_3-imbibed seeds at phase III. Endosperm cells of H_2O-imbibed seeds remained with intact protein bodies while those of GA_3-imbibed seeds demonstrated the formation of large vacuoles and the decrease of protein bodies. It was also possible to verify the disruption of its cell walls. The endosperm cells of *Camellia sinensis* were also characterized by the presence of vacuoles and their protein content had been substantially depleted during germination processes (Berjak et al. 1993). Proteases present within protein bodies have no activity because the internal pH is above the optimal pH required for these enzymes. An acidification caused by pumping of H+ ions, activated by GAs, promotes the coalescence of protein bodies and proteases activation resulting in the hydrolysis of the storage proteins and the release of amino acids which become available for the embryo growth. Thus, the vacuolation found in *C. sellowiana* GA_3 treated seeds evidenced the action mechanisms of GAs depleting the storage reserves consequently increasing the cells growth potential by the vacuole pressure against the cell wall. It was also demonstrated in *S. licopersicum*, *Nicotiana* spp. and *Capsicum annuum* where GAs increase the growth potential of embryo cells and it is necessary to overcome the mechanical restraint conferred by endosperm layers by weakening these tissues. (Finch-Savage and Leubner-Metzger 2006).

Conclusions

In the present work, we found two types of *C. sellowiana* seeds according to the seed coat colour: dark seeds (DS) and pale seeds (PS). Besides the higher fresh weight and relative water content, DS presented a seed coat full developed with cells showing the presence of neutral polysaccharides, proteins and phenolic compounds. Its fully developed embryo presented cells with evident nuclei and dense cytoplasm forming the shoot and root apical meristems in the extremities. Surrounding the embryo there was the endosperm composed by large cells with thick walls and proteins as storage reserve as well as many vacuoles. On the other hand, PS presented a seed coat not well developed, a stunted embryo and a retracted endosperm which implicated in a low germination rate. Even showing tissue viability, H_2O imbibed seeds showed a very low germination rate. So, it was suggested the presence of nondeep physiological dormancy as the GA_3-treated PS did not present satisfactory germination while in DS this plant growth regulator increased the germinative rate and germination speed index. Consequently, a tree-phase imbibition curve was observed only in GA_3-imbibed DS. Moreover,

during the radicular protrusion the PAs content was much higher than in the other germination phases. All Put, Cad, Spm and Spd were found in large amounts in the GA_3 imbibed DS on phase III, characterizing an association between GAs and PAs in *C. sellowiana* seeds. From the ultrastructure analysis it could be deduced that all cells of phase III GA_3 imbibed DS were highly active once it presented a high degree of protein mobilization and vacuolation. From the details of this work, future studies could be done to investigate the effects of exogenous application PAs on seed germination of *C. sellowiana* aiming the assess their role in breaking dormancy. Moreover, quantification of hormones during seed germination would be essential to infer about the physiological seed quality of this species.

Author contributions statement

LOZ—designed and performed experiments; LOZ, APL—performed microscopy analysis and statistical data; LOZ and DG—performed polyamine analysis; LOZ and NS— design experiments and wrote the manuscript.

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Capítulo II

Seed storage behaviour of *Calibrachoa sellowiana* (Sendtn.) Wijsman associated to Polyamines

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Abstract

Calibrachoa sellowiana (Sendtn.) Wijsman is a native, endemic ruderal species of South Brazil and has become an important plant for ornamental purposes. Aiming the conservation of their seeds, this study was undertaken to compare the physiological quality of *C. sellowiana* seeds to ultrastructural modifications as well to analyse changes in polyamines as a result of desiccation and chilling stresses during seed storage. Our data suggest that a range of cellular tolerance to desiccation and chilling injuries exist in *C. sellowiana* seeds. The seeds of *C. sellowiana* tolerated desiccation to a very low water content once their final germination rate did not differ statistically from fresh seeds. In the same way, fresh seeds stored at low and ultra-low temperatures remained viable evidencing the maintenance of their viability and vigour under desiccation and cold storage. Ultrastructure analysis have shown modifications in the mobilizations of protein and lipid reserves, aiming the cellular integrity during storage. The polyamines content of desiccated and cold stored seeds has suffered slight fluctuation once they are known to be involved in many physiological processes including resistance against stresses. Hence, this is the first report showing the relationships among physiological, ultrastructural and biochemical analysis in *C. sellowiana* seeds which may be useful for further studies on seed conservation of other herbaceous and endemic species.

Keywords: Solanaceae. Desiccation tolerance. Cold storage. Putrescine. Spermidine. Spermine.

Abbreviations

2,3,5 TTC	2,3,5 Triphenyl Tetrazolium Chloride
DAS	Days after sowing
DS	Desiccated seeds
FM	Fresh mass
GA_3	Gibberellic acid
GSI	Germination speed index
H_2O	Water
PAs	Polyamines
Put	Putrescine
PVS2	Plant Vitrification Solution number 2
Spd	Spermidine
Spm	Spermine
TEM	Transmission electron microscopy
WC	Water content

Introduction

The intensive, predatory extractivism of several plant species, and the unsustainable expansion of industry, agriculture, tourism, and urbanization have resulted in a lost at an unprecedent rate of many habitats and ecosystem services and consequently in the species that disperse and grow in these environments (Li and Pritchard 2009). In this sense, plant conservation strategies have been one of the main concerns of the megadiverse countries, such as Brazil. Conservation can be categorized into two main groups, *in situ* and *ex situ*. *In situ* is the conservation in the natural environment which is critically important as it allows the progression of evolutionary processes; *ex situ* conservation is managed preservation outside the natural habitat. *Ex situ* conservation acts as a back-up for certain segments of diversity that might otherwise be lost in nature and in human-dominated ecosystems (Li and Pritchard 2009). It includes conservation in gene banks, germplasm repositories, genetic resource centres and seed banks (Benson 2008). Plant conservation by seed banking aim tackling the challenges of food security, sustainable energy, loss of biodiversity and climate change by focusing seed conservation efforts on endangered species helping to conserve plant diversity that is most at risk of extinction (Liu et al. 2018). Besides, seed conservation is crucial to avoid the loss of endemic species which have, in addition to economical, ecological and intrinsic values. Moreover, the safest and most economical and convenient way of preserving the genetic resources of the majority of plants is by long-term seed storage (Roberts 1991; Liu et al. 2018).

Seed storage consists of storing the seeds trying to maintain their maximum physiological quality being an inexpensive method of *ex situ* conservation (Souza et al. 2016b). In the same way, a method that has been frequently used for seed conservation is cold storage. This technique is known as viable option for storage of plant cells, tissues, seeds and embryos (Reed 2008). The cryopreservation, a long-term seed storage technique, offers substantial practical and economic benefit to both custodians and end users of biodiversity as it requires minimal space, labour and maintenance, provided cells removed from storage resume normal functions and are fit-for-purpose (Benson 2008). However, cryobanking needs a reliable cryopreservation method for germplasm conservation and features need to be evidenced to suit different genera and species (Mathew et al. 2018). Studies of physiological, structural and biochemical information in relation to the seed-storage capacity of species have increased due to the need for viable seeds for conservation

programs. This information is strictly required once it is considered decisive for maintaining viability after exposure to cold temperatures. However, adequate storage conditions aiming the establishment of storage protocols have been made for only a few species and it is still needed for so many other endemic species (Souza et al. 2016b).

During storage, each step (desiccation in silica gel, pre-treatment, freezing/rewarming) may influence the viability and morphogenetic response of the cells (Kulus et al. 2018). However, the physiological, structural and biochemical status of the seeds might be able to determine the survival of cells and tissues. Before reaching maturity, seeds may acquire important properties, including desiccation tolerance, germination/dormancy and vigour (Bewley et al. 2013; Wang et al. 2015). Considering in terms of sensitivity or tolerance to desiccation, the seeds can be categorized as orthodox, intermediary and recalcitrant (Walters 2015). Orthodox seeds are tolerant in relatively extreme desiccation (~4% water content) and may survive in the dehydrated state for long periods. In contrast, recalcitrant seeds are desiccation sensitive and cannot survive drying during *ex situ* conservation. Intermediary seeds are relatively desiccation-tolerant but do not withstand removal of water to levels as low as orthodox seeds (Pammenter and Berjak 1999; Wang et al. 2015). Due to this dehydrated state, many seeds acquire resistance to stress and some molecules are synthesized late in seed development and are involved in tolerance against stresses (Pammenter and Berjak 1999; Castro et al. 2004)

A group of molecules that are associated with protective aspects is known as Polyamines (PAs). PAs are ubiquitous nitrogenous compounds implicated in the regulation of many physiological processes of which putrescine (Put), spermidine (Spd) and spermine (Spm) accumulate under stress conditions in plants, including during desiccation and chilling stresses (Bouchereau et al. 1999; Cheng et al. 2009). However, the mechanism of action of PAs under stress response is not clearly understood. The accumulating and the extensive variation in PAs content under stress conditions suggest that PAs function in adaptive responses to various environmental stresses. (Ebeed et al. 2017). It has been proposed that PAs involvement in stress adaptation could be due to their roles in osmotic adjustment, membrane stability, and free-radical scavenging (Liu et al. 2016). However, the role of PAs in enhancing the desiccation and cold tolerance of seeds of ruderal species is not yet known and so it is fundamental to understand whether there is any correlation between such characteristics.

Calibrachoa sellowiana (Sendtn.) Wijsman is a native, endemic ruderal species occurring in South Brazil and has become an important species for ornamental purposes (Waterworth and Griesbach 2001). Many *Calibrachoa* cultivars have been launched every year since then by several breeding companies. Today *Calibrachoa* is one of the most important ground cover plants, especially in temperate climates (Kanaya et al. 2010). Several studies have investigated the responses of *Calibrachoa* cultivars to environmental stresses factors (Kanaya et al. 2010), but we found no information regarding water and chilling stresses with seeds of *C. sellowiana*. In this context, this species has great potential for studies to support genetic conservation by mean seed storage. Thus, the objective of the present work was to determinate the threshold of desiccation tolerance and storage at low (8ºC, -20ºC) and ultra-low (-196ºC) temperatures of *C. sellowiana* seeds as well as to analyse the PAs profile content and ultrastructural alterations during desiccation and storage of these seeds. This is the first report showing the relationships among physiological, ultrastructural and biochemical features in *C. sellowiana* seeds, a promising ornamental and endemic species.

Materials and methods

Plant material

Capsules with *Calibrachoa sellowiana* seeds were collected in January 2018 from plants of a natural population located near Curitibanos (27º16'58" South and, 50º35'04" West), Santa Catarina State, Brazil.

Water content

Water content (WC) was measured by 3 samples of 500 seeds weighed, then oven dried at 103±2ºC for 24 hours and weighed again. Moreover, fresh seeds were distributed in *Germitest*® paper imbibed in water for 24 hours at 5ºC (Brasil 2009). The initial water content was calculated according to Sun (2002).

Tetrazolium tests

Tetrazolium test was realized with 3 samples of 25 seeds imbibed in distilled water for 12 hours, then reacted with 2,3,5 Triphenyl Tetrazolium Chloride (2,3,5 TTC) (1%) at 25±2ºC in the dark for 1 hour (Brasil 2009).

Electroconductivity

The electroconductivity was measured with 4 samples of 25 seeds weighed and submerged in 75.0-ml of distilled water. The samples were maintained in an incubation chamber (BOD) at 25±2ºC for 12 hours. At each hour the electroconductivity was measured with a mass conductivity meter and expressed by $\mu S.cm^{-1}g^{-1}$.

Desiccation and conditions of seed storage

The desiccation curve was measured according to Pammenter et al. (2002) where the seeds were desiccated in a hermetically sealed container with silica gel blue (Sigma-Aldrich, RJ, Brazil). It was weighted each 30 minutes until 6 hours and after that each 1 hour until 24 hours. Desiccated seeds were cold stored in polypropylene cryovials at 8ºC and -20ºC for 60 days; and frozen by direct plunging in liquid nitrogen (-196ºC), with or without Plant Vitrification Solution number 2 (PVS2) as cryoprotective treatment. After 24 hours of cryopreservation the cryovials were rapidly thawed in water bath at 40ºC for 1 minute and then the seeds germination tests, ultrastructural and polyamine analysis were carried out.

Germination tests

The seeds were previously disinfected in an ethanol solution (70%) for 30 seconds, then in a sodium hypochlorite solution (1%) for 1 minute and finally, washed 3 times in sterile water. Germination test was carried out with 8 samples of 25 seeds inoculated in 6-cm Petri dishes on *Germitest*® paper moistened with 2.0-ml water (H_2O) or Gibberellic Acid (GA_3) (50 µM) at 25±2ºC in the light (120 $\mu Em^{-2}s^{-1}$, 12/12 hours) for 50 days. The germination speed index (GSI) was calculated according to Maguire (1962).

Transmission electron microscopy

Embryo hypocotyl-radicle axis were fixed in glutaraldehyde (7.5%) containing sodium cacodylate buffer (0.3 M) and sucrose (2%) for 7 days. The material was post-fixed with sodium cacodylate buffer (0.1 M) containing osmium tetroxide (1%) for 4 hours. The material was dehydrated in an increasing ethanolic series and then embedded in LR White Acrylic Resin® (Merck KGaA, Darmstadt, Germany) for 15 days. Ultrathin sections (70 nm) were collected on grids recovered with Parlodion than stained with aqueous uranyl acetate followed by lead citrate. Two grids for treatment were examined in the JEM 1011

transmission electron microscopy (TEM) (JEOL Ltd., Tokyo, Japan) at 100 kV.

Polyamines determination

Polyamines analysis was carried out by 3 samples of 200 mg of seeds grounded in 1.6-ml of perchloric acid (5%). Free polyamines (PAs) were extracted, dansylated and identified by reverse phase HPLC, according to Steiner et al. (2007). PAs content was determined using a fluorescence detector at 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).

Statistical analysis

Data have been analysed for significance using one-way ANOVA (R® Software, version 3.4.1, 2017) with the Tukey's multiple range post hoc test to identify significant differences ($P<0.05$).

Results

Physiological tolerance behaviour during seed desiccation and storage of Calibrachoa sellowiana.

C. sellowiana fresh seeds showed 11.63% of water content (WC) and after 3 and 24 hours dehydration in silica gel seeds reached 6.96% and 4.02% WC, respectively (Fig. 1A). The seed desiccation affected germination dynamic in *C. sellowiana*. It was observed that all H_2O or GA_3 imbibed seeds germinated and both started to germinate at 7 days after sowing (DAS) (Fig. 1 B,C). Fresh seeds H_2O-imbibed reached 13% of germination in 38 days while 6.96% and 4.02% reached 13% and 18% in 39 days, respectively (Fig. 1B). On the other hand, fresh seeds GA_3-imbibed reached 77% in 37 days, while seeds with 6.96% and 4.02% WC reached 39% and 65% of germination in 39 and 37 days, respectively (Fig. 1C).

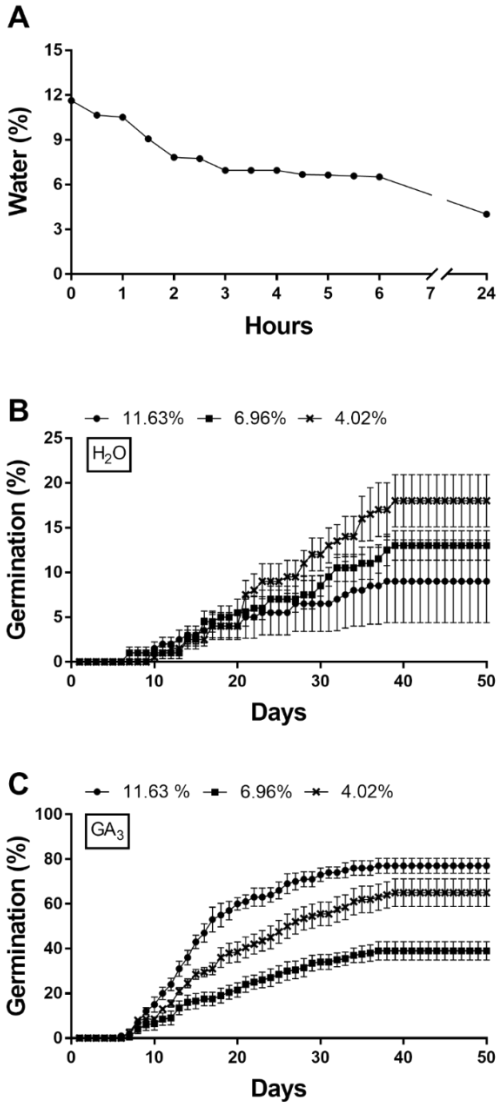

Fig. 1 Desiccation curve (%) (**A**) and seed germination of *Calibrachoa sellowiana* (Sendtn.) Wijsman represented by germination dynamics of fresh (11.63%) and desiccated seeds (6.96%, 4.02%) germinated in H_2O (**B**) or GA_3 (50 µM) (**C**) at 25±2ºC in the light (120 $\mu Em^{-2}s^{-1}$, 12/12 hours) for 50 days. The vertical bars indicate ±SD.

Independent of seed WC and storage temperature (8ºC, -20ºC, -196ºC) all the seeds germinated in H_2O showed low values of germination (Fig 2). The highest germination value (25%) in H_2O treatment was observed in seeds with 4.02% WC stored at -196ºC treated with PVS2 (Fig 2D). Previous work with this species reported the use of GA_3 to promote germination (Chapter I), however we keep the seed germination in water for all the desiccation and storage treatments as a control as well to compare with GA_3 (50µM) treatment. Independently of the storage temperature all seeds germinated in GA_3 and there were no statistical differences between different WC. The highest value of seed germination (78%) was observed in GA_3 imbibed seeds stored at -196ºC with 6.96% WC (Fig. 2D). This value did not differ from those obtained with 11.63% and 4.02% WC at the same storage temperature. On the other hand, the lowest germination value of GA_3 imbibed seeds (39%) was observed in desiccated seeds (DS) with 6.96% WC which were not stored (Fig. 2A).

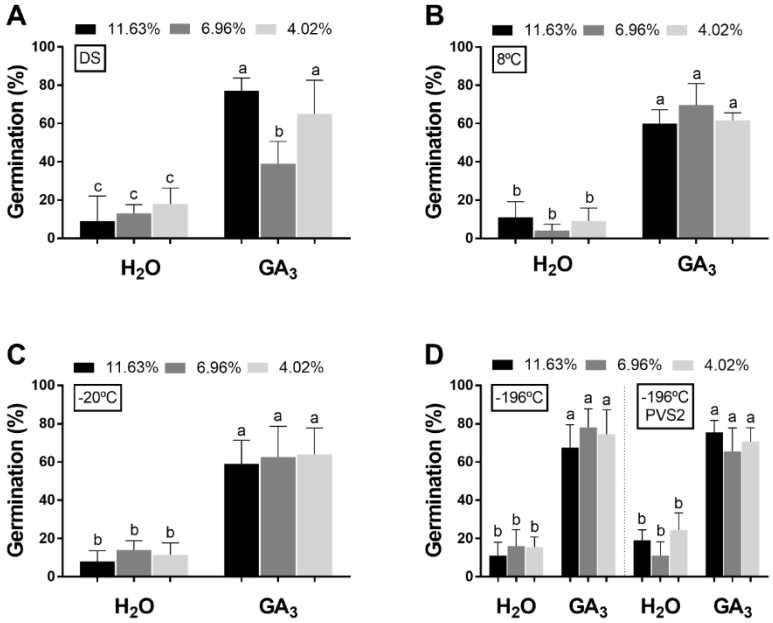

Fig. 2 Germination of fresh (11.63%) and desiccated (6.96%, 4.02%) (DS) *Calibrachoa sellowiana* (Sendtn.) Wijsman seeds in H_2O or GA_3 (50µM) treatments (**A**) and stored at low temperatures of 8ºC (**B**) and -20ºC (**C**); stored at ultra-low temperature of -196ºC (**D**). The vertical bars indicate ±SD.

Means followed by the same letters are not significantly different according to the Tukey test ($p < 0.05$).

The treatment with GA_3 imbibed seed showed an increase in the germination speed index (GSI). The highest GSI (1.76) was observed in 11.63% WC seeds stored at -196ºC with PVS2 treatment (Fig. 3D) while the lowest GSI (0.63) was observed in desiccated seeds (DS) with 6.96% WC (Fig. 3A). Similar to the germination dynamic data, H_2O imbibed seeds showed a decrease in their GSI and the highest GSI (0.42) was observed in 11.63% WC of seeds stored at -196ºC with PVS2 treatment (Fig. 3D), while the lowest GSI (0.13) was observed in fresh seeds (Fig. 3A).

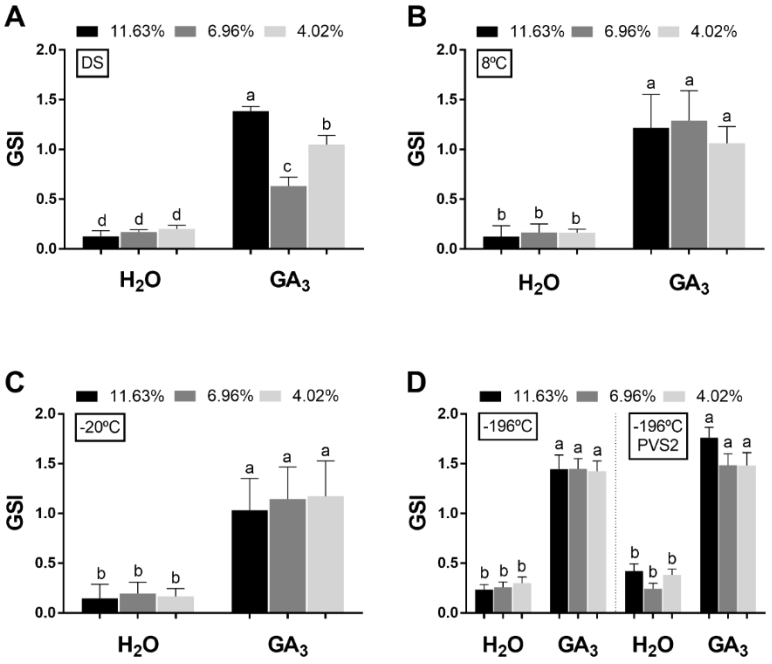

Fig. 3 Germination speed index (GSI) of fresh (11.63%) and desiccated (6.96%, 4.02%) (DS) *Calibrachoa sellowiana* (Sendtn.) Wijsman seeds germinated in H_2O or GA_3 (50µM) (**A**) and GSI of seeds stored at low temperatures of 8ºC (**B**) and -20ºC (**C**); and ultra-low temperature of -196ºC (**D**). The vertical bars indicate ±SD. Means followed by the same letters are not significantly different according to the Tukey test ($p < 0.05$).

Electroconductivity values ($\mu S.cm^{-1}g^{-1}$) indicated two categories of seed behaviour. Fresh seeds (11.63% WC) had an electroconductivity value of 251.78 $\mu S.cm^{-1}\ g^{-1}$. The other group was desiccated seeds (6.96 and 4.02% WC) (DS) which had similar values of 421.84 $\mu S.cm^{-1}\ g^{-1}$ and 392.09 $\mu S.cm^{-1}\ g^{-1}$, respectively (Fig. 4A). On the other hand, electroconductivity values of seeds stored at 8ºC and -20ºC was very similar. 11.63% WC seeds exhibited a low electrolyte leakage (255.785 $\mu S.cm^{-1}g^{-1}$ and 254.573 $\mu S.cm^{-1}g^{-1}$, respectively) if compared with desiccated treatments which presented an increase in its leakage (628.763 $\mu S.cm^{-1}g^{-1}$ at 8ºC and 590.818 $\mu S.cm^{-1}g^{-1}$ at -20ºC) (Fig. 4B,C). At -196ºC the electrolyte leakage was similar (398.026 $\mu S.cm^{-1}g^{-1}$) for all WC, with or without PVS2 treatment (Fig. 4D).

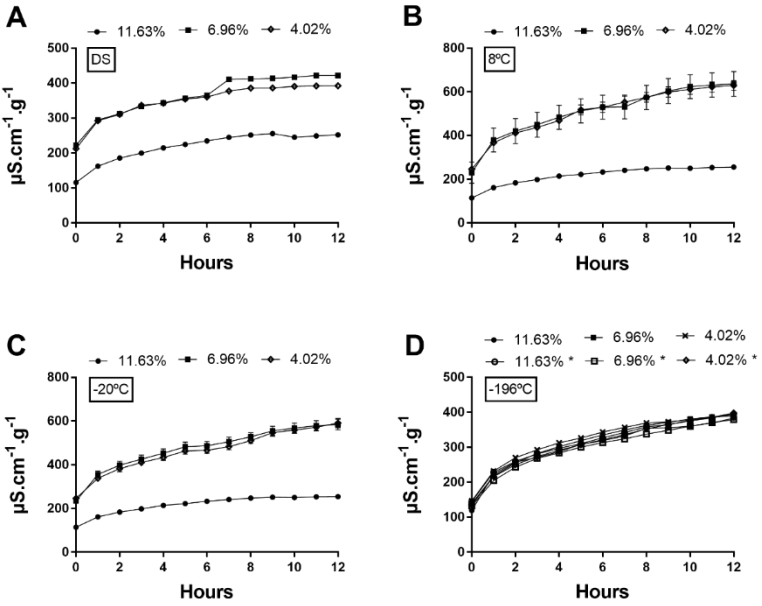

Fig. 4 Electrolyte leakage ($\mu S.cm^{-1}g^{-1}$) of fresh (11.63%) and desiccated (6.96%, 4.02%) (DS) *Calibrachoa sellowiana* (Sendtn.) Wijsman seeds (**A**) and seeds stored at low temperatures of 8ºC (**B**) and -20ºC (**C**); and ultra-low temperature of -196ºC (**D**). The asterisk (*) indicates cryoprotectant treatment with PVS2.

Indirect analysis of seed viability by tetrazolium (2,3,5 TTC) indicated that 11.63% WC seeds had the highest tissue viability value (72%) in DS as well as seeds stored at 8ºC and -20ºC, while seeds with 6.96% WC stored at 8ºC had the lowest tissue viability value (11%) (Fig. 5). However, these results did not agree with the results of the germination tests.

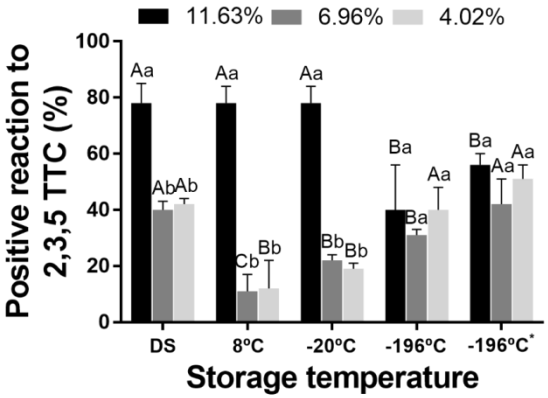

Fig. 5 Positive reaction (%) dynamics to the 2,3,5-Triphenyl Tetrazolium Chloride (2,3,5 TTC; 1%) of fresh (11.63%) and desiccated (6.96%, 4.02%) (DS) *Calibrachoa sellowiana* (Sendtn.) Wijsman seeds and seeds stored at low temperatures of 8ºC and -20ºC; and ultra-low temperature of -196ºC imbibed in H_2O for 12 hours at 25±2ºC in the light (120 $\mu Em^{-2}s^{-1}$). The asterisk (*) indicates cryoprotectant treatment with PVS2. The vertical bars indicate ±SD. Means followed by the same letters are not significantly different according to the Tukey test ($p < 0.05$). Capital letters compare different water contents at distinct storage temperatures and small letters compare different water contents at the same storage temperature.

Ultrastructural alterations could be perceived during desiccation and cold storage of Calibrachoa sellowiana embryo cells.

The ultrastructural analysis of C. sellowiana fresh embryos showed ground meristem cells with the presence of electron-dense protein bodies (PB) surrounded by populated rounded shape less electron-dense lipid bodies (LB) (Fig. A). However, 11.63% WC embryo cells stored at 8ºC presented cytoplasm filled with protein bodies organized in the cell wall (CW) periphery as well the presence of some large cluster of proteins. Moreover, lipid bodies had dramatically decreased in number. The cell wall presented a tiny and sinuous aspect and are separated by the middle lamella and a cell shrinkage may be perceived on this treatment (Fig. 7B). On the other hand, embryo cells of desiccated seeds at 4% WC and stored at -196ºC presented a dense cytoplasm showing a peculiar ripple shape of protein bodies while lipid bodies were found in coalescence in the cell wall periphery which were thicker by the deposition of compounds (Fig. 7C).

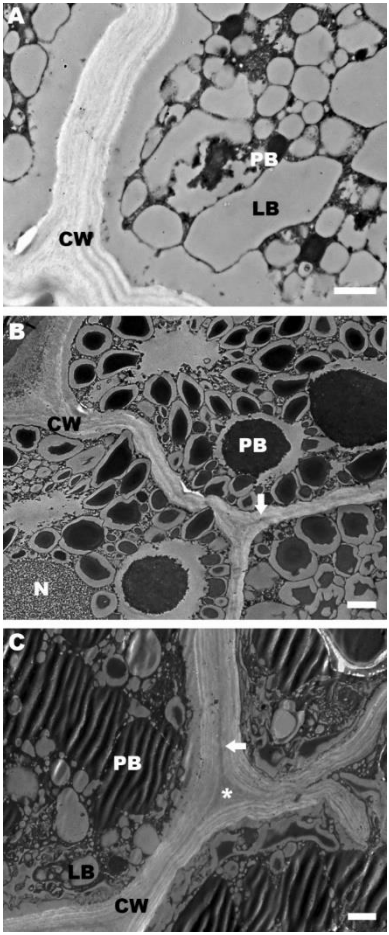

Fig. 6 Transmission electron microscopy (TEM) images of *Calibrachoa sellowiana* (Sendtn.) Wijsman seeds. Ground meristem embryo cells of fresh seeds presenting a cell wall (CW) well developed and a cytoplasm filled with electron-dense protein bodies (PB) surrounded by densely populated of less electron-dense lipid bodies (LB) (**A**). 11.63% WC embryo cells stored at 8ºC for 60 days showing a nucleus (N), the sinuous cell wall of the cells separated by the middle lamella (arrow) and the storage reserve of electron-dense protein bodies organized in the periphery (**B**). 4.02% WC embryo cells stored at -196ºC for 24 hours showing the cytoplasm filled with electron-dense protein bodies and less electron-dense lipid bodies in coalescence to the cell wall. The intercellular space (*) and the middle lamella (arrow) may be perceived (**C**). A, bar: 1 µm; B-C, bar: 2 µm.

Polyamines content fluctuation on the seed storage behaviour of Calibrachoa sellowiana.

Our results have demonstrated the fluctuation of putrescine (Put), spermidine (Spd) and spermine (Spm) content at different WC of C. sellowiana seeds (Fig. 7A). Put increased above eleven-fold in desiccated 6.96% WC seeds (0.133 µmol.g^{-1} FM) when compared with fresh seeds (11.96% WC) (0.012 µmol.g^{-1} FM). On the other hand, the fresh seeds increased almost two-fold (0.107 µmol.g^{-1} FM) the content of Spd than 6.96% WC seeds (0.054 µmol.g^{-1} FM) but did not differ statistically from the 4.02% WC seeds (0.060 µmol.g^{-1} FM). Moreover, Spm content did not differ statistically between the different WC and fresh seeds (11.96% WC) which showed 0.018 µmol.g^{-1} FM of Spm. Total free PAs of fresh seeds (20.36 µmol.g^{-1} FM) did not differ statistically from all desiccated seeds (Fig. 7B). However, PAs ratio of 6.96% WC seeds was higher (1.05 µg.g^{-1} FM) than the 4.02% WC seeds (0.2 µg.g^{-1} FM) or fresh seeds (0.06 µg.g^{-1} FM) (Fig. 8C).

We also analysed the endogenous free PAs content of fresh seeds (11.96% WC) as a control to compare to different cold storage temperature (Fig. 8D). Our results indicate that Put content was less abundant in seeds stored at -196ºC with PVS2 (0.009 µmol.g^{-1} FM) and it was similar to the control (0.012 µmol.g^{-1} FM). These two treatments differed statistically from the other treatments (8ºC, -20ºC, -196ºC). Spd presented the highest values in C. sellowiana seeds stored at -196ºC with PVS2 treatment (0.128 µmol.g^{-1} FM) which differed from storage at 8ºC (0.059 µmol.g^{-1} FM). On the other hand, Spm presented similar values between the storage temperatures and control (0.018 µmol.g^{-1} FM) but differed from the storage at -196ºC with PVS2 treatment which presented the lowest contents (0.007 µmol.g^{-1} FM). Total free PAs showed similarities between the control (20.36 µmol.g^{-1} FM) and all the others storage temperatures (Fig. 8E). However, PAs ratios were similar for seeds stored at -196ºC with PVS2 treatment (0.04 µg.g^{-1} FM) and control (0.06 µg.g^{-1} FM) but differed from seeds stored at 8ºC which showed higher values (0.17 µg.g^{-1} FM) (Fig. 8F).

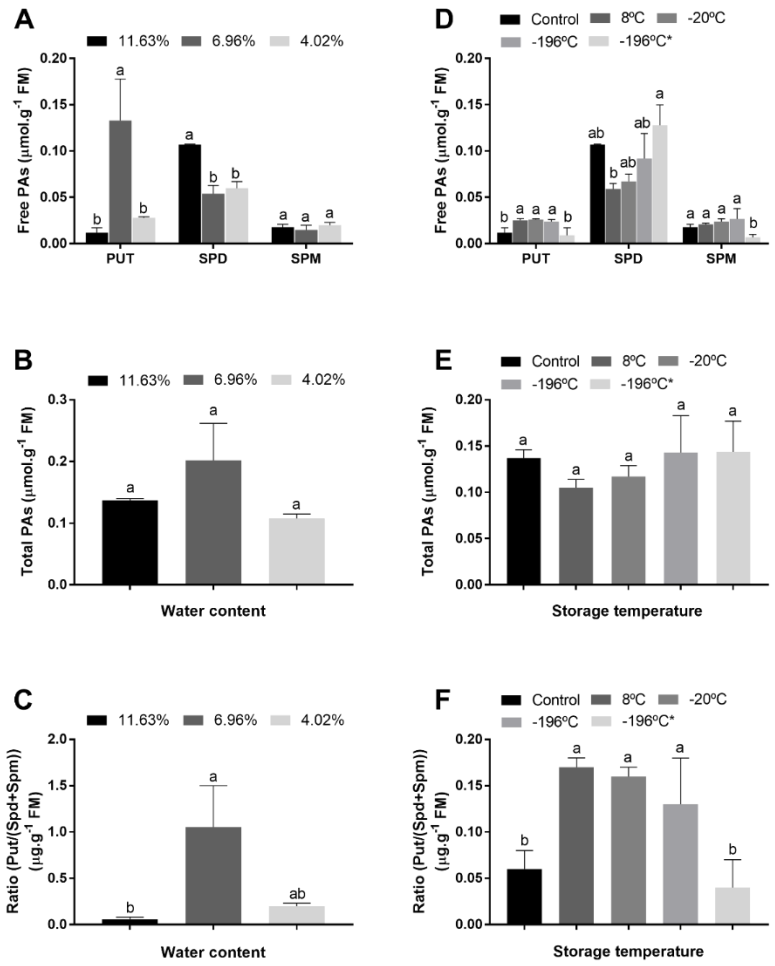

Fig 7 Endogenous free polyamines (PAs) (µmol.g^{-1} FM) putrescine (Put), spermidine (Spd) and spermine (Spm) of *Calibrachoa sellowiana* (Sendtn.) Wijsman seeds in three water content (WC) (11.63%, 6.96%, 4.02%) (**A**) as well their total PAs (**B**) and ratio (Put/(Spd+Spm)) (**C**) (µg.g^{-1} FM). Endogenous free PAs (µmol.g^{-1} FM) of fresh seeds (11.63% WC - control) and fresh seed stored at 8ºC, -20ºC, -196ºC, -196* (**D**) as well their total PAs (**E**) and ratio (Put/(Spd+Spm)) (**F**) (µg.g^{-1} FM). The asterisk (*) indicates addition of cryoprotectant treatments. The vertical bars indicate ±SD. Means followed by same letters are not significantly different according to the Tukey test ($p < 0.05$).

Discussion

Physiological data have demonstrated the maintenance of vigour and viability of Calibrachoa sellowiana seeds after exposure to desiccation and cold stresses.

Considered only in terms of desiccation tolerance which is typically described by a threshold or low water content limit to survival, seeds can be categorized as orthodox, intermediary and recalcitrant (Walters 2015; Wang et al. 2015). *C. sellowiana* fresh seeds showed low WC (11.63%) after-ripening and elevated germinability. Usually, orthodox seeds are capable of surviving to desiccation at maturity, which state they can remain for long periods and continue to growth after imbibition (Bewley et al. 2013). In our work, after 24 hours of *C. sellowiana* seeds desiccation it was observed the stability of desiccation (4.02% WC) and the germinability remained stable. However, H_2O imbibed seeds presented low values of germination which may be due to the physiological dormancy attributed to this species which is a block to the completion of germination of an intact viable seed under these favourable conditions (Finch-Savage and Leubner-Metzger 2006). It can be an adaptive strategy for seed to survive under adverse natural conditions, but it also creates an obstacle for ornamental seed production, where rapid germination and growth are required (Wang et al. 2015). Thus, fresh and desiccated seeds of *C. sellowiana* treated with GA_3 had considerably increased in its germination values, corroborating the possible physiological dormancy. Similar results were also found in other dormant species such *Annona cherimola* (Oliveira et al. 2010), *Amaranthus retroflexus* (Kepczynski and Sznigir 2014) and *Egletes viscosa* seeds that presented a low WC (8.7%) and had an increment of the germination when imbibed in GA_3 solution (Bezerra et al. 2006). This effect is due to the fact that Gibberellins play a key role in dormancy release and in the promotion of germination by increase the growth potential of the embryo and overcoming the mechanical restraint conferred by endosperm and seed coat (Kucera et al. 2005).

Our results indicate that *C. sellowiana* seeds are characterized to survive no only at low WC but also at low and ultra-low temperatures storage. Independently of the WC and storage temperature the seeds maintained the viability and do not showed significative differences in germination. Contrasts were found only in the comparison between seeds germinated in H_2O and germinated in GA_3 once the vigour (GSI) and germination increased considerably in the last one. The highest

germination value (78%) of *C. sellowiana* was observed in seeds with 6.96% WC stored at -196ºC, similar to the germination value observed in fresh seeds (77%). Similar results were found by Souza et al. (2016a) in the germination of *Physalis peruviana* seeds stored at 5ºC and -196ºC which did not affect final germination rate if compared with fresh seeds. In the same way, *Solanum lycopersicum* cryopreserved seeds for 28 days displayed about 60% of germination without any statistically significant differences from fresh seeds (Zevallos et al. 2013). These authors suggest that seeds with low WC can be stored at ultra-low temperatures and maintain their physiological quality. These finds confirm the data of the present work and indicate that Solanaceae seeds tend to be similar in physiological behaviour to seed desiccation and storage. Our study shows that the combined effects of desiccation and storage do not negatively affect seed viability and vigour. Specifically, our results indicate that storage temperature do not influence *C. sellowiana* seed response to germination.

During seed germination, imbibition is required for the resumption of metabolism and initiation of cellular events leading to physical and structural changes associated with the absorption of water (Bewley et al. 2013). In our work, the electroconductivity of *C. sellowiana* fresh seeds suggested the stable structural organization of cell membranes due to the low electrolyte leakage and keep the germination. On the other hand, desiccated and stored seeds reached an elevated electrolyte leakage. It is in part explained by the destabilisation of cell membranes during imbibition once desiccation enhances the electrolyte leakage by inducing cell membrane damage (Mckersie and Stinson 1980). Membranes are considered one of the primary sites of lethal damage by desiccation to cells (Bryant et al. 2001). In damaged seeds the cellular membranes are not intact and solutes are released into the apoplast. Once the imbibition is too fast in seeds with low WC, an internal cell tension can result in disruption of cell membranes and extrusion of cellular contents, often leakage solutes such as sugars, organic acids, ions, amino acids and proteins increasing the electroconductivity of the surrounding medium (Bewley et al. 2013). This indicates that imbibition damage and solute leakage are associated with rapid water uptake into seeds that are at low WC. It was observed in desiccated seeds of *Glycine max* which showed a greater electrolyte leakage at 12 hours of imbibition and consequently decreased germination (Senaratna and McKersie 1983) evidencing some degree of cell membrane destabilization. However, in *C. sellowiana* desiccated and stored seeds, the high electrolytes leakage did not affect seed germination. On the other hand, this may be explained

by the extrusion of biological compound that are result of metabolism activation during the first stage of seed germination.

Additionally, the germinative potential of desiccated and cold stored seeds was tested by the 2,3,5 TTC as an indirect test of seed viability. The 2,3,5 TTC give us the respiratory activity in living cells (Brasil 2009). Our results showed that after 12 hours of imbibition 11.63% WC seeds stored at 8ºC and -20ºC presented the highest values of viability while desiccated seeds decreased considerably their viability. However, the results found in the germination values of *C. sellowiana* were somewhat inconsistent with the viability results found in 2,3,5 TTC test indicating that even under dehydration and cold storage stress seed are able to germinate.

The different water states play an important and central role in cryopreservation and storage stability. Water status and cryoprotection are the most influential determinants of seed survival in combination with physiological factors after exposure of seeds to cold temperatures below 0ºC (Reed 2008) due to the fact that as temperature is reduced water molecules change its symmetry and ice is formed (Benson 2008). Additionally, when is applied a slow and controlled cold temperature, ice initially forms extracellularly and a difference water potential is created across cellular membrane. Consequently, intracellular water moves outside the cell causing a cryoprotective effect as it reduces the amount of water available to form damaging ice (Benson 2008). In *C. sellowiana* seeds stored at -196ºC, the vigour and viability rates remained stable independently of the WC. The principle of cryopreservation is almost universal because it uses ultra-low temperatures to slow, to the point of cessation, but not death, vital functions such as enzymatic activity and cell division being expected that liquid nitrogen arrest ageing at cellular, physiological and molecular levels (Benson 2008). Thus, while high temperature and WC increase cell respiration, low temperatures delay metabolic processes. It has been suggested that storage below -18ºC guarantees stabilization of viable cells *ad infinitum*, based on the premise that germplasm remains viable and, does not undergo long-term changes and can be recovered alive and unaltered after storage (Benson 2008). In this case, the metabolic processes are in a latent state, minimizing deterioration and allowing cell conservation. In cryopreservation some exogenous substances could be used to protect cells against damaging ice crystals formation. Plant Vitrification Solution number 2 (PVS2) is a cryoprotective treatment commonly used and is effective for both temperate and tropical seeds (Reed 2008). However, in our work PVS2 did not show statistically differences of control treatment. It makes

explicit that there is a mechanism in *C. sellowiana* seeds that maintain cellular integrity during freezing once it is in part explained by absence of damaging ice crystals inside the cell. Thus, when ultra-rapid cooling rates are applied, water molecules may not have the opportunity to create crystals (Benson 2008) allowing to keep cell and tissue integrity. *C. sellowiana* seeds could resist not only desiccation but also to the cryopreservation damage and this suggest that these seeds showed an orthodox seed behaviour.

Ultrastructural features have evidenced changes in the storage reserves configuration allowing viability during desiccation and cold storage of Calibrachoa sellowiana seeds.

Ultrastructural changes in ground meristem embryo cells of C. sellowiana were investigated under cold storage at different WC. Cells of fresh embryo (11.63% WC without storage) showed their cytoplasm filled with lipid bodies surrounding protein bodies. This kind of cell architecture was also found in *Helianthus annuus* (Walters et al. 2005), *Taxus mairei* (Chien et al. 1998) and *Arabidopsis thaliana* (Shimada et al. 2008) where lipid bodies and protein bodies had an orderly distribution. This pattern found in wild-type seed cells can be related with the promotion of the rapid degradation and utilization of lipids and proteins for maintenance of germination once this embryo is able to germinate (Shimada et al. 2017). However, a new organization could be observed on 11.63% WC *C. sellowiana* embryo cells stored at 8ºC due to the fact that their cytoplasm increased the quantity of protein bodies apparently decreasing lipid bodies. Thus, many physiological changes occur in plants in response to cold temperature, particularly in cellular membranes which can be attacked by free radicals suffering peroxidation (Gao et al. 2009). In this case, the decrease of lipid bodies could be associated to the fatty acid peroxidation during the temperature drop as observed by Rahman et al. (2013) in cold stored seeds (11% WC) of *Abelmoschus esculentus*, where the peroxidation of lipid bodies caused by cold storage degraded cell membrane and caused leakage from cells. On contrary, protein bodies appeared to be filling the entire cytoplasm of the *C. sellowiana* embryo cells as well as being juxtaposed to the sinuous cell wall. By the accumulation of proteins, embryo cells tend towards a more solid matrix and the structure is stabilized by solidification once solids structures maintain their shape (Walters 2015). With the decrease to ultra-low temperature (-196ºC) and extreme desiccation (4.02% WC), *C. sellowiana* embryo cells showed the coalescence of protein bodies into great protein clusters with peculiar ripple shape as well the presence of lipid bodies on the cell wall periphery. The accumulation of reserves, besides maintains cellular shape as seen before, it has a role in the protection against osmotic stresses. In embryogenic cultures of *Bactris gasipaes* a greater content of storage compounds was associated with a low content of free water, thus avoiding the formation of ice crystals, the main cause of cell collapse at cryogenic temperatures (Heringer et al. 2013). Thus, desiccated embryo cells with great quantities of reserves are able to face water loss without imposing water stress and then avoid the

formation of ice (Walters 2015). In transmission electron microscopy analysis of *C. sellowiana* embryo cells, these protein reserves acquired a peculiar ripple shape. These effects were probably attributed to the rapid freezing method used in the cryopreservation technique that collapsed the structures of proteins giving it that shape. Lipid bodies are storage compartments actively biosynthesized in seed embryo cells that are degraded during seed germination and seedling growth, providing energy and metabolic substrates during germination processes (Shimada et al. 2017). Moreover, in the process of cryopreservation, lipid bodies could be mobilized to sustain the cellular shape (Walters 2015).

Polyamines content have suffered slight fluctuation during desiccation and cold storage of Calibrachoa sellowiana seeds.

Desiccation and cold storage could lead seeds to loss their vigour and viability by injuries caused at cellular or molecular level. The major PAs Put, Spd and Spm are known to be involved in many physiological processes including resistance against stresses (Bouchereau et al. 1999). Remarkably, desiccation stress induces changes in PAs contents, which broadly correlate with drought resistance traits (Alcázar et al. 2011). In our experiments *C. sellowiana* fresh (11.63% WC) and desiccated (6.96% and 4.02% WC) seeds have suffered a low fluctuation in endogenous content of free PAs. Put was among the PAs found in smaller amounts in C. sellowiana fresh seeds. Put is known to be involved in the maturation of the embryo during seed development. It was observed that this molecule is linked to cell division and elongation during embryo development of *Pinus sylvestris* (Vuosku et al. 2006) and *Araucaria angustifolia* (Astarita et al. 2003). Furthermore, Put can modulate the expression of peroxidases and other related proteins at the seed development stage (Oliveira et al. 2016). However, in *C. sellowiana* desiccated seeds at 6.96% WC, Put content was considerably elevated comparatively to fresh (11.63% WC) seeds. Similar results were found in *Oryza sativa* plants subjected to PEG-induced drought stress that responded by increasing cellular Put contents significantly (Capell et al. 2004). These authors suggested that Put accumulation in tissues under stress might be a consequence of the reduction in the rate of Spd and Spm synthesis, the molecules that are believed to protect plants under desiccation stress. On the same way, generally Spd was observed in great quantities in seeds of *C. sellowiana* comparatively to the others free PAs. *S. lycopersicum* fruits also showed an increase in Spd content under water stress which showed greater antioxidant response (Sánchez-Rodríguez et al. 2016). Moreover, in embryogenic cultures of *A. angustifolia*, Spd was linked to the elongation of cells and formation of somatic embryos (Dutra et al. 2013). The desiccation-tolerant nature of *Ramalina farinaceae,* evidenced a strong increase in Spd content once this PAs could play a protective role against desiccation (Unal et al. 2008). So, in this work it is suggested that a high amount of Spd seems to be related to water stress tolerance. In contrast, Spm concentrations were lower compared with Put and Spd in all three WC percentages. This molecule is the final product of the PAs biosynthetic pathway and it is present in low amounts in most organisms (Imai et al. 2004). As in *C. sellowiana*, *Triticum aestivum* seeds submitted to drought stress presented a low concentration of Spm

content compared to Put or Spd (Ebeed et al. 2017). In *S. lycopersicum* fruits under water stress the Spm content also was lower comparatively to other PAs, despite exerting a positive effect on antioxidant systems analysis (Sánchez-Rodríguez et al. 2016). Moreover, high contents of Put and Spd were observed in *A. thaliana* when submitted to desiccation, which intriguingly, did not lead to Spm accumulation but to a progressive reduction in Spm pools (Alcázar et al. 2011). Spm was also associated with low cellular growth and alteration of cellular structure of in vitro embryogenic suspension cultures of *A. angustifolia* (Dutra et al. 2013). These authors suggest that this was due to the polycationic characteristic of PAs, especially in this long chain PAs, which have been implicated in a wide range of biological processes in plants, including resistance against stresses even at low concentrations.

C. sellowiana fresh seeds (11.63% WC) showed a slight increase of Put content when they were stored in cold temperatures. This also was observed in *Nicotiana tabacum*, which showed an increase of Put content in seed exposed at low temperature condition and keep viability (Xu et al. 2011). In the same way, Put as well Spm were the major PAs found in *Cucumis melo* fruits submitted to low temperatures (Zhang et al. 2017). Also, *T. aestivum* cultivars responded to low temperatures with a significant increase in Put content and this showed positive correlation with the chilling resistance (Racz et al. 1996). Theses finds were related to the fact of a range of biochemical processes that lead to cellular integrity linked to PAs which promote resistance against cold stress conferring adaptative and protective functions (Zhang et al. 2017). In this sense it was suggested that during chilling stress, Put can bounds to antioxidant enzymes or be conjugated to small antioxidant molecules allowing it to permeate to sites of oxidative stress within cells (Bouchereau et al. 1999) once the oxidation caused by the cold is damaging to the cellular membrane system. On the other hand, *C. sellowiana* seeds cold stored showed a decrease in Spd content compared to the control (11.63% WC without storage) and -196ºC with PVS2 treatment. This could indicate that low free Spd content seems to be inversely correlated with stresses injury parameters, and positively correlated with tolerance of seeds. Similar results were also found in *Zea mays* seedlings exposed to stress once Spd inhibited cold injury by retarding lipid peroxidation and preserving membrane integrity (Gao et al. 2009). However, Spd compared with Put and Spm, was found in the highest quantity in *C. sellowiana* seeds which indicate that it was related to desiccation tolerance. It was also observed in *Pinus radiata* orthodox seed, that Spd was the major PAs in the megagametophytes containing

the developing embryos, followed by Put and Spm (Minocha et al. 1999). This also lead us to relate that Spd content seems to be related to the cell desiccation tolerance and maintenance of seed viability. In general, Spm content was the lowest PA observed in *C. sellowiana* seeds. In cold stored seeds it did not differ statistically from control but differed from the treatment at -196°C with PVS2 which presented low values. In *T. aestivum* seeds exposed to low temperature stress, Spm content appeared to be the least responsive (Racz et al. 1996) while in *C. melo* fruits, high Spm content was associated to the conservation and tissue damage protection during cold stress (Zhang et al. 2017)**.**

Studies carried out in plantlets of *Pringlea antiscorbutica* have demonstrated that cold temperature induced the accumulation of all free PAs (Hummel et al. 2004). Due to the fact PAs are polycationic, they can bind strongly to negative charge cellular components such as nucleic acid, proteins and phospholipids leading to the stabilization of chromosomes, decrease of peroxidation and preservation of cell membrane integrity during the cold storage of seeds (Bouchereau et al. 1999). It was suggested that this effects lead to the inhibition of lipid peroxidation and stabilization of membranes through direct reactive oxygen species scavenging or indirect effect via activation of antioxidant enzymes (Sánchez-Rodríguez et al. 2016).

Conclusions

C. sellowiana mature seeds showed an orthodox seed behaviour once it was observed desiccation tolerance at 4.02% WC and the seed germination did not differ from the control (11.63% WC). Seed germination was low (~9%) in H_2O imbibed seed in comparison to the GA_3 imbibed seed as it was considerably increased (77%), and this were associated to the physiological dormancy of this seed (Chapter I). In all cold temperatures storage, the seeds maintained viability once the germination did not differ statistically from the control treatment. Ultrastructural alterations indicated a small cell shrinkage and lipid and protein reserves mobilization during desiccation and storage tolerance. The indirect analysis of viability by 2,3,5 TTC test did not validate the data of seed germination and this indicate that this test should be improved in future experiments. Our results indicate that the *C. sellowiana* seeds PAs content did not change significantly during desiccation tolerance and storage and that Spd was the most abundant PA which could be associated to the maintenance of seed viability. From the details of our work, future studies could be done intending to use PAs

inhibitors before cryopreservation of *C. sellowiana* seeds aiming to better understand how these molecules could be associated to cold tolerance.

Author contributions statement

LOZ—designed and performed experiments; LOZ, APL—performed TEM analysis and statistical data; LOZ and DG—performed polyamine analysis; LOZ and NS— design experiments and wrote the manuscript.

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4. CONCLUSÕES E PERSPECTIVAS

No Capítulo I, demonstramos que *Calibrachoa sellowiana* possui sementes ortodoxas uma vez que na maturação as sementes mostraram características fisiológicas e estruturais recorrentes na ortodoxia. Por meio do estudo morfológico nós encontramos dois tipos de sementes de *C. sellowiana* de acordo com a coloração do tegumento: sementes escuras (DS) e sementes pálidas (PS). DS apresentaram o tegumento completamente desenvolvido com a presença de polissacarídeos neutros, proteínas e compostos fenólicos. O embrião de formato linear levemente curvado estava circundado pelo endosperma composto por células grandes com paredes celular grossas e proteínas como reserva. No entanto, PS apresentaram tegumento incompleto, embrião atrofiado e endosperma retraído. Tais características morfológicas evidenciam a qualidade germinativa de sementes de *C. sellowiana* uma vez que PS não germinam eficazmente. Porém, mesmo apresentando alta viabilidade tecidual, DS embebidas em água apresentaram germinação extremamente baixa o que nos levou a suposição da presença de dormência fisiológica nesta espécie e que foi evidenciado pela aplicação exógena de Ácido giberélico (GA_3) nas sementes. GA_3 aumentou consideravelmente a taxa final e a velocidade, bem como antecipou a germinação das sementes. Consequentemente, uma curva trifásica de germinação foi observada em sementes tratadas com GA_3. Além disso, durante a protrusão radicular o conteúdo endógeno de putrescina, cadaverina, espermina e espermidina forma encontrados em grandes quantidades caracterizando ums associação entre Giberelinas e Poliaminas em sementes de C. sellowiana. A partir dos detalhes do nosso trabalho, estudos futuros poderiam ser realizados para investigar os efeitos da aplicação exógena de Poliaminas na germinação de sementes de *C. sellowiana* visando a quebra da dormência por meio destas moléculas. Além disso, a quantificação hormonal durante a germinação das sementes seria essencial para inferir a qualidade fisiológica das sementes desta espécie uma vez que investigar as causas da dormência é de nosso interesse e ajudará a melhorar a taxa final de germinação de sementes de *C. sellowiana*.

No Capítulo II, demonstramos que sementes de *C. sellowiana* dessecadas a 11. 63%, 6.96% e 4.02% do conteúdo de água e então armazenadas em baixas (8ºC, -20ºC) e ultrabaixas (-196ºC) temperaturas, mantêm a viabilidade uma vez que a taxa de germinação não diferiu estatisticamente do tratamento controle. Além disso, as alterações ultraestruturais indicaram uma pequena contração celular e mobilização de reservas lipídicas e proteicas durante a dessecação e tolerância ao

armazenamento. A análise indireta da viabilidade pelo teste de tetrazólio (2,3,5 TTC) não validou os dados de germinação de sementes e isso indica que este teste deve ser melhorado em experimentos futuros. Nossos resultados demonstraram que o conteúdo de Poliaminas em sementes de *C. sellowiana* não se alterou significativamente durante a tolerância à dessecação e armazenamento, mas indicam que a Espermidina foi a Poliamina mais abundante e que pode estar associada à manutenção da viabilidade das sementes. A partir dos detalhes de nosso trabalho, estudos futuros poderiam ser feitos visando o uso de inibidores de Poliaminas antes da criopreservação de sementes de *C. sellowiana*, com o objetivo de entender melhor como essas moléculas poderiam estar associadas à tolerância ao frio.

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APÊNDICE A- Principais diferenças estruturais, histoquímicas e fisiológicas entre DS e PS

Características	Dark Seeds (DS)	Pale Seeds (PS)
Características Estruturais		
Testa		
Coloração	Escura	Clara
Ornamentação	+	+
Textura	Firme	Macia
Endosperma	Preenche toda a cavidade	Retraído
Embrião	Completo	Incompleto
Cotilédones	+	-
Meristemas apicais bem desenvolvidos	+	-
Meristema fundamental	+	+
Características Histoquímicas		
Testa		
Polissacarídeos ácidos (TB-O)	+	+
Polissacarídeos neutros (PAS)	+	-
Compostos fenólicos (CF)	+	-
Proteínas (CBB)	+	-
Endosperma		
Polissacarídeos ácidos (TB-O)	+	+
Polissacarídeos neutros (PAS)	+	-
Compostos fenólicos (CF)	–	-
Proteínas (CBB)	+	+

Características	Dark Seeds (DS)	Pale Seeds (PS)
Embrião		
Polissacarídeos ácidos (TB-O)	+	+
Polissacarídeos neutros (PAS)	+	-
Compostos fenólicos (CF)	-	-
Proteínas (CBB)	+	+
Características Fisiológicas		
Número médio de sementes/fruto	42	44
Peso de 1000 sementes	0,239g	0,136g
Conteúdo Inicial de Água (IWC)	11,63%	11,32%
Conteúdo Relativo de Água (RWC)	20,31%	7,17%
Reação positiva ao Tetrazólio (2,3,5 TTC)	78%	12%
Eletrocondutividade	255,79 $\mu S.cm^{-1}g^{-1}$	709,71 $\mu S.cm^{-1}g^{-1}$
Início da Germinação (Dias após semeadura)		
H_2O	10	17
GA_3	6	-
Taxa Final de Germinação		
H_2O	9%	4%
GA_3	77%	-