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CRIOPRESERVAÇÃO DE SEMENTES E CARACTERIZAÇÃO MORFO-HISTOLÓGICA DA GERMINAÇÃO *IN VITRO* DE *Cattleya crispa* Lindl.

Dissertação submetida ao Programa de Pós-Graduação em Recursos Genéticos Vegetais da Universidade Federal de Santa Catarina, para obtenção do grau de Mestre em Ciências área de concentração Recursos Genéticos Vegetais.

Orientação: Dra. Rosete Pescador
Co-orientação: Dr. Angelo Schuabb Heringer

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por

Bruna Vargas Andriolli

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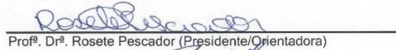

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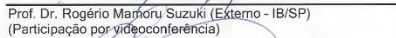

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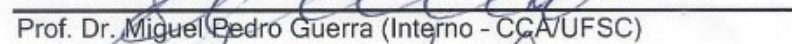

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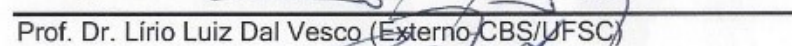

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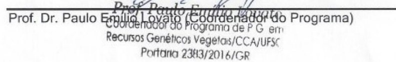

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A meus pais que nunca mediram esforços para minha formação,
DEDICO essa conquista.

O homem é a mais insana das espécies.
Adora um Deus invisível e mata a Natureza visível.
Sem perceber que a Natureza que ele mata,
é o Deus invisível que ele tanto adora.

Man is the most insane species.
Worships an invisible God and destroys a visible Nature.
Unaware that this Nature he is destroying,
is this God he worships so much.

~ Hubert Reeves

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RESUMO

Cattleya crispa é uma orquídea endêmica, com distribuição geográfica restrita à Mata Atlântica, que está seriamente ameaçada pela perda de habitat. *C. crispa* está presente na Lista Vermelha da Flora Brasileira e é protegida pelo anexo II da Convenção sobre o Comércio Internacional de Espécies Ameaçadas de Fauna e Flora Silvestres (CITES). A espécie em estudo possui valor de uso devido a sua rara beleza e pertence ao gênero *Cattleya* que possui ampla capacidade de recombinação genética, tendo potencial para melhoramento genético. Os objetivos desse trabalho foram desenvolver um protocolo de criopreservação de sementes, bem como estudar o desenvolvimento inicial *in vitro*, visando produzir dados que subsidiem o estabelecimento de estratégias de uso sustentável e conservação para a espécie. Sementes de *C. crispa* foram criopreservadas pela técnica de imersão direta em nitrogênio líquido, posteriormente foram descongeladas em banho maria, desinfestadas com hipoclorito de sódio e inoculadas em meio de cultura MS. Sementes submetidas a criopreservação resultaram em alta taxa de germinação (81,14%). Esse potencial de germinação, após a exposição ao NL, provavelmente, está relacionado ao baixo teor (5%) de água inicial da semente. Por outro lado, a porcentagem de sobrevivência dos protocormos não criopreservados (8,48%) e criopreservados (11,12%) avaliados cinco meses após a semeadura, foi reduzida. As análises de MEV revelaram que as sementes tem o tegumento persistente, que, apesar de não influenciar o início da germinação, comprime o protocormo ocasionando sua morte. Nas análises morfológicas e histológicas observa-se que o embrião tem uma germinação homogênea, que ocorre aos 7 dias de cultivo. O embrião é formado por protoderme e promeristema. Avaliações ultraestruturais do embrião revelaram que ele é composto principalmente de corpos lipídicos e proteicos e contém alguns pequenos grãos de amido. Os resultados obtidos no presente trabalho, configuram-se como o primeiro estudo de criopreservação de sementes e do processo de germinação de *C. crispa*.

Palavras-chave: Orchidaceae. Conservação. Tegumento. Protocormo. MET. MEV.

ABSTRACT

Cattleya crispa is an endemic orchid, with geographic distribution restricted to the Atlantic Forest, which is seriously threatened by the loss of habitat. *C. crispa* is on the Brazilian Red List and is protected by Annex II to the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). The species under study has a use value due to its rare beauty and belongs to the *Cattleya* genus, which has a large capacity for genetic recombination and has potential for genetic improvement. The objectives of this work were to develop a protocol for cryopreservation of seeds, as well as to study the initial in vitro development, aiming to produce data that support the establishment of sustainable use and conservation strategies for the species. Seeds of *C. crispa* were cryopreserved by direct immersion in liquid nitrogen, later thawed in water bath, disinfested with sodium hypochlorite and inoculated in MS culture medium. Seeds submitted to cryopreservation resulted in a high germination rate (81.14%). This germination potential, after exposure to NL, is probably related to the low content (5%) of initial seed water. On the other hand, the percentage of survival of non-cryopreserved (8.48%) and cryopreserved (11.12%) protocorms evaluated five months after sowing was reduced. SEM analysis revealed that the seeds have a persistent seed coat which, although it does not influence the germination, compresses the protocorm, causing its death. In the morphological and histological analyzes it is observed that the embryo has a homogeneous germination, which occurs at 7 days of culture. The embryo is formed by protoderm and promeristem. Ultrastructural evaluations of the embryo revealed that it is composed mainly of lipid and protein bodies and contains some small starch grains. The results obtained in the present work, are configured as the first study of seed cryopreservation and the process of germination of *C. crispa*.

Keywords: Orchidaceae. Conservation. Seed coat. Protocorm. TEM. SEM.

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LISTA DE ABREVIATURAS E SIMBOLOS

CAPES – Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
C. crispa – *Cattleya crispa* Lindl.
DAS – Days After Sowing
HMDS - 1,1,1,3,3,3-hexamethyldisilazane
IBt – Instituto de Botânica
ISTA – International Seed Testing Association
LFDGV – Laboratório de Fisiologia do Desenvolvimento e Genética Vegetal
LCME – Laboratório Central de Microscopia Eletrônica
LM – Light microscopy
LN – Liquid nitrogen
Min – Minutes
Mg – Miligrama
Ml - Mililitro
PAS – Periodic acid-Schiff
PBS – Phosphate buffered saline
PPG-RGV – Programa de Pós-Graduação em Recursos Genéticos Vegetais
SEM – Scanning electron microscopy
TEM – Transmission electron microscopy
TTC – Triphenyl tetrazolium chloride
UFSC – Universidade Federal de Santa Catarina
WC – Water content
μm – Micrômetro

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JUSTIFICATIVA E ANTECEDENTES

A Mata Atlântica é um dos biomas mais biodiversos e com mais endemismos do planeta, simultaneamente é um dos mais ameaçados pela ação antrópica, restando apenas cerca de 8% da sua cobertura original (RIBEIRO *et al*., 2009). Tal cenário, enquadra o bioma brasileiro entre os cinco primeiros no ranking mundial de “Hotspots” (MYERS *et al*., 2000). Entre as espécies que se encontram na última lista de espécies da flora brasileira ameaçadas de extinção, a maior parte delas mais de 50% pertencem ao bioma Mata Atlântica (MMA, 2014).

Orquídeas são espécies frequentes deste bioma e têm sofrido contínua ameaça pela destruição e fragmentação dos habitats naturais, aspecto este, que é agravado pelo fato da maioria delas serem epífitas (BENZING, 1998). Além de contribuir para a manutenção da estabilidade dos ecossistemas florestais em função do seu elevado grau de especialização e de sua adaptação às condições climáticas e oligotróficas extremas, as orquídeas são importantes comercialmente em decorrência da beleza de suas flores (BENZING, 1990).

A família botânica Orchidaceae é uma das maiores e mais diversas famílias de plantas entre as Angiospermas e entre as plantas ornamentais, compreendendo 899 gêneros e mais de 35.000 espécies (THE PLANT LIST, 2013). Apresenta distribuição cosmopolita, embora seja mais abundante e diversificada em florestas tropicais da Ásia e das Américas (RODRIGUES, 2011). Possui uma taxa de endemismo de 67% em nível específico. E constantemente novas espécies são descritas. Os representantes dessa família são plantas herbáceas perenes, bastante diversificadas em suas características, como tamanho, forma e cor das flores (SINGH, 1992). Estima-se que 2.451 espécies de orquídeas ocorrem no Brasil e dentre elas 102 são do gênero *Cattleya* e 95 são endêmicas (REFLORA, 2019).

As orquídeas são divididas em seis subfamílias: Apostasioideae, Cypripedioideae, Spiranthoideae, Orchidoideae, Epidendroideae e Vandoideae. As duas últimas se destacam por apresentarem grande número de espécies exploradas economicamente; são elas a Epidendroideae e a Vandoideae. A subfamília Epidendroideae, possui gêneros com enorme valor horticultural como *Cattleya, Guarianthe, Phalaenopsis* e *Rhyncholaelia* entre outros (VAN DEN BERG *et al*.,

2009), todos de ocorrência natural no Brasil, e que são muito utilizados para a produção de híbridos comerciais.

As espécies dessa família representam uma das mais importantes plantas de flor tanto para corte como para vaso, sua exuberância encanta e interessa as pessoas desde os primeiros tempos (SILVA, 2013). Muitas orquídeas com importância comercial no mercado florícola, são produzidas em diferentes escalas. Porém, algumas dessas espécies estão sobre grave risco de extinção na natureza, decorrente da coleta ilegal de suas flores tanto para fins medicinais como ornamentais, além disso, ocorrem em ambientes amplamente fragmentados e explorados por atividades antrópicas (COATES; DIXON, 2007).

As orquídeas são importantes plantas ornamentais e suas flores têm destaque no comércio mundial de flores de vaso e corte não só pela beleza exótica, como também por sua longa durabilidade. Além disso, diversos gêneros possuem espécies cultivadas principalmente por sua folhagem exuberante (CHUGH; GUHA; RAO, 2009). Embora sejam cultivadas majoritariamente para fins ornamentais, algumas espécies da família são utilizadas como ervas medicinais e alimentos por muitas culturas e tribos (ARDITTI, 1992). Sendo a baunilha um raro exemplo de orquídea utilizada como especiaria de elevado valor econômico.

O gênero *Cattleya* abrange aproximadamente 102 espécies de orquídeas principalmente epífitas, além de inúmeras variedades e híbridos. É um dos mais populares gêneros da família Orchidaceae, em decorrência do seu alto valor ornamental (THE PLANT LIST, 2013). As espécies desse gênero são predominantemente epífitas e para sobreviver adotam estratégias de retirada de umidade e nutrientes dos materiais orgânicos que são depositados perto de suas raízes. O seu habitat natural é a floresta subtropical e tropical (DEMATTÊ; DEMATTÊ, 1996).

Comercialmente, o cultivo de espécies do gênero *Cattleya* é de grande importância para o mercado, principalmente devido a ampla capacidade de recombinação genética, beleza, tamanho e durabilidade de suas flores (ZANENGA-GODOY; COSTA, 2003). Esse potencial tem levado a redução e desaparecimento de várias populações e consequentemente, várias destas espécies apresentam-se em sérias ameaças de extinção (CRUZ; BORBA; VAN DEN BERG, 2003).

Na Lista Oficial das Espécies da Flora Brasileira Ameaçadas de Extinção, publicada pelo Ministério do Meio Ambiente, das espécies de orquídeas que estão listadas, um número significativo é considerado com “dados insuficientes”, o que evidencia a falta de informações a respeito do estado de conservação das orquídeas brasileiras (MMA, 2014).

Cattleya crispa Lindl. (Fig. 1) é uma espécie de orquídea endêmica do Brasil que está presente na Lista Vermelha da flora brasileira, com a classificação de "Vulnerável" pelos padrões da IUCN (CNCFlora, 2012). A espécie consta ainda, em nível subnacional, na Lista Vermelha da Flora de Minas Gerais, como "Criticamente em perigo" (COPAM-MG, 1997).

Essa espécie tem ocorrência natural na Mata Atlântica, nos Estados de Minas Gerais, Espírito Santo e Rio de Janeiro (BARROS *et al*., 2012). No Parque Nacional do Itatiaia foi coletada há mais de 90 anos e acredita-se que esteja atualmente extinta nessa localidade. Há um registro para São Paulo (CNCFlora, 2012). Possui habito epifítico de crescimento e seu tempo de geração é estimado em dez anos. Em decorrência disso e considerando o declínio populacional da espécie, especula-se que *C. crispa* venha a sofrer uma redução no número total de indivíduos de pelo menos 30% nos próximos 30 anos (CNCFlora, 2012).

A família Orchidaceae, tem uma característica peculiar no que se refere as sementes, as quais são produzidas em grande número de tamanho reduzido, contendo reservas mínimas de nutrientes (ARDITTI; GHANI, 2000). Apesar do grande número de sementes produzidas, apenas 0,2-0,3% germina na natureza (SINGH, 1992). Por serem tão pequenas, as sementes são desprovidas de cotilédone e possuem um endosperma diminuto, não dispondo de todos os nutrientes necessários para o desenvolvimento do embrião (FERREIRA, *et al*., 2010). Como solução, as orquídeas dependem da associação com fungos micorrizos que auxiliam na absorção principalmente de recursos de carbono, possibilitando assim a germinação e o desenvolvimento do embrião (RASMUSSEN, 1995; VIANA, 2014).

Essa característica, aliada ao perigo de extinção que muitas espécies da família enfrentam, exigem o desenvolvimento de técnicas de propagação rápida para conservação de orquídeas. Sendo que a propagação *in vivo* através de sementes não é preferível, devido a heterozigosidade da semente, o pequeno tamanho da semente, a presença de endosperma reduzido e a necessidade de associação com fungos micorrizos (SAIPRASAD; POLISETTY, 2003).

Dessa forma, técnicas de cultura de tecidos vegetais *in vitro* são amplamente utilizadas para uma rápida e eficiente propagação de inúmeras espécies de orquídea com importante valor comercial (BHATTACHARJEE; ISLAM, 2014). Mesmo que os padrões de germinação e crescimento observados nas orquídeas, sejam similares nos

cultivos *in vivo* e *in vitro*, nesse último caso a associação com fungos micorrizos simbiontes não é necessária, devido ao aporte nutricional que é fornecido via meio de cultura.

O grande salto na propagação de orquídeas e na cultura de tecidos em geral foi após a descoberta de Knudson (1922), de que sementes de orquídeas podiam germinar na ausência da associação micorrízica, desde que o meio de cultura fosse suplementado com uma fonte de carbono. Após isso foi possível à obtenção de híbridos, a produção de plantas em larga escala e permitiu um grande avanço no cultivo de embriões zigóticos de outras espécies (RAGHAVAN, 2003).

O uso das ferramentas de micropropagação, permite a multiplicação de plantas em meio de cultivo específico, sob condições assépticas e ambiente controlado. Esta técnica oferece diversas vantagens como a possibilidade de multiplicação em larga escala, num curto período e espaço reduzido para obtenção de elevada quantidade de plantas. Além disto, viabiliza a obtenção de plantas livres de doenças e pragas, elevada precisão em cronogramas de produção e a homogeneidade das plantas obtidas (GUERRA *et al*., 1999).

A primeira aplicação comercial da micropropagação foi feita por Morel em 1960, ao multiplicar orquídeas através da cultura de ápices caulinares e regeneração de protocormos, que se diferenciavam e originavam embriões. A sucessiva divisão destes protocormos possibilitou acelerar o processo de propagação de orquídeas. Aproximadamente uma década depois, Smith e Murashige obtiveram plantas inteiras a partir de meristemas apicais em meio contendo sais minerais e vitaminas enriquecidas com fitorreguladores (GRATTAPAGLIA; MACHADO, 1990).

Na conservação da biodiversidade vegetal são consideradas as estratégias básicas de conservação: *in situ* e *ex-situ*. De acordo com a Convenção sobre a Diversidade Biológica, por conservação *ex-situ* entende se como a conservação dos componentes da diversidade biológica fora do seu hábitat natural (GONZÁLEZ-ARNAO; ENGELMANN, 2013). Na conservação *ex-situ*, os avanços na biotecnologia, especialmente aqueles associados à cultura *in vitro*, também têm fornecido ferramentas para apoiar e melhorar a conservação e manejo da diversidade vegetal (WITHERS, 1995).

Devido a elevada ameaça que as limitadas populações de orquídeas sofrem na natureza, isso as torna candidatas ideais para estratégias de conservação *ex situ* (HAY *et al*., 2010). Tradicionalmente a conservação de orquídeas é realizada através de coletas de plantas vivas e a propagação

das mesmas em estufas ou por cultivo *in vitro* de culturas embriogênicas. Porém, ambas técnicas são alternativas de conservação de curto a médio prazo. Atualmente, a criopreservação tornou-se uma ferramenta de grande potencial para a conservação a longo prazo do germoplasma de orquídeas, já que facilita o gerenciamento da coleta *in vitro* minimizando o risco de variação somaclonal e contaminação de culturas (PANDEY *et al*., 2008).

As sementes de orquídea costumam ser classificadas como ortodoxas e por isso são tolerantes a dessecação. Bancos de sementes ainda possuem escassos estudos e resultam em baixa longevidade do material vegetal (HAY *et al*., 2010). Estudos com armazenagem de sementes de orquídeas tropicais a temperatura de 4ºC, revelam elevadas perdas de viabilidade após 360 dias das quais, apenas 5% das sementes permaneceram viáveis (SUZUKI, *et al*., 2012). Em contrapartida a criopreservação de protocormos demanda protocolos complexos e custosos. Portanto, existe uma necessidade em desenvolver métodos mais eficientes e baratos, para maximizar a viabilidade de sementes após a criopreservação (SCHOFIELD *et al*., 2018).

A criopreservação é uma técnica de conservação de germoplasma a longo prazo sob temperatura ultra reduzida, em nitrogênio líquido a -196ºC ou na fase de vapor a -150ºC. Essas temperaturas reduzem eficientemente a movimentação de moléculas e o metabolismo celular é tão reduzido que a deterioração biológica é praticamente inexistente (SANTOS, 2000). Além disso, muitas amostras podem ser armazenadas em pequeno espaço (ENGELMANN, 2011). Existem inúmeros relatos de diferentes propágulos de orquídeas, como sementes, meristemas, protocormos e estruturas semelhantes a protocormos, que foram criopreservados com sucesso (HIRANO; ISHIKAWA; MII, 2005; SOPALUN; THAMMASIRI; ISHIKAWA, 2010; GALDIANO, *et al*., 2012; SILVA, 2013; LIN, *et al*., 2014; HUGHES; KANE, 2018; DIENGDOH; KUMARIA; DAS, 2019).

O uso de sementes para criopreservação de orquídea pode ser feito por meio da simples imersão direta em nitrogênio líquido, devido ao baixo teor de água encontrado nas sementes maduras, o que reduz o risco de dano intracelular por formação de cristais de gelo (KULUS; ZALEWSKA, 2014), reduzindo assim os custos com soluções crioprotetoras. O número de espécies de orquídeas que apresentaram sucesso com estratégias de criopreservação, tem aumentado desde o início dos estudos nos anos 1980 (POPOVA *et al*., 2016). Portanto, criobancos

de orquídeas oferecem uma excelente oportunidade para conservação desses exemplares com padrão de crescimento e germinação único do reino vegetal.

Fig. 1. Plantas e inflorescências de *Cattleya crispa*. Fonte: Dr. Rogério M. Suzuki.

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Seed cryopreservation and morpho-histological characterization of *in vitro* germination of *Cattleya crispa* Lindl.

Abstract

Cattleya crispa is an ornamental orchid species with geographic distribution restricted to the Atlantic Forest, which is seriously threatened by human-induced habitat loss. *C. crispa* is present in the Red List of Brazilian Flora and is protected under the appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). Procedures were developed for seed cryopreservation and improve the understanding of the germination pattern, aiming at enhancing its sustainable use and conservation. Seeds submitted to cryopreservation resulted in a high germination rate (81.14%), which is attributed to the seed initial low water (5%). However, the survival rate of protocorms non-cryopreserved (8.48%) and cryopreserved (11.12%) evaluated five months after sowing was reduced. SEM images revealed a persistent seed coat that does not influence the start of germination but compresses the protocorm leading to its death. Morphological and histological analyses showed that the globular embryo, has a homogeneously early germination, that occur by the 7 day. The embryo is formed by protoderm and promeristem. Within germination, the protocorm shows bipolarity, with shoot apex and basal region. Ultrastructure observations of the embryo revealed that it is composed mostly of lipid and protein bodies and contain a few small grains of starch. The data gathered from *C. crispa* in the present work, is the first detailed report of this endangered species.

Keywords: Orchidaceae. Conservation. Seed coat. Protocorm. SEM. TEM

1. Introduction

Orchidaceae represents one of the largest and most diverse families of flowering plants, consisting of about 35.000 species, which have fascinated botanists and plant enthusiasts over centuries (Barthlott et al., 2014), due to its extensive horticultural, medicinal and culinary uses. Nearly 3.140 orchid species are estimated to occur in Brazil and among them, 102 are *Cattleya* spp. (WCSP, 2019). The *Cattleya* genus covers the native orchids of tropical America, mainly epiphytes and several hybrids. It is one of the most popular and widely cultivated genera of the Orchidaceae family, the high ornamental value of its members and large ability for genetic recombination, are attractive for the market (Galdiano et al., 2017).

Cattleya crispa Lindl. is an ornamental epiphyte orchid, endemic from the Atlantic Forest of Brazil (CNCflora, 2012). It is distinct from all others *Cattleya* species, both in color and form of the labellum and other structures of the flower (Lindley, 1828). As a species threatened with extinction, *C. crispa* is protected internationally under the appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (UNEP-WCMC, 2014), is present in the Red List of Brazilian Flora (CNCFlora, 2012) and on sub-national level, appears in the Red List of Minas Gerais Flora (COPAM-MG, 1997), classified by the IUCN (2016) guidelines, as Critically Endangered (CR).

Despite the high diversity of orchids in Brazil and in other parts of the world, many species are subject of extractive exploitation, which together with habitat degradation, threaten their survival (Vendrame et al., 2014). Conservation of plant genetic resources, according to Convention on Biological Diversity (CBD, 1992), can be carried out in the natural habitats (*in situ*) or outside (*ex situ*). The *in situ* method, is about to promote the conservation of biological diversity in natural habitats. And the e*x-situ* conservation is the conservation of biological diversity components outside their natural habitat. *Ex situ* conservation is generally used to safeguard populations in danger of destruction.

Due to its epiphytic habit *C. crispa* presents slow growth and its generation time, is estimated in about ten years (CNCFlora, 2012). Thus, considering the strong population decline of the species, the application of plant tissue culture techniques can provide tools to support and improve its conservation (Mohanty et al., 2012). For long-term conservation, cryopreservation is a recommended technique for species

with vegetative propagation, recalcitrant seeds, rare germplasm, threatened species or wild plants (IBPGR, 1982).

In order to establish cryopreservation protocols, seeds are better suited as compared to other propagules, because they allow the maintenance of a wider genetic base (Chen et al., 2015). In orchids, cryopreservation has been successfully achieved for mostly well-dried seeds and pollen. However, the regrowth of other explants such as meristems, protocorms or protocorms-like bodies, are highly variable (Popova, et al. 2016). By consequence of only a few laboratories worldwide conduct seed science for orchids (Magrini, et al., 2019), further research is needed to fill existing gaps about seed structure characteristics, including those referring to anatomical, histochemical and ultrastructural aspects (Moura, et al., 2010).

An orchid embryo possesses highly specialized morphology, biology and developmental characteristics. Consequently, germination and development differ from other flowering plants (Veyret, 1974; Harrison, 1977). The aim of this work was to cryopreserve and characterize the developmental anatomy, histochemistry and ultrastructure of *C. crispa* seed during germination and initial development.

2. Material and methods

2.1. Seed collection

The seeds used in the present work were originated from the *ex situ* conservation collection of the *Orquidário Frederico Carlos Hoehne* - Institute of Botany (IBt), located in Vila Agua Funda neighborhood in São Paulo, Brazil (-23.642196, -46.621763).

The seeds were obtained from six 7-month old yellowish green cross-pollinated mature capsules. Flowering occurs in the entire summer and fruits at the beginning of dehiscence can be obtained from 6 to 10 months after pollination. In early February 2017 the cross-pollination was evaluated and in September six nearly dehisced mature capsules were collected.

2.2. Seed water content

The oven-method was used to determine water content (WC) and was calculated using the below formula (ISTA, 1985). The seeds were weighted and dried at 103 ± 2ºC for 17 h to constant weight. The moisture content was determined with three replicates measurements with 10 mg of seeds which and was expressed in percent.

$$WC\ \% = \frac{(W_2 - W_3)}{(W_2 - W_1)} \times 100$$

W1 = weight of aluminum boat, W2 = weight of aluminum boat + seeds before drying, W3 = weight of aluminum boat + seeds after drying.

2.3. Cryopreservation and viability assay

Seeds of seven-month old capsules, were frozen in 1.5 mL plastic cryotubes by direct immersion into liquid nitrogen, approached 20 mg of seeds were placed in each cryotube. After 48 h of storage in liquid nitrogen, the cryotubes were defrosted in a water bath at 40 ºC per 2 min. The thawed seeds were then taken out from the cryotubes and sterilized and cultured as described in the item below (2.4). Protocorms from cryopreserved seeds were cultured under the same conditions as protocorms from control seeds.

A subset of seeds was used in triplicate for the tetrazolium viability test. The seeds were soaked in distilled water for 17 h at 25 ± 2ºC, then the water was removed and a 0.5 % solution of 2,3,5-triphenyl tetrazolium chloride (TCC) were set for incubation for 15 h. After staining, seed viability was determined with a microscope (BX40: Olympus™). The assessment was realized for the treatment (cryopreservation) and control seeds.

Seed viability (%), seed germination (%), survival rate (%) and protocorms weight (mg) were evaluated 5 months after sowing. Viability was assessed by counting the red seeds as viable, and the white seeds as nonviable; four replications of approximately 300 seeds per treatment being analyzed. Seed germination was calculated dividing the germinated seeds per total amount of seeds inoculated in each plate (< 900) (Brasil, 1992). Survival rate was evaluated counting has live, the green protocorms and seedlings between the germinated ones. And for the protocorms weight, we measured 30 protocorms of each plate. Each

replicate was composed of a Petry dish (15mm×150mm) and organized as completely randomized experiment with four replicates.

2.4. Procedures for *in vitro* culture

Both *in vitro* sowing and disinfestation were realized under horizontal laminar flow cabinet (PA-220: Pachane™) for aseptic conditions. For disinfestation the seeds were imbibed in sterile-distilled water with a drop of surfactant detergent (Tween™ 20) during 10 min in constant agitation, this process was followed by sterilization using 0.5% sodium hypochlorite (NaClO) during 10 min and then it was rinsed three times using sterile-distilled water.

After disinfestation using a micropipette and sterile tips, the seeds were removed from the water and sowed on Murashige and Skoog (1962) medium supplemented with 30 g/L sucrose (P.A. Sigma™), solidified with 2 g/L gelling agent (Phytagel: Sigma™) and set into pH 5.5 before sterilized at 120 ºC for 15 min.

For the control (non-cryopreserved seeds) and the treatment (cryopreserved seeds), less than 1000 seeds were cultured in which sterile polystyrene Petri dish (100 × 15 mm) with 20 mL of culture medium. The experiment was conducted with 4 replicates per treatment and all the cultures were maintained under cool white fluorescent light (50-60 µmol m^{-2} s^{-1}) with 16h day / 8h night photoperiod, at 25 ± 2ºC.

2.5. Morphology description

After inoculation, the cultures were weekly examined under stereo microscope (SZH 10: Olympus™) from the germination and development of the embryo, through the protocorm phase to the seedling stage. The process of seed germination until seedling stage, were classified into six phases according to embryo development, which were adapted from Arditti (1967). The classification that we propose are presented in the following table.

Table 1. Stages of initial *in vitro* development from *Cattleya crispa* seeds.

Stage	Description
0	Unviable seeds.
1	Embryo swollen and green with ruptured testa (= germination).
2	Early globular protocorm.
3	Protocorm showing a pointed vegetative apex and rhizoids.
4	Protocorm with one leaf emerged.
5	Protocorm with two spreading leaves.
6	Seedling showing two or more leaves with root presence (= seedling).

*Adapted from Arditti (1967), in the original paper the loss of the seed coat was the defining event for the classification of general *Cattleya* spp. germination stage.

2.6. Microscopic analyses

All collections for microscopic analyzes were performed at 0, 7, 15, 30 and 60 days after sowing (DAS). The samples collected at 60 DAS, were separately analyzed between green and brown protocorms.

2.6.1. Light microscopy (LM)

For histological observation, microscope slides were made by employing historesin method, according to the manufacturer's recommendations. The fresh samples were immediately removed from the culture medium and dabbed dry on a piece of filter paper. The material was fixed in 2,5% glutaraldehyde and 0,1M phosphate-buffered saline (PBS) (1:1, v/v) in vacuum for 1 week followed by dehydration in an alcohol series. After dehydration, the samples were infiltrated with hydroxyethyl-methacrylate (Historesin: Leica™), according to the manufacturer's instructions. For sectioning (5 µm) we used a manual microtome (Slee Cut 4055: Mainz™). The histochemical tests performed were: periodic acid-Schiff reagent (PAS) for polysaccharides, that stain in purple-magenta color (Feder and O'Brien, 1968) and toluidine blue O (TB-O) for metachromasy (O'Brien et al., 1964). The images were obtained using light microscope (BX40: Olympus™) with a high-

resolution color digital camera (DP71: Olympus) and Capture Pro 5.1 Image Software from LFDGV - UFSC.

2.6.2. Scanning electron microscopy (SEM)

Selected samples for the SEM observations, after fixation and dehydration has described above, were placed on carbon strips of tape and affixed on the sample stub to continue dehydration using the low surface tension solvent 1,1,1,3,3,3-hexamethyldisilazane (HMDS). The dried samples were then covered with 20 nm of gold in metallizer (EM SCD 500: Leica™) from the Central Laboratory of Electronic Microscopy (LCME - UFSC) for the ultrastructural study in scanning electron microscope (JSM-6390LV: Jeol™).

2.6.3. Transmission electron microscopy (TEM)

The fresh material was collected and fixed with 2,5% glutaraldehyde, 0,1M sodium cacodylate buffer (pH 7.2) and 0,2M sucrose following descriptions in Schmidt et al. (2012). The samples were than post-fixed in 1% osmium tetroxide for 6h, dehydrated in a graded acetone series and embedded in Spurr resine (Leica™). After sectioning, the material was stained with aqueous uranyl acetate followed by lead citrate. The sections were examined under transmission electron microscope (JEM-1011: Jeol™) from the LCME-UFSC.

3. Results

The microscopic analyses revealed that cryopreservation had no effect on the viability and all cryopreserved seeds germinated like unfrozen seeds. Observations under stereo microscope and scanning electron microscope, contained well-formed protocorms and no abnormalities were apparent. While histological and ultrastructural observations, showed intact cells with all structures preserved.

3.1. Effect of seed cryopreservation

The data collected after *C. crispa* seed cryopreservation are presented in Table 2.

Table 2. Effects of cryopreservation by direct immersion in liquid nitrogen (LN) of *Cattleya crispa* seeds.

Variable	-NL	+NL
Seed viability (TCC) (%)	87,15 a	88,04 a
Seed germination (%)	78,32 a	81,14 a
Survival rate (%)	08,48 a	11,12 a
Protocorms weight (mg)	104,27 a	104,85 a

*Means values followed by the same letter do not differ by Tukey test at 5 %.

The initial WC of the seeds was 5%, which allowed the direct immersion of the plastic cryotubes into liquid nitrogen. The tetrazolium viability test percentages showed no significant differences between control and cryopreserved seeds. The results evidence that, the high viability of the seeds remained unaffected after cryopreservation (Fig. 2).

Fig. 2. *Cattleya crispa* seeds after the tetrazolium viability test. Viable embryos have a strong red color (A) and can be easily differentiated from an unviable embryo, due to its non-colored appearance (B). *Em* embryo; *Sc* seed coat. Scale bars: 200 µm.

After five months, the seed germination percentage showed non-significant differences, supporting the tetrazolium test results and suggesting that seeds of *C. crispa* were at optimum moisture content and

were in the correct maturation stage for cryopreservation. Even though protocorms derived from cryopreserved seeds appeared to grow faster than control protocorms, at the five months old weight evaluation, no significant differences in protocorm weight between the treatments were observed.

When survival rate of germinated seeds was assessed at five months, we consider the healthy green protocorms has alive for the counting, while the brown protocorms were evaluated has death. Although the high initial seed viability and the successful germination, survival rate was very low. No statically differences between cryopreservation and control were noticed, which discards the hypothesis that such effect is caused by the treatment.

3.2. Seed coat influence in survival rate

Despite the high initial viability and germination rates, most of the protocorms were turning brown and dying. So, at the 60 DAS, both SEM and TEM images that were distinctly performed in green alive protocorms and in brown dying ones, gave further insights into why this was happening. The figures obtained, showed that all live protocorms assessed, had totally lost the seed coat and all internal structures remained organized, such as nuclei and cell walls (Fig. 3A-D). While in brown protocorms, the seed coat was still attached and covering the structure (Fig. 3E-G). Ultrastructural analysis in the parenchyma of dying protocorms, confirm a generalized cell death, possibly occasioned by necrosis (Fig. 3H).

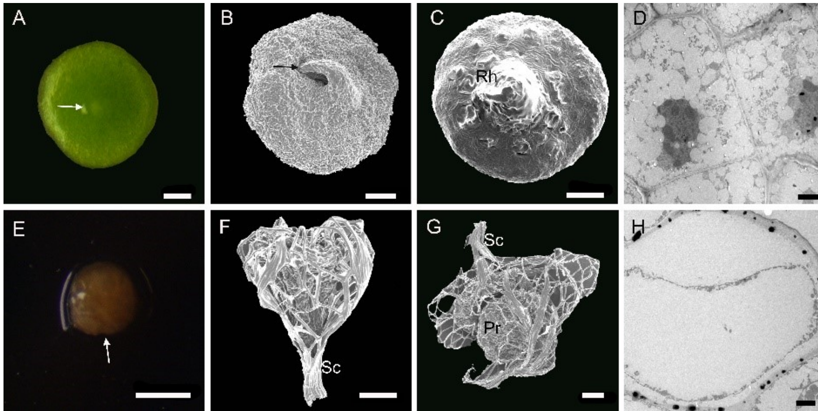

Fig. 3. Protocorms of *Cattleya crispa* collected 60 DAS. A-C (Stereo microscope): Green and alive protocorms without the seed coat; arrows indicate the vegetative apex with the leaf primordium in formation. D (MET): Intact parenchyma of a green protocorm. E-G (Stereo microscope): Brown and dying protocorms, with the seed coat attached to the structure; arrow indicate the protocorm with brown color. H (MET): Cell death of a brown protocorm. *Rh* rhizoids; *Sc* seed coat; *Pr* protocorm. Scale bars: 2 µm (D, H). 100 µm (B, C, F, G). 200 µm (A). 500 µm (E).

3.3. Seed morphology

Inside a mature *C. crispa* capsule, we found thousands of minute seeds that had light yellow color, typical wind-dispersed form and medium size of 0.7-0.9 mm. They are relatively undifferentiated seeds characterized by the presence of one ellipsoidal shaped embryo at the center and a slightly pigmented seed coat without any endosperm or cotyledon (Fig. 4A). Some seeds were infertile due to the absence or defect of the embryo. The chalaza extremity is tapered and closed, and the basal region has an opening at the micropylar end and in some seeds, the suspensor projects through the micropyle (Fig. 4B-D). According to Shushan (1959), it provides and entry point for water and fungi into the seed. The testa shape is reticulated with cells elongated rounded at the end, in conformity with the proposed for *Cattleya* genus in the "Phylogenetic trees: Shape of testa cells" of Barthlott et al. (2014).

3.4. Morphoanatomy of initial development

The morphological structures observed during the initial *in vitro* development of *C. crispa*, are presented in Figure 4. In the present work, at the 5 DAS on the culture medium, the embryo started to be swollen due to the imbibition process, but the alteration was slight and did not allow the differentiation of the seeds into viable and inviable ones. The germination process started by the 7 DAS (stage 1), when the embryo was swollen and green, visible different from unviable seeds which showed white color and no alterations in the embryo/seed coat ratio (stage 0) (Fig. 4E-F).

The seeds presented continuous growth of the chlorophyllous tuberculous structure, filling and stretching the central zone of the ruptured seed coat (15 DAS) (Fig. 4G). By the 30 DAS, this morphological aspect was more accentuated with the structure reaching a globular and characteristical green protocorm shape (stage 2). It was also visible, an early oxidation with reddish-brown color appearance in some protocorms (Fig. 4H). In *C. crispa,* the vegetative organs started to develop 60 DAS (stage 3). The upper part of the protocorms showed a vegetative apex that contained the leaf primordium in formation while at the base of the protocorms, rhizoids were observed (Fig. 4I-J). At the 70-80 DAS, the first leaf emerged in some protocorms (stage 4) (Fig. 4K). Although the initial germination process be quite homogeneous, we observed protocorms at different development stages over time. With 100-110 DAS, the protocorms showed a second leaf well spread (stage 5), while the first leaves became gradually wider and thicker. The root presence was observed around 150 DAS (stage 6), we define this morphological change as the ending mark of the protocorm stage and the beginning of the seedling phase (Fig. 4L-M).

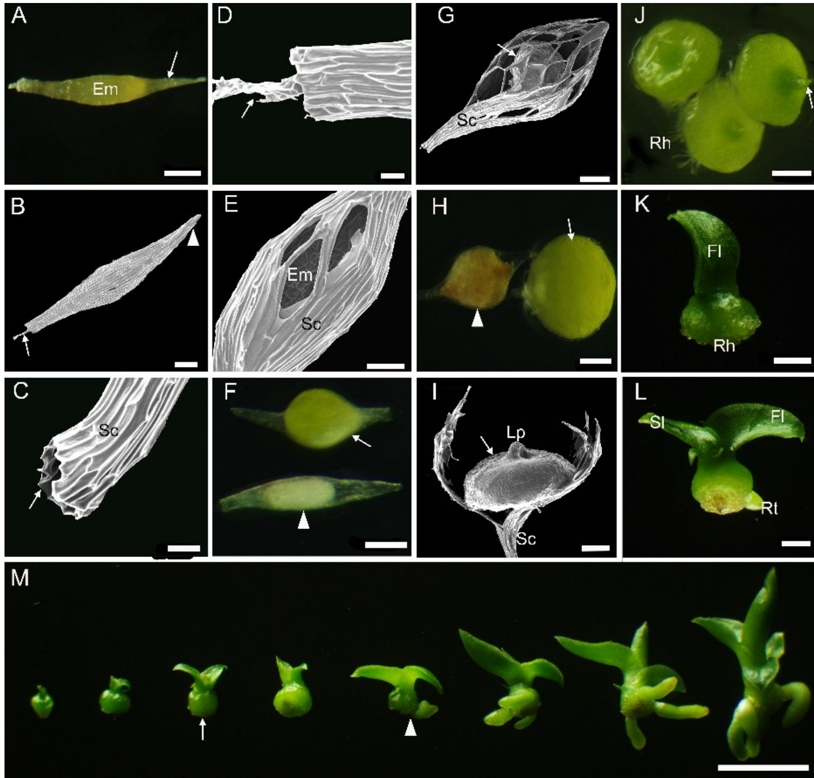

Fig. 4. Morphological structures observed during germination and initial *in vitro* development of *Cattleya crispa*. Stereo microscope (A,F,H,J,K,L,M). MEV (B,C,D,E,G,I). A-D. 0 DAS: General aspect of external seed morphology. A: Slightly pigmented seed coat (*arrowed*) with the embryo inside; B: Arrow indicate the suspensor projected through the micropylar end and arrow head indicate the chalaza extremity closed. C-D. Close view of the micropylar end of the seed. C: Arrow indicate the opening in the seed coat. D: Arrow indicate the suspensor. E-F. 7 DAS: E: Close view of the seed at the beginning of germination with the seed coat ruptured and the embryo inside. F: Arrow head indicate unviable seed with white embryo (Stage 0) and arrow points embryo swollen and germinated (Stage 1). G. 15 DAS: Protocorm development with broken but not lost seed coat. H. 30 DAS: Arrow head indicate a protocorm with signs of oxidation and arrow are pointed to the globular shaped protocorm (Stage 2). I-J. 60 DAS: I: Protocorm with totally broken seed coat (*arrowed*). J: Protocorms with leaf primordia indicated

by arrow and rhizoids (Stage 3). K. 80 DAS: Protocorm with the first leaf emerged (Stage 4). L. 110 DAS: Seedling with two leaves and root presence (Stage 6). M: Gathering of the development process, from the protocorm with leaf primordia, until complete seedling stage. Arrow indicate a protocorm with two spreading leaves and absence of root (Stage 5). Arrow head indicate the end mark of the protocorm stage (Stage 6) with complete seedlings forward. *Em* embryo; *Rh* rhizoids; *Fl* first leaf; *Sl* second leaf; *Rt* root; *Sc* seed coat; *Lp* leaf primordia. Scale bars: 20 µm (C-D). 50 µm (E). 100 µm (A, B, G, I). 200 µm (F, H). 500 µm (J). 1 mm (K-L).

3.5 Histology of initial development

The embryo is formed by a protoderm that delimits a promeristem (0 DAS). The protoderm is the outermost layer of partially differentiated cells. The promeristem is basically composed by cells that exhibit evidence of divisions on different planes (Fig. 5A). The longitudinal sections showed an embryo with elliptic shape and the chalazal-micropylar axis discernible, being that the terminal or chalazal region, are composed by smaller and denser cells and the basal or micropylar region, consists of larger cells with the suspensor attached. The suspensor is formed by large elongated and probably dead cells, mostly in a double row (Fig. 5B).

At the beginning of germination (7 DAS), the swelling embryo became initially more globous as a result of the increased cell number through both anticlinal and periclinal divisions. Changes in shape and volume were accompanied by histological differentiations, evidencing the embryo bipolarity: the shoot meristem cells were in divisions; right below, the parenchyma filling the central zone; and at the basal region, initial cells of the root apex, as the hypophysis are noted (Fig. 5C).

Within 15 DAS, the protocorm presented signs of lateral expansion. The shoot apex was at intense meristematic activity, with anticlinal divisions of the protoderm cells and divisions in several planes in the parenchyma cells, while the suspensor and the basal region cells, begin to disappear. The seed coat is largely broken (Fig. 5D). By the 30 DAS, the protoderm showed some recently formed rhizomes and trichomes, being needed a change in the expression, from protoderm to epidermis. The parenchymatous cells presented bigger vacuoles and the

mass increased through several divisions of the cells, ensuring to the protocorm a flat form (Fig. 5E).

60 DAS, due to the heterogeneous development previously reported, while some protocorms presented intense mitotic activity with the beginning of the differentiation at the shoot apex (Fig. 5F), other protocorms, already showed the leaf primordium that continued to enlarge (Fig. 5G). In other hand, the brown protocorm samples, showed generalized cell death (Fig. 5H).

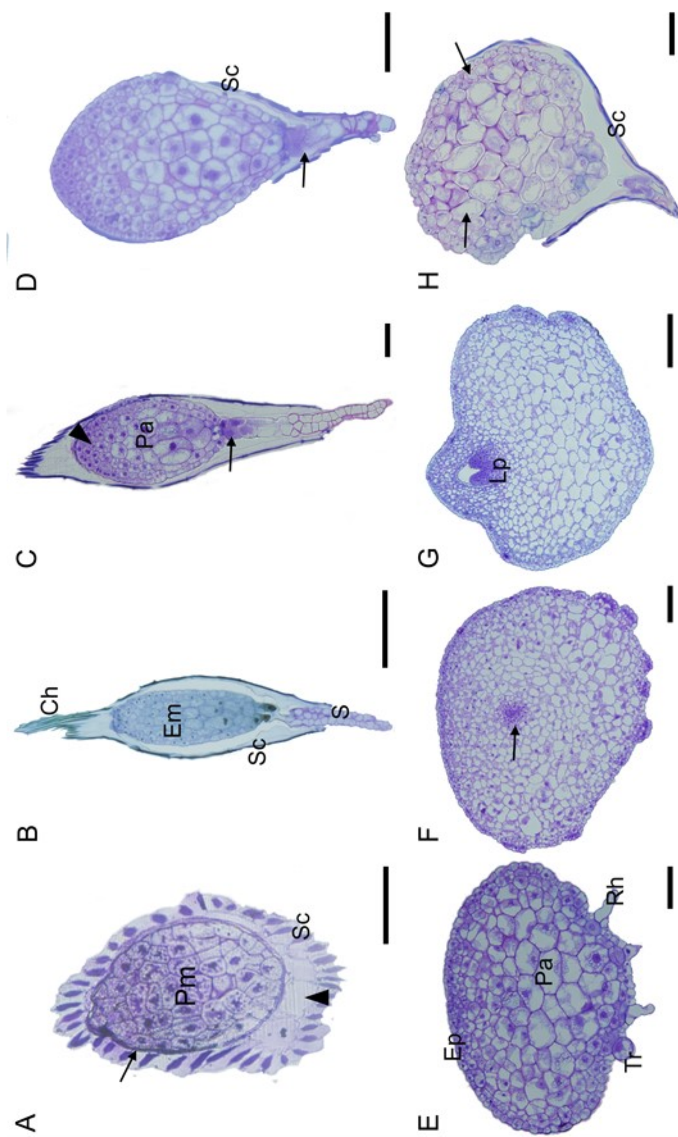
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Fig. 5. Histological aspects of *Cattleya crispa* under light microscopy analysis during germination and initial in vitro development. A. 0 DAS: Cross section of the seed: Embryo composed by a protoderm (*arrowed*) and a promeristem, with evidence of divisions in several planes. Seed coat involving the air space indicated by arrow head. B. 0 DAS: Longitudinal section of the seed: Embryo with elliptic shape and the chalazal-micropylar axis discernible. The chalaza end is closed and the suspensor is projected through the open micropylar end. C. 7 DAS: Germination accompanied by early histological differentiations evidencing the embryo bipolarity. The shoot apex cells in divisions are indicated by arrow head and the hypophysis cells are indicated by arrow. D. 15 DAS: Protocorm showing early signs of lateral expansion, with the shoot apex and the parenchyma cells in divisions, while the suspensor and the basal region cells were at the beginning of degradation (*arrowed*). E. 30 DAS: Protocorm with large lateral expansion due to anticlinal divisions of the epidermal cells and divisions in different planes in the increased mass of the parenchymatous cells, which present bigger vacuoles. Trichomes and rhizoids are present at the basal region. F-H. 60 DAS: F: Beginning of the differentiation of the shoot apex cells; G. 60 DAS: Developing shoot apex with visible leaf primordia; H: Brown protocorm sample, showing advanced cell death. *Pm* promeristem; *Sc* seed coat; *Em* embryo; *S* suspensor; *Ch* chalaza; *Pa* parenchyma; *Ep* epidermis; *Rh* rhizoids; *Tr* trichome; *Lp* leaf primordia. Scale bars: 50 µm (A,). 200 µm (B). 100 µm (C, D, E, F, G, H).

3.6. Reserve compounds

The cells of the embryo (0 DAS), were rich in lipid and protein bodies allotted in globoids structures of varying sizes, those, accompanied by limited early starch granules, are the primary reserves of *C. crispa* seeds (Fig. 6A-B). During the imbibition process (7 DAS), some events were observed: mitotic activity in the epidermal cells; rapid deposition of polysaccharides all over the embryo (Fig. 6C); and digestion of protein bodies, which are probably a result of the activation of the metabolism of the proteases. Also, the starch grains became bigger (Fig. 6D).

The polysaccharides granules tended to congregate around the nucleus of the cells (were observed) after 15 DAS (Fig. 6E). Due to the rapid digestion of the lipids, some bodies presumably glyoxysomes, are visible and tend to be found in proximity with the lipid bodies. Numerous large amyloplasts containing many starch grains are more evident (Fig. 6F). The parenchyma cells become largely vacuolated by the 30 DAS. An increasing gradient of polysaccharides was observed from the basal region of the protocorm, to the upper region, possibly due to mobilization of the free sucrose present in the culture medium (Fig. 6G-H). Polysaccharides at 60 DAS, were largely mobilized to the cells of the shoot apex in differentiation and to the cell layers close to the epidermis (Fig. 6I). These cells have dense cytoplasm and we note the formation and coalescence of lipidic bodies (Fig. 6J).

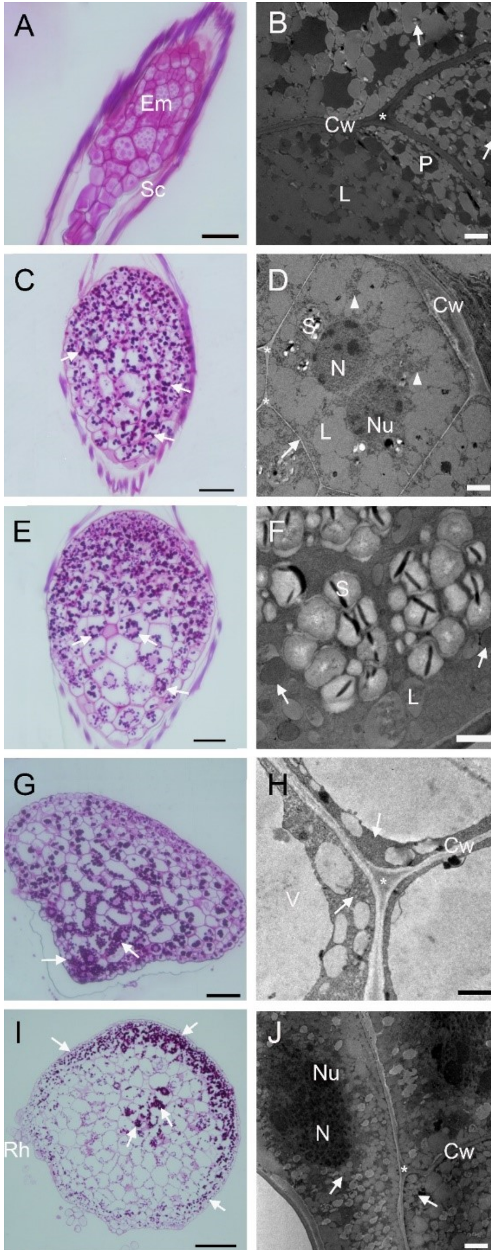
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Fig. 6. Reserve compounds of the initial *in vitro* development of *Cattleya crispa.* PAS staining (A,C,E,G,I). MET (B,D,F,H,J). A-B. 0 DAS: Embryo rich in lipid and protein bodies. Arrows indicate a few early starch granules that did not appear with PAS staining. C-D. 7 DAS: C. At the germination stage, stars a fast deposition of polysaccharides that stained purple-magenta color, apparently in the entire embryo (*arrowed*). D. Protoderm cells right after mitosis. The cell walls were still thin (*arrowed*). Arrow heads indicate the protein bodies largely digested. E-F. 15 DAS: E. The polysaccharides tend to congregate around the nucleus (*arrowed*). F. Presence of some bodies, presumably glyoxysomes found in proximity with the lipid bodies in process of digestion (*arrowed*). Numerous large amyloplasts containing many starch grains are evident. G-H. 30 DAS: G. A gradient of quantity of polysaccharides is visible from the basal region of the protocorm, to the upper region (*arrowed*). H. Parenchyma cells large vacuolated with disorganized protein (*arrowed*). I-J. 60 DAS: I. Polysaccharides mobilized to the cells of the shoot apex in differentiation and to the cell layers close to the epidermis (*arrowed*). J. These cells have dense cytoplasm, with largely lipidic bodies formation (*arrowed*). *Em* embryo; *Sc* seed coat; *Cw* Cell wall; *L* lipid bodie; *P* protein bodie; *S* starch grain; *N* nucleus; *Nu* nucleolus; *Rh* rhizoids; * Middle lamella. Scale bars: 50 µm (A, E,). 100 µm (C, G, I). 2 µm (B, D, J). 1 µm (F, H).

4. Discussion

4.1. Effect of seed cryopreservation

In general, for tropical orchid species, the germinability of cryopreserved seeds is high 70-100% (Popova et al. 2016). The water content of the seeds is an important factor for their successful cryopreservation, as low water level in the embryo cells, reduce the potential for lethal intracellular freezing injuries (Hirano et al. 2005). The high viability and germination of *C. crispa* in the present work, was directly related to the initial optimal value of water content of the seeds (5%), indicating their orthodox nature and allowing a simple cryopreservation technique without the use of cryoprotectants during freezing.

Pritchard (1984; 1985), Nikishina et al. (2001; 2007), Popova et al. (2003), Popov et al. (2004) and Suzuki et al. (2018) successfully

cryopreserved different orchid species with seed moisture content ranging from 5% to 14% and the germination rates after the liquid nitrogen storage, remained equal or greater. Even though Hughes and Kane (2018) study showed that seeds of *Bletia purpurea* and *Epidendrum nocturnum* with 55% moisture content prior to cryopreservation, did not had negative impact for both species, according to Popova et al. (2016), high post-cryopreservation germinability has been achieved in most studies and orchid seeds with moisture content below 13% can be successfully cryopreserved by direct immersion in liquid nitrogen.

The tetrazolium test that we performed on the solution concentration 0.5%, for overnight staining period at 25 ± 2ºC room temperature on *C. crispa* cryopreserved and control seeds, proves efficient for assessing the physiological quality of these seeds. In the present work as well as in similar studies with orchid seeds, the tetrazolium test results were consistent with the germination count (Alvarez-Pardo and Ferreira, 2006; Hosomi et al. 2012; Galdiano et al. 2012; Suzuki et al. 2018). Thus, tetrazolium test shows up as an important tool for a quick and efficient assessment without long waiting for seed germination.

A significantly increase in the germination rate after seed storage in liquid nitrogen, has been reported to occur in some orchids species (Nikishina, et al. 2007; Surenciski, et al. 2012; Hughes and Kane, 2018). Also, in Nikishina et al. (2001) study, cryopreserved seeds of the epiphytic orchids *Angraecum magdalenae* Schltr. & H.Perrier and *Miltonia flavescens* × *Brassia longissimi*, germinated 7–10 days faster than unfrozen seeds. Popov et al. (2004), noticed that protocorms from cryopreserved seeds developed larger during the first 45 d of *in vitro* culture compared to unfrozen seeds.

Our results demonstrate that germination and protocorm development (weight) of *C. crispa* seeds, were unaffected by direct LN immersion. Similarities were related by Jitsopakul et al., (2012), whereas seed cryopreservation of the Asiatic orchid *Vanda tricolor*, had no effect on the size and number of growing protocorms as well as in the morphological aspects of developing seedlings. As has been known, the cryopreservation effect on seed germination, protocorm development and seedling formation, is specific for the species and even the lot of seeds used.

Orchids present some requirements for germination, including a particular dependence on the culture media composition (Arditti and Ernst, 1993) with specific requirements for macro and micro elements

(Churchill et al., 1972). Pauw and Remphrey (1993), raise up an important information, that the requirements for germination and protocorm growth, can be very different. While some elements may improve the start in germination, they may have no effect on protocorms growth or seedling development.

So, it is possible that *C. crispa* protocorms may require more stringent culture medium conditions than what we provide for its continuous development, yet, cryopreserved and unfrozen seeds developed into normal seedlings with healthy shoot and root formation. Abrão et al., (2014) observed that MS medium results in highest germination and seedling survival for *Cattleya loddigesii*. Galdiano et al. (2014), recommend the use of ½ MS medium for initial *in vitro* development of *Cattleya walkeriana* however they did not test the entire MS formulation.

4.2. Seed coat influence in low survival rate

Seeds of *Cattleya* did not possess an inner integument known as a carapace, that is commonly present in temperate orchids and provides the physical dormancy (Custódio et al. 2016), however, morphological dormancy is a common characteristic found in the Orchidaceae family (Baskin and Baskin, 2014; Pierce et al. 2018). Our SEM images, linked with *in vitro* culture data, revealed that *C. crispa* seeds does not present integumentary dormancy, once the seed coat allows the entrance of water into the seed. Despite the seed coat did not affect the beginning of germination, it proves to be persistent, which affects further development of the protocorm by compressing it, which leads to a process of oxidation that ends up with the death of the protocorm. Cell death by necrosis occurs widely in plants, is an unprogrammed process, which remains poorly characterized and it is induced by a range of abiotic stresses (Van Doorn et al. 2011). Further investigation of the origin of the cell death in this subject, are still needed.

Our results are in accordance with previous findings for other orchids species. According to Dalzotto and Lallana (2015) the seed coat of *Bipinnula pennicillata* does not limit the asymbiotic germination of the seeds, instead it causes a delay in obtaining protocorms with apical bud and rhizoids, also by the 94 DAS, they began to observe a pronounced oxidation in the protocorms of all the treatments. Hossomi et al. (2012) study with ten *Cattleya* spp., showed that by the 63 DAS of culture,

seedling quality declined and some protocorms became somehow shrivelled and brown. Due to our finding, some procedures can be suggested to increase efficiency in raising the survival rate of the protocorms, through softening or breakdown the seed coat.

Seed morphological dormancy can be broken by cold treatments, lengthy imbibition, chemical scarification of the testa with sodium or calcium hypochlorite or by mechanical damage (Zeng et al. 2013). Germination of *Cypripedium* spp. can be improved by softening the testa with hypochlorite solution or cell wall degrading enzymes by leaching out the inhibiting substance with water imbibition (Bae et al. 2010). Chen, et al. (2015) observed that the highest germination was achieved after chemical scarification of *Paphiopedilum spicerianum* seeds pretreated with 1% sodium hypochlorite for 40 min. Hosomi et al. (2012) discuss that freezing (−18°C) may have caused injuries in the seed coat of four *Cattleya* spp., where germination after storage increased.

Further investigation is clearly required and the use of pretreatments in *C. crispa* seeds, such as soaking with sodium or calcium hypochlorite solutions, for longer periods or higher concentrations than what we have done, could lead to better results. As Arditti (1967) in his classic work suggests, orchid seeds despite their minuteness and frailty appearance, can resist and survive comparatively harsh treatments, also they often have a long life after dispersal.

4.3. Seed morphology

Size and number of orchids seeds are among the most noted characteristics of the Orchidaceae family. The seeds are usually produced in large numbers, which is a frequently mentioned characteristic of the Angiosperm plant group, reaching 4 million seeds per capsule (Arditti and Ghani, 2000; Rolfe, 1962). They are characterized by the tiny size containing only minimal reserves of nutrients and can measure from 0.05 to 6.0 mm in length and 0.01 to 0.9 mm in diameter (Gupta, 2016). The size refers to the length of the seed and can be classified into five categories according to Barthlott et al. (2014): Very small 100 – 200 µm; small 200 – 500 µm; medium size 500 – 900 µm; large 900 – 2000 µm; and very large 2000 – 6000 µm, being that, medium sized seeds represents the average in the Orchidaceae family.

Morphological aspects of seeds are related to be very different even between the genera. Studies with *Cattleya* seeds, (Knudson, 1921, 1929; Davis, 1946; Hoene 1940; White, 1945), showed variability in size,

from 150 µm to 910 µm and in colour, from white to yellowish-brown. Barthlott et al. (2014), assign the colour of the seeds to the seed coat and the embryo, which can be white, beige, yellow, orange, red-orange, brown, dark brown or even greenish in seeds that contain chlorophyll. The light-yellow color found in *C. crispa*, are also related for *Cattleya bicolor, C. loddigesii, C. purpurata* and *C. tigrine* (De Fatima Eggert and De Moraes, 2017).

In most of the orchids, the embryo is undifferentiated, suspended within a membranous, often transparent, but at times pigmented, seed coat (Arditti, 1967) and the endosperm development is suppressed (Cocucci and Jensen, 1969). In contrast to the widespread affirmation of the absence of endosperm in orchids, Kurzweil (1993) showed that in some *Diseae* species from the subfamily *Orchidoideae,* the seeds are unusually large and contain endosperm. This characteristic is attributed to an adaptation to the specific habitat from this specie that demand a quick germination from the seeds to prevent then from being carried by rain.

According to Barthlott et al. (2014), in contrast to various aspects of flower morphology, the seed coat is a well conserved structure that are barely susceptible to selection pressure, since the Orchidaceae family has a predominant seed dispersal mechanism. A connection between morphology aspects and dispersal properties was already suggested by Arditti et al. (1980), they affirm that the orchid seed morphology, their wettability and aerodynamics in turn affect the dispersion of the seeds. Therefore, seed dispersal is a fundamental life-cycle process of each threatened species and morphological descriptions are still insufficient, also these info's are useful for circumscribing subtribes in Orchidaceae.

4.4. Morphoanatomy of initial development

Categorization of development stages allows seedling development to be measured and the comparison between the effectiveness of different germination procedures (Hoang et al. 2016). Despite some authors consider the loss of the testa with posterior formation of a tuberiform structure called protocorm, as the characterizing event for orchid germination, we do not consider this has the main fact for *C. crispa* germination. Our SEM images showed that the seed coat did not prevent the formation of an early protocorm (Figure 2). So, we define germination as Salazar-Mercado and Contreras (2017) did for *Cattleya trianae*. The process of *C. crispa* germination (phase 1) starts

with the expansion of the embryo and chlorophyll production, at this stage the embryo was visible to the naked eye as small green propagule (Abraham et al., 2012).

The germination process of *C. crispa* started quickly and was homogeneous, however most of the protocorms presented slow and heterogeneous growth. Orchid seeds are quite heterogeneous, among sowed seeds, there is usually a population of strong seeds, which sharply overcomes the others in their development (Nikishina et al., 2007). We observed protocorms at different development stages over time, which could be repaired by transferring the protocorms to a fresh culture medium or adapting seed breaking dormancy methods for this goal.

The initial growth of *C. crispa* was similar to what Hossain et al., (2010) reported for *Cymbidium giganteum* and Galdiano et al., (2013) for *Cattleya violacea.* By the 30 DAS, the early globular protocorm was formed and by the 60 DAS, the protocorms develop in the upper portion a meristematic region responsible for the formation of the vegetative organs and in the lower portion, a few trichomes called rhizoids, with main absorption function. Abraham et al., (2012), noticed the emergence of the first small leaves in *Coelogyne nervosa* within 80 DAS and by the 100 DAS two or more elongated green leaves were visible, the same was observed in our work. They recommend the addiction of BA and NAA to MS medium in order to promote individual seedling elongation and root formation.

Morphological studies of initial development of native plants may provide support for the interpretation of germination tests and aid at the assessments of their vegetative cycle, also are very important for botanical, taxonomic, ecological studies and for development of efficient propagation techniques (Gentil and Ferreira 2005).

4.5. Histology of initial development

Orchid embryos are composed mostly by isodiametric and relatively undifferentiated cells. Two regions may be noticed, the posterior region formed by large and vacuolated, cells and the anterior region, consists of smaller and denser cells with quite evident nuclei, which will develop the shoot meristem (Knudson, 1929; Stoutamire, 1964; Arditti, 1967). Studies on the ontogeny in the genus *Vanda* showed that the embryo differentiation occurs early and three regions are formed, parenchymatous, meristematic and suspensor; the suspensor also

disappearing early, then the parenchyma fulfill the function of meristem nourishment (Alvarez, 1963).

In *Dendrophylax lindenii*, the embryo polarity was established by 20 DAG, as noted by development of the shoot meristem at the anterior region (Hoang et al., 2016). In *Cymbidium giganteum* the embryo is undifferentiated and occupies the center of the seed, however, a chalazal-micropylar axis is soon discernible, at the 15 DAG (Hossain et al., 2010). The present work with *C. crispa* showed similarities, where despite the presence of an early axis, the embryo is relatively undifferentiated. But at the beginning of the imbibition, the differentiation process is already evident. A hypothesis is that our seeds were probably collected at a more advanced stage of maturity.

Orchid embryos commonly start the development into globular protocorms and continued to increase in diameter, forming light-green protocorms with rhizoids along the basal region. Protocorm is a term used to describe the extension of the embryonic stage, that in other words, is an embryonic structure, ofter called proembryo, which completes the development outside of the seed (Barabé et al., 1993). In the Orchidaceae family there are several discussions regarding terms definition including variations between embryo and protocorm and germination itself. In the present work we found that germination occurs due to proembyo imbibition.

Subsequently the globular protocorm stage, intense meristematic activity is evident and the shoot meristem with the leaf primordia recently formed are mostly enclosed within the protocorm structure, that is in continuous enlargement (Hoang et al., 2016). Once the differentiation of the leaf primordia and the formation of the procambium tissue are advanced, the parenchymal tissue starts degeneration. This process along with the presence of roots marks the end of the protocorm phase and the transition into seedling (Alvarez and Sagawa, 1965; Veyret, 1974).

4.6. Reserve compounds

Mature seeds of all Orchidaceae species contain substantial reserves of lipid and protein in the cells of the embryo, but starch is absent (Knudson, 1929; Arditti, 1967; Manning and Van Staden, 1987). Reserves of orchid embryos consist of cellular inclusions like oil droplets and starch grains at levels that are not high in absolute terms (Arditti and Ghani, 2000). The embryo of a *C. crispa* mature seed was rich in lipid

and protein bodies, containing few small grains of starch. Their seeds probably accumulate lipids, to act as principal source of energy during the germination (Somerville, 2000).

As seeds are also reserve units, they can accumulate several highly energetic compounds that are mobilized in the beginning of germination and at the development of the seedling. The products of reserve mobilization are used as energy sources for tissue formation (Buckeridge et al., 2004). As seed germination require remobilization of nutrients on a large scale, and *C. crispa* seeds are rich in lipids and proteins, it is possible that the mobilization of these compounds, collaborate to the rapid germination. Whereas the availability of energy resulting from the hydrolysis of these reserves, is ample.

The evident presence of polysaccharides, including starch, during the initial development, is attributed to the mobilization of lipids and imbibition of the sucrose from the culture medium. Penfield et al., (2005) reported, that this mobilization leads to acetyl Co-A formation with posterior synthesis of sucrose through gluconeogenesis. If the levels of polysaccharides in the cytosol are too high, they can be stored as transient starch. We believe there is a correlation between the accumulation of starch grains during the germination and the degradation of primary reserves. The mobilization was probably greater than the required for these initial stages, therefore, to maintain the osmotic balance, the excess was converted into a starch reserve. Manning and Van Staden (1987), studied some African orchids by ultrastructural and histochemical techniques and reported similarities.

5. Conclusions

We achieve successful cryopreservation of this endangered species, through direct NL immersion of seeds with 5 % MC. Through the establishment and accompaniment of the development stages, we discover the persistent seed coat. Based on our study, we recommend for enhancing the obtention of viable protocorms, the assessment of different seed pretreatments. This work is the first morphological, histological and ultrastructural report of *C. crispa* seeds and protocorms and provides a basis for further conservation studies.

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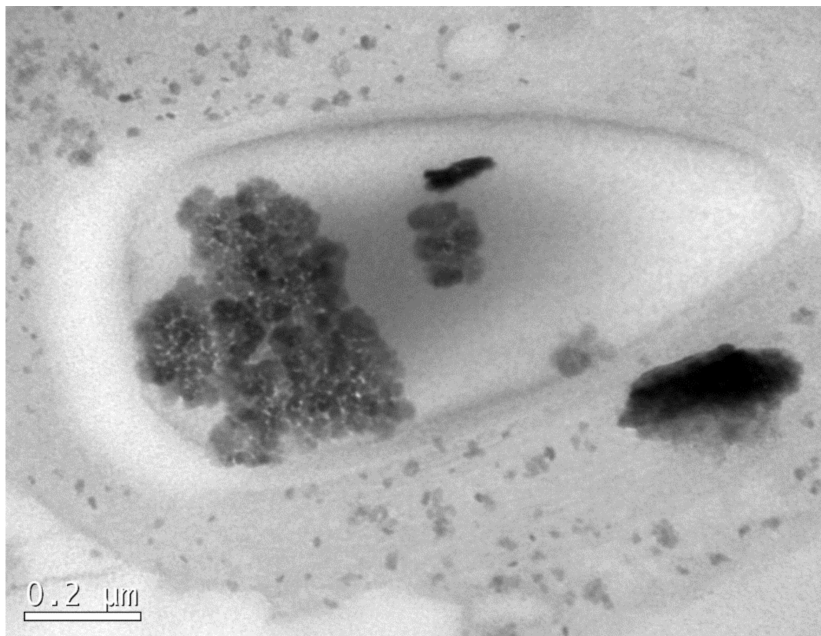
Appendix

Appx 1. *Cattleya crispa* “the curled-petaled *Cattleya*”. (Drawing found in Lindley, J. 1828. *Botanical Register* 14: t. 1172).



Appx 2. Ultrastructural analysis of a parenchyma cell from a 60 day old, green-healthy protocorm. Close look to a vesicle with possibly accumulation of phenolic compounds.