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Seeds cryopreservation of *Vriesea reitzii* Leme & A.F. Costa endemic bromeliad from Atlantic Rainforest[1]

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Running head tittle: Seeds cryopreservation of *Vriesea reitzii*

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[1] Part of the first Author's Master's Thesis

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ABSTRACT – (Seeds cryopreservation of *Vriesea reitzii* Leme & A.F. Costa endemic bromeliad from Atlantic Rainforest). *Vriesea reitzii* is an endemic bromeliad from the Atlantic Rainforest. The objective of this research was to evaluate the cryopreservation using the method of direct immersion of its seeds, collected from capsules at 120, 135, and 150 days after anthesis (DAA). The water content was determined before cryopreservation, while the germination percentage, germination speed index (GSI), and total soluble carbohydrates were quantified after cryopreservation. The highest percentage of moisture (17.6%) was observed in 120 DAA, while the highest percentage of germination (89.6%) and GSI (17.0) were observed in 150 DAA. Optical and transmission electron microscopy analyses were performed, and no cell damage or changes at the morpho-histological and ultrastructural levels were observed after the cryopreservation process. From these results, *V. reitzii* seeds can be classified as orthodox seeds and the cryopreservation (+LN) is an efficient tool for an *ex situ* conservation of this species.
Keywords: Bromeliaceae, carbohydrate, germination, moisture content, morpho-histology

RESUMO – (Criopreservação de sementes de *Vriesea reitzii* Leme & A.F. Costa bromélia endêmica da Mata Atlântica). *Vriesea reitzii* é uma bromélia endêmica da Mata Atlântica. O objetivo deste trabalho foi avaliar a criopreservação de sementes utilizando o método de imersão direta, coletadas de cápsulas aos 120, 135 e 150 dias após a antese (DAA). O teor de água foi determinado antes da criopreservação. A porcentagem de germinação, o índice de velocidade de germinação (GSI) e os carboidratos solúveis totais foram quantificados após a criopreservação. A maior porcentagem de umidade (17,6%) foi observada aos 120 DAA, enquanto a maior porcentagem de germinação (89,6%) e GSI (17,0) foram observadas aos 150 DAA. Em análises de microscopia ótica e eletrônica de transmissão não foram observados danos celulares ou alterações a nível morfohistológico e ultraestrutural após o processo de criopreservação. A partir desses resultados, as sementes de *V. reitzii* podem ser classificadas como ortodoxas e a criopreservação (+LN) é uma ferramenta eficiente para a conservação *ex situ* desta espécie.
Palavras-chave: Bromeliaceae, carboidratos, germinação, morfohistológia, teor de umidade

Introduction

The Bromeliaceae Juss. Family encompasses 78 genera and 3.336 know species. Also it is considered one of the most diverse in the Atlantic forest (Gouda *et al*. 2021). The genus *Vriesea* Lindl. comprises of 231 species, 219 out of a which are endemic in Brazil (Costa *et al*. 2021). The genus *Vrisea* is the richest species in terms of the Atlantic Forest biome, it has 169 species in total. a large number of species (145) are endemic, such as *Vriesea reitzii* Leme & A.F. Costa, which is currently endangered, as reported in the list of the Rio Grande do Sul State, Brazil (Martinelli *et al*. 2008).

Considering the ecological importance of bromeliads and endemism, it is of fundamentally important to establish conservation strategies that ensure the availability of genetic diversity (Costa *et al*. 2014, BFG 2015). In this context, available information on *ex situ* conservation procedures of bromeliad species in Brazil is still incipient, revealing the need to establish effective programs for the conservation of native flora (Rodrigues *et al*. 2014).

In the effort to protect Bromeliads, several strategies have been used to better understand and support future studies. In *V. reitzii*, certain studies use biotechnological techniques as conservation tools, such as *in vitro* cultivation with the nodular cultures (NCs) production. NCs can be considered as a morphogenic response pattern with high regenerative potential since they have the ability to generate more than 5300 micro sprouts g^{-1} in 10 weeks of cultivation, which can be generated from leaf base (Dal Vesco & Guerra 2010, Guerra & Dal Vesco 2010, Dal Vesco *et al*. 2014a) or seeds (Dal Vesco *et al*. 2014b). In addition to the morphological and histochemical characterizations regarding the induction and origin of nodular cultures (Corredor-Prado *et al*. 2015), proteomic analyzes and identification of differentially expressed proteins involved during the induction and regeneration of nodular cultures (Corredor-Prado *et al*. 2016, 2019), as well as in the dynamics of changes in the levels of proteins, carbohydrates and global DNA methylation during the induction of NCs. (Corredor-Prado *et al*. 2020).

However, an effective alternative is to establish a cryopreservation protocol, since it allows an effective and safe conservation of materials, suiting the long-term storage system. In these protocols, different materials can be used, for the most diverse plant species, such as seeds, zygotic embryos, apical buds, meristems, and others (Engelmann 2011). Direct cryopreservation is presented as a simple methodology, which consists of direct immersion of fresh or dehydrated plant material in liquid nitrogen (LN). when such material is removal in most cases whit reheating it in water at 40 °C and the subsequent regeneration of the material happens (Popova *et al*. 2016).

Seed conservation is the most efficient and economical way to store germplasm (Kaviani 2011). This technique has been studied in bromeliads with success in its application. Seeds of six species of the genus *Encholirium* and two of *Dyckia* cryopreserved without the use of cryoprotectants maintained the germination percentage after cryopreservation (Tarré *et al*. 2007), even as for *Encholirium spectabile* Mart. ex Schult. & Schult.f. seeds, which it was proven that the use of cryoprotectants is not necessary for cryopreservation (Ferrari *et al*. 2016), likewise, seeds of *Dyckia brevifolia* Baker and *Dyckia delicata* Larocca & Sobral showed respectively 92% and 79% germination after cryopreservation without cryoprotectants (de Paula *et al*. 2020).

In Bromeliaceae, the use of seeds of some species has shown a high tolerance for drying and freezing with the genera *Encholirium* Mart. ex Schult. & Schult. f. and *Dyckia* Schult. & Schult. f. (Tarré *et al*. 2007), in *Pitcairnia albiflos* Herbert (Pereira et al. 2010) and ten endemics and threatened Brazilian species (Rodrigues *et al*. 2014). This tolerance is related to the content and deposition of reserves, such as carbohydrates, lipids, and proteins, which are constituents of cells and membranes, providing protection during the seed maturation and dehydration phase (Mollo *et al.* 2011, Carvalho *et al*. 2019).

According to Mollo *et al*. (2011), in *Alcantarea imperialis* (Carrière) Harms (Bromeliaceae), carbohydrates undergo appreciable changes, in adaptations to the cold. The authors describe that the sucrose contents of plants under cold conditions can be exchanged for trehalose, raffinose, and

stachyose. They also report that starch concentrations changed with the presence of cold, possibly reduced to carbohydrates associated with tolerance and protection of the plant to cold.

Furthermore, intracellular ice crystal formation is the most damaging event during the cryopreservation process. The damage is irreversible. It can occur during freezing or thawing and may lead to cell wall rupture, epidermal rupture, protoplast egress, anomalous nuclei of surviving explants, or explant death (Kaviani 2011). Anatomical studies, such as the one carried out by Wesley-Smith *et al*. (2014, 2015), report the importance of understanding the interactions of water content and ice structure through studies for the atomization of cryogenic processes and cell survival since ice crystal size and cell damage can have late effects on cell death.

In this regard, *V. reitzii* seeds could be a plausible material for the conservation of germplasm with great genetic diversity and with protocols already established for large-scale propagation. Thus, the objective of the present work was to evaluate the viability, cell integrity, and presence of polysaccharides in *V. reitzii* cryopreserved and non-cryopreserved seeds using anatomical, biochemical and ultrastructural analyzes.

Material and Methods

Physiologically mature capsules of *V. reitzii* were collected from a natural population, in the municipality of Curitibanos, Santa Catarina State, Brazil (27º17'02.7"S, 50º32'05.5" W, 900 m altitude The collection was carried out at three different times, established by days after anthesis (DAA), according to the maturation curve, between physiological maturation and dispersion: 1) at 120 DAA, when the seeds reached physiological maturation and at the beginning of the reduction in the content of indoor water; 2) at 135 DAA, intermediate point of the natural dehydration process and; 3) at 150 DAA, beginning of carpel opening for seed release and dispersion. The experiments were conducted in the Laboratory of Developmental Physiology and Plant Genetics (LFDGV), Center

of Agricultural Sciences, of the Federal University of Santa Catarina (CCA/UFSC) in Florianópolis, Santa Catarina State, Brazil.

The moisture content (%) of the seeds was obtained from a pool of 1000 seeds of different fruit collection times. The oven method was used at 105 ± 3 ºC for 24 ± 1 hours, according to Brazil (2009), using four replications of 200 seeds. The moisture content, expressed on a wet basis, was obtained through the expression:

$X\% = \frac{Iw-Fw}{Iw} x\ 100$; Where: Iw: Initial weight of the sample (g); Fw: Final sample weight (g) and X%: water content in percentage of wet basis (w.b), %.

For the seeds conservation, a 2x3 factorial experimental design was used: Two conservation methods: 1) Cryopreservation (+LN; at -196 °C) and; 2) control, non-cryopreserved (-LN) stored in a refrigerator at 4 °C, combined with three collection times (120; 135 and 150 DAA). Each experimental unit consisted of a cryotube containing 30 seeds and four replicates arranged in random blocks. After 24 h of storage (-196 °C and Control), was performed the immersion in a water bath for two minutes at 42 °C. Afterward, the seeds were disinfected according to the method described by Guerra & Dal Vesco (2010).

The germination test was conducted *in vitro* and the seeds were placed in Petri dishes (60 x15 mm), on sterilized filter paper, soaked with 2.0 mL of liquid culture medium MS (Murashige & Skoog 1962), and added with Morel vitamins (Morel & Wetmore 1951) and 30 g L^{-1} of sucrose. The pH was adjusted to 5.8 before sterilisation in an autoclave for 20 minutes at 121 °C and 1.3 atm. The cultivations were kept in a BOD chamber at 25 ºC ± 2 ºC for a 16 hours photoperiod of light in a luminous intensity of 55 ± μmol m^{-2} s^{-1}.

Germination percentage data (%) and germination speed index (GSI) were daily obtained until the 15th day of cultivation. Germinated seeds were considered when they presented embryo swelling, seminal envelope rupture and, light green colour as a sign of active metabolism. The GSI was calculated according to Maguire (1962) and the formula:

GSI= ∑ (Gi/ni) where: Gi = number of germinated seeds and; ni = counting day.

The determination of total soluble carbohydrates levels (mg g^{-1}) was based on seed samples (200 mg) of each treatment, previously described, using the phenol-sulfuric method (Dubois *et al*. 1956). The samples, in triplicate, were macerated with liquid nitrogen and transferred to test tubes (15 mL), with an addition of 0.4 mL of ethanol (80% at -22 °C) and kept in a water bath at 70 °C for 5 minutes. The extracts were centrifuged at 3.000 rpm for 10 minutes and after its conclusion, the supernatants were collected. This process was repeated 3 times, followed by filtration with glass wool and had the volume adjusted to 2 mL with ethanol (80% at -22 °C). Afterward, 50 μL of the extract was added to 450 μL of distilled water, 0.5 mL of phenol (5%), and 2.5 mL of sulfuric acid (96%). The total carbohydrate content was estimated from a standard curve, determined based on a standard carbohydrate Glucose and with absorbance measured at 490 nm on a spectrophotometer (Pro-Analysis V-1600)

Representative samples of the seeds were fixed in paraformaldehyde solution in phosphate buffer (2.5%) 0.2M pH 7.2 for 72 hours at 4 °C, for anatomical analysis. The dehydration was in an increasing ethyl series (0-100 °Gl), and soon after the samples were pre-infiltrated in a solution of ethanol (100%) and historesin glycol methacrylate (GMA) (1:1 v v) for 6 hours, infiltrated in Historesin (Leica Historesin, Heidelberg, Germany) and arranged in histo-molds at 35 °C in an oven for 24 hours.

Sections (5 μm) were obtained using a hand-rotating microtome (Leica RM 2135) with tungsten razors. The sections were placed on slides and stained with Toluidine Blue (TBO) to identify acid polysaccharides and Lugol for starch identification. The sections were analysed under an optical microscope (Olympus BX40) and photographed with an image capture system (Olympus Q-Color-3C América Ind.).

Transmission electron microscopy (TEM) analysis was performed from seeds of each treatment, fixed in a glutaraldehyde solution (2.5%) in 0.1 M sodium cacodylate buffer pH 7.2 and

0.2 M sucrose at 4 °C overnight; followed by immersion in 0.3 M sodium cacodylate buffer solution, pH 7.2, 0.6 M sucrose and H_2O, five times, reducing the concentration of sucrose in each wash by 25% and in osmium tetroxide (2%) and 0.2 M sodium cacodylate, pH 7.2 (1: 1, v v) for 4 hours at room temperature. The dehydration in an increasing series of acetone (30 - 100 °GL) and pre-infiltration in series of acetone and Spurr resin solutions (3:1, 2:1, 1:1, 1:2, 1:3) and infiltrated in Spurr resin. Sections (60-90 nm) were obtained with a Power Tome XL ultramicrotome diamond razor and contrasted with uranyl acetate (1%) and lead citrate (1%). Images were obtained using a Jeol JEM1011 Transmission Electron Microscope (Tokyo, Japan) at CEML-UFSC, and the relevant events were recorded in micrographs.

Germination percentage data (%) and levels of total soluble carbohydrates (mg g^{-1}MF) were submitted to analysis of variance (ANOVA) and test of comparison of means (Tukey 5%), using the statistical program R (R Development Core Team, 2010).

Results

The different periods of fruit collection, days after floral anthesis (DAA), revealed statistically significant water levels (table 1). The highest ($p < 0.05$) average percentage of water (17.6%) present in the seeds was observed in seeds collected at 120 DAA. Seeds collected at 135 and 150 DAA did not show statistical differences in moisture content.

The average percentage of seed germination did not present any statistically significant difference when comparing conservation methods, cryopreserved (78.9%), and non-cryopreserved (78.3%) (table 1). Therefore, significant differences in the germination percentages of the seeds were observed in the different DAA. The highest and most significant ($p < 0.01$) average germination percentage (89.6%) was observed with seeds collected at 150 DAA (table 1).

Concerning the germination speed index (GSI), no significant differences were found between cryopreserved (+LN) and non-cryopreserved (-LN) seeds. Seeds submitted to the conservation

methods started to germinate on the 7th day of cultivation, and the maximum germination point was observed on the 15th day. The seeds collected at 150 DAA had an average GSI equal to 17.0, being, statistically, the largest and the most significant ($p<0,05$) when compared to the results of seeds collected at 120 and 135 DAA (table 2).

The average content of total soluble carbohydrates for *V. reitzii* seeds did not show a significant difference for the studied factors: collection period, and conservation methodology (table 3).

Anatomical analysis by light microscopy of different days of seed collection (DAA) and exposure to cryopreservation and non-cryopreservation, did not reveal mechanical damage, such as rupture of the cell wall as damage to the membrane system. However, differences were observed in the response to histochemical reactions of the contents of chemical compounds in the regions of the seed coat, the endosperm, the aleurone layer, and the embryo (figure 1).

In the micrographs of *V. reitzii* seeds, seeds with the embryo and plumose appendages at the time of conservation are noteworthy (figure 1a). In the embryo region, a reaction of the acid compounds to the TBO was observed, characterized by the procambium, formed by the nuclei of the embryonic cells, which presented a dark purple colour (figure 1b, see arrow).

The presence of a double seed coat was also observed, with a positive reaction to treatment with TBO, where the external seed coat, called testa, presented a dark green colour indicating the presence of tannic compounds (figure 1b). The internal seed coat, called tegmen, showed a greenish colour indicating phenolic composition and purple coloration in the plumose appendages, indicating rich in pectin composition (figure 1b).

The aleurone layer that surrounds the endosperm and the embryo is presented in a single layer of cells, of a thick wall, with the presence of acid polysaccharides in the cytoplasm and cell wall (figure 1c, see arrow). Concurrent, there was an absence of starch grains in the cytoplasm of the aleurone layer.

It was observed the presence of neutral polysaccharides in the endosperm region, as constituents of the cell wall, indicating the presence of cellulose. And, when stained with Lugol, neutral polysaccharide grains were observed, conferring a high concentration of starch grains distributed in amyloplasts (figure 1d, see arrow). Analyses of 120 DAA seeds, non-cryopreserved (figure 1c) and cryopreserved (figure 1f); 135 DAA non-cryopreserved seeds (figure 1d) and cryopreserved (figure 1g), as well as 150 DAA non-cryopreserved seeds (figure 1e) and cryopreserved (figure 1h), all showed structural integrity after both conservation treatments.

Through TEM, the seed ultrastructure's, observed in cells of the embryo of non-cryopreserved seeds (-LN), were identified with the presence of membranes, organelles, and nucleus in the natural state (figure 2a). Besides, in detail, cells with a large number of reserve structures in lipid bodies (figure 2b) and protein bodies (figure 2c). Additionally, analyses of embryonic cells, from cryopreserved seeds (+LN), presented cell integrity, in other words, the absence of damage, lysis or, injury to membranes, and cell walls (figure 2d). As depicted, in detail, the membranes of the nucleus and cell wall (figure 2e), in addition to the structures of protein and lipid reserves (figure 2f). TEM results added to light microscopy results confirm that the seeds preserved the structural integrity after cryopreservation.

Discussion

The time intervals established in seed collections (DAA), according to the maturation and viability curve, allowed the analyzes entrenched in this work, as well as enabled its use in protocols for multiplication and generation of nodular cultures (Guerra & Dal Vesco 2010, Dal Vesco *et al*. 2014a). The water content in the seeds of *V. reitzii*, appraised in the present work, revealed the occurrence of a natural desiccation process, which is directly related to its degree of physiological maturity. Starting with this factor, it can be inferred that the seeds of this species have a characteristic orthodox behaviour. In these species, the seed moisture tends to reduce as the seeds pass through the

maturation process in the mother plant, resulting in natural desiccation and conceding tolerance to artificial desiccation (Dousseau *et al*. 2011). Although the water content can be a variable when classifying orthodox seeds, Engelmann & Dussert (2000) report that the moisture content must remain between 10 to 20%. Walters (2015) indicate that the water content must be below 0.1 g H_2O g^{-1} at the time of conservation, which requires to be achieved by artificial desiccation. Thus, the hypothesis that the seeds of *V. reitzii* are to be contained in the orthodox category with regards to the percentage of moisture as laid out by these authors, is corroborated.

In this context, it is emphasized the relevance of observing the water content present in the plant material used in the cryopreservation protocols. This content can be decisive when defining strategies to avoid possible damage caused by the crystallization of intracellular water (Vendrame *et al*. 2014). Factor, particularly, not observed in the current work and, according to ultrastructural analyses, characterized by the absence of lysis or damage to membranes and cell walls. According to González-Arnao & Engelmann (2013), some low water content in the plant material results in the change of the water phase during the immersion in LN, called the "vitrification" phase. This phase allows the elimination of ice structures by inhibiting the union of water molecules, maintaining the physical properties of an amorphous solid (Kulus & Zalewska 2014).

The importance of water content is reported in several studies and for various plant materials, such as seeds of *Punica granatum* L. (Punicaceae) (Silva *et al*. 2015), seeds of *Encholirium spectabile* Martius ex Schultes f. (Bromeliaceae) (Ferrari *et al*. 2016), seeds of *Pyrostegia venusta* (Ker Gawl.) Miers (Salomão *et al*. 2020), as well as seeds, pollen, protocorms, structures similar to protocorms, apexes extracted from *in vitr*o plants and meristematic tissues of orchids (Popova *et al*. 2016).

Cryopreservation has advantages considering the possible storage time, as well as cryopreservation by direct immersion, which is the cheapest in the category by not using cryoprotectants or too many reagents. Concerning the storage of plant material for indefinite periods, cryopreservation in liquid nitrogen (-196 °C) is the only current method (Engelmann 2011). Likewise,

cryopreservation has vast advantages when compared to traditional conservation methods (Vendrame *et al*. 2014).

In the present study, it was observed that the anticipation of seed collection, at 120 DAA, resulted in a lower average germinative percentage (70%), when compared to seeds collected in the desiccation period (89.6%), at 150 DAA (table 1). Another important factor observed was that the cryogenic process did not affect the germinative power of the seeds. Yet, the moisture content was a key factor in survival and germination. Likewise, the cryopreservation of Ipê-Roxo (*Tabebuia impetiginosa* (Mart. ex DC.) Standl.) seeds, with the moisture content of 12.5%, 8.4%, and 4.2%, maintained vigour and germination capacity. However, at 18.3% of moisture content, there was a decline in germination (Martins *et al*. 2009).

Furthermore, there are reports of different species works that show gains in the germinative percentage of seeds after cryopreservation, of which, the cooling process at ultra-low temperatures can act to break seed dormancy. As an example, Rocha *et al*. (2009) found in their studies that the cryopreservation process of *Gossypium hirsutum* L. seeds promoted an increase in the percentage of seed germination and seedling vigor, after going through the cryopreservation process. Cryopreservation also advanced a higher germination percentage of *E. subsecundum* (Baker) Mez seeds with a moisture content of 12.7% (Tarré *et al.* 2007).

From these results, it can be inferred that the period of fruit collection and seed extraction in *V. reitzii* is an important factor to observed. Also, the highest percentage of germination and GSI are related to lower water content, greater accumulation of reserves, and embryo maturity. These factors were also observed in the germination in *Moringa oleifera* Lam. seeds (Agustini *et al.* 2015). Data that corroborate with those observed by Guimarães & Barbosa (2007), in which the increase in GSI in *Machaerium brasiliensi* Vogel seeds is related to the fruit maturation process. Therefore, the natural desiccation determines the quality and vigour of the seeds, an important factor to be considered in conservation programs, such as the use in cryopreservation.

Carbohydrates, on the other hand, are associated with the seed's capacity to withstand desiccation and storage at negative temperatures, among other structural and biochemical characteristics. For Mollo *et al*. (2011), the carbohydrates present in the seeds of *Alcantarea imperialis* (Carrière) Harms (Bromeliaceae), trigger mechanisms of adaptation to the cold, when seeds were submitted to low temperatures in growth chambers (5 ± 2 °C, 15 ± 2 °C, 15 °C dark / 30 °C light and 30 ± 2 °C, photoperiod 12-h). According to these authors, the sucrose contents in plants, at cold conditions, can be exchanged for trehalose, raffinose, and stachyose. Moreover, the starch concentrations changed with the presence of cold, possibly reduced to carbohydrates associated with the plant's tolerance and protection from cold (Mollo *et al.* 2011). Hence, it can be inferred that a longer permanence of the fruits connected to the mother plant, after anthesis, represents a greater accumulation of carbohydrates, since, according to the results of the present study, the higher the DAA for the fruit collection, the greater will be the loss of water in the seeds.

It was observed, in the present study, that cryopreserved seeds, when compared to those non-cryopreserved, do not show structural or biochemical differences, thus, corroborating that cryopreservation did not affect the seeds. Seen in these terms, non-reducing sugars, sucrose, and some oligosaccharides are important for the protection of membranes against the formation of crystals at the moment of freezing, as well as the accumulation of lipid bodies and starch grains (Zaritzky 2015). In embryonic corn cells, protein bodies were also observed (Wen 2009), while in the embryonic cells of *Livistona chinensis* (Jacq.) R.Br. ex Mart., the accumulation of these substances was not detected, probably because they are considered recalcitrant seeds (Wen 2010). Therefore, it was observed, concerning the present study, that the seeds of *V. reitzii* have reserve organelles in the form of lipid bodies, protein bodies, and orthamyloplasts, which can confer tolerance to desiccation and cooling and also reaffirm the orthodox behaviour of the seeds.

Cryopreservation is the only technique that guarantees the conservation of genetic material, theoretically for an indefinite period, safely and economically. (Engelmann 2011). In liquid nitrogen

storage (-196 °C), all metabolic activity and cell divisions are interrupted, preventing the degradation of the stored material and contamination (Cruz -Cruz *et al*. 2013). Direct immersion in liquid nitrogen has the advantage of not requiring the use of cryoprotectants in materials with tolerance to cooling and low moisture content, as seen in the present study, and with several examples, such as the one seen for *Dyckia brevifolia* Beker and *Dyckia delicata* Larocca & Sobral seeds (De Paula *et al*. 2020), mature seeds of *Cattleya guttata* Lindl (Vettorazzi *et al*. 2019) and for *Passiflora edulis* Sims seeds (Generoso *et al*. 2019). Although the present study hasn't observed a difference between the storage treatments, it is assumed that cryopreservation has more long-term advantages when compared to storage in a refrigerator, in which there may be degradation and contamination of the material in the medium term.

Conclusions

In conclusion, the method of immersing the seeds in liquid nitrogen for a period of 24 hours is sufficient to promote the interruption of metabolism. Hence, one can infer the tolerance potential and its indefinite storage projection. The use of the cryopreservation method for *V. reitzii* seeds, with direct immersion in liquid nitrogen, revealed to be an effective mechanism for long-term ex situ conservation system. Accentuating, however, the importance of the seed maturity state and water content, as important factors for the success in the conservation process. Moreover, *V. reitzii* seeds have a natural desiccation process in the mother plant and present the ability to tolerate desiccation and storage, key features to be classified as orthodox seeds.

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Author Contributions

Francisco S. Montoya-Serrano: Designed and performed the experiments, wrote the manuscript and prepared figures. The author read and approved the final version and reviewed the manuscript.

Rosete Pescador: Supervised the project and provided funding. The author read and approved the final version and reviewed the manuscript. Contribution to critical revision, adding intellectual content.

Lirio L. Dal Vesco: Supervised the project and collected the plant material. The author read and approved the final version and reviewed the manuscript. Contribution to critical revision, adding intellectual content.

Conflicts of interest

There is no conflict of interest

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Table 1. Germination speed index (GSI) from *Vriesea reitzii* Leme & A.F. Costa seeds collected on different days after anthesis -DAA (120, 135 and 150 days) and cryopreservation.

Days After Anthesis	Moisture Content (%)[1]	Germination (%)[2]		
		Cryopreserved (+NL)	Non-cryopreserved (-NL)	Mean
120	17.6a	72.5	67.5	70.0b
135	14.6b	75.0	77.5	76.3b
150	14.5b	89.2	90.0	89.6a
	Mean	78.9 A	78.3 A	78.6

*Means followed by different letters in the column (lowercase) are significantly different by Tukey´s test ($p<0.05$) and means followed by different letters in the line (Capital letter) are significantly ANOVA. CV (%) = 1 = 11.8%; 2 = 9.3%.

Table 2. Moisture content (%) and Effect storage temperature (cryopreserved and non-cryopreserved) and germination (%) from *Vriesea reitzii* Leme & A.F. Costa seeds collected on different days after anthesis - DAA (120, 135 and 150 days).

	Germination speed index (GSI)			
	Days after anthesis (DAA)			
Treatment	120	135	150	Mean
Cryopreserved (+NL)	13.8	14.3	16.9	15.0a
Non-cryopreserved (-NL)	13.5	14.5	17.0	15,0a
Mean	13.7 B	14.4 B	17.0 A	15.0

*Means followed by different letters in the column (lowercase) are significantly different by ANOVA and means followed by different letter in the line (capital letter) are significantly different by Tukey´s test ($p<0.05$). CV (%) = 15.2%.

Table 3. Average levels of total soluble carbohydrates (mg g^{-1}MF) of *Vriesea reitzii* Leme & A.F. Costa seeds at three different times of fruit collection (120, 135 and 150 days after anthesis - DAA) and subjected to cryopreservation (+ NL) and non-cryopreserved (-NL).

	Days after anthesis (DAA)			
Treatment	120	135	150	Mean
Cryopreserved (+NL)	7.94	8.240	9.45	8.55a
Non-cryopreserved (-NL)	6.74	7.800	9.41	7.98a
Mean	7.34A	8.02A	9.43A	8.27

*Values expressed in mg of glucose equivalent (mg g^{-1}MF) for each 1g of seeds, from a standard curve with R^2 = 0.9989. Means followed by different letters in the column (lowercase) are significantly different by ANOVA and means followed by different letter in the line (capital letter) are significantly different by Tukey´s test ($p<0,05$).

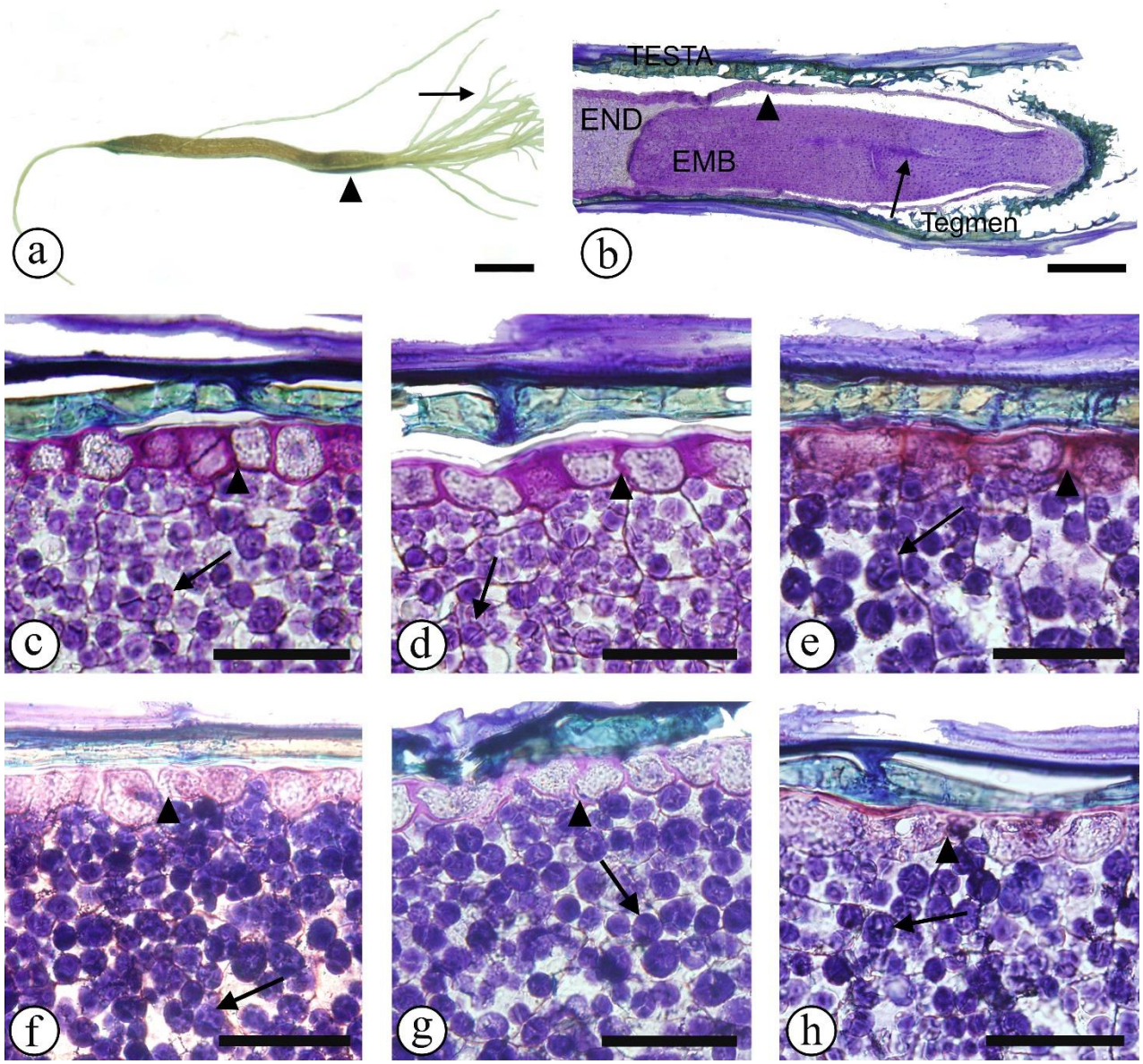

Figure 1. Morphological and histological aspects of cryopreserved *Vriesea reitzii* seeds Leme & A.F. Costa (+LN) and non-cryopreserved (-LN) subjected to double staining of toluidine blue (TBO) and Lugol. a. seed overview, the embryo (arrowhead), plumose appendages (arrow); b. TBO: seed cross section, the cross section of the seed containing the embryo, endosperm structure, aleurone layer (arrowhead) and procambium or nuclei of embryonic stem cells (arrow); c-h. it can observe cell wall of thickened aleurone layer (arrowhead) and compounds starch grains (arrow): c. TBO and Lugol: 120 DAA +LN seed; d. TBO and Lugol: 135 DAA +LN seed; e. TBO and Lugol: 150 DAA +LN seed; f. TBO and Lugol: 120 DAA -LN seeds; g. TBO and Lugol: 135 DAA -LN seeds; h. TBO and Lugol: 150 DAA -LN seeds. Bar: 50 µm. DAA: Days after anthesis. TBO: Toluidine Blue. LN: Liquid nitrogen.

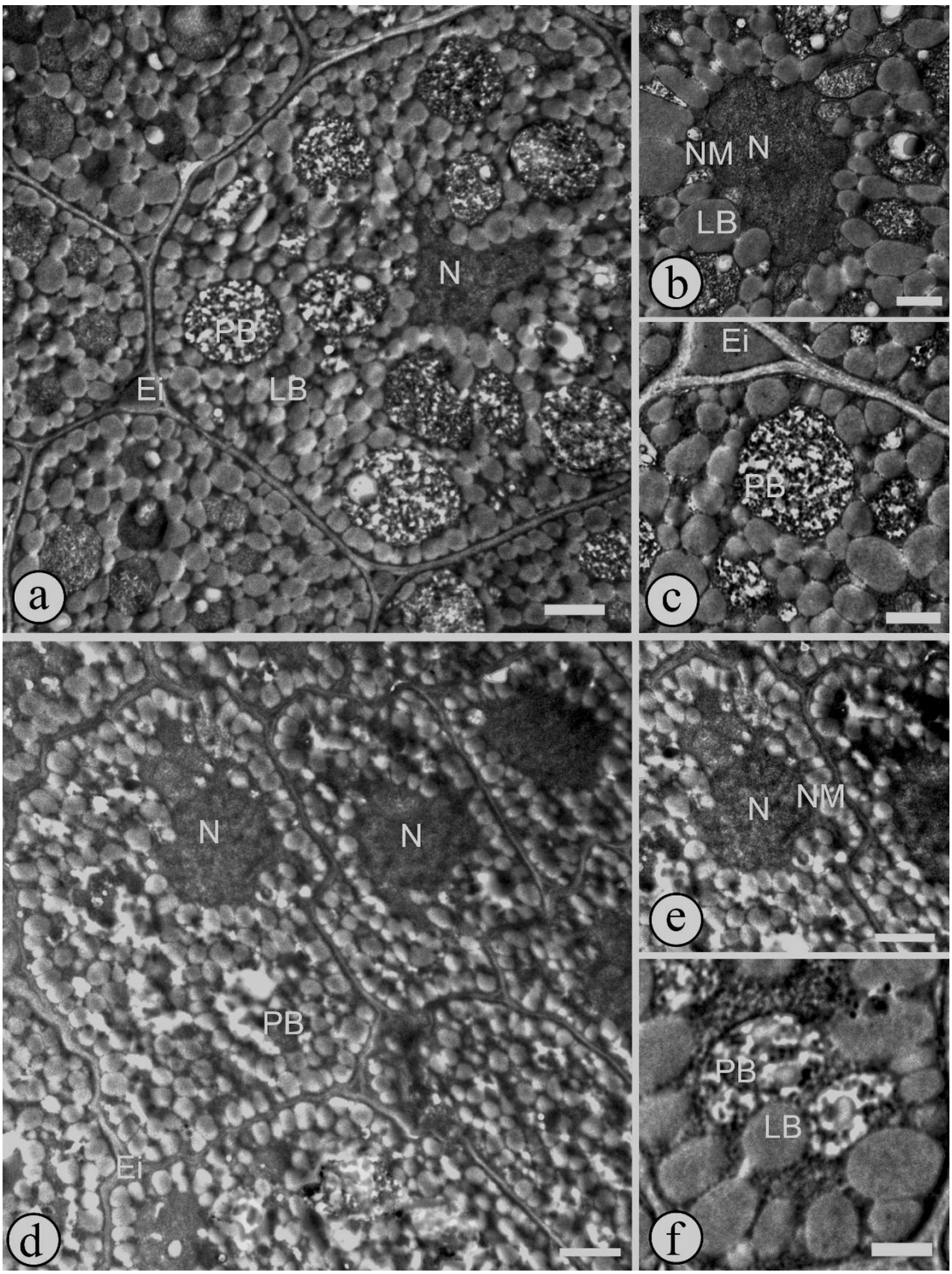

Figure 2. Transmission electron microscopy (TEM) images of *Vriesea reitzii* Leme & A.F. Costa seed. a. embryo non-cryopreserved cell structure. Protein bodies (PB), nucleus, and lipid bodies (LB); b. detail of the cell nucleus (N), nucleus membranes (NM) and lipid bodies (LB); c. detail of protein bodies (PB), intercellular space (IS); d. embryo cryopreserved cell structure, nucleus, protein bodies (PB), lipid bodies (LB), intercellular space (IS); e. detail of the cryopreserved cell nucleus, Nucleus (N), nucleus membranes (NM); f. detail of protein bodies (PB) and lipid bodies (LB). PB: protein bodies; LB: lipid bodies; IS, intercellular space; (NM), nucleus membranes; N, nucleus. Bar a,d = 2 µm; b, c, e = 1 µm.

Article
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