Biotechnologies Tools for Germination and Preservation of *Butia Eriospatha* Embryos (Arecaceae)

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Abstract

Butia eriospatha is an endemic species of Brazilian Atlantic Forest and due to the anthropic intervention, it is in the vulnerable conservation status. In this species, plants are only regenerate by seeds which have dormancy and slow as well low germination. To cope with the concern limitation of seedling establishment we study *B. eriospatha* embryo desiccation tolerance threshold as well the physiological requirement for in vitro germination and cryopreservation. Fresh embryos and desiccated ones were in vitro germinated using a culture medium with hormones and antioxidants. The embryo desiccation tolerance threshold was $0.14 \text{ gH}_2\text{O.gDW}^{-1}$ with 93.33% of germination. During embryos desiccation was observed a significant increase in PUT, which resulted in the ratio decrease $[(\text{SPD}+\text{SPM}). \text{PUT}^{-1}] \text{ DW}$, but the SPD was the most abundant polyamine overall. Significant increase in POD and APX activity led us to suggest that they are the main enzymes involved in cellular protection during desiccation. An increase of amino acids content, especially glutamic acid (Glu), leucine (Leu), lysine (Lys), glutamine (Gln), which are known as osmoprotectors, also was observed. A specific embryo desiccation stage ($0.14 \text{ gH}_2\text{O gDW}^{-1}$) associated to its biochemical state were successfully used in the cryopreservation protocol and result in more than 90% of recovery and in vitro germination. The physiological and biochemistry approach of this study associated to an applied protocol for plant genetic resources conservation of *B. eriospatha* embryo can be potentially used for other Arecaceae species.

Introduction

Arecaceae species are worldwide popular for their organoleptic and nutritional fruits as well for the ornamental plant characteristics (Hoffmann et al. 2014). However, is not popular known that around 26% of Arecaceae species are on the Red List - International Union for Conservation of Nature (IUCN) (IUCN, 2021). Unfortunately, some of them, such as *Corypha taliera* Roxb. and *Cryosophila williamsii* P.H. Allen are already "extinct in the wild" (EW) (Johnson 1988; IUCN 2021). *Butia eriospatha* (Martius Ex Drude) Beccari is a native Arecaceae of South Brazilian Atlantic Forest and is also on the IUCN list as "vulnerable" (VU) and on the Brazilian Endangered Species list (Instrução Normativa 06, MMA 2008, IUCN 2021). The worry conservation status of *B. eriospatha* is due the severe anthropic intervention, including the local and international illegal trade, overexploitation of fruits and habitat replacement by exotic tree and cattle farming (Nazareno and Dos Reis 2012, 2014b). As a consequence the small remnants of the *B. eriospatha* plant population have low levels of genetic diversity (Nazareno and Dos Reis 2012). On top on that plants are only regenerate by seeds and, because of the dormancy they can take up to a year to germinate only around 50% and this is a concerning limitation about seedling establishment and species conservation (Broschat 1998; Fior et al. 2011; Lopes et al. 2011; Magalhães et al. 2013; Schlindwein et al. 2013). Many article reported the worrying genetic situation of the *B. eriospatha* species (Nazareno et al. 2011; Nazareno and Dos Reis 2012, 2014b, a) and in vitro culture protocols have been studied to preserve its genetic diversity (Minardi et al. 2011; Waldow et al. 2013). Nonetheless, the knowledge regarding its reproductive biology is still limited, especially related to seed germination and desiccation tolerance physiology, which is essential for species conservation and restoration.

Whether or not a seed can survive drying has a direct influence on in situ and ex situ plant conservation methods (Walters and Pence 2020). Around 50% of the trees of tropical forest, including Arecaceae species, produce recalcitrant seeds that are not desiccation tolerant (Wyse and Dickie 2017; Royal Botanic Gardens Kew 2021). This seeds are shed with high water content and, if they do not find the right condition to germinate they die, since soil bank seeds are not observed for most of recalcitrant seeds species (Obroucheva et al. 2016; Gonçalves et al. 2020). In tropical ecosystems, ex situ conservation programs have been more and more important due the climate change and forest reduction. They have been largely use biotechnologies tools to preserve tissues, seeds and embryos from many plant species that cannot be storage in conventional seed bank, are from rare collection or have natural issues to germinate (Engelmann 2004; Walters and Pence 2020). Zygotic embryos are a source of genetic diversity and are a suitable target for cryopreservation protocols since they are young tissues (Normah and Makeen 2008; Wen and Wang 2010; Engelmann 2011). However, embryos from recalcitrant seed do not tolerate desiccation and the primary goal of any cryopreservation procedure is to reduce water content to achieve intracellular vitrification preventing the intracellular lethal ice crystals formation (Berjak and Pammenter 2013;

Walters et al. 2013). Plant cell tissue undergo to desiccation have to cope with cellular stress and the protective mechanisms are determinant to maintain cell viability (Walters 2015). Usually, it was observed chromatin compaction, late embryogenesis abundant proteins (LEAs) and heat shock proteins (HSPs) synthesis, reactive species oxygen (ROS) equilibration, lipids, polyamines (PAs), specific amino acid (AA) who act in cell membrane stabilization and deposition of non-reducing sugars as fillers that prevent mechanical cell collapse (Kleinwachter et al. 2014; Li et al. 2015; Leprince et al. 2017; Dussert et al. 2018). To cope with the lack of these mechanisms, over the last 30 years the use of plant vitrification solution (PVS₂ and PVS₃) has been applied in protocols of cryopreservation (Sakai et al. 1990; Yamada et al. 1991; Nishizawa et al. 1993). Also, new techniques based on high cooling rate (Walters and Koster 2008; Orjuela-Palacio et al. 2019) as well as droplet-vitrification (Kantha et al. 1982; Berjak and Pammenter 2014) have been developed to preserve challenging plant cell tissue. These techniques have allowed that during the cryostorage, biochemical metabolic and cell division activities are arrested, allowing the long-term storage (Benelli et al. 2013; Kulus and Zalewska 2014).

Particularly during the last 40 years many efforts have been made to establish cryopreservation protocols for Arecaceae species tissues, for instance *Elaeis guineensis* Jacq. (Grout et al. 1983), *Phoenix dactylifera* L. (Bagnio and Engelmann 1991), *Cocos nucifera* L. (Assy-Bah and Engelmann 1992), *Sabal* spp (Wen and Wang 2010), including *Butia capitata* (Dias et al. 2015), *B. odorata* (Fior et al. 2020) and *B. yatay* (Vargas et al. 2020). However, different species require different cryopreservation protocols and not all the previous Arecaceae studies have the goal to cryopreserve zygotic embryos. We are facing an alarming conservation status of *B. eriospatha* and the scientific community is sick to develop ex situ conservation strategies as well found out more about seed physiological behavior that can help to do restoration and preserve the remain plant population of this species. Our hypothesis is that there is specific biochemical status and desiccation tolerance threshold of *B. eriospatha* embryos which make them responsive to cryopreservation protocol and in vitro germination. This study analyzes and discusses the role and interaction of PAs, amino acids and antioxidant enzymatic defense in the maintenance of *B. eriospatha* embryo viability during dry. In vitro germination and cryopreservation protocols were for the first time established given an efficient tool for long-term ex situ conservation strategy and high germination seeds.

Material And Method

Plant material

Mature fruits were collected from 20 *B. eriospatha* wild plants in the Serrano Plateau of Santa Catarina State (S 27° 12', W 50° 36'), from January to March 2021. Fruits without insects or microorganisms attack were manually processed to get out seed. The seeds enveloped by the woody endocarp (pyrenes) were kept at 25°C for 3 days and then storage at 8°C for no more than 15 days before experiments start.

Seed and embryo morphology and morphometry

Seed and embryos measurements (diameter, length, and width) were performed in 100 seeds using caliper. They were pictured using stereo microscope (Olympus - SZH10) equipped with image capture system (Olympus - DP71) and DP Controller software.

Water relations

Water content (WC) and water potential (Ψ_w)

Water content (WC) was determined using the oven-drying method (ISTA 2004), measured gravimetrically by difference in weight before and after drying a $103 \pm 1^\circ\text{C}$ for 24 h. Water content value is expressed on a dry mass basis (gH₂O. gDW⁻¹

¹). Water potentials (Ψ_w) was determined by immersing embryos in solutions of polyethylene glycol 6000 (PEG 6000) à 25°C (Michel and Kaufmann 1973). Polyethylene glycol 6000 concentrations provided $\Psi_w = 0, -0.5, -1, -2, -3$ and -4 MPa. The fresh masses of the embryos were determined in their initial condition and after 24h of immersion in the osmotic solutions and their WC were gravimetrically measured.

Embryos drying

Butia eriospatha embryos were partial desiccated according to Engelmann (2004) with modification. Embryos were excised from the hard endocarp seed with forceps and scalpel and directly seat in a Petri dish with filter paper soaked in ascorbic acid (0.2 g l^{-1}) and citric acid (0.2 g l^{-1}) solution to prevent tissue oxidation. For desiccation, embryos were transferred to a dry filter paper in a laminar airflow cabinet at $25 \pm 2^\circ\text{C}$, $55 \pm 5\%$ relative humidity [RH] and an airflow of 0.46 m s^{-1} . Embryos were desiccate for 0 (control); 15; 30; 60; 120; 180; 240 and 300 min and then the desiccation curve was calculated (Hong and Ellis, 1996). Viability and vigor analyses were performed in embryos samples from all desiccation times, while biochemical and cryopreservation analyses were performed in embryos desiccated for 0; 15; 240 and 300 min. Embryos drying rate index (k) was calculated from the amount of water loss in dry basis ($\text{gH}_2\text{O g DW}^{-1}$) divided by correspondent elapsed time in minutes.

Viability and vigor

In vitro germination tests and germination speed index (GSI)

Seeds were immersed in ethyl alcohol ($70\% \text{ v v}^{-1}$) for 1 min followed by 15 min in sodium hypochlorite ($2.5\% \text{ v/v}^{-1}$) and then rinsed three times with sterilized distilled water. Embryos were excised according to previous description topic “*Embryos drying*”, and were immersed in a commercial sodium hypochlorite solution ($0.5\% \text{ v/v}^{-1}$) for 10 min and then rinsed three times in sterile distillate water (Magalhães et al. 2013). Immediately after that, embryos were inoculated in Petri dish containing 25 ml of MS ($75\% \text{ v/v}^{-1}$) culture medium (Murashige and Skoog 1962) supplemented with 30 g l^{-1} sucrose, 0.5 mg l^{-1} thiamine, 1 mg l^{-1} pyridoxine, 0.5 mg l^{-1} nicotinic acid, 3 g l^{-1} activated charcoal, $8 \mu\text{M GA}_3$ and 6 g l^{-1} agar (Ribeiro et al. 2011). The pH was adjusted to 5.8 and autoclaved at 121°C , 1.5 atm for 15 min. The germination was performed in growth room ($25 \pm 2^\circ\text{C}$; 55% RH) with white LED light (Green Power TLED W; Philips TM; $77 \mu\text{mol m}^{-2} \text{ s}^{-1}$) and 16/8 h photoperiod. Germinated embryos were recorded every day considering radicle protrusion. Germination percentage was calculated by the cumulative number of daily germinated seeds with respect of the total number of seeds evaluated (Ranal and De Santana 2006). The germination speed index (GSI) was calculated according to the Maguire’s index (Mangure 1962) as the total number of seeds germinated per day between sowing and germination divided by the number of days of the test.

Tetrazolium tests (TTC)

Embryos were soaked in distilled water for 12 h and after they were immersed in 2,3,5 triphenyl tetrazolium chloride (2,3,5 TTC) ($0.5\% \text{ w v}^{-1}$) at 35°C for 4 h in dark, and viability was assessed by staining intensity and location (Ribeiro; et al. 2010). Results were expressed in percentage of seed with positive reaction to TTC.

Seedling morphometry

Seedling measurements (primary root, cotyledonary petiole and leaf sheath) were performed after eight weeks. Seedlings were pictured in stereo microscope (Olympus - SZH10) equipped with image capture system (Olympus - DP71) and DP Controller software.

Cryopreservation

For the high cooling rate technique embryos were wrapped in aluminum foil envelopes (20 μm) and plunged directly into liquid nitrogen (LN) at -196°C for 24 h (Walters and Koster 2008; Orjuela-Palacio et al. 2019). For the droplet-vitrification technique embryos were conditioned in PVS_2 (Sakai et al. 1990) and PVS_3 (Nishizawa et al. 1993) solutions on an orbital shaker at 90 rpm at 4°C for 60 min (Karthi et al. 1982; Berjak and Pammenter 2014b) and then droplets (10 - 15 μL) were placed to aluminum foil strips (0.5 cm x 3.0 cm) and transferred to a sterile polypropylene cryovials (2 ml) before to be immersed in LN. After 24 h, aluminum foil strips and embryos removed from aluminum foil envelopes were thawed in a sucrose solution (1.2 M) at 45°C for 5 min (Walters et al. 2008) and then embryos were transferred to Petri dishes (90 mm diameter) containing the germination culture medium and kept under incubation condition described in topic "*In vitro germination tests and germination speed index (GSI)*" and seedlings morphometry were realized following the described in topic "*Seedlings morphometry*".

Antioxidant enzyme extraction and assays

For antioxidant enzyme assays, three embryos samples (300 mg FW, $\cong$ 300 embryos) of each treatment were homogenized on ice with 1 ml of potassium phosphate buffer (50 mM, pH 7.0) containing 1 mM ethylenediaminetetra acetic acid (EDTA) and 1% (p v^{-1}) polyvinylpyrrolidone (PVP) using an Ultra-Turrax Homogenizer, according to Bailly and Kranner (2011) with modifications. The homogenate was centrifuged at $15.000 \times g$ for 20 min at 4°C , and the supernatant was collected and placed on ice until analysis. Superoxide dismutase (SOD EC 1.15.1.1) activity was determined in a reaction mixture composed of 10 μL of enzyme extract and 290 μL of potassium phosphate buffer (50 mM, pH 7.8) containing 13 mM methionine, 75 μM nitroblue tetrazolium, 2 mM of riboflavin and 100 nM EDTA. Then, reaction mixtures were placed in a dark box and illuminated for 15 min. Duplicate samples kept in the dark for the same time were used as blanks. Absorbance was determined at 560 nm (Giannopolitis and Ries 1977). Ascorbate peroxidase (APX EC 1.11.1.11) activity was determined in a reaction mixture composed of 10 μL of enzyme extract and 290 μL of potassium phosphate buffer (50 mM, pH 7.0) containing 4.75 mM H_2O_2 , 5 mM EDTA and 50 mM ascorbic acid. The absorbance decreases at 290 nm (molar extinction coefficient $2.8 \text{ mM}^{-1} \text{ cm}^{-1}$) was recorded for 10 min at 25°C (Koshiba 1993). APX activity was expressed in $\mu\text{mol min}^{-1} \text{ mg}^{-1}$ protein. Guaiacol peroxidase (POD EC 1.11.1.7) activity was determined in a reaction mixture composed of 7 μL of enzyme extract and 293 μL of sodium phosphate buffer (10 mM, pH 6.0) containing 12.6 mM hydrogen peroxide, and 0.31 M guaiacol. The absorbance increases at 470 nm (molar extinction coefficient $26.6 \text{ M}^{-1} \text{ cm}^{-1}$) was recorded for 3 min at 32°C (Freitas and Stadnik 2015). Catalase (CAT EC 1.11.1.6) activity was determined in a reaction mixture composed of 10 μL of enzyme extract and 290 μL of potassium phosphate buffer (50 mM, pH 7.0) containing 13 mM H_2O_2 . The absorbance decreases at 240 nm (molar extinction coefficient $39.4 \text{ mM}^{-1} \text{ cm}^{-1}$) was recorded for 3 min at 25°C (Peixoto et al. 1999). Catalase activity was expressed as $\mu\text{mol. min}^{-1} \text{ mg}^{-1}$ protein. Protein content was determined according to Bradford (1976), using bovine serum albumin (BSA) as standard. Enzymatic activities were expressed in Katal, where 1kat represents the amount of enzyme converting 1M of substrate s^{-1} . Activity of enzymes and protein content were performed using a spectrophotometer Spectra-Max® 190 Microplate Reader

Free polyamines (PAs) determination

For polyamines (PAs) determination, three samples (200 mg FW, $\cong$ 200 embryos) of embryos from each treatment were ground in 1.6 ml of 5 % (v v^{-1}) perchloric acid. Free PAs were extracted, dansylated and quantified, according to Steiner et al. (2007) with modifications. Free and conjugated PAs were derivatized with dansyl chloride and quantified by HPLC using a 5- μm C_{18} reverse-phase column (Shimadzu Shin-pack CLC ODS). The gradient of absolute acetonitrile was programmed to 65% over the first 10 min, from 65 to 100% for 10 to 13 min, and 100 % for 13 to 21 min, using 1 ml min^{-1} flow rate at 40°C . PAs concentration was determined using a fluorescence detector with a wavelength of 340 nm (excitation) and 510 nm

(emission). Peak areas and retention times were measured by comparison with standard PAs: putrescine (PUT), spermidine (SPD) and spermine (SPM). The 1,7-diaminoheptane (DAH) was used as internal standard.

Free amino acids (AA) determination

For free amino acids (AA) determination three embryos samples (100 mg FW, $\approx$ 100 embryos) of each treatment were ground in 1.5 ml of 80% ($v v^{-1}$) ethanol and concentrated in 'speed vac'. Samples were resuspended in 0.5 ml Milli-Q water type and centrifuged at $20.000 \times g$ for 10 min. The supernatant was filtered through a 20- μm membrane. Amino acids were derivatized with o-phthalaldehyde (OPA) and identified by high-performance liquid chromatography (HPLC), according to Astarita et al. (2003). The samples using a 5- μm C₁₈ reversephase column (Shimadzu Shin-pack CLC ODS). The gradient was developed by mixing increasing proportions of 65% methanol to a buffer solution (50 mM sodium acetate, 50 mM sodium phosphate, 20 ml l⁻¹ methanol, 20 ml l⁻¹ tetrahydrofuran and pH 8.1 adjusted with acetic acid). The gradient of 65% methanol was programmed to 20% over the first 32 min, from 20% to 100% for between 32 min and 71 min, and 100% for between 71 min and 80 min, at 1 ml min⁻¹ flow at 40°C. Fluorescence excitation and emission wavelengths of 250 nm and 480 nm, respectively, were used for amino acid detection. Peak areas and retention times were measured by comparison with known quantities of standard amino acids

Statistical Analyses

Data on seed morphometry was measured in four replicates of 25 seeds each. For water relations analyses were used five replicates of 10 embryos/treatment. For viability and vigor analyses and cryopreservation experiments were used four replicates of 25 embryos/treatment. The data normality was evaluated using the Shapiro – Wilk test, and then submitted to analysis of variance in a completely randomized design. In the case of significance of the F values, means were compared using SNK test ($p < 0.05$) (Sokal and Rohlf 1995). For statistical processing, the data rate was log transformed. Statistical analysis was carried out with R 3.4.4 programming environment.

Result

Morphology characterization of seeds and embryos

B. eriospatha seeds have elliptic irregular shapes; a brown seed coat (sc) and an operculum (os) forming a small protuberance at its proximal extremity (Fig. 1a). Seed diameter (d) were 10.29 ± 0.84 mm and length (h) were 8.56 ± 0.92 mm (Fig. 1a). The white opaque endosperm (en) was covered by thin seed coat (sc) and have a cavity containing small embryo (Fig. 2b), located immediately under to the operculum (Fig. 2b). In the embryos proximal portion (pp) a cotyledonary petiole (cp) envelops a rudimentary embryonic axis while in the distal portion (dp) it is the haustorium (ha) (Fig. 2c). Embryos were of 1.1 ± 0.04 mm in length and 0.5 ± 0.008 mm in width.

Water relations

B. eriospatha mature seeds (Fig. 1a) and fresh embryos (Fig. 1c) showed a water content (WC) of $0.24 \text{ gH}_2\text{O gDW}^{-1}$ (19.87%) and $0.94 \text{ gH}_2\text{O gDW}^{-1}$ (48.52%), respectively. Fresh embryos excised and immersed in antioxidant solution (control) had its WC increase to $2.56 \text{ gH}_2\text{O gDW}^{-1}$ as a consequence of the initial imbibition (Fig. 2a). In the first 15 min of drying, we observed a significant decrease in the WC, probably due to the exit of the antioxidant solution (Fig. 2a). Embryos after 15 min of drying ($0.91 \text{ gH}_2\text{O gDW}^{-1}$) showed similar WC to the fresh embryos ($0.94 \text{ gH}_2\text{O gDW}^{-1}$) (Fig. 2a). After 60 min, embryos WC were at $0.25 \text{ gH}_2\text{O gDW}^{-1}$ and in 180 min embryos almost reached a basal stable WC ($0.17 \text{ gH}_2\text{O gDW}^{-1}$). At

300 min of drying, embryos loose around 94.53% of initial WC ($0.94 \text{ gH}_2\text{O gDW}^{-1}$) reaching $0.14 \text{ gH}_2\text{O gDW}^{-1}$. The drying rate (K) of *B. eriospatha* embryo was subdivided into three phases. In the initial 15 min the drying rate was higher ($0.110 \text{ g H}_2\text{O}^{-1} \text{ min}^{-1}$), from 15 min to 120 min decreased ($0.006 \text{ g H}_2\text{O}^{-1} \text{ min}^{-1}$) and almost stabilized from 120 min to 300 min ($0.004 \text{ g H}_2\text{O}^{-1} \text{ min}^{-1}$).

The water potential (Ψ_w) of fresh embryos was related to the ability of embryo to absorb or retain water (Fig. 2b). Fresh embryos ($0.94 \text{ gH}_2\text{O gDW}^{-1}$) that were immersing in pure distilled water ($\Psi_w = 0.00 \text{ MPa}$), for 24 hours, showed increase of approximately three-fold in their WC ($2.70 \text{ gH}_2\text{O gDW}^{-1}$). Increasing of embryos WC were also noted in the $\Psi_w = -0.5 \text{ MPa}$ solution ($1.71 \text{ gH}_2\text{O gDW}^{-1}$) and the reduction was observed at $\Psi_w = -1.0 \text{ MPa}$ ($0.51 \text{ gH}_2\text{O gDW}^{-1}$) and at $\Psi_w = -4.0 \text{ MPa}$ reached $0.03 \text{ gH}_2\text{O gDW}^{-1}$ (Fig. 2b).

Viability and vigor

Desiccation time did not have a significant effect on *B. eriospatha* embryos germination since after they were dried for up to 300 min it was observed a germination of 93.33% while the control germinates 100% (Fig. 3a). Germination results validated all the data observed in tetrazolium test indicating an adequate protocol for embryo viability in this species (Fig. 3a). However, significant effects were observed in the embryos germination dynamic. In control and embryos that went through drying for up to 60 min the protrusion of the radicle started at 7th day after inoculation and took 10 days to reach 100% of germination (Fig. 3b). Embryos dried for 120 and 180 min extended germination up to 12 days, and showed a small decrease in the germination percentage (96.67%). Embryos dried for 240 and 300 min showed a decrease in germination speed, obtaining 93.33% of germinated embryos only at 18 days after inoculation (Fig. 3b). Germination dynamic is directly associated to the germination speed index (GSI) which show the lowest value in embryos dried for 240 and 300 min (GSI = 0.43 and GSI = 0.42, respectively) compared to the control and embryos dried up to 180 min (GSI = 0.71 and GSI = 0.59, respectively) (Fig. 3c). *Butia eriospatha* seedlings morphometric analyses indicated an expressive length of the cotyledonary petiole (cp) (Average: $23.25 \pm 0.54 \text{ mm}$) but did not present significant difference between drying times (Fig. 3d). However, when embryos were dried for 300 min, we observed a decreased in both the lengths of primary root (rp) and leaf sheath (ls) (Fig. 3d) when compared to the control and embryos dried for 15 min.

Cryopreservation

Embryos soaked in antioxidant solution and dried for 15 min, regardless of having been incubated in PVS₂ and PVS₃ solution, did not germinate when were submitted to a high cooling rate and droplet vitrification technique (Table. 1). These embryos that did not germinate remained unchanged in size for more than eight weeks, without elongation or callus formation. Embryos dried for 180 and 300 min, followed by incubation in PVS₂ and PVS₃ solution, had an increase in their water content (Table. 1). Despite of the increase in the water content, embryos dried for 180 and 300 min, instead of the PVS₂ and PVS₃ incubation, submitted to a high cooling rate and droplet vitrification technique presented germination rates above $83.33 \pm 7.95\%$ and showed no significant difference (Table. 1). Germination speed index (GSI) values for embryos dried for 180 and 300 min with or without incubation in PVS₂ and PVS₃ solution did not show significantly difference (Table. 1).

Eight weeks after cryopreservation the presence of normal seedling development were seen in the embryos dried for 180 and 300 min and submitted to high cooling rate and droplet vitrification technique (PVS₂ and PVS₃ solution) (Table. 1; Fig. 4a). Embryos dried (180 and 300 min) incubated in PVS₂ solution and submitted to droplet vitrification technique showed high number of abnormal seedlings (29.17 ± 0.88 and 37.03 ± 1.05), respectively, following by the incubated in PVS₃ solution (14.81 ± 0.80 and 16.66 ± 0.81). These abnormal features include the absence of primary root (arrow) (Fig. 4b) and leaf

sheath (Fig. 4c), as well as the swelling of the cotyledonary petiole (cp). In this treatment it was also observed callus proliferation in the basal region of embryo (Fig. 4d).

Antioxidant system

Control and *B. eriospatha* embryos dried for 15 min showed a higher superoxide dismutase (SOD) activity (73.89 and 96.23 $\text{Umin}^{-1} \text{mg}^{-1}$ protein, respectively), compared to embryos that were dried for 180 and 300 min that had a reduction at SOD activity (68.05 and 96.23 $\text{Umin}^{-1} \text{mg}^{-1}$ protein, respectively) (Fig. 5a; Table. S1). In contrast to SOD enzyme behavior, ascorbate peroxidase (APX) and guaiacol peroxidase (POD) activity presented a decrease in the first 15 min of drying (0.69 and 0.031 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ protein, respectively), and exhibited an increase after 180 min (0.88 and 0.77 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ protein, respectively) (Fig. 5b – c; Table. S1). Guaiacol peroxidase activities values in embryos desiccated for 180 min were 30 times higher than observed in soaked embryos and embryos dried for 15 min (increment of 0.03 to 0.90 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ protein) (Fig 5c; Table. S1). Catalase activity increased during the first 180 min of drying, reaching the point of maximum activity (0.88 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ protein) and decreased drastically after 300 min of drying (0.19 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ protein) (Fig. 5d; Table. S1). These data suggest that in *B. eriospatha* embryos, POD together with APX are the main protective enzymes against the damage caused by reactive oxygen species (ROS) during desiccation.

Polyamines (PAs) content

In *B. eriospatha* embryos the total free PAs content decreased when the embryo were desiccated and presented the lowest contents of putrescine (PUT), spermidine (SPD) and spermine (SPM) (Fig. 6a – d). Control had higher contents of SPD and SPM compared to embryos desiccated for 300 min (Fig. 6c-d) and the opposite behavior was observed for PUT which show the highest value at the same desiccation time (Fig. 6b). Spermidine (SPD) was the most abundant polyamine, followed by SPM and both reached their maximum contents when desiccated for 180 min (329.89 and 157.26 $\text{mMol mg}^{-1} \text{DW}$, respectively) (Fig. 5 c-d; Table. S1). Due to the slight increase in free PUT during drying time, the PAs ratio (SPD + SPM). PUT⁻¹] DW indicated a decrease with the drying time increase, concomitant with a reduction in viability, seen by the lower GSI value of *B. eriospatha* embryos (Fig. 6e).

Free amino acid content

An interesting relation were observed in embryos dried for 300 min with a significant increase in total free amino acids content compared to the ones dried for 0; 15 and 180 min (Fig. 7a). Lysine (Lys) represents 42% of the total free amino acids profile observed during the whole drying time (Fig. 7b; Table. S3). Gamma-aminobutyric acid (Gaba), citrulline (Cit) and arginine (Arg), were amino acid which did not change significantly during drying times while lysine (Lys), leucine (Leu), isoleucine (Ile) glutamine (Gln) and glutamic acid (Glu) practically doubled their contents when the embryo was desiccated for 300 min (Fig. 7b; Tab. S1). These amino acids were approximately 97.85% of the total amino acids detected in embryos that were desiccated for 300 min. These results could suggest that in *B. eriospatha* embryos theses amino acid can be related to desiccation tolerance mechanisms and play a key role in maintaining viability of embryos, once dried embryos for 300 min presented a germination rate of 93.33% . The ratio of ornithine (Orn) and arginine (Arg) to total amino acids indicate that Orn increase preferentially to Arg with dehydration (Fig. 7c). Undesiccated embryos (control) had higher Arg content, which decreased at 15 min of desiccation and remained constant until 300 min of desiccation. The Orn content was higher in the 15 min of desiccation compared to the control, but did not differ from 180 min and 300 min of desiccation (Fig. 7c).

Discussion

Butia eriospatha fresh seeds had a WC of 0.24 gH₂O gDW⁻¹ (19.87%) and this result was similar to the observed in *Butia odorata* (Barb. Rodr.) Noblick (17.48%) (Schlindwein et al. 2019) and *Butia capitata* (Mart.) Becc. (20.93%) (Neves et al. 2010). Fresh excised embryos presented WC of 0.94 gH₂O gDW⁻¹ (48.52%) and a 100% of in vitro germination and this indicate the presence of a mature embryo with high physiological quality. Embryos (control) lost 94.53% of their WC when desiccates for 300 min, reaching 0.14 g H₂O g DW⁻¹ (11.98%) (-2.2 MPa). Although, embryos dried for 300 min exhibited high viability (germination and TTC test > 90%), the germination speed index (GSI) decreased substantially from 0.71 (control) to 0.42 (300 min), indicating a reduction of vigor. A study with *B. capitata* showed that whole seeds were able to tolerate moisture loss to 10 and 5%, whereas dehydration to 3.5% moisture content was harmful (Dias et al. 2015). Comparatively to these reports our results indicating that the *B. eriospatha* species produce high physiological quality seeds and the embryo desiccation tolerance threshold is close to the embryos from orthodox seeds (Hong and Ellis 1996; Hamilton et al. 2013; Pelissari et al. 2018). The majority of studies on palm seed still not precise about classification of seed desiccation tolerance giving rise a limited knowledge about life time storage in seed bank (e.g., Royal Botanic Gardens Kew 2015, Dias et al. 2015; Fior et al. 2020). Other aspects that have limited *B. eriospatha* seed conservation is the large size, dormancy, slow, low and uneven germination (Lopes et al. 2011; Fior et al. 2013; Waldow et al. 2013). In these cases, the ability to excise embryonic axis, cryopreserve and germinates them in vitro is a viable technique for ex situ conservation and was indicate for germplasm storage of *B. capitata*, *B. eriospatha*; *B. lallemantii*, *B. odorata*, *B. paraguayensis* e *B. yatay* (Neves et al. 2010; Dias et al. 2013; Taniguchi et al. 2020). Also, comparatively to whole seeds, isolated embryos made it easier in the cryopreservation protocols steps, since the smaller the explant size the greater chance of survive and regenerate (Assy-Bah and Engelmann 1992).

In our study, excised embryos of *B. eriospatha* were small (1.1 mm in length and 0.5 mm in width), but fresh embryos had a high WC (0.94 gH₂O gDW⁻¹). So, after 60 min of desiccation, the embryos still viable and the WC (<0.25 gH₂O gDW⁻¹) was reduced which increase the probability to not have lethal ice formation in cells when they are cooled at rates faster than ~10°C min⁻¹ (Wesley-Smith et al. 2001, 2004, 2014; Wolfe et al. 2002; Walters and Koster 2008). However, some Arecaceae species that are tolerant to dehydration are sensitive to low temperatures (Dickie et al. 1992; Orozco-Segovia et al. 2003; Dias et al. 2015). These led us to test cryopreservation techniques such as rapid freezing and droplet vitrification with incubation in PVS₂ and PVS₃, but even embryos drying for 15 min (0.91 gH₂O gDW⁻¹) or for 60 min (0.25 gH₂O gDW⁻¹) were not able to germinate after cryopreservation. Our results suggest that fresh embryos soaked in antioxidant solution (control) and the embryos dried for 15 min had a high WC (2.56 gH₂O gDW⁻¹; 0.91 gH₂O gDW⁻¹), respectively, and probably the osmotic stresses during cooling led to ice crystal formation and cell death (Fahy and Wowk 2015). However, the influx of PVS₂ and PVS₃ solution in embryos (control and dried for 15 min) resulted in the sharp decline in WC, but theses embryos did not germinate, probably due to the toxic effect of the vitrification solutions. The adverse effects of cryoprotective solutions are as relevant as their protective function, while the PVS₃ is composed of sucrose and glycerol, non-permeable agents the PVS₂ is characterized by its high chemical toxicity, due to the permeability of DMSO and ethylene glycol (Fahy 1986; Fahy et al. 1990, 2004). Few chemical toxicity events have been linked, but can be toxic due to the high osmotic pressure it exerts on plant cells (Grout 2007; Sakai and Engelmann 2007; Kim et al. 2009; Engelmann 2011; Elliott et al. 2017). It was reported that glycerol and sucrose led to increased vacuolization and autophagy in *Haemanthus montanus* Baker. zygotic embryo (Sershen et al. 2012). In *Syzygium mairae* (A.Cunn.) Sykes & Garn.-Jones zygotic embryos, the PVS₂ had a negative impact on embryo survival and plantlet formation (Van Der Walt et al. 2021). In contrast *Hevea brasiliensis* L. zygotic embryos had a high tolerance to PVS₂ in terms of survival, but are sensitive to osmotic stress induced by PVS₃ (Nakkanong and Nualsri 2018). In our study, embryos of *B. eriospatha* dried for 180 (0.17 gH₂O gDW⁻¹) and 300 min (0.14 gH₂O gDW⁻¹), regardless of whether or not they were incubated in PVS₂ and PVS₃ solution, showed high germination (>80%), after cryopreservation. However, embryos desiccated for 300 min (0.14 gH₂O gDW⁻¹) followed by immersion in LN (-196°C) showed an increase of normal seedlings (92.86%), suggesting that PVS₂ and PVS₃ was overall toxic. Our study

found out for the first time the desiccation tolerance threshold and a complete and successful cryopreservation protocol of *B. eriospatha* embryo allowing more than 90% of germination.

Our results suggest that the ability of *B. eriospatha* embryo to be desiccation tolerant and maintain high viability is associated to the oxidative stress control by the elimination of reactive oxygen species (ROS). The elevated levels of ROS might inhibits repair processes, which are linked, for the most part, to protein synthesis (Umezawa et al. 2006). In embryos not desiccation tolerant, the metabolic balance is disturbed and the generation of ROS is out of control, which means that protective antioxidant reactions are not able to remove ROS quickly enough (Leprince and Buitink 2015). Many studies have reported elevated rates of ROS production during drying of *Castanea sativa* Mill. (Roach et al. 2008, 2010), *Antiaris toxicaria* L. (Cheng and Song 2008), *Araucaria bidwillii* Hook (Francini et al. 2006) and *Acer platanoides* L (Pukacka and Ratajczak 2007) embryos. In *B. capitata* embryos under moderate water stress (-1MPa) presented a high concentration of hydrogen peroxide (H_2O_2) and superoxide radicals (O_2^-) (Gonçalves et al. 2020).

The ROS stress-induced under desiccation are usually neutralized by enzymatic antioxidant systems (Bailly 2004; Sahu et al. 2017). Superoxide dismutase (SOD) is in the first line of defense against ROS, which catalyzes O_2^- producing H_2O_2 and oxygen O_2 (Gill and Tuteja 2010) and has been reported in seed during the acquisition of desiccation tolerance (Huang and Song 2013; Feng et al. 2017; Zhang et al. 2019). In our study, *B. eriospatha* embryos had an increase of SOD activity in the first 15 min of desiccation ($0.91\text{ gH}_2\text{O gDW}^{-1}$), which could indicate that SOD was responding to the initial intracellular production of ROS. The increasing in the SOD activity also was reported in *B. capitata* embryos submitted to water stress (-1 and -2 MPa) (Gonçalves et al. 2020). However, as the desiccation levels increases, a decrease was observed in SOD activity, as seen in *B. eriospatha* embryos dried for 180 min ($0.17\text{ gH}_2\text{O gDW}^{-1}$) and 300 min ($0.14\text{ gH}_2\text{O gDW}^{-1}$). In fact, this behavior have already being observed in orthodox seed tissues and seem to be related with the reduction in the respiration rates that leads to reduction in ROS production (Leprince et al. 2000). Additionally, according to Vertucci and Farrant (1995) mitochondrial respiration decrease when WC is lower than $0.25\text{ gH}_2\text{O gDW}^{-1}$, what could justify the reduction in SOD activity observed in our study, since after 180 min drying the WC was lower than $0.17\text{ gH}_2\text{O gDW}^{-1}$. On the other hand, *B. eriospatha* embryos dried for 180 min showed an increase in the activity of the enzymes ascorbate peroxidase (APX), guaiacol peroxidase (POD) and catalase (CAT). It has been reported that the main product of SOD activity is H_2O_2 (Bailly 2004; Gill and Tuteja 2010). So, in our study the peak of SOD activity after 15 min of desiccation may also have generated H_2O_2 , which could be effectively converted into H_2O by the high APX, POD and CAT activity observed after 180 min of desiccation ($0.17\text{ gH}_2\text{O gDW}^{-1}$). This also make sense once we find out a report showing that in not desiccation tolerant *Antiaris toxicaria* L. embryos, the H_2O_2 could not be converted into H_2O effectively because of the less activities CAT and APX (Cheng and Song 2008). In the present work, CAT activity increases up to 180 min and its activity was dramatically reduced after 300 min of desiccation, achieving less activity than was observed in embryos without desiccation. In contrast, POD and APX showed the highest activities at 300 min of desiccation, which suggests that they play an important role in the desiccation tolerance with dehydration.

Water stress tolerance in plant tissues and seeds were also increased by biosynthesis and accumulation of PAs as was observed in *Arabidopsis thaliana* L., *Craterostigma plantagineum* Hochst. (Alcázar et al. 2011), and *Campomanesia xanthocarpa* (Mart.) O. Berg (Vieira et al. 2021). In *B. eriospatha* embryos the SPD and SPM content decreased when the embryos were desiccated while PUT content significant increased. In drought-sensitive wild chickpea species and in rice plants under water stress produced an increase in the three main PAs (PUT, SPD and SPM), which was related by the authors to protection against water stress (Capell et al. 2004; Nayyar et al. 2005; Do et al. 2013). Polyamines can interconvert from PUT to SPM and SPM to SPD and back (Pál et al. 2015). In our study, the increase in PUT and a decrease in SPD content, can be related to the absense of PUT conversion into other PAs. Increase in free PUT resulted in a decrease in the ratio $[(SPD + SPM). PUT^{-1}]$ as also the lowest GSI value. Pál et al. (2015) reported that the greater accumulation of PUT, leading to a low ratio $[(SPM + SPD). PUT^{-1}]$ may even injure plants. In aquatic species as *Nymphoides peltatum* L. (Wang et al.

2007) and *Potamogeton crispus* L. (Yang et al. 2010) sensitive to drought, also had their PUT content increased, as well as a decrease of (SPM+SPD). PUT⁻¹] ratio. Some authors argued that increases in the content of PUT is generally accompanied by the generation of ROS (Groppa and Benavides 2008; Paul et al. 2018) and that plants under moderate stress can overcome the ROS overflow through the activation of antioxidant enzymes system, which are induced by the accumulation and catabolism of SPM and SPD or by stress itself (Moschou et al. 2008; Yu et al. 2019). Exogenous SPD was able to stimulate the antioxidant system (SOD, CAT, APX and POD enzymes) that moderates the oxidative stress in *White clover* seed germination under drought stress (Li et al. 2014). A correlation between endogenous SPD and the activity of CAT and APX, indicating its complementary role in the antioxidant defense system also was found by in *C. xhantocarpa* seeds under desiccation (Vieira et al. 2021). In our study, despite of *B. eriospatha* embryos under desiccation showed a significant increase in PUT content, it is noteworthy that SPD was the most abundant polyamine, followed by SPM, parallel with an increase in the APX and POD activities. Our results showed that the interaction of PAs and antioxidant systems are involved in the *B. eriospatha* embryos desiccation tolerance. Corroborating our results, other authors describe the role of PAs in scavenging free radicals by increasing the activity of antioxidant enzymes, decreasing lipid peroxidation (Tang and Newton 2005; Vieira et al. 2021). Furthermore, studies have shown that PAs act by binding directly to membrane phospholipids; and acting on the osmotic adjustment, maintaining a cation-anion balance; maintaining cell viability and homeostasis (Kasukabe et al. 2004; Fariduddin et al. 2013; Pál et al. 2015; Saha et al. 2015). However, the exact points of interaction between PAs and ROS are far from being fully understood and remain one of the most curious and complex biochemical phenomena that occur in plant cell stress (Minocha 2014)

Many efforts have been made to describe the role of amino acids in abiotic stress tolerance (drought and salt stress) in plants. *i.e.*, *Zea mays* L. (Thakur and Rai 1985), *Oryza sativa* L. (Yang et al. 2000) *Glycine max* L. (Ramos et al. 2005), *Sporobolus stapfianus* (Stapf) Stent. (Martinelli et al. 2007), and *Arabidopsis thaliana* (L.) Heynh (Nambara et al. 1998). Nonetheless, there is a lack of studies considering endogenous amino acids behavior during embryos drying, and there are no mentions when it comes to tolerance or sensibility to desiccation in embryos or seeds and its possible relations to seed desiccation stress, antioxidant activity, PAs, and seed viability. Desiccation tolerance of *B. eriospatha* embryos observed in this study might be strongly related to the amino acids metabolism once the total contents of free amino acids in response to drying time increased by approximately six-fold. The analysis of the ratio of Orn and Arg in relation to the total free amino acid during desiccation revealed a decrease in the proportion of Arg and an increase in the proportion of Orn. Interestingly, the amino acid Arg is the precursor of arginine decarboxylase (ADC) and Orn is the precursor of ornithine decarboxylase (ODC), both responsible for PUT biosynthesis (Chen et al. 2019). By this way, our results suggest that Arg was being used by the ADC pathway for the biosynthesis of PUT, which had its contents increased during seed desiccation. Indeed, gene expression analysis showed that the biosynthesis of PAs via ADC responds much more strongly to abiotic stress than the ODC pathway (Do et al. 2013; Berberich et al. 2015). Our results additionally showed a significant increase in Methionine (Met) content in embryos dried for 300 min (481.82 µg g⁻¹ DW) compared with embryos not desiccated (124.59 µg g⁻¹ DW). Methionine is the precursor of S-adenosylmethionine (SAM) which are involved with the generation of SPD and SPM by the addition of one or two aminopropyl groups, respectively, to the PUT formed (Mustafavi et al. 2018). The increase in the Met content and the reduction of SPD and SPM content during desiccation, may be because this biosynthesis pathway is not highly active, also observed by the decrease in the ratio [(SPM + SPD). PUT⁻¹].

In our study, Lys was the most abundant amino acid (representing 42% of the total free amino acid profile) and its content increased about 33-fold when embryos were dried for 300 min (5928.69 µg g⁻¹ DW) as compared to the control (175.54 µg g⁻¹ DW), suggesting some role of this amino acid in the desiccation process of *B. eriospatha* embryos. In fact, previous studies have already reported an increase of Lys content in potatoes (Muttucumaru et al. 2015) and in sunflower (Behboudian et al. 2001) grown under water-deficient conditions. In *Raphanus sativus* seeds, exogenous Lys was used to overcoming the adverse effects of drought stress (Noman et al. 2018). Though all these studies reveal a potential role of Lys in desiccation tolerance through different modes, its effective concentration as well as the mechanisms are still to be explored (Ali et al. 2019). We also observed that embryos under desiccation had an increase in Glu and Gly content by 30

and 6-fold, respectively. Glutamic acid is a precursor of Pro and their high concentration was associated to wild chickpea drought-tolerant seeds (Behboudian et al. 2001; Rontein et al. 2002; Trombin-Souza et al. 2017). Like Glu, the Gly is also indirectly related to desiccation tolerance, since one of the glycinebetaine (GB) synthesis pathways is through glycine N-methylation (Chen and Murata 2002, 2011). Additionally, the putative role of Glu in the oxidative defense mechanism has been found to be due not only to its internal metabolism but also to its exogenous use, in soybean seeds priming, that increased the antioxidant activity of CAT, POD and SOD enzymes (Teixeira et al. 2017).

As a conclusion this study designs a schematic integration of the in vitro germination and cryopreservation protocols of *B. eriospatha* embryos and its biochemical status associated to plant population remnants as well to an ex situ conservation (germoplasm bank) strategy (Fig. 8). Our study points out for the first time that *B. eriospatha* embryos can have more than 90% of in vitro germination in a water content range of 2.56 to 0.14 gH₂O gDW⁻¹. This germination protocol using culture medium supplemented with hormones and antioxidants ensures the large-scale seed germination of remnant plant population to guarantee the genetic diversity preservation (Fig. 8). In addition to advances in understanding the physiological behavior of desiccation tolerance, we provide relationships between amino acids, antioxidant activity, and polyamines during desiccation stress. At a specific embryo desiccation threshold (0.14 gH₂O gDW⁻¹) compare to the previous embryos water content, the increase in the activity of APX and POD suggest they were highly efficient against the oxidative stress caused during desiccation (Fig. 8). Also, the increase in the endogenous contents of PUT and the decrease in the biosynthesis of SPD and PMS, in relation to the control (2.56 gH₂O gDW⁻¹), resulted in a reduction in the ratio [(SPD+SPM). PUT⁻¹] DW). This biochemical event combined with the increase in the content of Gln, Glu, Lys, Leu, Orn and Met seems to play a key role in the desiccation tolerance and maintenance of embryo viability. At this specific embryo WC (0.14 gH₂O gDW⁻¹) and biochemical state they do not only were able to germinate in vitro, but even tolerate low temperature storage. The combination of this embryo stage and rapid cooling in LN, allowed us successfully established a cryopreservation protocol with in vitro germination and normal seedling development above 90% (Fig. 8). In this study it was showed that the precise information about the cell state related to water content and its biochemical composition were essential for the establishment of cryopreservation protocols with the reduction of metabolism without cell damage of *B. eriospatha* embryos. In this way and known the loss of habitats of *B. eriospatha* combined with the seed dormancy and low germination is essential to develop accessible technologies that ensure propagation and conservation of this species. Thus, both, in vitro germination and the cryopreservation protocol here established are powerful tools directly applied and useful to preserve a vulnerable tropical species from Brazilian Atlantic tropical forest, which is one of the most important hotspots of biodiversity in the world.

Declarations

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Author contributions

D.G., N.S. and C.W. contributed to planning the experiments, analyzing the data, as well as writing and editing the manuscript. N.S. coordinated the study. D.G. and M.M.S. collected the mature seeds. D.G., R.A.E. and M.M.S. performed the experiments. N.S. and M.P.G. contributed to the writing of the manuscript.

Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest

Supporting information:

Additional supporting information may be found online in the supporting information section at the end of the article

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Tables

Table. 1 Water content, germination, germination speed index and seedlings morphological of *Butia eriospatha* cryopreserved embryos

Technique	Drying time (min)	Water content (H ₂ O.g DW ⁻¹)	Germination (%)	GSI	Seedlings morphometry			
					Cotyledonary petiole (cm)	Leaf sheath (cm)	Root (cm)	Abnormal (%)
High cooling rate	0	2.56 ± 1.15	0.00 ± 0.00 ^b	0.00 ± 0.00 ^b	0.00 ± 0.00 ^b	0.00 ± 0.00 ^b	0.00 ± 0.00 ^b	0.00 ± 0.00 ^b
	15	0.94 ± 0.15	0.00 ± 0.00 ^b	0.00 ± 0.00 ^b	0.00 ± 0.00 ^b	0.00 ± 0.00 ^b	0.00 ± 0.00 ^b	0.00 ± 0.00 ^b
	180	0.17 ± 0.07	91.67 ± 6.66 ^a	0.34 ± 0.04 ^a	2.00 ± 0.22 ^a	0.90 ± 0.19 ^a	1.30 ± 0.47 ^a	12.50 ± 0.75 ^d
	300	0.14 ± 0.10	92.44 ± 2.41 ^a	0.30 ± 0.02 ^a	1.90 ± 0.24 ^a	1.00 ± 0.16 ^a	1.30 ± 0.32 ^a	7.14 ± 0.31 ^e
Droplet vitrification (PVS ₂)	0	1.30 ± 0.05	0.00 ± 0.00 ^b	0.00 ± 0.00 ^b	0.00 ± 0.00 ^b	0.00 ± 0.00 ^b	0.00 ± 0.00 ^b	0.00 ± 0.00 ^b
	15	0.43 ± 0.09	0.00 ± 0.00 ^b	0.00 ± 0.00 ^b	0.00 ± 0.00 ^b	0.00 ± 0.00 ^b	0.00 ± 0.00 ^b	0.00 ± 0.00 ^b
	180	0.36 ± 0.07	86.11 ± 5.31 ^a	0.34 ± 0.02 ^a	2.00 ± 0.30 ^a	1.20 ± 0.38 ^a	1.30 ± 0.44 ^a	29.17 ± 0.88 ^b
	300	0.22 ± 0.10	86.67 ± 7.24 ^a	0.33 ± 0.02 ^a	1.80 ± 0.45 ^a	1.50 ± 0.43 ^a	1.00 ± 0.48 ^a	37.03 ± 1.05 ^a
Droplet vitrification (PVS ₃)	0	0.25 ± 0.10	0.00 ± 0.00 ^b	0.00 ± 0.00 ^b	0.00 ± 0.00 ^b	0.00 ± 0.00 ^b	0.00 ± 0.00 ^b	0.00 ± 0.00 ^b
	15	0.24 ± 0.02	0.00 ± 0.00 ^b	0.00 ± 0.00 ^b	0.00 ± 0.00 ^b	0.00 ± 0.00 ^b	0.00 ± 0.00 ^b	0.00 ± 0.00 ^b
	180	0.46 ± 0.10	86.11 ± 2.85 ^a	0.32 ± 0.01 ^a	1.70 ± 0.58 ^a	0.90 ± 0.43 ^a	0.70 ± 0.43 ^a	14.81 ± 0.80 ^c
	300	0.35 ± 0.08	83.33 ± 7.95 ^a	0.32 ± 0.06 ^a	2.40 ± 0.48 ^a	0.80 ± 0.28 ^a	0.90 ± 0.47 ^a	16.66 ± 0.81 ^c

Data mean ± SD (n = 100).

Different letters indicate significant differences at $P < 0.05$, according to the SNK test

Figures

Figure 1

Morphology of *Butia eriospatha* seed (a-b) and embryo (c). External morphology of whole of seeds showing operculum (os) and seed coat (sc); regions of diameter (d) and length (l) measurements (a). Longitudinally sectioned seed with embryo (em), endosperm (en) and seed coat (sc) (b). Mature embryo with cotyledonary petiole (cp) in the proximal portion (pp) and haustorium (ha) in the distal portion (dp) (c). Bars: a, b = 2.0 mm; c = 500 μ m

Figure 2

Variation of the water contents (gH₂O. gDW⁻¹) of *Butia eriospatha* embryos in response to drying for 0; 15; 30; 60; 120; 180; 240 and 300 min and drying rate (k). Box indicate the time (15 min) in that water content value resembles that in fresh embryo (a). Water content and water potential (Ψ_w) relationship levels of 0; - 0.5; - 1; - 2; - 3 and - 4 MPa (b). Data: means $\pm$ standard error (n = 50), five independent replicates, each being a pool of 10 embryos. Linear regression of water content in function to water potential $r^2 > 0.98$

Figure 3

Physiological behavior of *Butia eriopatha* embryos in response to drying time for 0; 15; 30; 60; 120; 180; 240 and 300 min **(a-c)** and embryo and seedlings cultured in vitro **(e-f)**. Embryo germination (%) (axis right) and positive reaction to 2,3,5-triphenyltetrazolium chloride (TTC) (%) – (axis left) **(a)**. Germination dynamics (%) during 30 days after inoculation, † Absolute values in the graphic legend indicate the percentage of seed germination 30 days after inoculation **(b)**. Content water (WC = gH₂O.g DW⁻¹) (axis left) and germination speed index (GSI) (axis right) **(c)**. Lengths of primary root (rp); cotyledonary petiole (cp) and leaf sheath (ls) **(d)**. Data mean ± standard error (n = 100), four independent replicates, each being a pool of 25 embryos. Means followed by different letters are significantly different (P < 0.05) according to the SNK test

Figure 4

Butia eriospatha embryo zygotic development cryopreserved after eight weeks of culturing medium (a) by droplet vitrification expose in PVS₃ for 60 min (b-c) by droplet-vitrification expose in PVS₂ for 60 min (d). Normal seedling showing primary root (pr), cotyledonary petiole (cp) and leaf sheath (ls) (a). Abnormal seedling, showing absence of primary root (arrow) (b). Abnormal seedling, showing without leaf sheath (ls) (c). Abnormal embryo (d). Bars: a, b = 2.0 mm; c, d = 500 μ m

Figure 5

Antioxidant enzyme activities in *Butia eriospatha* embryos in response to drying time (min) 0; 15; 180; and 300 minutes, (a-d). SOD (a). APX (b). POD (c). CAT (d). Data mean $\pm$ standard error ($n = 3$) independent replicates, each being a pool of $\cong 300$ embryos. Means followed by different letters are significantly different ($P < 0.05$) according to the SNK test. Absolute values Supporting Information – Table S1

Figure 6

Free polyamines contents ($\text{mMol mg}^{-1} \text{DW}$) in *Butia eriospatha* embryos in response to drying time (min) for 0; 15; 180; and 300 minutes **(a-d)**. Putrescine (PUT) **(a)**. Spermine (SPD) **(b)**. Spermidine (SPM) **(c)**. PAs $[(\text{SPD}+\text{SPM})/\text{PUT}]$ ratio **(d)**. Data mean $\pm$ standard error ($n = 3$) independent replicates, each being a pool of $\cong 200$ embryos. Means followed by different letters are significantly different ($P < 0.05$) according to the SNK test. Absolute values Supporting Information – Table S2

Figure 7

Total free amino acid contents ($\mu\text{g g}^{-1}\text{DW}$) in *Butia eriospatha* embryos in response to drying time 0; 15; 180 and 300 minutes **(a)**. Amino acids heatmap cluster according to embryos drying time and water content. Color gradients represent the differences value of amino acid contents **(b)**. The ratio of arginine (Arg) and ornithine (Orn) to total amino acids **(c)**. Data mean $\pm$ standard error ($n = 3$) independent replicates, each being a pool of ≈ 100 embryos. Means followed by different letters are significantly different ($P < 0.05$) according to the SNK test. For absolute values and statistical analysis see Supporting Information – Table S3

Figure 8

Schematic integration of the in vitro germination and cryopreservation protocol (a) and main biochemical changes (b) of *Butia eriospatha* embryos associated to plant population remnants as well to an ex situ conservation strategy. Excised fresh embryos immersed in antioxidant solution (ascorbic and citric acid 0.2 g l⁻¹, each) and the most desiccated ones (0.14 g H₂O gDW⁻¹) were in vitro germinate. These embryos showed a significant increase in the enzymatic activity of POD and APX, in the endogenous content of amino acids (Glu, Gln, Lys, Leu, Orn, Met) and reduction in PAs [(SPD+SPM) PUT⁻¹] ratio. Specific embryo stage (0.14 g H₂O gDW⁻¹) and its biochemical state were used to establish a highly efficient cryopreservation protocol (germination > 90%). Dried embryos were immersed in liquid nitrogen (LN) and thawed in sucrose (1.2 M) at 45°C for 5 min and then in vitro germinated using 3/4 MS medium with GA₃ (8μM) and activated charcoal (3g l⁻¹). Cryopreserved embryos can be long term storage in a germplasm bank and seedlings can be transferred to a green house and after to the field

Supplementary Files

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