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The cryoprotectant PVS2 plays a crucial role in germinating *Passiflora ligularis* embryos after cryopreservation by influencing the mobilization of lipids and the antioxidant metabolism

Débora de Oliveira Prudente[a,*], Renato Paiva[a], Débora Domiciano[a], Lucas Batista de Souza[a], Sebastien Carpentier[b,c], Rony Swennen[b,c,d], Luciano Coutinho Silva[e], Fernanda Carlota Nery[f], Wesley Pires Flausino Máximo[a], Bart Panis[b,c]

[a] Laboratório de Cultura de Tecidos de Plantas (LCTP UFLA), Departamento de Biologia, Universidade Federal de Lavras (UFLA), Lavras, MG, Brazil
[b] Laboratory of Tropical Crop Improvement, Division of Crop Biotechnics, Katholieke Universiteit Leuven (KU Leuven), W. De Croylaan 42, 3001 Heverlee, Belgium
[c] Bioversity International, W. De Croylaan 42, 3001 Heverlee, Belgium
[d] International Institute of Tropical Agriculture, POB 10, Duluti, Arusha, Tanzania
[e] Universidade Federal da Paraíba (UFPB), João Pessoa, PB, Brazil
[f] Universidade Federal de São João Del Rei (UFSJ), São João Del Rei, MG, Brazil

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ABSTRACT

Cryopreservation is a process whereby biological structures are preserved in liquid nitrogen (−196 °C) without losing their viability. Many cryopreservation techniques use the *Plant Vitrification Solution 2* (PVS2) for cryoprotection. This study will therefore evaluate the influence of different exposure times to the cryoprotectant PVS2 and discuss the importance of the mobilization of reserves and the antioxidant metabolism during the germination of cryopreserved *Passiflora ligularis* embryos. The composition of *P. ligularis* seeds was analytically determined. We tested the germination capacity and the Germination Speed Index (GSI) of embryos (that is, seeds without external tegument) which were exposed to different PVS2 exposure times (0, 30, 60 and 120 min) at 30 days after thawing. Proline content, hydrogen peroxide, activity of isocitrate lyase (ICL), malate synthase (MSy), lipid peroxidation and antioxidant enzyme activities (SOD, CAT, APX) were measured at 7, 14 and 21 days after cryopreservation. The germination from cryopreserved embryos was maximal (85%) after 60 min PVS2 exposure with a GSI of 0.6. At 60 min, the highest activity of the enzymes involved in the glyoxylate cycle, ICL and MSy were recorded. We hypothesize that a 60 min exposure to PVS2 accelerates the reserve mobilization which correlates positively with germination. Until 60 min, there was a positive correlation between the PVS2 exposure time and the proline content, as well as the activity of antioxidant enzymes (SOD, CAT, APX), and a negative correlation with the lipid peroxidation. This study enables us to optimize the long-term conservation of this species. In conclusion, fundamental research is necessary to optimize the cryopreservation procedure, and this study offers an effective and efficient workflow which can be extrapolated to other (oil-rich) species.

1. Introduction

Passiflora L. is the most representative genus of the Passifloraceae Juss. family, which comprises 18 genera containing approximately 630 species widely distributed throughout South America (Martin and Nakasone, 1970; Tiwari et al., 2015). *Passiflora ligularis* Juss., commonly known as sweet granadilla, grows in tropical regions at high altitudes in Brazil (Ocampo et al., 2015). The pulp of *P. ligularis* fruits has anti-diabetic and antimicrobial properties and is used for consumption *in natura* or as a commercial fruit (Saravanan and Parimelazhagan, 2014).

Despite their economic and medicinal potential, many species of *Passiflora* L. are threatened by destruction of their natural habitat (Cerqueira-Silva et al., 2014). Studies on the long-term storage of seeds through cryopreservation demonstrated success in preserving the viability of 22 *Passiflora* species belonging to intermediate and orthodox classes (Veiga-Barbosa et al., 2013; Posada et al., 2014).

The conventional conservation of wild Passiflora species, such as *P. ligularis*, in seed banks presents obstacles because of the reduction in germination potential with time (Pacheco et al., 2016).

* Corresponding author.
E-mail address: deboraoprudente@outlook.com (D.d.O. Prudente).

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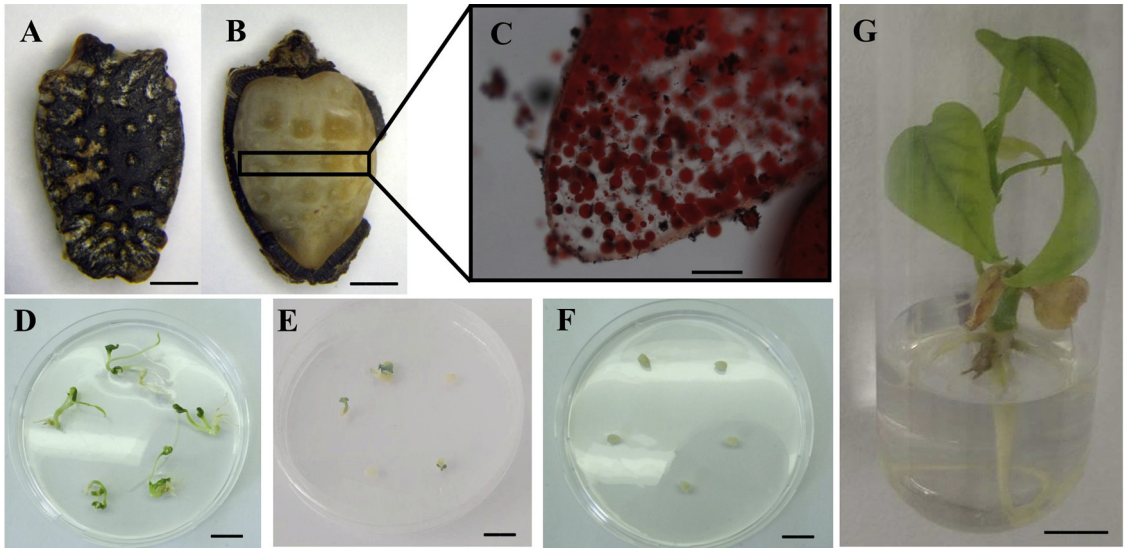

Fig. 1. General aspects of *P. ligularis* germination. Representative mature seeds with teguments (A) (Bar = 0.5 cm); without external tegument (B) (Bar = 0.5 cm); transverse section showing lipid bodies stained with Sudan IV (Bar = 100 μM) (C); embryos after 60 min of exposure to PVS2 followed by cryopreservation, 14 days after inoculation in germination medium (Bar = 0.5 cm) (D); embryos without exposure to PVS2 followed by cryopreservation, 14 days after inoculation in germination medium (Bar = 0.5 cm) (E); embryos of the control, 14 days after inoculation on germination medium (F) (Bar = 0.5 cm); completely regenerated plant 60 days after cryopreservation (60 min in PVS2) (Bar = 2 cm) (G).

Cryopreservation or conservation at ultralow temperatures (−196 °C) has been suggested as the ultimate long term storage method for biological materials (Engelmann, 2011; Coelho et al., 2017). Cryopreserved collections are maintained in small physical spaces, protected from contamination, and require minimal maintenance and little financial support during preservation compared to other available systems of plant material storage (Dulloo et al., 2009). Seed cryopreservation results in 175 times greater longevity than at temperatures used in conventional seed banks (Pritchard, 1995; Walters et al., 2004, 2005).

The use of cryoprotective compounds such as glycerol, ethylene glycol, dimethylsulfoxide (DMSO) and sucrose, which are all components of the Plant Vitrification Solution 2 (PVS2), proved to be quite effective in providing protection for a wide range of explants (Sakai et al., 1990; Matsumoto, 2017; Panis et al., 2011). Finding the optimal exposure time to PVS2 is crucial to the success of cryoprotection. In addition, the PVS2 compounds may also act as antioxidants, helping to regulate the balance between the production and detoxification of reactive oxygen species (ROS) (Reed, 2014).

The reduction in lipid peroxidation may favor the accumulation of low-molecular-weight compounds such as proline that act to protect membranes (Hossain et al., 2014). There are many reports indicating that exposure of embryos and seeds to ultralow temperatures following thawing may result in the modification of essential metabolic functions. Those modifications interfere with both the speed and the final percentage of germination (Salomão, 2002; Johnson et al., 2012; Kholina and Voronkova, 2012; Gantait et al., 2016).

To better understand the signaling pathways that trigger germination, we should understand the main type of reserves in each species (Rajjou et al., 2012). Seed reserves consist of complex carbohydrates, protein and lipid molecules that can vary in quantity and proportion (Bewley and Black, 1994). The oil-rich (lipid-rich) endosperm of some *Passiflora* L. species (Nyanzi et al., 2005; Tozzi and Takaki, 2011) causes specific metabolic characteristics which differ from other types of seeds which are rich in carbohydrate or protein reserves (Theodoulou and Eastmond, 2012).

Optimal germination of oilseeds is related to the fast and efficient use of lipid reserves at the start of germination through a coordinated induction of a number of biochemical pathways within different subcellular locations (Graham, 2008). The first step in lipid breakdown is catalysed by lipases, which provides free fatty acids (FFAs) and glycerol from triacylglycerol (TAG) molecules (Xu and Shanklin, 2016). The FFAs are then introduced into glyoxysomes, a membrane-bound organelle, where β-oxidation and the glyoxylate cycle occur (Li-Beisson et al., 2013). Glyoxysomes are structurally similar but metabolically distinct from peroxisomes and contain two unique enzymes linked to the glyoxylate cycle: malate synthase (MSy) and isocitrate lyase (ICL) (Graham, 2008). The products of these two enzymes are used respectively for cycle regeneration and for sucrose synthesis, of which sucrose will be transported to developing tissues to promote seedling growth (Geigenberger and Fernie, 2014).

Cryoprotection by exposure to PVS2 for prolonged periods can interfere with cellular homeostasis (Dietz et al., 2016) and, consequently, with the performance of key enzymes, such as MSy and ICL, which are both involved in the mobilization of lipids in oilseeds (Upchurch, 2008). This study therefore aims to evaluate the influence of the exposure time to the cryoprotectant PVS2 on the mobilization of reserves and on the antioxidant metabolism and its effect on germination of (cryopreserved) *P. ligularis* embryos.

2. Materials and methods

2.1. Moisture content, centesimal composition and histological analysis of seeds

Passiflora ligularis seeds were obtained from Tabutins sementes Brasil Ltda. (Gramado, RS, Brazil; http://www.tabutinssementes.com.br). The moisture content was determined using the drying oven method at 105 °C ± 3 °C for 24 h, with six replications of 25 seeds, in accordance with the rules for seed testing (Brasil, 2009). The centesimal compositions of fixed mineral residues, lipids, proteins, crude fiber and total carbohydrates were analytically determined using 100 g of freshly harvested *P. ligularis* seeds according to Horwitz (2007). For histological analysis, embryos were subjected to transverse sectioning with Sudan IV labeling for the identification of lipid bodies, according to Kraus and Arduin (1997). The preparation of semipermanent slides was performed as described by Johansen (1940). Observations were made using an Axiophot microscope equipped with DIC optics (Zeiss®, Oberkochen, Germany), and photographic documentation was carried out using a Powershot A640 digital camera (Canon®, Tokyo, Japan).

2.2. Germination procedures

Prior to surface sterilization, the seed tegument was removed with a mini-vise (Reis et al., 2007). This was followed by surface sterilization of 120 embryos per batch (Fig. 1A) in 200 mL of 70% (v/v) ethanol (30 s), 200 mL of a commercial sodium hypochlorite solution of 2.5% (v/v) (30 min), and with two drops of Tween-20 per 100 mL of solution. The material was rinsed three times in autoclaved distilled water. Different culture media were tested for *in vitro* germination of embryos: (1) MS (Murashige and Skoog, 1962) basal salts + B5 vitamins (Gamborg et al., 1968); (2) MS basal salts + MS vitamins; (3) half-strength MS basal salts + half-strength B5 vitamins; and (4) half-strength MS basal salts + half-strength MS vitamins. All media were supplemented with 0.01% (w/v) myo-inositol and solidified with 0.3% (w/v) Phytagel® (Sigma-Aldrich®, St. Louis, MO, USA). The pH was adjusted to 5.8 with 2 M NaOH and HCl prior to autoclaving at 121 °C for 20 min. Culture rooms were maintained at 25 °C ± 2 °C with a 16 h photoperiod and a photosynthetic photon flux of 36 μmol m^{-2} s^{-1} irradiance at the top of the culture vessels provided by Phillips Cool White 18-W fluorescent lamps. The percentage of germinated embryos with 2 mm rootlets was recorded in each treatment, and the Germination Speed Index (GSI) was calculated using the formula proposed by Maguire (1962):

$$GSI = \frac{G1}{D1} + \frac{G2}{D2} + \ldots \frac{Gn}{Dn}$$

where G1, G2, and Gn are the number of germination seeds at the first, second, and last count, respectively, and D1, D2, and Dn refer to the days after sowing at the first, second, and last count, respectively. After 30 days of *in vitro* culture, the percentage of germination, leaf number and shoot length were evaluated.

2.3. Cryopreservation procedures

Five embryos were placed into cryotubes (TruCool®, Sigma-Aldrich®, St. Louis, MO, USA) containing 2 mL of loading solution (LS; 2 M glycerol and 0.4 M sucrose) for 20 min at 25 °C. The LS was removed and replaced with 2 mL of PVS2 [30% (w/v) glycerol; 15% (w/v) ethylene glycol; 15% (w/v) DMSO and 0.4 M sucrose] (Sakai et al., 1990) at 0 °C for different exposure times (0, 30, 60 and 120 min), and then tubes were immersed into liquid nitrogen (LN) for 90 min. The rewarming of cryopreserved embryos was performed by transferring the cryotubes into a water bath at 40 °C for 2 min. Afterwards, the PVS2 was removed, and 2 mL of unloading solution (1.2 M sucrose) was added and maintained for 15 min at room temperature (~25 °C). The control treatment (without LN plunge) consisted of transferring cryoprotected but not frozen material into a water bath at 40 °C for 2 min followed by direct transfer to the unloading solution. All cryoprotectant solutions were dissolved in MS liquid basal medium (Murashige and Skoog, 1962), with the pH adjusted to 5.8 prior to filter sterilization, using a 0.22 μm pore size micropore filter (Millipore Filter Corp., Bedford, Mass.). Then, cryopreserved (LN +) and control (LN -) embryos were transferred to germination media (MS basal salts and B5 vitamins). The same conditions were applied as for germinating seeds (see above).

2.4. Protein content

Protein concentration was determined according to the standard Bradford protocol (Bradford, 1976) using commercially available reagents (Sigma-Aldrich®, St. Louis, MO, USA). Proteins were extracted from growing embryos using 0.1 M 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid and N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (HEPES) (pH 7.0) containing 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate (CHAPS), and sodium dodecyl sulfate bovine serum albumin (SDS) was used as a standard (all extracts measured separately).

2.5. Proline quantification

To quantify free proline content, 400 mg of growing embryos was sampled 7, 14 and 21 days after cryopreservation. The samples (only growing embryos) were homogenized in 3% (w/v) sulfosalicylic acid, after which the homogenate was filtered through a Whatman® (No. 4) filter paper. Two milliliters of filtrate were added to 2 mL of ninhydrin and 2 mL of glacial acetic acid in a microtube (Brand®, Sigma-Aldrich®, St. Louis, MO, USA) following incubation for 1 h at 100 °C, after which the reaction was terminated in an ice bath. The reaction mixture was extracted with 4 mL of toluene vigorously mixed using a test tube stirrer for 15–20 s. The chromophore containing toluene was aspirated from the aqueous phase, and this fraction was set aside for absorbance readings using a UV–VIS spectrophotometer (Shimadzu UVmini-1240, Kyoto, Japan) at a 520-nm wavelength (Bates et al., 1973). Proline was expressed in nanomoles per milligram of fresh weight (FW).

2.6. Hydrogen peroxide quantification

To determine hydrogen peroxide (H_2O_2) content, 200 mg of growing embryos was sampled 7, 14 and 21 days after cryopreservation. Samples were ground in LN, homogenized in 1.5 mL of 0.1% trichloroacetic acid (TCA) (w/v) and centrifuged at 12,000 × *g* at 4 °C for 15 min, and the supernatant was collected. H_2O_2 content was measured in 45 μL of 10 mM potassium phosphate (pH 7.0) and 90 μL of potassium iodide (1 M). The H_2O_2 content was assessed by measuring the absorbance at 390 nm of (Velikova et al., 2000).

2.7. Lipid peroxidation and antioxidant enzyme activity assays

For these analyses, 200 mg of growing embryos was sampled 7, 14 and 21 days after cryopreservation. Lipid peroxidation was evaluated through the quantification of species that are reactive to thiobarbituric acid (TBA), as described by Buege and Aust (1978). Samples were ground in LN, and 0.5% (w/v) TBA and 10% (w/v) TCA were added to the reaction medium followed by incubation at 95 °C for 30 min. The reaction was stopped by rapid cooling on ice, and readings were taken using a spectrophotometer at 535 nm and at 600 nm. TBA forms reddish complexes consisting of low-molecular-mass aldehydes, such as malondialdehyde (MDA), a peroxidation process by-product. The MDA/TBA complex concentration was calculated using the following equation: $[MDA] = (A^{535} - A^{600}) / (\xi.b)$, where ξ is the extinction coefficient equal to 1.56×10^{-5} cm^{-1} and b is the optical length equal to 1.

The antioxidant enzymes involved in hydrogen peroxide synthesis and metabolism extracts were prepared in 2 mL microtubes (Brand®, Sigma-Aldrich®, St. Louis, MO, USA) by adding 1500 μL of extraction buffer containing the following reagents: 375 μL potassium phosphate buffer at pH 7.8 (0.1 M), 15 μL EDTA (0.1 mM), 75 μL ascorbic acid (10 mM) and 1.035 μL distilled water. Next, the homogenous solution was centrifuged at 13,000 × *g* at 4 °C for 10 min. The supernatant was collected and used to measure the enzymatic activity of superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) (Biemelt et al., 1998).

The SOD activity was determined based on the ability of the enzyme to inhibit nitroblue tetrazolium chloride (NBT) photoreduction, as described by Giannopolitis and Ries (1977), with modifications. Ten microliters of enzymatic extract were added to 190 μL of incubation medium, which was composed of 100 μL of potassium phosphate buffer (100 mM; pH 7.8), 40 μL methionine (70 mM), 3 μL EDTA (10 μM), 31 μL distilled water, 15 μL NBT (1 mM) and 2 μL riboflavin (2 μM). Tubes containing the incubation buffer and the samples were illuminated (~250 μmol m^{-2} s^{-1}) with a fluorescent lamp (20 W, Osram, Barueri, Brazil) for 10 min. The control treatment was performed using the same reaction medium without the enzymatic extract and maintained for 10 min in a dark room at 25 °C ± 1 °C. Readings were performed at 560 nm, and the SOD activity was assessed using the

following equation: % inhibition = (A_{560} sample with enzymatic extract – A_{560} sample without enzymatic extract) / (A_{560} sample without enzymatic extract). One unit of SOD can inhibit 50% of the photoreduction of NBT under the assay conditions.

The CAT activity was quantitatively assessed every 15 s for 3 min as a decrease in absorbance at 240 nm, which was monitored by H_2O_2 consumption (molar extinction coefficient: 36 M^{-1} L^{-1} cm^{-1}) (Havir and McHale, 1987).

The APX activity was quantified every 15 s for 3 min by monitoring the ascorbate oxidation rate at 290 nm. One aliquot of 9 µL of enzymatic extract was added to 162 µL incubation buffer containing 90 µL of potassium phosphate (200 mM; pH 7.0), 9 µL ascorbic acid (10 mM) and 63 µL distilled water. Immediately prior to measurements, 9 µL H_2O_2 (0.1 mM) was added to the incubation medium. A molar extinction coefficient of 36 M^{-1} L^{-1} cm^{-1} was used (Nakano and Asada, 1981).

2.8. Glyoxylate cycle enzymatic assays

To determine the activity of isocitrate lyase (ICL) and malate synthase (MSy), 200 mg of growing embryos was sampled 7, 14 and 21 days after cryopreservation. Samples were ground in LN, and ICL was extracted on ice with 50 mM Tris-HCl buffer (pH 7.5) containing 5 mM $MgCl_2$, 1 mM EDTA and 2 mM cysteine. ICL samples were centrifuged at 15,000 × *g* for 10 min at 4 °C, and the supernatants were collected. ICL activity was measured in 50 mM Tris-HCl (pH 7.5) containing 5 mM $MgCl_2$, 2 mM L-cysteine, 10 mM L-isocitrate and 4 mM phenylhydrazine hydrochloride - PHY at 324 nm using the extinction coefficient of glyoxylic acid phenylhydrazone - GAPH (14.6 mM cm^{-1}), according to the methods of Eprintsev et al. (2014). MSy was extracted on ice using 0.1 M HEPES (pH 7.8) containing 5 mM $MgCl_2$, 1 mM EDTA and 2 mM 1,4-dithiothreitol (DTT). After centrifugation at 15,000 × *g* for 10 min at 4 °C, the supernatant was collected. MSy activity was determined in 0.1 M HEPES (pH 7.8) containing 6 mM $MgCl_2$, 5 mM sodium glyoxylate, 2.5 mM acetyl-CoA and 2 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) at 412 nm using the extinction coefficient of 2-nitro-5-thiobenzoic acid (TNB; 13.6 mM^{-1} cm^{-1}), according to the methods of Ma et al. (2016).

2.9. Experimental design and statistical analysis

The experiments were carried out in a completely randomized design with six replicates per treatment. A replicated unit consisted of a sterile polystyrene Petri dish (90 x 15 mm) (J. Prolab®, Paraná, Brazil) containing 25 mL of culture medium, with five embryos each. The Petri dishes were sealed with polyvinyl chloride film (Rolopac®, Santana do Parnaíba, Brazil). Data were subjected to analysis of variance (ANOVA) using the statistical software SISVAR 4.3 (System Analysis of Variance for Balanced Data, Lavras, Brazil) (Ferreira, 2014). Data were compared using Tukey's test ($p < 0.05$). The Principal Component Analysis (PCA) and its correlations produced eigenvectors and principal component scores which were used to measure the relative discriminative power of the axes and their associated characters. A dendrogram was constructed using the Ward method. The distance is expressed as average cluster distance (using Statistica® data analysis software system version 7).

3. Results

3.1. Seed characterization

The initial average moisture content of *P. ligularis* mature seeds was 11%. This finding is indicative that *P. ligularis* seeds may belong to the intermediate class instead of the orthodox one. Additional analyses must be performed to confirm this hypothesis.

Our results indicate that *P. ligularis* seeds contain a medium lipid content (average 31%) in their reserves, such as triacylglycerol (TAG), and can therefore be characterized as oilseeds (Table 1). This lipid content corresponds with that found in *P. edulis* (28.12%) as reported by Jorge et al. (2009).

Table 1
Centesimal composition of *Passiflora ligularis* seeds (g 100 g^{-1} dry weight).

Compounds	Seeds (%*)
Fixed mineral residue	03.05 ± 0.05
Lipids	31.15 ± 0.01
Proteins	13.17 ± 0.09
Crude fiber	22.99 ± 0.07
Total carbohydrates**	25.58 ± 0.34
Residual moisture content	04.06 ± 0.12

* Mean and standard deviation (±).
** The total level of carbohydrates contained in the samples corresponds to the sum of the amylaceous carbohydrates (available and resistant starches) excluding non-digestible carbohydrates (fibers).

Mature seeds of *P. ligularis* consist of a brown seed coat and a relatively thick layer of white endosperm, which surrounds the embryo. The predominant seed reserve content is stored in the endosperm as lipid bodies (Fig. 1C).

3.2. Germination procedures

Germination on MS basal medium supplemented with B5 vitamins was significantly higher (88%) compared to the other media (Fig. 2A). This combination also resulted in a significantly higher GSI (0.33), average number of leaves per germinating plant (2.56) and average shoot length per germinating plant (5.84 cm) (Fig. 2B–D).

3.3. Cryopreservation

The germination percentage of embryos treated with PVS2, without LN immersion, gradually decreased with increasing PVS2 exposure time (Fig. 3A). This is also the case with respect to the GSI, although to a lesser degree (Fig. 3B). For cryopreserved embryos, the germination rate increased with increasing PVS2 treatment, reaching a maximum (85%) at 60 min of PVS2 exposure (Fig. 3A). This is also observed with the GSI (Fig. 3B). A remarkable observation is that at 60 min, PVS2 treatment germination of cryopreserved seed is higher compared to the noncryopreserved seeds.

3.4. Proline content

The longer the PVS2 treatment, until 60 min of exposure, the higher the proline content. Samples exposed to 60 min PVS2 showed the highest proline content, especially at 7 and 14 days of cultivation (Fig. 4).

3.5. H_2O_2 content

Significant differences were observed regarding the content of H_2O_2. Both PVS2 exposure time before cryopreservation and germination days for each treatment were significant (Fig. 5).

3.6. Malondialdehyde (MDA) content and antioxidant enzymatic activity

The differences in antioxidant enzyme (SOD, CAT and APX) activities are provided in Fig. 6A–C. The enzymatic activity increases as the PVS2 time of exposure increases until 60 min. Regarding the cultivation period, the highest activity of SOD, CAT and APX is reached at 14 days. For lipid peroxidation, measured by the MDA content, the control treatment had the highest average at 21 days after germination. Conversely, the lowest MDA content was observed after 60 min PVS2

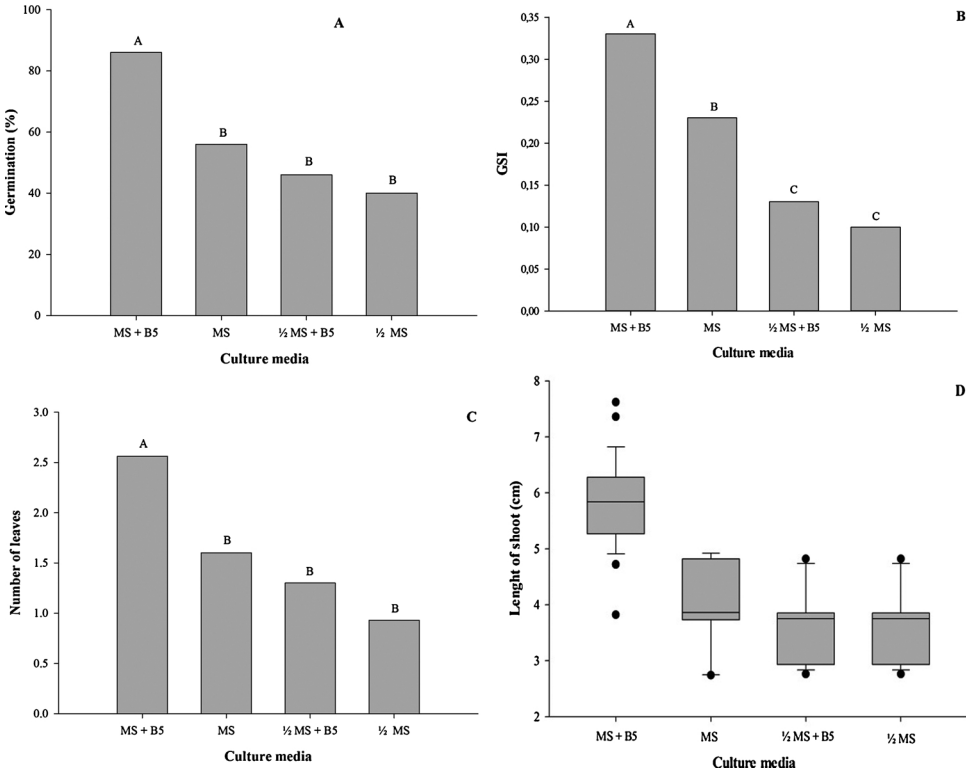

Fig. 2. Influence of different concentrations of MS media and types of vitamins (MS vitamins or B5 vitamins) on average values of percentage germination (A); Germination Speed Index (GSI) (B); the number of leaves (C); length of shoots (D) of *P. ligularis* cultivated *in vitro* for 30 days. Means followed by the same letter are not significantly different ($p > 0.05$) according to Tukey's multiple range test. MS + B5 = MS medium + Gamborg vitamins; MS = MS media + MS vitamins; ½ MS + B5 = half-strength MS + Gamborg vitamins; ½ MS = half-strength MS medium + MS vitamins.

treatment at 6 days after germination (Fig. 6D).

3.7. Activity of isocitrate lyase (ICL) and malate synthase (MSy)

The enzymatic activities of ICL and MSy showed significant differences among PVS2 exposure times as well as among days of culture (Fig. 7).

3.8. PCA classification

The first two principal components were responsible for 80.84, 83.33 and 84.23% of the total variation of data at 7, 14 and 21 days, respectively (Fig. 8A–C). These components were sufficient and accurate to assess the set of variables (Hongyu et al., 2015).

Based on this approach, the enzymatic activities of MSy, ICL, and antioxidant enzymes (SOD, CAT and Apx), as well as the content of proline (Pro), were highlighted on the first component at 7 days (Fig. 8A). Furthermore, the contrast between such variables and the lipid peroxidation (PER) was very clear. In the second component, the difference between the hydrogen peroxide (H_2O_2) and the other variables was also evident.

At 14 days, there was neither correlation between Pro and MSy nor ICL and the antioxidant system. All of these variables were correlated with each other for only a proportion of one set. At 21 days (Fig. 8C), the activities of the MSy and CAT were not correlated to the other variables, indicating the final consumption of the reserves for the germination process.

4. Discussion

For the majority of wild *Passiflora* species, no gene bank collections have been established, because there are no efficient *in vitro* regeneration and (cryo)preservation protocols (Veiga-Barbosa et al., 2013). In this study, the *in vitro* germination of *P. ligularis* seed was optimized. MS culture medium basal salts supplemented with B5 vitamins proved to be optimal (88%). However, for other *Passiflora* species, the *in vitro* germination and regeneration rates were obtained with further combinations of basal salts and vitamins: *Passiflora edulis* with ½ MS medium

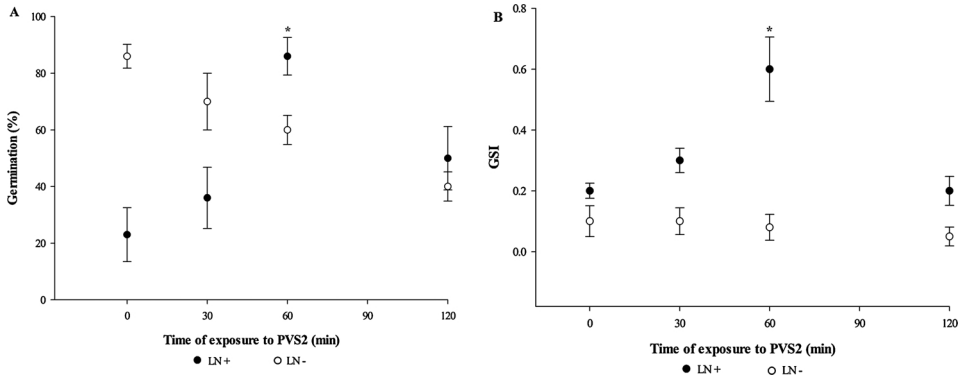

Fig. 3. Effect of time exposure to PVS2 (0, 30, 60 and 120 min) on *P. ligularis* embryos. Germination percentage (A) and GSI (B) of control (LN-) or cryopreserved (LN +) samples. (*) significantly different according to Tukey's multiple range test at $p < 0.05$ (n = 30). Bars represent standard error of the mean (SE).

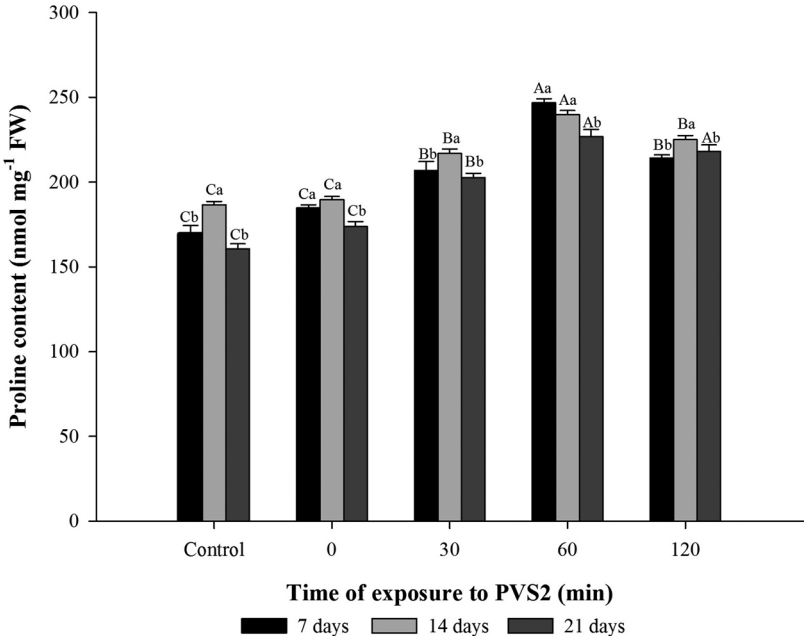

Fig. 4. Influence of PVS2 exposure time (0, 30, 60 and 120 min) on proline content of cryopreserved embryos after 7, 14 and 21 days on germination medium. Control is represented by no embryos exposed to LN. Means followed by the same upper case letter (for a given day) and lower case letter (for a given PVS2 time) are not significantly different according to Tukey's multiple range test at $p < 0.05$ (n = 8). Bars represent standard error of the mean (SE).

supplemented with B5 vitamins (Silva et al., 2011); *P. alata*, MS medium with MS vitamins (Pinto et al., 2010) and *P. suberosa* with ½ MS medium with ½ MS vitamins (Garcia et al., 2011). Hence, for each species the optimal conditions need to be determined. The main difference between the MS and B5 vitamin formulations is linked to the different concentrations of the components. For example, the vitamin thiamine is 10-fold more concentrated in the B5 formulation (Gamborg et al., 1968). This fact may be linked to a better performance of MS culture medium supplemented with this vitamin complex because thiamine is an essential cofactor for aerobic respiration enzymes in plants, acting as a greater stimulus for growth and germination (Goyer, 2010).

The second step for *in vitro* conservation consists of establishing a suitable cryopreservation technique for embryos without affecting their vigor. The increase of intracellular solute viscosity by highly concentrated solutions is often achieved using PVS2 (Gonzalez-Arnao et al., 2007). Therefore, the key for successful cryopreservation using the PVS2 vitrification protocol involves the determination of the optimal time of exposure of samples to that solution and the application of rapid cooling and thawing rates (Tsai et al., 2009; Panis et al., 2011).

It is known that sucrose, ethylene glycol (the simplest diol), glycerol (sugar alcohol with three hydroxyl groups) and DMSO (organosulfur compound) offer excellent protection against the damaging effects caused by freezing, especially as the hydrogen atoms of one water molecule are attracted towards the hydrogen-bonding with hydroxyl groups of ethylene glycol or glycerol molecules, thus stabilizing the hydration layer of the phospholipid membrane (Asahina and Takahashi, 1978; Harvey et al., 1983; Willhite and Katz, 1984; Robertson et al., 1988; Towey et al., 2013). Ethylene glycol and glycerol further share a hydroxyl functionality and have the ability to accept or donate hydrogen atoms (Boutron and Kaufmann, 1979). Both ethylene glycol and glycerol exhibit high viscosity, resulting in a vitreous state under the right cooling conditions (amorphous solid), and in small quantities, they can be rapidly reduced and incorporated into metabolic pathways

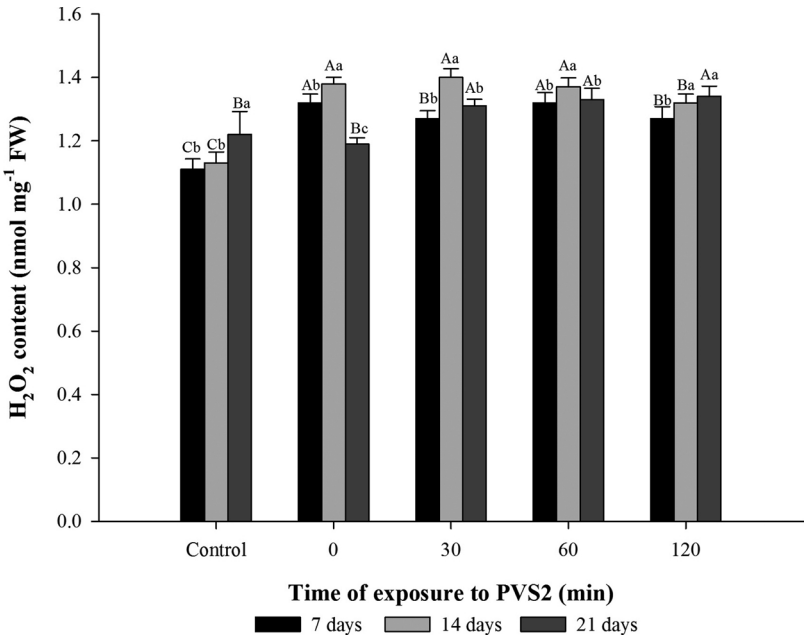

Fig. 5. Influence of PVS2 exposure time (0, 30, 60 and 120 min) on the H_2O_2 content of cryopreserved embryos after 7, 14 and 21 days on germination medium; the control is represented by embryos that were not cryopreserved. Means followed by the same upper case letter (for a given day) and lower case letter (for a given PVS2 time) are not significantly different according to Tukey's multiple range test at $p < 0.05$ (n = 8). Bars represent standard error of the mean (SE).

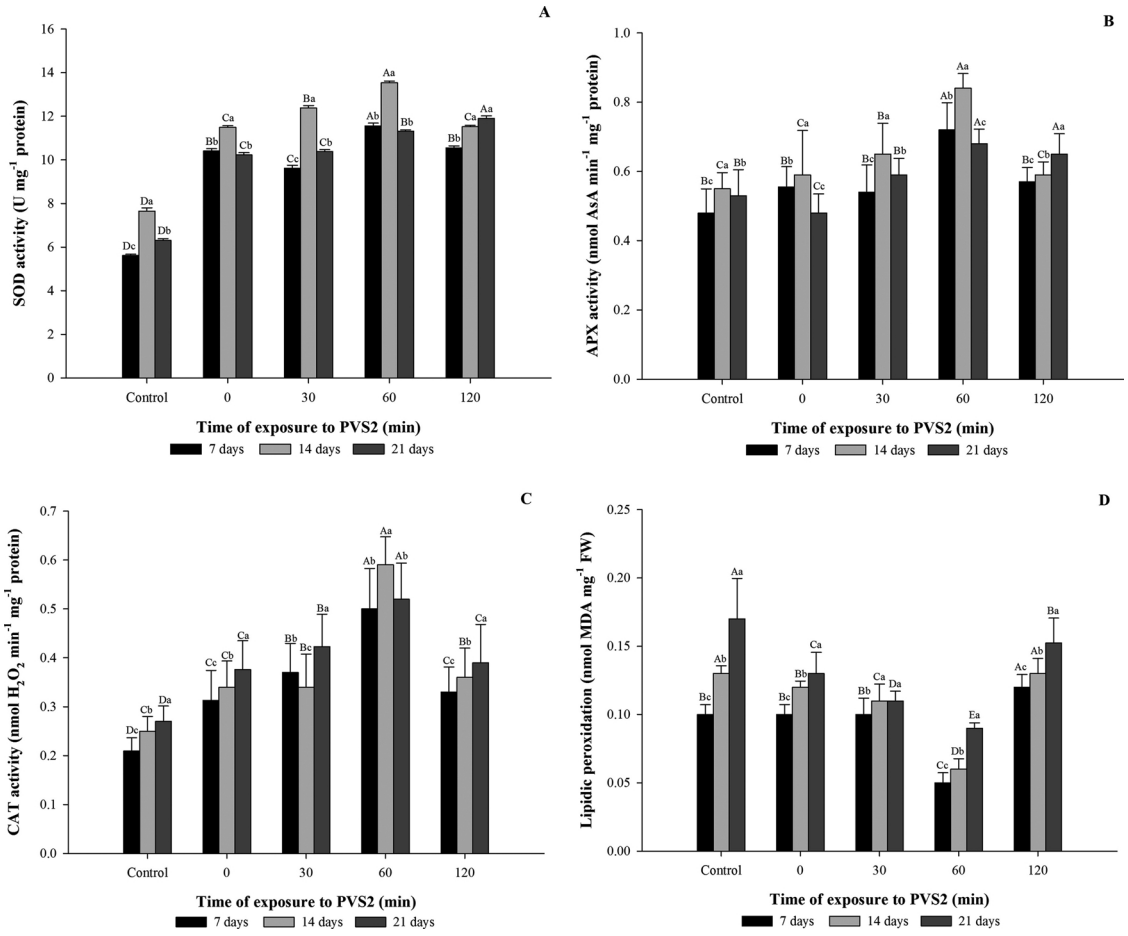

Fig. 6. Influence of PVS2 exposure time (0, 30, 60 and 120 min) on SOD activity (A), APX activity (B), CAT activity (C), and lipid peroxidation (D) of cryopreserved embryos after 7, 14 and 21 days on germination medium; the control is represented by embryos that were not cryopreserved. Means followed by the same upper case letter (for a given day) and lower case letter (for a given PVS2 time) are not significantly different according to Tukey's multiple range test at $p < 0.05$ (n = 8). Bars represent standard error of the mean (SE).

(Huang et al., 1995). When embryos were treated for 60 min with PVS2 and then cryopreserved, the germination process accelerated significantly, resulting in an increasing GSI (Figs. 1D and 2 B).

Oxidative stress commonly occurs during the use of cryoprotectant, generally when the time of exposure is insufficient or exceeds the appropriate cell balance for the vitrification process. The proposed hypothesis is that 60 min of PVS2 plays a crucial role in germinating *Passiflora ligularis* embryos after cryopreservation by influencing the reserve mobilization and antioxidant metabolism.

In this study, we show that the activity of the MSy is much higher than ICL, which indicates that the glyoxylate cycle is supplied with glyoxylate (substrate for activity of MSy) from other reactions. The metabolic pathways studied in this work are shown in Fig. 9.

Ethylene glycol (number 1 – Fig. 9) is primarily converted to glycolaldehyde by the action of alcohol dehydrogenase (ADH) and subsequently forms glycolate in the cytosol from (GDH) (Davies, 1960; Walder and Tyler, 1994). In the glyoxysome, through the glycolate oxidase (GOX) enzyme, glycolate can be converted to glyoxylate (Yue et al., 2012). In addition, protein reserves can be converted into glycine (number 2 – Fig. 9), and through transamination in the glyoxysome converted into glyoxylate (Dellero et al., 2016). During ß-oxidation, excessive production of H_2O_2 can occur when the reserve mobilization is active (number 3 – Fig. 9), which is broken down by the action of CAT inside the glyoxysome. APX acts only when excess H_2O_2 escapes from the glyoxysome to the cytosol (Corpas et al., 2015). Glycerol, originating from the breakdown of TAG, or as residuals from PVS2, can be phosphorylated and dephosphorylated by glycerol kinase (Hammerstedt et al., 1990). Glycerol can also be converted into pyruvate in the cytosol, which enters mitochondria by feeding the TCA cycle (number 4 – Fig. 9).

The oxidation of dicarboxylic acids by the TCA cycle generates reducing power, which is directed to the electron transport chain (*etc*) to allow ATP formation by reducing superoxide anion ($^{\cdot}O_2^-$). However, by incorporation of oxyacids into the TCA cycle (from other reactions during reserve mobilization), enhancement of partial reduction of O_2 and formation of $^{\cdot}O_2^-$ can occur (Ogawa and Iwabuchi, 2001). In addition, within the mitochondria, the enzyme SOD is capable of converting singlet oxygen ($^1O_2$) to H_2O_2 (number 5 – Fig. 9). When returning to normal conditions (stabilization of respiration), the amount of H_2O_2 produced may be greater than the ability of the antioxidant system (CAT and APX) to metabolize it (Corpas et al., 2015). In that case, H_2O_2 may be transported outside the mitochondria, and this process may function as a signaling mechanism (Bailly et al., 2008). Also in mitochondria, malate formed in the TCA cycle can be easily transported between organelles (Fernie et al., 2004), while in the cytosol, malate can be converted to oxaloacetic acid (OAA) by PEP carboxykinase (an enzyme of the C2 carbon cycle) to form sucrose *via* gluconeogenesis (Wiskich and Dry, 1985). The sucrose may protect membranes during cryopreservation or can be broken down and assist in embryo growth.

The cryoprotective effect of DMSO is associated with loosening membranes, resulting in electrostatic interactions between the polar sulfoxide moiety of DMSO and phosphatidylserine residues on phospholipid membranes. This effect favors the entrance of other

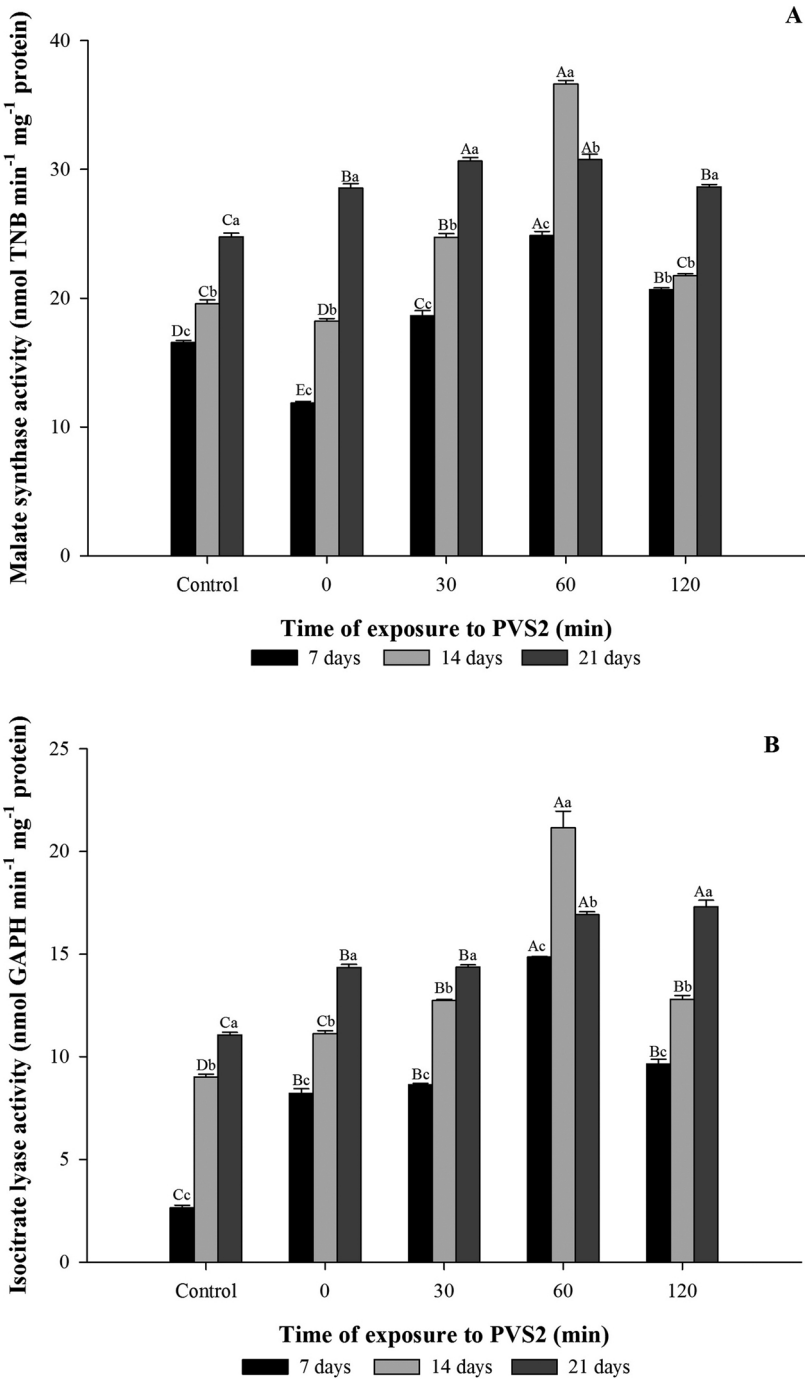

Fig. 7. Influence of PVS2 exposure time (0, 30, 60 and 120 min) on isocitrate lyase (ICL) activity (A) and malate synthase (MSy) activity (B) of cryopreserved embryos after 7, 14 and 21 days on germination medium; the control is represented by embryos that were not cryopreserved. Means followed by the same upper case letter (for a given day) and lower case letter (for a given PVS2 time) are not significantly different according to Tukey's multiple range test at $p < 0.05$ (n = 8). Bars represent standard error of the mean (SE).

cryoprotectants and the quick exit of water through cell membranes (Yu and Quinn, 1994). However, DMSO is moderately toxic to plant cells, depending on treatment temperature, duration of use and concentration (Fahy and Wowk, 2015). This is because after rewarming, the excess DMSO can result in the inhibition of mitochondrial respiration and in the increase of the cytosolic calcium (Galvao et al., 2014). The oscillatory Ca^{2+} activates phospholipases and proteases, affects the spatial organization of biological membranes (through mitochondrial depolarization) and amplifies the apoptotic signal (Zhivotovsky and Orrenius, 2011; Galvao et al., 2014).

The optimal osmoprotection is directly related to the ability of explants to equilibrate the quantity of DMSO and other compounds that favor vitrification, thus avoiding the toxicity. The percentage of germination in *P. ligularis* was increased by increased PVS2 exposure. We hypothesize that 60 min of exposure to PVS2 (Fig. 3) results in an ideal internal concentration of DMSO, since DMSO is related to the prevention of oxidative damage resulting from the production of abundant H_2O_2 (Fig. 5) and the consequent increase in lipid peroxidation (Fig. 6D).

ROS, such as H_2O_2, $^1O_2$ and hydroxyl radical (•OH), are produced within cells in small quantities during normal metabolic processes. However, the production of ROS often increases under oxidative stress

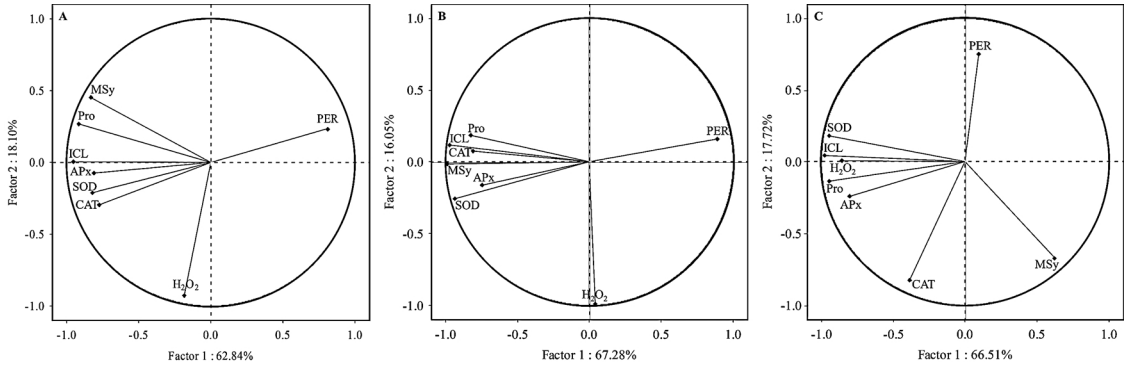

Fig. 8. Score plot of Principal Component Analysis (PCA) relationships among metabolic and enzymatic variables measured at 7 (A), 14 (B) and 21 (C) days after cryopreservation. Malate synthase (MSy), isocitrate lyase (ICL), proline (Pro), hydrogen peroxide (H_2O_2), superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and lipid peroxidation (PER) are represented.

(Smirnoff, 1993; Gutteridge and Halliwell, 2006). When ROS are produced at sufficient levels to overcome the antioxidant defenses, DNA, proteins and membrane fatty acids are oxidized. The latter can result in lipid peroxidation and loss of membrane function (Gutteridge and Halliwell, 2006).

Detoxification of ROS by antioxidants usually occurs at the site of production (cytosol, glyoxysome, peroxisome, mitochondria and chloroplasts). However, under stress-inducing conditions, the capacity of the detoxification system is not able to manage the levels of ROS produced, and H_2O_2 can spread to other cellular compartments, *e.g.*, the cytosol, and can affect the β-oxidation of fatty acids (Rashid et al., 2018).

H_2O_2 also modulates the expression of various genes involved in the transcription of proteins, such as protein kinases and calmodulin. The latter suggests longer-term effects in the cytoplasm (Bailly et al., 2008) and modulates the increase of cytosolic calcium, which influences the compatible osmolytes such as proline in the cytosol. Our recent study on oxidative stress and antioxidant metabolism during the cryopreservation of lateral buds in *Hancornia speciosa* demonstrated the importance of the antioxidant metabolism for a successful cryopreservation (Prudente et al., 2017).

The above findings suggest that the endogenous concentration of proline (Fig. 4) plays an important role during germination after cryopreservation, as well as with other abiotic stresses (Kishor et al., 2005; Wang et al., 2015; Yaish, 2015). Proline is an organic osmoprotectant synthesized from glutamate in the cytosol (Fig. 9), especially when the plant is exposed to osmotic stress (Szabados and Savoure, 2010). Additionally, proline can modulate antioxidant enzyme activities by increasing nonenzymatic antioxidant content, in addition to its potential role in osmotic adjustment, and can function as a molecular

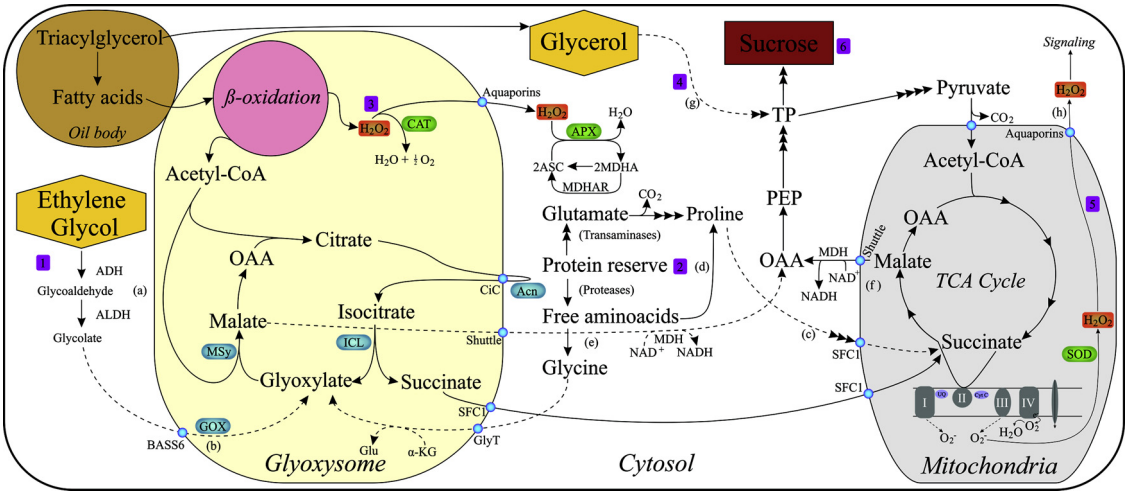

Fig. 9. Proposed pathways of fatty acid catabolism and PVS2 residual compounds during embryo germination in *P. ligularis*. Glyoxylate production begins with Acetyl-CoA that is produced through ß-oxidation reactions of fatty acids in the glyoxysome for generating citrate. Citrate is then transported to the cytosol by the Citrate Carrier (CiC) and converted by the aconitase enzyme (Acn) into isocitrate that returns to the glyoxysome to be cleaved in two molecules: succinate and glyoxylate. The ethylene glycol (1) passage to the glyoxysome, where it is broken down into glyoxylate, is shown by dashed lines; (a) Walder and Tyler (1994); Davies (1960); (b) Yue et al. (2012). The breakdown of reserve proteins (2) and the pathways for the formation of proline and glycine are also indicated. Proline can be converted to succinate and glycine can be converted to glyoxylate (dashed lines); (c) Takayama and Ezura (2015); (d) Bewley and Black (1994); (e) Murray (2013). During ß-oxidation, hydrogen peroxide is broken down by CAT in the glyoxysome or by APX in the cytosol (3). Glycerol can be incorporated into gluconeogenesis (dashed lines) and converted to sucrose, or triose phosphate (TP) can be converted to pyruvate, which enters the tricarboxylic acid cycle (TCA) (4); (f) Eastmond and Graham (2001) and Neill et al. (2002); (g) Penfield et al. (2006). Malate formed in the glyoxysome can be easily transported and converted to oxaloacetic acid (OAA) by MDH to form sucrose *via* gluconeogenesis (dashed lines). During the TCA cycle, excessive production of ROS can occur (dashed lines), generating H_2O_2 that can act on cytosolic signaling (5); (h) Bailly et al. (2008). Sucrose can be derived from gluconeogenesis and can represent a residual compound from the LS, PVS2 or RS solutions (6). ADH = Alcohol dehydrogenase; ALDH = Aldehyde dehydrogenase; GOX = Glycolate oxidase; ICL = Isocitrate lyase; MSy = Malate synthase; MDH = Malate dehydrogenase; MDHAR = Monodehydroascorbate reductase; SOD = Superoxide dismutase; CAT = Catalase; APX = Ascorbate peroxidase. Transporters: BASS6 = Bile Acid Sodium Symporter; CiC = citrate carrier; GLyT = glycine transporter; SFC1 = succinate–fumarate carrier. Dashed lines = possibilities to occur. Solid lines = pathways previously studied.

chaperone capable of protecting protein integrity against denaturation (Mattioli et al., 2008; Szabados and Savoure, 2010). Proline can be easily metabolized when the conditions normalize to also serve as a carbon source to provide energy for embryo growth after adverse conditions (Kishor et al., 2014; Zadehbagheri et al., 2014).

Corroborating this study, increases in proline content occur in many plant species during cold acclimation (Koster and Lynch, 1992; Xin and Browse, 2000). This compound contributes to cytoplasmic osmotic adjustment in response to water loss without interfering with normal cellular processes and biochemical reactions. In regenerated cryopreserved embryos of *Hypericum rumeliacum*, the concentration of free proline increased to twice that of the control (Georgieva et al., 2014).

5. Conclusion

The present study indicates the contributing role of the cryoprotectant PVS2 in germination of *P. ligularis* and reveals new aspects of the metabolic processes occurring during germination after cryopreservation using the PVS2 vitrification protocol. This study suggests that the components of PVS2 exert a residual effect after cryopreservation, increasing the speed of reserve mobilization and germination through antioxidant metabolism and the incorporation of some compounds into metabolic pathways. The high activity of the MSy enzyme demonstrates that the glyoxylate cycle in oilseeds may be important for the conversion of fatty acids to carbohydrates. In conclusion, the application of the cryopreservation technique by vitrification using PVS2, in addition to serving as a tool for conservation, can accelerate germination of this oilseed species.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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