PEG induces maturation of somatic embryos of *Passiflora edulis* Sims ‘UENF Rio Dourado’ by differential accumulation of proteins and modulation of endogenous contents of free polyamines

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Abstract
Sour passion fruit (*Passiflora edulis* Sims) has economic and social relevance and is an alternative crop mainly for family farming agriculture. The aim of this work was to evaluate the influence of polyethylene glycol (PEG) on the maturation of somatic embryos associated with differential accumulation of proteins and changes in the endogenous polyamine (PA) content during somatic embryogenesis of *P. edulis* ‘UENF Rio Dourado’. Maturation of somatic embryos was performed using embryogenic callus in MS culture medium with PEG 6% or without PEG (control). PEG 6% promoted the maturation of a significantly higher number of somatic embryos at globular and cotyledonal stages when compared to the control treatment. The higher somatic embryo formation induced by PEG 6% was associated with an increase in endogenous contents of free spermine, a PA with an important role in the maturation process of somatic embryogenesis cultures. Comparative proteomic analyses of PEG 6%/control revealed that PEG 6% treatment induced the up-accumulation of proteins related to the ATP metabolic, glycolytic, generation of precursor metabolite energy, and the response to light stimulus processes. The down-accumulated proteins were related mainly to the cellular metabolic process. The use of PEG induced the maturation and development of somatic embryos of *P. edulis* Sims ‘UENF Rio Dourado’ by the differential accumulation of proteins and modulation of endogenous contents of PAs.

Key message
PEG induces the maturation and development of somatic embryos of *P. edulis* Sims ‘UENF Rio Dourado’ by differential accumulation of proteins and modulation of endogenous polyamine contents.

Keywords *Passiflora edulis* · Polyethylene glycol · Somatic embryogenesis · Proteomics

Introduction
Sour passion fruit (*Passiflora edulis* Sims) is present in approximately 90% of Brazilian orchards (Faleiro et al. 2019) due to its superior qualities in relation to plant health, hybrid vigor and yield of pulp used in the manufacture of juices (Viana et al. 2016). With more than 100 endemic species, passion fruit is considered part of Brazilian biodiversity, presenting high economic and social relevance, with approximately 592,698 tons produced in 2020 (IBGE 2020), serving as an alternative crop mainly for family farming agriculture (Bernacci et al. 2005; Faleiro et al. 2019). *P. edulis* ‘UENF Rio Dourado’ is a new sour passion fruit cultivar developed by the UENF plant breeding program that...
The competence and development of embryonic cells offer a biological, biochemical and molecular aspects associated with (Botini et al. 2021). Thus, studies that investigate the physi- nous contents of some compounds, such as proteins and PAs somatic embryogenesis (Vale et al. 2014). (Mishra et al. 2012), simulating the desiccation step during sure and a reduction in the intracellular osmotic potential which leads to restricted water absorption, low turgor pres- which this high-molecular- weight molecule is not able to pass through the cell wall, PEG induces water stress once. Transcriptional, post-transcriptional and post-translational regulatory mechanisms contribute to decreasing the correlation between gene and mRNA levels and protein abundance (Rose et al. 2004; Laurent et al. 2010). In this sense, proteomics approaches have been considered a powerful tool for examining the physiological and biochemical conditions at the molecular level of in vitro plant tissues and organs.

The normal development and maturation of somatic embryos can be considered one of the main bottlenecks that limits the commercial application of somatic embryogenesis (Marquez-Martín et al. 2011; Mishra et al. 2012; Vale et al. 2014) because during the maturation phase, somatic embryos undergo morphological and biochemical changes, such as storage compound deposition (Marquez-Martín et al. 2011), synthesis and mobilization of proteins, carbohydrates and lipids, and alteration of endogenous contents of polyam- ines (PAs) (Silveira et al. 2004). Polyethylene glycol (PEG) has been used to promote maturation in somatic embryogenesis in various species, including Phoenix dactylifera (Alkhateeb 2006), Carica papaya (Vale et al. 2014; Almeida et al. 2019; Botini et al. 2021), Pinus (Stasolla and Yeung 2003; Salo et al. 2016), and Cicer arietinum (Mishra et al. 2012). PEG induces water stress once this high-molecular-weight molecule is not able to pass through the cell wall, which leads to restricted water absorption, low turgor pressure and a reduction in the intracellular osmotic potential (Mishra et al. 2012), simulating the desiccation step during somatic embryogenesis (Vale et al. 2014).

The osmotic stress induced by PEG can alter the endoge- nous contents of some compounds, such as proteins and PAs (Botini et al. 2021). Thus, studies that investigate the physi- ological, biochemical and molecular aspects associated with the competence and development of embryonic cells offer a strong potential to identify important molecules that can be used to monitor the development of somatic embryos and improve the understanding regarding the particularities of the process of somatic embryogenesis (Heringer et al. 2018). PAs have been reported to act in many processes during cell proliferation and differentiation, including embryogenesis (Pal Bais and Ravishankar 2002; Silveira et al. 2004).

More recently, attention has turned to revealing the differen- tial accumulation of proteins associated with somatic embryogenesis development (Aguilar-Hernández and Loy- ola-Vargas 2018; Heringer et al. 2018). Genes encode RNAs that have different mechanisms of transcriptional and post- transcriptional regulation, which generate modifications and variations in their stability and activity (Kuersten et al. 2013). Additionally, mRNAs are translated into proteins and protein function and activity can be altered according to their subcellular localization, interaction with other mole- cules and post-translational modifications, such as phospho- rylation, glycosylation and, ubiquitination (Wu et al. 2016).

Materials and methods

Plant material and induction of somatic embryogenesis

Mature seeds of P. edulis ‘UENF Rio Dourado’ were obtained from the collection of ripe fruits at the UENF Experimental orchard (21°40′ S, 42°04′ W and altitude of 76 m). The outer integuments of seeds were removed with the help of mini-vise as described by Silva et al. (2009),
and the seeds without the outer integuments were disinfested in a laminar flow chamber by immersion in 70% ethanol (Sigma–Aldrich, St. Louis, USA) for 1 min, followed by immersion in 30% commercial bleach (sodium hypochlorite from 0.6 to 0.75%; Qboa® Anhembi SA, Osasco, Brazil) supplemented with two drops of Tween® 20 (Sigma–Aldrich) for 30 min, followed by three rinses with distilled and autoclaved water. After the final rinse, the seeds were kept overnight in sterile distilled water to rehydrate and to facilitate zygotic embryo removal according to Silva et al. (2009). The zygotic embryos were aseptically removed from the seeds in a laminar flow chamber and transferred into Petri dishes (90 mm × 15 mm) containing MS basal culture medium with vitamins (Murashige and Skoog 1962) (M519; Phytotechnology Lab, Overland Park, USA) supplemented with 100 mg L⁻¹ myo-inositol (Sigma–Aldrich), 30 g L⁻¹ sucrose (Sigma–Aldrich), 20 μM 2,4-dichlorophenoxyacetic acid (2,4-D) (Sigma–Aldrich), 5 μM 6-benziladenin (BA) (Sigma–Aldrich), and 2 g L⁻¹ Phytagel® (Sigma–Aldrich). The pH of the culture medium was adjusted to 5.7 before Phytagel® was added and autoclaved at 121 °C for 15 min. The Petri dishes containing the zygotic embryos were kept in a growth chamber at a temperature of 25 °C ± 1 and in the dark for 45 days.

After 45 days, the inducted calli were separated into embryogenic and non-embryogenic according to their morphological characteristics. The embryogenic callus was characterized by its friable and yellowish appearance, according to observations in species of the genus Passiflora (Silva et al. 2009; da Silva et al. 2015). Aiming at multiplication, the embryogenic calli were submitted to three consecutive subcultures, with an interval of 21 days each, using the same culture medium and environmental conditions of the induction phase.

**Maturation experiment**

Three embryogenic calli (300 mg of fresh matter-FM each) were inoculated into Petri dishes (90 mm × 15 mm) containing 20 mL of MS culture medium supplemented with 30 g L⁻¹ sucrose, 2 g L⁻¹ Phytagel, 100 mg L⁻¹ myo-inositol, and without (control) or with 6% PEG (3,350 wt., Sigma–Aldrich). The experiment was carried out with seven biological replicates, with each being a Petri dish with three embryogenic calli. The cultures were kept in the growth room at 25 °C ± 1 in the dark for seven days. Thereafter, the cultures were grown at a temperature of 25 ± 1 °C, and a 16-h photoperiod was established with GreenPower TLED 20-W WmB (Koninklijke Philips Electronics NV, Amsterdam, Netherlands) at 55 μmol m⁻² s⁻¹ for up to 28 days of culture.

The experiment was performed using a completely randomized design. The number of somatic embryos at the globular and cotyledonary stages was evaluated at 14 and 28 days of maturation from seven biological replicates.

**Histomorphological analysis**

Samples from PEG 6% treatment were collected at 14 days of maturation for histomorphological analysis. Embryogenic calli were fixed in fixative solution containing 2.5% glutaraldehyde (Sigma–Aldrich) and 4% formaldehyde (Sigma–Aldrich) diluted in 0.1 M sodium cacodylate buffer (Merck Millipore, Darmstadt, Germany) pH 7.2 at room temperature for 48 h. Samples were dehydrated with an increasing ethanolic series (30, 50, 70, 90 and 100%) twice at each concentration for 12 h each. Subsequently, the samples were infiltrated in 1:1 (v/v) HistoResin® (Leica, Heidelberg, Germany) and 100% ethanol (Merck Millipore) for 24 h, followed by 100% HistoResin® for 48 h, and hardened in 100% HistoResin®. Sections (5 μm) were obtained on a Leica microtome (Leica, mounted on slides (Sail Brand, Zhejiang, China) and then stained with 1% aqueous toluidine blue solution (Sigma–Aldrich). The sections were observed in an Axiol Imager M2 microscope (Carl Zeiss®, Jena, Germany) with the AxioVision 4.8 program (Carl Zeiss). Images were obtained with an AxioCam MR3 camera (Carl Zeiss) coupled to the equipment.

**Free polyamine (PA) determination**

For free PA determination, samples at 14 and 28 days in both treatments (three biological replicates, 300 mg of FM per sample) were collected, frozen in liquid nitrogen and stored at − 80 °C until analysis. PA determination was performed according to Silveira et al. (2004). For PA extraction, samples were homogenized with 1.2 mL of 5% perchloric acid (PCA; Merck Millipore), incubated at 4 °C for 1 h and centrifuged for 20 min at 16,000xg and 4 °C. The supernatant containing the free polyamines was reserved, and the pellets were re-extracted with 0.3 mL. The supernatants were collected, and free PAs in the supernatant were analyzed directly by derivatization with dansyl chloride (Merck Millipore) and identified by high-performance liquid chromatography (HPLC; Shimadzu, Kyoto, Japan) using a 5-μm C18 reverse-phase column (Shin-pack CLC ODS, Shimadzu). The column gradient was achieved by adding increasing volumes of acetonitrile (Merck Millipore) to a 10% aqueous acetonitrile solution with the pH adjusted to 3.5 with hydrochloric acid (Merck Millipore). The acetonitrile concentration was maintained at 65% for the first 10 min, increased from 65 to 100% between 10 and 13 min, and maintained at 100% between 13 and 21 min at a flow rate of 1 mL min⁻¹ and 40 °C. The PA concentrations were
determined using a fluorescence detector at 340 nm excitation and 510 nm emission. The peak areas and retention times of the samples were measured through comparisons with PA standards putrescine (Put), spermidine (Spd) and spermine (Spm) (Sigma–Aldrich).

**Proteomic analysis**

Samples of embryogenic callus at 14 days of maturation for both Control and PEG 6% treatments (three biological replicates, 300 mg of FM per sample) were collected and macerated in a mortar and pestle using liquid nitrogen and stored at -80 °C until analysis. The samples were transferred to microtubes with 1 mL of extraction buffer consisting of 7 M urea (GE Healthcare, Piscataway, USA), 2 M thiourea (GE Healthcare), 2% Triton X-100 (GE Healthcare), 1% dithiothreitol (DTT, GE Healthcare), and 1 mM phenylmethanesulfonyl fluoride (PMSF, Sigma–Aldrich). Samples were vortexed for 30 min at 8 °C in a refrigerator, followed by centrifugation at 16,000×g for 20 min at 4 °C. The supernatants were collected, and the protein concentration was measured using a 2-D Quant Kit (GE Healthcare).

Before the trypsin digestion step, protein samples were precipitated using the methanol/chloroform methodology to remove any interference from the samples (Nanjo et al. 2012). After protein precipitation, samples were resuspended in 7 M urea/2 M thiourea solution for proper resuspension. Protein digestion of aliquots of 100 µg protein from each biological replicate was performed using the filter-aided sample preparation (FASP) method using trypsin (V5111; Promega, Madison, WI, USA; final ratio 1:100 enzyme:protein) as described by Wilsniewski et al. (2009) with modifications performed by Reis et al. (2021). The resulting peptides were quantified by the A205 nm protein and peptide methodology using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, USA).

**Analysis by mass spectrometry**

Mass spectrometry was performed using a nanoAcquity ultra-high performance liquid chromatograph (nanoUPLC) connected to a Q-TOF SYNAPT G2-Si instrument (Waters, Manchester, UK). Runs consisted of three biological replicates of 1 µg of digested proteins. During separation, samples were loaded onto the nanoAcquity UPLC M-Class Symmetry C18 5 µm trap column (100 Å, 5 µm, 180 µm×20 mm, 2D; Waters) at 5 µL min⁻¹ for 3 min and then onto the nanoAcquity M-Class HSS T3 1.8 µm analytical reversed-phase column (100 Å, 1.8 µm, 75 µm×150 mm; Waters) at 400 nL min⁻¹, with a column temperature of 45 °C. For peptide elution, a binary gradient was used, with mobile phase A consisting of water (Tedia; Fairfield, USA) and 0.1% formic acid (Sigma–Aldrich) and mobile phase B consisting of acetonitrile (Sigma–Aldrich) and 0.1% formic acid. Gradient elution started at 5% B, then ramped from 5% B to 40% B up to 91.12 min, and from 40% B to 99% B until 95.12 min, being maintained at 99% until 99.12 min, then decreasing to 5% B until 101.12 min, and kept 5% B until the end of experiment at 117.00 min. Mass spectrometry was performed in positive and resolution mode (V mode), 35,000 FWHM, with ion mobility (HDMSE), and in DIA mode; ion mobility separation used an IMS wave velocity ramp starting with 800 m s⁻¹ and ending with 500 m s⁻¹; the transfer collision energy ramped from 25 to 55 V in high-energy mode; cone and capillary voltages of 30 V and 3000 V, respectively; nano flow gas of 0.5 Bar and purge gas of 150 L h⁻¹; and a source temperature of 100 °C. For the TOF parameters, the scan time was set to 0.6 s in continuum mode with a mass range of 50–2000 Da. Human [Glu1]-fibrinopeptide B (Waters) at 100 fmol μL⁻¹ was used as an external calibrant, and lock mass acquisition was performed every 30 s. Mass spectra acquisition was performed by MassLynx v 4.1 software.

**Proteomics data analysis**

Spectral processing and database searching were performed using ProteinLynx Global Server (PLGS; version 3.0.2) (Waters) and the ISOQuant software workflow (Distler et al. 2014, 2016). The PLGS was processed using a low-energy threshold of 150 (counts), an elevated energy threshold of 50, and an intensity threshold of 750. In addition, the analysis was performed using the following parameters: two missed cleavages, a minimum fragment ion per peptide equal to 3, a minimum fragment ion per protein equal to 7, a minimum peptide per protein equal to 2, fixed modifications of carbamidomethyl and variable modifications of oxidation and phosphoryl. The false discovery rate (FDR) for peptide and protein identification was set to a maximum of 1%, with a minimum peptide length of six amino acids. The proteomics data were processed against Glycine max (ID: UP000008827) from UniProtKB.

Comparative label-free quantification analyses were performed using ISOQuant software v.1.7 using previously described settings and algorithms (Distler et al. 2014, 2016). The protein identification parameters in ISOQuant were set to an FDR of 1%, a peptide score greater than six, a minimum peptide length of six amino acids, and at least two peptides per protein. Label-free quantification was estimated using the TOP3 quantification approach (Silva et al. 2006). This is followed by the multidimensional normalization process implemented within ISOQuant (Distler et al. 2014). To ensure the quality of the results after data processing, only proteins present or absent (for unique proteins) in all biological replicates were considered for differential accumulation analysis in the PEG 6% Control comparison. Proteins.
with significant Student’s t test (two-tailed; \( p < 0.05 \)) results were considered differentially accumulated (DAP), as up-accumulated if the \( \log_2 \) fold change (FC) was greater than 0.6 and down-accumulated if the \( \log_2 \) FC was less than -0.6. The proteomics MS data have been deposited in the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al. 2019) partner repository with the dataset identifier PXD031175.

Finally, proteins were submitted to functional characterization by OmicsBox software (BioBam Bioinformatics S.L., Valencia, Spain). Sequences with biological processes not identified by OmicsBox were manually complemented with the online BLAST tools UniProtKB and NCBI. The predicted interaction networks of DAPs were constructed using Arabidopsis thaliana homologs that were identified through a STRING search followed by downstream analysis in Cytoscape (version 3.9) (Shannon et al. 2003). The gene IDs referring to the regulated proteins in each treatment were used as reference set entries for the enrichment analysis. The hypergeometric test with Bonferroni step down correction was used to assess enrichment categories in the Gene Ontology (GO) domains ‘Biological process’ from the A. thaliana database. In the resultant graph, the functional grouping was evaluated with #Genes/Term using the kappa statistic. Pairs of terms (nodes) with a kappa value of at least 0.5 related to edges in the network.

### Results

#### Effect of PEG on the maturation of somatic embryos

Morphologically, embryogenic callus presented both regions of meristematic cells (MC) and non-meristematic cells (NMC) in the same callus (Fig. 1A). The MC region presented a yellow color, and the NMC showed a white color and spongy appearance (Fig. 1A) at time 0 (before the maturation phase).

A significant effect of PEG 6% was observed regarding the maturation of somatic embryos of *P. edulis* ‘UENF Rio Dourado’ after 28 days of incubation (Table 1 and Fig. 1). The embryogenic callus incubated under PEG 6% treatment showed a significantly higher number of somatic embryos at

| Somatic embryo developmental stage | PEG 0% (control) | 6% |
|-----------------------------------|------------------|----|
|                                   | Day 14 | Day 28 | Day 14 | Day 28 |
| Globular                          | 6.9 Bb | 19.7 Ba | 20.6 Ab | 45.1 Aa |
| Cotyledonary                      | 0.0 Ab | 20.7 Ba | 3.3 Ab  | 33.7 Aa |

Capital letters denote significant differences between the maturation treatments at the same time of incubation. Lowercase letters denote significant differences between the time of incubation within the same maturation treatment. Means followed by different letters are significantly different according to the Student-Newman–Keuls (SNK) test (\( p < 0.05 \)). (n = 7; CV Globular = 19.35%; CV Cotyledonary = 38.53%)

Fig. 1 Morphological aspects of embryogenic callus of *P. edulis* ‘UENF Rio Dourado’ during maturation. A embryogenic callus before maturation treatments on control and PEG 6%; B embryogenic callus with 14 days under control treatment showing green points in the surface of callus; C embryogenic callus with 28 days under control treatment showing somatic embryos at the cotyledonary stage; D embryogenic callus with 14 days under PEG 6% treatment showing green points at the surface, and somatic embryos in the globular stage (arrow); E embryogenic callus with 28 days under PEG 6% treatment with somatic embryos in the globular and cotyledonary stage; F globular somatic embryo; G cotyledonary somatic embryo. Bars: A–E 0.1 mm; F–G 0.2 mm. (Color figure online)
the globular and cotyledonary stages compared to embryogenic callus in the control treatment at both 14 and 28 days of incubation (Table 1). Embryogenic callus treated with PEG 6% (Fig. 1D, E) presented a more compact form than that in the control treatment (Fig. 1B, C) during the time of incubation. The embryogenic callus showed somatic embryo differentiation on the callus surface, as the globular (Fig. 1F) and cotyledonary (Fig. 1G) stages were observed.

Histomorphological aspects of embryogenic callus under PEG in the maturation of somatic embryos

Embryogenic callus of *P. edulis* at 14 days of maturation was composed of different tissues (Fig. 2A) with a more compact appearance, yellowish color and a structure more organized (Fig. 2B), and some parts of the callus was less compact, with spongy appearance or soft mass with translucent aspect (Fig. 2C). The histomorphological analyses showed that the compact embryogenic callus was composed of meristematic-type cells (MC) with small and isodiametric cells, prominent nuclei and dense cytoplasm (Fig. 2D; yellow arrow). The soft mass of the callus showed nonmeristematic-type cells (NMC), which were larger and elongated cells and highly vacuolated (Fig. 2D; black arrow).

The development of somatic embryos occurs from the MC tissue of embryogenic callus of *P. edulis*. At 14 days of maturation was possible to observe the formation of somatic embryos at earlier stages of development, mainly in the periphery of the callus (Fig. 2E), observing the presence of globular (Fig. 2F) and heart somatic embryos (Fig. 2G).

PEG 6% induces changes in endogenous contents of polyamines (PAs) during maturation of somatic embryos

The contents of free PAs (Fig. 3) were affected by the maturation treatments (PEG 6% or control) and during incubation. Embryogenic callus incubated in PEG 6% presented significantly higher levels of total free PAs (Fig. 3A) at 28 days of culture than those incubated in the control treatment. PEG 6% induced a significantly higher content of Spm (Fig. 3C) in embryogenic callus than in that incubated in the control treatment at both incubation times. A significant reduction in the endogenous Spm contents was also observed for both treatments at 14 and 28 days of incubation.

**Fig. 2** Morphological and histomorphological aspects of *P. edulis* embryogenic callus matured under 6% PEG at 14 days. A Embryogenic callus showing both meristematic type cells (MC) and nonmeristematic-type cells (NMC); B MC; C NMC; D histomorphology of embryogenic callus showing MC (yellow arrow) and NMC (white arrow) tissues. E Embryogenic callus showing the MC from which the somatic embryos differentiate in the periphery of the callus; F globular somatic embryo; G heart somatic embryo (yellow asterisk). (Color figure online)
Differential accumulation of proteins (DAPs) and biological process enrichment proteins during the maturation of somatic embryos

The comparative proteomics analysis of embryogenic callus at 14 days of maturation allowed the identification of a total of 514 proteins (Supplementary Table S1). The comparison between embryogenic callus matured under PEG 6% with those from the control treatment (PEG 6%/control comparison) revealed 51 differentially accumulated proteins (DAPs), with 15 up- and 20 down-accumulated, 14 proteins unique to PEG 6% and two proteins unique to the control (Table 2).

The network of enriched biological processes by STRING for up-accumulated and unique proteins in PEG 6% resulted in the enrichment of the main processes, such as ATP metabolic process and glycolytic process, with proteins such as enolase (ENO1; I1L3K7), glyceraldehyde-3-phosphate dehydrogenase GAPCP1, chloroplastic (GAPCP-1; I1MM44), ATP synthase CF1 beta subunit (ATPB; Q2PMV0), triosephosphate isomerase, cytosolic (TPI; A0A368UHG1) and pyruvate dehydrogenase E1 component subunit alpha, mitochondrial (IAR4; I1N1B9) (Fig. 4A and Table 2), the generation of precursor and metabolites energy process with 6-phosphogluconate dehydrogenase, decarboxylating 3 isoform A (AT3G02360; I1N6I4), ras-related protein RABE1 c-like (AT5G59840; I1LK56), malate dehydrogenase [NADP], chloroplastic (I1LCM5; AT5G58330), TPI, succinate dehydrogenase [ubiquinone] flavoprotein subunit 1, mitochondrial (SDH1-1; I1J8W0) and IAR4 proteins, and the process related to response to light stimulus with chlorophyll a-b binding protein 21, chloroplastic (LHB1B1) and ribulose biphosphate carboxylase/oxygenase activase, chloroplastic (RCA; D4N5G3) proteins (Fig. 4A and Table 2).

Down-accumulated and unique proteins in embryogenic callus in the control treatment were enriched mainly for cellular metabolic processes with cell division cycle protein 48 homolog (ATCDC48B; P54774), UDP-glucose 6-dehydrogenase1 (UGD2; I1LWR5), UDP-glucose6-dehydrogenase4 (UGD2; I1J5Y9), NADP-dependent
Table 2  Differentially accumulated proteins (DAPs) in the embryogenic callus of *P. edulis* ‘UENF Rio Dourado’ at 14 days of maturation under PEG 6% treatment compared with the control treatment (without PEG)

| Accession | Description | Arabidopsis ortholog (String mapping) | Biological process |
|-----------|-------------|----------------------------------------|--------------------|
| **Up-accumulated** | | | |
| I1L3K7 | Enolase | ENO1 | ATP metabolic process, Glycolytic process, Carbohydrate metabolic process |
| I1M6E1 | Heat shock cognate protein 80 | HSP81-3 | Protein folding, response to temperature stimulus, cellular process |
| I1MM44 | Glyceraldehyde-3-phosphate dehydrogenase GAPCP1, chloroplastic | GAPCP-1 | ATP metabolic process, Glycolytic process, Carbohydrate metabolic process |
| I1N6I4 | 6-phosphogluconate dehydrogenase, decarboxylating 3 isoform A | AT3G02360 | Generation of precursor and metabolites energy, Carbohydrate metabolic process, Phosphate-containing compound metabolic process |
| A0A0R0EWS1 | Ubiquitin-activating enzyme E1 1 | UBA1 | Response to metal ion, cellular metabolic process, response to cadmium ion |
| I1LK56 | Ras-related protein RABE1c-like isoform X2 | AT5G59840 | Generation of precursor and metabolites energy, Carbohydrate metabolic process |
| I1LCM5 | Malate dehydrogenase [NADP], chloroplastic | AT5G58330 | Generation of precursor and metabolites energy, Carbohydrate metabolic process |
| I1NBU8 | Putative pectinesterase/pectinesterase inhibitor 41 | AT2G47550 | Cell wall modification |
| Q2PMV0 | ATP synthase CF1 beta subunit | ATPB(PB) | ATP metabolic process |
| Q43468 | HSP70-HSP90 organizing protein 1 | HOP2 | Protein folding, Stress response |
| A5JNV7 | Superoxide dismutase [Mn], mitochondrial | MSD1 | Response to metal ion, cellular metabolic process |
| A0A0R4J318 | Peptidyl-prolyl cis–trans isomerase CYP19-3 | ROC2 | Response to metal ion, Protein folding, cellular metabolic process |
| K7M8F8 | Probable fructokinase-6, chloroplastic | AT1G66430 | Cellular metabolic process, Carbohydrate metabolic process, phosphate-containing compound metabolic process |
| I1MQM4 | Glutamate decarboxylase | GAD5 | Glutamate metabolic process |
| A0A0R0JER2 | Peptidyl-prolyl cis–trans isomerase CYP40-like | SQN | Protein folding, cellular metabolic process |
| **Down-accumulated** | | | |
| P54774 | Cell division cycle protein 48 homolog | ATCDC48B | Cellular metabolic process |
| I1LYX2 | 5-methyltetrahydropteroyltriglutamate–homocysteine methyltransferase | ATMS1 | Amino-acid biosynthesis Methionine biosynthesis |
| A0A0R0FY49 | Heat shock 70 kda protein | HSP70b | Protein folding, Protein transport, Small GTPase mediated signal transduction |
| A0A0R0K398 | Guanosine nucleotide diphosphate dissociation inhibitor 2 | GDI2 | Protein transport, Small GTPase mediated signal transduction |
| I1J7T0 | Pectinesterase 2 | AT2G45220 | Cell wall modification, Pectin catabolic process |
| I1LWR5 | UDP-glucose 6-dehydrogenase 1 | UGD2 | Cellular metabolic process |
| I1J5Y9 | UDP-glucose 6-dehydrogenase 4 | UGD2 | Cellular metabolic process |
| I1J4J8 | NADP-dependent malic enzyme | NADP-ME4 | Cellular metabolic process, Malate metabolic process, Pyruvate metabolic process |
| Q6QZ28 | Phosphoenolpyruvate carboxylase 2 | PPC1 | Cellular metabolic process, Carbon fixation, Tricarboxylic acid cycle |
| I1JTY8 | Aminopeptidase M1 isoform X2 | APM1 | Proteolysis |
| A0A0R0G163 | Sucrose synthase 2 isoform X2 | SSA | Cellular metabolic process, Sucrose metabolic process |
| I1LW13 | Hypersensitive-induced response protein-like protein 2 | AT1G69840 | Unknow (Binding) |
| I1KSD7 | Mitochondrial dicarboxylate/tricarboxylate transp. DTC | AT5G19760 | Transport |
| I1KV07 | ATP-dependent 6-phosphofructokinase 3 | PFK3 | Cellular metabolic process, Glycolysis |
| I1JYA9 | Superoxide dismutase [Mn], mitochondrial | MSD1 | Response to metal ion, cellular metabolic process |
malic enzyme (NADP-ME4; I1J4J8), phosphoenolpyruvate carboxylase 2 (PPC1; Q6Q2Z8) and sucrose synthase 2 isoform X2 (SSA; A0A0R0G163) (Fig. 4B and Table 2).

Other biological processes elucidated by GO (Gene Ontology) by UniProt showed that up-regulated proteins in embryogenic callus matured in PEG 6%, such as cell wall modification with pectinesterase protein (AT5G27870), protein folding and stress response with HSP81-3, HSP60 and HOP2, and down-regulated proteins were observed in biological processes such as sucrose metabolic process (SSA), protein transport (GDI2), tricarboxylic acid cycle (PPC1) and malate metabolic process (NADP-ME4) (Table 2).

### Discussion

**Effect of PEG on the maturation of somatic embryos and changes in PA contents**

The use of 6% PEG promoted a significantly higher number of somatic embryos at the cotyledonary stage than the control (Table 1). Similarly, the use of PEG resulted in the highest number of somatic embryos reaching the maturation phase in other species, as observed for *Panax ginseng* (Langhansova et al. 2004), *Picea abies* (Hudec et al. 2016), *C. papaya* (Heringer et al. 2013; Vale et al. 2018), and *Texas ebony* (Ibarra-López et al. 2021). PEG molecules are large and unable to pass through cell walls, which leads to a restriction of water absorption
and reduced turgor pressure, reducing the intracellular osmotic potential and ultimately leading to desiccation (Misra et al. 1993; Vale et al. 2018). The effect of PEG mimics naturally occurring water stress on seeds during late stages of maturation, and the stress caused by PEG increases concentrations of ABA, which are essential for somatic embryo development (Stasolla and Yeung 2003; Bohanec et al. 2010). Our findings show that PEG 6% can be an efficient treatment of the maturation process, allowing the optimization of somatic embryogenesis protocols of *P. edulis* ‘UENF Rio Dourado’.

Embryogenic callus matured under PEG 6% showed a higher proportion of compact tissue, which contained MC, allowing them to be called embryogenic (Fig. 2). These cells are small and isodiametric, have cytoplasm-rich cells and allow the formation of somatic embryos (Fig. 2D). In contrast, the non-embryogenic tissue of the callus presented elongated and highly vacuolated cells that were dispersed throughout the callus (Fig. 2D). During subculture cycles, it is possible to separate embryogenic callus from non-embryogenic callus by morphological characteristics, as shown in somatic embryogenesis in sugarcane (Silveira et al. 2013). The presence of MC and NMC cells in the same callus was previously shown for several plant systems (Fehér et al. 2003; Silveira et al. 2013), including other species of the Passifloracea family (Silva et al. 2009; Paim-Pinto et al. 2011; Silva et al. 2015). It is known that the acquisition of embryogenic competence requires the presence of non-embryogenic cells that produce and secrete molecules in the culture medium (Hecht et al. 2001). These molecules could then be perceived by other cells, which in turn could become competent and develop into somatic embryos (Pennell et al. 1992; Santa-Catarina et al. 2004; Silveira et al. 2013). In this sense, the presence of non-embryogenic cells within embryogenic callus could be relevant to the development of somatic embryos in *P. edulis*.
In addition to morphological changes, genetic and biochemical factors are also important for understanding somatic embryogenesis in various plant somatic cells (Karami and Saidi 2010). Among the biochemical molecules, PA content could be reported as a marker for the acquisition of somatic embryogenesis competence (Santa-Catarina et al. 2004; Silveira et al. 2013). In our study, the total free PA levels showed that PEG 6% treatment was associated with an increase in the levels of total free PAs at 28 days of the maturation process (Fig. 3). In addition, a higher level of Spm was observed in embryogenic callus treated with PEG 6% compared to the control (Fig. 3C), suggesting the relevant role of Spm in the maturation of somatic embryogenesis in *P. edulis*. Osmotic stress also increased free Spm levels in *Pinus sylvestris* proembryogenic cell cultures under PEG (10%) treatment and had no effect on free Put and Spd levels (Muilu-Mäkelä et al. 2015). The increase in Spm levels was associated with a reduction in cell proliferation due to osmotic stress, suggesting the possible role of Spm in the inhibition of cell mass growth of *P. sylvestris* (Muilu-Mäkelä et al. 2015). In embryogenic callus of sugarcane, high levels of Spm and Spd were associated with the acquisition of embryogenic competence that allows the maturation of somatic embryos (Silveira et al. 2013). Further studies on the exogenous addition of PAs in the maturation process of *P. edulis* ‘UENF Rio Dourado’ embryogenic callus should be developed to verify the effects of exogenous PAs on somatic embryo maturation and development.

**Effect of PEG 6% on the differential accumulation of proteins during the maturation of somatic embryos**

The use of proteomics as a tool for understanding biochemical and molecular aspects has been important in the study of somatic embryogenesis (Campos et al. 2017; Heringer et al. 2018). Comparative proteomic analysis between embryogenic callus treated with PEG 6% and control treatments was performed in *P. edulis* cv ‘UENF Rio Dourado’, and the results are discussed based on the DAPs and unique proteins and their relationship with somatic embryogenesis development.

**ATP metabolic process, glycolytic process and generation of precursor metabolite energy**

The glycolytic process has been considered a central process in the development of somatic embryos and seeds in different species, especially in the maturation process (Carrari and Fernie 2006; Fait et al. 2006; Xu et al. 2012; Ge et al. 2014). We identified several glycolytic proteins unique to PEG 6% treatment or up-accumulated in embryogenic callus at 14 days of maturation with PEG 6% compared to the control treatment, such as ENO1, GAPCP-1, ATPB, TPI and IAR4, which modulate the glycolytic pathway.

In glycolysis, plant cells modify carbohydrates in energy by ATP, which is essential to generate intermediate metabolites that modulate the biosynthesis of molecules at the intra- and extracellular levels required by cells (Aguilar-Hernández and Loyola-Vargas 2018). During developmental processes, the cell requires a greater energy supply for the new formation of embryos, and the proteins of the glycolytic pathway are associated with the development of somatic embryos and embryogenic competence in different species, such as sugarcane (Heringer et al. 2015), *Zea mays* (Varhaníková et al. 2014) and *C. papaya* (Vale et al. 2014; Almeida et al. 2019).

In our study, the TPI protein was identified as a unique PEG 6% treatment. This glycolytic enzyme is essential for glycolysis, mainly for energy generation (Zhou et al. 2009; Zhao et al. 2015). The TPI enzyme was identified as a protein associated with the initial stages of somatic embryo formation in embryogenic callus (Xu et al. 2012; Zhao et al. 2015; Almeida et al. 2019) and in response to PEG treatments in the maturation process (Vale et al. 2014).

**Response to light stimulus**

In our study, we identified proteins related to photosynthesis biological processes in embryogenic callus at 14 days of maturation under PEG 6% (Fig. 4), including two proteins, RCA and LHB1B1 (Unique Peg 6%) (Table 2 and Fig. 4A). The initial differentiation of photosynthetic tissues during somatic embryogenesis seems to be associated with coordinated expression of mRNA for *rbcL*, *lhcb* and *por* in late torpedo-shaped embryos (Sato-Nara et al. 2004). Thus, the photosynthesis-related proteins that differentially accumulated in embryogenic callus matured under PEG 6% compared with the control treatment were relevant for photosynthetic apparatus differentiation.

**Response to stress-related proteins and Protein Folding**

The network process identified HOP2, HSP81-3 and HSP60 as up-regulated proteins that are related to the stress response. The stress response is a recurrent common protein group that is regulated in embryogenic cultures and is associated with the induction of somatic embryo formation (Heringer et al. 2018). As PEG acts as an osmotic agent that causes osmotic stress, the identification of up-regulated proteins could be associated with the stress response in embryogenic callus of *P. edulis* treated with PEG 6%. Stress response proteins are frequently reported in dividing cells or tissues, among which heat-shock proteins (HSPs) were found to be most representative in embryogenic callus (Zhao et al. 2015). HSP proteins have also been identified during somatic embryogenesis of *Vitis vinifera* (Zhang et al. 2001).
abundance mainly in zygotic embryo formation (Chollet et al. 2015 and Fraga et al. 2016). In this sense, the increase in the accumulation of HSP proteins in embryogenic callus of *P. edulis* treated with PEG suggests the relevance of these proteins for maturation processes in this species.

Another protein that was unique in Peg 6% was 14-3-3 protein (AT2G42590; K7LWG5) (Table 2). The 14-3-3 protein is a highly conserved phosphoserine/phosphothreonine-binding protein that regulates a wide range of target proteins in all eukaryotes and may play important roles in the response to environmental, metabolic and nutritional stresses (Roberts et al. 2002; Zhao et al. 2015).

Working with embryogenic and non-embryogenic tissues of *L. principis-rupprechtii*, regulation of ATP synthases by the 14-3-3 protein was observed, suggesting a mechanism for plant cells to adapt to environmental changes such as nutrient supply, especially exogenous plant growth regulators, during somatic embryogenesis (Zhao et al. 2015).

Up-accumulated proteins related to cell wall modification

During the transition of somatic embryos from embryogenic callus of *Manihot esculenta*, several expression genes were involved in the polysaccharide hydrolase of the cell wall and pectinesterase precursors (Kohli et al. 2015). Pectinesterase proteins are enzymes responsible for breaking the glycosidic bond of pectin substances in the cell wall through hydrolysis (Kohli et al. 2015; Kumaravel et al. 2020) and can catalyze the de-esterification of pectin to form a pectate gel (Micheli 2001; Kohli et al. 2015). In addition, pectinesterase enzymes can act to loosen the cell wall by pectin degradation and influence cell expansion during the maturation of somatic embryos (Kumaravel et al. 2020). In our work, we observed that one pectinesterase (AT5G27870; I1KMW7) was up-accumulated in embryogenic callus treated with PEG 6% compared with the control treatment, suggesting its involvement in the development of somatic embryos during the maturation process, possibly by modulating biosynthesis and cell wall expansion.

Cellular metabolic process

Among the DAPs, some proteins related to cellular metabolic processes, such as ATCDG48B, UGD2, NADP-ME4, PPC1 and SSA, were down-regulated in embryogenic callus incubated with PEG 6% compared to the control treatment (Table 2 and Fig. 4B). PPC1 is an enzyme with increased abundance mainly in zygotic embryo formation (Chollet et al. 1996) and catalyzes phosphoenolpyruvate to yield oxaloacetate (OAA) (Chollet et al. 1996). These enzymes were related to photosynthesis, but in the last decade, the higher number of studies showing PPC1 also increased abundance in non-photosynthetic conditions, especially in seeds, because these enzymes use HCO$_3^-$ liberated by respiration to yield oxaloacetate, which is converted into aspartate, malate and other intermediates of the TCA cycle (Leblón et al. 1991; Golombek et al. 1999; O’Leary et al. 2011; Noah et al. 2013). In general, the higher levels of the TCA cycle and oxidative phosphorylation enzymes in somatic embryos suggest a more active aerobic/respiration pathway (Noah et al. 2013).

In our study, PPC1 was down-accumulated in embryogenic callus matured under PEG 6% compared with the control treatment, suggesting that this enzyme may modulate the conversion of oxaloacetate in intermediates of the TCA cycle and may capture the energy necessary for the development of somatic embryos.

In the future, metabolic studies are recommended to verify whether the presence and absence of PEG 6% can cause a higher level of the intermediates of the TCA cycle that is related to cellular respiration because we now verify that PPC1 may be an indication that in the absence of PEG 6% treatment, the embryogenic callus may be performing more cellular respiration to produce energy to form somatic embryos than with PEG treatment in *P. edulis* ‘UENF Rio Dourado’.

Conclusion

The use of PEG 6% promoted the maturation of somatic embryos of *P. edulis* ‘UENF Rio Dourado’, significantly increasing the number of somatic embryos at the globular and cotyledonary stages compared to the control treatment. The addition of PEG 6% significantly increased the endogenous contents of Spm in embryogenic callus, which was related to the effect of PEG treatment on the development of somatic embryos during the maturation process. Embryogenic callus treated with PEG 6%, when compared to the control, showed an up-accumulation of proteins related to glycolytic processes and responses to light stimulus, which are necessary for somatic embryo development. This is the first report showing somatic embryo development for *P. edulis* ‘UENF Rio Dourado’.

Supplementary Information

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**Author contributions** KZCMC and VS designed the research; KZCMC, NB and EMV conducted the experiment. KZCMC, FAA and EMV carried out the proteomics and statistical analyses; KZCMC, RGV and CS-C performed the histomorphological analysis; KZCMC, RGV and RCS performed the PA analysis. All the authors read, reviewed and approved the manuscript.

**Data availability** The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD031175. The list of all identified proteins is available in the supplementary material.

**Declarations**

**Conflict of interest** The authors have no conflicts of interest to declare that are relevant to the content of this article. The authors declare they have no financial interests.

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