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 cotyledonary 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. PEG also promoted a significantly higher content of putrescine and total free PAs at the end of maturation, which was relevant for the increase in the number of somatic embryos. 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.

Introduction

Sour passion fruit (*P. 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 presents climate characteristics north and northwest of Rio de Janeiro (Viana et al. 2016).

Conventional propagation of *P. edulis* occurs via seminiferous (Faleiro et al. 2019). Micropropagation approaches based on *in vitro* tissue and organ culture of *Passiflora* species have been developed for scale-up clonal propagation and production of highly phytosanitary plantlets, germplasm conservation, protoplast development, somatic hybridization, genetic transformation and synthetic seed development (Otoni et al. 2013; Ozarowski and Thiem 2013; Faleiro et al. 2019). The first study using *in vitro* plant cells, tissue and culture with *Passiflora* was reported by Nakayama (1966) and involved shoot production from stem segments of mature plants of *Passiflora caerulea*. After some years, somatic embryogenesis studies have also been performed in the *Passiflora* genus (Otoni et al. 1995; Anthony et al. 1999; Silva et al. 2009; Paim-Pinto et al. 2011; Rosa et al. 2013; Silva et al. 2015; Prudente et al. 2017).

Somatic embryogenesis is a morphogenetic process analogous to zygotic embryogenesis, in which single cells or a small group of somatic cells act as precursors of embryos (Tautorius et al. 1991). The developmental stages of somatic and zygotic embryos are similar; however, in contrast to zygotic embryogenesis, somatic embryogenesis allows embryo differentiation from a diverse set of somatic tissues (Dodeman et al. 1997).

The normal development and maturation of somatic embryos can be considered one of the main bottlenecks that limits the commercial application of somatic embryogenesis (Márquez-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 (Márquez-Martín et al. 2011), synthesis and mobilization of proteins, carbohydrates and lipids, and alteration of endogenous contents of polyamines (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 endogenous contents of some compounds, such as proteins and PAs. Thus, studies that investigate the physiological, 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). PAs have been studied mainly as important routes for plant regeneration and as a potential model system for studying regulatory events of *in vitro* plant morphogenesis (Yadav and Rajam 1997).

More recently, attention has turned to revealing the differential regulation of proteins associated with somatic embryogenesis development (Aguilar-Hernández and Loyola-Vargas 2018; Heringer et al. 2018). Proteomics approaches have been considered a powerful tool for examining the physiological and biochemical conditions of plant tissues and organs. The differentially regulated proteins during the developmental stages of somatic embryos are closely related to various cellular processes, such as cell division, cell wall modifications and defense response (Kumaravel et al. 2020). Thus, identifying the differentially accumulated proteins associated with the maturation process has become an important strategy for understanding the molecular mechanisms related to somatic embryo development and identifying new biomarker candidates that could be used to develop strategies to improve somatic embryogenesis protocols (Isah 2019; Kumaravel et al. 2020; Botini et al. 2021). The aim of this work was to evaluate the influence of polyethylene glycol (PEG) on the maturation of somatic embryos associated

with the differential accumulation of proteins and changes in the endogenous polyamine (PA) content during somatic embryogenesis of *P. edulis* 'UENF Rio Dourado'.

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, 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 x 15 mm) containing MS (Murashige and Skoog 1962) culture medium (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-benziladenina (BA) (Sigma–Aldrich), and 2 g L⁻¹ Phytigel® (Sigma–Aldrich). The pH of the culture medium was adjusted to 5.7 before Phytigel® was added and autoclaved at 121°C for 15 minutes. 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 embryogenic callus was separated by a friable yellowish callus appearance (Campos et al. 2017). Aiming at multiplication, the embryogenic calli were transferred to new culture medium by three consecutive subcultures with 21 days between each under the same conditions and culture medium.

Maturation experiment

Three embryogenic calli (300 mg of fresh matter-FM- each) were inoculated into Petri dishes (90 mm x 15 mm) containing 20 mL of MS culture medium supplemented with 30 g L⁻¹ sucrose, 2 g L⁻¹ Phytigel, 100 mg L⁻¹ myo-inositol, and without (control) or with 6% PEG. 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 W_mB (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. Samples from PEG 6% treatment were collected at 14 days of maturation for histomorphological analyses. Samples from both the control and PEG 6% treatments were collected at 14 days of maturation for proteomic analyses. For PA determination, samples at 14 and 28 days in both treatments were collected. For PA and proteomic analysis, samples were frozen in liquid nitrogen and stored at -80°C until analysis.

Histomorphological analysis

Embryogenic calli from PEG 6% treatment at 14 days of maturation 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 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 AxioImager 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

Free PA determination was performed according to Santa-Catarina et al. (2006). 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 16,000 ×g and 4°C. 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 concentration was 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 macerated in a mortar and pestle using liquid nitrogen. The samples were transferred to microtubes with 1 mL of extraction buffer, vortexed for 5 min

and incubated on ice for 30 min. The extraction buffer consisted 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 phenylmethanesulfonylfluoride (PMSF, Sigma–Aldrich). Samples were vortexed for 30 min at 8°C and centrifuged 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 interferent 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 as described by Wisniewski 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 UPLC 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 x 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 x 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 (HDMS^E), 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 V 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 to 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. 2014a; Distler et al. 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. 2014a; Distler et al. 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. 2014b). 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.

Statistical analysis

All experiments were performed using a completely randomized design. The data were analyzed using analysis of variance (ANOVA) ($p < 0.05$) followed by the Student–Newman–Keuls (SNK) test using the R software environment (R Core Team 2014).

Results

Effect of PEG on the maturation of somatic embryos

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; Fig. 1). The embryogenic callus incubated under PEG 6% treatment showed a significantly higher number of somatic embryos at the globular and cotyledonary stages compared to embryogenic callus in the control treatment at both 14 and 28 days of incubation (Table 1).

Table 1

Number of somatic embryos (per Petri dish) of *P. edulis* ‘UENF Rio Dourado’ at the globular and cotyledonary stages from embryogenic callus matured under the control and PEG 6% treatments.

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%).				

Morphologically, embryogenic callus treated with PEG 6% (Fig. 1D-E) presented a more compact aspect than embryogenic callus 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 is composed of different portions of tissues (Fig. 2A) with a more compact appearance, yellowish color and a structure more organized (Fig. 2B), and some parts of the callus are 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). The soft mass of the callus showed nonmeristematic-type cells (NMC), which were larger and elongated cells and highly vacuolated (Fig. 2D).

The development of somatic embryos occurs from the MC portion of the embryogenic callus of *P. edulis*. At 14 days of maturation, the embryogenic callus allowed us 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) and free Put (Fig. 3B) at 28 days of culture than those incubated in the control treatment. In addition, PEG 6% induced a significantly higher content of Spm (Fig. 3C) in embryogenic callus compared to those incubated in the control treatment at both incubation times. The levels of endogenous Spd showed no significant changes (Fig. 3D).

PEG 6% induces a differential accumulation of 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 1). 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).

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).

Acession	Description	<i>Arabidopsis</i> 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, Carbon fixation in photosynthetic organisms, 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 RAB1c-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
A5JVZ7	Superoxide dismutase [Mn], mitochondrial	MSD1	Response to metal ion, cellular metabolic process

Acession	Description	<i>Arabidopsis</i> ortholog (STRING Mapping)	Biological 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
I1LXY2	5- methyltetrahydropteroyltriglutamate- -homocysteine methyltransferase	ATMS1	Amino-acid biosynthesis Methionine biosynthesis
A0A0R0FY49	Heat shock 70 kda protein	HSP70b	Protein folding
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
Q6Q2Z8	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
I1LWI3	Hypersensitive-induced response protein-like protein 2	AT1G69840	Unknow (Binding)

Acession	Description	<i>Arabidopsis</i> ortholog (STRING Mapping)	Biological Process
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
I1JHT1	Glutamate decarboxylase 1	GAD	Response to metal ion, Cellular metabolic process
A0A0R4J656	Chaperonin CPN60-2, mitochondrial	HSP60	Response to metal ion, Cellular metabolic process
Q5F2M9	Glutamate dehydrogenase 1	GDH1	Response to metal ion, Cellular metabolic process
I1LQD7	Fructokinase-2 isoform B	AT3G59480	Cellular metabolic process
A0A0R0FCD2	Elongation factor Tu, chloroplastic	RABE1b	Cellular metabolic process
UNIQUE PEG 6%			
D4N5G3	Ribulose biphosphate carboxylase/oxygenase activase, chloroplastic	RCA	Response to temperature stimulus, Response to light stimulus
A0A368UHG1	Triosephosphate isomerase, cytosolic	TPI	ATP metabolic process, Generation of precursor metabolites and energy, Glycolytic process
I1LZ47	60S ribosomal protein L12	AT2G37190	Cellular metabolic process, Response to temperature stimulus
I1KPN3	Luminal-binding protein 5	BIP2	Response to metal ion, Protein folding, Cellular metabolic process
P09755	Chlorophyll a-b binding protein 21, chloroplastic	LHB1B1	Photosynthesis, light harvesting in photosystem I, response to light stimulus
K7KXN7	Tubulin alpha-3 chain-like	TUA5	Cellular process, Microtubule-based process
K7LWG5	14-3-3-like protein D isoform X1	AT2G42590	Protein DNA binding
I1L0D6	Berberine bridge enzyme-like 21	AT4G20840	Oxidative stress

Acession	Description	<i>Arabidopsis</i> ortholog (STRING Mapping)	Biological Process
I1J8W0	Succinate dehydrogenase [ubiquinone] flavoprotein subunit 1, mitochondrial	SDH1-1	ATP metabolic process, Generation of precursor metabolites energy, Phosphate-containing compound metabolic process
I1N1B9	Pyruvate dehydrogenase E1 component subunit alpha, mitochondrial	IAR4	ATP metabolic process, Generation of precursor metabolites and energy, Glycolytic process, Carbohydrate metabolic process
I1L0H7	Berberine bridge enzyme-like 21	AT4G20840	Oxidative stress.
I1KMW7	Pectinesterase/pectinesterase inhibitor	AT5G27870	Cell wall modification, Pectin catabolic process.
I1LAL4	Chaperonin CPN60-2, mitochondrial	HSP60	Protein folding, Cellular metabolic process, Response to cadmium ion
Q43467	Elongation factor Tu, chloroplastic	RAB1b	Cellular metabolic process
UNIQUE CONTROL			
A0A0R0GAY5	Peroxidase 16	AT2G18980	Response to oxidative stress
A0A0R0H9T5	Peptidyl-prolyl cis-trans isomerase	ROC3	Protein folding

Considering the DAPs during the maturation of embryogenic callus at 14 days under PEG 6%/control, some proteins related to somatic embryogenesis development were up-accumulated, such as enolase (ENO1; I1L3K7), heat shock cognate protein 80 (HSP81-3; I1M6E1), glyceraldehyde-3-phosphate dehydrogenase GAPCP1, chloroplastic (GAPCP-1; I1MM44), 6-phosphogluconate dehydrogenase, decarboxylating 3 isoform A (AT3G02360; I1N6I4), superoxide dismutase [Mn], mitochondrial (MSD1; A5JVZ7), ubiquitin-activin enzyme E1 1 (UBA1; A0A0R0EWS1), peptidyl-prolyl cis-trans isomerase CYP19-3 (ROC2; A0A0R4J318), ras-related protein RAB1 c-like (AT5G59840; I1LK56), probable fructokinase-6, chloroplastic (AT1G66430; K7M8F8), glutamate decarboxylase (GAD5; I1MQM4), malate dehydrogenase [NADP], chloroplastic (I1LCM5; AT5G58330), putative pectinesterase/pectinesterase inhibitor 41 (AT2G47550; I1NBU8), ATP synthase CF1 beta subunit (ATPB; Q2PMV0) and and, HSP70-HSP90 organizing protein 1 (HOP2, Q43468) (Table 2).

Additionally, unique proteins in PEG 6%, related to somatic embryogenesis development, were observed, such as ribulose biphosphate carboxylase/oxygenase activase, chloroplastic (RCA; D4N5G3), triosephosphate isomerase, cytosolic (TPI; A0A368UHG1), 60S ribosomal protein L12 (AT2G37190; I1LZ47), luminal-binding protein 5 (BIP2; I1KPN3), chlorophyll a-b binding protein 21, chloroplastic

(LHB1B1; P09755), chaperonin CPN60-2, mitochondrial (HSP60; I1LAL4), tubulin alpha-3 chain-like (TUA5; K7KXN7), 14-3-3-like protein D isoform X1 (AT2G42590; K7LWG5), berberine bridge enzyme-like 21 (AT4G20840; I1L0D6 and I1L0H7), succinate dehydrogenase [ubiquinone] flavoprotein subunit 1, mitochondrial (SDH1-1; I1J8W0) and pyruvate dehydrogenase E1 component subunit alpha, mitochondrial (IAR4; I1N1B9) (Table 2).

In relation to down-accumulated proteins in PEG 6%/control comparison, some proteins related to somatic embryogenesis development were also observed, such as cell Division cycle protein 48 homolog (ATCDC48B; P54774), 5-methyltetrahydropteroyltriglutamate–homocysteine methyltransferase (ATMS1; I1LXY2), heat shock 70 kDa protein (HSP70-B; A0A0R0FY49), guanosine nucleotide disphosphate dissociation inhibitor 2 (GDI2; A0A0R0K398), superoxidase dismutase [Mn], mitochondrial MSD1; I1JYA9), pectinesterase 2 (AT2G45220; I1J7T0), glutamate decarboxylase 1 (GAD; I1JHT1), UDP-glucose 6-dehydrogenase1 (UGD2; I1LWR5), UDP-glucose6-dehydrogenase 4 (UGD2; I1J5Y9), NADP-dependent malic enzyme (NADP-ME4; I1J4J8), chaperonin CPN60-2, mitochondrial (HSP60; A0A0R4J656), glutamate dehydrogenase 1 (GDH1; Q5F2M9), fructokinase-2 isoform B (AT3G59480; I1LQD7), phosphoenolpyruvate carboxylase 2 (PPC1; Q6Q2Z8), elongation factor Tu, chloroplastic (RABE1b; A0A0R0FCD2), aminopeptidase M1 isoform X2 (APM1; I1JTY8), sucrose synthase 2 isoform X2 (SSA; A0A0R0G163), hypersensitive-induced response protein0like protein 2 (AT1G69840; I1LWI3), mitochondrial dicarboxylate/tricarboxylate transport DTC (AT5G19760; I1KSD7), ATP-dependent 6-phosphofructokinase 3 (PFK3; I1KV07) and, the peroxidase 16 (AT2G18980; A0A0R0GAY5) and peptidyl-prolyl cis-trans isomerase (ROC3; A0A0R0H9T5) was unique in the control treatment (Table 2).

Biological process enrichment proteins under PEG 6%/control at 14 days of embryogenic callus maturation of *P. edulis* 'UENF Rio Dourado'

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 ENO1, GAPCP-1, ATPB, TPI and IAR4, generation of precursor and metabolite energy with AT3G02360, AT5G59840, AT5G58330, TPI, SDH1-1, IAR4 proteins and response to light stimulus with LHB1B1 and RCA proteins (Fig. 4A; Table 2). Down-accumulated and unique proteins in embryogenic callus in the control treatment were enriched mainly for cellular metabolic processes with ATCDC48B, UGD2, NADP-ME4, PPC1 and SSA (Fig. 4B; 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 callus, which contained MC, allowing them to be called embryogenic callus (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 portion 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 *Passifloraceae* 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 and free Put at 28 days of the maturation process (Fig. 3). Similarly, embryogenic callus of sugarcane showed a higher level of total free PAs, which was associated with meristematic division cells and somatic embryo formation (Silveira et al. 2013). Moreover, the addition of exogenous Put in somatic

embryogenesis of sugarcane improved the number of somatic embryos, increasing the levels of endogenous total free PAs (Reis et al. 2016). In *Araucaria angustifolia*, higher endogenous contents of Put were observed in embryogenic callus under prematuration treatment in culture medium containing PEG and maltose (Farias-Soares et al. 2014). 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*. 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; 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.). 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. 2009), *C. papaya* (Vale et al. 2014), sugarcane (Reis et al. 2016), *Larix principis-rupprechtii* (Zhao et al. 2015) and *A. angustifolia* (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. ; Zhao et al. 2015).

Working with embryogenic and non-embryogenic tissues of price rupprecht's larch (*Larix 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 was involved in the polysaccharide hydrolase of the cell wall and pectinesterase precursors (Ma et al. 2015). Pectinesterases 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; Ma 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) 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 ATCDC48B, UGD2, NADP-ME4, PPC1 and SSA, were down-regulated in embryogenic callus incubated with PEG 6% compared to the control treatment (Table 2; 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(Leblová 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'.

Declarations

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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.

Financial interests

The authors declare they have no financial interests.

Conflicts of interest/Competing interests

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

Authors' contribution

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

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Figures

Figure 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.

Figure 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*) portions. **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*).

Figure 3

Endogenous contents of free PAs in embryogenic callus of *P. edulis* 'UENF Rio Dourado' at 14 and 28 days of maturation under control (without PEG) and PEG 6% treatments. **A)** Total PAs; **B)** Put; **C)** Spm; **D)** Spd. Capital letters denote significant differences between PEG treatments for each day of incubation. Lowercase letters denote significant differences between days of incubation in the same maturation treatment. Means followed by different letters are significantly different according to the Student-Newman-Keuls (SNK) test ($p < 0.05$); ($n = 3$; $CV_{\text{Total}} = 10.63\%$; $CV_{\text{Put}} = 15.60\%$; $CV_{\text{Spm}} = 38.93\%$; $CV_{\text{Spd}} = 18.12\%$).

Figure 4

Protein–protein interaction network between DAPs of embryogenic callus of *P. edulis* ‘UENF Rio Dourado’ at 14 days of maturation under PEG 6% compared to those from the control treatment. **A)** Up-accumulated proteins in PEG6%/control and unique proteins in PEG 6% treatment. **B)** Down-regulated proteins in PEG 6%/control and unique proteins in the control treatment. *Green arrows* indicate the biological process that increased in up-accumulated proteins, and *red arrows* indicate the biological

process that decreased in down-accumulated proteins. The analysis was performed using *A. thaliana* orthologs identified by STRING using Cytoscape.