RESEARCH ARTICLE

Morphological analyses and variation in carbohydrate content during the maturation of somatic embryos of *Carica papaya*

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Abstract Efficient protocols for somatic embryogenesis of papaya (*Carica papaya* L.) have great potential for selecting elite hybrid genotypes. Addition of polyethylene glycol (PEG), a nonplasmolyzing osmotic agent, to a maturation medium increases the production of somatic embryos in *C. papaya*. To study the effects of PEG on somatic embryogenesis of *C. papaya*, we analyzed somatic embryo development and carbohydrate profile changes during maturation treatments with PEG (6%) or without PEG (control). PEG treatment (6%) increased the number of normal mature somatic embryos followed by somatic plantlet production. In both control and PEG treatments, pro-embryogenic differentiation to the cotyledonary stage was observed and was significantly higher with PEG treatment. Histomorphological analysis of embryonic cultures with PEG revealed meristematic centers containing small isodiametric cells with dense cytoplasm and evident nuclei. Concomitant with the increase in the differentiation of somatic embryos in PEG cultures, there was an increase in the endogenous content of sucrose and starch, which appears to be related to a rising demand for energy, a key point in the conversion of *C. papaya* somatic embryos. The

endogenous carbohydrate profile may be a valuable parameter for developing optimized protocols for the maturation of somatic embryos in papaya.

Keywords Somatic embryogenesis · Polyethylene glycol · Maturation · Histomorphology

Introduction

The use of somatic embryogenesis, an in vitro culture technique in which single cells or small groups of somatic cells give rise to embryos (Tautorat et al. 1991), represents a valuable tool for developing cultures of economic importance, since it has great potential for the mass propagation of elite plants (Gupta et al. 1993; Kim et al. 2005).

Maturation is a crucial step for the success of somatic embryogenesis. Important processes of this stage include cell expansion, differentiation, and accumulation of reserve substances essential for germination and regeneration of somatic embryos (Mishra et al. 2012).

Since the early studies of somatic embryogenesis in *Carica papaya* L. (Caricaceae) (Bruijne et al. 1974; Yie and Liaw 1977), several studies have been conducted to develop efficient protocols to enable its use on a commercial scale. Recently, reports have been published on somatic embryogenesis of *C. papaya* with induction (Malabadi et al. 2011; Anandan et al. 2012) and maturation cultures (Heringer et al. 2013; Vale et al. 2014), the latter demonstrating the importance of polyethylene glycol (PEG) in the maturation of *C. papaya* somatic embryos.

PEG is a non-plasmolyzing osmoticum that induces hydric deficit; it has been used to stimulate somatic embryo maturation in several species, such as *Hevea brasiliensis* (Linossier et al. 1997), *Picea abies* (Svobodová et al.

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1999), *Glycine max* (Walker and Parrott 2001), *Aesculus hippocastanum* (Troch et al. 2009), and *C. papaya* (Mishra et al. 2010; Heringer et al. 2013; Vale et al. 2014). The effects of PEG action include the modulation and regulation of genes (Stasolla et al. 2003a, b) and proteins (Heringer et al. 2013; Vale et al. 2014). The addition of 6% PEG to MS culture medium increases somatic embryo formation and protein synthesis in *C. papaya* hybrid UENF/CALIMAN 01 (Heringer et al. 2013). In addition to promoting somatic embryo development, using PEG for maturation treatments leads to changes in protein abundance. Most of the proteins stimulated by PEG are related to carbohydrate and energy metabolism (18.4%) and stress response (18.4%) (Vale et al. 2014). Furthermore, PEG treatments induce differential expression of enolase, esterase and ADH3 proteins, which may play important roles in *C. papaya* embryo maturation (Vale et al. 2014).

Carbohydrate contents were also changed by PEG (Businge et al. 2013; Hudec et al. 2016) during the maturation of Norway spruce somatic embryos. Changes in the soluble carbohydrate levels of embryogenic cultures may be a response to the hydric stress induced by PEG addition. It has been shown that soluble carbohydrates regulate a range of developmental processes from embryo development to plant senescence (Gibson 2005).

In somatic embryogenesis, variations in soluble carbohydrate and starch levels may provide important information on the mechanisms by which embryos are converted to plantlets (Pescador et al. 2008). They may act as signaling molecules and/or gene expression regulators (Eveland and Jackson 2012). However, little is known about the effects of PEG on carbohydrate metabolism and the conversion of somatic cells to the somatic embryos, especially in *C. papaya*.

Based on these previous observations, to determine the effects of PEG on somatic embryogenesis in *C. papaya*, we assessed somatic embryo development and carbohydrate profiles during the maturation process in media with or without (control) PEG (6%).

Materials and methods

Plant material

To induce somatic embryogenesis in papaya, immature zygotic embryos were isolated from mature seeds of the hybrid papaya UENF/CALIMAN01 and used as explants. Immature fruit were kindly provided by the Caliman Agrícola Co., located in the city of Linhares, Espírito Santo (ES), Brazil (19°23'S 40°4'W).

Induction and multiplication of embryogenic cultures

Induction of embryogenic cultures was performed as described by Heringer et al. (2013) and Vale et al. (2014). Briefly, immature fruit were disinfected in 70% ethanol (Merck, Darmstadt, Germany) for 2 min and then in 50% commercial bleach (2–2.5% sodium hypochlorite) for 30 min, followed by three washes with autoclaved distilled water. Then, immature seeds were removed and sorted in a laminar flow cabinet; and immature embryos were isolated to be used as explants. These immature embryos were inoculated into test tubes (25 × 150 mm) containing 10 mL MS culture medium (Murashige and Skoog 1962) (Phytotechnology Lab, Shawnee Mission, KS, USA) supplemented with 3% sucrose (Vetec, São Paulo, Brazil), 20 µM 2,4-dichlorophenoxyacetic acid (2,4-D) (Sigma-Aldrich, St. Louis, USA) and 2.0 g L⁻¹ Phytigel (Sigma-Aldrich). The pH of the culture medium was adjusted to 5.8 before Phytigel was added. The culture medium was sterilized by autoclaving at 121 °C for 15 min. The tubes were then inoculated with explants and incubated in the dark at 25 ± 2 °C for 42 days. The induced embryogenic cultures were then isolated and subcultured in culture media with the same composition. Before maturation experiments, four subcultures were made at intervals of 21 days for embryogenic culture multiplication.

Maturation treatment

Three colonies with 300 mg fresh matter (FM) were inoculated into Petri dishes (90 × 15 mm) containing 20 mL MS culture medium supplemented with myo-inositol (Merck) (100 mg L⁻¹), Phytigel (2.0 g L⁻¹), sucrose (3%), and either 0 or 6% PEG 3350 (Sigma-Aldrich), hereafter referred to as control and PEG treatments, respectively. The pH of the culture medium was adjusted to 5.8 before adding Phytigel; the medium was sterilized by autoclaving at 121 °C for 15 min. The cultures were incubated in a growth chamber at 25 ± 1 °C in the dark for the first 7 days, followed by a photoperiod with 16 h of light (60 µmol m⁻² s⁻¹). Sixteen Petri dishes were used per treatment, each containing three colonies. Samples from four Petri dishes of each treatment were collected after 0, 7, 14, 21 and 28 days of culture. Sections of one colony from each culture were removed for histomorphological analysis, and the remaining colonies were stored at -20 °C for subsequent soluble carbohydrate and starch analyses. In addition, cellular growth and the number of somatic embryos were evaluated after 28 days of culture.

Growth analyses and the number of somatic embryos

For both treatments, cellular growth and the number of somatic embryos were measured at the beginning of the experiment (day 0) and after 7, 14, 21, and 28 days of culture. Cellular growth was measured in terms of the increase in fresh mass (FM) from the initial value (300 mg per colony). The number of somatic embryos of each developmental stage (globular, cordiform, torpedo, and cotyledonary) were counted. After 28 days of culture, the number of morphologically abnormal somatic embryos was counted, including fused embryos, embryos with fused cotyledons, and mono or poly-cotyledonary embryos.

Histomorphological analyses

Samples were collected at the beginning of the experiment (day 0) and after 7, 14, 21, and 28 days of culture and were then fixed in a solution containing 2.5% glutaraldehyde (Merck, Darmstadt, Germany) and 4% paraformaldehyde (Merck) in 100 mM sodium cacodylate (pH 7.2) (Merck) for 24 h at room temperature. The samples were subsequently washed with 100 mM sodium cacodylate (pH 7.2) for 45 min at room temperature.

Before microscopy, samples were dehydrated twice through an ethanol series of 30, 50, 70, 90 and 100% for 1 h each. After dehydration, the samples were infiltrated with HistoResin (Leica, Wetzlar, Germany) and 100% ethanol (1:1, v/v) for 12 h and, subsequently, with 100% HistoResin for 24 h before embedding in HistoResin. Sections (approximately 5 μ m thick) were cut and stained with 1% toluidine blue (Sigma-Aldrich). Samples were examined under a Zeiss Axioplan light microscope (Carl Zeiss, Jena, Germany) equipped with an AxioCam MRC5 digital camera (Carl Zeiss), interfaced with the AxioVisionLE 4.8 software (Carl Zeiss) for image analysis.

Determination of soluble carbohydrates and starch content

Soluble carbohydrates were quantified using high-performance liquid chromatography (HPLC), as described by Aragão et al. (2015). Samples (300 mg MF each) of *C. papaya* embryogenic cultures were macerated with 1 mL extraction solution consisting of 80% ethanol (Merck), 3% polyvinylpyrrolidone—PVPP (w/v) (Sigma-Aldrich), and 1% ascorbic acid (w/v) (Sigma-Aldrich) at 4 °C. After maceration, the samples were vortexed briefly and then transferred to a water bath at 70 °C for 90 min. Following centrifugation at 16,000 $\times$ g for 10 min, the supernatant was removed, and the pellets were then re-extracted with 1 mL extraction solution and centrifuged again at 16,000 $\times$ g for 10 min. The supernatants were combined, filtered through a

20 μ m membrane and stored at -20 °C until carbohydrate analysis. Carbohydrates were identified and quantified by HPLC (Shimadzu Corporation, Kyoto, Japan), using an evaporative light scattering detector (ELSD-LT II) at 40 °C, nitrogen gas pressure of 350 MPa, a gain of 9 and a filter setting of 4. A Prevail Carbohydrate ES (Alltech Associates, Deerfield, IL, USA) 5 μ m column (250 $\times$ 4.6 mm) and pre-column (7.5 $\times$ 4.6 mm) were used for separation. The gradient was achieved by mixing decreasing proportions of absolute acetonitrile (Merck) with water. The acetonitrile gradient was programmed as follows: 80% for the first 16 min, 80–70% between 16 and 23 min, and 70% from 23 to 30 min, with a flow rate of 1 mL min⁻¹ at 25 °C. A 5- μ L sample was injected, and the peak areas and retention times were measured by comparison with carbohydrate standards containing sucrose, fructose, and glucose (Sigma-Aldrich).

Starch extraction was performed according to the method described by McCready et al. (1950) with modifications. Pellets from soluble carbohydrate extracts were resuspended in 1 mL 30% perchloric acid (PCA-Sigma-Aldrich), stirring for 1 h and centrifuging at 16,000 $\times$ g for 15 min at 4 °C. Supernatants were collected, and the resulting pellet was resuspended and centrifuged with the same conditions. The supernatants were mixed, and starch quantification proceeded according to the method described by Colvin Jr. et al. (1961). Briefly, the samples (10 μ L) were mixed with a solution of 100 μ L 0.05% anthrone (Sigma-Aldrich) and 72% sulfuric acid (Vetec), (1:10; v/v). This mixture was vortexed and heated in a dry block heater at 100 °C for 10 min. Then, the samples were placed in water at room temperature for 3 min and then incubated in the dark for 30 min. Readings were performed in a 96-well Microplate Spectrophotometer (Bio-Tek, Winooski, VT, USA) at 620 nm. Starch concentrations were determined using a range of glucose concentrations as standards, multiplying the readings by 0.9 according to McCready et al. (1950).

Statistical analysis

All experiments were performed using completely randomized designs. Data was analysed using analysis of variance (ANOVA) ($P < 0.05$) followed by the Student–Newman–Keuls (SNK) test or *t* test using the R statistical software (R Core Team 2014) and the easyanova package (Arnhold 2013).

Results

Effects of maturation treatments on somatic embryo formation

Significant differences in the number and quality of somatic embryo production between treatments were

Table 1 Somatic embryo number (SE), fresh matter (FM) increment and abnormal mature somatic embryo (ASE) in embryogenic cultures of *C. papaya* after 28 days of culture under control and PEG maturation treatments

Treatment	SE	FM (mg)	ASE (%)
Control	115 ^b	600.0 a	24 a
PEG	150 a	361.6 b	4 b

^bMeans followed by the same letters are not significantly different according to the *t* test ($P < 0.05$). CV coefficient of variation. (n = 4; CV SE = 9.3%; CV FM = 24.9%; CV ASE = 32.9%)

observed (Table 1). PEG use increased the number of somatic embryos (Table 1; Fig. 1a, b). By contrast, the control had higher FM increment (600 mg) than did PEG treatment (361.6 mg) (Table 1). Furthermore, PEG treatment significantly reduced the number of abnormal somatic embryos (Table 1).

Next, the progression of somatic embryo development was evaluated during control and PEG incubation. Somatic embryos were counted according to their state of development: globular, cordiform, torpedo and cotyledonary (Fig. 1c–f), the last of which was able to germinate and regenerate plants (Fig. 1g, h).

Our results showed a significant increase in the number of globular somatic embryos within the first 7 days of incubation with PEG (63.4 embryos per colony) compared to control treatment (11.2 embryos per colony), with a similar number for PEG treatment after a 14-day incubation (62.4 somatic embryos per colony) (Fig. 2a). A significant decrease in the number of globular somatic embryos was observed from 14 to 21 days of incubation in PEG treatment compared to the control (Fig. 2a), while there was a significant increase in number of somatic embryos at the cotyledonary stage (Fig. 2d).

Histomorphological analysis during maturation

Embryogenic cultures incubated with PEG presented morphological differences compared to control cultures (Fig. 3a, b). Embryogenic cultures incubated in PEG had a higher abundance of cells with embryogenic characteristics, i.e., small isodiametric cells with dense cytoplasm and conspicuous nuclei. These cells were organized into small groups of meristematic cells called “meristemoids”, observed mainly in the peripheral regions of the cultures. These meristemoids quickly differentiated into globular somatic embryos and progressed to more advanced embryonal stages (Fig. 3b–g).

On the other hand, the control embryogenic cultures produced large numbers of non-embryogenic cells, which were more elongated and more dispersed, with large vacuoles relative to embryonic cells (Fig. 3a). Given these non-embryogenic cell clusters, the control cultures showed

lower histomorphological organization and consequently formed fewer somatic embryos (Fig. 3a).

In addition, the embryogenic cultures showed asynchronous differentiation during the 28 days of incubation, giving rise to embryos at different maturity stages: globular, cordiform, torpedo and cotyledonary (Fig. 3). Somatic embryo differentiation started with the formation of a small group of cells forming the pro-embryo (Fig. 3c), containing cells with prominent nuclei, densely stained cytoplasm and cells bounded by a cell wall, which preceded the globular embryo stage. Each globular embryo was characterized by protoderm formation, large suspensor, and a large meristematic cell cluster on the axial portion, suggesting increased division and the formation of juxtaposed cells around the embryo (Fig. 3d). Cordiform embryos also exhibited meristematic cells, which were more pronounced at the apex of the embryo, along with early cotyledon development (Fig. 3e). Embryos in the torpedo stage were characterized by full cotyledon development, while embryos in late torpedo and cotyledonary stages showed development of meristematic tissues such as the procambium (Fig. 3f).

Soluble carbohydrate and starch content during maturation

During the maturation of the embryogenic cultures, the carbohydrates sucrose, fructose, and glucose were identified and quantified in both control and PEG treatments (Fig. 4). The cultures showed significant differences with respect to endogenous sucrose content (Fig. 4a). The highest sucrose content was measured on the seventh day of incubation and was significantly higher for the PEG cultures. Sucrose then decreased through the rest of the culture period (Fig. 4a). Interestingly, this peak of sucrose level for PEG treatment coincided with a significant increase in the number of globular somatic embryos (Fig. 2a). For fructose and glucose, distinctive accumulation patterns were identified in cultures depending on the treatment applied (Fig. 4b, c). Embryogenic cultures without PEG (control) showed increasing amounts of hexoses throughout the incubation time (Fig. 4b, c). On the other hand, PEG cultures exhibited increasing contents of both glucose and fructose until the 14th and the 21st day of incubation, respectively, which were followed by significant decreases to the end of incubation on the 28th day (Fig. 4b, c).

The highest starch content was found in embryogenic cultures with PEG treatment, reaching a peak at day 7 of incubation, and then decreased through day 28 (Fig. 4d). Like sucrose (Fig. 4a), the peak of starch accumulation in cultures with PEG coincided with a significant increase in the number of globular-stage somatic embryos, thus

Fig. 1 Morphology of *C. papaya* embryogenic cultures after 28 days of maturation culture without (control) (a) or with PEG (b), and somatic embryo developmental stages: globular (c), cordiform (d),

torpedo (e), cotyledonary (f), germinating embryo (g) and regenerated plantlet (h). The arrowhead indicates an abnormal embryo. Bars: a and b = 0.5 mm; c, d, e, f and g = 0.2 mm; h = 1.0 cm

suggesting that sucrose and starch play important roles in somatic embryo formation in *C. papaya*. Moreover, embryogenic cultures incubated on control medium showed increasing contents of starch only in the first 14 days of incubation, but were still lower than the starch content observed in the first 7 days of incubation with PEG, and they decreased throughout the rest of the incubation period (Fig. 4).

Discussion

The use of PEG improved somatic embryo maturation in *C. papaya* (Table 1; Figs. 1, 2, 3). Consistently, among the currently used osmotic agents, PEG has been demonstrated to be important for the maturation of different species, including *H. brasiliensis* (Linossier et al. 1997), *G. max* (Walker and Parrott 2001), *A. hippocastanum* (Troch et al.

Fig. 2 Number of somatic embryos at globular (a), cordiform (b), torpedo (c) and cotyledonary (d) stages of *C. papaya* cultures during maturation, for control and PEG treatment. Lowercase letters denote significant differences between treatments for each day of culture. Uppercase letters denote significant differences between days of

culture for the same treatment. Means followed by different letters are significantly different by the SNK test ($P < 0.05$). CV coefficient of variation. (n = 4; CV globular = 26.2%; CV cordiform = 62.0%; CV torpedo = 21.9%; CV cotyledonary = 34.1%)

Fig. 3 Histomorphological aspects of *C. papaya* embryogenic cultures after 28 days of maturation in control (a) and PEG groups (b), pro-embryogenic masses (c) and somatic embryos at globular (d),

heart-shaped (e), torpedo (f) and cotyledonary (g) stages. Asterisks indicate embryos developing within the cultures. Arrowheads indicate SAMs, black arrows indicate procambium. Bars = 20 μ m

Fig. 4 Contents (mg g⁻¹ FM) of sucrose (**a**), glucose (**b**), fructose (**c**) and starch (**d**) through 28 days of culture in control and PEG treatments. Lowercase letters denote significant differences between treatments for each day of culture. Capital letters denote significant differences between days of culture in the same treatment. Means

followed by different letters are significantly different ($P < 0.05$) according to the SNK test. CV coefficient of variation. (n = 3; CV sucrose = 39.8%; CV glucose = 11.4%; CV fructose = 7.5%; CV starch = 15.3%)

2009), and *C. papaya* (Mishra et al. 2010; Heringer et al. 2013; Vale et al. 2014). Large PEG molecules are 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).

Supplementation of the culture medium with 6% PEG resulted in a significant improvement in the number and quality of somatic embryos produced (Table 1), promoting higher conversion from globular (Fig. 2a) to cotyledonary (Fig. 2d) somatic embryos. Similarly, a previous study showed that PEG affected the maturation of *P. abies* embryogenic tissues by accelerating somatic embryo formation, thus producing a higher number of mature embryos (Hudec et al. 2016). In *G. max*, PEG addition into culture media enhances the development of cotyledonary embryos and the regeneration of plantlets (Walker and Parrott 2001). In the maturation of genetically transformed *C. papaya* somatic embryos, cultures had a maximum conversion of globular somatic embryos into cotyledonary embryos when using 4.2% PEG, thereby increasing the rate of plant regeneration (Mishra et al. 2010). The same phenomenon was observed for the maturation of hybrid UENF/CALIMAN01 *C. papaya* somatic embryos (Heringer et al. 2013; Vale et al. 2014).

We observed that the embryogenic cultures grown with PEG produced more cells with embryogenic characteristics, i.e., small in size with dense cytoplasm, and containing large nuclei with prominent nucleoli and small vacuoles (Fig. 3), resulting in the development of many meristematic centers, which subsequently led to an increased number of somatic embryos (Fig. 2). Additionally, the formation of pro-embryogenic masses (Fig. 3c), which would later give rise to globular (Fig. 3d), cordiform (Fig. 3e), torpedo (Fig. 3f) and cotyledonary embryos (Fig. 3g) was observed. Histological analyses allowed the observation not only of the development of somatic embryos and their respective structural characteristics but also of their quality. Such histodifferentiation studies of dicot somatic embryos have contributed to the understanding of somatic embryo differentiation in various species (Kärkönen 2000; de Faria et al. 2003; Cangahuala-Inocente et al. 2004; Langhansova et al. 2005; Li et al. 2008; Correia and Canhoto 2010; Pinto et al. 2011).

At the molecular level, PEG may act to regulate the expression of many genes responsible for controlling cell division and differentiation, as well as the development of the shoot apical meristem (SAM) (Stasolla et al. 2003a). According to Liu et al. (1993), cotyledon development occurs predominantly through cell division in cotyledonary

tissues, and then, cotyledonary slit cells stop dividing, leading to the formation of heart-shaped embryos. Moreover, it has been proposed that the osmotic stress induced by PEG may also cause changes in DNA methylation, favoring the expression of genes important for differentiation (Smulders and Klerk 2011).

The addition of 6% PEG also improved the quality of *C. papaya* somatic embryos formed in culture (Table 1). El Dawayati et al. (2012) demonstrated the morpho-ontogenetic action of PEG on the normal development of somatic embryos of *Phoenix dactylifera*, suggesting the involvement of PEG in the activation of morphogenetic processes in these cultures. The improved morphogenetic responses of callus matured with PEG may be related to the control of SAM activity (Stasolla et al. 2003a), specifically in the expression of important genes for SAM development as Fiddlehead, AGM, and Knotted-like and ZWILLE (Moussian et al. 1998; Stasolla et al. 2003b).

In addition to these morphological aspects, the reduced osmotic potential caused by PEG may stimulate endogenous production of abscisic acid (ABA), which is responsible for synthesizing important storage molecules during somatic embryo development including late embryogenesis abundant (LEA) proteins (Stasolla and Yeung 2003). In addition, 6% PEG treatment increases the soluble protein content of *C. papaya* cv. UENF/CALIMAN01 embryogenic cultures (Heringer et al. 2013). Likewise, proteomic analyses have found a number of proteins that are differentially regulated between 6% PEG and control in papaya embryogenic cultures of the same cultivar (Vale et al. 2014).

In addition to the modulation of proteins, PEG can also induce and modulate the production of carbohydrates during somatic embryogenesis (Saranga et al. 1992; Lygin et al. 2012; Hudec et al. 2016). In the present study, PEG treatment influenced the endogenous content of sucrose, glucose, fructose, and starch during papaya somatic embryo development, suggesting the importance of carbohydrates for morphogenetic responses during maturation under osmotic stress (Fig. 4).

Endogenous sucrose contents were significantly increased by PEG treatment for the first 7 days of incubation (Fig. 4a). As such, we have shown that PEG-induced osmotic stress positively influences endogenous sucrose levels and promotes the continued development of somatic embryos in *C. papaya* (Fig. 2a). The mechanisms of signal transduction and gene regulation involved in the metabolism of carbohydrates in plants are not completely understood (Padilla-Chacón et al. 2010). However, soluble carbohydrates, such as sucrose and glucose, have been recognized to play roles in gene expression for a diverse range of processes, such as cell cycle control, stress responses, energy storage, cell differentiation and development. Thus, sucrose

cleavage is an essential reaction for plant growth (Sturm and Tang 1999). As such, the higher sucrose contents induced by PEG (Fig. 4a) in the early stages of incubation may be related to cell division and differentiation, which in turn increase the formation of *C. papaya* somatic embryos. These results corroborate those observed by Stasolla et al. (2003a). These authors demonstrated that PEG increased the expression of sucrose synthase genes, at specific developmental stages, in embryogenic cultures of *Picea glauca*. Therefore, increasing the sucrose content may be a cellular response to the osmolality changes caused by PEG, especially during the first few days of culture. In addition, Nieves et al. (2003) have attributed high hexose contents to the elevated activity of neutral and acidic invertase enzymes, which promote sucrose hydrolysis for reserve mobilization; their activities can increase intracellular hexose contents and the storage of metabolic compounds, promoting rapid cell proliferation (Blanc et al. 2002). Proteins related to carbohydrate metabolism have been found to be differentially expressed during the embryogenic maturation of papaya UENF/CALIMAN01 treated with PEG, which may play important roles in the maturation of these cultures (Vale et al. 2014). The authors indicated that enolase, a key protein in the glycolytic pathway, can be used as a marker of papaya embryonic maturation (Vale et al. 2014), reinforcing the role of carbohydrate metabolism in culture maturation for this species.

In the present study, the observed reductions in glucose and fructose content after 14 and 21 days of incubation, respectively, in embryogenic cultures treated with PEG may be related to the increased maturation of somatic embryos to the cotyledonary stage in *C. papaya* (Fig. 2c). This decrease in endogenous hexose contents may be an important factor for the reorientation of cellular metabolism (Blanc et al. 2002), because somatic embryos are ready for germination after the formation of cotyledonary embryo. Kubeš et al. (2014) found that the level of hexoses was higher than that of sucrose during the maturation of *P. abies* somatic embryos. These authors suggested that sucrose may exert important effects on the signaling cascade that triggers somatic embryogenesis; however, it must remain at low levels for a specific duration of the cultivation period. In *Medicago arborea*, Martin et al. (2000) also reported higher amounts of fructose and glucose and lower amounts of sucrose in embryogenic cultures, attributing the reduced sucrose concentration to its consumption during somatic embryo development.

Like sucrose, the starch contents in embryogenic cultures increased significantly after 7 days under PEG treatment (Fig. 4d). This observation suggests that greater sucrose levels may promote the accumulation of starch, since sucrose is a substrate for ADP-glucose pyrophosphorylase, an important enzyme for starch synthesis (James

et al. 2003). The increase in starch contents was highest when embryogenic cultures contained the greatest number of somatic embryos at the globular (Fig. 2a) and torpedo (Fig. 2c) stages. This suggests that starch hydrolysis may provide the energy required for cell differentiation during somatic embryo formation. Analyses of starch content during maturation have been previously reported for other species such as *Medicago sativa* (Lai and McKersie 1994), *P. abies* (Lipavská et al. 2000), *P. glauca* and *Dendrocalamus hamiltonii* (Kaur et al. 2012), identifying higher contents in the early stages of somatic embryo maturation. According to He et al. (2011), starch is a “temporary carbon pool” in cells and may be used at any time for the biosynthesis of other carbohydrates.

The somatic embryo is a powerful physiological drain that utilizes a large quantity of assimilates, which are partitioned between multiple storage components. An excess of assimilates may be temporarily converted to starch until enzymes and metabolic processes can transform them into their final storage forms (He et al. 2011). In some seed species, starch serves as a temporary store of carbon, available for use throughout development for the biosynthesis of other molecules such as lipids and proteins (Norton and Harris 1975).

Conclusions

Somatic embryo maturation in papaya was enhanced by PEG supplementation of the culture medium. It promoted differentiation of globular somatic embryos and improved their developmental progression, leading to increased cotyledonary somatic embryo production. PEG improved the formation of meristematic centers in the embryogenic cultures, containing cells with embryogenic characteristics and thus enabling the transition of somatic embryos from the pro-embryogenic to the cotyledonary stage. The elevated formation of somatic embryos in embryogenic cultures incubated with PEG may be related to the osmotic stress induced by PEG and the resulting alterations in carbohydrate profiles.

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Compliance with ethical standards

Conflict of interest The authors of the manuscript have no conflict of interest to declare.

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