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Putrescine induces somatic embryo development and proteomic changes in embryogenic callus of sugarcane

Ricardo Souza Reis [a], Ellen de Moura Vale [a], Angelo Schuabb Heringer [a], Claudete Santa-Catarina [b], Vanildo Silveira [a,*]

[a] Laboratório de Biotecnologia, Centro de Biociências e Biotecnologia (CBB), Universidade Estadual do Norte Fluminense Darcy Ribeiro (UENF), Av. Alberto Lamego, 2000, Campos dos Goytacazes, RJ 28013-602, Brazil
[b] Laboratório de Biologia Celular e Tecidual, CBB-UENF, Campos dos Goytacazes, RJ, Brazil

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ABSTRACT

Somatic embryogenesis, an important biotechnological technique, has great potential for application in sugarcane breeding and micropropagation. Polyamines have been associated with the regulation of several physiological processes, including the acquisition of embryogenic competence and somatic embryogenesis. In this study, we used a proteomic approach to evaluate the effects of exogenous polyamine on sugarcane somatic embryo development to better understand this process. Embryogenic cultures were treated with different concentrations of putrescine, spermidine, and spermine. Proteomic analyses combined the shotgun method and the nanoESI-HDMS^E technology. Among polyamines, 500 μM putrescine gave rise to the highest number of somatic embryos; however, no differences in the amount of fresh matter were observed between polyamines and control. Differences in protein abundance profiles resulting from the effect of 500 μM putrescine on sugarcane somatic embryo maturation were observed. Proteomic analyses of putrescine and control treatment showed differences in the abundances of proteins related to somatic embryogenesis, such as arabinogalactan proteins, peroxidases, heat shock proteins, glutathione s-transferases, late embryogenesis abundant proteins, and 14-3-3 proteins. These results show that putrescine and the identified proteins play important roles in protecting the cells against an in vitro stress environment, contributing to the formation of somatic embryos during the maturation treatment. *Biological significance:* Despite all studies with somatic embryogenesis, the molecular mechanisms controlling the process have not been completely understood. In this study, we highlighted the effects of the polyamine putrescine on somatic embryogenesis of sugarcane and the differentially abundant proteins related to somatic embryo development. We identified six groups of important stress related proteins that are involved in the adaptation of cells to the stress environment of in vitro culture and may also be part of the mechanisms associated to the somatic embryogenesis process. Therefore, our research is trying to understand the complexity of how one single somatic cell becomes a whole plant.

1. Introduction

Currently, all of the cultivated sugarcane worldwide is derived from the crossing of two main species: *Saccharum officinarum*, a domesticated species that accumulates high sugar content and presents a basic number of chromosomes equal to 10 and a chromosome constitution of 2n = 80, and *Saccharum spontaneum*, a wild species presenting a basic number of chromosomes equal to 8 and a chromosome constitution of 2n = 40–128, which has high resistance to biotic and abiotic stresses [1–3]. The hybrid derived from this cross shows a genomic constitution even more complex than the parental genomes, featuring a chromosome constitution of 2n = 100–130, where 60–70% of the chromosomes have been inherited from *S. officinarum* [3]. Sugarcane cultures allow several means of economic exploitation, such as sugar, ethanol, and biopolymers, as well as electricity generation and cellulosic ethanol from the bagasse and straw. This species has been cultivated on an industrial scale for sugar production in more than 90 countries worldwide for over 100 years, and the interest in its cultivation has increased due to the production of ethanol as a renewable energy source [4].

The potential for the application of biotechnological tools to improve sugar production and agronomic performance of sugarcane crops is relatively promising because the yield gains using conventional breeding may be reaching their limit due to the difficulties imposed by the complex genome of sugarcane [3]. Furthermore, the selection of superior genotypes within a population obtained by crossing two individuals is a long-term project that takes at least ten years to generate results [5].

* Corresponding author.
E-mail addresses: ricardoreisreis@gmail.com (R.S. Reis), ellenmoura27@gmail.com (E.M. Vale), angeloheringer@gmail.com (A.S. Heringer), claudete@uenf.br (C. Santa-Catarina), vanildo@uenf.br (V. Silveira).

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Therefore, biotechnological tools have been considered particularly important for sugarcane crops due to the insertion of new genes conferring advantageous agronomic characteristics [6]. One possible morphogenetic pathway for this process is through somatic embryogenesis, an in vitro culture system in which a single somatic cell, or small group of cells, gives rise to a somatic embryo [7]. Several research programs using in vitro cultures of sugarcane have been conducted using various applications, such as micropropagation, breeding, germplasm conservation, and genetic engineering [3,8]. Moreover, the comparison of proteomics [9] and metabolomics profiles [10,11], dehydrin proteins [12], antioxidant enzyme activity [13], polyamine contents and morphological studies [14] between embryogenic and non-embryogenic callus in several cultivars of sugarcane has been undertaken to understand the complete route that triggers the de-differentiation, re-differentiation, and development of somatic cells into embryos.

In the process of somatic embryogenesis, various molecules, including polyamines, have been described as important induction signals in plants. The effects of polyamines on somatic embryogenesis have been described for several species [15–17], including sugarcane [14,18,19]. Polyamines have been considered a class of plant growth regulators; they are small, polycationic aliphatic molecules bearing amino groups that are capable of electrostatically interacting with macromolecules such as nucleic acids, phospholipids, cell wall components, and proteins [20,21]. Putrescine, spermidine and spermine are the main polyamines in plants, and they have been associated with the regulation of physiological processes, such as organogenesis, embryogenesis, flower development, senescence, fruit maturation and development, as well as responses to biotic and abiotic stresses [22]. In embryogenic and non-embryogenic callus from sugarcane var. SP79-1011, the changes in endogenous polyamines profile, especially in spermine contents, may be important for the acquisition of embryogenic competence and somatic embryo maturation in embryogenic callus [14].

To gain a better understanding of the biochemical, physiological, and morphological changes that these molecules may cause in plant development, proteomic tools might be useful for studying gene expression products through the identification of differentially abundant proteins and, potentially, their interactions.

The development of new technologies in the field of mass spectrometry has allowed the acquisition of reliable and high quality data, which is of particular importance for the analysis of highly complex protein mixtures. During MS^E acquisition, the mass detector alternates between a low-energy scanning mode (MS), for accurate mass peptide precursor identification, and an elevated-energy mode (MS^E), for generation of accurate mass multiplex peptide fragmentation data, from which both quantitative and qualitative characterization of complex proteomic samples can be obtained [23,24]. The use of traveling wave-based ion mobility separation (IMS) has provided an additional dimension of separation, improving system peak capacity while reducing chimeric and composite interferences, thus increasing the resolving power of the IMS-enhanced MS^E analyses (high definition MS^E, $HDMS^E$) [25].

The main objective of this work was to study the effects of exogenous polyamines on somatic embryo induction and differential abundance of proteins during the somatic embryogenesis of sugarcane cv. SP80-3280 to acquire a more comprehensive understanding of the mechanisms underlying this complex process. In our study, we used a high-throughput proteomic approach combining the shotgun method and the nanoESI-$HDMS^E$ (data-independent acquisition, with ion mobility) technology.

2. Materials and methods

2.1. Plant material

Sugarcane plants cv. SP80-3280 were obtained from the Universidade Federal Rural do Rio de Janeiro (UFRRJ), Campus Leonel Brizola, localized in Campos dos Goytacazes, RJ, Brazil (21° 48'S and 41° 17'W). This variety was chosen based on a search using The Sugarcane EST Project (SUCEST) protein databank (http://sucest-fun.org/), which helped with the acquisition of more reliable $HDMS^E$ data.

Callus induction was performed as previously described [14]. Internodes with axillary buds were planted in plastic trays containing the commercial substrate PlantMax (DDL Agroindustria, Paulínia, São Paulo, Brazil) for a period of two months. Subsequently, plants were processed by removing the mature leaves. The resulting leaf rolls were surface sterilized in 70% ethanol for 1 min, then in 30% commercial bleach (2–2.5% sodium hypochlorite) for 15 min, and subsequently washed three times in autoclaved distilled water. As explants, leaf rolls were transversely sectioned into 2–4 mm-thick slices and cultured in test tubes (150 × 25 mm) containing 10 mL of MS [26] (Phytotechnology Lab, Overland Park, KS, USA) culture medium, supplemented with 20 g/L sucrose, 2 g/L Phytagel® (Sigma-Aldrich, St. Louis, MO, USA) and 10 μM 2,4-dichlorophenoxyacetic acid (2,4-D - Sigma-Aldrich). The pH of the culture medium was adjusted to 5.8 before Phytagel was added. The culture medium was sterilized by autoclaving at 121 °C for 15 min, and after inoculation, the cultures were kept in the dark at 25 ± 1 °C.

After 45 days in culture, samples of induced callus were transferred to Petri dishes (90 × 15 mm) containing 20 mL of the same culture medium, kept in the dark at 25 ± 1 °C, and then subcultured every 21 days. During this multiplication period, embryogenic callus was separated according to morphological characteristics as previously described [14].

2.2. Effects of exogenous polyamines in somatic embryo induction

For analysis of polyamine effects on somatic embryo induction, five Petri dishes containing 20 mL of MS medium supplemented with 30 g/L sucrose and 2 g/L Phytagel were inoculated with three colonies of 200 mg fresh matter (FM) of embryogenic callus per Petri dish. The pH of the culture medium was adjusted to 5.8 before Phytagel was added. Various concentrations (0, 10, 100 and 500 μM) of the polyamines putrescine, spermidine, and spermine were used separately. Polyamine solutions were adjusted to pH 5.8 and filter-sterilized before addition into the autoclaved MS medium. The culture medium was sterilized by autoclaving at 121 °C for 15 min, and the cultures were kept at 25 ± 1 °C in the dark for 7 days and transferred to light for an additional 21 days of culture, with a photoperiod of 16 h (90 μmol/m^2/s). Before (time 0) and after 7, 14, 21, and 28 days of culture, the FM increment and the number of somatic embryos formed were evaluated from the embryogenic callus.

The best treatment in terms of the production of somatic embryos and the control treatment were utilized for subsequently polyamine and proteomic analyses. For these analyses, callus colonies were homogenized, and samples with 300 mg FM were stored at −20 °C. For polyamine analysis, samples were collected before (time 0) and after 7, 14, 21 and 28 days of culture, whereas for proteomics, samples from 14 and 28 days in culture were utilized.

The somatic embryos were regenerated on MS culture medium supplemented with 30 g/L sucrose and 2 g/L Phytagel. The pH of the culture medium was adjusted to 5.8 before Phytagel was added. The culture medium was sterilized by autoclaving at 121 °C for 15 min, and after inoculation, were incubated at 25 ± 1 °C, with a photoperiod of 16 h (90 μmol/m^2/s) for 30 days. For acclimatization, sugarcane plants were transferred to 50 mL plastic cups containing plant substrate and vermiculite (1:1) and kept at 25 ± 1 °C under a photoperiod of 16 h (90 μmol/m^2/s). Cups were placed in plastic trays covered with PVC film for 7 days to maintain high humidity; after 30 days of cultivation, they were transferred to larger trays and kept in a greenhouse.

2.3. Free polyamine analysis

The analysis of free polyamines was carried out using high-performance liquid chromatography (HPLC - Shimadzu, Japan) as

previously described [27]. Five biological samples (300 mg FM each), from five separated Petri dishes, were pulverized in liquid nitrogen. Then, 1 mL of 5% perchloric acid (Merck, Darmstadt, Germany) was added, and the samples were agitated and then incubated on ice for 60 min. Next, samples were centrifuged at 16,000 g for 20 min at 4 °C, and supernatants were collected. Extracted polyamine samples were derivatized with dansyl chloride (Sigma-Aldrich), vacuum dried in CentriVap® (Labconco, Kansas, MO, USA) and resuspended in pure acetonitrile (Merck) prior to analysis by HPLC using a reversed phase Shinpack CLC ODS 5 μm column (Shimadzu). The gradient was developed by mixing increasing proportions of absolute acetonitrile (Merck) with 10% acetonitrile in water (pH 3.5). The gradient of absolute acetonitrile was programmed as follows: 65% over the first 10 min, from 65% to 100% between 10 and 13 min, and 100% between 13 and 21 min, with a constant flow rate of 1 mL/min at 40 °C. Free polyamine detection was performed with a fluorescence detector (Shimadzu) using 340 nm excitation and 510 nm emission, and the concentrations of putrescine, spermidine, and spermine (Sigma-Aldrich) were determined using standard curves.

2.4. Proteomic analysis

2.4.1. Protein extraction

For total protein extraction, a previously described protocol was used [28]. The extraction buffer consisted of 7 M urea, 2 M thiourea, 2% triton X-100, 1% dithiothreitol (DTT), 1 mM phenylmethanesulfonyl fluoride (PMSF), and 5 μM pepstatin. Five biological samples (300 mg FM each sample), from five separated Petri dishes, were pulverized using a mortar and pestle in liquid nitrogen. Then, in microtubes, 1 mL of extraction buffer was added to the sample powder. Samples were vortexed and incubated on ice for 30 min, followed by centrifugation at 16,000 *g* for 20 min at 4 °C. The supernatants were collected, and protein concentration was measured using a 2-D Quant Kit (GE Healthcare, Piscataway, NJ, USA).

2.4.2. Protein digestion

For protein digestion five biological replicates were combined to yield one pooled sample [29] of 100 μg of proteins per treatment (Fig. S1). Before the trypsin digestion step, pooled samples were desalted on 5000 MWCO Vivaspin 500 membranes (GE Healthcare, UK) using 50 mM ammonium bicarbonate (Sigma-Aldrich) pH 8.5, as buffer. Membranes were filled to maximum capacity with ammonium bicarbonate and centrifuged at 15,000 g for 20 min at 8 °C. This procedure was repeated at least three times, resulting in approximately 50 μL per sample.

The methodology used for protein digestion was as previously described [30]. For each sample, 25 μL of 0.2% (*v*/*v*) RapiGest® (Waters, Milford, CT, USA) was added, and samples were briefly vortexed and incubated in an Eppendorf Thermomixer® at 80 °C for 15 min. Then, 2.5 μL of 100 mM DTT (Bio-Rad Laboratories, Hercules, CA, USA) was added, and the tubes were vortexed and incubated at 60 °C for 30 min under agitation. Next, 2.5 μL of 300 mM iodoacetamide (GE Healthcare) was added, and the samples were vortexed and then incubated in the dark for 30 min at room temperature. The digestion was performed by adding 20 μL of trypsin solution (50 ng/μL; V5111, Promega, Madison, WI, USA) prepared in 50 mM ammonium bicarbonate, and samples were incubated at 37 °C overnight. For RapiGest® precipitation, 10 μL of 5% (*v*/*v*) trifluoroacetic acid (TFA, Sigma-Aldrich) was added and incubated at 37 °C for 90 min, followed by a centrifugation step of 30 min at 16,000 g. Samples were transferred to Total Recovery Vials (Waters).

2.4.3. Mass spectrometry analysis

A nanoAcquity UPLC connected to a Synapt G2-Si HDMS mass spectrometer (Waters, Manchester, UK) was used for ESI-LC-MS/MS analysis. The chromatography step was performed by injecting 1 μL of digested samples to normalize them before the relative quantification of proteins. To ensure standardized molar values for all conditions, normalization among samples was based on stoichiometric measurements of total ion counts of scouting runs prior to analyses. Runs consisted of three technical replicates per pooled sample. During separation, samples were loaded onto the nanoAcquity UPLC 5 μm C18 trap column (180 μm × 20 mm) at 5 μL/min during 3 min and then onto the nanoAcquity HSS T3 1.8 μm analytical reversed phase column (100 μm × 100 mm) at 600 nL/min, with a column temperature of 60 °C. For peptide elution, a binary gradient was used, with mobile phase A consisting of water (Tedia, Fairfield, Ohio, 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 7% B and was held for 3 min, then ramped from 7% B to 40% B up to 90.09 min, and from 40% B to 85% B until 94.09 min, being maintained at 85% until 98.09 min, then decreasing to 7% B until 100.09 min and kept 7% B until the end of experiment at 108.09 min. Mass spectrometry was performed in positive and resolution mode (V mode), 35,000 FWHM, with ion mobility, and in data-independent acquisition (DIA) mode; IMS wave velocity was set to 600 m/s; the transfer collision energy ramped from 19 V to 45 V in high-energy mode; cone and capillary voltages of 30 V and 2800 V, respectively; and a source temperature of 70 °C. In TOF parameters, the scan time was set to 0.5 s in continuum mode with a mass range of 50 to 2000 Da. The human [Glu1]-fibrinopeptide B (Sigma-Aldrich) at 100 fmol/μL was used as an external calibrant and lock mass acquisition was performed every 30 s.

2.4.4. Bioinformatics

Spectra processing and database searching conditions were performed by Progenesis QI for Proteomics Software V.2.0 (Nonlinear Dynamics, Newcastle, UK). The analysis used the following parameters: one missed cleavage, minimum fragment ion per peptide equal to 1, minimum fragment ion per protein equal to three, minimum peptide per protein equal to 1, fixed modifications of carbamidomethyl (C) and variable modifications of oxidation (M) and phosphoryl (STY), and a default false discovery rate (FDR) value at a 4% maximum, score greater than five, and maximum mass errors of 10 ppm. The analysis used the SUCEST database (http://sucest-fun.org) of ESTs databank. Label-free relative quantitative analyses were performed based on the ratio of protein ion counts among contrasting samples. After data processing and to ensure the quality of results, the following protein refinement parameters were used: only proteins present in 3 of 3 runs and with a coefficient of variation lesser than 0.5 were included. For unique proteins, only those present in 2 of 3 runs were considered regardless of the coefficient of variation. Furthermore, differentially abundant proteins were selected based on a max fold change of at least 2. Functional annotation was performed using Blast2Go software v. 3.0 PRO [31] and UniProtKB (http://uniprot.org).

2.5. Statistical analysis

The experiment testing the effect of exogenous polyamines was conducted in a completely randomized factorial design with five biological replicates represented by five Petri dishes and three colonies of 200 mg FM per Petri dish. The resulting data were submitted to analysis of variance (ANOVA), and the means were compared using the Student–Newman–Keuls (SNK) test (significant level, $P < 0.01$) using the statistical analysis software R [32] with the easyanova packet [33].

3. Results

3.1. Effects of polyamine on somatic embryo induction

Compared to the control (Fig. 1B), 500 μM putrescine showed the best results among treatments used, in terms of the number of somatic embryos, presenting an average of 55 embryos per callus after 28 days of culture (Fig. 1C–D), compared with 19 somatic embryos per callus

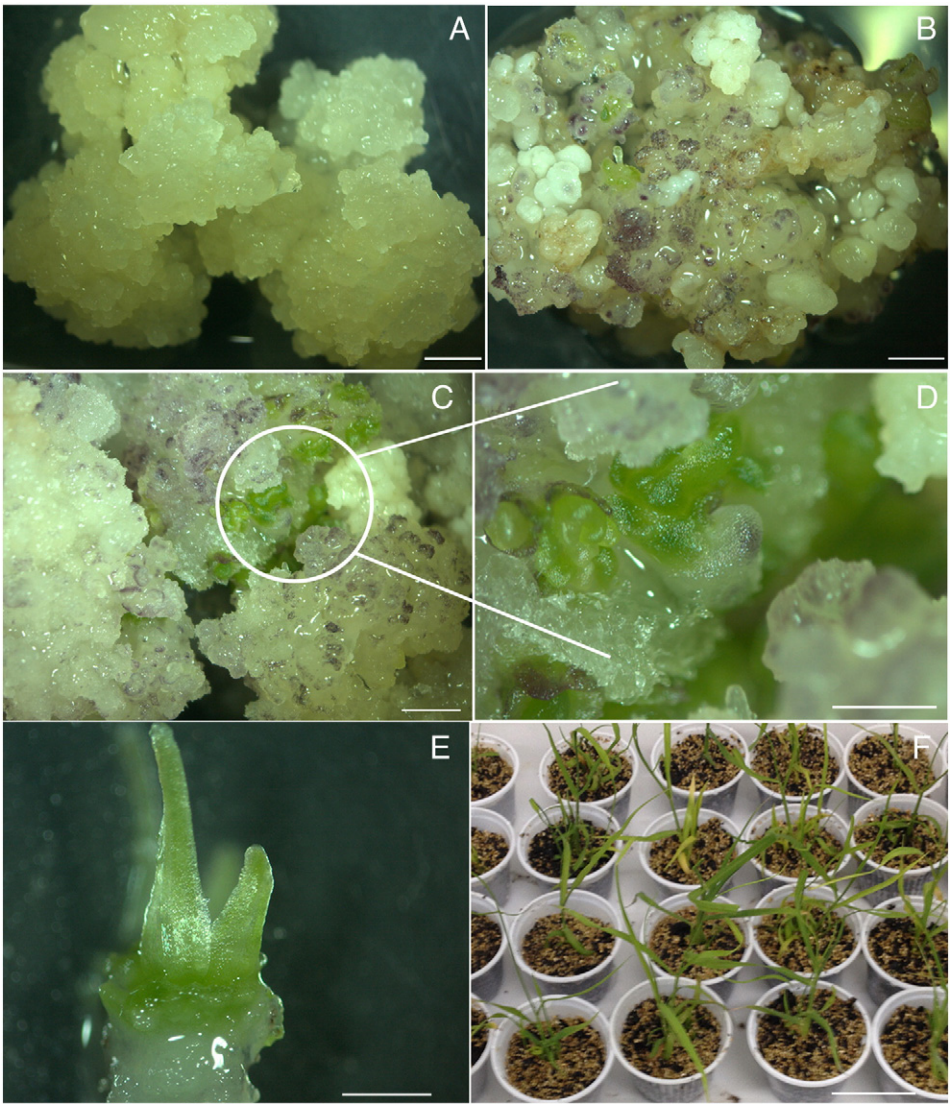

Fig. 1. Morphological characteristics of embryogenic cultures of sugarcane var. SP80-3280 at time 0 (A) and subjected to maturation treatments (control) (B); or to 500 μM putrescine treatment (C) and (D). Morphological characteristics of somatic embryos (E); and regenerated plantlets (F). Bars: (A–D): 0.5 mm; (E): 0.2 mm and (F): 15 mm.

in the control treatment (see also Table 1). The addition of polyamines to the culture medium, as well as control treatment without polyamines, showed no significant effect on the FM increase of callus (data not shown). Both treatments, 500 μM putrescine and control, enabled the conversion of somatic embryos into plantlets, and 100% of acclimatized plants survived (Fig. 1E–F).

Based on the results shown in Table 1, further analyses of endogenous free polyamines and proteomics analyses were limited to samples obtained from the control and 500 μM putrescine conditions.

Table 1
Average number of embryos per callus after 28 days of culture.

Control	Putrescine			Spermidine			Spermine		
0 μM	10 μM	100 μM	500 μM	10 μM	100 μM	500 μM	10 μM	100 μM	500 μM
19bc	35bc	37bc	55a	30bc	16c	24bc	39b	34bc	25bc

Means followed by different letters are significantly different ($P < 0.01$) according to the SNK test. n = 5 and coefficient of variation equal to 23.92%.

During the maturation period with light exposure (at 7 and 28 days of culture), morphological observations showed that all callus cultures presented anthocyanin pigments, which were induced by light exposure, and that no pigments were observed for the first seven days in the dark (Fig. 1A). However, it was observed that somatic embryos developed from areas close to these anthocyanin pigments.

3.2. Endogenous free polyamine content

The analyses of free endogenous polyamines contents were performed in 7-day intervals until the end of the 28-day period of culture during maturation of callus in the control and 500 μM putrescine treatments (Fig. 2).

The results of free endogenous polyamines demonstrated that sugarcane callus cultures treated with 500 μM putrescine presented considerably higher endogenous contents of this free polyamine, which peaked on day 14 (Fig. 2A). Spermidine contents showed no differences among treatments, but they changed during the culture period, with the highest content observed at 7 day in culture and decreasing until the end of the maturation period (Fig. 2B). Spermine had the lowest

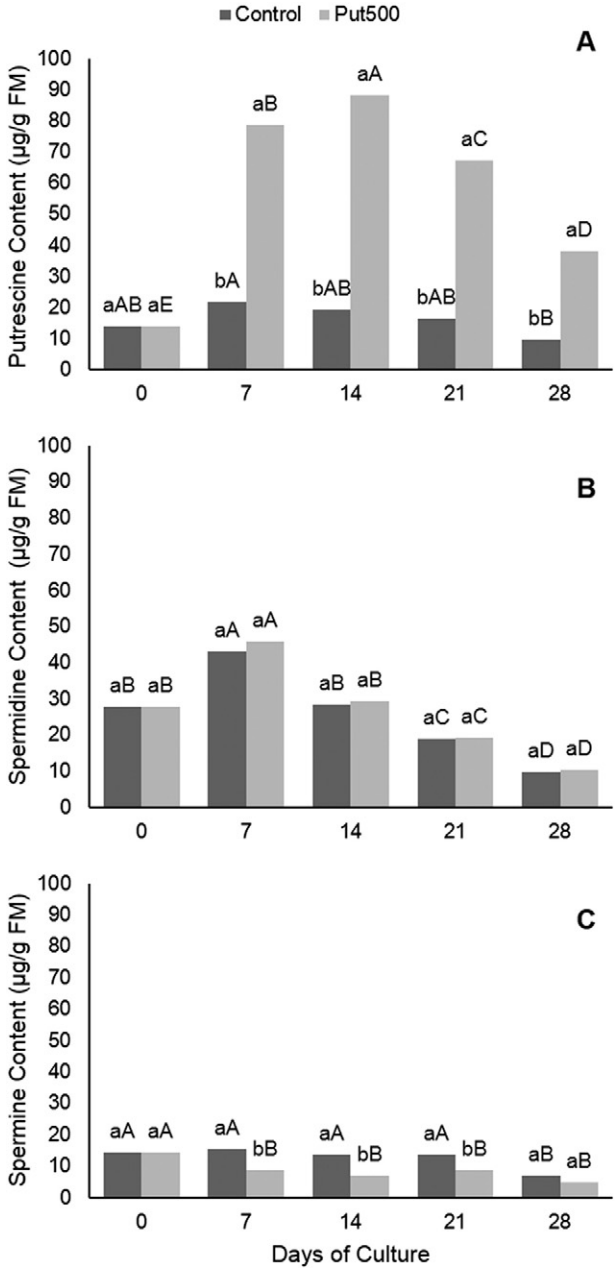

Fig. 2. Content of polyamines during 28 days of culture in control and 500 µM putrescine treatment (Put500). A, putrescine, CV = 13.62%; B, spermidine, CV = 13.31%; and C, spermine, CV = 22.56%. Lowercase letters denote significant differences among treatments in the same period. Capital letters denote significant differences among days of culture within the same treatment. Means followed by different letters are significantly different ($P < 0.01$) according to the SNK test ($n = 5$). FM (Fresh Matter).

contents among the three analyzed polyamines, and the control treatment showed higher contents of endogenous spermine on days 7, 14, and 21 compared to the condition treated with 500 µM putrescine (Fig. 2C).

3.3. Protein identification by LC–MS/MS

Proteomic analysis was performed for samples from 14 and 28 days of culture for control and 500 µM putrescine treatments. Day 14 was used because it was the first week of culture in a light exposure period, in contrast to the first 7 days, in which the cultures were still in the dark. Day 28 was the end of the maturation period and was thought to be critical for a better comprehension of the somatic embryogenesis process.

As a relative quantification analysis, this proteomics study was based on comparative analyses among samples, where each comparison resulted in a list of proteins identified in both samples and lists of proteins unique to one of the samples. In each list, protein refinement was performed to ensure the quality of the results, consisting of establishing a cutoff for proteins with a coefficient of variation greater than 0.5, thus automatically excluding proteins present in only 1 or 2 replicates, aside from the exclusion of false positives. The only exceptions to this refinement were the unique proteins because these were considered, according to their presence or absence in the samples, as an important characteristic for studying somatic embryogenesis without considering their quantification.

During the analysis, $HDMS^E$ data were contrasted between the putrescine 500 µM and control treatments within the same day (days 14 and 28). The raw list of differentially abundant proteins for the control and 500 µM putrescine treatments on days 14 and 28 presented a total of 2611 identified proteins and, after refinement, remained at 1291 and 1161 proteins, respectively, on days 14 and 28. Another cutoff was established to show only proteins with differences in abundance levels of at least 2-fold. For Blast2Go analysis, in each time period, functional annotation was divided between the two groups, up- and down-regulated proteins, for 500 µM putrescine treatment in relation to the control. Thus, after 14 days of culture, treatment with 500 µM putrescine had 61 up-regulated and 97 down-regulated proteins when compared to the control, whereas on day 28, treatment with 500 µM putrescine showed 85 up-regulated and 56 down-regulated proteins; 12 and 10 unique proteins were identified on days 14 and 28, respectively. Of the 12 unique proteins at day 14, 11 belonged to the 500 µM putrescine treatment group and only 1 to the control group. Of the 10 unique proteins at day 28, seven belonged to the 500 µM putrescine treatment group and three to the control group. See Supplementary Table S1 for a complete list of all differentially abundant proteins.

Proteins were grouped according to several biological processes (Table 2). Because a protein may be classified in more than one group, functional classification results are presented sequentially for each protein, emphasizing the main functional groups for that protein.

After processing the data, several stress and detoxification-related proteins were identified, such as eight peroxidases, three heat shock proteins (HSPs), three 14-3-3 proteins, and nine glutathione s-transferases (GSTs) as well as five late embryogenesis abundant (LEA) proteins and two arabinogalactan proteins (AGPs), which were differentially abundant on days 14 and 28 (Table 2) in the 500 µM putrescine treatment in relation to the control. These proteins were chosen based on known relationships to some type of stress and on having been described previously in other works as being important during somatic embryogenesis.

On day 14, all of the identified AGPs and HSPs showed increased abundance in the 500 µM putrescine treatment compared to the control, whereas only one GST of nine was up-regulated; meanwhile, three peroxidases also showed increased abundance (Table 2). The protein showing the most marked up-regulation was a ubiquitin-like protein, which was up-regulated by 61.3-fold. In addition, another ubiquitin-conjugating enzyme was observed to be uniquely present in the 500 µM putrescine treatment, indicating that these proteins might play an important role during putrescine-induced somatic embryogenesis in sugarcane (Table S1). Two 14-3-3 and three LEA proteins and peroxidases were down-regulated in the 500 µM putrescine treatment (Table 2).

On day 28, the abundance of one of the two AGP proteins increased in the presence of 500 µM putrescine compared with the control. Furthermore, three LEA proteins, including a dehydrin, were also up-regulated in the 500 µM putrescine treatment group (Table 2). One peroxidase was considered unique, another showed increased abundance, and two showed decreased expression with putrescine treatment (Table 2). The most up-regulated protein during this stage was an indole-3-acetic acid-amido synthetase, which showed a 55.8-fold

Table 2
Max fold change of somatic embryogenesis-related proteins in putrescine treatment compared to control on days 14 and 28.

Accession	Peptide count	Unique peptides	Score	Description	Biological process	Down on day 14	Up on day 14	Down on day 28	Up on day 28
SCCCCL3001E12.b	8	6	50.68	Arabinogalactan protein	Unknown		7.0		
SCEZRT3069B05	1	1	5.81	Arabinogalactan protein precursor	Unknown		3.7		7.0
SCCCRZ3004A06	4	3	29.85	Heat shock 70 kDa protein	Protein metabolic process; response to stress		7.6		
SCRFST1041E06	3	1	11.77	Stromal 70 kDa heat shock-related chloroplastic-like	Protein metabolic process; response to stress		2.3		
SCCCST3C11C04	6	1	33.31	20 kDa heat shock mitochondrial-like	Protein metabolic process; response to stress		2.1		
SCJFLR1035D05	2	1	16.8	Peroxidase	Carbohydrate metabolic process; response to abiotic stimulus; metabolic process; biosynthetic process; transport; response to stress				Unique*
SCJLRT1014B03	9	1	63.22	Loc100286338 (peroxidase 16-like)	Response to stress; iron ion transport	13.0			
SCCCAD1001C08	1	1	6.60	Peroxidase 42 precursor	Catabolic process; metabolic process; response to stress				5.6
SCEQRT1025E05	11	1	116.36	Cytosolic ascorbate peroxidase	Metabolic process; response to stress		5.1		
SCRLAD1099B04	6	5	39.82	Class III peroxidase 66	Metabolic process; response to stress		2.4		
SCCCLB1002D05	8	1	61.36	Loc100286338 (peroxidase 16-like)	Response to stress; iron ion transport	2.3			
SCEPRZ1011A06	3	2	18.95	Peroxidase 72 precursor	Response to stress; lignin biosynthetic process	2.1		2.9	
SCCCCL7C05F08	1	1	7.98	Class III peroxidase 66	Metabolic process; response to stress		2.0		
SCCCCL5003C11	7	2	57.51	Glutathione s-transferase 4	Metabolic process; response to stress			4.0	
SCJLRT1020A09	13	4	90.65	Glutathione s-transferase 31	Metabolic process; response to toxic substances; toxin catabolic process	2.8			
SCCCCL4003D01	7	5	39.93	Glutathione s-transferase 30	Response to stress, toxic substances, and growth hormones; regulation of growth; amino acid transport	2.5			
SCSFCL6068E03	10	3	127.14	Glutathione s-transferase parA	Metabolic process; auxin-activated signaling pathway			2.6	
SCJFRT1008A09	11	5	73.55	Glutathione s-transferase gstu6	Metabolic process; response to stress and toxic substances; toxin catabolic process			2.5	
SCCCCL4014B12	5	2	35.40	Glutathione s-transferase 31	Metabolic process; response to toxic substance; toxin catabolic process		2.5		
SCCCLR1048D04	20	8	253.48	Glutathione s-transferase parA	Metabolic process; auxin-activated signaling pathway	2.0		2.1	
SCCCCL4015B02	3	2	16.94	Glutathione s-transferase	Response to stress and toxic substances	2.0			
SCCCCL4007F05	12	6	85.82	Glutathione s-transferase gstu6	Metabolic process; response to stress and toxic substances; toxin catabolic process	2.0			
SCVPCL6061E12	5	5	33.73	Late embryogenesis abundant protein 14-a	Response to abiotic stimulus; response to stress	4.8			2.7
SCACLR1126F12	3	2	22.00	Late embryogenesis abundant protein 1	Response to stress		2.2		
SCCCCL4006B06	4	1	46.65	Late embryogenesis abundant protein group 3 variant 1	Response to stress				2.4
SCCCCL4007G11	10	4	92.35	Late embryogenesis abundant protein group 3 variant 2	Response to stress	2.7			
SCCCCL6001A04	5	2	53.12	Dehydrin 11	Response to abiotic stimulus; response to stress	2.8			2.5
SCEQRT2094B01	13	1	129.15	14-3-3-like protein A	Regulation of metabolic processes	2.3			
SCEQRT1031D02	16	3	190.60	14-3-3-like protein	Regulation of metabolic processes	2.2			
SCMCRT2102A01	14	1	136.60	14-3-3-like protein A	Regulation of metabolic processes			3.9	

* Protein present only in the putrescine treatment.

increase in putrescine-treated embryogenic cultures (Table S1). In contrast, the 40S ribosomal protein s15 was the most down-regulated protein. Moreover, an auxin-induced protein pcnt115 was found to be unique to the control treatment during this developmental stage (Table S1).

Interestingly, although it would be logical to believe that somatic embryogenesis development in sugarcane would demand the increased synthesis of new proteins, our results showed that most of the ribosomal proteins were down-regulated in the 500 μM putrescine treatment (Table S1).

4. Discussion

Exogenous polyamines have been studied as growth regulators in tissue culture as well as stress-mitigating compounds in plants or seeds of many species. In in vitro cultures, they have been tested on somatic embryos [34,35] and organogenesis induction [36,37], as well as during plant development under a variety of stresses [38,39].

Treatment with 500 μM Putrescine showed the best results regarding the production of somatic embryos (Fig. 1C–E and Table 1). The exogenous putrescine can be incorporated, resulting in a high intracellular content of free putrescine, which may be necessary for inducing the best performance during the maturation of somatic embryos in sugarcane. Endogenous polyamines appear to show similar patterns among several species under somatic embryogenesis induction, as demonstrated by the presence of a high abundance of putrescine, followed by an intermediate content of spermidine and a lower content of spermine in *Vitis vinifera* [40], *Coffea canephora* [41], *Pinus sylvestris* [42], and sugarcane [14]. In contrast, spermidine was the most abundant of the polyamines in somatic and zygotic embryos of *Quercus ilex* [43] and *Pinus radiata*

[44], and in zygotic embryos of *P. sylvestris* [45]. Farias-Soares et al. [46] reported high conversion rates of pro-embryogenic masses to somatic embryos and higher contents of polyamines in cultures of *Araucaria angustifolia* under pre-maturation treatment in culture medium containing polyethylene glycol and maltose as osmotic agents, with putrescine observed at higher contents.

These results demonstrated that polyamine metabolism may be quite different and show distinct responses depending on the species or culture condition. The best rates of somatic embryo induction might be due to the action of putrescine in higher exogenous concentrations, which could play a dual role in sugarcane somatic embryogenesis. One role is as a continuous supply for spermidine synthesis, and the other is to help cells withstand the oxidative stress induced by an excessive production of reactive oxygen species (ROS), by which putrescine can modulate the expression of peroxidases and other related proteins. This scenario would be different in the control treatment due to the low concentration of endogenous putrescine; thus, the control would not be able to tolerate the oxidative stress and produce embryos at the same efficiency as cultures treated with 500 μM putrescine.

Other than differences in the numbers of somatic embryos, no significant effect of polyamine treatments was observed on the FM increment of callus during the maturation period. By contrast, in somatic embryogenesis of *Ocotea catharinensis* [16] and *A. angustifolia* [15], exogenous putrescine also showed no effect on culture growth, but spermidine and spermine demonstrated an inhibitory effect, which might be explained by the inhibition of proton pumps [47]. On the other hand, Paul et al. [35] reported a positive effect of all polyamines on the FM increment in *Momordica charantia* embryogenic callus, and putrescine presented the best results in both FM and somatic embryo development.

Beyond the involvement of polyamines in various cellular processes such as cell growth, embryogenesis, and stress in whole plants and in vitro cultures [48], the precise mechanism by which polyamines can induce somatic embryogenesis has not been elucidated. Proteome analysis is a promising possibility for identifying the molecular mechanisms that trigger somatic embryogenesis in sugarcane, via tracking the pathways that cells follow to become embryogenically competent and form somatic embryos induced by polyamines.

Exogenous putrescine at a concentration of 500 μM, which promoted the best results regarding somatic embryo induction, was able to change the protein abundance profile of the treated embryogenic cultures compared to the control treatment, thereby modulating the expression of several proteins related to somatic embryogenesis. Among the differentially abundant proteins, the six classes of proteins shown in Table 2 have been reported previously in cultures that have been submitted to somatic embryogenesis induction or in embryogenic and non-embryogenic callus.

One class of these proteins is the AGPs, which are an abundant group of plant glycoproteins, which have been implicated in various biological processes such as cell division, programmed cell death, embryo development, growth, abscission, signaling, and stress responses and which may interact with plant growth regulators [49,50]. Two AGPs were up-regulated in the 500 μM putrescine treatment on day 14 (3.7- and 7.0-fold), and one of them further increased its abundance from 3.7- to 7.0-fold on day 28 (Table 2), suggesting the possible action of AGPs on somatic embryogenesis in sugarcane. This hypothesis is based on the fact that the addition of AGPs in to embryogenic callus cultures stimulated somatic embryogenesis development in *Gossypium hirsutum* [51] and *Quercus bicolor* [52] as well as shoot organogenesis in *Triticum aestivum* cultures [53]. The use of Yariv reagent (β-glucosyl), a synthesized chemical antibody, has been described to interfere with AGP action, causing loss of function and thus reducing the somatic embryo formation in a β-glucosyl dose-dependent manner in peach palm [54].

Plants must employ essential mechanisms to eliminate harmful molecules, such as ROS, and protect plant cells and their organelles against the toxic effect of these species [55]. The ROS detoxification systems include the enzymatic action of peroxidases [55]. In our study, a wide variety of peroxidases were found to be up- and down-regulated on days 14 and 28 (Table 2), demonstrating that these enzymes are related to responses to stress, as well as to the biosynthesis of lignin and iron ion transport, as shown by functional classification. These results suggest that there is a requirement for peroxidases during early stages of sugarcane embryogenic culture growth because a wider variety of enzymes was expressed on day 14 (Table 2), possibly to protect and prepare cells for further development. Differences in peroxidase activity between embryogenic and non-embryogenic callus have been reported in some species as possible somatic embryogenesis markers, as in date palm (*Phoenix dactylifera*), lettuce (*Lactuca sativa*) and *Medicago truncatula*, in which peroxidases are more active in embryogenic callus [56–58]. In contrast, Gallego et al. [59] showed lower peroxidase activity in embryogenic callus when compared to non-embryogenic callus in *Medicago arborea*.

Hydrogen peroxide has previously been described as a somatic embryogenesis inducer in some cultures [53,60]. Thus, the key for increased somatic embryo induction might not be the presence of peroxidases but rather the hydrogen peroxide levels inside cells, which might trigger a signaling cascade and promote the expression of various stress-related genes and thus, via a still unknown route, may stimulate somatic embryogenesis in a growth regulatory manner.

HSPs are a large family of proteins considered as molecular chaperones that play roles in maintaining cellular homeostasis both for optimal growth and in response to stress conditions [61]. They are divided among five major families, i.e., HSP60, HSP70, HSP90, HSP100, and small HSP (sHSP), localized in various cellular compartments, and have been implicated in protein folding, assembly, translocation, and degradation under normal cellular conditions. Furthermore, they also function in the stabilization of proteins and membranes and may assist in protein refolding under stress conditions [62]. In our study, three types of HSPs were identified with increased abundance in sugarcane embryogenic cultures from 14 to 28 days: HSP70 (7.6-fold), a possible chloroplastic HSP70 (2.3-fold), and a hypothetical protein functionally classified as a mitochondrial HSP of 20 kDa, which would be considered a sHSP (2.1-fold; Table 2).

In general, members of the HSP90, HSP70, and HSP60 families are typical energy-dependent proteins that use ATP binding and hydrolysis to support refolding of damaged proteins once a stress condition subsides, whereas sHSPs are energy-independent chaperones that prevent aggregation without supporting refolding [63], and HSP100 proteins work by removing or reactivating aggregated, misfolded, or non-functional polypeptides [61]. The increased abundance of HSPs in our study suggests that these proteins would be responsible for either folding newly synthesized proteins or refolding damaged and unstable proteins, the latter caused by the stress of in vitro culture in sugarcane embryogenic callus. Thus, when it is not possible to refold damaged or misfolded proteins, HSPs would direct these proteins to degradation in an ubiquitin-proteasome system, therefore avoiding cellular damage.

Ubiquitin-like protein was the most up-regulated protein in embryogenic callus from the 500 μM putrescine treatment on day 14 (Table S1). This protein plays roles in the modification and targeting of misfolded and damaged proteins to 26S proteasomes or other pathways for degradation [64,65]. The increased abundance of HSPs may be related to the degradation of unnecessary or damaged proteins, in conjunction with ubiquitin-like protein and the proteasome, therefore avoiding cellular damage, as well as promoting the folding of newly synthesized proteins that are needed by newly differentiated cells in somatic embryos in sugarcane. Meanwhile, mitochondrial sHSP might help to stabilize partially stress-unfolded proteins caused by ROS produced during metabolic processes that might otherwise cause damage to mitochondria. The decreased abundance of these proteins in control might suggest an intracellular instability condition to the proteome, which means that cells must further synthesize new proteins, as explained below.

Thus, the decreased abundance of ribosomal proteins in embryogenic callus from 500 μM putrescine treatment on days 14 and 28 (Table S1) might also be related to the degradation process promoted by the ubiquitin-proteasome system, which would then result in the decreased synthesis of new proteins. These results show that exogenous putrescine dramatically altered the protein profile in embryogenic callus when compared to the control treatment. In contrast, embryogenic callus from the control treatment showed higher abundance of ribosomal proteins (Table S1), which would be necessary for newly synthesized proteins to help cells overcome the stress condition promoted by the in vitro environment and to replace the stress-damaged or unstable proteins degraded by the ubiquitin-proteasome system, which requires energy expenditure. In contrast, putrescine-treated embryogenic cells would be more prepared for this stress condition because polyamines are involved in stress tolerance, which would allow these cells to direct their energy toward developing somatic embryos.

GSTs are a family of stress-induced enzymes responsible for detoxifying xenobiotic compounds and ROS by conjugating these molecules to the tripeptide glutathione (GSH), thus tagging them for vacuolar import by specific ATP-binding transporters [66]. In addition to these functions, GSTs also play roles in normal cellular metabolism, in response to auxin, in the metabolism of plant secondary products, such as anthocyanin, and in the stress caused by pathogen attack [67], and its expression has been described during early stages of direct somatic embryogenesis in an interspecific chicory hybrid (*Cichorium intybus* var. *sativum* × *C. endivia* var. *latifolia*) [68]. Some GSTs may be induced by lipid peroxidation, hydrogen peroxide, and either natural or synthetic auxins [67]. Furthermore, GST expression has been reported to be up-regulated by high temperature, heavy metal (mercuric chloride), herbicide, and hydrogen peroxide and down-regulated by spermidine, likely due to the stress-alleviating action of polyamines [69].

Similarly, embryogenic callus of sugarcane from the 500 μM putrescine treatment presented eight down-regulated GSTs, and only one was found to be up-regulated (Table 2), suggesting that putrescine may support sugarcane cells in overcoming the stress of in vitro culture conditions, helping them develop more somatic embryos. On the other hand, the increased abundance of GSTs in control treatment might be due to the lack of alternative mechanisms to overcome the excessive production of ROS, while putrescine might facilitate the cellular mechanisms used for ROS scavenging, avoiding the damage caused by the stress.

LEA proteins, as their name suggests, were first observed in the late stages of embryogenesis in *G. hirsutum* seeds during desiccation [70] and are related to stress conditions including desiccation, low temperature, light, and osmotic stress [71,72], and they can prevent freezing- and desiccation-induced protein aggregation [73]. On day 14, three LEA proteins were found to be down-regulated in embryogenic callus in the 500 μM putrescine treatment compared with the control, whereas one was up-regulated. Two of these down-regulated LEA proteins were later up-regulated on day 28 of embryogenic callus culture together with a new protein (Table 2), observed to be a dehydrin. Dehydrins have been localized mainly in chromatin of the nuclei of embryogenic callus during sugarcane somatic embryogenesis development and were not identified in non-embryogenic callus [12].

The 14-3-3 proteins are a family of phosphoserine-binding proteins that are capable of regulating, via protein-protein interactions, several target proteins related to metabolism, signal transduction, chromatin function, ion transport, and vesicle trafficking and are also involved in stress responses [74]. Swatek et al. [75] reported that a specific 14-3-3 isoform interacts with indole-3-acetic acid-amido synthetase, which, in our study, was the most up-regulated protein (55.8-fold) on day 28 in embryogenic callus treated with 500 μM putrescine (Table S1). The 14-3-3 proteins were down-regulated in embryogenic callus at 28 days of culture in putrescine treatment and were up-regulated in the control, suggesting that 14-3-3 proteins may have a negative regulatory action on indole-3-acetic acid-amido synthetase, thus decreasing its abundance in the control treatment.

Indole-3-acetic acid-amido synthetase is involved in auxin homeostasis by conjugating excess auxin to amino acids, thus inactivating them [76]. High auxin concentration is important for the acquisition of embryogenic capacity and for the initial stimulus of somatic embryogenesis; however, eliminating or reducing auxin concentration is also important for the further development of somatic embryos [77]. Silveira et al. [14] reported a negative effect of 2,4-D on somatic embryo maturation in sugarcane callus culture when compared to cultures grown on plant growth regulator-free medium supplemented with activated charcoal. Furthermore, the identification of an auxin-induced protein pcnt115, which was unique to the control treatment (Table S1), might indicate a high intracellular auxin level. Thus, the auxin-conjugating enzyme indole-3-acetic acid-amido synthetase may play an important role during somatic embryogenesis development in putrescine-treated embryogenic callus.

5. Conclusions

Among the polyamines tested, the addition of 500 μM putrescine in the culture medium promoted the highest number of sugarcane somatic embryos and induced proteomic changes in six classes of proteins (AGPs, peroxidases, HSPs, GSTs, LEA, and 143-3 proteins), which have previously been associated with somatic embryogenesis and responses to stress conditions.

It is also possible that somatic embryogenesis acts as a survival mechanism in response to stress conditions faced by cells in tissue culture, which would explain the diversity in the stress response proteins found in several studies. Thus, to continue the lineage of offspring, mother cells trigger signaling pathways that culminate in the formation of somatic embryos and subsequently a new plant.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2015.09.029.

Conflict of interest

The authors declare no conflict of interest.

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