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Involvement of polyamines in the maturation of grapevine (*Vitis vinifera* L. 'Mencía') somatic embryos over a semipermeable membrane

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ABSTRACT

The endogenous content of polyamines and the expression of genes involved in their metabolism were analyzed in grapevine (*Vitis vinifera* L. 'Mencía') somatic embryo aggregates to determine the effect of a semipermeable membrane on their maturation in differentiation medium. The endogenous polyamine content in the somatic embryo aggregates was higher in those cultured over the semipermeable membrane and significantly increased with culture time due to an increase in the free polyamine fraction. Free putrescine represented more than 95% of the total free polyamine fraction and significantly peaked in the second week of culture of the somatic embryo aggregates over the semipermeable membrane. This finding appears to be supported by active expression of the *VvADC* gene and the low free spermidine level. Another significant peak of free putrescine was detected at the end of culture over the membrane, in which free spermidine level remained low despite the V*vSPDS2* gene was upregulated. Hence, it is advisable that this increase in free putrescine was supported by back conversion from spermidine through *VvPAO* expression. As the semipermeable membrane successfully avoided precocious germination of the grapevine somatic embryos, the results support that polyamine metabolism, particularly putrescine metabolism, is involved in their correct maturation.

1. Introduction

Somatic embryogenesis is a method that has multiple applications in plant improvement. In grapevine (*Vitis vinifera* L.), the first protocol of somatic embryogenesis was developed in the 1970s (Martinelli and Gribaudo, 2009) and is currently the most widely used tool for *in vitro* manipulation of species in the genus *Vitis*. Despite the usefulness of this method, there are still some limitations, such as variations related to genotype, which implies the need for protocol improvement in virtually every cultivar tested, particularly those related to the differentiation and maturation of embryos and their subsequent conversion into plantlets (Acanda et al., 2013, 2020; Prado et al., 2010).

Precocious germination of grapevine somatic embryos has been observed during their culture in differentiation medium, a phenomenon that was detrimental to the proliferation of embryos by secondary embryogenesis (Prado et al., 2010). The comparison of plant conversion between those precociously germinated embryos and embryos normally germinated in germination medium showed that precocious

Abbreviations: ABA, abscisic acid; ADC, arginine decarboxylase; dcSAM, decarboxylated S-adenosylmethionine; DAPI, 4′,6-diamidino-2-phenylindole; DAO, diamine oxidase; 2,4-D, 2,4-dichlorophenoxyacetic acid; DM, differentiation medium; DW, dry weight; EF1-α, elongation factor 1-alpha; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ODC, ornithine decarboxylase; PBS, phosphate-buffered saline buffer; PAO, polyamine oxidase; SAM, S-adenosylmethionine; SAMDC, S-adenosylmethionine decarboxylase; SPDS, spermidine synthase; SPMS, spermine synthase.

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germination in differentiation medium reduced plant conversion even at high rates depending on the cultivar (Prado et al., 2010). The absence of a peak of endogenous ABA appears be involved in the abnormal maturation and precocious germination of grapevine somatic embryos (Faure et al., 1998; Prado et al., 2014).

Different strategies have been used to reduce the maturation problems of somatic embryos (Dhekney et al., 2020). An improvement of maturation has been obtained with the incorporation of a semipermeable membrane between the somatic embryo aggregates and the differentiation medium (Acanda et al., 2020). The use of these membranes has emerged as a very effective strategy in the maturation of somatic embryos of citrus (Niedz et al., 2002), olive (Cerezo et al., 2011) and avocado (Palomo-Ríos et al., 2013). The effectiveness of this technique is associated with the ability of the membranes to restrict the availability of water to embryos and thus subject them to conditions of water stress, as shown by Acanda et al. (2020). Modification of the hydric state during maturation improves the quality of embryos by promoting the synthesis of abscisic acid (ABA), which in turn is related to an increase in ABA levels (Palomo-Ríos et al., 2013). In grapevine, Acanda et al. (2020) described an increase in endogenous ABA levels in somatic embryos that differentiated over a semipermeable membrane due to activation of both ABA synthesis and deconjugation-related genes. Their results demonstrated that some degree of water deficit contributes to adequate maturation of somatic embryos, thus avoiding the occurrence of precocious germination.

Recent studies have revealed the existence of coregulation between endogenous levels of ABA and polyamines. Thus, it has been verified that the upregulation of polyamine biosynthesis genes, observed when subjecting *Arabidopsis* plants to water stress, does not occur in ABA-deficient or ABA-insensitive mutants, which is consistent with the fact that ABA response elements have been found in the promoters of several of these genes (Alcázar et al., 2006). Similarly, analysis of insertion mutants with mutations in polyamine biosynthesis genes revealed that the loss of functionality of these genes altered the endogenous content of ABA and the ABA-dependent response under cold stress (Cuevas et al., 2009).

In plants, the most common polyamines are putrescine, spermidine and spermine (Bagni and Tassoni, 2001; Zhang and Huang, 2013), which are involved in a wide range of physiological processes, such as germination, organogenesis, flowering, development of the fruit, embryogenesis and, above all, the response to different types of stress (Anwar et al., 2015; Bouchereau et al., 1999; Kuznetsov and Shevyakova, 2007). A number of studies have found relationships between polyamines and plant tolerance to abiotic stress (Alcázar et al. 2020 and references included). Changes in endogenous polyamine levels have been described in grapevine in response to multiple types of abiotic stress, including water stress (Liu et al., 2011; Toumi et al., 2010).

Unlike mammals and fungi, where putrescine is synthesized only from ornithine by the enzyme ornithine decarboxylase (ODC), in plants and bacteria, arginine also is a metabolic precursor of this polyamine through the action of arginine decarboxylase (ADC). Sequential addition of propylamino groups to putrescine and spermidine by spermidine synthase (SPDS) and spermine synthase (SPMS) produces spermidine and spermine polyamines, respectively. The propylamino groups originate from decarboxylated S-adenosylmethionine (dcSAM), a molecule derived from S-adenosylmethionine (SAM) in a decarboxylation reaction catalyzed by the enzyme S-adenosylmethionine decarboxylase (SAMDC), which connects the biosynthesis of polyamines with that of ethylene by competing for common precursors (Bagni and Tassoni, 2001). Endogenous polyamine levels are maintained through balance with catabolism carried out by diamine oxidases (DAOs) and polyamine oxidases (PAOs), releasing H_2O_2 as a signaling molecule. PAO enzymes are also involved in the reversion of the polyamine pathway back to putrescine (Pál et al., 2021).

In grapevine, polyamine involvement in somatic embryogenesis has also been verified, with the putrescine-to-spermidine ratio being a good indicator of the state of somatic embryo development (Bertoldi et al., 2004; Faure et al., 1991). Polyamines seem to play an important role during grapevine somatic embryo differentiation and maturation, contributing to an improvement in the overall conversion rates of plantlets (Bertoldi et al., 2004; Nookaraju et al., 2008).

Due to the involvement of polyamines in somatic embryogenesis processes and their potential metabolic relationship with ABA, in this work, we analyzed the endogenous content of polyamines and the expression patterns of genes involved in critical steps of their metabolism (*VvODC, VvADC, VvSAMDC, VvSPDS, VvSPMS, VvDAO* and *VvPAO*) during the differentiation of 'Mencía' grapevine somatic embryos in DM1 medium with or without the incorporation of a semipermeable membrane. The main goal was to investigate whether the culture of grapevine aggregates over a semipermeable membrane has any influence on the role polyamines play in the differentiation of grapevine somatic embryo aggregates and the maturation of the derived somatic embryos.

2. Materials and methods

2.1. Plant material and embryogenic cultures

Adult field-grown *Vitis vinifera* L. 'Mencía' plants were selected from grapevine collection at the Centro de Formación y Experimentación de Viticultura y Enología de Ribadumia (Galicia, northwestern Spain). Inflorescences at stage H of the Baggiolini (1952) phenological scale corresponding to separate clusters were collected. The flowers were at the late binucleate microspore stage, as determined microscopically by squashing the anthers in the presence of 1 μg mL^{-1} DAPI (4′,6-diamidino-2-phenylindole) and 1% Triton X-100 in phosphate-buffered saline (PBS) buffer and viewing the microspores using an MZ10F fluorescence stereomicroscope (Leica Microsystems GmbH, Wetzlar, Germany). The collected inflorescences were washed twice for 5 min with 200 mL of distilled water containing a drop of detergent (Mistol®, Henkel Ibérica, S. A., Barcelona, Spain), rinsed twice for 5 min with distilled water and then chilled at 4°C for 4-6 d. The inflorescences were then sterilized as described by Kikkert et al. (2005) prior to dissection of immature stamens (anthers plus filaments) to be used as explants for the induction of somatic embryogenesis. Embryogenic cultures were induced from filaments of stamens cultured on medium containing (Nitsch and Nitsch, 1969) salts supplemented with 0.1 μM $CoCl_2$, (Murashige and Skoog, 1962) vitamins, 1 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 4.5 μM thidiazuron, 0.1% casein hydrolysate and 6% sucrose. The medium pH was adjusted to 5.8 before being autoclaved at 98 kPa and 121 °C, and the medium were solidified using 0.3% Gelrite (Duchefa Biochemie, Haarlem, Netherlands). Twenty-five stamens per plate were placed on 90 mm-diameter polystyrene Petri plates containing 25 mL of medium. The cultures were maintained under continuous darkness at 24 ± 1 °C and subcultured onto fresh medium every 30 d.

The differentiation of the somatic embryo aggregates into maturing embryos was accomplished in DM1 medium consisting of the induction medium described above without phytohormones and casein hydrolysate and supplemented with 0.25% activated charcoal in 90 mm-diameter polystyrene Petri plates. The effect of a semipermeable membrane on the differentiation of somatic embryos was tested by transferring somatic embryo aggregates maintained on induction medium to a membrane (dialysis tubing cellulose acetate membrane, 12,000 molecular size limit, Sigma, St. Louis, MO, USA) extended over 25 mL of DM1

medium. The membranes were prepared following the manufacturer's instructions and autoclaved twice in distilled water at 121 °C and 98 kPa for 20 min.

2.2. Determination of the endogenous content of polyamines

Free, acid-soluble and acid-insoluble polyamines were extracted, hydrolyzed and dansylated following the protocol of Marcé et al. (1995). The polyamines were extracted from 50 to 100 mg dry weight (DW) of lyophilized somatic embryo aggregates cultured in DM1 differentiation medium with and without a semipermeable membrane and collected weekly.

The dansylated polyamines were separated by a 6290 Alliance chromatograph (Waters, Milford, MA, USA). A Brownlee ODS Spheri-5 model (C18, 5 μm particle size, 80 Å pore size, 220 × 4.6 mm internal diameter, PerkinElmer, Waltham, MA, USA) was coupled to the instrument. The separated polyamines were detected fluorometrically by a Waters model 474 fluorescence detector. The temperature of the column was kept constant at 15 °C using an Ultimate 3000 Column Compartment thermostated module (Dionex, Sunnyvale, CA, USA).

For chromatographic separation, 20 μL of refrigerated samples was injected at 15 °C, and elution was carried out using a gradient of mobile phases, with a 1% solution of formic acid in distilled water and pure acetonitrile used as eluents (Table S1). Chromatograms were recorded for 15 min, and detection was performed at excitation and emission wavelengths of 252 and 500 nm, respectively. Quantification was performed using a calibration line with pure standards of putrescine, spermidine and spermine (5, 10, 20, 30, 50 and 75 μM). All the measurements were performed in triplicate.

2.3. Histological preparation

Samples of somatic embryo aggregates cultured in DM1 differentiation medium with and without a semipermeable membrane were collected weekly and fixed in 4% paraformaldehyde in PBS buffer (8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.14 M NaCl, 2.7 mM KCl; pH of 7.2) for 24 h. Then, the samples were transferred to 0.1% paraformaldehyde in PBS buffer and stored at 4 °C until use. The fixed samples were dehydrated in an acetone series and embedded in Technovit 8100 (Heraeus Kulzer GmbH, Wehrheim, Germany). Sections (2 μm thick) were stained with 0.075% toluidine blue in water for 5 min for general histological examination or with 1 μg mL^{-1} DAPI and 1% Triton X-100 for 10 min in PBS buffer for nuclear staining. The sections stained with toluidine blue were then mounted in Eukit (Fluka, Buchs, Switzerland), and those stained with DAPI were mounted in 50% glycerol in PBS. The sections were examined under an Eclipse 800 (Nikon, Tokyo, Japan) microscope equipped with an MRC 1024 confocal system (Bio-Rad, Hercules, CA, USA) and a DS-U2 digital camera (Nikon). The sections stained with DAPI were examined using a UV2A (Nikon) fluorescence filter.

2.4. Total RNA extraction and cDNA synthesis

Three independent samples (biological replicates) of 65 mg fresh weight (FW) of somatic embryo aggregates cultured in DM1 medium with and without a semipermeable membrane were collected weekly. The samples were frozen in liquid nitrogen prior to total RNA extraction using an Aurum™ Total RNA Mini Kit (Bio-Rad) according to the manufacturer's instructions. The RNA concentration and purity (260/280 nm and 260/230 nm ratios) were determined with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and analyzed on an Agilent 2100 Bioanalyzer RNA 6000 Nano LabChip (Agilent, Mississauga, ON, Canada) to assess the RNA quality. cDNA was synthesized from the total RNA at a ratio of 1 μg per 20 μL reaction volume using an iScript™ cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions.

2.5. Primer design and real-time qPCR

qPCR primers for the analysis of relative gene expression were designed according to sequences in the 12X grapevine reference genome in the Ensembl Plants database, and their presence in the genome of 'Mencía' grapevine was verified by conventional PCR. The primers used (Table 1) were designed using Gene Runner software (v3.01, Hasting Software, Inc., Las Vegas, USA) and the NCBI primer design tool.

The relative abundance of the studied gene transcripts was determined on a weekly basis during five weeks of somatic embryo culture in DM1 medium with or without a semipermeable membrane. *EF1-α(m)* and *GAPDH(m)* were used as reference genes (Acanda et al., 2020). Somatic embryo aggregates collected at the beginning of culture in DM1 medium were considered the calibrator group. Three biological samples were included per treatment, and each sample was tested in duplicate.

Gene expression analyzes were performed following the Minimum Information for publication of Quantitative real-time PCR Experiments (MIQE) guidelines (Bustin et al., 2009). qPCR was carried out on 20 μL reaction mixtures that comprised 1X SsoFast™ EvaGreen® Supermix (Bio-Rad), each primer at 0.4 μM and 1.66 ng of cDNA in 96-well plates in an iCycler iQ™ real-time thermal cycler (Bio-Rad). The reactions were performed as follows: 1 min at 98 °C, followed by 40 cycles of 5 s at 98 °C and 20 s at 58 °C for annealing and extension. Dissociation curves to verify the specificity of each amplification reaction were generated by heating the amplicons from 65 to 90 °C with a ramp-up setting at 0.5 °C/10 s. Duplicate nontemplate controls were included for each plate.

2.6. Data analyzes

The endogenous polyamine content data were statistically analyzed using one-way ANOVA with Tukey's HSD post hoc test ($p = 0.05$). Statistical analyzes were performed using SPSS statistical software (version 18 for Windows, SPSS, Inc., Chicago, IL).

The qPCR data were analyzed using iCycler iQ™ software (Real-Time Detection System Software, version 3.0 for Windows, Bio-Rad). The raw

Table 1
Primer sequences, amplicon lengths and efficiency for qPCR assays.

Gene description	Accession code	Primer sequence (forward / reverse)	Amplicon length (bp)	qPCR efficiency
VvADC	VIT_03s0038g00760	GTTCGATTGCCTGATGTGCTC / AAACACCTTGGTAGTGAGATTCGTAG	103	1.92
VvSPDS1	VIT_01s0026g00240	GACATTGTCACAAACTGCCG / TGAAATCAACAGCAGGTCCC	130	2.00
VvSPDS2	VIT_17s0000g08030	GCTCTGTCAACTATGCCTGG / CTACACCATGATTCGGCACT	140	2.00
VvSPMS1	VIT_16s0050g02670	TGGTTTTTGAGTCGTCAGCA / ACCACCAACAACCAGAACTC	152	1.99
VvSAMDC	VIT_14s0083g00580	TTCTCTCAATCCCACCCATC / TGCACCAGGAAATATGAAGC	98	1.90
VvODC	VIT_18s0001g00740	TATTATTGGGAAGCGTGTGAGG / GTCACAGTTGCATGGTCGTTTAG	102	_
VvDAO1	VIT_00s0225g00090	AAGGCTTCCAACGTCTTGC / CGGATAAAGGGTCCAAAGGG	199	1.97
VvPAO	VIT_17s0053g00880	AAAGTTGGGATCAGGAGCAT / CCACCACCTTTTTACACCCA	150	2.00

fluorescence data were analyzed using LinRegPCR software (Ruijter et al., 2009) to obtain the mean PCR efficiency for each primer pair. Relative gene expression was determined and statistically analyzed ($p < 0.05$) using REST-2009© software (ver. 2009, Pfaffl et al. 2002); PCR efficiency correction and normalization were performed via two reference genes, and the results were compared with those of the $2^{-\Delta\Delta Cq}$ method.

Samples were randomly collected from independent Petri plates, and the experiments were repeated at least twice independently to ensure the reproducibility of the results.

3. Results

3.1. Morphohistological characterization of 'Mencía' grapevine somatic embryo aggregates during maturation

The somatic embryo aggregates cultured in DM1 differentiation medium with or without the semipermeable membrane presented morphological variations visible to the naked eye beginning from the second week of culture. The presence of the semipermeable membrane

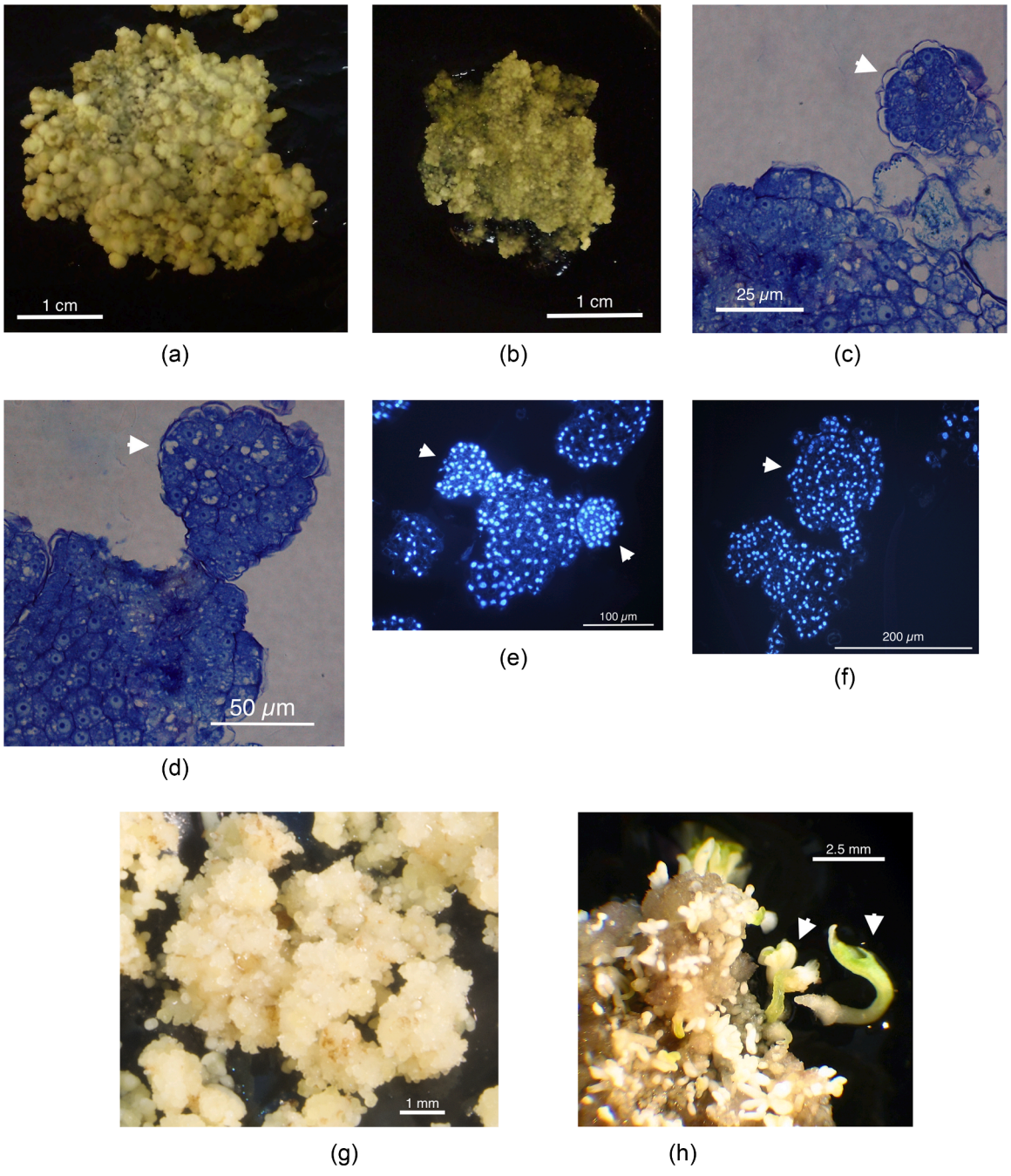

Fig. 1. Morphological (A, B, G and H) and microscopic (B, C, E and F) analysis of somatic embryo aggregates of 'Mencía' grapevine cultured on differentiation medium (DM1) with (A, C, E and G) or without (B, D, F and H) a semipermeable membrane. (A, B) Somatic embryo aggregates after two weeks of culture with (A) or without (B) a semipermeable membrane. (C, D) Toluidine blue staining showing globular somatic embryos after one week in DM1 medium. The globular embryos were smaller when formed in grapevine somatic embryo aggregates cultured over a semipermeable membrane (C) than in those cultured without the membrane (D). (E, F) DAPI staining showing somatic embryos formed by small cells with bright nuclei after two weeks of culture of the somatic embryo aggregates over a semipermeable membrane (E) or somatic embryos formed by larger cells with less bright nuclei in somatic embryo aggregates cultured without a semipermeable membrane (F). (G, H) Somatic embryo aggregates after five weeks of culture with precocious germination being completely absent (G) or present (H, arrowheads) when cultured with or without a semipermeable membrane, respectively. Arrowheads in C, D, E and F indicate the somatic embryos.

caused dehydration of the somatic embryo aggregates, making them whitish and dehydrated (Fig. 1A) instead of the characteristic yellowish color and hydrated appearance of the aggregates grown in the same medium but without the membrane (Fig. 1B).

At a microscopic scale, a strong process of cell proliferation was observed in the first week of culture of the somatic embryo aggregates in DM1 medium with or without a semipermeable membrane, with the presence of cells with high nuclear activity and dense cytoplasm inside the aggregates. This process of division gave rise to embryonic structures, including proembryos and small globular embryos (Fig. 1C and D). These embryos were smaller (25 μm in diameter; Fig. 1C) when formed in the aggregates cultured over the semipermeable membrane than when formed in the aggregates cultured without the membrane (50 μm in diameter; Fig. 1D).

During the next weeks of culture, the somatic embryos originating in the aggregates cultured over the semipermeable membrane doubled in size and comprised small cells with bright nuclei (Fig. 1E). In contrast, in the aggregates cultured without the semipermeable membrane, the embryos increased in size to a greater degree, with some globular embryos being up to four times larger (up to 200 μm in diameter; Fig. 1F) formed by larger cells with nuclei less bright than those observed in the aggregates cultured over the membrane (Fig. 1E).

Finally, after five weeks of culture, the somatic embryo aggregates cultured on DM1 medium over the semipermeable membrane maintained their whitish and dehydrated appearance (Fig. 1G), with no precocious germination. The aggregates cultured without the semipermeable membrane also maintained their yellowish and hydrated appearance, although they presented some precociously germinated embryos over their surface (Fig. 1H).

3.2. Endogenous polyamine content in 'Mencía' grapevine somatic embryo aggregates

Analysis of polyamines in grapevine somatic embryo aggregates grown in DM1 differentiation medium with or without a semipermeable membrane revealed no spermine in most of the analyzed samples. Only low spermine levels were detected in the free polyamine fraction, and the levels were inconsistent; hence, the contribution of spermine could not be taken into consideration in the analysis of the results.

High concentrations of polyamines (putrescine plus spermidine) were determined in somatic embryo aggregates of grapevine 'Mencía' at the beginning of their culture in differentiation medium, and the free fraction (228.1 nmol g^{-1} DW) was 56% of the total polyamine concentration (405.4 nmol g^{-1} DW). The acid–soluble- (118.2 nmol g^{-1} DW) and acid–insoluble-bound (59.1 nmol g^{-1} DW) fractions represented only 29% and 15% of the total polyamines, respectively. Differences in the endogenous content of polyamines in the somatic embryo aggregates were observed during differentiation in DM1 medium with or without a semipermeable membrane. Thus, the total endogenous content of polyamines significantly increased (1771.4 nmol g^{-1} DW) in somatic embryo aggregates cultured for five weeks in DM1 differentiation medium over the semipermeable membrane, in contrast with the somatic embryo aggregates cultured without the semipermeable membrane (861.6 nmol g^{-1} DW). The contribution of each fraction to the total endogenous polyamine content in the somatic embryo aggregates cultured over the semipermeable membrane markedly changed after five weeks, with the free fraction constituting 89% (1578.0 nmol g^{-1} DW) and the acid–soluble- and acid–insoluble-bound fractions constituting approximately 5% (100.6 and 92.8 nmol g^{-1} DW, respectively). In contrast, in the somatic embryo aggregates cultured without the membrane, the contribution of each fraction to the total endogenous polyamine content only slightly changed, with the free fraction constituting 64% (553.6 nmol g^{-1} DW), the acid–soluble-bound fraction constituting 26% (226.5 nmol g^{-1} DW) and the acid-insoluble fraction constituting 10% (81.5 nmol g^{-1} DW).

Free putrescine (Table 2) was initially present at higher levels than those of free spermidine (Table 2). In the somatic embryo aggregates cultured in DM1 media over the semipermeable membrane, free putrescine (Table 2) presented its greatest values in the second (1032.8 nmol g^{-1} DW) and fifth (1525.9 nmol g^{-1} DW) weeks of culture. These concentrations were significantly higher than those in aggregates cultured in DM1 medium without the membrane, in which the free putrescine level did not significantly change during the culture period, reaching its highest level at the end of the culture (526.9 nmol g^{-1} DW). At this time of culture, putrescine accounted for more than 95% of the total free fraction both in aggregates cultured over the membrane (1578.0 nmol g^{-1} DW) and without it (553.6 nmol g^{-1} DW).

In contrast to free putrescine, the free spermidine concentration (Table 2) was low and significantly decreased with time of culture in both membrane treatments. However, the final free spermidine content was significantly higher in somatic embryo aggregates cultured in DM1 medium over the semipermeable membrane (52.1 nmol g^{-1} DW) than in those cultured in DM1 medium without the membrane (26.7 nmol g^{-1} DW).

Acid-soluble putrescine (Table 2) was also initially present at higher levels than those of spermidine (Table 2). The acid–soluble-bound fraction of putrescine (Table 2) significantly increased until the fourth week of culture in somatic embryo aggregates cultured in DM1 medium over the semipermeable membrane, which were also significantly higher than those of somatic embryo aggregates cultured without the membrane. In the last week of culture, the concentration of acid–soluble-bound putrescine (Table 2) decreased to its initial values in somatic embryo aggregates cultured over the semipermeable membrane; these levels were significantly lower than those in the aggregates cultured without the semipermeable membrane. In this fraction, the concentration of spermidine (Table 2) remained low and constant throughout the culture of the aggregates in DM1 medium, with no significant differences between the cultures with or without the semipermeable membrane.

On the other hand, the endogenous concentrations in the acid-insoluble fraction of both polyamines (Table 2) were similar (approximately 30 nmol g^{-1} DW) and did not show significant variations in the somatic embryo aggregates cultured in both semipermeable membrane treatments. The only exception was observed in the fourth week of culture, when the endogenous level of both polyamines in this fraction was significantly higher in aggregates cultured without the membrane than in those with the membrane.

The high putrescine-to-spermidine ratio in the free and acid-soluble (Table 3) fractions obtained for somatic embryo aggregates cultured with both membrane treatments reflected their high content of putrescine, with values that were up to 15-30 times higher than those of spermidine, mostly detected in cultures over the semipermeable membrane.

Finally, in the acid-insoluble fraction, the putrescine-to-spermidine ratio was lower than 1 (Table 3), corresponding to a slightly higher concentration of spermidine than putrescine in this fraction. However, the ratio was also higher in aggregates cultured over the semipermeable membrane, again indicating that the level of putrescine in this fraction was higher in somatic embryo aggregates cultured over the semipermeable membrane than in those cultured without it.

3.3. Expression of polyamine metabolism-related genes during the differentiation of 'Mencía' grapevine somatic embryo aggregates

To further understand the role of polyamines in the effect that the semipermeable membrane has on the differentiation of grapevine somatic embryo aggregates, the relative expression of genes (*VvADC, VvODC, VvSAMDC, VvSPDS1, VvSPDS2, VvSPMS1, VvDAO1* and *VvPAO*) encoding key enzymes for polyamine metabolism was studied using qPCR. The *VvODC* gene transcripts were below the detection threshold and thus not quantified via qPCR.

The *VvADC* gene was expressed in 'Mencía' grapevine somatic

Table 2
Endogenous polyamines content (mean ± S.E., nmol g-1 DW) of 'Mencía' grapevine somatic embryo aggregates cultured for 5 weeks with (Membrane) or without (No membrane) a semipermeable membrane on DM1 differentiation medium. The different letters indicate significant differences between weeks of culture in each membrane treatment (one-way ANOVA with Tukey's post hoc test, $p = 0.05$). The times of culture at which significant differences between membrane treatments were observed are indicated by asterisks.

		Weeks of culture					
		0	1	2	3	4	5
Free putrescine	No membrane	159.1 ± 54.8 a	95.0 ± 27.0 a	378.0 ± 152.2 a *	244.6 ± 104.7 a	220.8 ± 123.7 a	526.9 ± 113.9 a *
	Membrane	159.1 ± 54.8 a	26.5 ± 2.0 a	1032.8 ± 245.0 bc *	500.4 ± 51.4 bc	202.2 ± 24.9 a	1525.9 ± 196.5 bc *
Soluble-bound putrescine	No membrane	96.4 ± 21.3 a	29.0 ± 4.1 a	68.2 ± 17.0 ab	85.9 ± 17.7 ab *	147.3 ± 20.6 bc *	207.6 ± 7.6 c *
	Membrane	96.4 ± 21.3 a	31.5 ± 4.4 a	105.9 ± 31.5 a	194.7 ± 36.5 ab *	337.6 ± 23.9 b *	72.6 ± 9.0 a *
Insoluble-bound putrescine	No membrane	30.0 ± 5.2 a	28.3 ± 1.5 ab	42.3 ± 5.4 abc	44.7 ± 3.5 bc	54.1 ± 6.1 c *	31.2 ± 2.6 ab
	Membrane	30.0 ± 5.2 a	40.0 ± 4.9 a	33.6 ± 2.0 a	36.4 ± 8.4 a	27.4 ± 5.9 a *	36.3 ± 4.4 a
Free spermidine	No membrane	69.0 ± 17.8 a	33.6 ± 5.8 bc	40.4 ± 11.3 bc	67.7 ± 6.8 ab	41.1 ± 6.7 bc	26.7 ± 4.6 c *
	Membrane	69.0 ± 17.8 a	20.7 ± 0.8 b	64.0 ± 11.3 ab	43.6 ± 3.3 b	26.3 ± 4.7 b	52.1 ± 9.1 b *
Soluble-bound spermidine	No membrane	21.8 ± 0.9 a	22.5 ± 3.1 a	23.9 ± 4.9 a	38.6 ± 6.0 a	29.7 ± 9.9 a	19.0 ± 1.6 a
	Membrane	21.8 ± 0.9 a	22.5 ± 0.5 a	29.0 ± 4.7 a	23.8 ± 1.1 a	22.7 ± 1.8 a	28.0 ± 5.9 a
Insoluble-bound spermidine	No membrane	29.2 ± 4.0 a	48.0 ± 8.3 bc	53.3 ± 17.5 bc	63.6 ± 10.7 bc	84.9 ± 13.2 c *	50.3 ± 8.0 bc
	Membrane	29.2 ± 4.0 a	55.2 ± 10.9 a	42.4 ± 8.4 a	34.2 ± 7.5 a	36.5 ± 9.9 a *	56.5 ± 11.4 a

Table 3
Putrescine to spermidine ratios in grapevine ('Mencía') somatic embryo aggregates cultured for five weeks over or not a semipermeable membrane on DM1 differentiation medium.

	Free putrescine to free spermidine ratios		Soluble-bound putrescine to soluble-bound spermidine ratios		Insoluble-bound putrescine to insoluble-bound spermidine ratios	
Weeks of culture	- Membrane	+ Membrane	- Membrane	+ Membrane	- Membrane	+ Membrane
0	2.3	2.3	4.4	4.4	1.0	1.0
1	2.8	1.3	1.3	1.4	0.6	0.7
2	9.4	16.1	2.9	3.6	0.8	0.8
3	3.6	11.5	2.2	8.2	0.7	1.1
4	5.4	7.7	5.0	14.9	0.6	0.8
5	19.7	29.3	11.0	2.6	0.6	0.6

embryo aggregates cultured in DM1 differentiation medium with and without the semipermeable membrane. In aggregates cultured over the semipermeable membrane its expression was high during the first three weeks of culture (Fig. 2A), with a peak in the second week, in which a significant peak of free putrescine specifically was detected (Table 2). However, *VvADC* gene expression decreased in the fourth and fifth weeks of culture, despite the high free putrescine content observed in the last week of culture (Table 2).

As was observed for the *VvADC* gene, the *VvSAMDC* gene was significantly expressed in grapevine somatic embryo aggregates during their differentiation over the semipermeable membrane in the first three weeks of culture (Fig. 2B). In particular, the highest expression of the *VvSAMDC* gene was in the second week of culture when was significantly different with respect to its expression in aggregates cultured without the membrane. *VvSAMDC* gene expression decreased in the following weeks until it was the lowest in the fourth week of culture of the somatic embryo aggregates over the membrane (Fig. 2B), in which it was significantly lower than in aggregates cultured without the membrane. However, in contrast to *VvADC, VvSAMDC* gene expression increased significantly in the fifth week of culture over the membrane, in which low levels of free spermidine were detected (Table 2), while the free putrescine level increased again (Table 2).

As the propylamino groups generated by the activity of the SAMDC enzyme encoded by the *VvSAMDC* gene are used to generate spermidine and spermine, we analyzed the expression of the grapevine genes encoding spermidine synthase (*VvSPDS1* and *VvSPDS2*) and spermine synthase (*VvSPMS1*). The expression of the *VvSPDS1* gene was repressed throughout the measured culture period of the somatic embryo aggregates both over- and without a semipermeable membrane (Fig. S1). The *VvSPDS2* gene (Fig. 2C) was significantly upregulated in the aggregates cultured for two weeks without a semipermeable membrane, whereas its expression did not change in the aggregates cultured over a semipermeable membrane, in which the *VvSAMDC* gene was significantly upregulated (Fig. 2B). The *VvSPDS2* gene was significantly downregulated in somatic embryo aggregates both over and without the semipermeable membrane in the fourth week of culture but was significantly upregulated by the fifth week of culture in the same samples, similar to the expression of the *VvSAMDC* gene (Fig. 2B), although low levels of free spermidine were detected (Table 2). Moreover, the *VvSPMS1* gene was downregulated throughout the culture period of the somatic embryo aggregates in both the presence and absence of the semipermeable membrane (Fig. S2), which was in accordance with the undetectable levels of spermine.

In addition, the expression patterns of the *VvDAO1* (Fig. 2D) and *VvPAO* (Fig. 2E) polyamine catabolism-related genes were determined. The expression of *VvDAO1* increased during culture in differentiation medium in both the presence and absence of semipermeable membranes, although its expression was significantly lower in the second week in somatic embryo aggregates cultured over the membrane, in which the putrescine level increased (Table 2). In contrast, *VvDAO1* was upregulated in aggregates by the fourth and fifth weeks of their culture in differentiation media with or without the semipermeable membrane. It is remarkable that the level of free putrescine was high in aggregates cultured over the membrane by the fifth week of culture (Table 2).

The expression of the *VvPAO* gene (Fig. 2E) did not change in the aggregates cultured in the first weeks without a semipermeable membrane, but it was significantly downregulated in aggregates cultured over the membrane. However, it was significantly upregulated by the fourth and fifth weeks of culture in somatic embryo aggregates cultured over the membrane, but only by the fifth week in aggregates cultured without the membrane, coinciding with low levels of free spermidine (Table 2) in the aggregates cultured with either with or without the membrane.

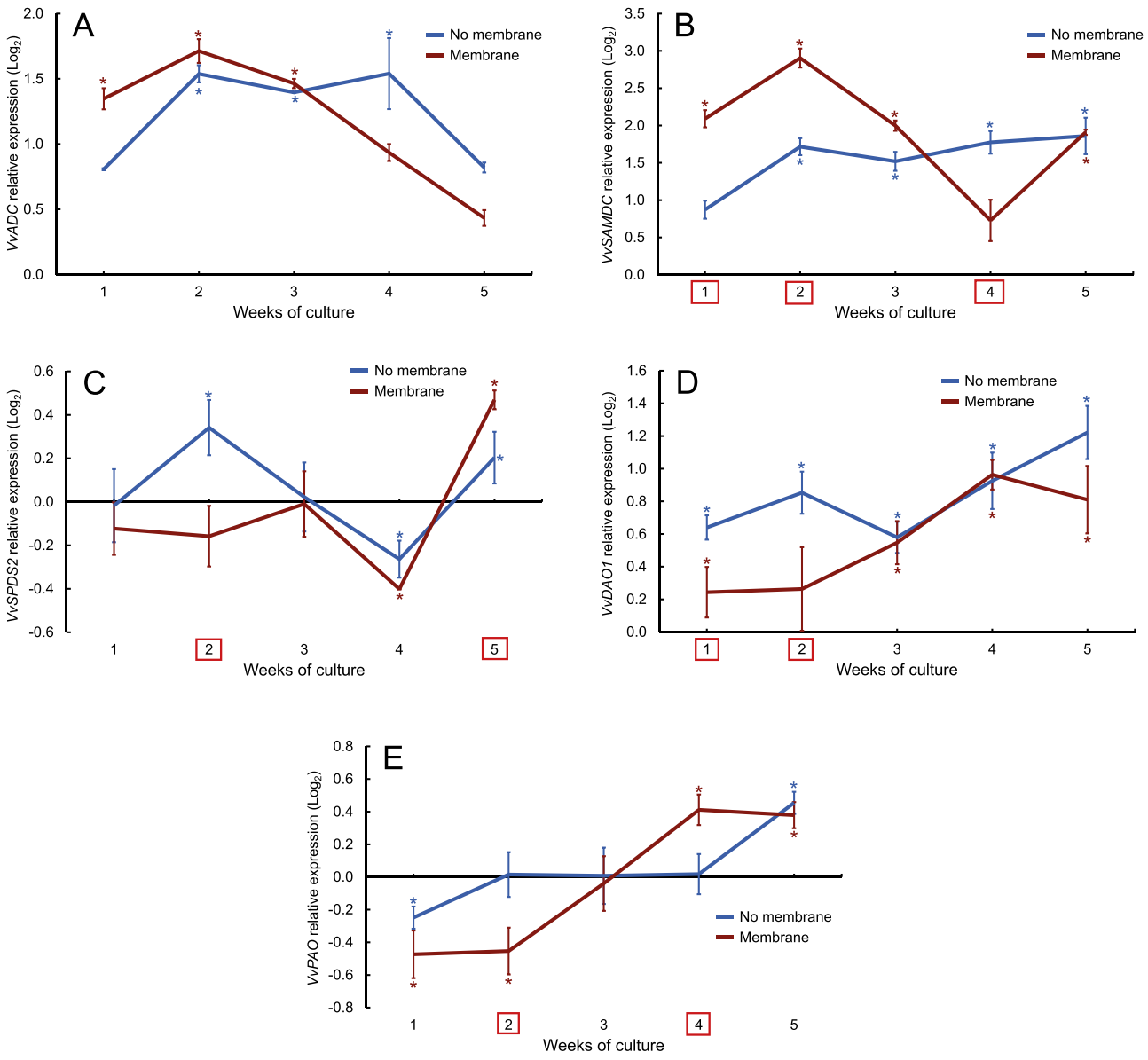

Fig. 2. Relative expression profiles of polyamine metabolism-related genes (A, *VvADC*; B, *VvSAMDC*; C, *VvSPDS2*; D, *VvDAO1*; and E, *VvPAO*) in 'Mencía' grapevine somatic embryo aggregates cultured for 5 weeks with (red line) or without (blue line) a semipermeable membrane on DM1 differentiation medium. Somatic embryo aggregates collected just at the time of transfer to DM1 medium (nontreated samples) were used as the calibration group for relative expression analyzes. *GAPDH(m)* and *EF1-α(m)* were used as reference genes for normalization. The data are presented as the means (± standard errors) of two independent experiments. The asterisks indicate statistically significant differences between the calibrator group and the analyzed group, whereas red squares framing weeks in the X-axes indicate statistically significant differences between treatments with or without the membrane, which in both cases were calculated using REST-2009© software ($p < 0.05$).

4. Discussion

Polyamine metabolism has been studied in relation to its role in somatic embryogenesis and in the stress response in numerous species (Baron and Stasolla, 2008; Pang et al., 2007). In our experimental somatic embryogenesis system, the total endogenous content of polyamines in the somatic embryo aggregates of the 'Mencía' grapevine increased during differentiation in DM1 medium after five weeks of culture, but this increase was significant only in differentiation media with a semipermeable membrane, in which reached four times the initial concentration. The concentration of total polyamines in the 'Mencía' grapevine somatic embryo aggregates grown in DM1 differentiation medium increased mostly due to the increase in the free polyamine fraction, which was the most abundant fraction throughout the culture with both membrane treatments. In particular, the free polyamine fraction in the somatic embryo aggregates cultured over a semipermeable membrane was approximately threefold higher than that in the aggregates cultured without the membrane after five weeks. Increased levels of free polyamines have been found in several species subjected to water stress (Alcázar et al., 2006; Flores and Galston, 1984; Willadino et al., 1996), a condition that induces variations in polyamine concentrations (Groppa and Benavides, 2008; Yamaguchi-Shinozaki and Shinozaki, 2006). We previously showed that the semipermeable membrane generates a water deficit that causes partial dehydration of somatic embryo aggregates (Acanda et al., 2020). This was also evidenced by the marked differences in the appearance of the somatic embryo aggregates cultured over or without the semipermeable membrane (Fig. 1).

Putrescine concentrations constituted more than 95% of the total free fraction in somatic embryo aggregates after five weeks of culture in differentiation medium with both membrane treatments. The free putrescine level peaked in the second and fifth weeks of culture, and these

values were significantly higher in the aggregates cultured over the semipermeable membrane than in those without the membrane. In fact, the somatic embryo aggregates cultured in the second week over the membrane presented globular embryos that exhibited higher proliferative activity and that were smaller than those cultured without the membrane. In the fifth week of culture the aggregates cultured over the semipermeable membrane developed normally without precociously germinating embryos (Fig. 1G). On the contrary, somatic embryo aggregates cultured without the membrane showed abnormally large and precociously germinated embryos (Fig. 1H), coinciding with their substantially lower levels of free putrescine. This suggest the existence of deficiencies in the maturation of these grapevine somatic embryos, and confirm the results of Acanda et al. (2020) who related this process with the metabolism of ABA.

This variation in free putrescine levels during the differentiation and maturation of grapevine somatic embryos coincides with the findings of previous works in other grapevine cultivars (Bertoldi et al., 2004; Faure et al., 1991), in which it was observed that the free putrescine concentration was also high during the initial differentiation phases, after which the concentration decreased as it progressed and increased again in the final stages of maturation of grapevine somatic embryos. Similar results have been obtained in other species, such as carrot (Feirer et al., 1984), cotton (Cheng et al., 2015), *Phaseolus coccineus* L. (Nagl, 1990) and *Picea abies* L. (Gemperlová et al., 2009; Vondráková et al., 2015), in which somatic embryogenesis is related to the accumulation of large amounts of polyamines. On the other hand, the application of polyamine biosynthesis inhibitors has been shown to block embryogenesis in carrot and *Citrus* (Mengoli et al., 1989; Robie and Minocha, 1989; Wu et al., 2009), while the exogenous application of polyamines (mainly putrescine) to proembryogenic masses improves maturation and increases the normal germination rates of somatic embryos in grapevine (Nookaraju et al., 2008), *Citrus* x *sinensis* Osbeck. (Wu et al., 2009) and *Triticum aestivum* L. (Aydin et al., 2016). The existence of such a conserved physiological pattern demonstrates that polyamines are important in the differentiation of somatic embryos.

The question of which of the putrescine synthesis pathways, ADC or ODC, play a greater role in accumulation in plants has not been resolved and seems to be dependent on multiple factors, such as genotype, tissue and experimental conditions (Tiburcio et al., 2014). In particular, a specific role for either of the two pathways in the somatic embryogenesis has not been established, although the ADC pathway has been related to embryo differentiation (Feirer et al., 1984). In our experimental system, the synthesis of putrescine seemed to depend on *VvADC* gene expression, as the transcript encoded by the *VvODC* gene was under the detection threshold. In grapevine somatic embryogenesis, no expression studies have been reported, although (Faure et al., 1991) observed greater ADC enzymatic activity during the differentiation of somatic embryos. Contrasting results have been obtained by Bertoldi et al. (2004), who detected higher ODC enzymatic activity in all the embryogenic phases they analyzed.

The peak of free putrescine observed in the second week of culture of the somatic embryo aggregates over the semipermeable membrane coincides with high relative expression of the *VvADC* gene. This peak also coincides with a significant decrease in water content, with a strong peak in endogenous ABA content, and with a significant upregulation of the *VvNCED1* ABA-synthesis gene that we have previously shown in grapevine somatic embryo aggregates cultured over a semipermeable membrane (Acanda et al., 2020). In addition, free putrescine do not seem to be utilized in the free spermidine synthesis, taking into account its low level and the lack of expression of the spermidine synthesis gene *VvSPDS2*. Beginning at the second week, the expression of *VvADC* progressively decreased, coinciding with the evolution of the concentration of free putrescine until the fourth week. However, in the fifth week of culture the endogenous content of free putrescine increased significantly, which could be explained through the activity of an already present ADC enzyme or through release from its conjugated molecules. Although other authors have questioned the involvement of conjugated polyamines in the development of grapevine somatic embryos, since these molecules were scarce or even null (Bertoldi et al., 2004; Faure et al., 1991), we observed a dramatic reduction in the putrescine levels of the soluble bound fraction in the fifth week of culture (Table 2). This also suggests that at the end of the culture period, the acid–soluble--bound fraction of putrescine could be used to meet in part the cellular demand for free putrescine generated by a water deficit induced in somatic embryo aggregates by the semipermeable membrane (Acanda et al., 2020).

Moreover, putrescine can be produced from spermidine via polyamine oxidase (PAO) activity (Pál et al., 2021), whose encoding gene (*VvPAO*) is upregulated in the somatic embryo aggregates in the fifth week of culture over the semipermeable membrane (Fig. 2E). Hence, this would help explain the high free putrescine levels of the somatic embryo aggregates over the semipermeable membrane at the fifth week of culture. In fact, their endogenous content of free spermidine was low whereas their spermidine synthesis genes *VvSAMDC* and *VvSPDS2* were upregulated. A low spermidine content appears to be inversely related to the maturation of somatic embryos, as has been demonstrated in other grapevine cultivars (Bertoldi et al., 2004; Nookaraju et al., 2008).

In plants, a consequence of the oxidation of polyamines by amine oxidases is the production of H_2O_2, one of the most important abiotic stress signaling molecules (Neill et al., 2002). As abiotic (water) stress seems to be involved in grapevine somatic embryo maturation (Acanda et al., 2020), it can be speculated that this would favor the activation of genes encoding amine oxidase with the consequence of the production of H_2O_2 and the observed increase in free putrescine. The stress-induced expression of PAO-coding genes in *Arabidopsis* supports this hypothesis (reviewed in Chen et al. 2019). Cheng et al. (2015) studied the dynamics of H_2O_2 production during cotton somatic embryogenesis and observed that the levels of this molecule were reduced in nonembryogenic cotton calli, increased when they acquired embryogenic competence and remained high during the differentiation of the embryos. In the same work (Cheng et al., 2015), the recorded *GhSAMDC* gene expression values did not explain the levels of spermidine and spermine. Taken together, this evidence supports the hypothesis that in our experimental system, degradation of spermidine and spermine may have occurred, which would explain the reduced endogenous content of these polyamines while the *VvSAMDC* and *VvSPDS2* genes were upregulated and the free putrescine level increased in the fifth week of culture over the semipermeable membrane.

The putrescine-to-spermidine ratio calculated for the free fraction in the grapevine somatic embryo aggregates during the five weeks of culture under both membrane treatments is a consequence of the accumulation of putrescine. Putrescine was higher in the somatic embryo aggregates cultured over the semipermeable membrane than in those without the membrane, particularly in the second and fifth weeks of culture. The ratio of putrescine to polyamines (spermidine plus spermine) has been used in various species — both gymnosperms and angiosperms — as an indicator of growth patterns (Theiss et al., 2002), morphogenic competence (Paschalidis and Roubelakis-Angelakis, 2005; Rey et al., 1994), and regenerative capacity (Baron and Stasolla, 2008). Therefore, the ratio could indicate the occurrence of differentiation processes in the somatic embryo aggregates in the second week of culture and of maturation of the derived somatic embryos in the fifth week of culture, both over the membrane. In general, high values of this ratio seem to be characteristic of the process of differentiation of somatic embryos in the grapevine (Bertoldi et al., 2004). In this work we observed that better maturation of grapevine somatic embryos, leading to elimination of the detrimental phenomenon of their precocious germination, can be accomplished by culturing somatic embryos over a semipermeable membrane. Taken together, this evidence could indicate that this improvement in the differentiation and maturation of grapevine somatic embryos could be related to high free putrescine levels and the putrescine-to-spermidine ratio, and suggest a relationship with the

ABA metabolism involved in this experimental system as previously described by Acanda et al. (2020).

Spermine was not detected in the grapevine somatic embryo aggregates during culture in DM1 differentiation medium with or without a semipermeable membrane, according to the undetected expression of the *VvSPMS1* gene involved in its synthesis. These results suggest that this molecule does not play a specific or direct role in the differentiation of grapevine somatic embryo aggregates or in the regulatory mechanism in relation to the type of water deficit applied. Unlike putrescine and spermidine, spermine has been shown not to be essential for the survival of some plant species (Imai et al., 2004), and many of its functions can be compensated for by the production of other polyamines (Bouchereau et al., 1999; Kusano et al., 2008).

5. Conclusion

The culture of 'Mencía' grapevine somatic embryo aggregates over a semipermeable membrane had a positive effect on the maturation of somatic embryos, allowing their synchronous development and avoiding the negative phenomenon of precocious germination. This positive effect seems to be related with endogenous polyamines since a higher polyamine content, particularly free putrescine, was detected in somatic embryos cultured over membrane than in those cultured without the membrane, probably due to the water stress conditions induced by the semipermeable membrane. A first peak of free putrescine was detected in the second week of culture over the semipermeable membrane that correlated with an upregulation of the *VvADC* gene for putrescine synthesis. In addition, a second peak of free putrescine at the end of culture over the membrane, correlating with an upregulation of the *VvSPDS2* gene for spermidine synthesis and the *VvPAO* gene for spermidine catabolism. As the level of free spermidine was low and the *VvADC* gene was not significantly expressed, these results may indicate that the increase of free putrescine is due to synthesis from spermidine via *VvPAO* gene expression. The dynamics of polyamine metabolism during 'Mencía' grapevine somatic embryo maturation supports a relationship with the metabolism of abscisic acid as revealed by Acanda et al. (2020) and contributed to understand the underlying mechanisms of the maturation of somatic embryos in *V. vinifera*.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article Table S1. Gradient of mobile phases for chromatographic analysis of the polyamine content Fig. S1. Relative expression profile of the *VvSPDS1* gene Fig. S2. Relative expression profile of the *VvSPMS1* gene.

CRediT authorship contribution statement

Cristina Domínguez: Conceptualization, Data curation, Writing – original draft. **Óscar Martínez:** Conceptualization, Data curation, Writing – original draft. **Óscar Nieto:** Formal analysis. **Yolanda Ferradás:** Formal analysis, Data curation. **María Victoria González:** Conceptualization, Data curation, Writing – original draft. **Manuel Rey:** Conceptualization, Data curation, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data sharing is not applicable to this article, as all newly created data are already contained within this article.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.scienta.2022.111537.

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