Journal of Agricultural Science and Technology B 6 (2016) 387-399
doi: 10.17265/2161-6264/2016.06.004

Cryopreservation by Vitrification of *Vitis vinifera* cv. "Red Globe" Zygotic Embryos and Effect on the Expression of DNA Methyltransferase Genes

Heriberto García-Coronado[1], Maria-Elena Báez-Flores[2], Rosalba Troncoso-Rojas[1], Marisela Rivera-Domínguez[3] and Martín-Ernesto Tiznado-Hernández[1]

1. Coordinación de Tecnología de Alimentos de Origen Vegetal, Centro de Investigación en Alimentación y Desarrollo A.C. (CIAD), Hermosillo, Sonora, CP 83304, México

2. Facultad de Ciencias Químico Biológicas, Universidad Autónoma de Sinaloa, Culiacán, Sinaloa, CP 80013, México

3. Coordinación de Ciencia de los Alimentos, Centro de Investigación en Alimentación y Desarrollo A.C. (CIAD), Hermosillo, Sonora, CP 83304, México

Abstract: Mexico is a large producer of table grape (*Vitis vinifera* L.) and therefore it is important to develop protocols to store the grape varieties germplasm. The objective of the present work was to design a protocol for the cryopreservation by vitrification of zygotic embryos of *V. vinifera* cv. "Red Globe" and evaluate possible epigenetics changes. The plant vitrification solution 2 (PVS2) was utilized before the utilization of liquid nitrogen (LN). The effect of this protocol on embryo viability was tested by the triphenyl-tetrazolium chloride solution, as well as by the *in vitro* development of grape embryos into plantlet. A cDNA expression library of grape zygotic embryos was created to isolate expressed sequence tags of several DNA methyltrasferases. Gene expression of domains rearranged methyltransferase type 1 (DMR1) and DNA (cytosine-5)-methyltransferase 1 (MET1-2) isozymes was analyzed by quantitative reverse transcriptase PCR. The optimal conditions for vitrification were 10 min in 50% PVS2, followed by 10 min in 100% PVS2. Under these conditions, about 30% of plantlet was obtained from embryos after cryopreservation. It was recorded a reduction in the MET1-2 gene expression, which plays a role in the maintenance of DNA methylation. It is possible to cryopreserve viable grape zygotic embryos, although the treatment seems to induce alterations in the normal DNA methylation pattern of the zygotic embryo genome.

Key words: *Vitis vinifera*, zygotic embryo, cryopreservation, vitrification, DNA methyltransferase.

1. Introduction

The grape (*Vitis vinifera L.*) is a crop with great economic importance in the world mainly due to the diverse range of foods and beverages that can be prepared with the fruit and the nutritional and health benefits [1-3]. Mexico is an important producer and exporter of table grape in the world, and Sonora state accounts for 75% of the total table grape production of Mexico. The Sonora's grape production is based in four varieties: "Flame", "Perlette", "Superior" and "Red Globe". Because of this, it is critical to design strategies to store the grape germplasm.

Cryopreservation is a good alternative to preserve the grape germplasm [4]. In this procedure, plant tissues are frozen at temperatures of -196 °C by using liquid nitrogen (LN) [5]. There are several protocols available for cryopreservation, but the cryopreservation by vitrification is one of the most utilized [6]. In this procedure, the formation of ice crystal is suppressed to avoid membrane damage and eliminate the negative effects on the viability of the plant tissues [7]. However, it had been reported that the cryopreservation of plant tissues can induce

Corresponding author: Martín-Ernesto Tiznado-Hernández, Ph.D., research fields: biochemistry and plant molecular biology.

changes in the plant DNA methylation pattern and alter the final phenotype of the plant [8, 9]. The modification of the gene expression induced by changes in the DNA methylation pattern is known as epigenetic alteration. This alteration in the genome regulation is not related with changes in the DNA sequence [10]. There are several epigenetic mechanisms, such as histone acetylation and deacetylation, chromatin remodeling, interference of RNA and DNA methylation [11]. Further, DNA methylation can control replication, transcription, recombination, DNA repair and gene transposition. Also, it is involved in tissue differentiation, gene silencing and genome responses to abiotic or biotic stress [12]. From above, it is very important to analyze the possible effects of any cryopreservation treatment on genomic DNA methylation.

The plant vitrification solution 2 (PVS2) had been utilized in the cryopreservation of different plant species since 1980 [13]. The changes in the DNA methylation pattern had been reported due to cryopreservation protocols in both vegetable [8] and animal cells [14].

The genomic DNA methylation is catalyzed by enzymes known as plant cytosine DNA methyltransferases. They are classified into four families: 5-cytosine DNA methyltransferases type 1 family (MET1), chromatin organization modifier 5-cytosine methyltransferase (CMT), domains rearranged methyltransferases (DRM) and DNA methyltransferase homologue 2 (DNMT2). The enzymes of the families DRM and CMT play a role in the methylation *de novo* of the genomic DNA, whereas the MET family functions by keeping the methylation pattern. Furthermore, the role of the DNMT2 family is not well understood [15, 16].

It was reported that the DNA methyltransferases play a role in several phenomena of plant development, such a cell division in carrot [17], peach [18] and rice [19]. Also, they were shown to play a role in the growth and development of wheat [15]. In agreement with this, the inhibition of DNA methyltransferases alteration in the normal growth and cell differentiation in *Physcomitrella patens* [20].

From above, it was decided to work in the vitrification cryopreservation of grape zygotic embryos to avoid methylation changes related with tissue development. The grape cultivar "Red Globe" was chosen, because the seeds of this cultivar are known to be recalcitrant [21] and it had been reported a reduction in the germination of seeds stored in LN [22]. Thus, the objective of this research was to develop a vitrification cryopreservation treatment for zygotic embryos of grape (*V. vinifera* L. cv. "Red Globe") and study the treatment effect on the expression of genes encoding DNA methyltransferases.

2. Materials and Methods

2.1 Plant Materials

The experiment was carried out using zygotic embryos of the grape variety "Red Globe". The grape berries were obtained from the Association of Local Agricultural Producers of Table Grape at Hermosillo (México). Before the removal of the embryos, the seeds were pre-treated with 1 mg/mL of gibberellic acid (AG3) during 24 h at 25 °C with the goal to break embryo dormancy [23]. After this treatment, in a laminar flow hood, the seeds were surface sterilized using sequentially alcohol solution at 70% during 1 min and a solution containing 1.5% of sodium hypochlorite in water during 7 min. After, they were washed with sterilized distilled water and collocated under a stereo microscope to remove the embryos with a sterile disposable surgical scalpel.

2.2 Vitrification Treatments

The vitrification treatments were carried out utilizing the plant vitrification solution 2 (PVS2) consisting of 30% (w/v) of glycerol, 15% (w/v) of ethylene glycol, 15% (w/v) of dimethyl sulfoxide in a 0.4 M sucrose solution with a pH of 5.8 [24]. The

vitrification of the embryos was done by embryo immersion in the PVS2 solution using a 1.5 mL cryotubes. Five vitrification treatments were designed and other two were obtained from literature: V_1 [25] and V_4 [26]. V_1 treatment was 180 min in 100% of PVS2 at 25 °C; V_2 treatment was 60 min in 50% of PVS2 at 25 °C and 120 min in 100% of PVS2 at 0 °C; V_3 treatment was 60 min in 50% of PVS2 at 25 °C and 60 min in 100% of PVS2 at 0 °C; V_4 treatment was 50 min in 50% of PVS2 at 25 °C and 30 min in 100% of PVS2 at 0 °C; V_5 treatment was 10 min in 50% of PVS2 at 25 °C and 10 min in 100% of PVS2 at 0 °C; V_6 treatment was 5 min in 50% of PVS2 at 25 °C and 5 min in 100 % of PVS2 at 0 °C; V_7 treatment was 1 min in 50% of PVS2 at 25 °C and 1 min in 100% of PVS2 at 0 °C.

2.3 Cryopreservation Treatments

After the vitrification treatment V_4, the PVS2 solution was almost completely eliminated, and the embryos kept in the Eppendorf tube were cryopreserved by immersion in LN [7] during 10 s, 1 h, 1 d, one week or one month. Also, treatments V_5 and V_7 were cryopreserved in the same way but only by 30 min. The positive control was grape zygotic embryos without vitrification and without cryopreservation, and the negative control were embryos cryopreserved without vitrification treatment.

2.4 Assessment of the Effect of Vitrification and Cryopreservation Treatments on Grape Embryo Viability

The embryo viability in this experiment was evaluated by either using the 2,3,5-triphenyl-tetrazolium chloride solution (TTC) as described by Grzybowski et al. [27] or by inducing the embryo development into a plantlet *in vitro* [28]. The TTC protocol was utilized to evaluate embryo viability after vitrification with the treatments V_1, V_2, V_3 and V_4. Also, this protocol was utilized after vitrification and cryopreservation with the V_4 treatment, followed by immersion in LN for 10 s, 1 h, 1 d, one week and one month. For the TTC protocol, embryo viability percentage was calculated by using the next Eq. (1):

$$\text{Embryo viability percentage} = \frac{\text{Abs530 of sample} \times 100}{\text{Abs530 of control}} \quad (1)$$

where, Abs530 corresponds with the solution absorbance at 530 nm wavelength.

The experiment of analysis of embryo viability by using the TTC protocol was carried out in triplicate.

The protocol for the *in vitro* grape embryo development was carried out essentially as reported in Ref. [28]. This protocol was carried out to evaluate the effect of the vitrification treatments V_4, V_5, V_6 and V_7 on embryo viability. The experimental unit in this experiment was 12 embryos and it was carried out in duplicate.

2.5 mRNA Extraction and cDNA Expression Library Creation

Zygotic embryos were frozen with LN and powdered using a micropistil. Total RNA was isolated from the tissue using the RNeasy kit, following the instructions of the manufacturer (QIAGEN México, S. de R.L. de C.V.). The concentration of RNA was assessed with a spectrophotometer NanoDrop ND-1000 UV-Vis (NanoDrop products, 3411 Silverside Road, Bancroft Building, Wilmington, DE 19810, USA) and the quality by agarose gel electrophoresis with 1% of buffer TAE and observation with UV-transilluminator after GelRed staining. The cDNA expression library was created using the SMARTer PCR cDNA synthesis kit, following the instructions of the manufacturer (Clontech Laboratories, Mountain View, CA 94043, USA).

2.6 Isolation of Expressed Sequence Tags (ESTs) Coding for DNA Methyltransferase Enzymes

In order to find ESTs encoding grape DNA methyltransferases, an alignment using the ClustalW

software [29] was carried out with gene sequences from *Arabidopsis thaliana* (NM_124293.3), *Fragaria ananassa* (FJ804059.1; FJ804058.1) and genes of *V. vinifera* identified by bioinformatics analysis with the gene prediction method Gnomon (XM_002267164.1, XM_002267248.2, XM_002268202.2, XM_002273936.2, XM_002264190.1 and XM_002283319.2) of sequences available in the GenBank. Oligonucleotides designed were analyzed with the software Oligo Analyzer 3.1 (integrated DNA technologies) to calculate annealing temperature, GC percentage, primer melting temperature, tendency to form primer-dimers and secondary structures.

The amplification was carried out from the cDNA expression library created. The fragments obtained with the different oligonucleotides pairs designed (Table 1) were cloned into PGEM-T vector and sent for sequencing to the Genomic Analysis and Technology Core Facility, University of Arizona, AZ, USA. Some of the amplified DNA fragments not inserted in a vector were sent for sequencing to Genewicz (Genewiz Global Headquarters, 115 Corporate Boulevard South Plainfield, NJ 07080, USA) and Macrogen Inc. (1002, 254 Beotkkot-ro, Geumcheon-gu Seoul, 153-781, Republic of Korea).

2.7 Quantification of DNA Methyltransferases Gene Expression

The DNA sequences obtained were analyzed with the deduced aminoacid sequence using the BLASTX algorithm [30] against the non-redundant database of the National Center for Biotechnology Information (NCBI). It was isolated two ESTs belonging to the family of DRM , designated as DRM1 and DRM2 with NCBI accession numbers of JZ923779 and JZ923780, respectively. Also, two ESTs of the family of MET, designated as MET1 and MET1-2 with NCBI accession numbers of JZ923781 and JZ923782, respectively, as well as one EST belonging to the family of CMT designated as CMT3 with NCBI accession number of JZ923778. Other characteristics of the ESTs sequences are included in Table 2.

With the sequences of the grape DNA methyltransferase genes obtained, oligonucleotides were designed using the software PrimerQuest from Integrated DNA Technologies to carry out the quantification of two genes expression level: one from the family MET1 and one from the family DRM1. The effects of vitrification and cryopreservation on gene expression was evaluated by the protocol of the $2^{-\Delta\Delta Ct}$ method, with the Brilliant II SYBR Green QRT-PCR Master Mix kit, 1-Step (Agilent Technologies México, S. de R.L. de C.V.) in the StepOne^{TM} real time PCR system (Applied Biosystems de México, S. de R.L. de C.V.). Data normalization was carried out using the gene of *V. vinifera* encoding the glyceraldehyde-3-phosphate dehydrogenase enzyme as a reference [31].

Table 1 Characteristics of the oligonucleotides designed to amplify DNA fragments of genes encoding DNA methyltransferase enzymes from zygotic embryos of *V. vinifera*.

Oligonucleotide ID	Orientation	Sequence (5'-3')	Oligonucleotide size (bp)
DRM1 F	Sense	CAGGTGGCATCACTCTCTTATC	22
DRM1 R	Antisense	TCATCCAGCAGCCTTTCATC	20
DRM2 F	Sense	CAGCAGAGAGACATCCTTCATC	22
DRM2 R	Antisense	TCCAGTATTATGCCACCACTTC	22
MET1 F	Sense	TATTTGCTGGTTGTGGTG	18
MET1 R	Antisense	CTCACCTGATAACCCATTTC	20
MET1-2 F	Sense	GAGCAAAGTTCAGTGTGAAATGAT	24
MET1-2 R	Antisense	GCTCCGAACAGCAGCATA	18
CMT3 F	Sense	AGGTCCTCCATGTCAAGG	18
CMT3 R	Antisense	GGCGATGGTCATAAAGCA	18

DRM1 = domains rearranged methyltransferase type 1; DRM2 = domains rearranged methyltransferase type 2; MET1 and MET1-2 = DNA methyltransferase 1; CMT3 = DNA (cytosine-5)-methyltransferase type 3; F: forward; R: reverse.

Table 2 Results of the analysis using the deduced aminoacid sequence obtained from the nucleotide sequences of the EST.

EST	Length (bp)	Accession No. form NCBI	DNA methyltransferase encoded/organism	Expected value	Identity	Query coverage
DRM1	535	JZ923779	Domains rearrenged methyltransferase type 1/ *Theobroma cacao*	4e-51	82%	56%
DRM2	354	JZ923780	Domains rearrenged methyltransferase type 2/ *Glycine soja*	1e-46	70%	93%
MET1	495	JZ923781	DNA methyltransferase 1/*Malus domestica*	9e-79	90%	87%
MET1-2	408	JZ923782	DNA methyltransferase 1/*Morus notabilis*	8e-32	64%	93%
CMT3	589	JZ923778	DNA (cytosine-5)-methyltransferase type 3/ *Medicago truncatula*	4e-70	76%	95%

The analysis was carried out using the BLASTX algorithm (E > 1e-06) against the non redundant database of the NCBI.

2.8 Statistical Analysis

The effect of vitrification and cryopreservation on embryo viability and relative gene expression was analyzed by one way variance analysis based on a completely randomized design with 95% of confidence. Data percentage was arc sine transformed to get variable normal distribution. When variance analysis showed significant differences among means, it was carried out the Fisher test (LSD) to analyze the embryo viability data and Dunnett test to compare gene relative expression data.

It was utilized the software NCSS 2007 for all statistical analysis with the exception of the gene relative expression data that was analyzed with the program GenEX 6.

3. Results

3.1 Effect of the Vitrification on Embryo Viability

In Fig. 1, it is shown the results of the vitrification effect carried out by four different treatments on the embryo viability percentage, evaluated by using the TTC protocol. Clearly, the V_4 treatment showed the highest viability with 62.92% ± 2.79%, being statistically different with the V_3 treatment with 46.75% ± 1.64% ($P > 0.05$).

Based on the results of the experiment just described, it was decided to test the vitrification cryopreservation using the conditions of the V_4 treatment at different times in LN. As shown in Fig. 2, cryopreservation effect on the viability percentage of the embryos, evaluated with TTC, after vitrification and after 10 s in LN was 62.92% ± 2.79% and 64.53% ± 3.22%, respectively ($P > 0.05$). Further, the embryo viability after 1 h and 1 d in LN was 41.94% ± 7.03% and 46.78% ± 4.63%, respectively. There was no statistical difference between them ($P > 0.05$), but statistically lower ($P < 0.05$) as compared with the viability after 10 s in LN. The embryo viability after one week was 24.4% ± 4.46% and after one month was 25.81% ± 4.46% ($P > 0.05$). However, these last two periods of cryopreservation showed the lowest embryo viability as compared with the other cryopreservation times (Fig. 2). Based on the results of the last experiment described, it was decided to reduce the vitrification time.

3.2 Effect of the Vitrification on Plantlet Regeneration

As shown in Fig. 3, the treatment with the highest plantlet development was the V_7 treatment with 58.33% ± 8.33%. It was statistically lower as compared with the control which showed a 79.13% ± 3.8% of plantlet regeneration, but higher ($P < 0.05$) than V_5 treatment which showed a 37.17% ± 3.8%. Based on the results, it was decided to test the effect of the best two vitrification treatments in combination with cryopreservation on embryo assessed by the protocol of plantlet development.

Based on the results of the embryos without vitrification and cryopreserved (negative control) in Fig. 4, it is clear that the vitrification treatments protect the negative effects of the cryopreservation. By other side, it can be seen that the best vitrification treatment

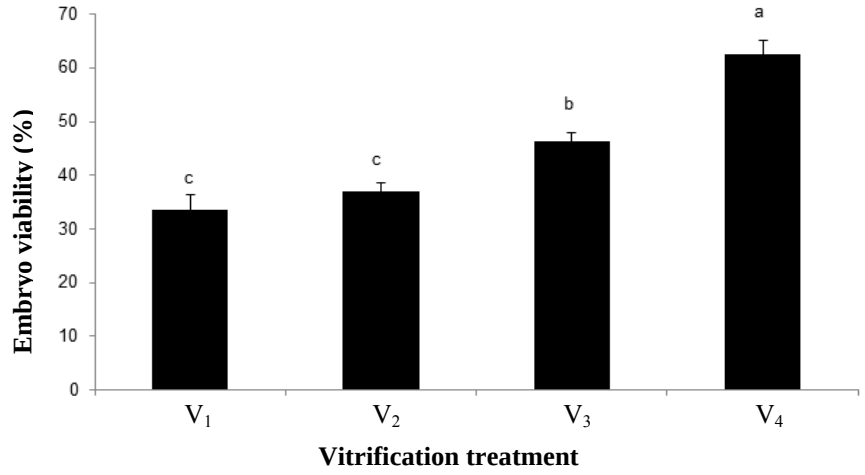

Fig. 1 Effect of different vitrification treatments on the embryo viability (%) evaluated by using the TTC protocol.
The height of bar corresponds with the mean and vertical lines on bars with the standard error. Bars with different letters are significantly different ($P < 0.05$).

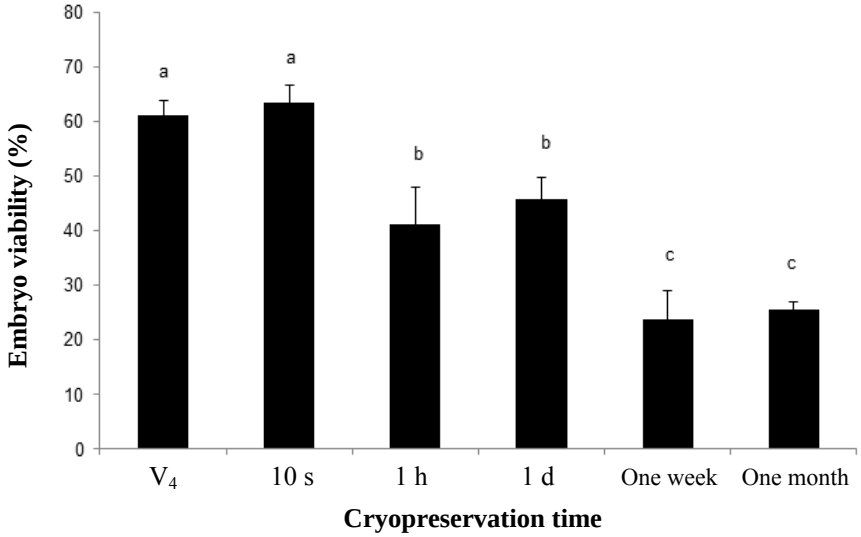

Fig. 2 Effect of cryopreservation in LN during 10 s, 1 h, 1 d, one week and one month after V_4 vitrification treatment over the zygotic embryo viability assessed using the TTC protocol.
The height of bar corresponds with the mean and vertical lines on bars with the standard error. Bars with different letters are significantly different ($P < 0.05$).

was V_5. After vitrification, V_5 treatment showed an embryo development percentage of 37.94% ± 4.16%, and after 30 min of cryopreservation, the percentage was 29.16% ± 4.16% which was not statistically different ($P > 0.05$). In contrast, after vitrification with V_7 treatment, it was recorded a value of 58.33% ± 8.33% and after cryopreservation for 30 min, it was recorded 4.16% which is statistically lower ($P < 0.05$). Also, both V_5 and V_7 vitrification treatment showed a lower value ($P < 0.05$) as compared with the positive control.

3.3 Effect of the Vitrification on the Expression Level of DNA Methyltransferases

In order to measure the gene message levels using the approach of comparative ΔΔCt, a dilution series of the genes MET1-2, DRM1 and the reference gene glyceraldehyde 3-phosphate dehydrogenase (GADPH)

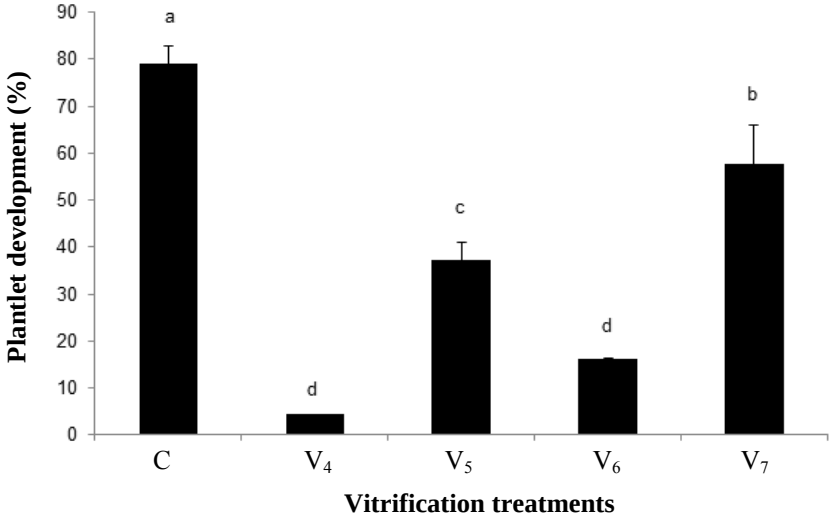

Fig. 3 Effect of different vitrification treatments on the embryo viability assessed by the induction of plantlet development.
C: control group without vitrification treatment.
The height of bar corresponds with the mean and vertical lines on bars with the standard error. Bars with different letters are significantly different ($P < 0.05$).

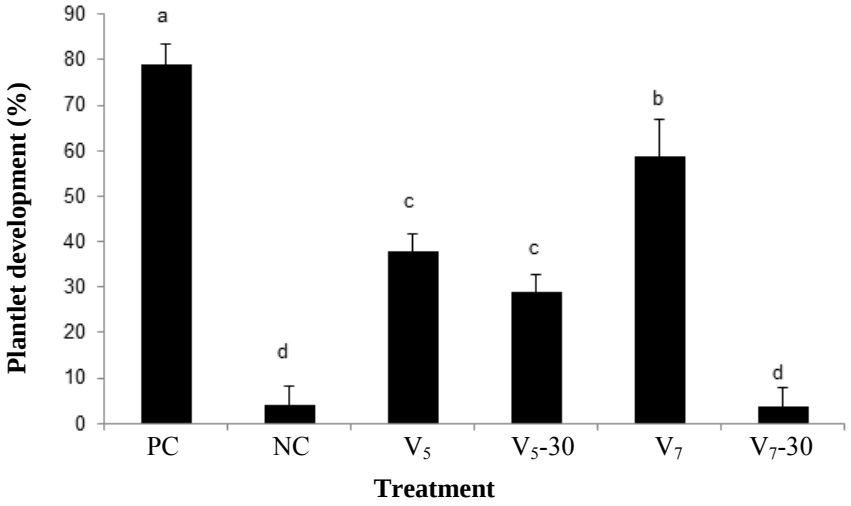

Fig. 4 Effect of the vitrification with V_5 and V_7 treatments and cryopreservation during 30 min on the embryo viability assessed by the induction of plantlet development.
PC: embryos without vitrification and without cryopreservation; NC: embryos without vitrification and cryopreserved during 30 min; V_5-30 or V_7-30: V_5 or V_7 vitrification treatment + 30 min of cryopreservation.
The height of bar corresponds with the mean and vertical lines on bars with the standard error. Bars with different letters are significantly different ($P < 0.05$).

were carried out to measure the reaction efficiency. The efficiency for DRM1 and MET1-2 was between -3.179 and -3.32, which is within the ideally reaction efficiency range of -3.58 and -3.10. By other side, the efficiency of the GADPH reference gene was 3.0901, which is close to the lower side of the reaction efficiency range. All regression lines showed a coefficient of determination higher than 0.98 (data not shown).

The changes in the expression level of the gene MET1-2 in response to V_5 and V_7 vitrification treatments and cryopreservation during 30 min and 2

h were shown in Fig. 5. It was recorded lower expression level in V_7-120 and the same expression in the case of the V_5-30 treatments with respect to the control treatment. Further, it was recorded down regulation of the gene for V_7, V_7-30, V_5 and V_5-120 treatments.

The changes in the expression level of the gene DRM1 in response to V_5 and V_7 vitrification treatment and cryopreservation during 30 min (V_5-30 and V_7-30) and 2 h (V_5-120 and V_7-120) were shown in Fig. 6. It was recorded the same expression level for the treatments V_7-30 and V_7-120 and a down regulation in the case of the treatments V_7, V_5, V_5-30 and V_5-120.

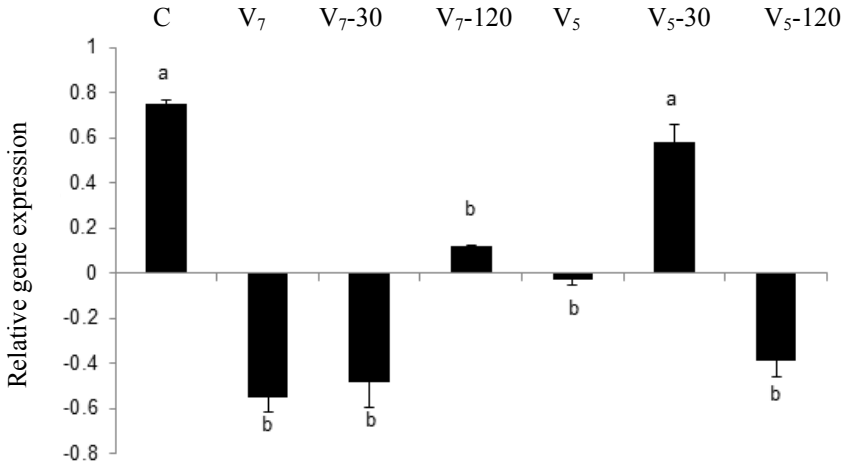

Fig. 5 Gene expression level of the gene MET1-2 in grape zygotic embryos after vitrification with V_5 and V_7 treatments.
C: embryos without vitrification and without cryopreservation (control); V_5-30 or V_5-120: V_5 vitrification treatment + 30 min or + 120 min of cryopreservation; V_7-30 or V_7-120: V_7 vitrification treatment + 30 min or + 120 min of cryopreservation.
The height of bar corresponds with the mean and vertical lines on bars with the standard error. Bars with different letters are significantly different ($P < 0.05$).

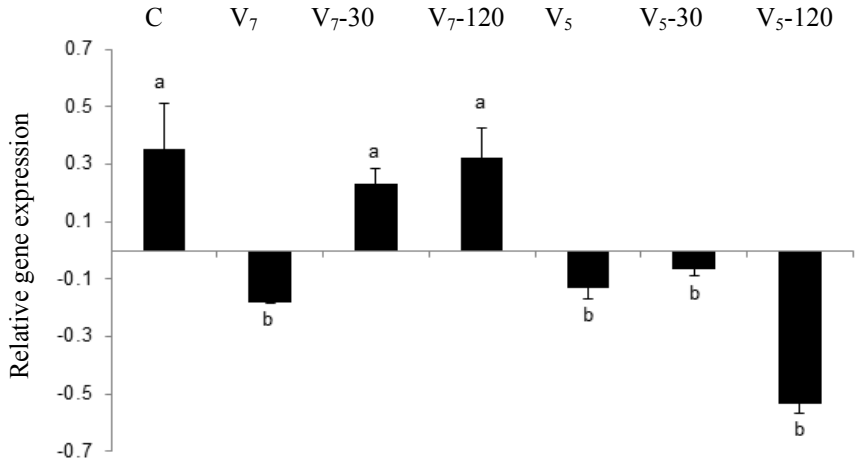

Fig. 6 Level of the gene DRM1 in grape zygotic embryos after vitrification with V_5 and V_7 treatments.
C: embryos without vitrification and without cryopreservation (control); V_5-30 or V_5-120: V_5 vitrification treatment + 30 min or + 120 min of cryopreservation; V_7-30 or V_7-120: V_7 vitrification treatment + 30 min or + 120 min of cryopreservation.
The height of bar corresponds with the mean and vertical lines on bars with the standard error. Bars with different letters are significantly different ($P < 0.05$).

4. Discussion

Because there are very few manuscripts, to our knowledge, describing the cryopreservation of grape zygotic embryos, the results of this work will be discussed with results of similar experiments carried out in other plant species and tissues. In this experiment, for first time, a vitrification cryopreservation protocol was developed for grape zygotic embryos, to our knowledge. It was found that the utilization of 100% PVS2 solution only for longer times can damage the embryos as assessed by the TTC protocol. Further, it was recorded better embryo viability by using 50% of PVS2 before exposure to 100% of PVS2 by using the TTC protocol (Fig. 1). However, after finding the best vitrification conditions among four different treatments using the TTC protocol (V_4 treatment), it was recorded that this treatment reduced the viability of embryos after either 1 h or one month in LN (Fig. 2).

In contrast with this experiment data, shoot tips of *Carica papaya* cultivars "70" and "B2" exposed to 20% PVS2 solution for 60 min, followed by 100% PVS2 for 20 min and stored at LN during 2, 6, 12 and 18 months showed no effects on leaf and shoot development from shoot tips [32]. It was reported that the amount of ice formation is much lower using 60 min of PVS2 exposure time [33]. However, in the present experiment, it was recorded a better embryo viability using lower concentration of PVS2 and short exposure time. The negative effects observed with larger exposure times to the PVS2 solution can be explained by the fact that glycerol is more toxic to plant cells in the presence of ethylene glycol and dimethyl sulfoxide, which are components of the PVS2 [34]. Besides, it appears that 15% of dimethyl sulfoxide in the PVS2 solution can damage the plant tissues [35]. Indeed, the reduction in the amount of dimethyl sulfoxide from 15% to 10% and 5% in the PVS2 solution, improved the regeneration of *Loxocarya cinerea* shoot tips genotype SXH404 from 20% to almost 60%, respectively [33]. In contrast, the exposure of shoot tips of apple cultivars to 10% of dimethyl sulfoxide diluted in MS media containing 30 g/L of sucrose at pH 5.7 reduced the regeneration percentage of several *Malus domestica* Borkh cultivars [36]. The results with the TTC protocol do not directly correlate with the number of living cells [37]. Because of that, plantlets were induced from embryo to test the embryo viability. By using this protocol, it was found better results by reducing time and concentration of PVS2. The treatment with the highest plantlet development percentage was exposed during 1 min with 50% PVS2, and after that, exposure during 1 min with 100% PVS2 (V_7 treatment), as compared with V_4, V_5 and V_6 treatment conditions, which includes higher exposure times to the two PVS2 solutions at the same concentration as described for V_7 treatment (Fig. 3). In agreement with these data, embryogenic calli developed from tissues of *Anemarrhena asphodeloides* Bunge treated with a solution of 2 M glycerol and 0.4 M sucrose before a treatment with 100% of PVS2 during 10, 20, 30, 40, 50 and 60 min showed a reduction ($P < 0.05$) in the percentage of plant development after 50 min and 60 min of exposure as compared with 40 min [19]. Also, when the grape rootstock shoot tips of the cultivar "Kober 5BB" (*Vitis berlandieri* × *Vitis riparia*) were exposed to 100% of PVS2 during 0, 30, 60 and 90 min, it was recorded a 94.2%, 57%, 14% and 0% of tissue viability, respectively. Furthermore, it was noticed malformations in 44% of the plant tissues developed from shoot tips under 60 min exposure or more [38]. In this experiment, it was also recorded plantlets with smaller leaves, yellow leaves and without leaves and the presence of callus tissue only in some treatments (data not shown). In contrast, shoot tips pre-cultured with 0.5 M of sucrose during 24 h, of the apple cultivars "Colmar", "Florina", "Idared" and "Rebra" before exposure to 100% of PVS2 solution for up to 60 min, did not show any alterations in the tissue morphology [36].

The V_5 and V_7 treatments induced less damage to the embryos as evaluated by plantlet development (Fig. 3). However, after testing the cryoprotective properties of V_5 and V_7 treatments using LN during 30 min, it was recorded that V_5 treatment protected better the embryos against the cryopreservation damage (Fig. 4). These results clearly showed that longer times of PVS2 exposure protect better the plant tissue. In agreement with this results, shoot tips of *C. papaya* genotypes "Z6", "97", "TS2" and "35" treated during 20 min with 20% PVS2 and during 20 min with 100% PVS2 before cryopreservation in LN showed a 60% of plantlet recovery [39]. In contrast, it was recorded an inverse relationship between the exposure time to PVS2 during 30, 60 and 90 min and the development of *C. papaya* zygotic embryos, in such a way that after 90 min, the embryo failed completely to develop [40].

There are not reports in the literature studying the effect on genes encoding DNA methyltransferase enzymes of grapes in response to vitrification and cryopreservation, to our knowledge. Because of this, the similar studies in other plant species and tissues will be utilized.

It is clear that cryopreservation can induce alterations at the level of genome DNA methylation. It was recorded alteration in the methylation of a 2.55 kb fragment of the rDNA region in shoot cultures of *Solanum tuberosum* cultivar "Golden Wonder" by using restriction fragment length polymorphism. The tissues were treated during 1 h with MS 1× including 10% of dimethyl sulfoxide before cryopreservation [41]. Also, analysis of 5-methylcytosine changes of the cryotolerant *Ribes nigrum* cv. "Ben More" and "Ben Tron" and the cryosensitives species *R. sanguineum* cv. "King Edward VII" and *R. ciliatum* showed an increase and decrease of the methylation percentage of the genomic DNA, respectively, after encapsulation-dehydration and cryopreservation [42].

It was recorded lower expression in V_7-120 treatment and down regulation for V_7 and V_7-30 treatments, with respect to the positive control in the gene MET1-2. This gene plays a role in the maintenance of DNA methylation pattern. Further, no statistical differences ($P > 0.05$) were found for V_5 treatment after cryopreservation during 30 min (Fig. 5). However, it was recorded down regulation in V_5 and V_5-120 treatments. These results suggest that both V_5 and V_7 vitrification cryopreservation treatments could induce a change in the normal DNA methylation pattern, by reducing the enzyme activity which maintains the DNA methylation pattern, although more experimental evidences are needed to support this statement. However, shoot tips of *C. papaya* cultivar "97" treated during 20 min with 20% PVS2 and during 20 min with 100% PVS2 before cryopreservation showed a methylation marker variation of 2.13%, evaluated by using amplified DNA methylation polymorphism technique [39]. Also, analyses using the protocol of methylation-sensitive amplified polymorphism shoot tips in *Malus pumila* cv. "M26" subjected to encapsulation-dehydration procedure and cryopreservation with 100% PVS2 solution found a reduction in the methylation in five sites of the genomic DNA [43]. Besides, reduction in the methylation in one site of genomic DNA was found in shoot tips of *M. pumila* cv. "Joho" subjected to same treatment [43].

In the case of the DRM1 gene, which plays a role altering the DNA methylation pattern *de novo*, it was recorded the same level of expression in V_7 treatment or a down regulation in the case of V_5 treatment (Fig. 6). These results suggested that no changes in the DNA methylation pattern *de novo* were induced by the vitrification and cryopreservation treatments of this experiment, although more experimental evidences are needed. In agreement, analyses using the protocol of methylation-sensitive amplified polymorphism in shoot tips in *M. pumila* cv. "M26" and "Joho" subjected to encapsulation-dehydration procedure and cryopreservation with 100% PVS2 solution did not find *de novo* changes in the level of genome methylation [43, 44].

5. Conclusions

It was possible to develop a vitrification cryopreservation procedure for zygotic embryos of *V. vinifera* cv "Red Globe" using the PVS2 solution at two different concentrations. Furthermore, it was possible to demonstrate the transcriptional activity of several DNA methyltransferase genes in the grape zygotic embryo. Also, data suggest that the vitrification cryopreservation treatment induced changes in the genome methylation by lowering the DNA methyltransferase expression which plays a role in the maintenance of the normal DNA methylation pattern.

Acknowledgments

This work was financially supported by the National Council of Science and Technology of Mexico, grant No. 168895.

References

[1] Basli, A., Soulet, S., Chaher, N., Mérillon, J. M., Chibane, M., Monti, J. P., and Richard, T. 2012. "Wine Polyphenols: Potential Agents in Neuroprotection." *Oxid. Med. Cell. Longev.* doi: 10.1155/2012/805762.

[2] Huang, Y. L., Chu, H. F., Dai, F. J., Yu, T. Y., and Chau, C. F. 2012. "Intestinal Health Benefits of the Water-Soluble Carbohydrate Concentrate of Wild Grape (*Vitis thunbergii*) in Hamsters." *J. Agric. Food Chem.* 60 (19): 4854-8.

[3] Tomé-Carneiro, J., Gonzálvez, M., Larrosa, M., Yáñez-Gascón, M. J., García-Almagro, F. J., Ruiz-Ros, J. A., García-Conesa, M. T., Tomás-Barberán, F. A., and Espín, J. C. 2012. "One-Year Consumption of a Grape Nutraceutical Containing Resveratrol Improves the Inflammatory and Fibrinolytic Status of Patients in Primary Prevention of Cardiovascular Disease." *Am. J. Cardiol.* 110 (3): 356-63.

[4] Engelmann, F. 2011. "Use of Biotechnologies for the Conservation of Plant Biodiversity." *In Vitro Cell. Dev. Biol.—Plant* 47 (1): 5-16.

[5] Marco-Medina, A., and Serrano-Martínez, F. 2012. "Cryopreservation: Tool for the *Ex Situ* Conservation of Plant Material." *Biodiversity Notebooks* 38: 9-12.

[6] Martinez-Montero, M. E., and Harding, K. 2015. "Cryobionomics: Evaluating the Concept in Plant Cryopreservation." In *Plant Omics: The Omics of Plant Science.* New Delhi, India: Springer, 655-82.

[7] Sakai, A., Kobayashi, S., and Oiyama, I. 1990. "Cryopreservation of Nucellar Cells of Navel Orange (*Citrus sinensis* Osb. var. *brasiliensis* Tanaka) by Vitrification." *Plant Cell Rep.* 9 (1): 30-3.

[8] Harding, K. 2004. "Genetic Integrity of Cryopreserved Plant Cells: A Review." *Cryo Letters* 25 (1): 3-22.

[9] Miguel, C., and Marum, L. 2011. "An Epigenetic View of Plant Cells Cultured *in Vitro*: Somaclonal Variation and Beyond." *J. Exp. Bot.* 62 (11): 3713-25.

[10] Rapp, R. A., and Wendel, J. F. 2005. "Epigenetics and Plant Evolution." *New Phytol.* 168 (1): 81-91.

[11] Achrem, M., Skuza, L., Kalinka, A., Szućko, I., Filip, E., Słominska-Walkowiak, R., and Rogalska, S. M. 2012. "Role of Epigenetic Mechanisms in Plant Response to Low Temperature." *Acta Biol. Cracov.—Bot.* 54 (1): 7-15.

[12] Vanyushin, B. F. 2005. "Enzymatic DNA Methylation Is an Epigenetic Control for Genetic Functions of the Cell." *Biochemistry (Moscow)* 70 (5): 488-99.

[13] Kulus, D., and Zalewska, M. 2014. "Cryopreservation as a Tool Used in Long-Term Storage of Ornamental Species: A Review." *Sci. Hortic.* 168: 88-107.

[14] Chatterjee, A., Saha, D., Niemann, H., Gryshkov, O., Glasmacher, B., and Hofmann, N. 2017. "Effects of Cryopresevation on the Epigenetic Profile of Cells." *Cryobiology* 74: 1-7.

[15] Dai, Y., Ni, Z., Dai, J., Zhao, T., and Sun, Q. 2005. "Isolation and Expression Analysis of Genes Encoding DNA Methyltransferase in Wheat (*Triticum aestivum* L.)." *Biochim. Biophys. Acta* 1729 (2): 118-25.

[16] Pavlopoulou, A., and Kossida, S. 2007. "Plant Cytosine-5 DNA Methyltransferases: Structure, Function and Molecular Evolution." *Genomics* 90 (4): 530-41.

[17] Bernacchia, G., Primo, A., Giorgetti, L., Pitto, L., and Cella, R. 1998. "Carrot DNA-Methyltransferase Is Encoded by Two Classes of Genes with Differing Patterns of Expression." *Plant Journal* 13 (3): 317-29.

[18] Giannino, D., Mele, G., Cozza, R., Bruno, L., Testone, G., Ticconi, C., Frugis, G., Bitonti, M. B., Innocenti, A. M., and Mariotti, D. 2003. "Isolation and Characterization of a Maintenance DNA-Methyltransferase Gene from Peach (*Prunus persica* (L.) Batsch): Transcript Localization in Vegetative and Reproductive Meristems of Triple Buds." *J. Exp. Bot.* 54 (393): 2623-33.

[19] Teerawanichpan, P., Chandrasekharan, M. B., Jiang, Y., Narangajavana, J., and Hall, T. C. 2004. "Characterization of Two Rice DNA Methyltransferase Genes and RNAi-Mediated Reactivation of a Silenced Transgene in Rice Callus."*Planta* 218 (3): 337-49.

[20] Malik, G., Dangwal, M., Kapoor, S., and Kapoor, M. 2012. "Role of DNA Methylation in Growth and Differentiation in *Physcomitrella patens* and Characterization of Cytosine DNA Methyltransferases." *FEBS Journal* 279 (21): 4081-94.

[21] Engelmann, F. 2004. "Plant Cryopreservation: Progress and Prospects." *In Vitro Cell. Dev. Biol.—Plant* 40 (5): 427-33.

[22] Hassan, N. A., Gomaa, A. H., Shahin, M. A., and El-Homosany, A. A. 2013. "*In Vitro* Storage and Cryopreservation of Some Grape Varieties." *J. Hortic. Sci. Ornamen. Plants* 5 (3): 183-93.

[23] Ellis, R. H., Hong, T. D., and Roberts, E. H. 1983. "A Note on the Development of a Practical Procedure for Promoting the Germination of Dormant Seed of Grape." *Vitis* 22: 211-9.

[24] Marković, Z., Chatelet, P., Sylvestre, I., Kontić, J. K., and Engelmann, F. 2013. "Cryopreservation of Grapevine (*Vitis vinifera* L.) *in Vitro* Shoot Tips." *Centr. Eur. J. Biol.* 8 (10): 993-1000.

[25] Lazo-Javalera, M. F., Tiznado-Hernández, M. E., Vargas-Arispuro, I., Valenzuela-Soto, E., Rocha-Granados, M. D. C., Martínez-Montero, M. E., and Rivera-Domínguez, M. 2015. "Data on Antioxidant Activity in Grapevine (*Vitis vinifera* L.) Following Cryopreservation by Vitrification." *Data in Brief* 5: 549-55.

[26] Matsumoto, T., and Sakai, A. 2003. "Cryopreservation of Axillary Shoot Tips of *in Vitro*-Grown Grape (*Vitis*) by a Two-Step Vitrification Protocol." *Euphytica* 131 (3): 299-304.

[27] Grzybowski, C. R. D. S., Ohlson, O. D. C., Silva, R. C. D., and Panobianco, M. 2012. "Viability of Barley Seeds by the Tetrazolium Test." *Rev. Bras. Sementes* 34 (1): 47-54.

[28] Tiznado-Hernández, M. E., Miranda-Jiménez, A., Ojeda-Contreras, Á. J., Sánchez-Estrada, A., Arreola-Ortiz, H. J., and Martínez-Díaz, G. 2015. "Development of New Seedless Grape Varietes (*Vitis vinífera*) by Embryo Rescue." *Rev. Mex. Cienc. Agríc.* 6 (5): 917-28.

[29] Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J., and Higgins, D. G. 2007. "Clustal W and Clustal X Version 2.0." *Bioinformatics* 23 (21): 2947-8.

[30] Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. 1997. "Gapped BLAST and PSI-BLAST: A New Generation of Protein Database Search Programs." *Nucleic Acids Res.* 25 (17): 3389-402.

[31] Reid, K. E., Olsson, N., Schlosser, J., Peng, F., and Lund, S. T. 2006. "An Optimized Grapevine RNA Isolation Procedure and Statistical Determination of Reference Genes for Real Time (RT)-PCR during Berry Development." *BMC Plant Biology* 6 (1): 27.

[32] Kaity, A., Drew, R. A., and Ashmore, S. E. 2013. "Genetic and Epigenetic Integrity Assessment of Acclimatised Papaya Plants Regenerated Directly from Shoot-Tips Following Short- and Long-Term Cryopreservation." *Plant Cell, Tissue and Organ Cult.* 112 (1): 75-86.

[33] Kaczmarczyk, A., Funnekotter, B., Turner, S. R., Bunn, E., Bryant, G., Hunt, T. E., and Mancera, R. L. 2013. "Development of Cryopreservation for *Loxocarya cinerea*: An Endemic Australian Plant Species Important for Post-mining Restoration." *Cryo Letters* 34 (5): 508-19.

[34] Volk, G. M., Harris, J. L., and Rotindo, K. E. 2006. "Survival of Mint Shoot Tips after Exposure to Cryoprotectant Solution Components." *Cryobiology* 52 (2): 305-8.

[35] Wang, Z. C., and He, Y. X. 2009. "Effect of Cryopreservation on the Development and DNA Methylation Patterns of *Arabidopsis thaliana*." *Life Sci. J.* 6 (1): 55-60.

[36] Halmagyi, A., Deliu, C., and Isac, V. 2010. "Cryopreservation of *Malus* Cultivars: Comparison of Two Droplet Protocols." *Sci. Hortic.* 124 (3): 387-92.

[37] Mikuła, A., Niedzielski, M., and Rybczyski, J. J. 2006. "The Use of TTC Reduction Assay for Assessment of *Gentiana* spp. Cell Suspension Viability after Cryopreservation." *Acta Physiol. Plant.* 28 (4): 315-24.

[38] Ganino, T., Silvanini, A., Beghé, D., Benelli, C., Lambardi, M., and Fabbri, A. 2012. "Anatomy and Osmotic Potential of the *Vitis* Rootstock Shoot Tips Recalcitrant to Cryopreservation." *Biol. Plant.* 56 (1): 78-82.

[39] Kaity, A., Ashmore, S. E., and Drew, R. A. 2009. "Field Performance Evaluation and Genetic Integrity Assessment of Cryopreserved Papaya Clones." *Plant Cell Rep.* 28 (9): 1421-30.

[40] Nadarajan, J., and Pritchard, H. W. 2014. "Biophysical Characteristics of Successful Oilseed Embryo Cryoprotection and Cryopreservation Using Vacuum Infiltration Vitrification: An Innovation in Plant Cell Preservation." *PLoS ONE* 9 (5): e96169.

[41] Harding, K. 1991. "Molecular Stability of the Ribosomal RNA Genes in *Solanum tuberosum* Plants Recovered from Slow Growth and Cryopreservation." *Euphytica* 55 (2): 141-6.

[42] Johnston, J. W., Benson, E. E., and Harding, K. 2009. "Cryopreservation Induces Temporal DNA Methylation Epigenetic Changes and Differential Transcriptional Activity in Ribes Germplasm." *Plant Physiol. Biochem.* 47 (2): 123-31.

[43] Hao, Y. J., Liu, Q. L., and Deng, X. X. 2001. "Effect of Cryopreservation on Apple Genetic Resources at Morphological, Chromosomal and Molecular Levels."

Cryobiology 43 (1): 46-53.

[44] Hao, Y. J., You, C. X., and Deng, X. X. 2002. "Analysis of Ploidy and the Patterns of Amplified Fragment Length Polymorphism and Methylation Sensitive Amplified Polymorphism in Strawberry Plants Recovered from Cryopreservation." *Cryo Letters* 23 (1): 37-46.