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Genetic Stability of Cryopreserved Grapevine (*Vitis vinifera* L.) Genome by Vitrification Method

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Abstract: Grapevine (*Vitis* spp.) is an economically important fruit crop worldwide. In Mexico, Sonora State leads the table grape production and exportation to international markets. In this regard, it is important to preserve the grape varieties during long time without phenotypical or genotypical changes. Cryopreservation is a good alternative, although it very often can induce changes in genome and phenotype. In this study, grapevine cv. "Flame Seedless" axillary buds were cryopreserved by vitrification using the plant vitrification solution 2 (PVS2) and stored in liquid nitrogen (LN) for one hour, one week and one month, respectively. Genetic stability of buds cryopreserved under all treatments was evaluated using inter-simple sequence repeats (ISSR) markers. Ten ISSR primers were evaluated, but only two primers were possible to amplify distinct and reproducible bands with sizes between 300 bp and 2,000 bp. Different ISSR fragment patterns were recorded in cryopreserved buds as compared with control. These results suggest that cryopreservation by LN and vitrification-cryopreservation affect genetic stability in grapevine.

Key words: Table grape, axillary buds, liquid nitrogen, vitrification, PVS2, inter-simple sequence repeats.

1. Introduction

Grapevine (*Vitis vinifera* L.) is considered as one of the most economically important crops in the world [1], largely because it is grown for wine and juice production, and consumed as fresh fruit. Species conservation requires efficient and cost-effective *ex situ* methods, which can be complemented with *in situ* preservation programs [2]. However, grapevine conservation techniques are not particularly efficient for all species, suggesting the need to explore new methods for conservation. The cryopreservation by liquid nitrogen (LN) is a very efficient alternative for the long-term storage of germplasm, due to the large reduction of the metabolic functions. Application of cryopreservation combined with *in vitro* methodologies has offered new opportunities for long-term conservation of vegetative propagated crops [3, 4].

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Protocol for cryopreservation in *Vitis vinifera* L. was first reported by Dussert et al. [5] using embryogenic cell suspension. Later, different cryopreservation techniques, such as encapsulation-dehydration and vitrification [1, 6, 7], had been tested in grapevine tissues, but no analyses of the genome stability were carried out and only recovery, viability and regrowth parameters were recorded. Cryopreservation of axillary buds in grapevine has been developed by Zhao et al. [8] in four grape varieties using encapsulation-dehydration techniques, and the regrowth percentage ranged between 15% and 40%. Later, Pathirana et al. [9] obtained 60% of recovery of cryopreserved tissues in axillary and apical buds of six diverse *Vitis* accessions. In addition, the regeneration of 37% and 78%, respectively, have been recorded in the cryopreservation of grapevine using embryogenic cell suspensions [1, 10-12] and shoot-tips tissue [6, 13].

Cryopreservation process involves many tissue manipulations, such as culture initiation, proliferation,

acclimation, dehydration, cryoprotection, LN exposure, vitrification, rewarming, recovery and regeneration. All these tissue manipulations can induce cell injury and alteration in the genome [14]. For that reason, it is desirable to assess the genetic integrity of the plants after cryogenic storage, in order to determine if they are true-to-type after cryopreservation. Therefore, the study of genomic alterations becomes essential [14]. In the field of plant cryopreservation, some researches, which assess the genetic stability in plants recovered after cryostorage, have reported genome changes, but did neither show any significant variation nor morphological changes in plants recovered after cryopreservation [15-18].

To the authors' knowledge, up to date, there are only two reports studying the effect of cryopreservation in the grapevine genome. Shoot-tips from "Cabernet franc", "Chardonnay", "Fengh 51" and "LN3"' cultivars were cryopreserved with the encapsulation-dehydration method. Furthermore, no differences were found when comparing the DNA patterns of control and cryopreserved plantlets using random amplified polymorphic DNA (RAPD) marker [19]. In addition, Marković et al. [20] tested the efficiency of the droplet-vitrification cryopreservation protocol. The cryopreservation protocol led 50% of recovery in the cultivar "Portan", and the genetic stability of regenerated plants was studied using amplified fragment length polymorphism (AFLP) markers and found polymorphic fragments. However, there is the need for more studies to evaluate the effect of cryopreservation protocols in the genetic stability of grapevine germplasm. The present research was carried out to evaluate the effect of cryopreservation on genetic stability by vitrification protocol of grapevine cv. "Flame Seedless" axillary buds using inter-simple sequence repeats (ISSR) marker.

2. Materials and Methods

2.1 Plant Materials

Axillary buds of grapevine (*Vitis vinifera* L.) cv. "Flame Seedless" were used. Sample collection of grapevine rootstocks containing 5-7 axillary buds were obtained from the "Casas Grandes" vineyard, located 40 km by the Highway 36, north to the coast of Hermosillo, Sonora, México (29°02′41.0″ N, 111°43′59.3″ W). The rootstocks were randomly selected, washed, disinfected with a solution containing commercial chlorine (1% NaOCl), and washed three times with distilled water. In horizontal laminar hood, the axillary buds were dissected with a sterile razor blade, and disinfected in commercial chlorine solution at 20% containing 50 drops/L of Triton® X-100 for 60 min, and then rinsed three times with sterile distilled water [21].

2.2 Vitrification-Cryopreservation Procedure

The buds were treated according to the procedure described by Matsumoto and Sakai [13] with some modifications. The plant vitrification solution No. 2 (PVS2) contained 0.4 M sucrose, 30% (w/v) glycerol, 15% (w/v) ethylene glycol, and 15% (w/v) dimethyl sulfoxide (DMSO) in Murashige and Skoog (MS) medium at pH 5.8 [22]. The buds were disinfected as described above and transferred into 2 mL cryovials (five replicates per treatment with five buds each, n = 25) containing 1 mL of PVS2 solution previously sterilized by filtration. The samples were incubated at 25 ± 2 °C with agitation for 180 min (T_1). This was the best incubation time according to a previous viability assay using grapevine buds (data not shown). The control buds (C) or the buds only stored in LN treatment did not include the PVS2 solution. The cryovials containing bud tissues with and without PVS2 were directly immersed in LN and stored for one hour, one week and one month, respectively. Total eight treatments were included in the experiment as following: control buds without treatment (C); buds after 180 min in PVS2 (T_1); 1 h in LN without PVS2 (T_2); PVS2 and 1 h in LN (T_3); one week in LN without PVS2 (T_4); PVS2 and one week in LN (T_5); one month in LN without PVS2 (T_6); PVS2 and one

month in LN (T_7).

After each treatment, freezing buds were thawed in a water-bath at 38 °C for 3 min. The PVS2 solution was removed, and the buds were washed with sterile distilled water.

2.3 Genetic Stability Assessment

DNA was extracted from 0.5 g tissue using the hexadecyltrimethylammonium bromide (CTAB) method according to Japelaghi et al. [23]. The extracted nucleic acids were quantified with an ultralow volume spectrophotometer (Nanodrop), and the integrity of total DNA was verified by running samples on 1% agarose gel containing ethidium bromide (1 μg/mL). For ISSR analysis, a set of 10 primers (Table 1) reported by Seyedimoradi et al. [24] was used. PCR amplification was carried out in a total volume of 50 μL containing 5 μL of 10× PCR buffer, 1 μL of 10 mM PCR nucleotide mix, 3 μL of 25 mM $MgCl_2$ solution, 80 pmol primer, 0.25 μL GoTaq® DNA polymerase and 25 ng genomic DNA [25]. All amplifications were achieved in a Swift™ MaxPro (ESCO, Singapur) as follows: 4 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at annealing temperature (Ta) (Table 1) and 2 min at 72 °C and 8 min at 72 °C for final extension. Amplified products were electrophoresed in 1.2% agarose gels, running for 1.5 h at 50 V in 1× TAE buffer, and visualized by ethidium bromide staining.

2.4 Data Analysis

Scoring of ISSR data was performed using a 1.2% agarose gel electrophoresis profile. The gel was visualized under a UV transilluminator (Cleaver Scientific Ltd., U.K.). Amplified bands obtained with all the molecular markers were quantified for the presence and absence of bands for all the treatments evaluated using the ImageJ software (ImageJ 1.50i, developed at the National Institute of Health, USA, by Wayne Rasband). Polymorphic information values were calculated for each ISSR primers in all treatment according to Saleh [26].

3. Results and Discussion

In this study, ISSR markers were employed to analyze the genetic variability of cryopreserved axillary buds of grapevine (*Vitis vinifera* L.) cv. “Flame Seedless”. ISSR markers have been tested to analyze grapevine diversity because of their properties of genetic co-dominance, high reproducibility, high abundance, high overall mutation rate and high polymorphism [27-29]. Eight treatments of grapevine buds cryopreservation were analyzed. In order to assess their genetic variability of cryopreserved buds, ISSR patterns were compared with control buds. Out of the 10 primers tested, eight (80%) generated amplicons. Of these eight primers, only two (20%) produced reproducible and good quality bands, which

Table 1 Primers used in ISSR analyses.

Marker	Ta (°C)	Sequence (5’ to 3’)
ISSR1	50	AGAGAGAGAGAGAGAGT
ISSR2	52	TGTGTGTGTGTGTGTGA
ISSR3	54	GGATGGATGGATGGAT
ISSR4	44	CTCTCTCTCTCTCTCTG
ISSR5	52	CACACACAC ACACACAG
ISSR6	45	GAGAGAGAGAGAGAGAA
ISSR7	50	GGAGAGGAGAGGAGA
ISSR8	48	GAAGAAGAAGAAGAAGAA
ISSR9	48	CACACACACACACACAT
ISSR10	52	ATGATGATGATGATGATG

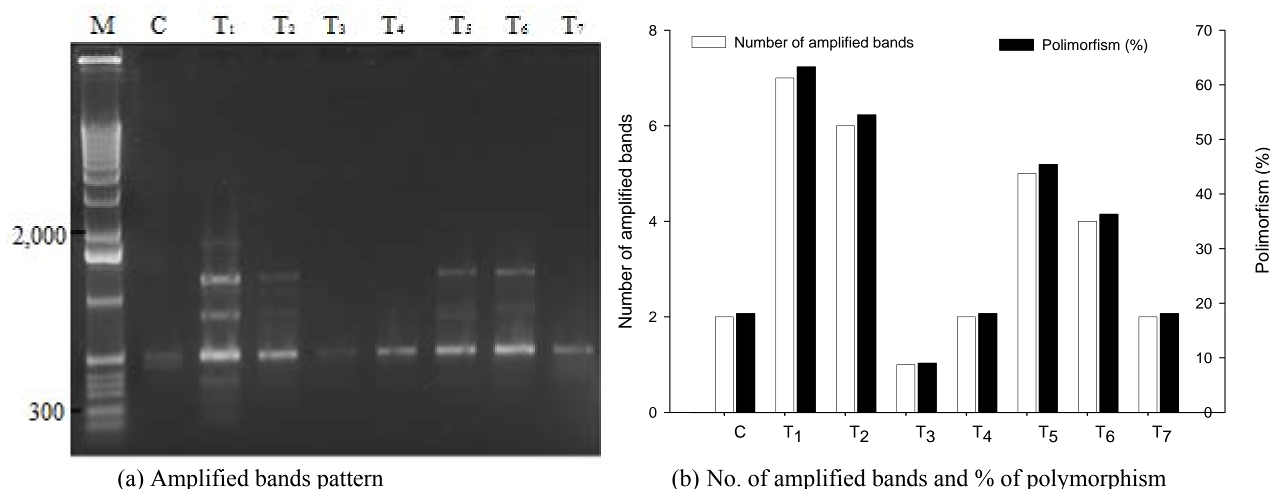
were used in the present study to analyze the genetic stability. One of these markers was ISSR1 that generates from one to seven bands, and the other was ISSR6 primer that generates between five to six bands (Table 1, Figs. 1 and 2). The size of the amplified products ranged from 300 bp to 2,000 bp.

3.1 The Amplified Band Pattern Produced by ISSR1 Primer

Fig. 1a represents the amplified band pattern produced by ISSR1 primer in the different cryopreservation treatments with and without PVS2 in all the times of storage in LN. Differences in the pattern of bands were recorded when comparing the control and the cryopreserved samples after storage in LN, with and without the cryoprotection step. The percentage of polymorphism increase after 180 min in PVS2, one hour, one week and one month in LN without cryoprotection. In Fig. 1b, it is included the number of amplified bands in the different treatments using the ISSR1 primer as well as the polymorphism percentage. It was recorded a large increase in polymorphism percentage between control and buds after PVS2 treatment. The same trend was observed between buds treated with PVS2 and one week of storage in LN (T_5) and buds treated with LN during one week without cryoprotection (T_4). Further, a reduction in polymorphism percentage was detected between buds treated with PVS2 and stored during one hour (T_3) and one month in LN (T_7) and buds stored in LN only (T_2 and T_6), respectively. Similar to the results in this study, in the experiment of shoot tips of grapevine cv. "Portan" cryopreserved by droplet-vitrification using AFLP markers, polymorphic fragments were observed in non-cryopreserved and cryopreserved samples treated with PVS2 solution, and the number of polymosphism increased with the exposure to PVS2 [20]. Likely, the use of the cryoprotective solution PVS2 is one of the main factors in the changes observed with the ISSR markers. Aronen et al. [17] in cryopreserved embryogenic cultures of *Abies cephalonica* found genetic variation in the DMSO treated samples. Thus, it can be concluded that the cryoprotectants may cause a risk of genetic fidelity, and monitoring it is important.

3.2 The Amplified Band Pattern Produced by ISSR6 Primer

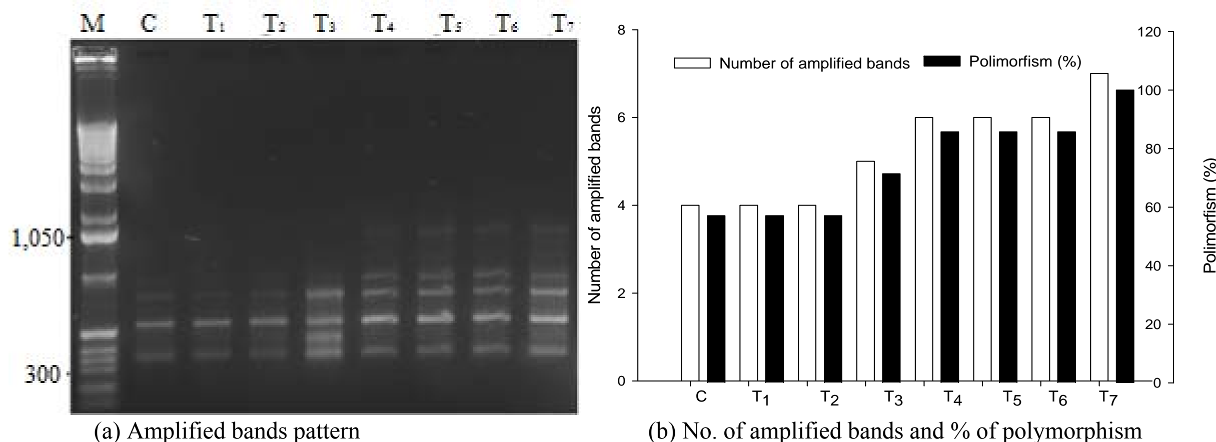
In Fig. 2a, it is included the amplified bands pattern produced by the ISSR6 primer in different cryopreservation treatments with and without PVS2 in all the times of storage in LN. A slightly different band



(a) Amplified bands pattern (b) No. of amplified bands and % of polymorphism

Fig. 1 ISSR1-PCR profiles of buds of grapevine (*Vitis vinifera* L.) cv. "Flame Seedless".

M: 1 kb plus ladder marker; C: control buds without treatment; T_1: buds after 180 min in PVS2; T_2: one hour in LN without PVS2; T_3: PVS2 and one hour in LN; T_4: one week in LN without PVS2; T_5: PVS2 and one week in LN; T_6: one month in LN without PVS2; T_7: PVS2 and one month in LN.



(a) Amplified bands pattern (b) No. of amplified bands and % of polymorphism

Fig. 2 ISSR6-PCR profiles of buds of grapevine (*Vitis vinifera* L.) cv. "Flame seedless".

M: 1 kb plus ladder marker; C: control buds without treatment; T_1: buds after 180 min in PVS2; T_2: one hour in LN without PVS2; T_3: PVS2 and one hour in LN; T_4: one week in LN without PVS2; T_5: PVS2 and one week in LN; T_6: one month in LN without PVS2; T_7: PVS2 and one month in LN.

pattern was recorded between treatments in LN without PVS2 as compared with treatments including vitrification method. In Fig. 2b, it is included the number of amplified bands in the different treatments using the ISSR6 primer, as well as the polymorphism percentage. A slight increase in polymorphism percentage was observed between buds treated with PVS2 and stored during one week (T_5) and one month (T_7) as compared with buds without PVS2 treatment and stored during the same time (T_4 and T_6) in LN.

At the present, increasing evidences have demonstrated that materials maintained genetic stability during the period of cryopreservation [19, 30, 31], maybe due to that in most cases, only a very small fraction of the genome (0.001%-1%) was analyzed [32]. Nevertheless, at the time, the research suggests that the most plant materials recovered from cryopreservation procedure maintain the genetic stability. In the analysis of this study, significant genomic changes were found when buds were treated with PVS2 solution and stored in LN for several periods of time (Figs. 1 and 2). In this work, axillary bud tissues were used, because they are programmed to directly develop into shoots and can avoid changes for effect of tissue culture. However, the changes found in bands profiles in the grapevine buds are very evident in the treatments of cryopreservation with and without PVS2. Like the results from this study, few cases of genetic variability were observed at RAPD or ISSR loci [15-17, 33-35], but it has been attributed to the toxic effect of PVS2 and/or the regeneration phase [36].

However, these genetic alterations can be due to the different type of stresses, like dehydration, osmotic pressure and low temperature that the tissue should face throughout the cryopreservation process [37, 38]. In this context, Harding [39] found unexpected polymorphism and it was not attributed to cryopreservation treatment *per se*, but was more likely the result from the whole process (i.e., tissue culture, pre-growth, cryoprotection, freezing, thawing, recovery and plant regeneration). More in-depth research is needed to assay the possible effect on the genetic stability for the cryopreservation procedure, because with the evidence at the time, only a low cover of the full plant genome to discard any mutagenic effects of the cryopreservation procedure was recorded [16]. Results from the present study show the importance of carrying out genetic stability studies in plant tissues stored by vitrification in cryopreservation protocols.

4. Conclusions

The results from the present study showed that the

process of cryopreservation by vitrification caused genetic variability in axillary buds of grapevine (*Vitis vinifera* L.) cv. "Flame seedless" stored during different times in LN. Further research is needed using next generation tools in order to determine the level of the changes occurring in the genome due to cryopreservation methodologies.

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