Cryopreservation of *Vitis vinifera via* Droplet- vitrification

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ABSTRACT

In the present study, two Egyptian grapevine cultivars (Red romy and Ghariby) and a Chinese variety (Cabernet sauvignon) were successfully cryopreserved by droplet vitrification. Axillary shoot tips were excised from two months old plantlets cultured on solidified ½MS medium with 0.5mg benzyladenine, 3% sucrose and 0.7% agar (pH 5.8) at 25 ˚C, under a 12 h light/12 h dark photoperiod with a light intensity of 40 µE m^{-2} s^{-1}*. For vitrification, excised shoot tips were precultured on half strength MS solidified medium supplemented with 0.1 M sucrose for 3 days in darkness and then treated with a mixture of 2 M glycerol and 0.4 M sucrose (LS solution) for 20 min at 25 ˚C. Shoot tips were then dehydrated with half-strength PVS2 vitrification solution (30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15 % dimethylsulfoxide and 0.4 M sucrose in MS basal medium, for 30 min. This was followed by full strength PVS2 for (25min, 50 min or 60 min) at 0 ˚C before direct immersion in liquid nitrogen. The results showed that the mean percentage of survived shoot tips was not significantly different among the three genotypes, i.e., 66.67, 54.72 and 58.06% for Cabernet sauvignon, Red romy, and Ghariby, respectively. Also, the mean number of shoot tips regrowth was not significantly different, i.e. 57.50, 50.50 and 50.50 for Cabernet sauvignon, Red romy and Ghariby, respectively. The optimal duration of dehydration with PVS2 for survival and regrowth was 50 min. Ten ISSR primers were used for assessing the stability of the cryopreserved genotypes compared to the noncryopreserved. A negligible percentage of polymorphism was detected in the two cultivars Red romy (6.52%) and Ghariby (2.26%) with no morphological changes after cryopreservation. While, the cryopreserved plantlets of the cultivar Cabernet sauvignon did not exhibit any variability at the morphological or molecular levels compared to the control (noncryopreserved plantlets).*

Key words: *Grapevine, cryopreservation, Droplet-vitrification, ISSR markers.*

INTRODUCTION

The common grapevine (*Vitis vinifera* L.) is a species of the vitis genus which is considered one of the most important economical fruits worldwide nowadays. It is characterized by a chromosome number of 2n= 4x=38 (Xu and Lu, 2004 and Chu *et al*., 2018) with a genome size of 504.6 Mb (Velasco *et al.,* 2007). The cultivated grapevine, *Vitis vinifera* subsp. Vinifera, has been domesticated from the dioecious, *V. vinifera* subsp. Sylvestris (Zohary, 1996; McGovern, 2003 and Laucou *et al.,* 2018). The cultivated genetic pool has been shaped by the combined action of spontaneous hybridization, somatic variation, selection and propagation through cuttings or seeds (Laucou *et al*., 2018).The wide use of the most interesting parents during domestication and early selection favored the emergence of groups of related cultivars

(Myles *et al*., 2011; Zenilabidine *et al*., 2015 and Laucou *et al*., 2018).Nowadays, about 5000 cultivars of *Vitis vinifera* are available worldwide (Bi *et al*., 2017).

Many grapevine cultivars are now endangered and international efforts aiming at preserving grapevine biodiversity have been undertaken (Markovic, *et al*., 2013). Conservation of plant genetic resources is one of the main activities of gene banks. These plant materials represent the reservoirs for germplasm that can be used for crop improvement and food security (Kaviani, 2011 and Bi *et al*., 2017). Conservation of plant genetic resources can be carried out *in situ* (in the natural habitats) or *ex situ* (outside). Seed storage is the most convenient method for long term conservation for plant genetic resources (Kaviani, 2011).However, clonally propagated crops are much more difficult to store for long term (Reed, 2018).

Nowadays, biotechnology is offering a broad range of tools for conservation of genetic resources. Cryopreservation has become the preferred option for the long-term conservation of vegetatively propagated germplasm by storing the explants at the ultra-low temperature of liquid nitrogen (LN) and or vapor phase at a temperature of – 196 to -140^{o} C (Benson, 2008; Keller *et al.,* 2008; Nukari *et al.,* 2009 ;Engelmann, 2011, Kaviani, 2011 and Markovic *et al.,* 2015). Different cryopreservation protocols have been described for grapevine including encapsulation-dehydration (Plessis *et al*., 1991 and 1993; Wang *et al.,* 2000 and Zhao *et al*., 2001) and vitrification (Matsumoto and Sakai, 2003; Shatnawi, 2011 and Markovic *et al.,* 2013).Also Hassan and Haggag, (2013) used a two steps vetrification protocol to cryopreserve two Egyptian grape cultivars. More recently, the droplet-vitrification protocol has been established for grapevine (Markovic *et al.,* 2013 and 2015). The droplet-vitrification technique has been successfully applied for the cryopreservation of different plant materials including potato (Yoon *et al.,* 2006); yams (Leunufna and Keller, 2005), lily (Chen *et al*., 2011), garlic and chrysanthemum (Kim *et al*., 2011) and grapevine (Markovic *et al.,* 2013 and 2015). The success of this technique in recovering high percentage is due to the direct contact between samples and LN during cooling and between samples and the unloading solution during rewarming which led to very high cooling and rewarming rates (Markovic et *al.,* 2013). However, cryopreservation protocols are highly genotype dependent (Ashmore *et al.,* 2007) and some cultivars will demand a precisely adapted protocol (Markovic et *al.,* 2015). In addition, there is an increasing need to determine if cryopreserved germplasm is 'true to type' and to measure the extent of the near 'normal phenotype'(Harding, 2004). Molecular markers are useful tools for characterizing and estimating the genetic stability among different genotypes. Different studies have been conducted to assess the variations in *in vitro* derived plantlets using ISSR (Dhanorkar *et al.* 2005; Alizadeh and Singh 2009 ;Seyedimoradi *et al.* 2012 and Rayan *et al.* (2014) in grapevine. Also, the genetic stability has been evaluated in regenerates recovered from cryopreservation using random amplified polymorphic DNA (RAPD) (Zhai *et al*., 2003; Wang *et al.,* 2017 and Bi *et al*., 2018). In addition, the genetic stability of recovered cryopreserved plantlets has been assessed by ALFP (Markovic *et al.,* 2015 and Wang *et al*., 2014). In the present study, the efficiency of the droplet – vitrification technique for the cryopreservation of axillary shoot tips of two Egyptian grape cultivars (Red romy and Ghariby) in addition to a Chinese variety (Cabernet sauvignon) was evaluated. Moreover, the genetic stability of the recovered plantlets after cryopreservation was assessed using the ISSR molecular markers.

MATERIALS AND METHODS

Plant Material

Two Egyptian grape cultivars (Red romy and Ghariby) and a Chinese variety (Cabernet sauvignon) were used in this investigation. Egyptian grape cultivars were provided from the accessions of grape germplasm collection at National Gene Bank, Giza, Egypt. While, the Chinese variety was kindly provided by Prof. Qiaochun Wang, A&F University in China.

Methods

In vitro culture and sterilization

Axillary buds were collected from greenhouse-grown plants. Sterilization was conducted with 70% ethanol for 1 min followed by 10% bleach for 10 min. Axillary buds were cultured on solidified ½ MS medium with 0.5 mg benzyladenine, 3% sucrose and 0.7% agar (pH 5.8) at 25°C, under a 12 h light/12 h dark photoperiod with a light intensity of 40 µE m^{-2} s^{-1}. *In vitro* plantlets were kept without subculture for 2 months before shoot tips excision. Axillary shoot tips, about 1 mm in length, consisting of an apical dome with three to five tiny primordial leaves, were excised and maintained on modified Murashige and Skoog (1962) basal medium composed of half strength MS mineral elements with 1mg benzyladenine, 3% sucrose and 2.5 g Gellan gum at pH 5.8.

Droplet-vitrification procedure

The Droplet-vitrification procedure has been carried out according to Markovic *et al.,* (2013).Excised shoot tips (1 mm) were pre cultured on solid half strength MS medium with 0.1M sucrose for 3 days in darkness. Pre cultured shoot tips were treated with a loading solution (LS) containing 2M glycerol + 0.4M sucrose in MS medium for 20 min at room temperature (25°C). Then, the shoot tips were dehydrated with half-strength Plant Vitrification Solution 2 (PVS2) at room temperature for 30 min, followed by the full strength PVS2 at 0°C.PVS2 is composed of (30% (w/v) glycerol, 15% (w/v) ethylene glycol (EG), 15% dimethyl sulfoxide (DMSO) and 0.4 M sucrose) in MS medium. Three different durations of exposure to PVS2, i.e. 25, 50 or 60 min, were investigated. Shoot tips were placed on aluminum strips in 3µl droplets of PVS2 and directly immersed in LN for at least 1 h. For rewarming, the aluminum strips with the shoot tips were immersed in unloading solution containing 1.2M sucrose for 20 min at room temperature (Fig.1). Finally the shoot tips were transferred onto recovery medium comprised of half-strength MS supplemented with 1mg benzyladenine.

Assessment of survival and regrowth

Survival of the shoot tips was evaluated two weeks after cryopreservation by counting the number of growing shoots. While, regrowth was identified by the development of apices into shoots with expanded leaves 8 weeks after rewarming. The survival and regrowth percentages were calculated relative to the total number of shoot tips treated.

Assessments of genetic stability

ISSR molecular markers were used to investigate the genetic stability of the cryopreserved plantlets subjected to PVS2 for 50 min as the optimal duration. After recovery, regenerated plantlets were maintained in tissue culture conditions for 2 months before samples were taken for DNA isolation. The DNA representing each cultivar was isolated from 5 recovered plantlets and pooled. Plantlets ltivated for 2-months were considered as control samples.

Table (1): Name, sequence and annealing temperature (Ta) of ISSR primers used to detect the genetic stability of the three grapevine cultivars before and after cryopreservation.

Primer name	Sequence	Ta(C)	Primer name	Sequence	Ta(C)
17899-B	(CA)6GG	40 ºC	17899-A	(CA)6 AG	40 ºC
807	(AG) 8T	42 ºC	BEC	(CA) 7 TC	48 ºC
3	(CA) 8 AT	46 ºC	17898-A	(CA)6 AC	40 ºC
CHR	(CA) 7 GG	51 ºC	ISSR-34	(AG)8 TG	53 ºC
834	(AG) 8CT	53 ºC	ISSR-35	TCGA(CA)7	53 ºC

Total DNA was extracted using the DNA easy Plant Mini Kit (Qiagen, Santa Clarita, CA), according to the manufacturer's protocol. DNA quality was determined visually on 0.8 % agarose gel. Also, the DNA concentration was quantitatively measured on a Bio photometer (Eppendorf, Germany) at wave length 260 nm and adjusted to 50 ng / µl by adding sterile double distilled water. A set of ten ISSR (Inter Simple Sequence Repeats primers) was used for the detection the genetic stability (Table 1).These primers were synthesized by Bioron Corporation, Germany. The amplification reaction was carried out in a volume of 25µl containing 12.5 master mix (Emeralad Amp), 2 µl primer (20 pmol), 1µl DNA (50 ng) and 9.5µl ddH2O.

The PCR amplification was performed in an Eppendorph Master Cycler programmed at 95ºC for 5 min as an initial denaturation cycle. This was followed by 35 cycles of : denaturation step at 94ºC for 1min, annealing temperature (Ta) for 1 min, then an extension step at 72°C for 1 min, and a final extension step at 72°C for 10 min. The PCR products were resolved by electrophoresis in 2 % agarose gel (Seakem, USA) in 1X TBE running buffer, containing ethidium bromide at 100 volts. ISSR products were visualized on UV transilluminator, and photographed using a Gel Documentation System (Alpha Innotech).

Data Analysis

Statistical analysis of the survival and regrowth data were performed according to Snedecor and Cochran, (1980) using the Duncan's multiple range (Duncan, 1955) methods at 0.05% level of significance. The banding patterns generated by ISSR molecular markers for each of the three genotypes before and after cryopreservation were compared. Clear and distinct amplification products of ISSR were scored as (1) for present and (0) for absent bands. Bands of the same mobility were scored as identical.

RESULTS AND DISCUSSION

Effect of duration of dehydration with PVS2 on shoot recovery and growth of the three genotypes

Cryopreservation technique is an effective approach for storage of plant cells, tissues, seeds and embryos. This can be a perfect and effective method for long term preservation of a wide range of cells (Engelmann, 2011; Kalaiselvi *et al.,* 2017 and Wang *et al.,* 2018). In the present study, the droplet- vitrification technique was applied (Fig.1) and the recovery and shoot growth from shoot tip cultures of the three cultivars were examined after exposing to PVS2 for three different durations (25 min, 50 min or 60 min). As shown in Table (2) the results revealed that there was no significant differences in the mean percentage of survival and mean number of shoot tips growth among the genotypes across the three different duration treatments. Also, the mean percentage of survived shoot tips was not significantly different among the three genotypes, i.e. 66.67, 54.72 and 58.06 % for Cabernet sauvignon, Red romy, and Ghariby, respectively. Also, the mean number of shoot tips regrowth was not significantly different, i.e., 57.50, 50.50 and 50.50 for Cabernet sauvignon, Red romy and Ghariby, respectively. The treatment duration 50 min revealed the highest shoot tips survival rate% (71.67%, 68, 33 and 65.83 %) for Cabernet sauvignon, Red romy and Ghariby, respectively compared with the other durations. While, increasing or decreasing the duration of the dehydration with PVS2 resulted in a decrease of the survival and regrowth. Therefore, our results showed that the optimal duration of dehydration with PVS2 for survival and regrowth was 50 min. This result is in consistence with the findings of Matsumoto and Sakai, 2003 and Markovic *et al.,* 2013). From Table (2) it could be also deduced that the different genotypes responded differently to the dehydration treatment. The genotype Cabernet sauvignon revealed the highest survival rates at the three treatment durations (60.00, 71.67 and 68.33) compared to the two Egyptian genotypes, Red romy and Ghariby (42.50, 68.33 and 53.33& 54.17, 65.83 and 54.17, respectively). Similary, the mean number of shoot regrowth was highest in the Cabernet sauvignon genotype compared to Red romy and Ghariby (Table 2).

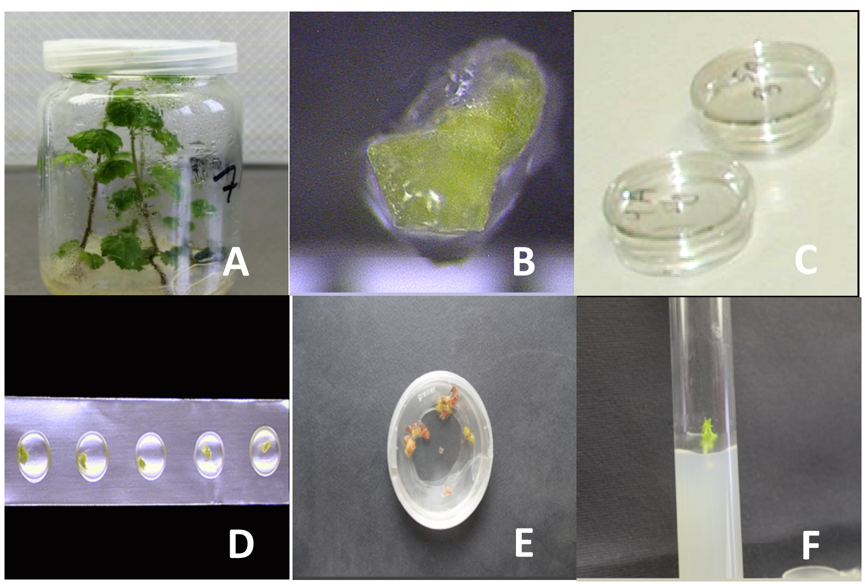

Fig.(1): Steps of the Vitis shoot tip droplet-vitrification cryopreservation and recovery processes for Red romy (A). In vitro culture (8 weeks) , (B)Shoot tip (1.0 mm) containing 5–6 leaf primordia used for cryopreservation,(C) Rewarmed shoot tips (D) PVS2 droplets containing shoot tips (E) Surviving shoot tip after one week (F) A regrowing plant recovered from cryopreserved shoot tips after 8 weeks.

Table (2): Effect of exposing time to PVS2 solution on survival and regrowth (%) of cryopreserved (+LN) grapevine cultivars.

Time	Survival (%)				Shoot growth			
	25 min	**50 min**	**60 min**	**Mean**	**25 min**	**50 min**	**60 min**	**Mean**
Cabernet sauvignon	60.00 ab	71.67 a	68.33 a	**66.67 a**	51.67 ab	62.50 a	58.33 ab	**57.50 a**
Red Romy	42.50 b	68.33 a	53.33 ab	**54.72 a**	38.33 b	60.00 a	51.67 ab	**50.50 a**
Ghariby	54.17 ab	65.83 a	54.17 ab	**58.06 a**	53.33 ab	59.17 ab	37.50 b	**50.50 a**
Mean	**52.22 a**	**68.61 a**	**58.61 a**		**47.78 a**	**60.56 a**	**49.17 a**	

Figures followed by different letters are significantly different according to Duncan's Multiple Range Test ($P < 0.05$).

In this respect, Ashmore *et al.* (2007) and Markovic *et al.* (2015) pointed out that cryopreservation protocols are highly genotype dependent. Nevertheless, at 50 min dehydration treatment we reached a survival rate ranging from 66% to 72% and a mean regrowth from 59.2 to 62.5. The present survival rate is satisfactory for grapevine as it is considered as one of the recalcitrant plants. Different authors attempted to improve the efficiency of cryopreservation for the long term storage of grapevine germplasm worldwide. Grapevine apices have been cryopreserved by Matsumoto and Sakai (2003) and Plessis *et al.* (1991) using encapsulation-dehydration with recovery range from 24-40%, while Plessis *et al.* (1993) obtained a recovery range from 40-60%. In addition, the recovery range obtained by Wang *et al.*(2000) reached 47-85% for four grapevine cultivars. By improving the droplet-vitrification protocol *via* incorporating a two-step dehydration procedure, Barroco *et al.* (2011) obtained a shoot recovery range of 60-80%. Furthermore, Markovic *et al.* (2013) obtained 50% regrowth with the droplet-vitrification procedure. Moreover, Bi *et al.* (2018) obtained an average shoot regrowth level of 50 %.

Vitrification-based cryoprocedures, dehydration can be performed, usually by exposure of samples either to PVS or to air drying. In PVS-mediated cryoprocedures, the type of PVS, duration and temperature of exposure need to be distinct (Bi *et al.,* 2018).In the present investigations PVS2 has been used at three different exposure periods. Moreover, other investigation proved that dehydration with PVS2 increased the tolerance to freezing in grapevine (Markovic *et al.,* 2013 and 2015 and Bi *et al.,* 2018) and other plant species such as sweet potato shoot tips (Hairai and Sakai, 1999). To protect the tissue the cryoprotectants have to penetrate the plant tissue and each cell, therefore also the minimal exposure time has to be determined. Thus, the acquisition of osmotolerance for shoot tips to PVS2 is essential in obtaining successful cryopreservation by vitrification (Hassan and Haggag, 2013). Panis *et al.* (2005) stated that droplet-vitrification, combines advantages of droplet protocols with vitrification. To date, droplet-vitrification has been applied to a number of vine cultivars, and rootstocks (Hassan and Haggag, 2013; Marković *et al.,* 2015; Pathirana *et al.,* 2016 and Bi*, et al.*, 2017). It has also been demonstrated to be the most applicable to diverse genotypes of a given species and considered the most promising solution to overcome species- or genotype specific limitations, which is often a bottleneck for the establishment of cryo-banks (Panis *et al.,* 2005; Reed, 2008 and Wang *et al.,* 2014).However, our results elucidate the fact that optimization of the cryopreservation conditions for the same protocol may be necessary to achieve optimum cryopreservation of the different genotypes.

Assessment of the genetic stability using ISSR markers

The aim of successful cryopreservation is to maintain genetically stable plant material (Kaczmarczyk *et al.*, 2012). Molecular markers were employed to evaluate the genetic variability in recovered plantlets after cryopreservation of different germplasm (Choudhary *et al.,* 2013; Merhy *et al.,* 2014; Kaya *et al.,* 2017 and Bi *et al.,* 2018). In the present study ten ISSR primers were used to assess the genetic stability of the three grapevine cultivars under investigation before and after cryopreservation. As shown in Table (3) and Fig. (2) the total number of amplified amplicons generated by the ten primers was 92 fragments with an average of the 9.2 amplicons/ primer from both the cryopreserved and the control in two varieties Cabernet sauvignon and Red romy. Out of these 92 amplicons, 6 bands (one/ primer) were found to be polymorphic (6.52%) in Red Romy. Whereas, the Cabernet sauvignon genotype did not show any polymorphic bands. In this respect, Bi *et al.* (2018) reported that they did not detect any polymorphic amplicon in the Cabernet sauvignon from cryopreserved shoot tips compared to the non-cryopreserved plantlets. The total number of bands amplified in the cultivar Ghariby was 86 with only three amplicons revealing polymorphism between the cryopreserved and the non-cryopreserved regenerants. The presence of non- significant variation in the banding patterns of regenerants recovered from cryopreserved shoot tips in Vitis has been reported by several authors (Zhai *et al*., 2003; Wang *et al.,* 2017 and Bi *et al*., 2018). Our results showed that the percentage of polymorphism (6.52%) for Red romy and (3.26 %) Ghariby is negligible (not significant) and there was no morphological changes. This could be due to other factors including the genotype, the freezing procedure employed and regrowth pattern achieved (Harding,1996). Similarly, several authors, who assessed the genetic stability in plants recovered after cryostorage, have reported genome changes, but did neither show significant variation nor morphological changes (Castillo *et al.,* 2010; Preetha *et al.,* 2015 and García-Coronado *et al.,* 2016).

Table (3): Primers name, total number of amplicons, size range of amplified fragments, and polymorphic amplicons for the three grape cultivars.

Primer name	Size of amplified fragments (bp)	Cabernet sauvignon		Red Romy		Ghariby	
		Total number of amplicons	Polymorphic bands	Total number of amplicons	Polymorphic bands	Total number of amplicons	Polymorphic bands
17899-B	463-1486	8	0	7	0	7	0
17898-A	606-1495	9	0	7	1	7	0
807	435-1071	8	0	6	0	7	0
3	670-1542	6	0	6	0	6	0
ISSR-34	404-1633	13	0	15	1	11	1
ISSR-35	512-1666	13	0	14	1	11	1
834	401-1648	18	0	18	1	17	0
17899-A	509-1790	7	0	8	0	6	0
BEC	552-1349	5	0	6	1	10	1
CHR	495-1393	5	0	5	1	4	0
Total	-	92	0	92	6	86	3
Average		9.2	0	9.2	0.65	8.6	0.32
% of polymorphism	-	-	0%	-	6.52	-	3.26

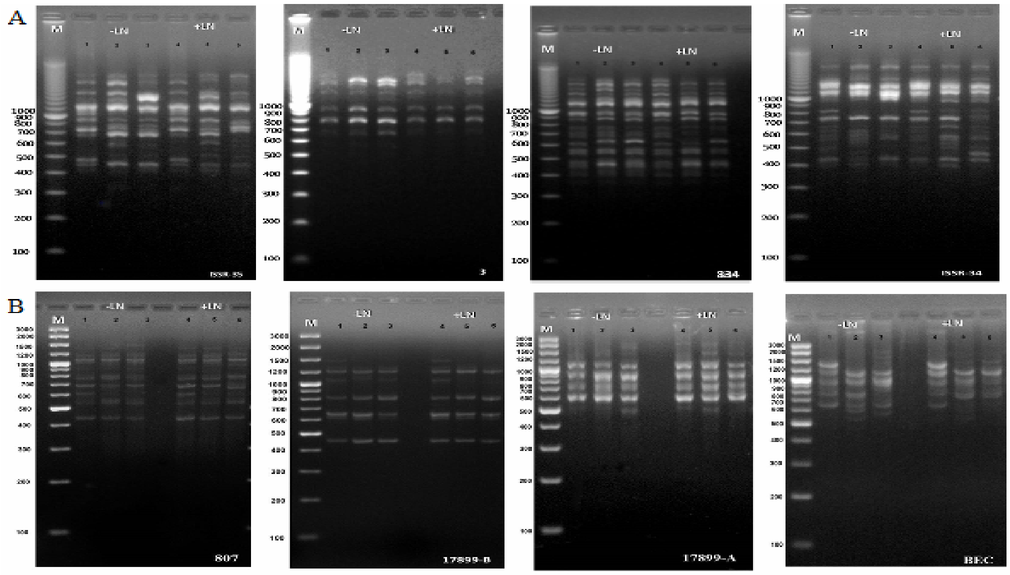

Fig. (2): ISSR profiles of the three grape cultivars Cabernet sauvignon, Red Romy and Ghariby, before (Lanes: 1,2 and 3) and after (lanes 4,5 and 6) cryopreservation as detected by ISSR primers (A= ISSR34, 3, 834 and CHR and M=1kp , B= 807, 17899-B, 17898-A , BEC and M = 100bp Marker).

CONCLUSION

In the present study a successful cryopreservation protocol was applied for two Egyptian and one Chinese cultivars and reached a mean number of shoot tips regrowth ranging from 50.5 to 57.5. The recovered plantlets showed no morphological variations, while negligible polymorphism was detected in the Egyptian cultivars using ISSR. Further modifications in the present protocol need to be attempted to improve the cryopreservation efficiency.

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الملخص العربي

الحفظ طويل المدى لبعض اصناف العنب باستخدام تقنية Droplet-vitrification

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في هذه الدراسة تم إجراء الحفظ بنجاح لصنفين من العنب المصري (الرومى الاحمر والغريبى) والصنف الصيني (Cabernet sauvignon) بتقنيةdroplet vitrification بإستخدام قمم البراعم الابطية . تم إستئصال قمم البراعم الابطية من نباتات عمر شهرين والتى نميت علي بيئة MS ½ صلبه تحتوي علي 0.5 ملجرام بنزيل ادنين و ٣% سكروز و 0.٧% جرام أجار (pH 5.8) لمدة ١٢ ساعه ضوء و١٢ ساعه ظلام حيث كانت شده الاضاءه $40\ \mu E\ m^{-2}\ s^{-1}$ علي درجة ٢٥ درجة مئوي .للحفظ قد نميت البراعم الابطيه المستأصله علي بيئة ½ MS صلبة مضاف لها 0.1M سكروز لمدة ٣ ايام في الظلام ثم عوملت بمخلوط مكون من 2M جليسرول و 0.4M سكروز لمدة ٢٠ دقيقة علي درجة ٢٥ درجة مئوية. ثم تم معامله البراعم المستأصله لمدة 30 دقيقة فى بيئه MS اساسيه تحتوى على PVS2 ½ يتكون من (محلول 30% جليسرول (حجم/وزن) و15% ايثيلين جليكول و 15% داي ميثيل سلفوأوكسيد و 0.4M سكروز) .ثم نقلت الي محلول PVS2 لثلاث فترات زمنيه مختلفه وهى (٢٥ دقيقة ، ٥٠ دقيقة ، ٦٠ دقيقة) علي درجة صفر مئوي قبل ان تغمس في النتروجين السائل . اظهرت النتائج انه لا توجد اختلافات معنويه بين الثلاث تراكيب وراثية من حيث متوسط النسبة المئوية للبقاء حيث كانت ٦٦.٦٧و ٥٤.٧٢ و٥٨,٠٦ % للصنف الصيني والغريبي والرومي الاحمر علي التوالي . ايضا، لم يظهر متوسط إعادة النمو اى اختلافات معنوية حيث كانت ٥٧.٥٠ و ٥٠.٥٠و ٥٠.٥٠ للصنف الصيني والغريبي ثم الرومي الاحمر علي التوالي. وكانت أفضل فترة للبقاء واعاده النمو عند الغمس في PVS2 هي ٥٠ دقيقة . تم دراسة الثبات الوراثى للتراكيب الوراثية الثلاثة بعد الحفظ ومقارنتها بالغير محفوظه بإستخدام عشرة بوادئ من ISSR .حيث اظهرت النتائج نسبة ضئيله من التباين الوراثي يمكن اهمالها فى الصنفين الرومي الاحمر(٦.٥٢%) والغريبي(2.26%) مع عدم وجود أي تغير مورفولوجي بعد الحفظ . بينما اظهرت النباتات الخاصه بالصنف الصينى عدم وجود اى اختلافات وراثيه او مورفولوجيه بعد عمليه الحفظ مقارنه بالنباتات الغير محفوظه .