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In vitro Medium-Term Germplasm Conservation and Genetic Stability of Grape (*Vitis vinifera* L.)

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Abstract: The objective of the present investigation was to find out a medium-term *in vitro* conservation protocol of local grape (*Vitis vinifera* L. var. Black Matrouh) and molecular identification of the conserved cultures. The medium term conservation study was initiated at 5°C under complete darkness conditions using *in vitro* grown nodal cutting explants. Explants were subjected to different osmotic regulators (glucose, mannitol or sorbitol) at different concentrations (0, 0.5, 1.5, 2.5, 3.5, 4.5, 5.5, 6.5, 7.5, or 8.5% (w/v)) which added to three-quarter strength MS basal medium without growth regulators. Results concerning the effect of different conservation periods indicated that survival percentage decreased gradually with increasing the conservation period gradually from 3, 6, 9 to 12 months. Up to 77.78, 77.78 and 88.89% of nodal cutting explants remained green and healthy when conserved for 12 months on conservation medium with 3.5 or 4.5% glucose, 2.5% mannitol and 5.5% sorbitol, respectively. Results regarded recovery and regrowth cleared that regeneration percentage, number of shoots per explant and their length (cm) decreased with increasing conservation period from 6 to 12 months. Determination of stability was performed by assessment of Inter Simple Sequence Repeat amplification (ISSR). The analysis of ISSR markers did not show any variation among the conserved and non-conserved material with the primers used.

Key words: Grape · *In vitro* conservation · Glucose · Mannitol · Sorbitol · ISSR analysis

INTRODUCTION

Grape (*Vitis vinifera* L.) is one of the extensively grown fruit crops in the world and the second crop in production after citrus in Egypt [1]. Some of grape varieties are being vanished owing to genetic drift, bad agronomic practices and short of conservation strategies [2]. Biotechnological strategies, based on concepts of *in vitro* plant cell, tissue and organ culture, have been developed as an alternative and an ancillary measure in response to the problems related to the conservation of plant germplasm in the field [3]. Among the different methods of *in vitro* conservation are the short-and medium-term storage to increase the interval period between subcultures from the normal 2 to 6 weeks to a much longer period (*e.g.* 3 to 12 months) by reducing growth [4]. The growth rate of *in vitro* cultures can be limited by various methods including manipulation of the nutritive elements in the culture medium and the use of osmotic regulators and growth retardants, as well as incubation at reduced temperature and/or low light intensity [5, 6]. *In vitro* culture temperatures between 4 and 20°C have been used for the conservation of various fruit species. Temperate crops, such as raspberry, blackberry, apple and pear, can be conserved at temperatures above 4°C with relative success [3]. There are several reports on the successful suppression of growth in shoot and plantlet cultures, such as *Vitis* exposed to temperatures between 0 and 15°C [7-10]. On the other hand, the addition of osmotic agents or growth retardants to the medium has proved efficient for reducing growth rates of different plants species such as grape [2]. Osmotic agents gradually increase the osmotic pressure of the medium and reduce water availability to

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growing cultures. High concentrations of osmotic agents in the medium cause a negative water potential and reduce the optimal turgor pressure needed for cell division and inhibit growth [5]. On the other hand, it may lead to some toxicity [11].

The application of DNA technology in agricultural research has progressed rapidly, especially in the area of variety identification. PCR based DNA markers provide powerful tools for genetic analysis because of their simplicity and ease of handling. Markers generated by Inter Simple Sequence Repeat amplification (ISSR) have been shown to be useful for detecting polymorphisms and overcome many technical limitations of RFLP and RAPD analyses [12]. In grapes, ISSR approach has been applied so far to the analysis of a limited number of varieties [13].

The present study aims to develop a procedure for *in vitro* medium-term germplasm conservation of Black Matrouh grape variety by investigate the effect of adding different osmotic regulators (glucose, mannitol or sorbitol) to culture medium and incubation at low temperature under complete darkness. Also, the genetic stability of conserved cultures was determined by using ISSR analysis.

MATERIALS AND METHODS

These studies were carried out during the period from 2011 to 2013 at the laboratory of Tissue Culture and laboratory of Genetic Molecular in the National Gene Bank and Genetic Resources, Giza, Egypt.

Plant Material: Actively growing shoots of about 5-10 cm long were taken from potted plants of local grape (*Vitis vinifera* L. var. Black Matrouh) grown in the greenhouse and surface sterilized by 70% ethanol for 1min followed by sodium hypochlorite solution which prepared using commercial bleach "Clorox" (5.25 % available chlorine) at 10% concentration for 10 min and then rinsed three times with sterilized distilled water. Nodal cutting explants were excised and cultured onto glass jars (300 ml) contained full strength MS basal medium plus 3.0% sucrose and 0.7% agar and supplemented with benzylamino purine (BAP) 1.0 mgl^{-1} [14]. The culture jars were covered with autoclavable polypropylene lids and incubated at 25±2°C and photoperiods of 16 h day and 8 h night with light intensity of 3000 lux. Shoots proliferated from nodal cutting explants were subcultured monthly into fresh proliferation medium consisting of three-quarter strength MS basal medium plus 3.0% sucrose and 0.7% agar and supplemented with BAP 0.75 mgl^{-1} and IBA (indole-3-buteric acid) 0.5 mgl^{-1} to get aseptic plant materials for *in vitro* medium-term conservation treatments.

***In vitro* Medium-Term Conservation:** To assess osmotic regulator effect on medium–term conservation of *in vitro* grown grape cultures, nodal cuttings from proliferated shoots were transferred and separately cultured into glass tubes (100x25 mm) containing three-quarter MS basal medium plus 0.7% agar without growth regulators and supplemented with different concentrations (0.5, 1.5, 2.5, 3.5, 4.5, 5.5, 6.5, 7.5 or 8.5%) of glucose, mannitol or sorbitol. Also, three-quarter strength MS plus 0.7% agar without growth regulators and with 3.0% sucrose (standard concentration) was used as a control medium. The culture tubes were covered with autoclavable polypropylene lids and incubated at 5°C under complete darkness. Each treatment was repeated three times (three replications), six explants for each replicate. Survival percentage of the cultures was assessed at the end of each conservation period (3, 6, 9 and 12 months) according to Reed [15].

***In vitro* Recovery:** In this respect, explants from different medium-term conservation treatments were aseptically transferred at the end of different conservation periods (6 and 12 months) and recultured onto glass jars (300 ml) contained fresh proliferation medium mentioned before. The cultures were incubated under normal conditions described above. Regeneration percentage, number of proliferated shoots per explant and their length (cm) were estimated after 4 weeks.

Statistical Analysis: The experiments were arranged as factorial experiment in completely randomized design with two factors. The obtained results were statistically analyzed according to Waller and Duncan [16]. The percentages of means were transformed arcsine to find the biometrical according to Steel and Torrie [17].

ISSR Analysis: DNA extraction was carried out using leaf materials collected from each treatment. Genomic DNA was extracted and purified using the DNeasy plant Mini Kit following the manual instructions (QIAGEN, Chatsworth, CA). Inter Simple Sequence Repeats (ISSRs) was carried out in a total volume of 25 μl reaction volume containing 2X ready mix (EmeraldAmp Max PCR master mix), 25 pM oligonucleotide primer and 50 ng genomic DNA. The temperature profile composed of initial denaturation of the template DNA at 95°C for 5 min,

Table 1: Name, sequence and annealing temperature of the ten primers used in ISSR analysis

Primer Name	Primer Sequence	Ta (°C)
17899-B	(CA)6GG	42°C
17898-A	(CA)6AC	40°C
ISSR-35	TCG(CA)7	53°C
834	(AG)8CT	53°C
841	(GA)8TC	53°C
809	(AG)8G	53°C
ISSR-4	CGA(CA)7	53°C
17899-A	(CA)6AG	40°C
HB-10	(GA)6CC	40°C
BEC	(CA)7TC	42°C

followed by 35 cycles of 1 min denaturation at 94°C, 1 min annealing at (Ta) according to the primer and 1 min extension at 72°C and finally followed by 10 min of additional extension at 72°C. Name, sequences and annealing temperature of the ten ISSR primers are listed in Table (1).

Results Analysis: The amplification products were visualized in an ultraviolet transilluminator, after horizontal electrophoresis in 2.5% agarose gel with ethidium bromide. ISSR bands were scored as present (1) or absent (0) for each treatment. The molecular results were analyzed using the Phoretix 1D Pro software from nonlinear Dynamics.

RESULTS AND DISCUSSION

***In vitro* Conservation Using Osmotic Regulators at 5°C under Complete Darkness:** With regard to the effect of different glucose concentrations, results in Table (2) and Fig. (1A) revealed that the highest mean value of survival percentage was noticed in medium stressed with either 3.5 or 4.5% glucose (91.67%). Up to 77.78% of nodal cutting explants remained green and healthy after 12 months of conservation in medium with 3.5 or 4.5% glucose, while the lowest mean value of survival percentage (44.44%) was observed in medium amended with 0.5% glucose after 12 months of conservation.

Concerning the effect of different conservation periods, results in Table (2) indicate clearly that survival percentage decreased gradually from 92.22, 87.78, 71.11 to 60.00% with increasing conservation period from 3, 6, 9 to 12, respectively. It could be due to depletion of nutrients as mentioned by Ahmad and Anjum [18].

As for the effect of different mannitol concentrations, results in Table (3) and Fig. (1A) showed that medium stressed with 2.5% mannitol resulted in the highest mean value of survival percentage (91.67%). Up to 77.78% of nodal cultures survived after 12 months of conservation in this medium, while the lowest mean value of survival percentage (22.22%) was noticed after 12 months of conservation in medium stressed with high mannitol concentration 8.5%. In this respect, Ahmad and Anjum [18] found that low concentrations of MS medium and the lower concentration of mannitol (2.5%) successfully increased subculture duration of pear genotype.

Concerning the effect of different conservation periods, results in Table (3) indicate clearly that survival percentage decreased gradually from 91.11, 85.56, 64.45 to 54.45% with increasing conservation period from 3, 6, 9 to 12, respectively.

Concerning the effect of different sorbitol concentrations, results in Table (4) and Fig. (1A) revealed that adding 5.5% sorbitol to the conservation medium resulted in the highest mean value of survival percentage (94.44%). Up to 88.89% of nodal cutting explants remained green and healthy after 12 months of conservation in this medium, while the lowest mean value of survival percentage (44.44%) was noticed after 12 months of conservation in medium stressed with low sorbitol concentration 0.5%. in this concern, Hassan *et al.* [19] reported that 66.66% of shoot tip explants were able to survival when conserved for 12 months on MS medium with 40 or 50 gl^{-1} sorbitol for Red Romy grape variety.

With regard to the effect of different conservation periods, results in Table (4) indicate clearly that survival percentage decreased gradually from 93.33, 93.33, 81.11 to 68.89% with increasing conservation period from 3, 6, 9 to 12, respectively.

***In vitro* Recovery:** Concerning the effect of different glucose concentrations, results in Table (5) and Fig. (1B) clearly indicate a significant variation between different conservation media. Conservation medium with 3.5 and 4.5% glucose resulted in the highest regeneration percentage (66.67%) after recultured in recovery medium and incubation under normal conditions for 4 weeks. The maximum number of new shoots produced per explant (2.5 shoots/ explant) and their length (1.44 cm) was observed in medium with 3.0% sucrose (control medium). Explant conserved in medium with 0.5% glucose showed the lowest regeneration percentage (16.67%), shoot number/explant (0.61 shoots/explant) and shoot length (0.68 cm). While, explants conserved for 12 months in medium with 0.5% glucose failed completely to develop.

Table 2: Effect of different glucose concentrations and conservation periods (month) on survival percentage of "Black Matrouh" nodal cutting explants conserved at 5°C under complete darkness

	Conservation period (month)									
Glucose%	3		6		9		12		Mean	
Control (3.0 sucrose)	77.78	abc	77.78	abc	55.56	cd	55.56	cd	66.67	C
0.5	88.89	ab	77.78	abc	55.56	cd	44.44	d	66.67	C
1.5	88.89	ab	88.89	ab	55.56	cd	55.56	cd	72.22	BC
2.5	100.00	a	88.89	ab	88.89	ab	66.67	bcd	86.11	AB
3.5	100.00	a	100.00	a	88.89	ab	77.78	abc	91.67	A
4.5	100.00	a	100.00	a	88.89	ab	77.78	abc	91.67	A
5.5	100.00	a	88.89	ab	77.78	abc	55.56	cd	80.56	ABC
6.5	88.89	ab	88.89	ab	66.67	bcd	55.56	cd	75.00	ABC
7.5	88.89	ab	88.89	ab	66.67	bcd	55.56	cd	75.00	ABC
8.5	88.89	ab	77.78	abc	66.67	bcd	55.56	cd	72.22	BC
Mean	92.22	A	87.78	A	71.11	B	60.00	B		

Means followed by a letter in common in the same column are not significantly different at 0.05 level of probability

Table 3: Effect of different mannitol concentrations and conservation periods (month) on survival percentage of "Black Matrouh" nodal cutting explants conserved at 5°C under complete darkness

	Conservation period (month)									
Mannitol %	3		6		9		12		Mean	
Control (3.0 sucrose)	77.78	abc	77.78	abc	55.56	cde	55.56	cde	66.67	BCD
0.5	100.00	a	100.00	a	66.67	bcd	44.44	def	77.78	ABC
1.5	100.00	a	100.00	a	66.67	bcd	66.67	bcd	83.33	AB
2.5	100.00	a	100.00	a	88.89	ab	77.78	abc	91.67	A
3.5	88.89	ab	88.89	ab	66.67	bcd	66.67	bcd	77.78	ABC
4.5	88.89	ab	88.89	ab	66.67	bcd	66.67	bcd	77.78	ABC
5.5	88.89	ab	88.89	ab	66.67	bcd	55.56	cde	75.00	ABC
6.5	88.89	ab	77.78	abc	55.56	cde	55.56	cde	69.45	BCD
7.5	88.89	ab	77.78	abc	55.56	cde	33.33	ef	63.89	CD
8.5	88.89	ab	55.56	cde	55.56	cde	22.22	f	55.56	D
Mean	91.11	A	85.56	A	64.45	B	54.45	B		

Means followed by a letter in common in the same column are not significantly different at 0.05 level of probability

Table 4: Effect of different sorbitol concentrations and conservation periods (month) on survival percentage of "Black Matrouh" nodal cutting explants conserved at 5°C under complete darkness

	Conservation period (month)									
Sorbitol %	3		6		9		12		Mean	
Control (3.0 sucrose)	77.78	abc	77.78	abc	55.56	cd	55.56	cd	66.67	D
0.5	88.89	ab	88.89	ab	66.67	bcd	44.44	d	72.22	CD
1.5	100.00	a	100.00	a	77.78	abc	66.67	bcd	86.11	ABC
2.5	100.00	a	100.00	a	88.89	ab	77.78	abc	91.67	A
3.5	100.00	a	100.00	a	88.89	ab	77.78	abc	91.67	A
4.5	100.00	a	100.00	a	88.89	ab	77.78	abc	91.67	A
5.5	100.00	a	100.00	a	88.89	ab	88.89	ab	94.44	A
6.5	100.00	a	100.00	a	88.89	ab	66.67	bcd	88.89	AB
7.5	88.89	ab	88.89	ab	88.89	ab	66.67	bcd	83.34	ABC
8.5	77.78	abc	77.78	abc	77.78	abc	66.67	bcd	75.00	BCD
Mean	93.33	A	93.33	A	81.11	B	68.89	C		

Means followed by a letter in common in the same column are not significantly different at 0.05 level of probability.

Table 5: Effect of different glucose concentrations and conservation periods on regeneration (%), average shoot number/explant and shoot length (cm) of "Black Matrouh" nodal cutting explants conserved at 5°C under complete darkness on recovery medium and incubation under normal conditions for four weeks

	Regeneration (%)						Shoots no./ explant						Shoots length (cm)					
	Conservation period (month)						Conservation period (month)						Conservation period (month)					
Glucose %	6		12		Mean		6		12		Mean		6		12		Mean	
Control (3.0 sucrose)	77.78	a	44.44	bcd	61.11	A	2.67	a	2.33	b	2.50	A	1.42	ab	1.47	a	1.44	A
0.5	33.33	cd	0.00	e	16.67	B	1.22	gh	0.00	i	0.61	E	1.36	abc	0.00	g	0.68	D
1.5	55.56	abc	22.22	de	38.89	AB	1.78	cd	1.56	def	1.67	C	1.25	a-d	1.28	a-d	1.26	AB
2.5	77.78	a	44.44	bcd	61.11	A	1.67	cde	1.56	def	1.61	C	1.42	ab	1.39	ab	1.40	A
3.5	77.78	a	55.56	abc	66.67	A	2.33	b	1.89	c	2.11	B	1.14	a-e	1.25	a-d	1.19	AB
4.5	77.78	a	55.56	abc	66.67	A	2.22	b	1.89	c	2.06	B	1.22	a-d	1.22	a-d	1.22	AB
5.5	66.67	ab	44.44	bcd	55.56	A	1.56	def	1.67	cde	1.61	C	0.97	def	1.11	b-f	1.04	BC
6.5	66.67	ab	33.33	cd	50.00	A	1.33	fgh	1.44	efg	1.39	CD	1.03	c-f	1.00	def	1.01	BCD
7.5	66.67	ab	33.33	cd	50.00	A	1.44	efg	1.33	fgh	1.39	CD	0.83	ef	1.03	c-f	0.93	BCD
8.5	44.44	bcd	33.33	cd	38.89	AB	1.11	h	1.11	h	1.11	D	0.78	f	0.83	ef	0.81	CD
Mean	64.44	A	36.67	B			1.73	A	1.48	B			1.14	A	1.06	A		

Means followed by a letter in common in the same column are not significantly different at 0.05 level of probability

Table 6: Effect of different mannitol concentrations and conservation periods on regeneration (%), average shoot number/explant and shoot length (cm) of "Black Matrouh" nodal cutting explants conserved at 5°C under complete darkness on recovery medium and incubation under normal conditions for four weeks

	Regeneration (%)						Shoots no./ explant						Shoots length (cm)					
	Conservation period (month)						Conservation period (month)						Conservation period (month)					
Mannitol %	6		12		Mean		6		12		Mean		6		12		Mean	
Control (3.0 sucrose)	77.78	abc	44.44	def	61.11	AB	2.67	a	2.33	b	2.50	A	1.42	a-d	1.47	abc	1.44	A
0.5	66.67	bcd	0.00	g	33.33	CD	1.00	g	0.00	h	0.50	D	0.89	ef	0.00	g	0.44	B
1.5	66.67	bcd	44.44	def	55.56	ABC	1.11	fg	1.22	efg	1.17	C	1.50	abc	1.25	cd	1.38	A
2.5	100.00	a	44.44	def	72.22	A	1.78	c	1.78	c	1.78	B	1.47	abc	1.42	a-d	1.44	A
3.5	77.78	abc	33.33	ef	55.56	ABC	1.44	de	1.44	de	1.44	C	1.69	a	1.58	ab	1.64	A
4.5	88.89	ab	22.22	fg	55.56	ABC	1.56	cd	1.33	def	1.44	C	1.53	abc	1.50	abc	1.51	A
5.5	55.56	cde	22.22	fg	38.89	BCD	1.44	de	1.33	def	1.39	C	1.31	bcd	1.44	a-d	1.38	A
6.5	33.33	ef	0.00	g	16.67	D	1.44	de	0.00	h	0.72	D	1.14	de	0.00	g	0.57	B
7.5	33.33	ef	0.00	g	16.67	D	1.00	g	0.00	h	0.50	D	0.72	f	0.00	g	0.36	B
8.5	33.33	ef	0.00	g	16.67	D	1.00	g	0.00	h	0.50	D	0.75	f	0.00	g	0.38	B
Mean	63.33	A	21.11	B			1.44	A	0.94	B			1.24	A	0.87	B		

Means followed by a letter in common in the same column are not significantly different at 0.05 level of probability

With regard to the effect of different mannitol concentrations, results in Table (6) and Fig. (1B) clearly revealed that conservation medium with 2.5% mannitol resulted in the highest regeneration percentage (72.22%) after recultured in recovery medium and incubation under normal conditions for 4 weeks. Also, control medium (medium containing 3.0% sucrose) surpassed mannitol supplemented media in number of new shoots (2.5 shoots/ explant). Conservation medium with 3.5% mannitol showed the highest shoot length (1.64 cm). While conservation medium with 7.5 and 8.5% mannitol resulted in the lowest regeneration percentage (16.67%), shoot number/explant (0.50 shoots/explant) and shoot length (0.36 and 0.38 cm, respectively). Also, explants conserved for 12 months on medium with 0.5, 6.5, 7.5 and 8.5% mannitol could not be able to regenerate new shoots when recultured in recovery medium for 4 weeks.

As for the effect of different sorbitol concentrations, results in Table (7) and Fig. (1B) showed that conservation medium with 4.5 or 5.5% sorbitol enhanced plant recovery as 83.33% of explants were able to regenerate new shoots after transferring and reculturing on recovery medium and incubating under normal conditions for 4 weeks. Conservation medium with 5.5% sorbitol resulted the highest average number of shoots (2.83 shoots/ explant) and shoot length (1.60 cm). The lowest regeneration percentage (44.44%) was observed when explants conserved in medium with 0.5 or 8.5%

Table 7: Effect of different sorbitol concentrations and conservation periods on regeneration (%), average shoot number/explant and shoot length (cm) of "Black Matrouh" nodal cutting explants conserved at 5°C under complete darkness on recovery medium and incubation under normal conditions for four weeks

	Regeneration (%)						Shoots no./ explant						Shoots length (cm)					
	Conservation period (month)						Conservation period (month)						Conservation period (month)					
Sorbitol %	6		12		Mean		6		12		Mean		6		12		Mean	
Control (3.0 sucrose)	77.78	abc	44.44	de	61.11	AB	2.67	abc	2.33	cde	2.50	ABC	1.42	a	1.47	a	1.44	A
0.5	88.89	ab	0.00	f	44.44	B	1.67	f	0.00	g	0.83	E	1.36	a	0.00	c	0.68	B
1.5	100.00	a	33.33	e	66.67	AB	2.33	cde	2.44	bcd	2.39	BC	1.33	a	1.33	a	1.33	A
2.5	88.89	ab	44.44	de	66.67	AB	2.56	a-d	2.44	bcd	2.50	ABC	1.33	a	1.36	a	1.35	A
3.5	100.00	a	55.56	cde	77.78	A	2.78	ab	2.78	ab	2.78	AB	1.53	a	1.47	a	1.50	A
4.5	100.00	a	66.67	bcd	83.33	A	2.67	abc	2.78	ab	2.72	AB	1.42	a	1.44	a	1.43	A
5.5	100.00	a	66.67	bcd	83.33	A	2.78	ab	2.89	a	2.83	A	1.61	a	1.58	a	1.60	A
6.5	100.00	a	44.44	de	72.22	A	2.22	de	2.22	de	2.22	CD	1.31	a	1.33	a	1.32	A
7.5	88.89	ab	33.33	e	61.11	AB	2.33	cde	2.22	de	2.28	CD	1.28	a	1.31	a	1.29	A
8.5	55.56	cde	33.33	e	44.44	B	2.00	ef	1.78	f	1.89	D	0.89	b	0.92	b	0.90	B
Mean	90.00	A	42.22	B			2.40	A	2.19	B			1.35	A	1.22	A		

Means followed by a letter in common in the same column are not significantly different at 0.05 level of probability.

Table 8: Statistics of the ISSR fragments for Black Matrouh grape (*Vitis vinifera* L.) variety based on the ten ISSR primers

ISSR primers	No. of amplification products	Size range (bp)	No. of monomorphic bands
17898-A	3	529 – 809	3
17899-B	5	449 – 1275	5
ISSR-35	7	292 – 1185	7
834	10	296 – 1373	10
841	7	392 – 1125	7
809	7	262 – 1046	7
ISSR-4	6	340 – 1199	6
17899-A	5	478 – 1150	5
HB-10	6	326 - 872	6
BEC	5	485 – 1022	5

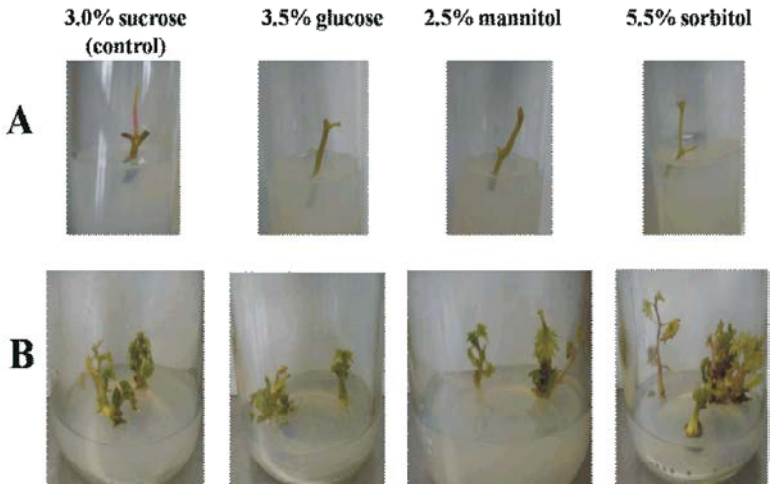

Fig. 1: (A) survived "Black Matrouh" nodal cutting explants conserved in media containing different concentrations of osmotic regulators at 5°C under complete darkness for 12 months. (B) Regrowth and regeneration of survived "Black Matrouh" nodal cutting explants after transferring and of reculturing on recovery medium and incubating under normal conditions for 4 weeks

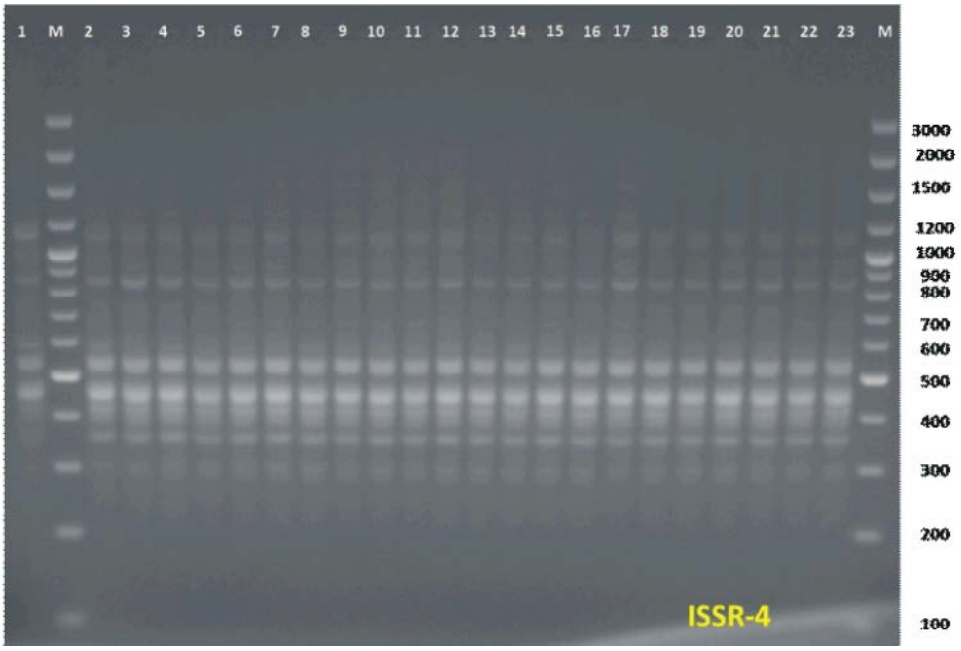

Fig. 2: Gel electrophoresis of ISSR fragments detected with the primer ISSR-4: (M) Molecular marker, (1) non conserved explants, (2) 3% sucrose, (3) 1.5% glucose, (4) 2.5% glucose, (5) 3.5% glucose, (6) 4.5% glucose, (7) 5.5% glucose, (8) 6.5% glucose, (9) 7.5% glucose, (10) 85% glucose, (11) 1.5% mannitol, (12) 2.5% mannitol, (13) 3.5% mannitol, (14) 4.5% mannitol, (15) 5.5% mannitol, (16) 1.5% sorbitol, (17) 2.5% sorbitol, (18) 3.5% sorbitol, (19) 4.5% sorbitol, (20) 5.5% sorbitol, (21) 6.5% sorbitol, (22) 7.5% sorbitol, (23) 8.5% sorbitol

sorbitol. The lowest shoots number (0.68 shoots/explant) and shoot length (0.68 cm) were noticed when explants conserved in medium with 0.5% sorbitol. Explants conserved for 12 months in medium with 0.5% sorbitol showed no response after transferring and reculturing in recovery medium for 4 weeks.

In general, results presented in Tables (5, 6 and 7) showed that the ability of conserved explants to regenerated shoots after transferring and reculturing on recovery medium and incubating under normal conditions, as well as, the average number of proliferated shoots per explant and their length (cm) decreased significantly with increasing conservation period from 6 to 12 months, regardless of osmotic regulators. The highest regeneration percentage, shoots number per explant and shoots length (cm) were recorded after conservation for 6 months in medium with sorbitol (90.0% regeneration percentage, 2.40 shoots/explant, 1.35cm shoot length). Meanwhile the lowest regeneration percentage, shoots number per explant and shoots length (cm) were observed after conservation for 12 months in medium stressed with mannitol (21.11% regeneration percentage, 0.94 shoots/ explant, 0.87 cm shoot length). These results are in agreement with those of Golmirzaie and Toledo [20] which demonstrated that the use of sorbitol as an osmotic agent can be metabolized by the plantlets after few months of storage and exhibition an incremental growth rate. In the same line, Bekheet *et al.* [21] showed that health shoot bud cultures of date palm were obtained after 6 months of storage in medium containing $40gl^{-1}$ sorbitol. Espinoza *et al*. [22] stated that addition of mannitol reduced the growth of *Ipomoea batatas* plants.

ISSR-PCR Analysis for Genetic Stability: PCR based ISSR markers were employed to assess genetic stability in the experimental samples that belong to Black Matrouh grapes. Ten ISSR primers were individually used to amplify DNA. A total of 61 DNA bands were amplified in the control and conserved plants ranging in size from 262 bp in (809) to 1373 bp in (834). The number of scorable markers produced per primer ranged from 3 in (17898-A) to 10 in (834) as shown in Table (8).

According to ISSR analysis, results presented in Table (8) showed that all the ten primers produced uniform amplification profiles of DNA of conserved plants. Thus, these treatments did not affect or mutated the genomic DNA of "Black Matrouh" variety. Also, plantlets derived from the *in vitro* conserved nodal

cutting explants were genetically identical to the control (non-conserved plant). ISSR marker profile produced by the primer ISSR-4 in 2.5% agarose gel was shown in Fig. (2).

In this concern, ISSR marker analysis has been used to study the degree of genetic changes in plants regenerated *in vitro* such as taro and *Rauvolfia serpentine* [23, 24].

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