Journal of Genetic and Environmental Resources Conservation, 2022, 10(1):60-66.
www.gercj.com Print ISSN: 2306-8663, Online ISSN: 2306 – 8280

Effect of osmotic regulators on *In vitro* conservation of grape

Rehab AlMousa* and Neveen Hassan
Department of Biotechnology, General Commission for Scientific Agricultural Research, Damascus, Syria.
National Gene Bank and Genetic Resources, Ministry of Agriculture and Land Reclamation, Giza, Egypt
***Corresponding author: bebo_moussa13@yahoo.com**
Received: September 23th 2021; Accepted: November 22nd 2021

Abstract

Some of grape varieties are being vanished owing to genetic drift, bad agronomic practices and short of conservation strategies. Biotechnological strategies have been developed as an alternative and ancillary method. So, the objective of this study was to find out a medium-term conservation protocol of 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 ¾ 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.

Keywords: Grape, Medium-term conservation, Osmotic regulators, ISSR analysis.

Introduction

Grape (*Vitis vinifera* L.) belongs to the family *Vitaceae* (Sajid and Ahmed, 2008). Some of grape varieties are being vanished owing to genetic drift, bad agronomic practices and short of conservation strategies (Tehrim and Sajid, 2011). Conservation of plant biodiversity is essential for classical and modern plant breeding. Biotechnological strategies, based on concept of *in vitro* plant cell tissue and organ culture, have been developed as an alternative and ancillary mearure in response to the problems related to the conservation of plant germplasm in the field (Scherwinski-Pereira and Costa, 2010). Medium- term storage based on increasing the interval period subcultures from the normal (2 -6 weeks) to a much longer period (*e.g.* 3 to 12 months) by reducing growth (Perez-Tornero et al., 1999), either by manipulation the nutritive elements in the culture medium or the use of osmotic regulators, as well as incubation at reduced temperature and/or low light intensity (Tahtamouni, 2001; Engelmann, 2010). 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 (Hagagy, 1998; Miaja, 2000; Rousseva, 2001; Silva et al., 2012).

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 RAPDanalyses (Zietkiewicz et al., 1994). In grapes, ISSR approach has been applied so far to the analysis of a limited number of varieties (Herrera et al., 2002).

This research aims to develop a procedure for medium-term conservation of Black Matrouh grape variety and genetic stability of conserved cultures using ISSR analysis.

Materials and Methods

Plant material and *In vitro* Medium-Term Conservation: *In vitro* nodal explants of Egyptian grape "Black Matrouh" were cultured on ¾ MS 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, ¾ MS (Murashige, and Skoog, 1962) plus 0.7% agar without growth regulators and with 3.0% sucrose (standard concentration) was used as a control medium. The cultures were 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 (Reed, 1992).

***In vitro* Recovery:** Explants from different medium-term conservation treatments were aseptically transferred at the end of different conservation periods (6 and 12 months) and recultured on fresh proliferation medium and incubated under normal conditions according to Almousa (Almousa et al., 2015). 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 withtwo factors. The obtained results were statistically analyzed according to Waller and Duncan (Waller and Duncan, 1969). The percentages of means were transformed arcsine to find the biometrical according to Steel and Torrie (1980).

ISSR Analysis: DNA extraction was carried out using leafmaterials 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, followed by 35 cycles of 1 min denaturation at 94°C, 1 minannealing 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.

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

Result and Discussion

***In vitro* Conservation Using Osmotic Regulators:** The highest survival percentages were noticed on medium with 5.5% sorbitol (94.44%), 3.5 or 4.5% glucose, 2.5% mannitol (91.67%). After 12 months of conservation, up to 88.89% of nodal cutting explants remained green and healthy on medium with 5.5% sorbitol, 77.78% on medium with 3.5 or 4.5% glucose and 2.5% mannitol. While the lowest survival percentages were noticed after 12 months of conservation on medium with 8.5% mannitol (22.22%), 0.5% glucose or sorbitol (44.44%). (Tables 1, 2, 3 and Fig 1A).

Survival percentage decreased gradually 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 (Ahmad and Anjum, 2010).

Table 1: Survival percentage of conserved explants on medium with different glucose concentrations.

Glucose%	Conservation period (month)				Mean
	3	6	9	12	
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**	

Table 2: Survival percentage of conserved explants on medium with different mannitol concentrations.

Mannitol %	Conservation period (month)				Mean
	3	6	9	12	
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**	

Table 3: Survival percentage of conserved explants on medium with different sorbitol concentrations.

Sorbitol %	Conservation period (month)				Mean
	3	6	9	12	
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**	

***In vitro* Recovery:** The highest regeneration percentages were noticed from explants conserved on media with 4.5% or 5.5% sorbitol (83.33%), 2.5% mannitol (72.22%), 3.5 or 4.5% glucose (66.67%). The maximum number of new shoots (2.83) were produced from explants conserved on medium with 5.5% sorbitol, while the highest shoot length (1.64 cm) was noticed from explants conserved on medium with 3.5% mannitol. All explants conserved for 12 months on media with 0.5% glucose or sorbitol, 0.5, 6.5, 7.5 and 8.5% mannitol failed completely to develop after transferring to recovery medium (Tables 4, 5, 6 and Fig 1B).

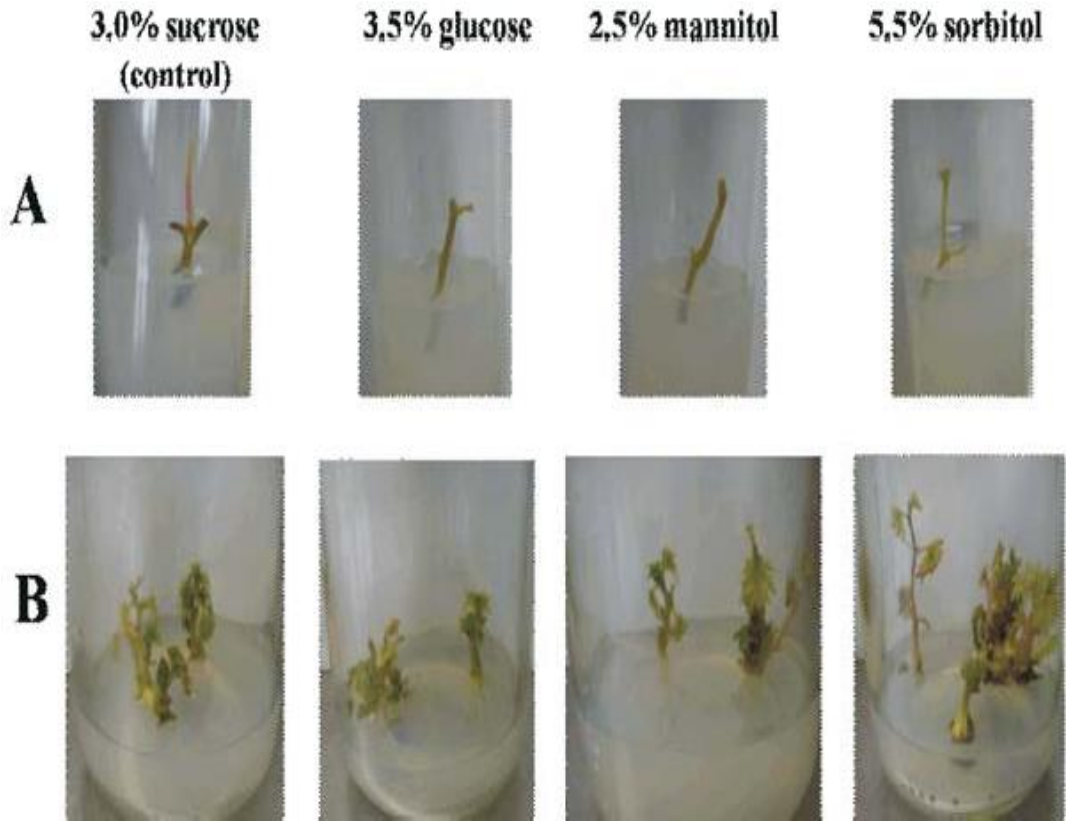

Fig. 1: (A) survived explants conserved on different concentrations of osmotic regulators. (B) Regrowth and regeneration of survived explants on recovery medium.

Table 4: Effect of different glucose concentrations on *in vitro* recovery

Glucose %	Conservation period (month)								
	Regeneration%			Shoot no./explant			Shoot length (cm)		
	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**	

Table 5: Effect of different mannitol concentrations on *in vitro* recovery

Mannitol %	Conservation period (month)								
	Regeneration%			Shoot no./explant			Shoot length (cm)		
	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**	

Table 6: Effect of different sorbitol concentrations on *in vitro* recovery

Sorbitol %	Conservation period (month)								
	Regeneration%			Shoot no./explant			Shoot length (cm)		
	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	100.00a	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**	

ISSR-PCR Analysis for Genetic Stability: 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 (7). According to ISSR analysis, results presented in Table (7) 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 conserved plant). ISSR marker profile produced by the primer ISSR-4 in 2.5% agarose gel was shown in Fig. (2).

Table 7: Statistics of the ISSR fragments for Black Matrouh grape variety.

ISSR primers	Primer Sequence	Ta (°C)	AB	Size (bp)	MB
17898-A	(CA)6GG	42	3	529 – 809	3
17899-B	(CA)6AC	40	5	449 – 1275	5
ISSR-35	TCG(CA)7	53	7	292 – 1185	7
834	(AG)8CT	53	10	296 – 1373	10
841	(GA)8TC	53	7	392 – 1125	7
809	(AG)8G	53	7	262 – 1046	7
ISSR-4	CGA(CA)7	53	6	340 – 1199	6
17899-A	(CA)6AG	40	5	478 – 1150	5
HB-10	(GA)6CC	40	6	326 - 872	6
BEC	(CA)7TC	42	5	485 – 1022	5

AB: Amplified bands, MB: monomoprphic bands

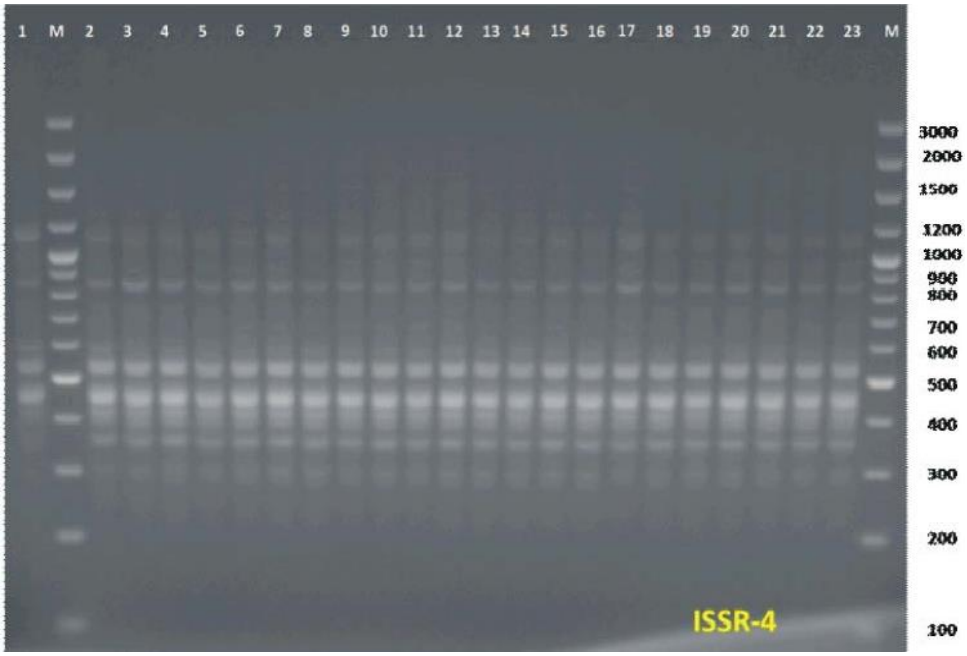

Fig. 2: Gel electrophoresis of ISSR fragments detected with the primer ISSR-4: (M) Molecular marker, (1) non conserved explants, (2) 3% sucrose, (3) 1.5% glucose, (4) 2.5% glucose, (5) 3.5% glucose, (6) 4.5% glucose, (7) 5.5% glucose, (8) 6.5% glucose, (9) 7.5% glucose, (10) 85% glucose, (11) 1.5% mannitol, (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

References

Sajid, G.m. and Z. Ahmed, 2008. Evaluation of various levels of mineral nutrients and plant growth regulators for *In vitro* culture of grape. Pakistan Journal of Botany 40(1): 329-336.

Tehrim, S. and G.M. Sajid, 2011. *In vitro* establishment, conservation and its implications for grape germplasm biodiversity. Romanian Biotechnological Letters, 16(6): 6781-6789.

Scherwinski-Pereira, J.E. and F.H.S. Costa, 2010. *In vitro* conservation of plant genetic resources: stratigies, principles and applications. *In vitro* culture of plants, Brasilia. Embrapa Technological Information, pp: 177-234.

Perez-Tornero, O., F. Ortin-Parraga, J. Egea and L. Burgos, 1999. Medium-term storage of apricot shoot tips in vitro by minimal growth methods. Hort Sci., 34(7): 1277-1278.

Tahtamouni, R.W., R.A. Shibli and M.M. Ajlouni 2001. Growth responses and physiological disorders in wild pear (*Pyrus syriaca* Boiss) during slow growth *in vitro* preservation on osmo-stressing media. Plant Tiss. Cult., 11: 415-23.

Engelmann, F. 2010. Use of biotechnologies for conserving plant diversity. Acta Hort., (ISHS), 812: 63-82.

Hagagy, N.A.A., 1998. *In vitro* conservation of some fruit germplasm. Annals of Agricultureal Science. Moshtohor, 36(3): 1667-1681.

Miaja, M.L., I. Gribaudo, R. Vallania and L.F. Fernandez, 2000. Low temperature storage and cryoconservation of a *Vitis vinifera* L. germplasm collection: first results. Acta Hort., 538(1): 177-181.

Rousseva, R., 2001. Study of the possibilities for *in vitro* storage of grape (*Vitis vinifera* L.) explants at low temperatures. Rasta days Science, 38: 374-376.

Silva, R., C. De, Z.G. Zanderluce and J.E. Scherwinski-Pereira, 2012. Short term storage *in vitro* and large scale propagation of grape genotypes. Pesq. agropec. bras., Brasília, 47(3): 344-350.

Zietkiewicz, E., A. Rafalski and D. Labuda, 1994. Genome fingerprinting by simple sequence repeats (SSR) anchored polymerase chain reaction amplification. Genomic, 20: 176-183.

Herrera, R., V. Cares, M.J. Wilkinson and P.D.S. Caligari, 2002. Characterization of genetic variation between *Vitis vinifera* cultivars from central Chile using RAPD and Inter Simple Sequence Repeat Markers. Euphytica, 124: 139-145.

Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plantarum, 15: 473-497.

Reed, B.M., 1992. Cold storage of strawberries in vitro: A comparison of three storage systems. Fruit Var. J., 46: 93-102.

Almousa, R., N. Hassan, R. Stino and A. Gomaa, (2015). Effect of plant growth regulators and medium constituents on *in vitro* propagation of grape (*Vitis vinifera* L.) cv. Black Matrouh. The Arab Journal of Arid Environments, 8(1-2): 12-24.

Waller, R.A. and D.B. Duncan, 1969. A basic rule for thaw symmetric multiple comparison problems. Amer. States. Assoc. J., pp: 1485-1503.

Steel, R.G.D. and J.H. Torrie, 1980. Principles and procedures of statistics. A biometrical approaches. McGraw-Hill, New York.

Ahmad, M. and M.A. Anjum, 2010. *In vitro* storage of some pear genotypes with the minimal growth technique. Turk J. Agric., 34: 25-32.