Journal of Horticultural Science & Ornamental Plants 5 (3): 183-193, 2013
ISSN 2079-2158

DOI: 10.5829/idosi.jhsop.2013.5.3.1126

In vitro Storage and Cryopreservation of Some Grape Varieties

[1]*Neveen A. Hassan,* [2]*Amina H. Gomaa,* [2]*Mohamed A. Shahin and* [1]*Ahmed A. El Homosany*

[1]National Genebank and Genetic Resources, Agricultural Research Center, Giza, Egypt
[2]Department of Pomology, Faculty of Agriculture, Cairo University, Giza, Egypt

Abstract: This study was carried out to evaluate three local grape varieties for *In vitro* conservation and genetic stability. 66.66% of shoot tip explants were able to survival when conserved for 12 months on MS medium with 20 g/l sucrose or 10 g/l sorbitol for Bez El-Anza, 40 g/l sucrose, 40 or 50 g/l sorbitol for Red Romy and 40 g/l sorbitol for Black Matrouh. Also, 66.66% of shoot tip explants of Black Matrouh conserved for 9 months on medium with 75µM CCC were able to survive. Conservation on media with different concentration of sucrose or sorbitol did not affected shoots number/explant and shoot length compared with those conserved on medium with 30 g/l sucrose (standard concentration). Increasing the conservation period from 3, 6, 9 to 12 months decreased gradually the shoots no. and shoot length. Conservation on media with different concentration of ABA or CCC reduced the shoot number/explant and shoot length compared with those conserved on ABA or CCC-free medium. Cryopreservation of seeds with liquid nitrogen after 6 hours desiccation showed highest germination percentage (70, 60 and 50 for Bez El-Anza, Red Romy and Black Matrouh respectively. The obtained results showed no significant differences between mother plant (control) and shoot tip explants conserved with different treatments in the three varieties under investigation using ISSR analysis.

Key words: ABA · CCC · Cryopreservation DNA Fingerprint · Grape · Medium term preservation · Sorbitol · Sucrose.

INTRODUCTION

***In vitro* Conservation:** The use of tissue culture techniques can be of great interest for germplasm collection, multiplication and storage of recalcitrant and vegetatively propagated species. Preservation of plant genetic resources has become extremely important for crop improvement to face the increasing depletion of natural resources. Moreover, tissue culture systems greatly facilitate the international exchange of germplasm [1]. Among the different methods of *In vitro* preservation is the medium and long-term conservation. Medium–term conservation there is a need to lengthen the period between subcultures by reducing growth rate. Whereas, with long –term conservation (cryopreservation) where plant material is stored in liquid nitrogen [2]. *In vitro* conservation of plant germplasm has been using slow growth procedures or cryopreservation [3]. Slow growth is usually achieved by Engelmann [1] reducing the culture temperature [4], low light intensity or complete darkness [5, 6], in the same trend, Rousseva [7] revealed that twelve grapevine microcuttings were conserved at low temperatures of 2, 4, 6 and 8°C. Who, also found that longest conservation term (12 months) and a high percentage of survival (83.50%) were achieved by gradual adjustment to 4°C. Benelli *et al.* [8] revealed that grape rootstock "Kober 5BB" (*Vitis berlandieri* x *V. riparia*) was preserved shoots kept at 10°C having higher survival (80 %) than those kept at 4°C conservation. Engelmann and Engels [2] modifying culture media with supplements of osmotic regulators and growth inhibitors or by removing growth promoters to reduce the cellular metabolism of the material, striving to maximize the time between subcultures [3,9-12]. Li *et al.* [13] stored ten grape genotypes at room temperature for up to 18 months with 10 to 20 ppm of CCC in B5 growth medium but other CCC concentrations, TIBA (2,3,5-Triiodobenzoic acid) and ABA were not effective. Hagagy [14] mentioned that shoot tips of grape (cv. Banati) can be stored for long periods at 5°C in one quarter strength medium with cummarin, whereas rooted plantlets of grape can be preserved in one quarter strength medium with cummarin for up to 12 months.

Cryopreservation involves storage of plant material at ultra-low temperatures in liquid nitrogen (-196°C). At this temperature, cell division and metabolic activities

Corresponding Author: Neveen A. Hassan, National Genebank and Genetic Resources, Agricultural Research Center, Giza, Egypt.

remain suspended and the material can be stored without changes for long periods of time. Cryopreservation is a part of biotechnology. Biotechnology plays an important role in international plant conservation programs and in preservation of the world's genetic resources [15, 16] and for genetic resources of vegetatively maintained crops [17]. Advances in biotechnology provide new methods for plant genetic resources and evaluation [18]. However, cryopreservation based Vitrification involve removal of most or all freezable water by physical or osmotic dehydration of explants, followed by ultra-rapid freezing which results in vitrification of intracellular soluted. Ezawa *et al.* [19] stated that shoot tips survival rates of 9 years old *Vitis labrusca* veins of variety Buffalo shoot tips was 80 % after 18 months storage in LN using vitrification. Matsumoto and Sakai [20] and Matsumoto and Sakai [21] indicated that *In vitro* grown axillary shoot tips of Vitis were successfully cryopreserved by vitrification (three-step vitrification procedure) and the recovery rate was improved to 85% and 80%, respectively.

Desiccation method (air drying) is the simplest method and consists of hydrating explants and freezing them rapidly by direct immersion in LN. Explants which are dried by a flow of sterile air under the laminar airflow cabinet or by air drying using a closed vial containing silica gel is more reproducible [22]. This method is mainly applied to most common agricultural and horticultural species, orthodox seeds, zygotic embryos, embryogenic axes and pollen grain [23, 24]. In France, in the national grape genetic resources conservation project, seeds of several hundred accessions are being cryopreserved after partial desiccation [25].

Genetic Stability Genetic Stability Assessments: Science occurrence of somaclonal variations in *In vitro* cultures is available in the literature, so it is important to ascertain the genetic fidelity of *In vitro* conserved germplasm. Also, Cryopreservation causes cryo-injury [26] the extent of which may be genotype. The effects of cryo- injury upon the genome are often unknown and may be attributed, in part to tissue culture, resulting in somaclonal variation [27]. It is particularly, important to assess if *In vitro* conserved germplasm and cryogenic storage surviving is genetically identical to its donor material prior to medium term conservation or cryo- storage. Therefore, there is an increasing need to determine if medium term conservation or cryopreserved germplasm is 'true to type' and to measure the extent of the near 'normal phenotype' and degree of closeness to the 'true' parental genotype. Cell and tissue culture manipulations have an essential role in cryopreservation and their routine application presents issues regarding the genetic integrity of cultures during long-term maintenance for research and other related activities [28]. There are many challenges regarding the detection of genomic change at the phenotypic, cytological and biochemical levels [29-33].

Over the last decade, less focus has been placed on phenotypic, morphological, histological, cytological and biochemical applications and more emphasis on the use of molecular 'tools' to assess genetic stability. It is possible, following molecular analysis of plants recovered from medium term conservation or cryopreservation germplasm, that DNA fragment polymorphic profiles may not detect genetic instability but more likely 'stability' in selected sequences. There is a need for guidance before the selection and application of specific techniques. Guidelines do exist for the selection of markers and their application to assess genetic diversity but there is little assurance that these will also provide adequate information for genetic stability assessments. Hassan and Bekheet [34] evaluated genome stability for six strawberry varieties which preserved under medium term *In vitro* preservation in 4°C and in dark condition and showed no significant differences between preserved and non preserved materials.

MATERIALS AND METHODS

***In vitro* Conservation:**

Explant Source: Shoot tip explants from the three local grape varieties (Bez El-Anza, Red Romy and Black Matrouh) (1 cm long) were taken from *In vitro* cultures after 1st subculture and separately cultured onto glass tubes (100 x 25 mm). Effect of different osmotic agents (sucrose or sorbitol), growth retardants (ABA or CCC) concentrations and conservation periods of shoot tip explants conserved and conservation periods (month) on survival percentage of shoots tip explants conserved at 10°C under complete darkness. Glass tubes (100 x 25) contained full strength MS plus 0.7 % agar and different concentrations of sucrose or sorbitol at (10, 20, 40, 50, 60, 90 and 120 g/l) or different concentrations of abscisic acid (ABA) at 5.0, 10.0, 15.0, 20.0 or 25.0 µM or cycocel (CCC) at 10.0, 25.0, 50.0, 75.0 and 100.0 µM separately. Also, full strength of MS medium plus 0.7% agar and 30 g/l sucrose (standard conservation) was investigated as control medium. All cultures were conserved at 10°C in a complete darkness. Survival percentage of shoot tip explants of each treatment was recorded at the end of each conservation period (four conservation periods 3 months for each one). The experiment was arranged as factorial experiment in a completely randomized design of {8 treatments x 4 conservation periods (3, 6, 9 and 12 months)} for each variety with four replicates, three

explants for each. The survived shoot tip explants from the three varieties were taken at the end of each conservation period and recultured in Jars 250 mm filled with 25 ml of recovery medium (normal growth medium) which consists of full strength MS plus 3% sucrose, 0.7% agar, BA at 1.0 mg/l and IBA at 0.1 mg/l. The cultures were incubated under normal growth conditions at 25 ± 2°C and photoperiods of 16 hour day and 8 hour night supplied by fluorescent lamp (four lamps per shelf) to provide light intensity of 3000 lux at explants level (30 cm from light). Average number of new proliferated shoots and length (cm) of shoots were recorded after 4 weeks.

Cryopreservation of Seeds Using Desiccation Method: The seeds were excised from mature fruits of the three grape varieties. The excised seeds were putted in wet sand inside a refrigerator for at least 10 to 12 weeks at 4°C before exposed to the air of laminar flow hood for 2, 4 and 6 hours then placed in 2.0 ml cryotube and immersed into liquid nitrogen for 1 day. Thawing was done for 1 min at 40°C, in a water bath after which the seeds were removed from the cryotubes and cultured in plastic 10 x 15 cm. pots filled with a mixture of Peat moss : Sand (1:1 by volume) and maintained in greenhouse (22°C±1). Survival germination of seeds was observed after 4 weeks. The seeds which success after cryopreservation was acclimatization in the greenhouse. The experiment was arranged as factorial experiment in a completely randomized design with four replicates, three explants for each.

Molecular Marker:

Isolation of Plant Genomic DNA: DNA extraction was carried out using leaf materials collected from each variety. Genomic DNA was extracted and purified using the DNeasy plant Mini Kit following the manual instructions (QIAGEN, Chatsworth, CA). Molecular fingerprinting of Grape varieties Based on Inter Simple Sequence Repeats (ISSRs) was carried out according to the procedure given by Sharma *et al*. [35]. ISSR analysis was carried out in a total volume of 50µl containing 5µl of 10x buffer, 10µl Q solution, 5µl of 2 mM dNTPs, 80 pmol primer, 0.5µl hot start taq polymerase and 25 ng DNA. The temperature profile composed of initial denaturating cycle at 95°C for 25 min followed by 10 touchdown cycles of 95°C/30 sec, 65-55°C/1 min, 72°C 90 sec. This was followed by 30 cycles of 95° C/0 sec, 55° C/1min, 72° C/90 sec and then a final extension cycle at 72°C for 7 min. The sequences of the ten ISSR primers (17899-B, 17898-A, 17899-A, 807, AW-3, TE, BEC and HAD were synthesized by Metabion, while BC 827 and BC 848 were synthesized by Pioneer are presented in Table 1.

Table 1: Name and sequence of the primers used in ISSR detection.

Primer code	Nucleotide sequences 5`_____3`
17899-B	(CA)6 GG
17898-A	(CA)6 AC
17899-A	(CA)6 AG
807	(AG)8 T
AW-3	(GT)7 AG
TE	GT (GGT)3 GAC
BEC	(CA)7 TC
HAD	CT (CCT)3 CAC
BC 827	(AC)8 G
BC 848	(CA)8 AG

Scoring of Data: Scoring of ISSR data was performed using 1% agarose gel electrophoresis profile, as clear and distinct fragment were scored as (1) for presence and (0) for absence. The molecular results were analyzed using the Phoretix 1D Pro software from nonlinear Dynamics.

Statistical Analysis: The obtained data were statistically analyzed according to Snedecor and W.G. Cochran [36]. Least significant difference (LSD) at $p \leq 0.05$ was employed to estimate the significant of differences among the treatment means.

RESULTS AND DISCUSSION

Effect of Different Sucrose Concentrations and Conservation Periods (Month) on Survival Percentage of "Bez El-anza, Black Matrouh and Red Romy" Shoot Tip Explants Conserved at 10°c under Complete Darkness: Data presents in Table 2 show that up to 66.6 % of shoot culture remained healthy and green after 12 months storage on MS medium supplemented with 20 g sucrose for Bez El-Anza variety, while Red Romy variety showed the highest survival rate (66.6%) on medium amended with 40 g/L sorbitol. However, up to 50 % of shootlets stored on medium with 20, 40 and 50 g/L sucrose for the same storage period (12 months) survived for variety Black Matrouh. While the lowest survival rate (25, 8.3 and 16.6%) were observed on medium contained 120g/L sucrose for Bez El-Anza, Red Romy and Black Matrouh, respectively (Table 2 and Figs. 1- 4).

Effect of Different Sorbitol Concentrations and Conservation Periods (Month) on Survival Percentage of "Bez El-anza, Black Matrouh and Red Romy" Shoot Tip Explants Conserved at 10°C under Complete Darkness: Up to 12 months 66.6 % of shoot cultures remain healthy and green on medium with 10g /L sorbitol, while the lowest survival rate 16.6 % was observed on medium with 120 g/L sorbitol (Table 3) for Bez–El Anza variety. Both

Table 2: Effect of different sucrose concentrations and conservation periods (month) on survival percentage of "Bez El-Anza, Black Matrouh and Red Romy" shoot tip explants conserved at 10°C under complete darkness.

	Grape varieties														
	Bez El-Anza					Red Romy					Black Matrouh				
Sucrose (g/l)	3	6	9	12	Mean	3	6	9	12	Mean	3	6	9	12	Mean
30 sucrose (control)	100.0a	100.0a	75.0bc	41.6e	79.16AB	100.0a	100.0a	75.0bcd	41.6fg	79.0AB	100.0a	100.0a	75.0bc	41.6def	79.1A
10	100.0a	100.0a	83.3ab	41.6e	81.25AB	100.0a	100.0a	83.3abc	50.0ef	83.3AB	100.0a	100.0a	75.0bc	41.6def	79.1A
20	100.0a	100.0a	91.6a	66.6cd	89.58 A	100.0a	100.0a	91.6abc	50.0ef	85.4AB	100.0a	100.0a	75.0bc	50.0 de	81.2A
40	100.0a	100.0a	83.3ab	41.6 e	79.16AB	100.0a	100.0a	100.0 a	66.6cde	91.6 A	100.0a	100.0a	75.0bc	50.0de	81.2A
50	100.0a	100.0a	75.0bc	41.6 e	79.16AB	100.0a	100.0a	58.3def	25.0 gh	70.8BC	100.0a	91.6ab	75.0bc	50.0 de	79.1A
60	100.0a	100.0a	66.6cd	33.3ef	75.00AB	100.0a	83.3abc	50.0 ef	16.6 h	62.5 C	100.0a	83.3ab	58.3cd	33.3efg	68.7AB
90	100.0a	100.0a	66.6cd	33.3ef	75.00AB	100.0a	75.0bcd	41.6 fg	16.6 h	58.3 C	100.0a	83.3ab	50.0de	25.0 fg	64.5AB
120	100.0a	83.3ab	58.3d	25.0 f	66.66 B	100.0a	66.6cde	41.6 fg	8.3 h	54.1 C	100.0a	75.0bc	33.3efg	16.6 g	56.2B
Mean	100.0a	97.9A	75.0B	40.6 C	--	100.0A	90.6 A	67.7 B	34.3 C	--	100.0A	91.6A	64.5 B	38.5c	--

Table 3: Effect of different sorbitol concentrations and conservation periods (month) on survival percentage of "Bez El-Anza, Black Matrouh and Red Romy" shoot tip explants conserved at 10°C under complete darkness.

	Grape varieties														
	Bez El-Anza					Red Romy					Black Matrouh				
Sorbitol (g/l)	3	6	9	12	Mean	3	6	9	12	Mean	3	6	9	12	Mean
30 sorbitol (control)	100.0a	100.0a	75.0bc	50.0de	81.2AB	100.0a	100.0a	75.0bcd	41.6fg	79.16AB	100.0a	100.0	75.0bcd	41.6 fgh	79.1A
10	100.0a	100.0a	83.3ab	66.6bc	91.6AB	100.0a	100.0a	83.3abc	50.0ef	83.33AB	100.0a	100.0	75.0bcd	50.0efg	81.2A
20	100.0a	100.0a	83.3ab	58.3cd	85.4AB	100.0a	100.0a	83.3ab	50.0ef	83.33AB	100.0a	100.0	75.0bcd	50.0efg	81.2A
40	100.0a	100.0a	100.0a	50.0de	83.3A	100.0a	100.0a	100.0a	66.6cde	91.67A	100.0a	100.0	83..3abc	66.6cde	87.5A
50	100.0a	100.0a	100.0a	41.6ef	79.1 A	100.0a	100.0a	100.0a	66.6bcd	91.67A	100.0a	91.6	83.3abc	50.0efg	83.3A
60	100.0a	100.0a	75.0bc	33.3ef	68.7AB	100.0a	100.0a	75.00	50.0def	81.25AB	100.0a	83.3	83.3abc	41.6fgh	81.2A
90	100.0a	83.3ab	58.3cde	25.0gh	60.4B	100.0a	83.3abc	58.3cde	33.3fg	68.75B	100.0a	83.3	58.3def	33.3gh	70.8A
120	100.0a	83.3ab	50.0def	16.6h	54.1B	100.0a	83.3ab	50.0def	25.0g	64.58B	100.0a	75.0	58.3def	25.0h	68..7A
Mean	100.0A	95.8A	78.1B	40.6 C	--	100.0A	95.8 A	78.12B	47.9C	--	100.0A	91.6	73.95B	44.7C	--

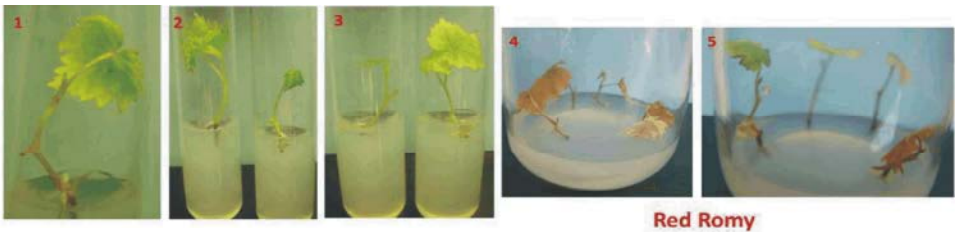

Fig. 1: Effect of (sucrose or sorbito) and (ABA or CCC) on shot tip explants conserved at 10°C after 12 months under completed darkness. 1-conrol 2-40gl^{-1} sorbito 3-50µ CCC 5- 40g l^{-1} sucrose 6- 10µM ABA

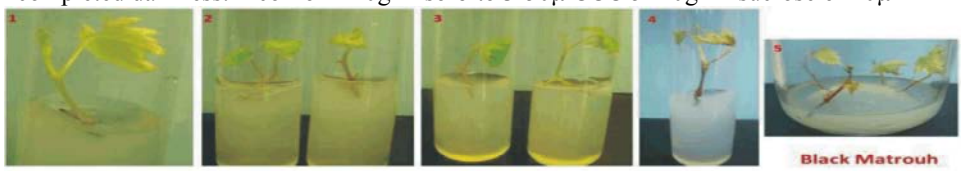

Fig. 2: Effect of (sucrose or sorbito) and (ABA or CCC) on shot tip explants conserved at 10°C after 12 months under completed darkness. 1-conrol 2-40gl^{-1} sorbito 3- 75µ CCC 4- 50 gl $^{-1}$ sucrose 5- 10µM ABA

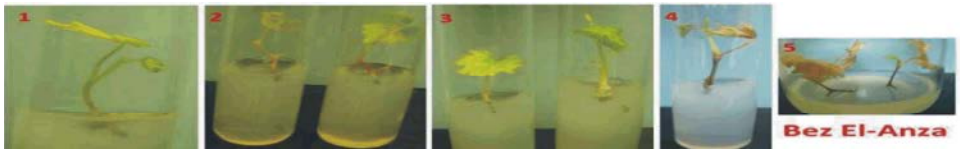

Fig. 3: Effect of (sucrose or sorbito) and (ABA or CCC) on shot tip explants conserved at 10°C after 12 months under completed darkness. 1- Conrol 2- 40gl^{-1} sorbito 3- 25µ CCC 4- 20 gl $^{-1}$ sucrose 5- 15µM ABA

Table 5: Effect of different CCC concentrations and conservation periods (month) on survival percentage of "Bez El-Anza, Black Matrouh and Red Romy" shoot tip explants conserved at 10°C under complete darkness.

	Grape varieties														
	Bez El-Anza					Red Romy					Black Matrouh				
CCC (µM)	3	6	9	12	Mean	3	6	9	12	Mean	3	6	9	12	Mean
CCC-free medium	100.0a	100.0a	75.0bc	41.6ef	79.1A	100.0a	100.0a	75.0bc	41.6ef	79.1A	100.0a	100.0a	75.0bcd	41.6e	79.1A
10	100.0a	100.0a	50.0de	25.0f	68.7A	100.0a	100.0a	50.0de	25.0f	68.7A	100.0a	91.6ab	75.0bcd	25.0fg	72.9A
25	100.0a	91.6ab	58.3cde	41.6ef	72.9A	100.0a	91.6ab	58.3cde	41.6ef	72.9A	100.0a	91.6ab	75.0bcd	25.0fg	72.9A
50	100.0a	91.6ab	50.0de	25.0f	66.6AB	100.0a	91.6ab	50.0de	25.0f	66.6AB	100.0a	91.6ab	75.0bcd	33.3ef	75.0A
75	100.0a	91.6ab	50.0de	25.0f	66.6AB	100.0a	91.6ab	50.0de	25.0f	66.6AB	100.0a	83.3abc	66.6cd	33.3ef	66.6A
100	100.0a	66.6cd	25.0f	00.0g	47.9B	100.0a	66.6cd	25.0f	00.0g	47.9B	100.0a	83.3abc	58.3d	8.3g	62.5A
Mean	100.0A	90.2A	51.3B	26.3C	--	100.0A	90.2B	51.3B	26.3C	--	100.0A	90.2A	70.8B	25.0C	--

Fig. 4: *In votro* shoot tip explants of three grape varieties under conservation conditions at 10°C in the incubators

Red Romy and Black Matrouh varieties showed 66.6% survival rate on medium with 50 g /L and 40 g/L sorbitol, respectively whereas the lowest survival rate (25.0%) were observed on medium with 120 g/L sorbitol for both varieties (Figs. 1-4).

Effect of Different Aba Concentrations and Conservation Periods (Month) on Survival Percentage of "Bez El-anza, Red Romy and Black Matrouh" Shoot Tip Explants Conserved at 10°c under Complete Darkness: Survival rate for both of Bez El Anza and Red Romy displayed 8.3% on medium containing 15 µm ABA. While, using 20 µm ABA showed 25 % survival rate for Black Matrouh variety after 12 months.On the other hand, the other treatment showed no survival rate for both of varieties.

The addition of ABA indicated low survival rate after 12 months preservation period for Bez El-Anza and Red Romy in addition to the weak and yellow shootlets after 12 months of storage which lead to reduction of the recovery percentage. The previous results concerning the effect of conserved shoot tip explants from different varieties under investigation on media supplemented with different ABA concentrations at 10°C for 12 months revealed that for "Bez El-Anza" variety over 50% (58.33%) of explants conserved on medium with 15 µM ABA and 50% of explants conserved on medium with 5, 10, 20 or 25 µM ABA for 6 months can able to survived, for "Red Romy" over 50% (75%, 83.33%, 83.33%, 91.69% and 91.67%) of explants conserved on medium with 5, 10, 15, 20 or 25 µM ABA respectively, for 6 months can able to survived. While, for "Black Matrouh" 50% of explants conserved on media with 15 or 20 µM ABA for 9 months can able to survived.In this concern, Engelmann [37] revealed that growth regulators are routinely used for *In vitro* germplasm conservation, with ABA being one of the most used (Table 4 and Figs. 1-4).

Effect of Different Ccc Concentrations and Conservation Periods (Month) on Survival Percentage of "Bez El-anza, Red Romy and Black Matrouh" Shoot Tip Explants Conserved at 10°c under Complete Darkness: Bez El Anza and Red Romy varieties revealed 41.6% survival rate on medium containing 25 µM CCC whereas no survival rate was observed on medium having 100 µM CCC.In contrast, using 50 and 75 µM CCC showed 33.3 % survival rate and the low percentage (8.3%) of survival rate observed on medium containing 100 µM CCC for Black Matrouh variety (Table 5 and Fig. 1- 4). In general, explants with 3 months old, up to 9 months revealed healthy and green shoots on medium with 10,20,40 and 50 g/L sucrose and 10, 25 and 50 CCC, also using medium containing 40,50 and 60 g/L sorbitol revealed high survival rates and viability decreased after 12 months. Lowest percentage was obtained on medium consist of ABA g/L sucrose and remained fully viable up to 3 months (100 % survival) and decrease after 9 months in the three varieties (Table 4). Using Sucrose and sorbitol as osmotic stress regulator seems to be more suitable for slow growth preservation of grape tissue cultures since it showed high survival rates of shootlets compared with ABA and CCC. With this respect, Schenck and Hildebrandt [38] stated that as sucrose concentration in the medium is increased above 4.5%, sucrose begins to have an inhibitory but non toxic effect on plant cell

Table 6: Effect of desiccation periods on seed germination percentage of (Bez El-Anza, Red Romy and Black Matrouh) after storage in liquid nitrogen at -196°C.

	Grape varieties			
Period (hour)	Bez El-Anza	Red Romy	Black Matrouh	Mean
Without desiccation (Control)	00.00 F	00.00 f	00.00 f	00.00 D
2	20.00 E	20.00 e	20 00 e	20.00 C
4	50.00 C	40.00 d	40.00 d	43.33 B
6	70 00 A	60.00 b	50.00 c	60.00 A
Mean	35.00 A	30.00 B	27.50 C	--

Means followed by the same letter (s) are not significantly different from each other at 5% level.

growth. Therefore, high sucrose level can be used to maintain cultures in a dormant condition for long periods. Wanas [39] found that for storage of the germplasm of apple rootstock cultures (M9, M26 and MM106). The cultures of M9 and MM106 were increased in survival percentage after 18 months storage at 4% sucrose. Tarmizi *et al.* [40] revealed that high concentration of sucrose was found to reduce growth, moisture content and also induced proline accumulation in the cultures. Also, Fortes *et al.* [11] and Shibli *et al.* [12] revealed that osmotic regulators act as growth retardants by causing osmotic stress to the material under conservation. When added to the culture medium, these carbohydrates reduce the hydric potential and restrict the water availability to the explants. Concerning with these results, Li *et al.* [13] stored ten grape genotypes at room temperature for up to 18 months and stated that ABA was not effective. Moreover, Moriguchi *et al.* [41] reported that adding ABA to the storage medium of Japanese pears did not improve their survival. They also, revealed that 1.0 M ABA improved survival of pear shoots stored at 10 and 15C for 1 year. Whereas, addition of 10 µM ABA reduced survival.

Cryopreservation: Effect of Desiccation Periods on Germination Percentage of Bez El-Anza, Red Romy and Black Matrouh Grape Seeds after Storage in Liquid Nitrogen at -196°C: Data in Table 6 indicated that the highest germination percentage (35.00%) was noticed with Bez El-Anza compared to Red Romy and Black Matrouh (30.00% and 27.50% respectively) with significant differences between them. As for the effect of desiccation periods showed 6 hours desiccation gave the highest significant germination percentage (60.00%). Also, increasing the desiccation periods from 2 to 4 hours increased the germination percentage from 20.00% to 43.33% respectively. Meanwhile, the seeds without

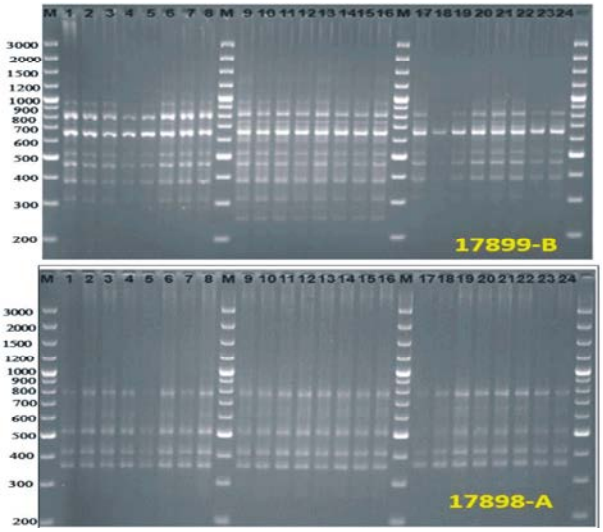

Fig. 5: ISSR profiles of 3 grape varieties as detected by 17899-B and 17898-A primers.

M = 100bp marker

1- Control Bez El-Anza	9- Control Red Romy	17- Control Black Matrouh
2- 20 g/l sucrose	10- 20 g/l sucrose	18- 20 g/l sucrose
3- 40 g/l sucrose	11- 40 g/l sucrose	19- 40 g/l sucrose
4- 10 g/l sorbitol	12- 20 g/l sorbitol	20- g/l sorbitol
5- 20 g/l sorbitol	13- 40 g/l sorbitol	21- 40 g/l sorbitol
6- 40 g/l sorbitol	14- g/l sorbitol	22- 50 g/l sorbitol
7- 15 µM ABA	15- 15 µM ABA	23- 20 µM ABA
8- 25 µM CCC	16- 25 µM CCC	24- 50 µM CCC

desiccation (control) cannot able to germinate (00.00%). Concerning the interaction between the varieties and desiccation periods data cleared that after 6 hours desiccation "Bez El-Anza" gave the highest significant germination percentage (70.00%) followed significantly by germination percentage (60.00%) of "Red Romy" desiccation for 6 hours. Desiccation "Bez El-Anza" for 4 hours and "Black Matrouh" for 6 hours resulted in the same germination percentage (50.00%). Meanwhile, Bez El-Anza, Red Romy and Black Matrouh after 2 hours desiccation gave the same germination percentage (20.00%). These results are in agreement with those obtained by Chaudhury and Chandel [42], who mentioned that the excised embryonic axes of almond desiccated between 10 to 4 % moisture could survive after liquid nitrogen exposure with viability percentage ranging from 100 to 40. The highest survival after cryopreservation was obtained for axes desiccated to about 7% moisture. Also, Bajaj [43] reported that survival and growth rates of embryonic axes of *Prunus persica* about 70-90% were similar for control and cooled axes after 1, 2, 3 and 4 hours dehydration, corresponding to moisture contents of 35.4, 30.1, 11.9 and 8.9%, respectively.

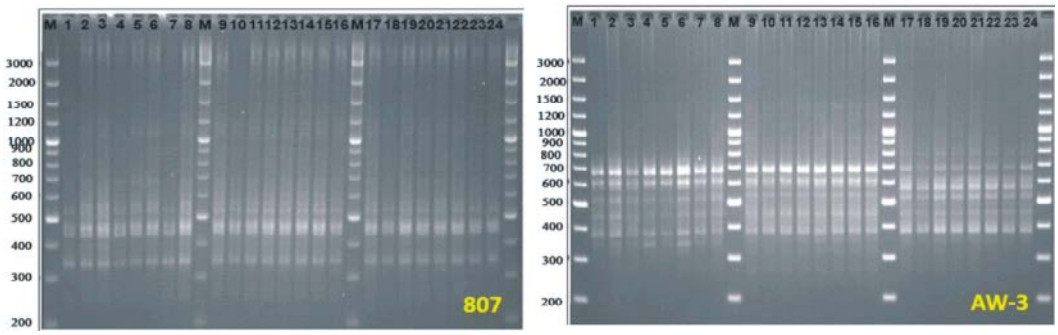

Fig. 6: ISSR profiles of 3 grape varieties as detected by 807 and AW-3 primers.

M = 100bp marker

1- Control Bez El-Anza	9- Control Red Romy	17- Control Black Matrouh
2- 20 g/l sucrose	10- 20 g/l sucrose	18- 20 g/l sucrose
3- 40 g/l sucrose	11- 40 g/l sucrose	19- 40 g/l sucrose
4- 10 g/l sorbitol	12- 20 g/l sorbitol	20- 20 g/l sorbitol
5- 20 g/l sorbitol	13- 40 g/l sorbitol	21- 40 g/l sorbitol
6- 40 g/l sorbitol	14- 50 g/l sorbitol	22- 50 g/l sorbitol
7- 15 µM ABA	15- 15 µM ABA	23- 20 µM ABA
8- 25 µM CCC	16- 25 µM CCC	24- 50 µM CCC

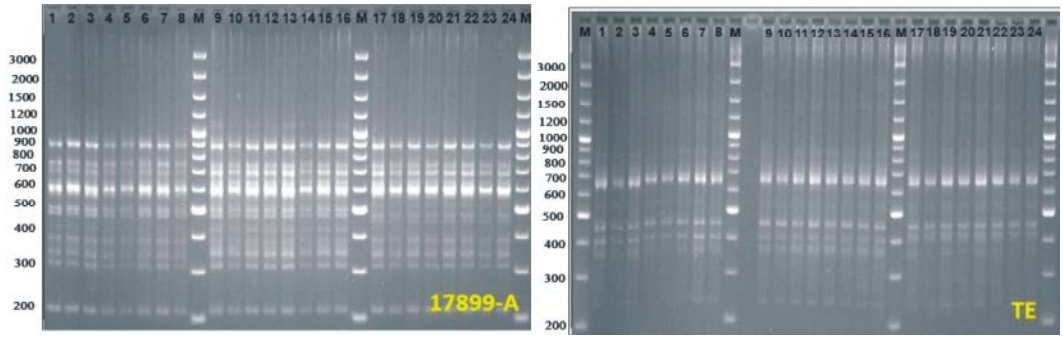

Fig. 7: ISSR profiles of 3 grape varieties as detected by 17899-A and TE primers.

M = 100bp marker

1- Control Bez El-Anza	9- Control Red Romy	17- Control Black Matrouh
2- 20 g/l sucrose	10- 20 g/l sucrose	18- 20 g/l sucrose
3- 40 g/l sucrose	11- 40 g/l sucrose	19- 40 g/l sucrose
4- 10 g/l sorbitol	12- 20 g/l sorbitol	20- 20 g/l sorbitol
5- 20 g/l sorbitol	13- 40 g/l sorbitol	21- g/l sorbitol
6- 40 g/l sorbitol	14- 50 g/l sorbitol	22- 50 g/l sorbitol
7- 15 µM ABA	15- 15 µM ABA	23- 20 µM ABA
8- 25 µM CCC	16- 25 µM CCC	24- 50 µM CCC

Genetic Stability Using ISSR Analysis: The effects of growth regulators, growth retardants, photoperiod and cold acclimatization on genetic stability under osmotic stress storage conditions were examined. Assessment of genetic stability was performed by ISSR analysis with DNA extracted from different *In vitro* explants preserved at 10°C in the dark. Amplification patterns of preserved material were compared with the non-preserved *In vitro* explant. The results showed no differences among control varieties plantlets and storage treatments within the three varieties. So the different treatments were effective for medium term storage and no genetic alteration caused by all the treatments a s assayed by ISSR analysis (Figs.5-9). ISSR profiled generated by the ten primers are shown in Figs. (5-9). Ten primers could produce multiple band profiles and the number of amplified DNA bands ranged from 165 bp to 944 bp (Figs 5-9). The genetic stability of micropropagated *Z. rubens* from sprouted buds of rhizomes using RAPD and ISSR analysis. Genetic integrity by RAPD and ISSR analysis has been reported earlier in many plant species [44-47]. Hussain and Tyagi [48] stated that on the basis of morphological, RAPD and ISSR markers tested no significant difference was observed among the Taro plants generated from shoot tip explants.

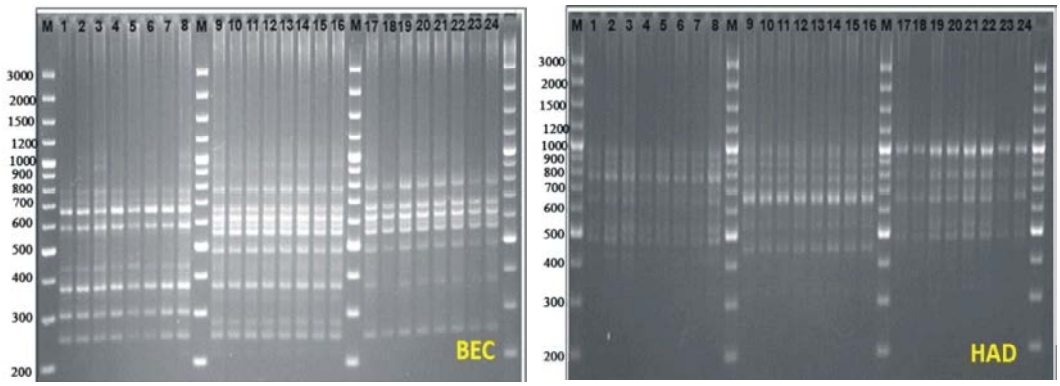

Fig. 8: ISSR profiles of 3 grape varieties as detected by BEC and HAD primers.

M = 100bp marker

1- Control Bez El-Anza	9- Control Red Romy	17- Control Black Matrouh
2- 20 g/l sucrose	10- 20 g/l sucrose	18- 20 g/l sucrose
3- 40 g/l sucrose	11- g/l sucrose	19- 40 g/l sucrose
4- 10 g/l sorbitol	12- 20 g/l sorbitol	20- 20 g/l sorbitol
5- 20 g/l sorbitol	13- 40 g/l sorbitol	21- 40 g/l sorbitol
6- 40 g/l sorbitol	14- 50 g/l sorbitol	22- 50 g/l sorbitol
7- 15 µM ABA	15- 15 µM ABA	23- 20 µM ABA
8- 25 µM CCC	16- 25 µM CCC	24- 50 µM CCC

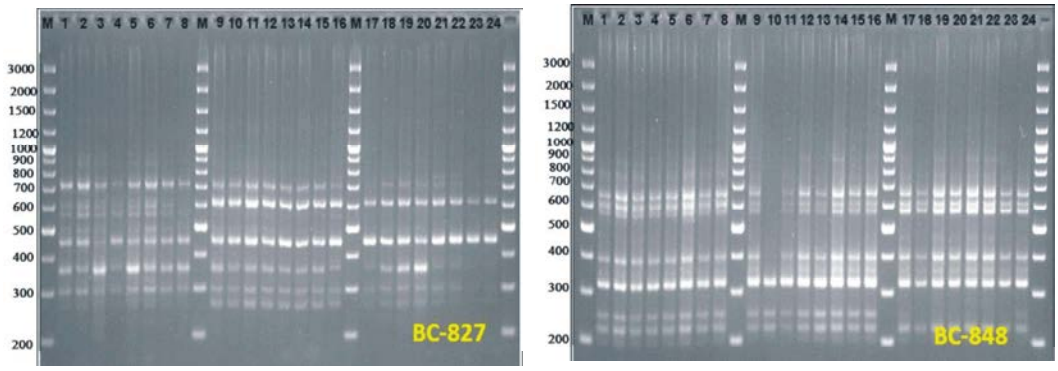

Fig. 9: ISSR profiles of 3 grape varieties as detected by BC-827 and BC-848 primers.

M = 100bp marker

1- Control Bez El-Anza	9- Control Red Romy	17- Control Black Matrouh
2- 20 g/l sucrose	10- g/l sucrose	18- 20 g/l sucrose
3- 40 g/l sucrose	11- 40 g/l sucrose	19- 40 g/l sucrose
4- 10 g/l sorbitol	12- 20 g/l sorbitol	20- 20 g/l sorbitol
5- 20 g/l sorbitol	13- 40 g/l sorbitol	21- 40 g/l sorbitol
6- 40 g/l sorbitol	14- 50 g/l sorbitol	22- 50 g/l sorbitol
7- 15 µM ABA	15- 15 µM ABA	23- 20 µM ABA
8- 25 µM CCC	16- 25 µM CCC	24- 50 µM CCC

Also, Bekheet and Aly [49] determine the genetic stability of preserved tissue cultures of globe artichoke and were identical to control plants. These results are in line with those obtained by Saker *et al.* [50], who mentioned that no significant variations were observed in tissue cultures derived date palm plantlets. Moreover, Bekheet *et al.* [51] concluded that, plantlets derived from cryopreserved cultures were identical to that derived from non-treated cultures and both were similar with the field grown plants i.e. no genetic variability of the frozen-thawed nodular cultures of date palm. Hassan and Bekheet [34] evaluated genome stability for six strawberry varieties which preserved under *In vitro* preservation in 4°C and in dark condition and showed no significant differences between preserved and non preserved materials.

REFERENCES

1. Engelmann, F., 1991. *In vitro* conservation of tropical plant germplasm a review. Euphytica, 57: 227-243.
2. Engelmann, F. and J.M. Engels, 2002. Technologies and strategies for *ex situ* conservation. In Engels, J.M.M. Ramanatha Rao, V. Brown, A.H.D. and Jackson, M.T. (eds) Managing plant Genetic Diversity. Wallingford and Rome, CAB International and IPGRI, pp: 89-104.
3. Scherwinski-Pereira, J.E. and F.H.S. Costa, 2010. *In vitro* conservation of plant genetic resources: strategies, principles and applications. *In vitro* culture of plants, Brasilia. Embrapa Technological Information, pp: 177-234.
4. Moges, A.D., N.S. Karam and R.A. Shibli, 2003. Slow growth *In vitro* preservation of African violet (*Saintpaulia ionantha* Wendl.) shoot tips. Advanced Hort Science, 17: 1-8.
5. Wang, P.J. and A. Charles, 1991. Micropropagation through Meristem Culture. In: Bajaj, Y.P.S. Eds. The Biotechnology in Agriculture and Forestry. High Tech. and propagation, Vol. 17, Springer Verlag Berlin HeideIberg, pp: 32-52.
6. Zandvoort, E.A., M.J.H. Hulshof and G. Staritsky, 1994. *In vitro* storage of *Xanthosoma* spp under minimal growth conditions. Plant Cell, Tissue and Organ Culture, 36: 309-316.
7. Rousseva, R., 2001. Study of the possibilities for *In vitro* storage of grapevine (*Vitis vinifera* L.) explants at low temperatures. Rasta Days Science, 38(7/10): 374-376.
8. Benelli, C., M. Lambardi and A. Fabbri, 2003. Low temperature storage and cryopreservation of the grape rootstock "Kober 5BB". Acta Horticulturae, 623: 249-253.
9. Goncalves, S. and A. Romano, 2007. *In vitro* minimum growth for conservation of *Drosophyllum lusitanicum*. Biologia Plantarum, 51: 795-798
10. Lata, H., R.M. Moraes, B. Bertoni and A.M.Pereira, 2010. *In vitro* germplasm conservation of *Podophyllum peltatum* L. under slow growth conditions. *In vitro* Cellular and Developmental Biology Plant, (46):22-27.
11. Fortes, G.R.L. and J.E. Scherwinski-Pereira, 2001. Preservation *In vitro* potato with acetylsalicylic acid and two carbohydrate sources. Brazilian Agricultural Research, 36: 1261-1264.
12. Shibli, R.A., M.A. Shatnawi, M.M. Ajlouni, A. Jaradat and Y. Adham, 1999. Slow growth *In vitro* conservation of bitter almond (*Amygdalus communis* L.). Advances in Horticultural Science, 13(3): 133-134.
13. Li, W., Z. Cao and L. Zhang, 1992. Effects of some plant inhibitors on the growth of grape plantlets in test tube. Acta Horticulturae Sinica, 19: 215-220.
14. Hagagy, N.A.A., 1998. *In vitro* preservation of some fruit germplasm. Annals of Agricultural Science. Moshtohor, 36(3): 1667-1681.
15. Bajaj, Y.P.S., 1995. Cryopreservation of Plant cell, Tissue and Organ Culture for the Conservation of Germplasm and Biodiversity. In: Biotechnology in Agriculture and Forestry Cryopreservation of Plant Germplasm. Bajaj Y.P.S. - New York, Springer-Verlag, pp: 3-18.
16. Benson, E.E., 1999. Cryopreservation. Plant Conservation Biotechnology, Taylor and Francis-London, pp: 83-95.
17. Kaczmarczyk, A., N. Shvachko, Y. Lupysheva, M.R. Hajirezaeiand and E.R.J. Keller, 2008. Influence of altering temperature preculture on cryopreservation results for potato shoot tips. Plant Cell Report, 27: 1551-1558.
18. Paunesca, A., 2009. Biotechnology for endangered plant conservation: A critical overview. Romanian Biotechnological Letters, 14(1): 4095-4104.
19. Ezawa, T., T. Harada and T. Yakuwa, 1989. Studies on freeze preservation of fruit tree germplasm. III. Freeze-preservation of grape shoot tips. Journal of the Faculty of Agriculture, Hokkaido University, 64(1): 51-55.
20. Matsumoto, T. and A. Sakai, 2000. Cryopreservation of *In vitro* cultured axillary shoot tips of *Vitis* by vitrification. Acta Horticulturae, 538(1): 173-175.
21. Matsumoto, T. and A. Sakai, 2003. Cryopreservation of axillary shoot tips of *In vitro* grown grape (*Vitis*) by a two step vitrification protocol. Euphytica, 131: 299-304.
22. Panis, B., R. Swennen and F. Engelman, 2001. Cryopreservation of plant germplasm. Acta Horticulturae, 650: 79-86.
23. Uragami, A., A. Sakai, M. Nagai and T. Takahashi, 1989. Survival of cultured cells and somatic embryos of *Asparagus officinalis* L. cryopreserved by vitrification. Plant Cell Report, 8: 418-421.
24. Engelmann, F., 2004. Plant cryopreservation: Progress and prospects. *In vitro* Cellular Developmental Biology Plant, 40: 427-433.
25. Engelmann, F., 2011. Use of biotechnologies for the conservation of plant biodiversity. *In vitro* Cellular Developmental Biology Plant, 47(1): 5-16.
26. Benson, E.E. and D.H. Bremner, 2004. Oxidative Stress in the Frozen Plant. In: Life in the Frozen State. Fuller, B. Lane, N. and Benson, E.E. (eds.) CRC Press LLC, Florida, pp: 205-242.

27. Harding, K., 1996. Approaches to assess the genetic stability of plants recovered from *In vitro* culture. In: Proceedings of the International Workshop on *In vitro* Conservation of Plant Genetic Resources. Normah, M.N. Narimah, M.K. and Clyde, M.M. (eds.) Plant Biotechnology Laboratory, University Kebangsaan Malaysia, Kuala Lumpur, Malaysia, pp: 137-170.
28. Harding, K., E.E. Benson and K. Clacher, 1997. Plant conservation biotechnology: an overview. Agro-food-Industry, 8(3): 24-29.
29. Harding, K., 2004. Genetic integrity of cryopreserved plant cells: a review. CryoLetters, 25: 3-22.
30. Harding, K., J. Johnston and E.E. Benson, 2005. Plant and Algal Cell Cryopreservation: Issues in Genetic Integrity, Concepts. In: 'Cryobionomics' and Current European Applications. In: Contributing to a Sustainable Future. Benett I.J. Bunn E. Clarke H. & McComb J.A. (eds.) Proceeding Australian Branch of the IAPTC & B, Perth, Western Australia, pp: 112-119.
31. Johnston, J.W., S. Dussert, S. Gale, J. Nadarajan, K. Harding and E.E. Benson, 2006. Optimization of the azinobis-3-ethyl-benzothiazoline-6-sulphonic acid radical scavenging assay for physiological studies of total antioxidant activity in woody plant germplasm. Plant Physiology and Biochemistry, 44: 193-201.
32. Johnston, J.W., K. Harding and E.E. Benson, 2007a. Antioxidant status and genotypic tolerance of Ribes *In vitro* cultures to cryopreservation. Plant Science, 172: 524-534.
33. Johnston, J.W., S. Horne, K. Harding and E.E. Benson, 2007b. Evaluation of the 1-methyl-2-phenylindole colorimetric assay for aldehydic lipid peroxidation products in plants: malondialdehyde and 4-hydroxynonenal. Plant Physiology and Biochemistry, 45: 108-112.
34. Hassan, Neveen, A. and S.A. Bekheet, 2008. Mid-term storage and genetic stability of strawberry tissue cultures. Egyptian Journal of Genetics and Cytology, 37(2): 289-301.
35. Snedecor, G.W. and W.G. Cochran, 1982. Statistical Methods. 7th Ed. The Iowa State University in Ames, Iowa, USA.
36. Sharma, P.C., B. Huttel, P. Winter, G. Kahl, R.C. Garender and K. Weising, 1995.The potential of microsatellites for hybridization and Polymerase chain reaction based DNA fingerprinting of chickpea (*Cicer arietinum* L.) and related species. Electrophoresis, 16: 1755-1761.
37. Engelmann, F., 1998. *In vitro* germplasm conservation. Acta Horticulturae, 461: 41-47.
38. Schenck, R.V. and A.C. Hildebrandt, 1972. Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Canadian Journal of Botany, 50: 199-204.
39. Wanas, W.H., 1992. *In vitro* storage of proliferated apple rootstock shoot tip cultures. Annals Agricultural Science, Ain Shams Univ. Cairo, 37(2): 501-510.
40. Tarmizi, A.H., M. Marziah and A.H. Halim, 1993. Effect of various concentrations of sucrose on growth and proline accumulation in oil palm polyemberyogenic cultures. Biotechnology in Agriculture Proceedings of the First Asia Pacific Conference on Agricultural Biotechnology, Beijing, China, pp: 365-368.
41. Moriguchi, T., I. Kozaki, S. Yamaki and T. Sanada, 1990. Low temperature storage of pear shoots *In vitro*. Bulletin of the Fruit Tree Research Station, 17: 11-18. (CAB Abst.).
42. Chaudhury, R. and K.P.S. Chandel, 1995. Cryopreservation of embryonic axes of almond (*Prunus amygdalus* Batsch.) seeds. Cryo Letters, 16: 51-56.
43. Bajaj, Y.P.S., 2002. Biotechnology in agriculture and forestry. Springer Verlag- Berlin. Heidelberg, New York. 50: 287-311.
44. Rout, G.R. and P. Das, 2002. *In vitro* Studies of Ginger: A review of Recent Progress. In: Goril, J.N. Anand Kumar, P. Singh, V.K. (ed.): Recent Progress in Medicinal Plants. Vol.4. Biotechnology and Genetic Engineering. Pp. 307-326. Studium Press, Houston.
45. Joshi, P. and V. Dhawan, 2007. Assessment of genetic fidelity of micropropagated *Swertia chirayita* plantlets by ISSR marker assay. Biology of Plants, 51: 22-26.
46. Panda, M.K., S. Mohanty, E. Subudhi, L. Acharya and S. Nayak 2007. Assessment of genetic stability of micropropagated plants of *Curcuma longa* L. by cytophotometry and RAPD analysis. International Journal of Integrative Biology, 1: 189-195.
47. Venkatachalam, L., R.V. Sreedhar and N. Bhagyalakshmi, 2007.Genetic analysis of micropropagated and regenerated plantlets of banana as assessed by RAPD and ISSR markers. *In vitro* cellular and developmental Biology, 43: 267-274.
48. Hussain, Z. and R.K. Tyagi, 2006. *In vitro* corm induction and genetic stability of regenerated plant in taro (*Colocasia esculenta* L. Schott). Indian Journal of Biotechnology, 5: 535-542.

49. Bekheet, S.A. and U.I. Aly, 2007. *In vitro* Conservation of Globe Artichoke (*Cynara scolymus* L.) Germplasm. International Journal of Agriculture and Biology, 9(3): 404-407.
50. Saker, M.M., S.A. Bekheet, H.S. Taha, A.S. Fahmy and H.M. Moursy, 2000. Detection of somaclonal variation in tissue culture-derived date palm plants using isozyme analysis and RAPD fingerprints. Biologia Plantarum, 43: 347-351.
51. Bekheet, S.A., H.S. Taha, M.M. Saker and M.E. Solliman 2007. Application of cryopreservation technique for *In vitro* grown Date Palm (*Phoenix dactylifera* L.) cultures. Journal Applied Science Research, 3: 859-866.