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Slow-Growth *in vitro* Conservation of Garlic Cultivars Grow in Egypt: Chemical Characterization and Molecular Evaluation

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Abstract: *In vitro* preservation of vegetatively propagated plant genetic resources provides an effective conservation system to guarantee adequate food supplies. In the present investigation, effective protocol for *in vitro* conservation of garlic cultivars (*Allium sativum* L.) grown in Egypt based on the use of osmotic stabilizers such as sucrose and sorbitol was developed. Bulblets, of the Balady and Seds 40 garlic cultivars, were divided evenly and culture into propagation medium consisted of Murashige and Skoog (MS) salts with the addition of 120 g L^{-1} sucrose, 5 g L^{-1} charcoal and 0.01 mg L^{-1} BA. Cultures were incubated at 22±2°C with a photoperiod of 16 h to produce virus free bulblets. The resultant bulblets were cultured in conservation media composed of MS salts supplemented with sorbitol or sucrose of three concentrations (0.1, 0.2 and 0.4 M). Cultures intended for preservation were incubated at 4°C and dark conditions. The developments of shoot and root growth as well as percentage of survival were evaluated during18 months of *in vitro* storage. No shoot or root growth was observed at 3 months of *in vitro* conservation. Results showed cultivar differences in response to the different osmoticums concentrations. Media supplemented with 0.1 sorbitol effectively retarded shoot and root length (mm) and number of developed roots of the Balady cultivar at 6, 12 and 18 months of *in vitro* preservation. Whereas, the addition of 0.1 or 0.2 M sucrose to the medium effectively retarded shoot and root growth of the Seds 40 cultivar during the same periods. Results also, indicted no increase in number of bulblets under all conditions. The maximum plant length was noticed when the medium was supplemented with 0.2 M sorbitol for both cultivars following 18 months of preservation at 4°C. Media contained 0.2 and 0.4 M sorbitol induced the highest number of roots for the Seds 40 and Balady cultivars, respectively. After 18 months of *in vitro* preservation, the percentage of survival was 100% and the recoveries of the *in vitro* cultures were assessed. Chemical characterization of vitamin C and total carbohydrates was also detected for the two cultivars before and after preservation. Genetic stability of the two cultivars was estimated at the molecular level using Random Amplified Polymorphic DNA (RAPD) techniques under all preservation conditions.

Key words: Garlic . genetic resources . *in vitro* preservation . RAPD . slow .growth . sucrose

INTRODUCTION

Garlic (*Allium sativum* L.) is consider as a one of the most important bulb vegetables grown and used as spice and flavoring agent for foods [1]. Cultivated garlic known at least 5000 years [2] and its proposed ancestor, *Allium longicuspis* originated in central Asia [3] and it has been spread west, south and east [4]. Garlic has been clonally propagated for thousands of years because it does not produce seed under standard cultivated conditions and is used as a popular condiment all over the world. Because its medicinal properties and benefits in lowering total plasma cholesterol, reducing blood pressure and decreasing platelet aggregation, garlic products have become popular in recent years and a variety of culinary and pharmaceutical preparations are now available in market [1, 5, 6].

The elaboration of some vegetable cells, tissues and organs conservation methods is necessary to preserve the germplasm sources in the conditions of progressive extension of monoclonal cultures or selected genotypes. Most attempts to store cultures have had dual objectives of stabilizing experimental material in terms of special characteristics and to avoid the demands and risks of subculture. Two basic approaches are followed to maintain germplasm collections *in vitro*: (i) minimal growth and (ii) cryopreservation [7].

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Medium term preservation (slow growth preservation) is used to preserve plant tissue for period of 1-5 years. It is therefore of value for the preservation of valuable germplasm which cannot be stored by conventional means as seed. In addition, using the slow growth preservation with the vegetatively (clonally) propagated plants like garlic is very valuable, as reproduction by seed does not yield a true-to-type plant and many garlic accessions do not produce seed and do not store well as bulbs. The term "slow growth" is used to cover "growth limitation", "inhibition" or "minimal growth" and other similar terms which imply a modification of the culture conditions, as well as naturally slowly growing material. Minimal growth conditions for short to medium term storage can be followed in several ways such as induction of osmotic stress with sucrose or mannitol [8], reduced temperature and/or light [9] and incorporation of sub-lethal levels of growth retardant [10, 11]. In general, the most widely applied technique for slow growth preservation is temperature reduction, which can be combined with a reduction in the concentration of nutritive elements and a decrease in light intensity or storage in the dark [12]. Notably, no success could be achieved at temperatures below 6°C and there was a variation in recovery percentage with different genotypes. The first report of successful *in vitro* storage of shoot tips of *Vitis rupestris* where in at 9°C, cultures could be stored up to 290 days. Modification of culture medium has also been used to reduce the subculture period for meristem cultures [13, 14]. Survival of cultures maintained at-10°C for one year could be increased from 39 to 56 percent following elevation of sucrose from 3 to 8% and it could be further increased to 30 percent by increasing culture volume from 3.5 to 6.0 ml. Incorporation of mannitol at 6 percent or abscissic acid (5 mg l^{1}) in the medium for cultures maintained at 22°C resulted in 63 and 43 percent survival respectively, compared with 14 percent without supplements, after one year's storage. In *Solanum* species, particularly in potato, Henshaw *et al.* [14] reported that there was 14 percent survival of meristems, stored for one year at 22°C, whereas it was 61 percent at 6°C for the same length of storage period. Survival rate could be increased to 83 percent by alternating day and night temperatures (12 and 6°C, respectively. Barlass and Skene [15] demonstrated in several *Vitis* species and cultivars that cultures could be maintained either as proliferating shoots or as single rooted shoots at 9.5°C for 6 to 12 months. Single shoots responded better than the proliferating shoot cultures, towards plantlet production. Roca *et al.* [16] have successfully shown that nodal cuttings from meristem derived plantlets of cassava (*Manihot esculentum*) could be maintained for two years on a medium with low osmotic concentration and activated charcoal. In garlic (*Allium sativum*) the shoot tips could be stored for a period of 16 months following reduction of temperature from 25 to 4°C and an increase in sucrose concentration to 10 percent [17]. The use of low temperature 9°C in dark for three years was used to stored taros (*Colocasia esculenta)* cultures successfully [18]. In more recent years, Wang *et al.* [19] have been used the atmospheric modification and the growth at a reduced temperature for long term preservation of the plant cell and in addition, they reported that the use of growth inhibiting chemicals (natural and synthetic hormones and osmotically active agents) and moderate reductions in the temperature to which cultures are maintained is the prominent among any *in vitro* preservation technical approaches.

Marker based estimates of genetic variability within and between accessions allows a more rational and efficient sampling of gene banks. Compared to morphological and protein markers, DNA-based genetic markers are often considered to be the most useful for genetic diversity studies because they are highly polymorphic and heritable (their expression is not affected by environmental variability. RAPD is a polymorphism assay which is based on the amplification of random DNA segments using sets of primers of arbitrary nucleotide sequence (20 and 21). The RAPD analysis has several advantages over isozymes and RFLP methods. It is simple, cost-effective and does not require radioactive labelling. Also, it reveals higher degree of polymorphism [22-24].

The aim of the present study is to develop an effective and applicable protocol for medium term *in vitro* preservation of bulbs of two Egyptian garlic cultivars. Evaluation of the reproductivity and chemical contents (vitamin C and total carbohydrates) before and after preservation has been shown. In addition, assessment of the genetic stability after preservation, have been performed.

MATERIAL AND METHODS

Materials

Initiation of bulbs and explants preparation: In this investigation two garlic cultivars (Balady and Seds 40) were used. *In vitro* culture was represented by bulbs. Pretreatment of the garlic bulbs at 4°C for approximately 8 weeks before preparing the stem discs to enhance bulbet formation. The mature foliage leaves removed before sterilization. The bulbs were initially sterilized according to [25] protocol, the bulbs were surface sterilized by 70% ethanol for 1 min followed by 30% sodium hypochlorite solution for 30 min and then

rinsed three times with sterile distiller water. Bulbs were cut and divided the stem disc into four parts and cultured in gars contained MS medium [26] supplemented with BA 0.01 mg L^{-1} 120 g L^{-1} sucrose, 6 g L^{-1} agars and 5 g charcoal. The pH was adjusted to 5.7 before autoclaving. The normal incubation conditions were at 25±2°C, photoperiod 16 h. 1500 lux with Philips white fluorescent tubes for 4 weeks for producing the bulbs.

Effects of sorbitol and sucrose concentrations on bulbs conservation: The produced *in vitro* bulbs were used for cold storage (4°C) and to asses the importance of increased sorbitol and sucrose content and its osmotic stress in med-term storage. The bulbs were individually transferred to MS medium without hormones and supplemented with different concentration of sucrose and sorbitol (0.1, 0.2 and 0.4 M L^{-1}) respectively, then incubated at 4°C in the dark. Fifteen cultures were taken after 4, 6, 10, 14 and 15 months and survival percentage was recorded. The cultures were transferred to fresh medium containing hormones and placed under standard culture room condition for four weeks and then survival percentage were assessed. Each treatment consisted of 15 replicate. All experiments were designed in complete randomized design and obtained data were statistically analyzed using the method described by Waller and Duncan [27].

All chemicals which used in determination of propagation and medium term preservation were purchased from SIGMA-ALDRICH Chem. Co. and DUCHEFA BIOCHIMIE B.V.

Determination of vitamin C: Vitamin C was determined by HPLC. A volume of 5 gm of each garlic bulb was homogenized with 4 ml of an extraction solution (30 g L^{-1} meta-phosphoric acid +80 g L^{-1} acetic acid). The resulting mixture was filtered under suction and adjusted up to 10 ml with distilled water. Samples were filtered through a 0.45 μm membrane filter and duplicates of 20 μm for each extract were analyzed by HPLC. Results are expressed as milligrams of ascorbic acid per 100 ml.

Separation of ascorbic acid was performed by HPLC using a Hypersil BDS C8 (5 μm) stainless steel column (250 mm x 4.6 mm) (Thermo Electron, United Kingdom). The solvent system used was an isocratic gradient of a solution 70% Buffer (0.85% v/v H_2SO_4 in 17.5 mM KH_2PO_4, pH 1.8) and 30% Methanol. The flow rate was fixed at 1.5 ml min^{-1}. A UV-vis detector was set at 245 nm; chromatographic data and UV-vis spectra were collected, stored and integrated using a chromostar light software. The calibration curve was built with one concentration level an ascorbic acid standard solution (100 mg L^{-1} in a solution 30 g L^{-1} meta-phosphoric acid +80 g L^{-1} acetic acid) [28].

Separation of carbohydrates: One gram of fresh bulb of garlic was homogenized and hydrolyzed by 2 M HCl on boiling water bath for 120 min. Carbohydrates were performed by HPLC using a Hypersil APS_2 (5 μm) stainless steel column (250×4.6 mm) (Thermo Electron, United Kingdom). The solvent system used was an isocratic gradient of a solution 80% Acetonitrile (ACN) and 20% water (H_2O). The flow rate was fixed at 0.5 ml min^{-1}. ELSD (Evaporative Light Scattering detector) was set at 30°C nebolization, 90°C evaporation and 1.6 SLM gas. Chromatographic data was collected, stored and integrated using chromostar light software. The calibration curve was built with one concentration level [29].

All chemicals which used in determination of vitamin C and separation of carbohydrates were purchased from SIGMA-ALDRICH Chem. Co.

DNA extraction: DNA was isolated from approximately 100 mg of garlic bulbs according to the protocol described previously [30]. The material was ground with a mortar and pestle to a fine powder in liquid nitrogen. The frozen powder was quickly added to extraction buffer (100 mM Tris pH 8.0, 50 mM EDTA pH 8.0, 500 mM NaCl, 10 mM mercaptoethanol). After the addition of 20% SDS (1:15, v/v), the mixture was maintained at 65 °C for 20-30 min. One third volume of 5 M potassium acetate was added and the mixture was placed on ice for 30 min. The mixture was centrifuged at 12000 rpm for 20 min and the DNA was precipitated with 0.6 volume of isopropanol and dissolved in TE buffer (50 mM Tris, 10 mM EDTA, pH 8.0).

Amplification of RAPD markers: Polymerase Chain Reactions (PCRs) were carried out in 25 μl volume. A reaction tube contained 50 ng of genomic DNA template, 2.0 μM primers, 0.2 μM each of dATP, dCTP, dGTP and dTTP, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5-2.5 mM MgCl2, 0.01% gelatin and 2 units of Taq polymerase (Promega). The PCR reaction mixture was performed in a Biometra T1 thermal cycler programmed as follows: an initial strand separation at 94°C (5 min) followed by 40 cycles of profile 94°C (1 min), 35°C (1 min), 72°C (1 min) and a final extension at 72°C (7 min). Amplification products were analyzed by electrophoresis in 1.7% agarose gels stained with ethidium bromide and photographed by Gel Documentation system. PCR reactions were conducted

Table 1: Names and sequences of the 5 decamer arbitrary primers

Primer name	Sequence
A4	5' AATCGGGCTG 3'
A7	5' GAAACGGGTG 3'
C5	5' GATGACCGCC 3'
G12	5' CAGCTCACGA 3'
N4	5' GACCGACCCA 3'

using five RAPD primers. Names and sequences of these primers are listed in Table 1.

RESULTS AND DISCUSSION

Initiation of the bulbs: Stem discs (cloves) sprouted within 4 weeks on MS medium supplemented with BA 0.01 mg L^{-1}, 120 g L^{-1} sucrose, 6 g L^{-1} agars and 5 g charcoal. Cloves development in four stages, expansion of tissue zones surrounded by the basal parts of the immature foliage leaves, formation of dome-shaped structures, bud differentiation directly from each dome and development into bulblets [31].

Effect of sorbitol and sucrose concentrations after 3 months under (4°C) and darkness: The data presented in Table 2 showed that the effect of sorbitol and sucrose concentrations on bulbs number, plant height (mm), root number and root length of two cultivars of garlic (Balady and Seds 40) after 3 months under low temperature (4°C) and darkness. There were insignificant differences in case of bulbs numbers between the different studied factors for the two cultivars. However, there were significant differences for the Seds 40 cultivar on the levels of average plant height (mm). The highest plant height (0.5) for Seds 40 cultivar was recorded with sorbitol 0.2 M, whereas sorbitol 0.1 M gave the lowest value (0.0), while Balady cultivar showed insignificant differences between the two studied factors.

Concerning the effect of sucrose and sorbitol levels on root number, there were no significant differences among the different osmotic concentrations for Balady cultivar. On the other hand, Seds 40 showed the highest root number was achieved with sorbitol at 0.2 M. Meanwhile, sorbitol at 0.2 M and sucrose at 0.4 M gave the lowest value.

In case of the root length, the highest root length for Seds 40 cultivar was recorded with sorbitol 0.2 M and sucrose 0.2 M, whereas sorbitol 0.1 M and Sucrose 0.4 M gave the lowest value (0.0). Alternatively, Balady cultivar showed insignificant differences between the two osmotic concentrations.

Effect of sorbitol and sucrose concentrations after 9 months under (4°C) and darkness: Table 3 shows the

Table 2: Effect of sucrose and sorbitol concentrations on bulb no., Plant height (mm), root no. and root length of two cultivars of garlic (Balady and Seds 40) after 3 months under low temperature (4°C) and darkness

	Balady				Seds 40			
Treatment	Bulb no.	Plant height (mm)	Root no.	Root length	Bulb no.	Plant height (mm)	Root no.	Root length
Sorbitol 0.1 M	1.0^{A}	0.4^{A}	0.8^{A}	0.1^{A}	1.0^{A}	0.0^{C}	0.0^{C}	0.0^{C}
Sorbitol 0.2 M	1.0^{A}	0.4^{A}	0.8^{A}	0.1^{A}	1.0^{A}	0.5^{A}	1.0^{A}	0.1^{A}
Sorbitol 0.4 M	1.0^{A}	0.5^{A}	1.0^{A}	0.1^{A}	1.0^{A}	0.1^{B}	0.3^{B}	0.03^{B}
Sucrose 0.1M	1.0^{A}	0.4^{A}	0.8^{A}	0.1^{A}	1.0^{A}	0.1^{B}	0.3^{B}	0.03^{B}
Sucrose 0.2 M	1.0^{A}	0.4^{A}	0.8^{A}	0.1^{A}	1.0^{A}	0.5^{A}	1.0^{A}	0.1^{A}
Sucrose 0.4 M	1.0^{A}	0.4^{A}	0.9^{A}	0.1^{A}	1.0^{A}	0.0^{C}	0.0^{C}	0.0^{C}

Means having the same letter(s) are not significantly different ($p<0.05$)

Table 3: Effect of sucrose and sorbitol concentrations on bulb no., Plant height (mm), root no. and root length of two cultivars of garlic (Balady and Seds 40) after 9 months under low temperature (4°C) and darkness

	Balady				Seds 40			
Treatment	Bulb no.	Plant height (mm)	Root no.	Root length	Bulb no.	Plant height (mm)	Root no.	Root length
Sorbitol 0.1 M	1.0^{A}	5.2^{AB}	6.0^{BC}	4.6^{AB}	1.0^{A}	1.1^{DEF}	1.3^{DE}	0.6^{EF}
Sorbitol 0.2 M	1.0^{A}	3.7^{BC}	2.6^{DE}	3.5^{ABCD}	1.0^{A}	6.6^{A}	8.0^{AB}	5.4^{A}
Sorbitol 0.4 M	1.0^{A}	0.6^{EF}	2.3^{DE}	1.4^{DEF}	1.0^{A}	4.8^{AB}	3.5^{CD}	3.7^{ABC}
Sucrose 0.1M	1.0^{A}	1.5^{CDEF}	3.3^{CD}	2.3^{CDE}	1.0^{A}	4.5^{AB}	5.6^{BC}	1.3^{DEF}
Sucrose 0.2 M	1.0^{A}	3.0^{BCDE}	9.0^{A}	3.0^{BCD}	1.0^{A}	4.0^{BC}	7.0^{AB}	2.1^{CDE}
Sucrose 0.4 M	1.0^{A}	3.5^{BCD}	6.0^{BC}	2.9^{BCD}	1.0^{A}	0.0^{F}	0.0^{F}	0.0^{F}

Means having the same letter(s) are not significantly different ($p<0.05$)

Table 4: Effect of sucrose and sorbitol concentrations on bulb no., Plant height (mm), root no. and root length of two cultivars of garlic (Balady and Seds 40) after 15 months under low temperature (4°C) and darkness

	Balady				Seds 40			
Treatment	Bulb no.	Plant height (mm)	Root no.	Root length	Bulb no.	Plant height (mm)	Root no.	Root length
Sorbitol 0.1 M	1.0^{A}	16.6^{CD}	16.0^{A}	10.3^{A}	1.0^{A}	10.0^{E}	9.0^{B}	7.0^{CD}
Sorbitol 0.2 M	1.0^{A}	25.6^{A}	8.3^{BC}	8.6^{ABC}	1.0^{A}	21.3^{B}	10.0^{B}	9.0^{ABC}
Sorbitol 0.4 M	1.0^{A}	7.1^{E}	5.6^{CD}	3.5^{E}	1.0^{A}	8.8^{E}	4.0^{D}	4.1^{E}
Sucrose 0.1M	1.0^{A}	15.6^{D}	9.6^{B}	5.1^{DE}	1.0^{A}	20.6^{BC}	10.3^{B}	8.0^{BC}
Sucrose 0.2 M	1.0^{A}	21.0^{D}	5.6^{CD}	9.0^{ABC}	1.0^{A}	22.0^{AB}	17.0^{A}	10.1^{AB}
Sucrose 0.4 M	1.0^{A}	14.5^{D}	5.6^{CD}	7.0^{CD}	1.0^{A}	0.0^{F}	0.0^{E}	0.0^{F}

Means having the same letter(s) are not significantly different ($p<0.05$)

Table 5: Recovery of the bulbs after 15 months storage at different concentrations of osmotic stress induced by sorbitol and sucrose under low temperature (4°C) and darkness

	Recovery (%)	
Treatment	Balady	Seds 40
Sorbitol 0.1 M	20.5^{C}	25.2^{C}
Sorbitol 0.2 M	00.0^{E}	10.0^{D}
Sorbitol 0.4 M	35.7^{E}	35.0^{B}
Sucrose 0.1 M	20.9^{C}	10.0^{D}
Sucrose 0.2 M	10.0^{D}	10.0^{D}
Sucrose 0.4 M	21.0^{C}	90.0^{A}

Means having the same letter(s) are not significantly different ($p<0.05$)

effect of sorbitol and sucrose concentrations on bulbs number, plant height (mm), root number and root length of two cultivars after 9 months under low temperature (4°C) and darkness. The storage data cleared that both Seds 40 and Balady cultivars showed insignificant differences at different concentrations treatments on bulb numbers. Regarding the effect of sucrose and sorbitol levels on average plant height (mm), data cleared that there were significant differences between the different osmotic concentrations for Seds 40 cultivar. The highest plant height (6.6 mm) was obtained with sorbitol at 0.2 M. The lowest value (0.0 mm) was obtained with sucrose at 0.4 M. In contrast, Balady cultivar showed the highest value of plant height (5.2 mm) with sorbitol at 0.1 M, while, the lowest value obtained by sorbitol at 0.4 M (0.6 mm). In case of the effect of sorbitol and sucrose on root number, it is cleared that the highest mean root number (9.0)/bulb was recorded with Balady at sucrose 0.2 M compared to Seds 40 (8.0) at 0.2 M of sucrose or sorbitol. In contrast, the lowest value (2.6 and 2.3) was obtained by at 0.2 M and 0.4 M of sorbitol concentration, respectively. For the effect of sucrose and sorbitol levels on the average of root length/bulb, there were significant differences between the different osmotic concentrations as shown in Table 3. The highest root length for Seds 40 cultivar was 5.4 and recorded with sorbitol at 0.2 M. On the contrary, sorbitol at 0.1 M gave the highest value (5.2) of root number for Balady cultivar. The lowest root length (0.0) was obtained by sucrose at 0.4 M for Seds 40 cultivar and also Balady cultivar showed the lowest root length (1.4) when we used sorbitol at 0.4 M.

In general, Data in Table 3 showed that the highest significant mean plant height (mm), root numbers and root length were recorded with Balady cultivar compared to Seds 40 cultivar.

Effect of sorbitol and sucrose concentrations after 15 months under (4°C) and darkness: The effect of osmotica on average of bulbs number, plant height (mm), root number and root length of two cultivars after 15 months are showing in Table 4. Concerning the bulbs number, there were insignificant differences between the two cultivars. However, the plant height for Balady cultivar showed the highest significant mean (25.6 mm) compared to Seds 40 cultivar.

The highest plant height was achieved with sorbitol at 0.2 M for Balady cultivar followed by sucrose at 0.2 M for Seds 40 cultivar. The lowest value (0.0 mm) was obtained when treated with sucrose 0.4 M for Seds 40 cultivar followed by sorbitol at 0.4 M for Balady cultivar.

Concerning the effect of sucrose and sorbitol on average root number/bulb and root length, there were significant differences between osmotica concentrations. The highest root number 17 and 16 and root length 10.3 and 10.0 were recorded with both sorbitol at 0.1 M and sucrose at 0.2 M for Balady and Seds 40 cultivars, respectively.

In general, from the data presented in Table 4, we can summarize that the highest significant mean plant

Table1 6: Effect of sucrose and sorbitol concentrations on the vitamin c and carbohydrates content

	mg/100 g							
	Balady				Sides 40			
Treatment	Vitamin C	Fructose	Glucose	Sucrose	Vitamin C	Fructose	Glucose	Sucrose
Control	28.81	103.52	86.75	325.11	46.32	109.78	92.69	334.04
Sucrose 0.1 M	0.47	59.45	35.67	83.29	6.99	54.45	19.14	68.98
Sucrose 0.2M	0.929	117.911	22.55	81.26	6.78	83.91	6.79	65.60
Sucrose 0.4 M	0.688	53.57	14.84	53.48	5.08	216.91	38.99	140.50
Sorbitol 0.1M	0.402	34.394	65.77	237.02	12.08	54.638	23.95	86.33
Sorbitol 0.2M	0.424	151.397	38.52	138.82	1.01	189.26	7.49	81.30
Sorbitol 0.4M	972.00	41.453	31.54	113.60	1.69	63.58	6.52	75.28

Table 7: Number of monomorphic bands as revealed by each RAPD primer

Primer	Monomorphicband in Balady	Monomorphic band in Sydes
A4	13	11
A7	5	4
C5	5	5
G12	6	4
N4	8	7

height (mm), root number and root length were recorded with Balady cultivar compared to Seds 40 cultivar.

Bulbs recovery: Bulbs of Seds 40 cultivar which treated with sucrose at 0.4 M carry on normal growth when placed in a culture medium without sucrose as osmotic stress. In contrary, bulbs of Balady which treated with sorbitol at 0.2 M showing a drop off bulb recovery when placed in a culture medium, while using sorbitol at 0.4 M continue growth with 35.7% recovery (Table 5). These results are in agreement with those found by El-Gizawy *et al*. [17]. They reported that shoot tips of garlic could be stored for a period of 16 months following reduction of temperature from 25 to 4°C and an increase in sucrose concentration to 10 percent. Also, Viterbo *et al*. [32] examined the *in vitro* conservation of basal leaf explants at 4-6°C for up to 16 months using standard medium or modified media containing 10% sucrose and the survival rate ranged from 80 to 100% recorded after 8 and 16 months cold storage, respectively.

Moreover, Botau *et al.* [33] showed that the plantlets of garlic incubated at low temperature on MS media without sucrose medium decreasing the regenerative capacity from 17 to 39% and slow down plants growth between 42 and 85%. Furthermore, Keller *et al*. [34] analyzed the garlic plants in slow growth culture cycles at reduced temperature (2 or 10°C) on medium without hormones for 12 months.

Chemical analysis: Table 6 shows the effect of sucrose and sorbitol concentrations on the vitamin c and carbohydrates content. The results of treatments show a great decrease of vitamin c between control and treatments with various concentrations of sucrose and sorbitol. The results obtained clearly separate Sides 40 with sorbitol 0.1 M from other treatment, which contain 12.08 mg 100^{-1} gm from vitamin c. In Balady, the maximum content of vitamin c was recorded in sorbitol 0.4M (0.972 mg 100^{-1} gm) and the minimum content of vitamin c was recorded in sorbitol 0.1M (0.402 mg/100gm) whereas, the minimum content of vitamin c in Sides 40 was recorded in sorbitol 0.2M (1.01 mg 100^{-1} gm). These results suggested that large amount of vitamin c was exhausted by a strong low temperature stress. Vitamin C is highly sensitive to air, water and temperature. Vegetables can be lost large amount of vitamin C by strong under low temperature stress [35].

The data also revealed that variation of carbohydrates content by using sucrose and sorbitol concentrations. Accumulation of soluble carbohydrates in temperate plants has been demonstrated during cold acclimation, either in nature or under experimental conditions. Many studies have shown that accumulation of these soluble carbohydrates in plant tissues correspond to the period when they were most tolerant to freezing [36]. For instance, the level of raffinose family oligosaccharides (RFO), namely raffinose and stachyose concentration increases in the tissues of some species in response to low temperature [36].

Soluble carbohydrate concentration in garlic cultivars shows in Table 6. Glucose and sucrose concentration decreased when used different concentration of sucrose and sorbitol in the

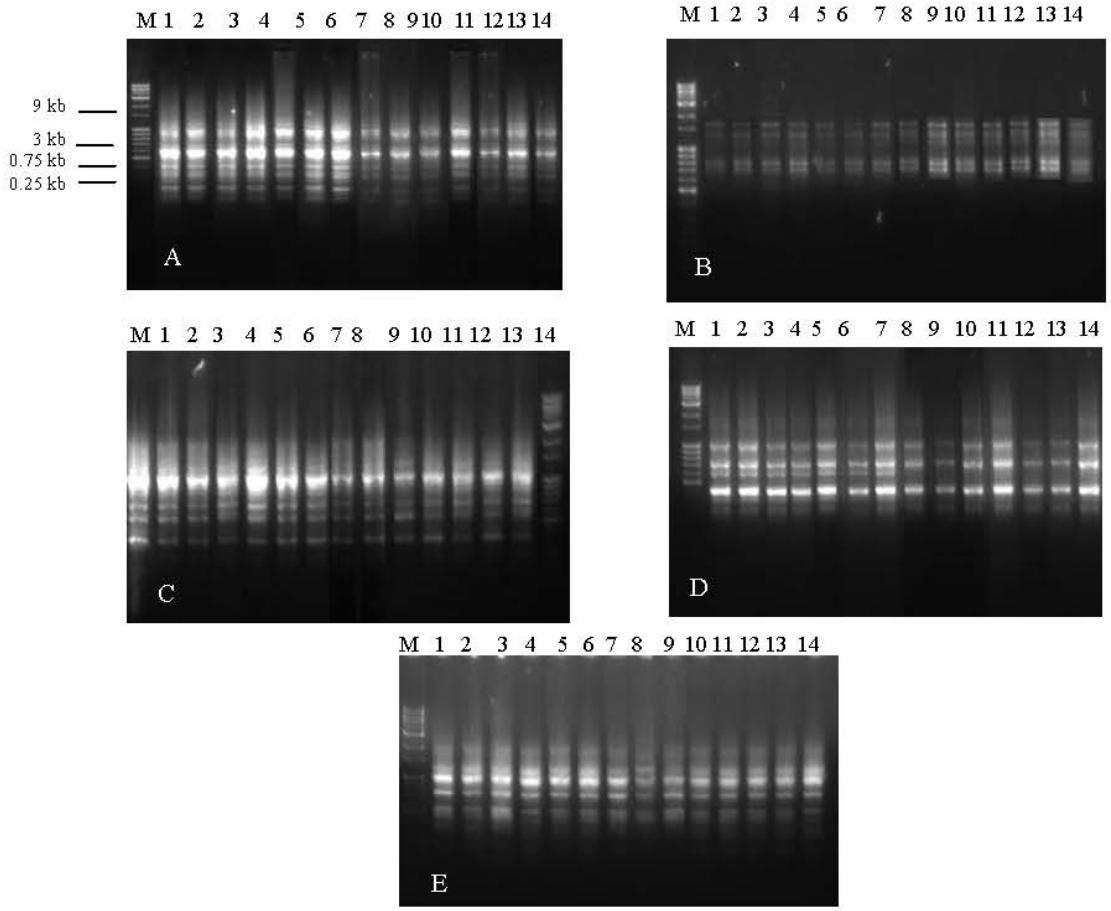

Fig. 1: RAPD analysis of fourteen *Allium sativum L* treatment with 5 random primers
Panels from A to E representing the RAPD products with the 5 primers (A4, A7, C5, G12 and N4), respectively. Columns from 1 to 7 represent the seven Balady cultivar treatments (control, Sucrose 0.1 M, Sucrose 0.2 M, Sucrose 0.3 M, sorbitol 0.1 M, sorbitol 0.2 M, sorbitol 0.3 M) and columns from 8 to 14 represent the seven Sides40 cultivar treatments (control, Sucrose 0.1 M, Sucrose 0.2 M, Sucrose 0.3 M, sorbitol 0.1 M, sorbitol 0.2 M, sorbitol 0.3 M). While M column represents the molecular DNA standard

conservation media. The results of treatments show increase of fructose concentration when used sucrose 0.2 M and sorbitol 0.2 M in both two cultivars, Balady and Sides 40 in the conservation media. The decrease of glucose and sucrose concentration might be involved in increase in reffinose and stachyose in large amounts [37].

Genetic diversity using RAPD analysis: The DNA of the 12 treatments and two control (Balady and Seds 40) extracted and amplified using five decamer primers to estimate the genetic similarity and variability between these treatments. All primers were successfully used as a fingerprinting tool and reproducibility was confirmed for each primer before gel documentation scanning. Figure 1 shows that the five primers could produce multiple band profiles and the number of amplified DNA bands ranged from 5 to 13 (Fig. 1 and Table 7). The data presented in Fig. 1 and Table 7 showed that each seven treatment from both cultivars have the genetic similarity, thus these treatments did not affect or mutated the genomic DNA of both cultivars. The results showed no significant differences between control bulbs and different storage treatments in the two cultivars (Balady and Seds 40). This result is in agreement with the Viterbo, *et al*. [32], when they reported that isozyme polymorphism of 13 proteins was used to assess the genetic stability of cold-stored explants. Considerable differences in zymograms were found between the fertile and the sterile varieties. However, no differences in isozyme profiles were detected between the control field-grown plants and those which were established from *in vitro* stored leaf base explants. The only exception was plantlets exposed to a high paclobutrazol concentration in

storage. The latter exhibited a doubling in the alcohol dehydrogenase profile.

In a conclusion, the protocol described in this study was effective for medium-term storage and no genetic alteration caused by all the treatments as assayed by RAPD analysis.

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