الحفظ الحيوي بالتجميد للسوسن (*Iris bostrensis, Iris aurantica*) بواسطة تقنية الكبسلة- التجفيف

Cryopreservation Storage of *Iris Bostrensis* and *Iris Aurantica* by Encapsulation-Dehydration for Long-Term.

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Abstract

Encapsulation- dehydration techniques of *Iris bostrensis* and *Iris aurantica* plants has been established for the first time in Syria. Shoot-tips were excised from *in vitro* grown plants and incubated for 3 days on solid Murashige and Skoog (HF-MS) media supplemented with 0.3 M sucrose under complete darkness at 25 ±1ºC. The precultured shoot-tips were suspended in Hormone free-Murashige and Skoog media (HF-MS) containing 3 % sodium alginate and 0.3 M sucrose for 20 minutes in which calcium was omitted from all media components. The water content of beads decreased during preculture from 78% to 23% and 17% after 4 and 6 h of dehydration, respectively. survival percentage increased rapidly from 67% (unacclimated shoot tips) to (77, 82%) after 4, 8 weeks of cold acclimation, respectively, with significant difference compared to the control (unacclimated shoot tips), and the best recovery percentage was 39% after 8 weeks with significant difference compared to the control. The Genetic stability studied by using 22 ISSR primers were used in this study. The genetic stability was high (from 95 to 99%) before and after storage in Liquid Nitrogen of *Iris bostrensis*. Therefore, storage of *Iris bostrensis, Iris aurantica* in Liquid Nitrogen was found to have no adverse effect (genetic variation) on the regeneration rates.

Key words: Cryopreservation, encapsulation-dehydration, Genetic stability, Shoot-tips, *Iris bostrensis, Iris aurantica.*

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الملخص

تم في هذا البحث وضع تقانة لحفظ سوسن بصرى والذهبي بالتجميد لأول مرة في سورية بواسطة تقنية الكبسلة- التجفيف، تم أخذ القمم النامية من النباتات المزروعة مخبريا، وزرعت في وسط الزراعة الأولية Murshige وSkoog (MS) صلب ومضافا له 0.3 مول سكروز لمدة ثلاثة أيام في الظلام وفي درجة حرارة 24±1°م، غمرت البراعم في وسط MS سائل خال من الهرمونات والكالسيوم ويحوي 3% ألجينات الصوديوم + 0.3 مول سكروز لمدة 20 دقيقة. تبين النتائج التي حصلنا عليها ان محتوى الماء في الكبسولات قد تناقص من 78% إلى 23 و17% بعد 4 و6 ساعات من التجفيف على التوالي. توضح النتائج زيادة في نسبة الحيوية من 67% لقمم الأفرع غير المؤقلمة إلى 77% بعد 4 أسابيع من الأقلمة الباردة، كما ارتفعت الى 82% بعد 8 أسابيع وبفروق معنوية بالمقارنة مع القمم غير المؤقلمة، كما تم الحصول على أفضل نسبة لإعادة النمو 39% بعد الأسبوع الثامن من عملية الأقلمة وبفروق معنوية مع الشاهد دون أقلمة.

تم دراسة الثبات الوراثي في النباتات المتجددة بعد الحفظ بالكبسلة، باستخدام تقنية الـ ISSR باستخدام 22 بادئ وقد كانت نسبة التشابه الوراثي عالية (95-99%) مما يدل عدم حدوث أي تباين وراثي في النباتات المحفوظة بالكبسلة بالمقارنة مع النباتات الأم.

الكلمات المفتاحية: الحفظ بالتجميد، الكبسلة - التجفيف، الثبات الوراثي، ، القمم النامية، السوسن الذهبي، سوسن بصرى.

Introduction

The Syrian flora has 3247 species (Mouterde, 1966). There are many endemic species, some of which belong to genus Lilium, Crocus, Tulipa and Iris. *Iris* is the largest and most complicated genus of iridaceae, which includes over 300 species. In Syria, *Iris* is considered as a wild perennial herbaceous plant that subjected to strict protection, though *Iris* grows naturally in many regions of Syria. It presents some 30 species grown in Syria (Mouterde, 1966). There are five subgenus found in the world. *Apogon, Pogonias, Xiphion, Guno* and *oncocyclus* which includes most of the Syrian species, that are considered as rare endemic plants, characterized by special, beautiful forms that have a great importance in applied studies for genetic biodiversity.

The principle of cryopreservation, is the storage of plant material at ultra low temperature (-196 ºC) that takes a place in a cryogenic condition which is liquid nitrogen, At this temperature, all forms of cellular divisions and metabolic activities of plant cell are ceased and consequently plant material can be stored unaltered for an indefinite time (Wen and Wang, 2010).

Cryopreservation is based on eradication of all freezable water from tissues using physical or osmotic dehydration, followed by ultra rapid. freezing (Reed, 2008).

Encapsulation-dehydration, developed by (Fabre and Dereuddre, 1990) in which shoot tips, somatic embryos or callus cells are encapsulated within alginate beads and subsequent culture in a medium containing elevated concentrations (0.7–1.5 M) of sucrose (Gonzalez-Arnao and Engelmann, 2006;Younes, 2012) for 1 to 3 days .The beads are then allowed to dehydrate using silica gel or by air under the laminar air flow until the moisture content drops to 20-30% (Fabre and Dereuddre, 1990), before being immersed in liquid nitrogen. Encapsulation-dehydration is practiced in a large scale because it is simple, inexpensive and provides a high level of genetic stability (Shibli, *et al.*, 2006). Also the encapsulating material keeps the tissue in a vitrified state that will reduce the hazard of ice crystal formation (Scottez *et al.*, 1992).

Inter simple sequence repeats (ISSRs) markers are very simple, fast, cost-effective, highly discriminative and reliable combines most of the advantages of SSRs and AFLP with the universality

of RAPD (Pradeep *et al.,* 2002). They are found to be more useful and reproducible than isozymes and RAPD, and less cumbersome and cost effective for routine application than RFLP (Fang *et al.*, 1997).

Beside conservation, cryopreservation aims to maintain the genetic stability of plant material. Metabolic activities are theoretically ceased ones the explant is stored cryogenically and consequently plant material is expected to be true to type after rewarming from cryopreservation (Panis *et al.*, 2002; Kaczmarczyk et *al.,* 2012). So, validity of crypreservation is only achieved if genetic stability is unchanged after exposure to liquid nitrogen (Zarghami *et al.*, 2008). As a result, only differentiated tissues such as shoot tips are targeted as plant material for cryogenic storage instead undifferentiated plant material like to avoid any genetic alteration (Harding, 2004).

Most ofthe studies detected no genetic variation among *in vitro* cryopreserved and non- cryopreserved material of many plants (Yin and Hong, 2009).

The Research Aims

The aim of this study has been to propagate and preserve endemic and wild Iris species found in Syria from deterioration by using Encapsulation- dehydration techniques, Beside conservation, cryopreservation aims to maintain the genetic stability of plant material.

Materials and Methods

The work was conducted at the National Commission for Biotechnology, Damascus, Syria and Faculty of Agriculture, Damascus University, Syria during the years 2015-2017.

Plant Materials

The plants of *Iris* species used in this study were collected from the wild in Syria Fig (1). This species are perennial and herbaceous which belong to Iridaceae family.

Iris aurantiaca Dinsm : It is an endemic plant and can be found in Djebel Druze, Tell Qouleib, Kafer, Tell Jaffna, Mayamas, Sahwet-el-Khodr (Mouterde, 1966).

Iris bostrensis Mout: It is distributed in Hauran and Djebel Druze (Mouterde, 1966).

Fig 1. The plants of *Iris* species collected from the wild in Syria

In Vitro Culture of Plant Material:

Shoot tips of rhizomes (after surface disinfection by chlorox 3% for 30 minutes) were cultured on solidified MS (Murashige and Skooge, 1962) medium containing 30 g/l sucrose supplied with 2 mg/l BAP, and 0.2 mg/l IBA for the two species in *Iris bostrensis* and *Iris aurantica*.

Sub culturing was performed every four weeks using a fresh growth medium to establish sufficient STs stock for experimentations. All cultures were incubated at 25±1 °C under a 16/8 (light/dark).

Cryopreservation

Cold acclimation of *in vitro* shoots was performed at 1℃ for (0,2,4,8,10 weeks).

For cryopreservation, shoot tips were precultured on a hormone-free solid MS medium containing 0.3 M sucrose for 3 days under complete darkness at 25 ±1ºC. Precultured ST were then suspended in a solution containing 3% sodium alginate in calcium-free and hormone-free in a modified liquid MS minerals and vitamins solution (in which calcium were omitted) supplemented with 0.1 M sucrose. Shoot tips were picked up individually using 1 ml wide mouth sterile pipette with some alginate solution and then were dropped into a liquid MS medium containing 100 mM calcium chloride and 0.1 M sucrose to form beads of about 4 mm in diameter. The beads were kept in the calcium chloride solution for 30 min for polymerization.

Beads, each containing one shoot tip, were transferred to a hormone-free liquid dehydration MS media with various concentrations (0.5, 0.75 and 1.0 M) of sucrose; then were incubated on a rotary shaker (100 rpm) at 25ºC for 3 days. The dehydration solution were then removed by sterile pipette and beads were subjected to air dehydration on sterilized filter paper in uncovered 9ºcm Petri dishes in a laminar airflow cabinet for 0, 2, 4 or 6 h at room temperature(25 ºC). Half of the beads were then transferred to a recovery medium for rehydration and growth while the other half of beads were placed in 2-ml sterile cryovials and directly were immersed into liquid nitrogen.

Two cooling procedures were used rapid cooling by direct immersion in liquid nitrogen and two –step freezing by progressive cooling (from -20 to -80 c before immersion in liquid nitrogen.

After cryopreservation, cryogenic vials containing beads were thawed in a water bath at 38 ºC for 2–3 min. Encapsulated non-cryopreserved (-liquid nitrogen) and encapsulated cryopreserved (+liquid nitrogen) beads were inoculated onto a solid MS recovery media containing 1.0 mg/l isopentenyladenin (2iP) and 0.1 M sucrose, then kept in the dark for 3 days. Survival rates were tested for a half of non–frozen and frozen encapsulated shoot tips using 2,3,5-triphenyl tetrazolium chloride salt solution (TTC) test (Lutts *et al*., 1996). The other half of the shoot-tips (STs) were transferred to the normal growth conditions as described previously and kept for further recovery. After 4 weeks, shoot tips (STs) Were checked under a binocular microscope for any recovery signs.

For Shoot-tips viability testing: (TTC) test Were performed. Ten shoot-tips were incubated in cryotube along with 2 ml of 0.5 % (w/v) TTC salt dissolved in 50 mM K2HPO4 at pH 7.0 for 16 h at 24 ± 1 ºC incomplete darkness. The shoot tips were then examined under a binocular microscope to obtain the survival percentages of each treatment. The viable shoot tips appeared in red, color which resulted from the reduction of tetrazolium salt to formazan by the action of hydrogen ions that released from the viable cells respiration. The following formula were applied to measure the survival percentages of the treated shoots:

Survival percentage = (∑red shoot – tips) / (∑ number of shoot – tips) ×100.

For moisture content (MC) determination of the beads during dehydration, beads devoid of shoot tips were transferred to a hormone-free liquid dehydration MS medium with various concentrations (0.5, 0.75, 1.0 M) of sucrose then incubated on a rotary shaker (100 rpm) at 24ºC for 3 days.

After each dehydration period, beads were weighed, dried in an oven at 90ºC for 16 h and then reweighed (Subaih *et al*., 2007). The moisture content was determined using the following formula:

MC % = [(Fresh weight - Dry weight) /Fresh weight]×100.

Stability analysis :

DNA extraction:

Total genomic DNA was isolated from fresh young leaves (4 months) before and after cryopreservation with (CTAB) (Murray and Thompson,1980), DNA concentration and purity was monitored spectrophotometrically.

DNA amplification and visualization by ISSR analysis:

Plantlets were evaluated using the ISSR (inter simple sequence short repeats) analysis, and primers of arbitrary sequence 22 for ISSR, (Table 1) were used for PCR.

Table 1. the primers used in ISSR analysis.

Primers	Primer sequence 5′ → 3′	Ta (ºC)
$ISSR_1$	(AG)8 T	50
$ISSR_2$	(GA)8 C	52
$ISSR_3$	(CA)8 T	50
$ISSR_4$	(AC)8 G	52
$ISSR_5$	(AC)8 T	50
$ISSR_6$	(GA)8 CG	56
$ISSR_7$	(TC)8 GA	54
$ISSR_8$	(TC)8 AG	54
$ISSR_9$	(AC)8 GG	56
$ISSR_{10}$	CCAG (GT)7	56
$ISSR_{11}$	(GT)4 (GA)5	54
$ISSR_{12}$	(AC)7 (AT)3	54
$ISSR_{13}$	KVR (TG)6	50
$ISSR_{14}$	C(CT)4 (GT)4 G	56
$ISSR_{15}$	(TG)8AA	52
$ISSR_{18}$	(AC)8T	50
$ISSR_{34}$	(CT)8 G	50
$ISSR_{40}$	(AC)8 TT	50
$ISSR_{41}$	(AC)8 CG	50
ISSR230/17	(CT)8 G	52
ISSR230/44	(CA)6AC	50
ISSR230/46	(TG) 8 G	50
K: G/T, V: G/C/A, R: G/A		

Thermo cycling profile

The amplification profile consisted of initial denaturation of the template DNA at 95ºC for 5 min, followed by 35 cycles of 1 min denaturation at 94°C, 1 min annealing at (Ta) according to the primer Table (1), and 1 min extension at 72°C, and finally followed by 10 min of additional extension at 72°C.

Gel electrophoresis and visualization of DNA bands

The amplification products were visualized in an ultraviolet trans illuminator, after horizontal electrophoresis in 2.5% agarose gel (SIGMA company), and photographed by gel documentation system (Alpha Innotech).

Experimental Design

Treatments in each experiment were arranged in a complete randomized design (CRD) where 30 shoot tips were used in each treatment. Each treatment was replicated three times with ten shoot tips per replicate. The collected data were statistically analyzed using XLSTAT. Means were separated according to the least significant difference (LSD) at 0.01 probability level.

ISSR bands were scored as present (1) or absent (0) for each treatment. Each band was assumed to represent a unique genetic locus. The molecular results were analyzed using the Phoretix 1D Pro software from nonlinear Dynamics by using Total Lab program.

Results and Discussion

Effect of Dehydration and sucrose concentration:

The water content of beads decreased from 78% to 23% and 17% after 4 and 6 h of dehydration, respectively. The highest survival percentage (100 %) was obtained when using non-cryopreserved shoot tips after (0, 2 h) dehydration, while the value was decreased to (87.6, 80%) after (4, 6 hour) dehydration Fig (2). Similar findings were obtained by (Shibli, 2000) who obtained the full survival percentage (100%) of encapsulated none-cryopreserved *Iris bostrensis* somatic embryos after 0 dehydration. Survival was decreased (62.67%) with increasing the dehydration period to 6 h dehydration in media provided by (0.5 M, 1M sucrose).

Survival of cryopreserved encapsulated shoot tips was (0 %) in control without sucrose concentration and dehydration, This agrees with (Shibli *et al*., 2006, 2009), (Sharaf, 2010) when they reported that moisture content of the encapsulated shoot-tips was reduced as well as the sugar concentration increased and reduction in moisture content (MC) is essential for successful cryopreservation (Uragami *et al*., 1990), and other studies have been noted that dehydration under laminar air flow cabinet was very effective factor to increase survival percentage and increase the survival (Tessereau *et al*., 1994). Results in Fig (2, b) showed, that survival of encapsulated shoot-tips increased from 43% to 67% as the sucrose concentration increased from 0 to 0.75 M with 4 hours dehydration, with significant difference between them, and then decreased to 6% when the sugar concentration increased to 1M, Similar results were obtained by (Shibli, 2000) in encapsulated somatic embryos when it has been reported that the highest survival was obtained when preculturing on media supplemented with 0.75 M sucrose was used.

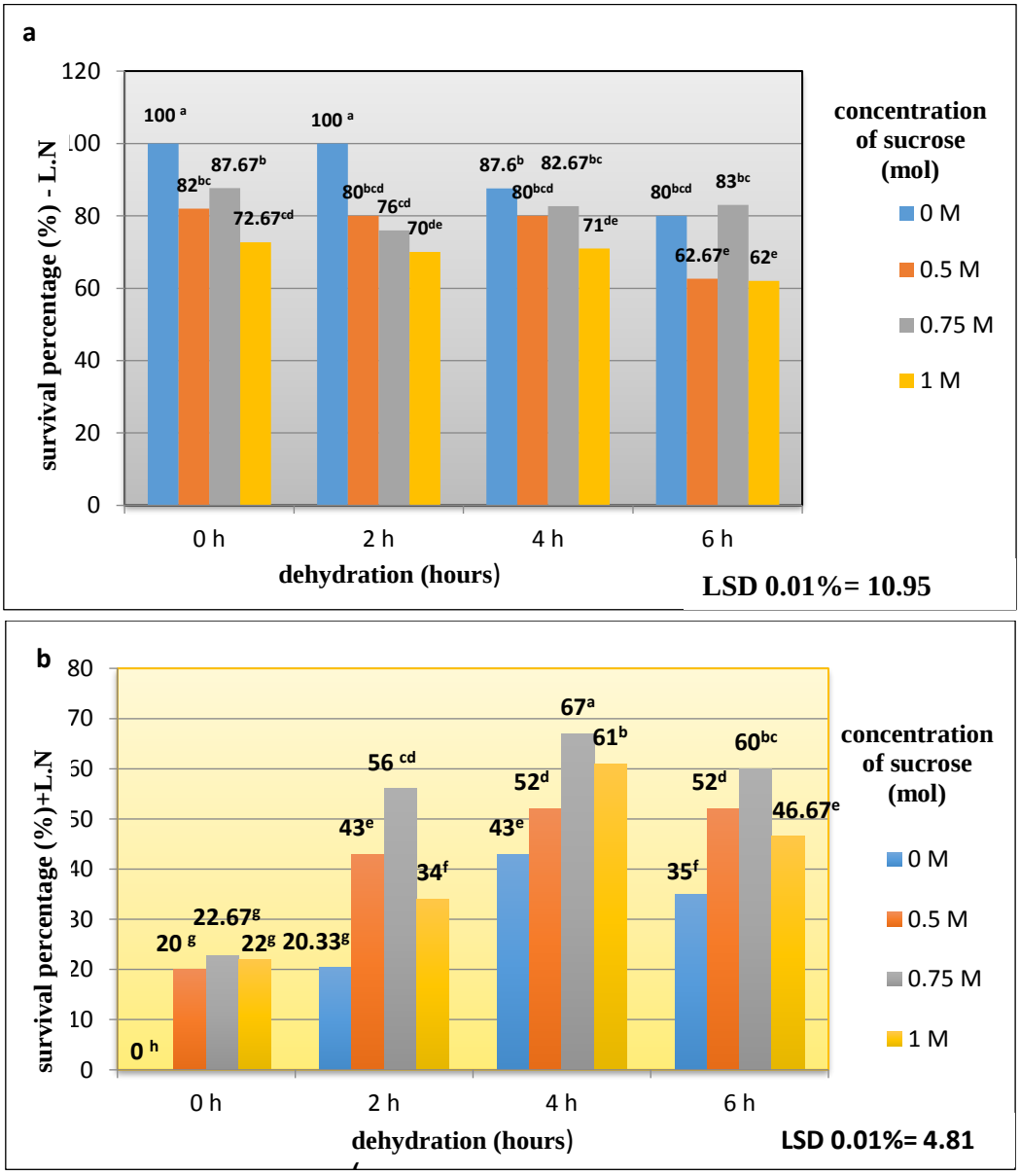

Fig 2. Effect of Dehydration and sucrose concentration of survival (a: non cryopreserved plants, b: cryopreserved plants).

High concentration of sucrose inside plant cells was beneficial in establishing a vitrification state during freezing; so preventing the damage caused by dehydration and freezing (Wang *et al*., 2002). For other species like grape (Plessis *et a*l., 1991) and solanum (Fabre and Dereuddre, 1990), Preculture with a high concentration of sucrose did not appear to be sufficient to induce this tolerance.

The protective effect of sucrose might be due to osmotic dehydration, this might be attributed to osmotic stress resulting from increased sucrose concentration, to decrease the water content of the cells and to increase their dry weight (Shibli *et al*., 2006, 2009; Sharaf, 2010; Rabba'a *et al*., 2012).

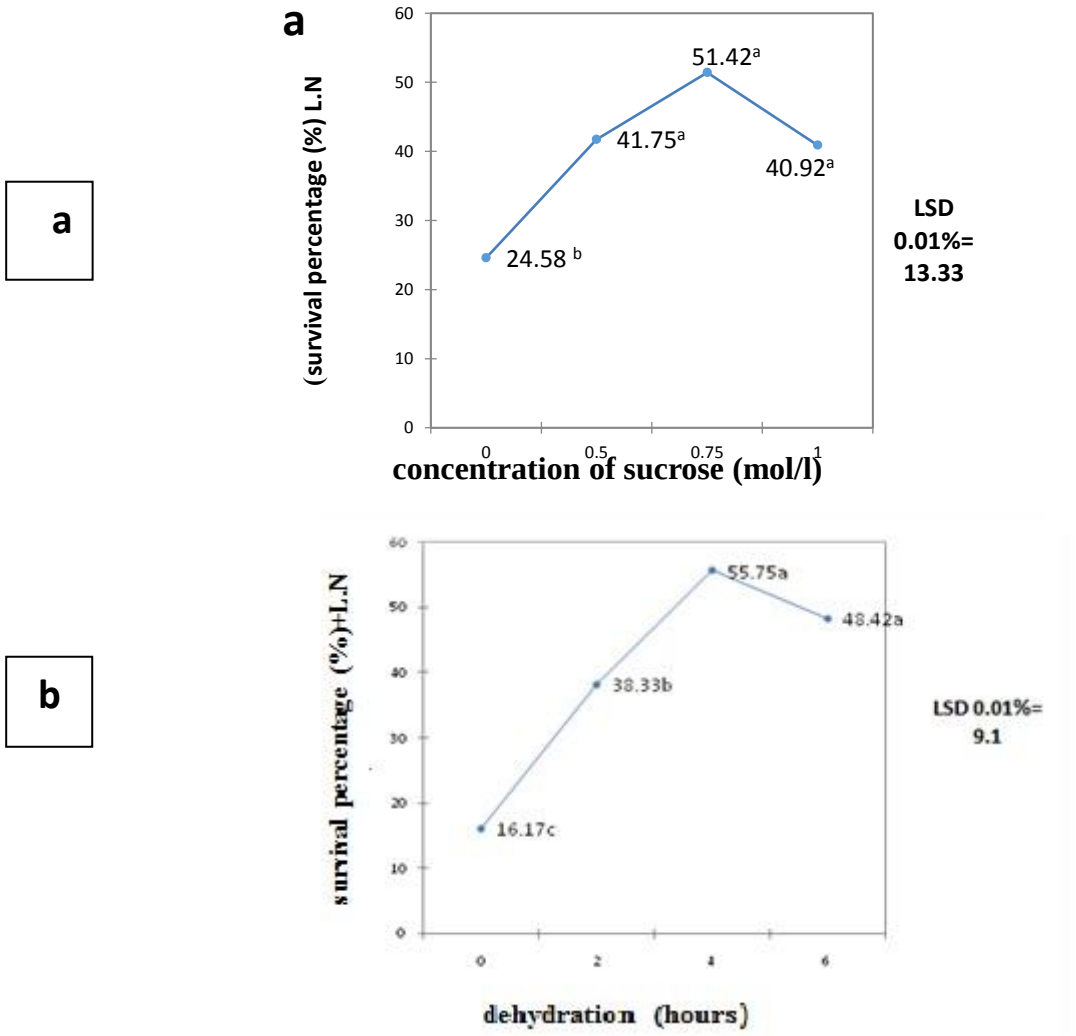

Fig 3. Effect of Dehydration and sucrose concentration of cryopreserved plants survival (+ L.N), a: concentration of sucrose(mol/l), b: dehydration (hours)

Al-Ababneh (2002) mentioned that survival encapsulated non-cryopreserved sour orange shoot tips were decreased with increasing sucrose concentration and dehydration duration.

Also, results of the current study reported fig (3) that the best survival encapsulated cryopreserved shoot tips was 55.75% after 4 h with significant difference compared to the control, then decreased to 48.42% after 6 h without significant difference compared with 4 h Similar result was reported by (shibli, *et al*., 2009) in crocus and (Al-Ababneh *et al.*, 2002) in *citrus aurantium* L.

The highest survival percentage in cryopreserved shoot-tips (67%) fig (2) was obtained when using 0.75 M sucrose and 4 hours dehydration with significant difference compared to other treatments, this

agrees with (Scottez *et al.,* 1992) where it has been obtained the highest survival 80 % when using 0.75 M sucrose and 4 hours dehydration.

Effect of cold acclimation of *in vitro* shoots on survival and shoot recovery

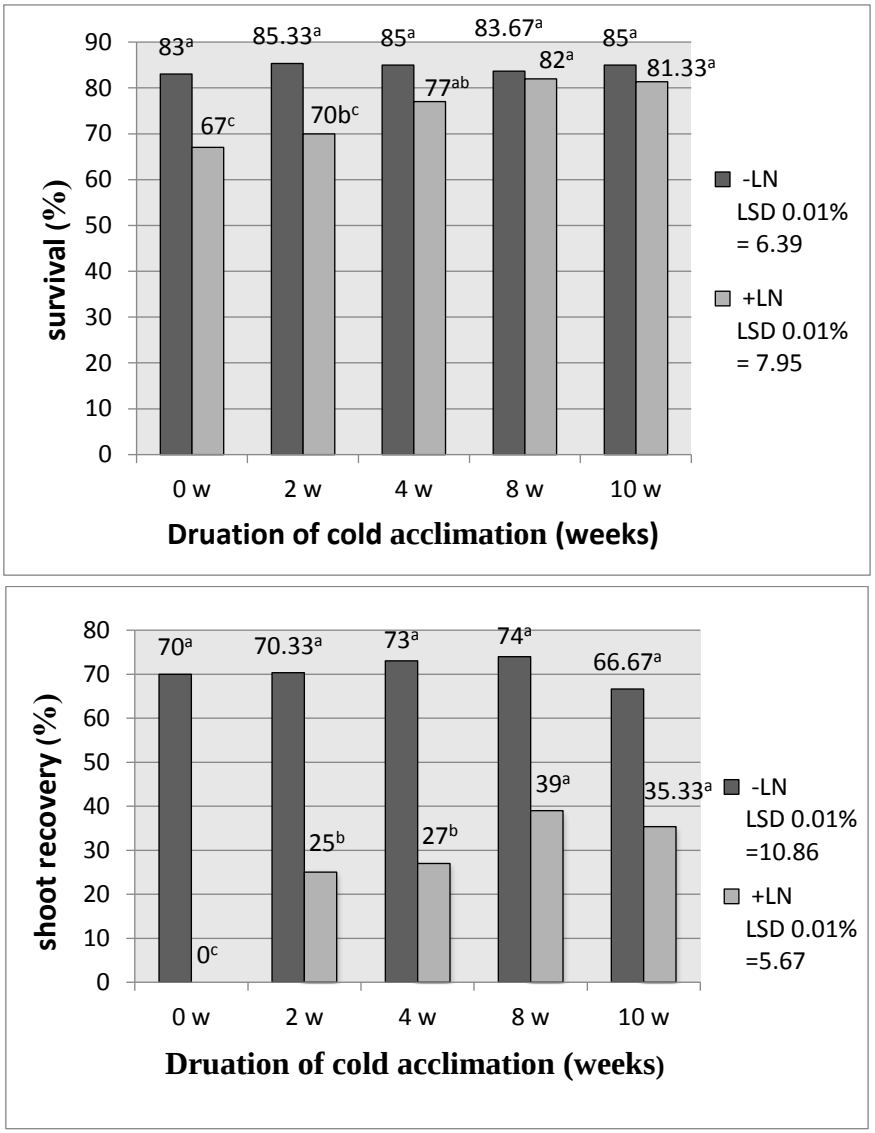

Fig 4. Effect of cold acclimation of *in vitro* shoots at 1°C on percentage of survival and shoot recovery (-L.N:cryopreserved plants , +L.N: non cryopreserved plants).

Cold treatment had no effect on survival and percentage shoot recovery from control shoot tips (Fig 4), Similar findings were obtained by (Scottez *et al*.,1992). After dehydration, shoot survival increased rapidly from 67% (unacclimated shoot tips) to (77% , 82%) after 4 and 8 weeks of cold acclimation, respectively to significant difference compared to control (unacclimated shoot tips), and shoot recovery increased from 0% (control) to 25, 27% after 2, 4 weeks of cold acclimation, respectively, and the best recovery percentage was 39% after 8 weeks to significant difference compared to control, then decreased to 35.33 after 10 weeks without significant difference compared to 8 weeks, the findings here agreed to those obtained by (Scottez *et al*.,1992) which noted that shoot recovery increased rapidly from 2% (unacclimated) to 44% , reaching to 82% by 12 weeks, and then decreased slightly during the

following weeks. Cold acclimation seemed essential for good survival and shoot recovery from encapsulated shoot tips. One week of cold treatment generally enhanced significantly the post liquid nitrogen survival rates of cryopreserved shoot tips excised from *in vitro* cultures of different species of *Rubus* (Dereuddre *et al*., 1990). Further improvement of survival can be obtained by increasing the duration of the treatment at low temperature to several months.

Effect of cooling rate:

In these experiments, there is no significant difference on survival between control shoot tips compared to rapid cooling shoot tips in liquid nitrogen, and shoot survival decreased rapidly from 82% (rapid cooling shoot tips) to (41.67) in two step cooling shoot tips with significant difference. The high recovery rates of shoot tips were obtained (39%) after direct immersion in liquid nitrogen with significant difference after two step cooling (23.33%) in Fig (5).

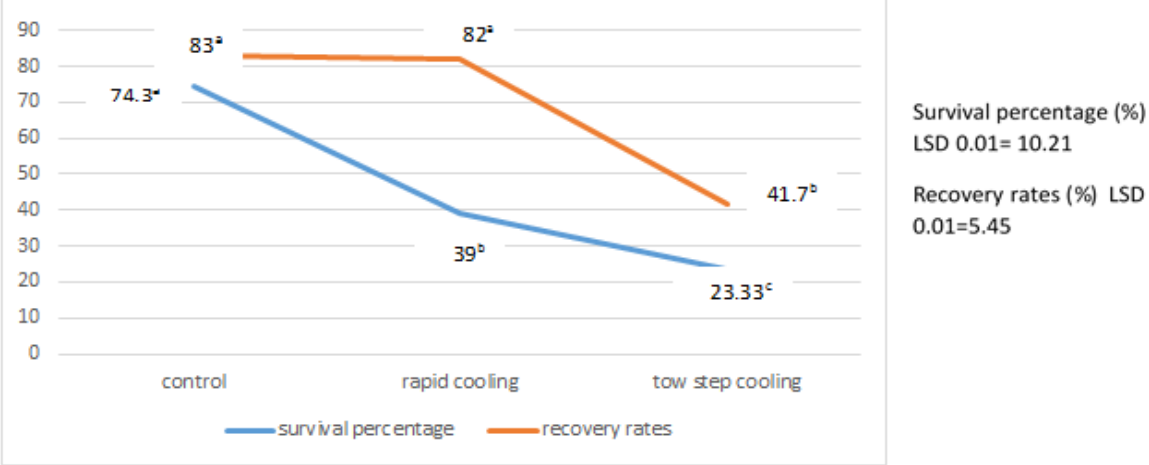

Fig 6. Effect of cooling rate from cryopreserved plants on survival and shoot recovery.

This property may be due to the ability of encapsulating material and plant organs to vitrify during cooling and to remain rewarming (Dereuddre *et al*., 1991).

The finding here disagreed with those obtained by (Gonzalez-Arnao *et al*., 1998) who recorded that the slow freezing achieved better results than rapid freezing, and that fortifier freeze-induced dehydration during slow prefreezing was necessary to achieve optimal survival. Similar results were noted with encapsulated grape apices for which slow freezing was necessary to achieve optimal survival (Paulet *et al*, 1993), nevertheless, the resistance of encapsulated and dehydrated shoot tips to liquid nitrogen did not depend on cooling rate (Scottez *et al.*, 1992). The reduction in regrowth compared to survival percentage Fig (5) may be attributed to partial damage of the calli due to osmotic shock after rehydration and ice crystallization of some cells in the calli (Al-Ababneh *et al*., 2003); or maybe due to unfavorable growth condition (Al-Ababneh, 2002; Moges *et al*., 2004).

Genetic stability:

Amplification patterns of cryopreserved shoot tips were compared to the non- cryopreserved shoot tips (control) from *in vitro* plantlets of *Iris bostrensis* and *Iris aurantica.* Table (2) showed that 13 primers produced multiple band profiles. A total of 59 amplification DNA bands, with an average frequency of

4.53 bands per primer, were produced in the cryopreserved shoot tips and non-cryopreserved shoot tips. The total number of scrabble markers produced per individual primer ranged between 1 in (ISSR5-ISSR6) and 9 in (ISSR-9), as shown in Table (2).

57 bands were monomorphic with an average of 4.38 bands/primer, while only 2 bands were polymorphic. The monomorphism percentages ranged between 100% in primers (ISSR2, ISSR5, ISSR6, ISSR9, ISSR10, ISSR11, ISSR12, ISSR15, ISSR18, ISSR40, $ISSR_{230/46}$) and 66.7% in primers ($ISSR_{230/44}$), with an average of 96.6 %. On the other hand, nine primers out of the twenty two used primers did not show any specific markers Table (2).

The lowest monomorphism percentages (66.7%) in primer ISSR230 44, then (83.3%) in primer ISSR4.

Fig (6) shows ISSR profiles as detected by primers ISSR18.

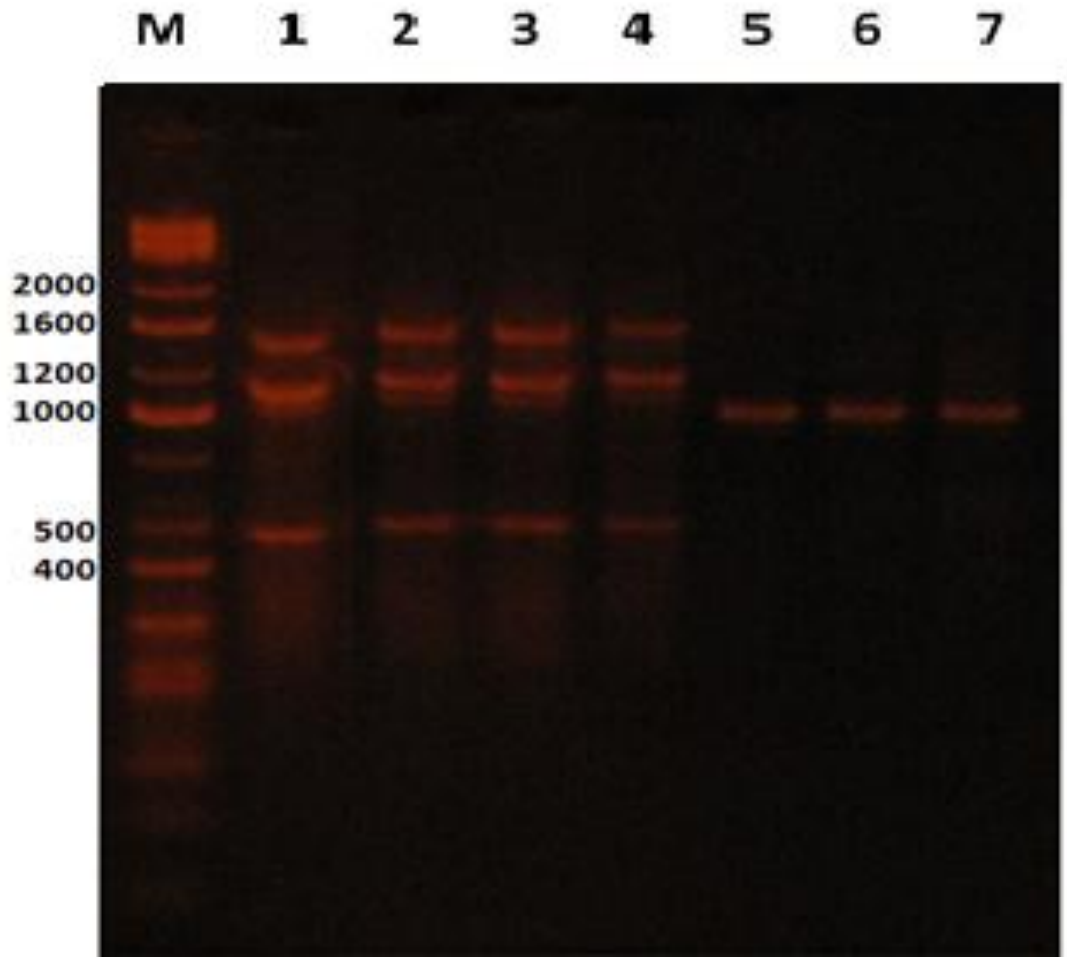

Fig 6. ISSR profiles as detected by primers ISSR18 (M = marker, 1: non cryopreservation *Iris bostrensis*, 2: cryopreservation *Iris bostrensis*, 3: cryopreservation *Iris bostrensis*, 4: cryopreservation *Iris bostrensis*, 5: non cryopreserved *Iris aurantica*, 6: cryopreservation *Iris. aurantica*, 7: cryopreservation *Iris aurantica*

Table 2. Statistics of the ISSR fragments for *Iris* based on the twenty-two ISSR primers.

ISSR primers	No. of amplification products	Monomorphic bands	Monomorphism %
$ISSR_1$	-	-	-
$ISSR_2$	7	7	100
$ISSR_3$	-	-	-
$ISSR_4$	6	5	83.3
$ISSR_5$	1	1	100
$ISSR_6$	1	1	100
$ISSR_7$	-	-	-
$ISSR_8$	-	-	-
$ISSR_9$	9	9	100
$ISSR_{10}$	6	6	100
$ISSR_{11}$	4	4	100
$ISSR_{12}$	4	4	100
$ISSR_{13}$	-	-	-
$ISSR_{14}$	-	-	-
$ISSR_{15}$	4	4	100
$ISSR_{18}$	5	5	100
$ISSR_{34}$	-	-	-
$ISSR_{40}$	5	5	100
$ISSR_{41}$	-	-	-
$ISSR_{230/17}$	-	-	-
$ISSR_{230/44}$	3	2	66.7
$ISSR_{230/46}$	4	4	100
Total	**59**	**57**	**-**
Average	**4.53**	**4.38**	**96.6**

The genetic stability of *in vitro* cryopreservated and non- cryopreserved plants based on ISSR analysis.

The genetic similarity coefficient of the variation plants with treatments was between 98% and 100% in *Iris bostrensis* Table (3) and 97-100% in *Iris aurantica* table (4). from Table (3) The genetic similarity between non-cryopreserved plants (control) and cryopreserved plants was 0.99 between treatments (2,3) (2,4) (3,4) and 0.98 between treatments (1,2) (1,3) (1,4) table (3) and 0.97 between treatments (1,3) (2,3), and 0.98 between (control, 2). Therefore, storage *Iris bostrensis, Iris aurantica* in liquid nitrogen was found to have no adverse effect (genetic variation) on the regeneration rates.

Table 3. Genetic Similarity coefficient matrix of *in vitro Iris bostrensis* between control and cryopreserved plants.

treatment	1: control (non-cryopreserved *Iris bostrensis)*	2:cryopreserved *Iris bostrensis*	3:cryopreserved *Iris bostrensis*	4:cryopreserved *Iris bostrensis*
1: control (non-cryopreserved *Iris bostrensis*	**1.00**			
2: cryopreserved *Iris bostrensis*	**0.98**	**1.00**		
3: cryopreserved *Iris bostrensis*	**0.98**	**0.99**	**1.00**	
4: cryopreserved *Iris bostrensis*	**0.98**	**0.99**	**0.99**	**1.00**

Table 4. Genetic similarity coefficient matrix of in vitro *Iris aurantica* between control and cryopreserved plants.

Treatment	1: control (non-cryopreserved *Iris aurantica)*	2: cryopreserved *Iris aurantica*	3: cryopreserved *Iris aurantica*
1: control (non-cryopreserved *Iris aurantica)*	**1.00**		
2: cryopreserved *Iris aurantica*	**0.98**	**1.00**	
3: cryopreserved *Iris aurantica*	**0.97**	**0.97**	**1.00**

Sopalun *et al* (2010) found no significant differences between control plant and shoot tip explants cryopreserved in *Grammatophyllum speciosum* using ISSR analysis. Also, other studies detected no genetic variation among *in vitro* cryopreserved and non- cryopreserved material of *Vanda coenulea*, *Dendrobium candida* and *Rhododendron* (Van Huylenbroeck and calsyn, 2009; Yin and Hong, 2009). On the contrary, there is some articles found that retrieved from *in vitro* cryopreserved and compared to original clones, resulted in showing remarkable DNA differences. Four accessions were found different from the original clones by morphological and molecular analyses; and one accession was found different at the molecular level (Martin and Gonzalez-Benito, 2006, 2009).

conclusions

The highest survival percentage (67%) was obtained when using 0.75 M sucrose and 4 hours dehydration with significant difference compared to all treatments, and the Survival percentage increased rapidly from 67% (unacclimated shoot tips) to (77, 82%) after 4, 8 weeks of cold acclimation, respectively, with significant difference compared to control (unacclimated shoot tips).

The best recovery percentage was 39% after 8 weeks with significant difference compared to control, and the genetic stability was high (from 97 to 99%) before and after storage in liquid nitrogen of *Iris aurantica* and *Iris bostrensis.* Therefore, storage of them in ABA and LN was found to have no adverse effect (genetic variation) on the regeneration rates.

Suggestions

- cryopreservation of another wild Iris species in Syria as genetic resources of degradation and loss through tissue culture and plant germplasm conservation.
- use another Method of *in vitro* cryopreservation for germplasm as vitrification and Encapsulation-Vitrification,

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