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Cryopreservation of *Capparis spinosa* Shoot Tips via Vitrification, Encapsulation Dehydration and Encapsulation Vitrification

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Abstract: *In vitro* shoot tips of *Capparis spiunosa* were cryopreserved using the vitrification, encapsulation/ dehydration and encapsulation vitrification techniques. In the vitrification procedure, a maximum of 70% regrwoth was obtained when shoot tips were treated with the Plant vitrification solution (PVS2) for 60 min at 0°C and plunged directly into liquid nitrogen. In encapsulation dehydration, shoot tips from 4 weeks cold hardend plants were encapsulated in calcium-alginate before preculture on MS medium supplemented with 0.75 M sucrose for 1 day, desiccated to 21% moisture content and immersed in liquid nitrogen. Maximum regrwoth using this technique was up to 83%. In encapsulation vitrivifaction, maximum regrwoth rate of 30% was obtained, when encapsulated shoot tips were exposed for 60 min in PVS2 at 0°C. Finally, no callus formation and fastest shoot elongation were obtained when cryopreserved shoot tips were post cultured on MS media free of hormone. The methods studied her are promising techniques for *in vitro* cryogenic storage of *C. spinosa* shoot tips.

Key words: *Capparis spinosa* · Cryopreservation · Encapsulation-dehydration · Encapsulation vitrification · Vitrification

INTRODUCTION

Caper (*Capparis spinosa* L.) belongs to the Capparidaceae family native to the Mediterranean region. *C. spinosa* is a perennial crop one of the most common aromatic plants that grow along the roadside, on the slopes, rocky and stony area and generally well adapted to dry areas basin [1, 2, 3]. The plant is not cultivated and grows wild and has been known for centuries in traditional phytomedicine, which exploited its properties for several purposes [1, 3, 4, 5]. Propagation of capper through seed is not preferable, because germination rates of this species are very low due to seed dormancy as seed coat contains inhibitors. Moreover, cross pollination behavior of capper plant causes high degree of heterozygosity in the seed [4, 5].

Capper plant has not been subjugated to its full potential either by the scientists or by the local farmers. Many of them are endangered due to many causes, such as the environmental changes, deforestation, wild orchid trade and other factors. Preservation of *C. spinosa* in the field requires land and constant maintenance and is costly. Moreover, the susceptibility of plants to insects, disease and environmental stress is a major constraint on the conservation of these genetic resources [6-10]. Therefore, there is an urgent need for long term conservation of capper plants. Tissue culture has the advantage over other propagation method in that it provides clonal material for rapid propagation [5, 11-17].

Cryopreservation is an important tool for long-term storage of biological materials. It offers a safe and cost-effective option for long-term conservation of genetic resources in many plant species [9, 10, 12, 14, 16, 17, 18]. At the temperature of liquid nitrogen (LN, -196°C), all the metabolic activities of cells are at a standstill. Thus, they can be preserved in such a state for a long period [8, 11, 16, 17]. Many new cryopreservation techniques such as vitrification, encapsulation/ dehydration and encapsulation/vitrification have been reported form successful use for tissues and organs of plant species. Cryopreservation provides greater security for the collection, require small storage space, has low maintenance and only a small number of replicates are needed to conserve an accession [6, 10, 19-22].

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Vitrification refers to the physical process by which a concentrated aqueous solution solidifies into metastable glass in the tissue, at sufficiently low temperatures, without the occurrences of ice crystallization [6, 10, 15, 22]. Successful vitrification requires careful control of highly concentrated vitrification solution, as it prevents injury associated with toxicity or excess osmotic stress during dehydration.

Encapsulation dehydration, involves encapsulation of explant in alginate beads, followed by pregrwoth treatment in a medium containing a level of sucrose, then dehydrated under laminar air flow over silica gel, followed by rapid cooling in liquid nitrogen [6, 10. 22, 23, 24, 25]. This procedure provides much greater flexibility and ease of handling a large sample of shoot. In the encapsulation-vitrification technique, the explants are encapsulated in alginate beads, loaded and dehydrated with a vitrification solution before rapid immersion in liquid nitrogen [6, 10, 24, 26, 27, 28]. To our knowledge there has been no report on long term germplasm conservation of *C. spinosa*. However, for successful cryopreservation, many factors are involved, such as starting materials, pretreatment conditions, cryopreservation procedures and post-thaw treatment. Therefore the goal of this investigation were to study the potential of long-term conservation (cryopreservation) by the application of vitrification, encapsulation dehydration and encapsulation vitrification method to define a reliable method for germplasm conservation.

MATERIALS AMD METHODS

Plant Materials and Culture Conditions: Shoots of *C. spinosa* (*local cultivar*) were obtained from the field of Al-Balqa' Applied University, Al-Salt-Jordan. Healthy shoot tips were selected and trimmed to approximately 15 mm in length. The leaflets were removed prior to sterilization. Shoot tips were sterilized in 70% alcohol for 30 seconds and placed in 1% NaOCl for 30 minutes, two drops of Tween 20 were added to decrease surface tension. Disinfestations with NaOCl took place under vacuum to reduce trapped air around the explants, thus increasing surface contact with plant tissue. After sterilization, each explant was rinsed 3 times in sterile deionized water and cut to a single bud under sterile conditions in a laminar air flow cabinet.

Explants were initially cultured on Murashige and Skoog medium (1962) [29] supplemented with 0.5 mM myo-inositol, 0.34 mM thiamine HCl, 2.4 mM pyridoxine HCl, 4.1 mM nicotinic acid and 3% sucrose. The pH was adjusted to 5.8 and 7 g/L agar agar (Sharlau, Germany) was added prior to autoclaving. 60 mL of medium were dispensed into each of (250 Duran flasks). The breather hole of each flask was then plugged with cotton wool to facilitate gas exchange and the media were autoclaved for 20 minutes at 121°C. Five explants were incubated at 24 ± 2°C with a 16 hour photoperiod and irradiance of (50 µmol $m^{-2}s^{-1}$) supplied by cool white florescent lamps. Shoots produced from these explants were subcultured every six weeks onto MS medium supplemented with 1.0 mg/L (6-benzylaminopurine) BAP and 0.1 mg/L (indole acetic acid) IAA to initiate enough plant material.

Shoot Tips Excisions: Shoot tips (2-3 mm) were excised under a binocular microscope from healthy *in vitro* shoots. The meristematic dome appeared as a glossy spot, surrounded by two or three leaf primordia. Excised shoot tips were precultured on solid MS medium supplemented with 0.4 M sorbitol and 0.1 M sucrose for 1 d in the dark

Cryogenic Storage by Vitrification Methods: Precultured shoot tips were placed in cryotubes (10 tips per replicate and loaded with 1.0 mL of the loading solution with a mixture of 2 M glycerol and 0.6 M sucrose for 20 min at 0°C. To check the effect of loading solution on the growth of the shoot tips, subsequently shoot tips were cultured on MS medium with 3% sucrose and incubated in the dark for one week and then transferred to the growth chamber at 24 ± 2°C. The loading solution was then removed and replaced with iced PVS2 or MPVS2, (PVS2 contained 30% (w/v) glycerol, 15% (w/v) DMSO, 15% ethylene glycol and 14% (w/v) sucrose dissolved in full strength MS medium; while MPVS2 which contained 20% (w/v) glycerol, 20% (w/v) DMSO, 20% ethylene glycol and 20% (w/v) sucrose dissolved in full strength MS medium). Shoot tips were exposed to the vitrification solution for varying times (0, 20, 40, 60, 80, 100, 120, 150, or 180 min), at 0°C. The vitrification solution was replaced once at the end of the exposure time and then the tips were transferred to 2 mL cryotubes and suspended in 0.5 mL of fresh PVS2 prior to immersion in liquid nitrogen (LN). Cryotubes containing the shoot tips were plunged directly in LN and maintained at -196°C for a period of 1 h to 1 d. After storage in LN, cryotubes were rewarmed rapidly in a water bath at 45ºC for 2 min. Subsequently, the vitrification solution was drained from the cryotubes and replaced with MS liquid medium supplemented with 14% (w/v) sucrose, in which the tips were washed for 15-20 min. Then shoot tips were transferred to solid hormone- free MS medium supplemented with 3% (w/v)

sucrose. Cultures were then incubated in the dark for one week and then transferred to the growth room conditions. Regrowth was defined as the percentage of shoot tips resuming growth six weeks after plating out. Recovery of shoot tips was observed at weekly intervals. The young plantlets were subcultured on the optimal multiplication medium defined. Surviving plantlets were then subcultured into a hormone-free MS medium every 3 weeks. Approximately ten shoots were tested and each experiment was repeated five times.

Cryogenic Storage Using Encapsulation Dehydration: Three-week-old shoot cultures were exposed to 4°C for 4 or 8 weeks with a ten hour photoperiod (15 μmol m^{-2} S^{-1}). Shoot tips were dissected from cold-hardened shoots and precultured on MS medium supplemented with 0.1 M sucrose and 7g/L agar agar (Difco Bitek) for 1 day under dark condition, at 24 ± 2°C. Then shoot tips of *C. spinosa* were suspended in a calcium-free liquid MS medium with 3% (w/v) Na-alginate (medium viscosity). Tips were pipetted with some alginate solution, which was dispensed as drops into a liquid MS medium containing 0.1 M calcium chloride, where the alginate formed solid beads through polymerization with calcium. After encapsulation, excess calcium chloride solution was poured off and the beads were then left for 30 min to polymerise. The encapsulated tips were then precultured in a liquid MS medium supplemented with 0.75 M sucrose for one day before dehydration in the dark. Beads were dehydrated under the air current of a laminar airflow cabinet, in 9 cm Petri dishes. Bead moisture content was measured for sucrose and sorbitol after 0, 2, 4, 5, 6, 7, 8 and 9 h desiccation. The oven dry weights of four extra samples were determined at 80°C over the same periods of desiccation. Bead moisture content (MC %) was determined on a fresh weight basis:

Percentage bead moisture content = {(Fresh weight-dry weight)/Fresh weight} X 100%

For each dehydration period, ten shoot tips were placed in 2 mL cryo-tubes and rapidly immersed in liquid nitrogen (LN) for a period from 1 to 24 h. Meanwhile, control (non immersed samples) were transferred to MS hormone-free medium and placed in the growth chamber. Encapsulated shoot tips were rapidly thawed from LN in a water bath at 45°C for 1 min. The encapsulated shoot tips were then directly transferred to hormone-free MS medium supplemented with 30 g/L sucrose and 7g/L agar agar and were incubated for one week with a 16- h photoperiod (50 μmol m^{-2} S^{-1}) at 24 ± 2°C to recover from freezing. Shoot tips showing any signs of survival after six weeks were extracted from the beads and the necrotic basal part was removed as necessary. The tips were allocated directly on hormone-free MS medium for further regrowth and cultured with a 16- h photoperiod (50 μmol m^{-2} S^{-1}) at 24 ± 2°C. Survival was recorded as the percentage of shoot tips that produced at least one new shoot six weeks after thawing. Shoots were then transferred to MS excluding plant growth regulators, for maintenance as stock cultures.

Cryogenic Storage by Encapsulation-Vitrification: Shoot tips (3 mm long) were excised from *in vitro* grown plants under sterile conditions and incubated for 3 d on solid MS medium hormone free supplemented with 0.3 M sucrose and incubated under darkness at 24 ± 1°C. Shoot tips were then encapsulated on alginate according to the method described above; before dehydrating them with plant vitrification solution 2 (PVS2) or MPVS2 as mention above. Encapsulated shoot tips were then transferred to sterile cryovials and loaded for 20 min with the loading solution (0.6 M sucrose and 2 M glycerol in full strength liquid MS media). After that the loading solution was removed and replaced with PVS2 for 20 min at 0°C. Half of the treated cryovials along with the encapsulated shoot tips and PVS2 were plunged directly in LN for at least 1 hr, whereas the other half was left without LN. After thawing, the unloading solution (MS media with 1.2 M sucrose) was replaced instead of the vitrification solution for 1 min. The encapsulated shoot tips were transferred to recovery MS media supplemented with 0.1 M sucrose, stored in dark conditions for 1 week and then transferred to the normal growth conditions. Regrowth was defined as the percentage of shoot tips resuming growth six weeks after plating out. Recovery of shoot tips was observed at weekly intervals. The young plantlets were subcultured on the optimal multiplication medium defined. Surviving plantlets were then subcultured into a hormone-free MS medium every 3 weeks. Approximately ten shoots were tested and each experiment was repeated five times.

Statistical Analysis: Data were subjected to one-way analysis of variance ANOVA; differences between individual means were determined by least significant difference (LSD) test at 0.05 level of probability. The layout of the different experiments was a complete randomized design (CRD). Collected data were statically analyzed using Statistica version [30].

RESULTS AND DISCUSSION

Vitrification: In the present study, a very efficient and reliable cryopreservation precedure is described for the first time for *C. spinosa using* vitrification, In general, cells will only survive low temperature in intracellular solution vitrify during cooling. Therefore, for successful cryopreservation via vitrification, it is vital to dehydrate cells sufficiently to survive after cooling in LN through vitrification. Therefore, shoot tips were loaded with a mixture of 2 M glycerol and 0.6 M sucrose for 20 min at 0°C in the dark to initiate acclimation to osmotolerance. It was found that using loading solution was essential to allow shoot formation. A similar finding was reported by Takagi *et al*., (1997), preserving tips of *Colocasia esculenta* [26].

Cryopreserved tips treated with PVS2 for 40 min showed 25% regrwoth. Longer exposures to PVS2 for 150-200 min caused significant reduction in regrowth for frozen and unfrozen tips (Fig. 1). Frozen shoot tips, once thawed, developed into shoots, after rapid thawing in a water-bath at 45°C. It was found that using a loading solution was essential to allow shoot formation after storage in liquid nitrogen (LN).

70% regrowth rates of cryopreserved shoot tips was obtained after 60 min exposure duration to PVS2 at 0°C, demonstrated that complete vitrification of the cryopreserved plant tissues avoids by intra and extracellular ice crystallization [25, 26]. Increasing time of exposure to vitrification solutions leads to increase solute concentrations inside the plant tissue [6, 10, 22]. Therefore, shoot tips have to be timed precisely exposure with PVS2 or MPVS2 application due to the rather narrow time window for the best survival.

To avoid the destabilization of the non-crystalline solid produced; thawing in this study performed rapidly by placing the cryovials in a water bath at 45°C for a few minutes. Warming was carried out rapidly, there is insufficient time for devitrification to occur and post thaw recovery is more likely to occur Cryopreservation using classical protocols is based on the dynamics of freeze dehydration. Optimization of the extracellular crystallization process is the key step during cooling and implementing a high warming rate is the critical step to prevent recrystallization during thawing of samples [31, 32, 33]. Vitrification is achieved by direct immersion in liquid nitrogen of samples which have been dehydrated at a non-freezing temperature. Therefore, the freeze-induced dehydration step characteristic of conventional procedures was eliminated. The vitrified state is achieved in systems that become sufficiently concentrated after a drastic desiccation process and that are cooled sufficiently rapidly so that the increase in cellular viscosity inhibits molecular rearrangement of water into a crystalline pattern [32, 33].

Encapsulation Dehydration: Cryopreservation using encapsulation/dehydration was studied for shoot tips of *C. spinosa*. This technique combined three treatments; precultured with sucrose and air dehydration and cold hardening. In this study both cold hardening and duration of dehydration where used to inducing cytoplasmic vitrification in order to avoid the formation of intracellular ice crystals during rapid cooling in liquid nitrogen (LN) [7, 16, 17]. Regrowth of encapsulated shoot tips decreased with decreasing bead water content and increasing dehydration time (Table 1). For frozen encapsulated shoot tips, there was a dramatic reduction in regrowth

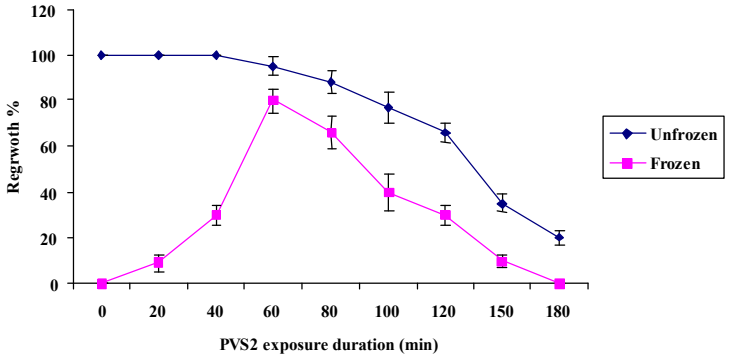

Fig. 1: Effect of exposure time with PVS2 at 0°C on regrowth percentages of unfrozen (-LN) and frozen (+LN) shoot tips of *Capparis spinosa.* Shoot tips were loaded with a mixture of 2 M glycerol and 0.6 M sucrose for 20 min at 0°C. Regrowth was defined as the percentage of cryopreserved shoot tips resuming growth six weeks after plating out. Error bars represented stander error of the mean. n = 5. Approximately 10-12 shoot tips were tested for each replicate

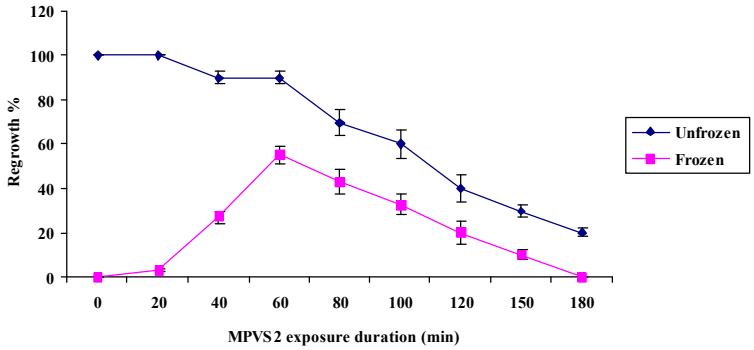

Fig. 2: Effect of exposure time with MPVS2 at 0°C on regrowth percentages of unfrozen (-LN) frozen (+LN) shoot tips of *Capparis spinosa.* Shoot tips were loaded with a mixture of 2 M glycerol and 0.6 M sucrose for 20 min at 0°C. Regrowth was defined as the percentage of cryopreserved shoot tips resuming growth six weeks after plating out. Error bars represented stander error of the mean. n = 5. Approximately 10-12 shoot tips were tested for each replicate

Table 1: Effect of cold hardening on regrowth percentage of frozen and unfrozen encapsulated shoot tips of *Capparis spinosa;* precultured in medium containing 0.75 M sucrose for one day

		Regrowth% Weeks of cold hardening		
Dehydration duration (h)	Bead moisture content %	0	4	8
Unfrozen encapsulated shoot tips (-LN)				
0	82.1	100a	100a	95.0a
2	73.0	95.0b	90.3a	90.3a
4	43.0	83.0b	83.3b	83.0b
5	26.0	76.0b	80.5b	83.0b
6	21.0	72.0b	77.3b	80.2b
7	19.5	45.0d	60.0c	55.6c
8	18.4	25.4e	40.3d	33.0d
9	16.3	20.0e	23.3e	20.6e
Frozen encapsulated shoot tips (+LN)				
0	82.1	0.0f	0.0f	0.0ef
1	73.0	0.0f	0.0f	0.0f
3	43.0	0.0f	0.0f	0.0f
5	26.0	20.0e	23.6e	15.3e
6	21.0	76.3c	83.0b	67.7c
7	19.5	40.6d	63.3c	63.6c
8	18.4	15.0e	25.3e	15.3e
9	16.3	0.0f	0.0f	0.0f

Means followed the same superscript(s) within the column are not significantly different according to LSD at P≤ 0.05. n = 5. Approximately 10-12 shoot tips were tested for each replicate

values between 0-3 h of dehydration, corresponding to rapid decrease of bead water content (Table 1). However, a dehydration step was necessary to avoid the formation of intracellular ice crystals even though increasing dehydration time led to damage of encapsulated shoot tips. The low values of regrwoth may result from severe dehydration and/or occur from uneven distribution of water in the tissue. Thus, drying may not be necessarily beneficial for cryopreservation, if uneven distribution of water results in different freezing responses among cells in the same tissue [16, 17, 22].

Regrowth percentage of encapsulated shoot tips also decreased with increased dehydration time. Regrwoth of unfrozen shoot tips decreased with increased dehydration duration (Table 1). Regrowth of all LN-treated beads were significantly lower than values of non LN-treated beads (p = 0.01). It confirms that the damage of ice crystal occurs in the cells. However, increasing sucrose concentration in preculture treatment and dehydration times could improve the survival ratio and regrowth. Therefore, regrwoth percentage of precultured beads after plunging into LN were slowly increased until 6-8 h of dehydration and then decreased again. As the dehydration time (> 6 h) increased, the regrwoth ratio of encapsulated shoot tips

were decreased until zero regrwoth. Regrowth of LN-treated shoot tips were reported after 5 to 8 h of dehydration when precultured on 0.75 M sucrose with clod hardening (Table 1). Maximum regrowth (83%) after cryopreservation were found when precultured on 0.75 M sucrose and after 6 of dehydration, with four weeks hardening periods.

Regrowth of encapsulated and osmotically-dehydrated shoot tips was not affected by the duration of cold acclimation. Cold acclimation for four weeks was significantly better than eight weeks, this because increased cold acclimation did not cause increased sugar level in the tissue. Reed (1988, 1993) indicated that cold acclimation is accompanied by biochemical changes such as solute accumulation and sugar has been considered to be one of the most important factors in freezing tolerance [7, 20]. On the other hand, previous studies, reported that cold acclimation could improving the efficiency of cryopreservation for many plant species such as, Ribes species [9, 20]. Cold acclimation treatments simulate the biochemical base of cold acclimation have been applied to enhance the cryopreservation survival of *C. Spinosa*.

Encapsulation Vitrification: Encapsulated shoot tips of *C. spinosa* shoot tips were successfully cryopreserved after various treatment durations with PVS2 and MPVS2 vitrification solutions (Fig. 3 and 4). Shoot formation of vitrified unfrozen shoot tips decreased significantly ($P<0.05$) with increasing times of exposure. Statistical analysis indicated that for each vitrification solution, there was a significant interaction between shoot formation (frozen and unfrozen) and time of exposure to the vitrification solution. Maximum regrowth (30%) of the frozen shoot tips was obtained when the exposure time was 60 min. However, longer exposure duration decreased shoot formation significantly of frozen and unfrozen shoot tips (Fig. 3). Following exposure to MPVS2 for 60 to 120 min at 0°C, regrwoth of frozen shoot was 10% at

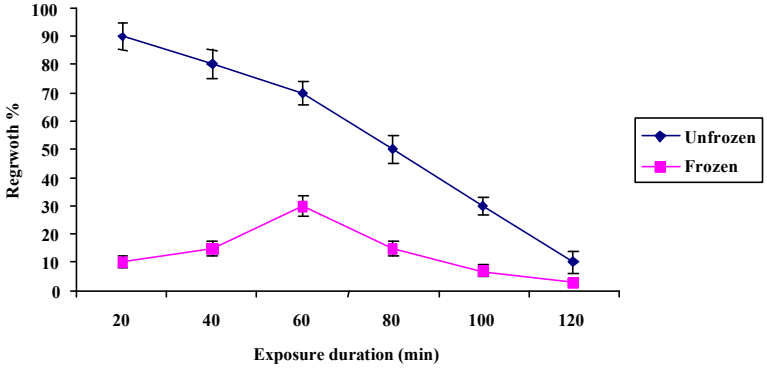

Fig. 3: Survival percentage of encapsulated unfrozen (-LN) and frozen (+LN) *Capparis spinosa* shoot tips as influenced by dehydration duration by using concentrated PVS2. Error bars represented stander error of the mean. n = 5. Approximately 10-12 shoot tips were tested for each replicate

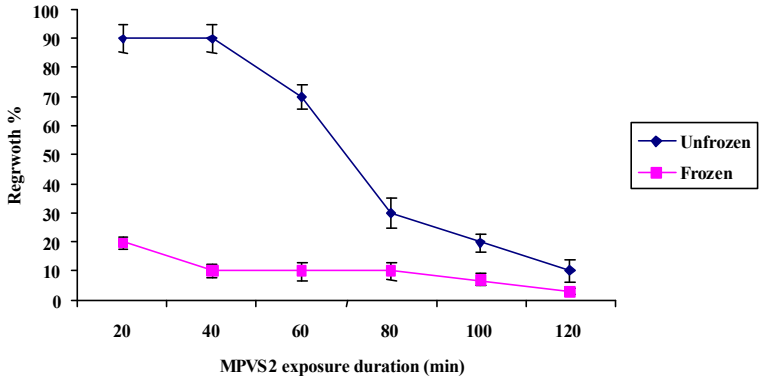

Fig. 4: Growth percentage of encapsulated unforzen (-LN) and frozen (+LN) *Capparis. spinosa* shoot tips as influenced by dehydration duration by using concentrated MPVS2. Error bars represented stander error of the mean. n = 5. Approximately 10-12 shoot tips were tested for each replicate

maximum. Thus, the optimum exposure duration to a particular vitrification solution has a critical effect on shoot formation after freezing in LN. Increasing time of exposure to vitrification solutions leads to the increase of solute concentrations inside the plant tissue [9, 22]. Sakai *et al.,* (1991) demonstrated that complete vitrification of the cryopreserved plant tissues eliminates effects of intra and extracellular ice crystallization [25]. PVS2 was successful in this study with a mean of 30% regrwoth of encapsulated shoot tips compared with 20 % by using MPVS2. Therefore, vitrification procedure requires highly concentrated solutions to dehydrate the cells without causing injury and produces a stable glass when plunged in LN. On the other hand, vitrification solutions are potentially injurious to shoot tips due to the phytotoxic effects of individual chemical components or their combined osmotic effects on cell viability.

Rapid thawing in a water bath at 45°C 1-2 min provide to suitable protecting shoot tips from the damaged for protecting the frozen shoot tips from the damaging effect of ice crystal formation in the cell, which may accor during warming [9, 11, 12]. Results obtained in this study suggest that long-term storage of shoot tips is possible, using the cryogenic methods study her. Hormone-free MS medium was used in this study; in a successful effort to avoid any callus formation and preserve true-to-type regrowth. Plantlets surviving cryopreservation were subcultured on MS medium without growth regulators for two weeks and then placed on MS media supplemented with 0.3 mg/L BA. Times to regreening and developing new growth were close to those reported by Niino *et al*., (1992), after cryopreservation of apple and pear shoot tips by vitrification methods [24]. The media successfully induced new shoot in all treatments. Cryopreserved shoot tips were generally regenerated into normal plantlets after thawing and continuous subculture of surviving shoot tips was employed to obtain healthy shoots. In this study, cryopreserved shoot tips produced shoots identical in appearance with those of the controls and plants regenerated from vitrified shoot tips were morphologically uniform. The methods developed in this study appear to be suitable for cryopreservation of shoot tips from *in vitro* cultures of *C. spinosa*. Additional studies are still needed to increase recovery of tips after cryopreservation and to standardize the protocols and evaluate genetic stability of cryogenic stored plant tissues.

In conclusion, our results demonstrate that shoot of *C. spinosa* successfully cryopreserved using vitrification, encapsulation dehydration, or encapsulation vitrification. Furthermore when different techniques were compared, maximum regrwoth after cryopreservation ranged between 30% and 83%. Maximum regrwoth rate (83%) was obtained with encapsulation dehydration. Studies are underway to assess the genetic stability of cryopreserved plants. The method develop her seems promising for a wide range of plant materials, if dehydration tolerance can be induced.

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REFERENCES

1. Chalak, L. and A. Elbitar, 2006. Micropropagation of *Capparis spinosa* L. subsp. *Rupestris* Sbith and Sm by nodal cuttings. Ind. J. Biotech., 5: 555-558.
2. Inocencio, C., D. Rivera, F. Alcaraz and A. Tomas-Barberan, 2000. Flavoniod content of commercial capper (*Capparis spinosa, C. sicula* and *orientalis*) produced in Mediterranean countries. Euro. Food Res. Techn., 212: 70-74.
3. Sozzi, C., 2006. Capper and caperberies. In: Peter v (eds). Handbooks of herb and spices(1 eds). CRC Press. New York. USA pp: 235-237.
4. Özkahraman, I., 1997. Caper. Forest Ministry, AGM Publications, Various Publication Series, No: 2. Ankara-Turkey
5. Musallam, I., M. Duwairy and R. Shibli, 2011. Micropropagation of capper (*Capparis spinosa* L.) from wild plants. Fuct. Plant Sci. Biotech., 5: 71-21.
6. Shibli, R.A., M.A. Shatnawi, W. Subaih and M.M. Ajlouni, 2006. *In vitro* conservation and cryopreservation of plant genetic resources: a review. W. J. Agri. Sci., 2(4): 372-382.
7. Reed, B.M., 1993. Responses to ABA and cold acclimation are genotype dependent for cryopreserved blackberry and raspberry meristems. Cryobiology, 30: 179-184.
8. Suzuki, M., M. Ishikawa and T. Akihama, 2005. Cryopreservation of encapsulated gentian axillary buds following 2 step preculture with sucrose and desiccation. Plant Cell, Tiss. Org. Cult., 83: 115-121.
9. Wang, Q.C. and J. Laamanen, 2005. Cryopreservation of *in vitro*-grown shoot tips of raspberry (*Rubus idaeus* L.) by encapsulation-vitrification and encapsulationdehydratation. Plant Cell Rep., 24: 280-288.

10. Engelmann, F., 1997. In vitro conservation methods. In: Ford-Lloyd B.V., J.H. Newburry. and J.A. Callow (eds), CABI, Wellingford, pp: 119-162.
11. Gonzalez-Arnao, M.T. and F. Engelmann., 2006. Cryopreservation of plant germplasm using the encapsulation-dehydration technique: Review and case study on sugarcane. Cryoletters., 27: 155-168.
12. Gonzalez-Arnao, M.T., A. Panta, W.M. Roca, R.H. Escobar and F. Engelmann, 2008. Development and large scale application of cryopreservation techniques for shoot and somatic embryo cultures of tropical crops. Plant Cell, Tiss. Org. Cult., 92: 1-13.
13. Shatnawi, M.A., R.A. Shibli, S.M. Abu-Romman, M.S. Al-Mazra'awi, Z.I. Al Ajlouni, W.A. Shatanawi and W. Odeh, 2011a. Clonal propagation and cryogenic storage of the medicinal plant *Stevia rebaudiana*. Spanish J. Agri. Res., 9(1): 213-220.
14. Shatnawi, M., G. Anfoka., R. Shibli., M. Al-Mazra'awi, W. Shahrour and A. Arebiat, 2011b. Clonal propagation and cryogenic storage of virus free grapevine (*Vitis Vinifera* L.) via meristem culture. Turkish J. Agri. For., 35: 173-184.
15. Shatnawi, M.A., R.A. Shibli, I. Qrunfleh, K. Bataeineh and M. Obeidat, 2007. *In vitro* propagation and cryopreservation of *Prunus avium* using vitrification and encapsulation dehydration methods. J. Food, Agri. Environ., 5(2): 204-208.
16. Shatnawi, M.A., K.A. Johnson and F. Torpy, 2004a. *In vitro* p ropagation and cryostorage of *Syzygium francisii* (Myrtaceae) by encapsulation-dehydration method. In vitro Cell, Devel. Biol-Plant., 40(4): 403-407.
17. Shatnawi, M.A. and K.A. Johnson, 2004b. Cryopreservation by encapsulation-dehydration of 'Chritsmas bush' (*Ceratopetalum gummiferum*) shoot tips. *In vitro* Cell, Devel. Biol-Plant., 40(2): 239-244.
18. Bachiri, Y., C. Gazeau, J. Hansz, C. Morisset and J. Dereuddre, 1995. Successful cryopreservation of suspension cells by encapsulation dehydration. Plant Cell, Tiss. Org. Cult., 43: 241-248.
19. Dumet, D., A. Grapin, C. Bailly and N. Dorion, 2002 Revisiting crucial steps of an encapsulation/ desiccation based cryopreservation process: Importance of thawing method in the case of *Pelargonium* meristems. Plant Sci., 163: 1121-1127.
20. Reed, B.M., 1988. Cold acclimation as a method to improve survival of cryopreserved *Rubus* meristems. Cryoletters., 9: 166-171.
21. Fabre, J. and J. Dereuddre., 1990. Encapsulation-dehydration: a new approach to cryopreservation of Solanum shoot-tips. Cryoletters., 11: 413-426.
22. Panis, B. and M. Lambardi, 2005 Status of cryopreservation technologies in plants (crops and forest trees). Paper presented on the Role of Biotechnology, Villa Gualino, Turin, Italy-5-7 March 2005. pp: 34-45.
23. Kaviani, B., 2010. Cryopreservation by encapsulation -dehydration for long-term storage of some important germplasm: seed of lily [*Lilium ledebourii* (Baker) Bioss.], embryonic axe of persian lilac (*Melia azedarach* L.), and tea (*Camellia sinensis* L.). Plant Omics J., 3(6): 177-182.
24. Niino, T. and A. Sakai., 1992. Cryopreservation of alginate-coated *in vitro* grown shoot tips of apple, pear and mulberry. Plant Sci., 87: 199- 206.
25. Sakai, A., S. Kobayashi and I. Oiyama, 1990. Cryopreservation of nucellar cells of navel orange (*Citrus sinensis* Osb. var. *brasiliensis* Tanaka) by vitrification. Plant Cell Rep., 9: 30-33.
26. Takagi, H., H. Tien-Thinh, O.M. Islam, T. Senboku and A. Sakai, 1997. Cryopreservation of in vitro grown shoot tips of taro [*Colocasia esculenta* (L) Schott] by vitrification. 1. Investigation by basic conditions of the vitrification procedure. Plant Cell Rep., 16: 594-599.
27. Volk, G.M. and C. Walters, 2006. Plant vitrification solution 2 lowers water content and alters freezing behavior in shoot tips during cryoprotection. Cryobiol., 52: 48-61.
28. Suzuki, M., M. Ishikawa and T. Akihama, 2005. Cryopreservation of encapsulated algentian axillary buds following 2 steppreculture with sucrose and desiccation. Plant Cell, Tiss. Org. Cult., 83: 115-121.
29. Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bioassays with tobacco cell cultures. Physiol. Plant., 15: 473-497.
30. Statsoft Inc., 1995. Statistica for Windows. Tulsa, Ok, USA
31. Engelmann, F., 2000. Importance of cryopreservation for the conservation of plant genetic resources. In: Engelmann, F. and H. Takagi (eds), Cryopreservation of tropical plant germplasm: Current Research Progress and Application, JIRCAS, Tsukuba/IPGRI, Rome, pp: 8-20.
32. Gonzalez-Arnao, M.T. and F. Engelmann, 2006. Cryopreservation of plant germplasm using the encapsulation-dehydration technique: Review and case study on sugarcane. Cryoletters., 27(3): 155-168.
33. MacFarlane, D.R., M. Forsyth and C.A. Barton, 1992. Vitrification and devitrification in cryopreservation. In: Advances in Low temperature Biology, vol 2, JAI Press, Greewish, CT, pp: 221-278.