JJBS

Volume 9, Number 3 September .2016
ISSN 1995-6673
Pages 147 - 154

Jordan Journal of Biological Sciences

# Experimenting Two Cryopreservation Techniques (Vitrification and Encapsulation-Dehydration) as Approaches for Long- term Conservation of in vitro Grown Shoot Tips of Wild Fennel

Rida A. Shibli[1,*], Fayha Al Hawmdeh[1], Mahmud Duwayri[1], Nazir Hadidi[1], Tamara S. Al-Qudah[2] , Reham W. Tahtamouni [3], Laila S. Younes [4]and Ahmad Zateemeh[5]

[1] *Department of Horticulture and Agronomy, Faculty of Agriculture, University of Jordan, Amman, Jordan*

[2] *Hamdi Mango Center for Scientific Research (HMCSR), University of Jordan, Amman, Jordan*

[3] *Department of Applied Sciences, Princess Alia University College, Al- Balqa Applied University, Amman, Jordan*

[4]*Agricultural and Animal Research Center,Tripoli, Libya*

[5] *Department of Applied Sciences, Ajloun University College, Al- Balqa Applied University, Ajloun, Jordan.*

*. Received: March 24, 2016 Revised: June 20, 2016 Accepted: June 28, 2016*

**Abstract**

Wild fennel (*Foeniculum vulgare* Mill.) is a medicinal plant that is native to Jordan as well as to most of Mediterranean region. Wild fennel is considered an aromatic medicinal plant and is used in cooking as a spice, and in folk medicine for the treatment of gastrointestinal and respiratory tract diseases. Due to the high international demand for this plant, the extensive uncontrolled collection of wild fennel became highly threatened species. In the present study, two cryopreservation techniques (vitrification and encapsulation-dehydration) were experimented as approaches for long term conservation of in vitro grown shoot tips of wild fennel. The results obtained from vitrification experimental part showed that survival and regrowth rates varied with type of loading solution, and the maximum survival (80%) and regrowth (65 and 60%) rates were recorded in shoot tips (STs) preloaded with either (2M glycerol + 0.4M sucrose) or (5% Dimethylsulfoxide, DMSO + 0.5 M sucrose), respectively. Also, data revealed that survival and regrowth rates of wild fennel STs were determined by the type of plant vitrification solution, as the highest survival and regrowth rates (85 and 60%, respectively) were obtained in STs cryoprotected with plant vitrification solution type 2 (PVS2) before exposure to Liquid Nitrogen (LN). Moreover, the best regrowth rate (65%) was recorded in encapsulated STs of wild fennel with 21.1% moisture content obtained after chemical dehydration in MS liquid media supplemented with 0.75 M sucrose for 1 day followed by air dehydration for 6 hrs. However, more research is still needed to optimize the pretreatments protocols prior exposure to LN in terms of ingredients, concentration and duration to improve regrowth rates.

**Keywords:** Cryopreservation, Encapsulation- dehydration, Vitrification, Wild fennel.

## 1. Introduction

For decades, people have used the vegetation around them for food, fuel and medicinal purposes. Consequently, the extensive use of plant resources resulted in environmental degradation. So, many strict regulations were developed to conserve plant resources, such as preventing cutting down indigenous trees and over grazing in addition to strict enforcement of existing rules/regulations of nature reserves (Ghazanfar et al., 2015). But, despite these regulations, plants are still a way from being safeguarded.

Wild fennel (Foeniculum vulgare Mill.) is a medicinal plant that belongs to Apiaceae family (He and Huang, 2011). This plant is native to Southern Europe and Mediterranean region including Jordan. Through history, wild fennel was considered a well-known aromatic medicinal plant, as all parts of it including shoots, leaves and fruits were used in cooking as a spice, and in folk medicine for the treatment of gastrointestinal and respiratory tract ailments (Raffo et al., 2011). Also, wild fennel is considered an important economic crop, and is

* Corresponding author. e-mail: *r.shibli@ju.edu.jo*

traded internationally to be utilized in phytoindustry (Raffo et al., 2011). However, the medicinal potential of wild fennel is due to the presence of many distinguished compounds, such as essential oil, fatty acid, phenylpropanoids, monoterpenids, sesquiterpenes and saponins (He and Huang, 2011) in most plant parts (shoots, roots, leaves, and seeds). Additionally, some elite compounds that were isolated from wild fennel, such as, trans-anethole, estragole, fenchone, sesquiterpenoids, coumarins and polyphenolics, were found to possess antimicrobial activities (Gulfraz et al., 2008). Meanwhile, in Jordan, wild fennel is suffering from serious degradation threats, as it is the case of many wild medicinal plants, due to the extensive uncontrolled collection by the locals for food and medicinal purposes (Royal Botanic Garden (RBG), 2015). This makes conservation of this plant of a high priority.

The ultimate task of plant conservation strategies is how is to ensure a sustainable supply of plant resources without altering the variety of genes or destroying the natural habitats and ecosystems (Kasagana and Karumuri, 2011). To fulfill this task, biotechnology protocols have been practiced to conserve plant genetic resources from loss and decline. These protocols include, slow growth conservation and cryopreservation. In slow growth conservation, inhibited or minimal growth is achieved by exposing the stored plant material to retardants, such as ABA, low environmental storage conditions (pressure, oxygen, temperature or light intensity), minimal growth media and the addition of osmotic agents, such as sucrose, mannitol or sorbitol to the culturing media (Shibli et al., 2006; Rabba'a et al., 2012; Sharaf et al., 2012; Tahtamouni et al., 2015). This can keep the plant material preserved for 1-15 years without the need for frequent subculturing (Rao, 2004). Cryopreservation is another storage technique that involves storage of plant material of a wide range of plant genotypes at ultra-low temperatures in liquid nitrogen (LN; -196°C) where cell division, metabolic and biochemical activities remain stopped, and yet plant material can be stored for unlimited time without alteration and deterioration (Engelmann, 2004; Shibli et al., 2006; Tahtamouni et al., 2015). For vegetatively propagated species, the best organs for cryopreservation are shoot apices or meristems, as they are virus-free, true to type, genetically stable and usually record high and recovery rates after cryopreservation (Micula et al., 2011).

In the last three decades, different protocols for cryopreservation were developed to meet the cryogenic needs of most plants species. These protocols include vitrification, encapsulation-dehydration, encapsulation-vitrification, and droplet-vitrification (Engelmann, 2004). Vitrification protocol involves supercooling of plant materials at ultra-low temperature after exposing them to a highly concentrated cryoprotectant, and yet the cryoprotectant inside the plant cell will solidify into a glass state without formation of ice crystals after exposure to liquid nitrogen (Sakai and Englemann, 2007). Encapsulation-dehydration is another cryopreservation protocol, in which shoot tips, somatic embryos or callus cells are encapsulated within alginate beads and cultured in a medium containing elevated concentrations of an osmotic agent (Fabre and Dereuddre, 1990). Encapsulation-dehydration is widely practiced on plants, because it is easily handled and maintains high rates of recovery after cryopreservation, this is due to the beads surrounding the explant which reduces the shock of exposure to liquid nitrogen (Shibli et al., 2006; Shatnawi et al., 2011).

The present study aims to experiment two cryopreservation techniques (vitrification and encapsulation- vitrification) as approaches for long term conservation of in vitro grown wild fennel shoot tips to ensure a sustainable true to type supply of this important plant for research and industry. ..

## 2. Materials and Methods

### *2.1. Mother Stock Establishment of Wild Fennel*

Sterilized seeds of wild fennel were inoculated on a full strength (4.4.g/L) hormone free MS (Murashige and Skoog, 1962) solid media supplemented with (30 g/L) sucrose. Then, cultures were kept in the growth room under daily regime of 16/8hr light/ dark regime (photosynthetic photon flux density (PPFD) = 40-45 µmole/m-2/s-1, light and 8-h dark photoperiod and 24 ± 1 °C till seed germination. After seed germination, the resulted microplants were transferred to a fresh hormone free MS media, prepared as described above for one month and kept under the same growth room conditions described earlier. Next, a preliminary experiment was conducted to decide the type and constituents of a shoot multiplication media that would yield the best multiplication rate by subculturing wild fennel microshoots into MS media, prepared as described earlier and supplemented with various types and levels of cytokinins plus 0.01 mg/L NAA and kept under the same growth conditions, as described before, and the best multiplication rate was obtained in MS media supplemented with 1.5 mg/L BA and 0.01 mg/L NAA (data not shown). Subculturing was performed every 4 weeks by subdividing the microshoots aseptically until a massive mother stock was obtained.

### *2.2. Cryopreservation*

All the conducted cryopreservation experiments have followed the protocol reported by Rabba et al. (2012).

#### *2.2.1. Cryopreservation Using Vitrification Technique*

**Effect of Loading Solution**

Shoot Tips (STs) were subculutred into a preculture media consisted of MS hormone free media plus (0.3 M) sucrose and kept under complete

dark conditions for three days. Then the STs were transferred into cryovials and loaded with 1 ml of different cocktails of loading solutions composed of Hormone Free (HF)-liquid media supplemented with either of (1 M sucrose, 0.4 M sucrose + 2 M glycerol, or combinations of sucrose [0.25, 0.5 M] + dimethylsulfoxide (DMSO) [5 or 10%] at 25 $^0$C for 20 min before being exposed to plant vitrification solution 2 (PVS2) which consisted of 30% glycerol, 15% ethyleglycol (EG), 15% DMSO (w/v%), 4.4 g/L MS media salts and 0.4 M sucrose for 20 min.

Then half of the treated cryovials were plunged directly in LN for at least 2 hrs., while the other half was not frozen with LN and it was unloaded with unloading solution (MS media with 1.2 M sucrose) to remove PVS2. Next, the STs were inoculated into a recovery media (hormone free MS media plus 0.1 M sucrose), and stored under dark conditions for 1 week before being transferred to the normal growth conditions for 1 week. After four weeks, the STs were examined for any regrowth signs to growth room condition. For the cryopreserved STs, the STs were thawed for 2-3 min at 37-38 ℃. Then unloaded by exposing the STs to MS liquid media supplemented with (1.2 M) sucrose, then transferred to the recovery media and incubated under dark conditions for 1 week then transferred to the normal growth conditions. After four weeks, the STs were examined for any regrowth sign. For determination of survival percentage, STs from each of none cryopreserved (-LN) and cryopreserved with liquid nitrogen (+LN) were examined using of 2, 3, 5-Triphenyl Tetrazolium Chloride (TTC) assay and survival percentages of the STs were determined according to the following equation: Survival percentage = (number of red shoots /total number of shoots) ×100%

**Effect Plant Vitrification Solution Type**

Fennel STs were precultured in preculture media for 3 days under dark. Next, the precultured STs were loaded with 1 ml either of different loading solution types consisted of [10% DMSO and 0.75 M sucrose in HF- liquid MS media], [10% DMSO and 0.5 M sucrose in HF- liquid MS media] or [2 M Glycerol+ 0.4M sucrose in HF- liquid MS media] at room temperature for 20 min. Then each loading solution type was replaced by 1 ml of either of the following plant vitrification solutions:[15% DMSO and 1 M sucrose in HF- liquid MS media], [30% DMSO and 1 M sucrose in HF- liquid MS media], [PVS3 solution consisted of 40% (w/v) glycerol and 40% (w/v) sucrose in HF- liquid MS media] or PVS2, prepared as earlier. The STs were incubated at room temperature for 20 min.

Then half of the treated cryovials were plunged directly in +LN for at least 1 hr., while the other half was left -LN and unloaded with he unloading solution (MS media with 1.2 M sucrose) three times to wash out each PVS solution three times. After that, the STs were transferred to recovery media, and kept under dark conditions for 1 week, and subsequently transferred to the normal growth conditions for 1 week. After four weeks, the STs were examined for any regrowth signs at room condition. For the cryopreserved STs, the STs were thawed for 2-3 min at 37-38 ℃. Then unloaded as described earlier, then transferred to recovery media, and incubated as described above. The viability test of the treated STs was performed as described earlier.

*2.2.2. Encapsulation-Dehydration*

To encapsulate the STs inside the beads, two liquid media were prepared. The first medium consisted of 3% sodium alginate plus calcium free-MS salts. The second medium included MS media with 100 mM calcium chloride (CaCl2) and 0.3 M sucrose. Next, the STs were precultured into the preculture media, as described above and kept in dark for 3 days. After that, STs were taken individually with some alginate solution, and then soaked into liquid MS medium provided with 100 mM CaCl2 and 0.3 M sucrose to produce the beads and then the beads polymerized for 30 min with stirring.

Next, the resulted beads were transferred into MS liquid medium containing 0.5 or 0.75 M sucrose and rotated on a shaker for 1 or 3 days. After that, the media containing 0.5 M or 0.75 M sucrose was removed, and then the beads were air dehydrated under laminar air-flow cabinet for 0, 2, 4, and 6 hrs. Half of the beads were then transferred to 2 ml sterile cryovials and dipped into LN for at least 3 hrs. Next, the cryovials were rapidly thawed, while the other half of beads were not exposed to the cryogenic treatment. All STs were transferred to the recovery media and incubated, as described above. For the determination of beads moisture content, fresh weight of beads was measured after each dehydration period; then beads were dried at 80 ℃ in an oven for 16 hrs and then reweighed.

Moisture Content (MC) was calculated using the following formula:

MC % = [(Beads fresh weight - Beads dry weight) / Beads fresh weight] ×100.

*2.3. Experimental Design and Data Analysis*

The treatments in each experiment described above were arranged in a Completely Randomized Design (CRD), and each treatment was replicated four times with five explants/ replicate. The collected data were statistically analyzed using Statistical Package for Social Sciences (SPSS version 20.0 for windows analysis system). The means and the standard errors for all experimental groups were calculated.

## 3. Results and Discussion

*3.1. Vitrification*

**Effect of Loading Solution Combination on F. Vulgare Shoot Tips Survival and Regrowth**

The obtained results showed that survival and regrowth rates varied with the type of the loading solution combination. For example, in none cryopreserved (-LN) shoot tips, full rates of survival

 © 2016 Jordan Journal of Biological Sciences. All rights reserved - Volume 9, Number 3

and regrowth were obtained in all treatments except in STs pretreated with either [10% DMSO + 0.25M sucrose] or [10% DMSO + 0. 5M sucrose] before exposure to PVS2 as they recorded (75 and 65%) survival and (45 and 25%) regrowth, respectively (Table 1). This might be a result of doubling DMSO concentration in the loading solution up to 10%, which might resulted in increasing sensitivity of the plant tissues to this highly toxic compound. Moreover, the exposure to PVS2 itself is a risky process in which STs suffer of being exposed to viscous hyperconcentrated solution. Meanwhile, the variation in survival and regrowth rates were more obvious in the cryopreserved STs (Table 1). The maximum survival (80%) and regrowth (65 and 60%) rates were recorded in STs preloaded with either (2M glycerol + 0.4M sucrose) or (5% DMSO + 0.5M sucrose). In many related studies, a combination of 2 M glycerol and 0.4 M sucrose was routinely used as a loading solution due to the high recovery rates obtained in many plant species after cryogenic exposure. Indeed, this combination described as the most popular loading solution type (Sakai and Engelmann, 2007). High recovery rates were also obtained in many wild medicinal plants in Jordan, such as mint, crocus, felty Germander, Shih and Qaysūm by using 2 M glycerol + 0.4 M sucrose as a loading solution before cryopreservation (Baghdadi *et al*., 2011; Rabba *et al*., 2012; Sharaf *et al*., 2012; Younis, 2012; Al- Baba *et al*., 2015). Also, in the present study using low concentration of DMSO (5%) in the loading solution might succeed in a supplementing wild fennel STs with a protective none toxic protection against PVS2 and LN shock which was translated into high survival and regrowth rates (80 and 60%) (Table 1).

**Table 1.** Survival and regrowth percentages of the non-cryopreserved (-LN) and cryopreserved with liquid nitrogen (+LN) wild fennel shoot tips as influenced by loading solution type

| Cryoprotectant | Survival% | Regrowth% |
|---|---|---|
| Non- cryopreserved (-LN) | | |
| 1M sucrose | 100± 0.0* | 100± 0.0 |
| 2M glycerol + 0.4M sucrose | 100± 0.0 | 100± 0.0 |
| 5% DMSO + 0.25M sucrose | 100± 0.0 | 100± 0.0 |
| 5% DMSO + 0.5M sucrose | 100± 0.0 | 95± 3.2 |
| 10% DMSO + 0.25M sucrose | 75± 9.3 | 45± 3.8 |
| 10% DMSO + 0.5 M sucrose | 65± 6.9 | 25± 11.4 |
| Cryopreserved (+LN) | | |
| 1M sucrose | 0.0± 0.0 | 0.0± 0.0 |
| 2M glycerol + 0.4M sucrose | 80± 4.3 | 65± 5.3 |
| 5% DMSO + 0.25M sucrose | 45± 6.8 | 0.0± 0.0 |
| 5% DMSO + 0.5M sucrose | 80± 6.1 | 60± 7.1 |
| 10% DMSO + 0.25M sucrose | 0.0± 0.0 | 0.0± 0.0 |
| 10% DMSO + 0.5 M sucrose | 0.0± 0.0 | 0.0± 0.0 |

* values represent means ± standard error

**Effect of the Loading Solution and Vitrification Solution Types**

Data revealed that survival and regrowth rates of wild fennel STs were determined by types of loading and plant vitrification solutions (Tables 2, 3, 4). Full survival and regrowth rates were recorded when none cryopreserved STs of wild fennel were preloaded with (2M glycerol+ 0.4M sucrose) followed by 20 min exposure to either PVS2 or (15%DMSO+ 1M sucrose) (Table 2). A similar trend was obtained after cryopreservation, as the highest survival and regrowth rates (85 and 60%) were obtained in STs pretreated with (2M glycerol+ 0.4M sucrose) and PVS2 (Table 2). On the other hand, both rates were adversely affected in (-LN) and (+LN) STs when higher concentration of DMSO (30%) was used in the plant vitrification solution, which indicated that DMSO was very toxic at this level to plant tissues (Table 2). Meanwhile, exposing the STs to PVS3 was most deleterious before and after cryopreservation, as shown in Table (2), which might refer to the high chemical toxicity of PVS3 which might be confounded with the insufficient pretreatment duration with the loading solution before exposure to PVS3 (Subaih *et al*., 2007). Also, our results showed that PVS2 was the best plant vitrification solution type in -LN and +LN STs. For example, the best survival and regrowth rates (70, 45 and 20, 5%) in (+LN) STs were obtained in explants cryoprotected with PVS2 after being loaded with either (10% DMSO+ 0.5M sucrose) or (10% DMSO+ 0.75M sucrose), respectively (Tables 3, 4). On the other hand, data recorded in (+STs) cryoprotected with (15% DMSO + 1 M sucrose) before cryopreservation indicated that this combination failed to be a proper cryoprotectant, and the resulted the chemical dehydration was not enough to prevent ice crystallization and cryogenic injury (Sakai and Engelmann, 2007).

Meanwhile, all STs died after exposure to LN when pretreated with the other PVS combinations (Tables 3, 4), which indicated failure of these treatment to overcome the hazards of chemical toxicity and/or the cryogenic injury (Shatnawi *et al*., 2011). This was in full agreement with Markovic *et al.* (2013) who reported maximum regrowth percentages when grapevine STs exposed to PVS2 before cryopreservation. The same study showed the complete death of grapevine STs exposed to PVS3, which indicates the high toxic nature of PVS3.

**Table 2:** Survival and regrowth percentages of the non-cryopreserved (-LN) and cryopreserved with liquid nitrogen (+LN) shoot tips of wild fennel as influenced by vitrification solution combination

| Loading solution type | Vitrification solution type | Survival % | Regrowth % |
|---|---|---|---|
| Non- cryopreserved (-LN) | | | |
| 2M glycerol+ 0.4M sucrose | PVS2 | 100± 0.0 | 100± 0.0 |
| | 30% DMSO+ 1M sucrose | 60± 9.1 | 40± 8.5 |
| | 15% DMSO+ 1M sucrose | 100± 0.0 | 100± 0.0 |
| | PVS3 | 50± 6.6 | 15± 7.3 |
| Cryopreserved (+LN) | | | |
| 2M glycerol+ 0.4M sucrose | PVS2 | 85± 4.3 | 60± 6.7 |
| | 30%DMSO+ 1M sucrose | 20± 8.4 | 0± 0.0 |
| | 15%DMSO+ 1M sucrose | 70± 5.1 | 55± 6.1 |
| | PVS3 | 0± 0.0 | 0± 0.0 |

* values represent means ± standard error

**Table 3:** Survival and regrowth percentages of the non-cryopreserved (-LN) and cryopreserved with liquid nitrogen (+LN) shoot tips of wild fennel different vitrification solution combinations

| Loading solution type | Vitrification solution type | Survival % | Regrowth % |
|---|---|---|---|
| Non- cryopreserved (-LN) | | | |
| 10% DMSO+ 0.5M sucrose | PVS2 | 100± 0.0 | 100± 0.0 |
| | 30%DMSO+ 1M sucrose | 30± 7.1 | 5± 7.8 |
| | 15%DMSO+ 1M sucrose | 90± 4.3 | 80± 4.3 |
| | PVS3 | 40± 6.7 | 0± 0.0 |
| Cryopreserved (+LN) | | | |
| 10% DMSO+ 0.5 M sucrose | PVS2 | 70± 5.4 | 20± 8.0 |
| | 30%DMSO+ 1M sucrose | 0± 0.0 | 0± 0.0 |
| | 15%DMSO+ 1M sucrose | 30± 7.4 | 0± 0.0 |
| | PVS3 | 0± 0.0 | 0± 0.0 |

* values represent means ± standard error

**Table 4:** Survival and regrowth percentages of the non-cryopreserved (-LN) and cryopreserved with liquid nitrogen (+LN) shoot tips of wild fennel different vitrification solution combinations

| Loading solution type | Vitrification solution type | Survival % | Regrowth % |
|---|---|---|---|
| Non- cryopreserved (-LN) | | | |
| 10% DMSO+ 0.75M sucrose | PVS2 | 90± 3.2 | 80± 3.6 |
| | 30%DMSO+ 1M sucrose | 25± 5.3 | 0±0.0 |
| | 15%DMSO+ 1M sucrose | 70± 4.2 | 55± 4.7 |
| | PVS3 | 15± 6.1 | 0±0.0 |
| Cryopreserved (+LN) | | | |
| 10% DMSO+ 0.75M sucrose | PVS2 | 45± 4.3 | 5± 6.7 |
| | 30%DMSO+ 1M sucrose | 0± 0.0 | 0±0.0 |
| | 15%DMSO+ 1M sucrose | 5± 6.2 | 0±0.0 |
| | PVS3 | 0± 0.0 | 0±0.0 |

* values represent means ± standard error

### *3.2. Encapsulation Dehydration*

The obtained results showed that regrowth rate of the encapsulated beads was determined by sucrose concentration, chemical incubation duration, air dehydration duration and bead moisture content after air dehydration. Also, it was obvious from the data that bead moisture content decreased with increasing sucrose concentration, chemical incubation and air dehydration durations (Tables 5-8). This in turn had a negative effect on regrowth rates of the encapsulated STs in (-LN) samples (Tables 5-8). However, after cryopreservation, the obtained regrowth results were completely opposite to those recorded in the (-LN) STs. For example, full death rates were recorded in all the encapsulated (+LN) STs with moisture content above (33.1 %) (Table 5-8). This might be a result of lethal ice crystallization which was formed due to the presence of freezable cellular water that indicates inadequate dehydration. Also, a complete death prevailed in STs with MC% less than (18.8 %) (Tables 5-8) which indicated that the STs were exposed to over desiccation due to preculture in high sucrose levels and/ or prolonged air dehydration durations. Hence, it might be very damaging to plant cells even before exposure to LN (Englemann, 2011).

© 2016 Jordan Journal of Biological Sciences. All rights reserved - Volume 9, Number 3

Moreover, the best regrowth rate (65%) was recorded in wild fennel STs in +LN with 21.1% MC obtained after chemical dehydration in MS liquid media supplemented with (0.75 M) sucrose for 1 day followed by air dehydration for 6 hrs (Figure 1, Table 6), which completely agrees with (Shatnawi *et al*., 2011), as best regrowth rate was recorded in *Capparis spinosa* STs exposed to similar treatment before cryopreservation. Removal of the extracellular water and increasing osmotic potential inside the plant cells are the main goal of all pretreatment protocols applied before cryopreservation in order to avoid the hazard of ice crystallization upon exposure to LN. However, most plant species were reported to withstand the cryogenic exposure at bead moisture content range of (17- 37 %) (Englemann, 2011), which agrees to a certain extent with our results, as regrowth was recorded only in beads with MC% ranged from 33.1-18.8% (Tables 5-8). However, the obtained range indicated that it is specific to wild fennel, as optimum MC% was always reported to be dependent on plant species (Englemann, 2011). Examples on other related researches that agree with wild fennel results are those reported by Marco-Mediana *et al.* (2010), as (22%) was the optimum MC% for the cryopreserved STs of *Thymus moroderi*, while Markovic *et al.* (2013) indicated that 22.28% MC % was best for the encapsulated +LN STs of grapevine.

**Table 5:** Regrowth percentages of encapsulated dehydrated shoot tips of fennel as affected by dehydration duration after pretreatment with 0.5 M sucrose concentration for 1 day

| Sucrose conc. (M) | Dehydration duration (hr) | Regrowth % | MC% |
|---|---|---|---|
| Non- cryopreserved encapsulated shoots tips (-LN) | | | |
| 0.5M | 0 | 100± 0.0 | 82.4 |
| | 3 | 100± 0.0 | 65.2 |
| | 6 | 100± 0.0 | 33.1 |
| | 9 | 65± 4.0 | 21.6 |
| Cryopreserved encapsulated shoots tips with liquid nitrogen (+LN) | | | |
| 0.5M | 0 | 0± 0.0 | 82.4 |
| | 3 | 0± 0.0 | 65.2 |
| | 6 | 25± 5.6 | 33.1 |
| | 9 | 55± 4.3 | 21.6 |

* values represent means ± standard error

**Table 6:** Regrowth percentages of encapsulated dehydrated shoot tips of fennel as affected by dehydration duration after pretreatment with 0.75 M sucrose concentration for 1 day

| Sucrose conc. (M) | Dehydration duration (hr) | Regrowth % | MC% |
|---|---|---|---|
| Non- cryopreserved encapsulated shoots tips (-LN) | | | |
| 0.75M | 0 | 100± 0.0 | 60.3 |
| | 3 | 100± 0.0 | 31.6 |
| | 6 | 85± 2.2 | 20.1 |
| | 9 | 40± 6.1 | 18.8 |
| Cryopreserved encapsulated shoots tips with liquid nitrogen (+LN) | | | |
| 0.75M | 0 | 0± 0.0 | 60.3 |
| | 3 | 30± 6.7 | 31.6 |
| | 6 | 65± 4.2 | 20.1 |
| | 9 | 5± 8.1 | 18.8 |

* values represent means ± standard error

**Table 7:** Regrowth percentages of encapsulated dehydrated shoot tips of fennel as affected by dehydration duration after pretreatment with 0.5 M sucrose concentration for 3 days

| Sucrose conc. (M) | Dehydration duration (hr) | Regrowth % | MC% |
|---|---|---|---|
| Non- cryopreserved encapsulated shoots tips (-LN) | | | |
| 0. 5M | 0 | 100.0± 0.0 | 65.6 |
| | 3 | 100.0± 0.0 | 33.5 |
| | 6 | 85.0± 3.0 | 22.1 |
| | 9 | 50.0± 4.8 | 19.2 |
| Cryopreserved encapsulated shoots tips with liquid nitrogen (+LN) | | | |
| 0. 5M | 0 | 0.0± 0.0 | 65.6 |
| | 3 | 15± 6.4 | 33.5 |
| | 6 | 55± 4.4 | 22.1 |
| | 9 | 100± 0.0 | 19.2 |

* values represent means ± standard error

**Table 8:** Regrowth percentages of encapsulated dehydrated shoot tips of fennel as affected by dehydration duration after pretreatment with 0.75 M sucrose concentration for 3 days

| Sucrose conc. (M) | Dehydration duration (hr) | Regrowth % | MC% |
|---|---|---|---|
| Non- cryopreserved encapsulated shoots tips (-LN) | | | |
| 0.75M | 0 | 100± 0.0 | 58.3 |
| | 3 | 100± 0.0 | 29.7 |
| | 6 | 40± 6.0 | 18.1 |
| | 9 | 10± 7.4 | 17.8 |
| Cryopreserved encapsulated shoots tips with liquid nitrogen (+LN) | | | |
| 0.75M | 0 | 0± 0.0 | 58.3 |
| | 3 | 35± 4.0 | 29.7 |
| | 6 | 0± 0.0 | 18.1 |
| | 9 | 0± 0.0 | 17.8 |

* values represent means ± standard error

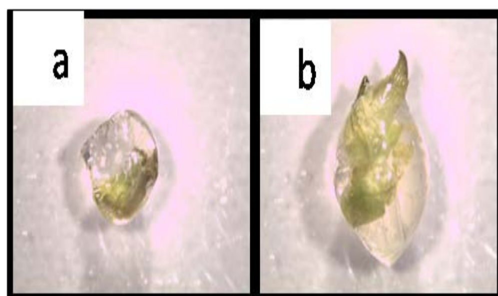


**Figure 1**: Encapsulated shoot tips of *F. vulgare* chemically dehydrated in MS liquid media supplemented with (0.75 M) sucrose for 1 day followed by air dehydration for 6 hrs. a: Directly after exposure to liquid nitrogen (LN). b: Two weeks after exposure to LN)

## 4. Conclusion

The obtained results indicated that long term conservation of wild fennel is possible. The results in both cryopreservation techniques (vitrification and encapsulation- dehydration) were very encouraging. However, more research is still needed to optimize the pretreatments protocols prior exposure to LN in terms of ingredients, concentration and duration to improve regrowth rates.

## Acknowledgment

Authors would like to thank the University of Jordan, The Deanship of Academic Research, for granting fund for the present study. Further thanks are extended to Hamdi Mango Center for Scientific Research, the Faculty of Agriculture: the Department of Horticulture and Crop Sciences at the University of Jordan.

## References

Al- Baba H, Shibli RA, Akash M , Al-Qudah T, Tahtamouni RW and Al- Ruwaiei H 2015. Cryopreservation and Genetic Stability Assessment of Threatened Medicinal Plant (*Ziziphora tenuior* L.) Grown Wild in Jordan. JJBS, **8**: 247 – 256.

Baghdadi S, Shibli RA, Syouf M, Shatnawi M, Arabiat A and Makhadmeh I. 2011. Cryopreservation by vitrification of embryogenic callus of wild crocus (*Crocus hyemalis* and *Crocus moabiticus*). *Acta Horticulturae*, **908**: 239-246.

Engelmann F. 2004. Plant cryopreservation: progress and prospects. *In Vitro Cell.Dev.Biol.-Plant,* **40**: 427- 433.

Engelmann F. 2011. Encapsulation – dehydration for cryopreservation: Past, present and future. *Acta Hor,* **908**: 165-170.

Fabre J. and Dereuddre J. 1990. Encapsulation-dehydration: A new approach to cryopreservation of Solanum shoot tips. *CryoLetters***, 11**: 413-126.

Ghazanfar SA, Taifour H, Al-Oqlah A, Borosova R and Osborne J. 2015. *Checklist of the Vascular Plants of Jordan*. Kew Publishing, London.

Gulfraz M, Mehmood S, Minhas N, Jabeen N, Kausar R, Jabeen K and Arshad G. 2008. Composition and antimicrobial properties of essential oil of *Foeniculum vulgare*. AJB, **7**: 4364-4368.

He W. and Huang B. 2011. A review of chemistry and bioactivities of a medicinal spice: *Foeniculum vulgare***.** JMPR**, 5:** 3595-3600.

Kasagana VN and Karumuri SS. 2011. Conservation of medicinal plants (past, present & future trends). JPS*R,* **3:**1378-1386.

Marcco-Medina A, Casas JL, Swnnen R and Panis B. 2010. Cryopreservation of *Thymus moroderi* by droplet vitrification. *CryoLetters,* **13:** 14-21.

Markovic Z, Chatelet P, Sylvertre I, Kontic JK and Engelmann F. 2013. Cryopreservation of grapevine (*Vitis vinifera* L.) *in vitro* shoot tips. CENT EUR J BIOL, **8:** 993-1000.

Mikuła A, Tomiczak K and Rybczyn´ski JJ. 2011. Cryopreservation enhances embryogenic capacity of Gentianacruciata (L.) suspension culture and maintains (epi)geneticuniformity of regenerants. PLANT CELL REP, **30:** 565-574.

Murashige T. and Skoog F. 1962. A revised media for rapid growth and bioassays with tobacco tissue cultures. PHYSIOL PLANTARUM, **15**: 473-479.

Rabba'a M, Shibli RA and Shatnawi M A. 2012. *In vitro* medium term conservation of felty germander *(Teucrium Polium L.)* micro-shoots. JJAS, **8 :** 523-544.

Raffo A, Nicoli S and Leclercq C. 2011. Quantification of estragole in fennel herbal teas: Implications on the assessment of dietary exposure to estragole**.** *Food Chem. Toxicol,* **49:** 370-375.

Rao NK. 2004. Plant genetic resources: Advancing conservation and use through biotechnology. AJB, **3:** 136-145

Royal Botanic Garden. (www.royalbotanicgarden.org.). **Cited on 5-10-2015.**

© 2016 Jordan Journal of Biological Sciences. All rights reserved - Volume 9, Number 3

Sakai A and Engelmann F. 2007. Vitrification, encapsulation- vitrification and droplet- vitrification: A review. *CryoLetters*, **28 :** 151-172.

Sharaf SA, Shibli RA, Kasrawi MA and Baghdadi SH. 2012. Cryopreservation of wild shih (*Artemisia herba-alba*) Asso. shoot-tips by encapsulation-dehydration and encapsulation-vitrification. PCTOC, **108:** 437-444.

Shatnawi M, Shibli R, Al-Dmoor H, Ateyat M and Abubaker S. 2011. *In vitro* propagation and cryopreservation of *Stevia rebudian* using vitrification method. *Acta Horticulturae*, **908:** 319-324.

Shibli R, Shatnawi M, Subaih W and Ajlouni M. 2006. *In vitro* conservation and cryopreservation of plant genetic resources: a review. WJAS, **2:** 372-382.

Subaih WS, Shatnawi MA and Shibli RA. 2007. Cryopreservation of date palm (*Phoenix dactylifera*) embryogenic callus by encapsulation-dehydration, vitrification and encapsulation-vitrification. JJAS, **3:**156-171.

Tahtamouni R, Shibli R, Al- Abdallat A, Al-Qudah T, Younis L, Al- Baba H and Al- Ruwaiei H. 2015. *In Vitro* Conservation and Cryopreservation of Medicinal and Aromatic Plants: A Review. JJAS, **11**: 147-167.

Younes L. 2012. *In vitro* multiplication and conservation of *Achillea fragrantissima* Forssk. Sch. Bip. Ph.D. Dissertation. College of Agriculture, University of Jordan, Amman, Jordan.