Rescuing Endangered *Moringa peregrina* (frossk) fiori by Cryopreservation Using Vitrification and Encapsulation- Vitrification Protocols

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**ABSTRACT**

Cryopreservation was applied to assess the possibility of using long-term conservation of *Moringa peregrina* (frossk) fiori using vitrification and encapsulation-vitrification techniques. In addition, the genetic stability of the recovered shoot tips after cryogenic storage was assessed using Amplified Fragment Length Polymorphism (AFLP) technique. Data obtained from the vitrification experiments revealed that using the 0.4 M sucrose plus 2 M glycerol as a loading solution yields the highest recovery rate (20%) compared to the other loading solutions cocktails. In another vitrification experiment, the maximum survival and recovery rates, i.e. 75% and 35% respectively were obtained in a combination of loading and vitrification solutions that consisted of 10% DMSO + 0.75 M sucrose and PVS2. In the encapsulation-vitrification experiment, cryopreserved *M. peregrina* shoot tips recorded the highest survival and recovery rates, i.e. 75% and 20%, respectively. Moreover, no genetic variation was found between the control samples and preserved shoot tips.

**Keywords:** Cryopreservation, encapsulation-vitrification, genetic stability, *Moringa peregrine*, vitrification.

**INTRODUCTION**

Jordan is distinguished with its rich and intensively diversified flora that exceeds 2,600 species (Ghazanfar *et al.*, 2015). The variation in topography and climate are the main causal agents behind the diversity in Jordanian flora. In spite of this botanical richness, Jordanian flora is endangered by overgrazing, limited water supplies, low rainfall, agricultural practices, fire hazards, and urbanization. However, climate change is also responsible for the loss of many plant species in Jordan, as the increase in temperature and the consequent drought have highly contributed to the fragmentation of the biodiversity (Harrison, 2009).

The Jordan Valley is one of the thirteen natural habitats in Jordan; it extends from Lake Tiberias to the Dead Sea and hosts a wide range of wild plants (Royal botanic garden (RBG), 2015). Unfortunately, the natural vegetation in the Jordan Valley is seriously exposed to loss as a result of unplanned agriculture practices, the mining industry, and climate change (Royal botanic
Moringa peregrina (Forssk.) Fiori (Figure 1) is one of Moringa species which grows wild in the Jordan Valley, the Dead Sea area, in addition to Wadi Araba, and Wadi Feynan, where it is named as Al-Ban or Al-Yassar by the locals (Lalas et al., 2012). The oil extracted from seeds is of nutritional and medicinal importance and is used in cooking by Bedouins, while the seeds are used as feed (Hegazy et al., 2008).

Many studies have indicated that Moringa peregrina (Forssk.) Fiori is a novel medicinal tree due to the presence of many pharmacological and phytochemical constituents, such as flavonoids, tannins, and alkaloids (Elbatran et al., 2005). This plant was also used for treating many illnesses such as burns, headache, abdominal pains, constipation, back and muscle pain, hypertension, asthma, stomach disorder, and diabetes (Mekonnen et al., 1999, Boulos, 2000). It was also used in traditional medicine as an anti-oxidant and wound healer (Nawash and Al-Horani, 2011). Furthermore, the oil extracted from the seeds was found to have significant anti-bacterial activities against many bacteria strains, such as Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumonia, Enterobacter cloacae, and Candida albicans (Lalas et al., 2012). According to Royal Botanic Garden (2015), M. peregrina (Forssk.) Fiori is listed as one of the most extremely threatened plants in Jordan. Thus, it is vitally important to find a proper and reliable plan of long-term maintenance and conservation of this valuable plant to avoid losing it.

Plant tissue culture provides a chance for the conservation of plant genetic material in vitro, using the med-term and long-term (cryopreservation) storage techniques that are widely applied in plant gene banks all over the world. Long-term storage methods (cryopreservation) are based on cooling and storing cells, tissues, or organs at ultra-low temperatures to maintain their viability (Sakai and Englemann, 2007). Cryopreservation is considered the most suitable method for long-term conservation of plant genetic resources (Kaczmarczyk et al., 2011). As all physiological and biochemical activities are significantly reduced during cryopreservation (Kavianí, 2011), plant genetic material stored this way is maintained true to type for indefinite time (Kaity et al., 2013). However, some genetic alterations were reported by few research studies after cryopreservation of in vitro-grown plant material. These alterations were considered to be accidental and were characterized by one or two new markers (Martínez and González-Benito, 2005; Micula et al., 2011). Therefore, the assessment of plant genetic uniformity became an interesting topic to insure the fidelity of the plants after exposure to cryopreservation (Micula et al., 2011).

There are four types of cryopreservation protocols, including: encapsulation-dehydration, vitrification, encapsulation-vitrification, and droplet vitrification. Each species varies in terms of which cryopreservation protocol is optimum for its conservation. Vitrification protocol is a widely used technique for germplasm conservation (Shibl et al., 2006). In the vitrification technique, the explants are loaded with a loading solution then exposed to cryoprotectants (plant vitrification solutions (PVS)) which acts as an antifreeze to eliminate the hazard of lethal ice formation inside the cells when exposed to liquid nitrogen (Withers, 1991). So optimizing the type and the exposure duration to the cryoprotectants and the loading solutions is a must for proper antifreeze and toxicity avoidance (Shibl et al., 2006, Tahtamouni et al., 2015).

Encapsulation-vitrification is another cryopreservation protocol which consists of the goods of vitrification and encapsulation dehydration techniques for an ultra-antifreeze action. In this protocol, the explants are encapsulated in alginate beads before treatment with PVS prior to being plunged in liquid nitrogen (Sakai and Englemann, 2007).

According to previous studies, there are no reports on the cryopreservation of M. peregrina. Therefore, this study was conducted to examine the possibility of developing a
reliable protocol for the cryopreservation of *M. peregrina* (Forssk.) Fiori, using vitrification and vitrification encapsulation-dehydration techniques. The genetic stability of the recovered shoot tips after cryogenic storage was also assessed, using Amplified Fragment Length Polymorphism (AFLP) technique.

**Figure 1**: Wild *M. peregrina* (Forssk.) Fiori tree growing near the Dead Sea area- Jordan (2014).

**MATERIALS AND METHODS**

This research study was conducted at Hamdi Mango Center for Scientific Research (HMCSR), Jordan University (Amman, Jordan). Plant material was supplemented as *in vitro* grown microshoots by Dr. Wesam Al Khateeb (Al Khateeb *et al.*, 2013). Plant materials were obtained on the summer of 2010 from seeds and were collected near the Dead Sea, Jordan (31°74’880”N; 35°59’378”E). The plant was identified by Dr. Ahmad El-Oqlah and Dr. Jamil Lapham (Department of Biological Sciences, Faculty of Science / Yarmouk University, Irbid- Jordan).

**In vitro multiplication of *M. peregrina* (Forssk.) Fiori for mother stock establishment**

A full strength Murashige and Skoog (MS) solid media was prepared as MS premix (Sigma Aldrich Murashige and Skoog Basal salt mixture) (Murashige and Skoog, 1962) at the concentration of 4.4g/L and supplemented with 30g/L sucrose, and 1.0mg/L Benzyl Amino Purine (BAP) was added. *In vitro* multiplication of *M. peregrina* was carried out as described by Al Khateeb *et al.* (2013); nodal segments were cut under completely sterile conditions (laminar air-flow cabinet), then transferred on the surface of (MS) media prepared as described above and supplemented with 1.0mg/L BAP. After that, flasks were transferred to the growth room under daily regime of 16-hr (photosynthetic photon flux density (PPFD) = 40-45 µmole/m²/s⁻¹, light and 8-h dark photoperiod and 24 ± 1 °C). Newly formed microshoots, (10-12mm) with two leaves, were sub-cultured to fresh media supplemented with the same previous media. Sub-culturing was performed every 4 weeks by subdividing the microshoots (10-12mm) under completely sterile conditions (laminar air-flow cabinet). After that, cultures were maintained in growth room conditions.

**Cryopreservation using vitrification technique**

In this experiment, the effects of loading solution and plant vitrification solutions (PVS) on the survival and recovery of the vitrified cryopreserved shoot tips were investigated.

**The effect of loading solution type**

Microshoots were cultured on pre-culture media as
described above, then shoot tips (STs) were transferred to cryovials and were loaded with 1ml loading solution composed of 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] + DMSO [5 or 10%] at 25 °C for 20 min) before being exposed to PVS2 for 20 min.

Then half of the treated cryovials were plunged directly in +LN for at least for 1hr, while the other half was left -LN and unloaded with unloading solution (MS media with 1.2 M sucrose) to wash out PVS2 three times for 10 min/time. Following that, the STs were transferred to recovery media, were stored in dark conditions for 1 week, and then were transferred to normal growth conditions for 1 week. After four weeks, the STs were examined for any regrowth signs. For the cryopreserved STs, the STs were thawed for 2-3 min at 37-38 °C. Then, they were unloaded by exposing the shoot tips to MS liquid media supplemented with 1.2M sucrose, then transferred to the recovery media that consisted of MS media supplemented with 0.1M sucrose, and incubated under dark conditions for 1 week, and then were transferred to normal growth conditions. After four weeks, the STs were examined for any regrowth sign. To determine the survival percentage, shoot tips from each of -LN (none cryopreserved) and LN (cryopreserved) were examined using 2, 3, 5-triphenyl tetrazolium chloride (TTC) assay by applying (TTC) assay on four cryovials with 5 STs/ cryovial taken from +LN and -LN shoot tips. The following formula was applied to measure the survival percentages of the treated shoots:

\[
\text{Survival percentage} = \left( \frac{\text{number of red shoots}}{\text{total number of shoots}} \right) \times 100\%
\]

The effect of plant vitrification solution type

M. peregrina STs were pre-cultured in pre-culture media for 3 days in the dark. Two modified MS media were prepared; one consisted of HF-MS liquid media, containing 0.3M sucrose and 3% sodium alginate, while the second solution was a modified HF-MS liquid media with 0.3M sucrose and 100 mM calcium chloride (CaCl₂). Next, pre-cultured STs were suspended in the modified liquid HF-MS media with 3% sodium alginate and 0.3M sucrose (first stock solution). After that, the STs were taken individually, using 1ml sterile micropipette including sodium alginate solution, then were dipped into the liquid HF-MS media with 100 mM calcium chloride and 0.3M sucrose (second solution) to form beads of 5mm in diameter. Next, the beads were allowed to polymerize for 30 min.

A- 10% DMSO and 0.75 M sucrose.  
B- 10% DMSO and 0.5 M sucrose.  
C- 2 M Glycerol+ 0.4M sucrose.  

Then, each loading solution type was replaced by 1ml of either of the following plant vitrification solutions:

1- 15% DMSO and 1 M sucrose  
2- 30% DMSO and 1 M sucrose  
3- PVS3 solution consisted of 40% (w/v) glycerol and 40% (w/v) sucrose  
4 - PVS2 described earlier in experiment 2

Following that, half of the treated cryovials were plunged directly in +LN for at least 1hr, while the other half was left -LN and unloaded with the unloading solution (MS media with 1.2 M sucrose) to wash out PVS2 three times for 10 min/time, then the STs were transferred to recovery media that were stored in dark conditions for 1 week, and then were transferred to normal growth conditions for 1 week. After four weeks, the STs were examined for any regrowth signs at growth room conditions. For the cryopreserved STs, the STs were thawed for 2-3 min at 37-38 °C. They were 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 above.

Encapsulation-vitrification

Shoot tips of M. peregrina were pre-cultured on pre-culture media for 3 days in the dark. Two modified MS media were prepared; one consisted of HF-MS liquid media, containing 0.3M sucrose and 3% sodium alginate, while the second solution was a modified HF-MS liquid media with 0.3M sucrose and 100 mM calcium chloride (CaCl₂). Next, pre-cultured STs were suspended in the modified liquid HF-MS media with 3% sodium alginate and 0.3M sucrose (first stock solution). After that, the STs were taken individually, using 1ml sterile micropipette including sodium alginate solution, then were dipped into the liquid HF-MS media with 100 mM calcium chloride and 0.3M sucrose (second solution) to form beads of 5mm in diameter. Next, the beads were allowed to polymerize for 30 min.
After encapsulation, the beads were transferred to sterile cryovials and then loaded with loading solution, as described earlier in experiment 2 for, 20min. Next, the loading solution was replaced with 100% PVS2 for different periods: 0, 10, 20, 40, and 60 min. at room temperature (25 ± 2 °C).

Then, half of the treated cryovials were plunged directly in +LN for at least 1hr, while the other half was left -LN and unloaded with the unloading solution (MS media with 1.2 M sucrose) to wash out PVS2 three times for 10 min/time. The beads were then transferred to the same recovery media as described earlier, were stored in dark conditions for 1 week, and then were transferred to normal growth conditions for 1 week. After four weeks, the STs were examined for any regrowth signs at growth room condition. For the cryopreserved STs, the STs were thawed for 2-3 min at 37-38°C. Then they were unloaded as described earlier, then transferred to recovery (regrowth) media, and incubated as described above. The viability test of the treated STs was performed as described earlier.

**Genetic stability assessment**

Ten microshoots were collected randomly from *in vitro* grown plant samples before and after cryopreservation. Genetic stability examination was performed using AFLP analysis technique as discussed by Blears *et al.* (1998) to determine if there were any genetic variations between *in vitro* propagated and cryopreserved plant.

**Genomic DNA extraction from plan tissues**

For DNA extraction from *M. peregrina*, about 1 g of plant tissues was grounded in a small amount of LN in 1.5 ml eppendorf tube. Then, about 40mg of finely grounded plant tissues were transferred to 1.5 ml tube and 600μL of Nuclei Lysis solution was added. Then, the solution was vortexed one to three seconds to uniform wet the plant powder. The samples were incubated at 65°C for 15 min. After that, 3μL of RNase solution was added to the cell lysate, and the samples were mixed homogenously by inverting the tube three to five times and incubated it for 15 min at 37°C.

Following that, the samples were left until cooling down at room temperature. After that, protein precipitation agent (200 μL) was supplemented and vortexed vigorously at high speed for twenty seconds. Solution was then centrifuged for three min at 13,000-16,000 rpm until the precipitated proteins were created at the bottom of pellet. The layer contained the DNA was extracted and transferred to 1.5 ml tube, having 600μL isopropanol. Subsequently, the solution was combined by inversion until thread-like strands of DNA shaped a visible mass. After that, the solution was centrifuged at the speed of 13,000-16,000 rpm for one min.

The supernatant was shifted to a new tube and DNA was rinsed by adding 600μL 70% ethanol. The tube containing the mixture was inverted for several times then centrifuged at 13,000-16,000 rpm for one min. After that, the ethanol was removed, using a drawn Pasteur pipette and the tube was overturned on the top of clean permeable paper and then the pellet was air-dried for 15 min until it dried. Subsequently, 100μL of DNA rehydration solution were added and rehydrated to tube and incubated at 65°C for 1 hr. The solution was mixed periodically by softly tapping the tube. Samples were then stored in refrigerator at 2-8°C.

**Quantification of genomic DNA concentration**

After DNA extraction, Agarose gel electrophoresis (0.7 %) was used to verify the quality and quantity of the DNA extracted from the *M. peregrina* tissues. The size and intensity of the sample was identified with 1kb DNA mass ladder (Promega) to determine genomic DNA concentration.

**Amplified fragment length polymorphism**

Two marked EcoRI (AAC IRDye 700 and ACT IRDye 800) with six selective primers were used for DNA amplification and five MseI unmarked primers (M-pr-CTT, CAC, CAT, CTC and CGT) were used to make AFLP profile as described according to Vos, *et al.* (1995). DNA samples were digested in the EcoRI and MseI.
restriction enzymes (Biolabs, EcoRI, Mse, T4 ligase, USA) and appropriate oligonucleotide adapters were ligated in the DNA ligase (Integrated Device Technology IDT, USA) by incubation at 37 °C for about 150 min. Double-stranded adapters were arranged by mixing individual synthetic oligonucleotides: EcoRI-adapter was set by mixing 7.0µL of the upper strand oligonucleotide (2µg/µL) with 7.5µL of the lower strand oligonucleotide (2µg/µL) in 486.1µL of TE buffer. MseI adapter was also set by adding and mixing 63.5µL of the upper strand oligonucleotide (2µg/µL) to 54.5µL of the lower strand oligonucleotide (2µg/µL) in 382µL of TE buffer.

A pre-amplification step was achieved using primers E-pr-A and M-pr-C designed to amplify the DNA fragment between the adapter sequence and one additional nucleotide. Then, for checking the quality of the reaction, 10µL of the reaction mixture was used to run on 1.5% agarose gel and the rest (10 µL) was diluted with 40µL of TE (10 mM) Tris and 1mM EDTA, (pH 8), which was adequate for about 30 AFLP-reactions. The diluted reaction was stored at -20°C. The results of pre-amplified DNA were selectively amplified using a combinations of six primers EcoRI+MseI (E-ACC 700 with M-CTC, M-CTA, and M-CAG) and (E-AAG 800 with, M-CTC, M-CTA, and M-CAG).

Touchdown program was used for PCR: thirty cycles of consequently reducing the annealing temperature (65 °C) by 0.7 °C per cycle, while keeping denaturation at 94 °C for 30 seconds and extension at 72°C for 60 seconds. This was followed by 23 cycles of denaturation at 94°C for 30 seconds, annealing at 56 °C for 30 seconds and extension at 72 °C for 60 seconds. After PCR finished, four µL of Blue Stop Solution were added directly prior to storage at -twenty °C.

**Polyacrylamide gels**

Outputs of AFLP reaction were analyzed using automated sequencer (LI-COR, Inc. Lincoln, Nebraska, USA). For gel electrophoresis of AFLP, 6% polyacrylamide gel was used, 200ml of polyacrylamide were prepared with 84g urea, 30ml acrylamide / bisacrylamide 40% solution (SIGMA), and 20ml of 10X Trisborate-EDTA (TBE) buffer. Gels were molded at least 60min prior to use and were concentrated by pre-run for 25min before loading. As counseled by LI-COR, the pre-running and running electrophoretic conditions were fulfilled at 1500V, 40W, 40 mA, 45 ºC, and 4 scan speed. TBE buffer (1X, 89 (mM) Tris, 89 (mM) borate and (2.2 mM) EDTA pH 8.3) was used as the running buffer. The objective of pre-running was to “warm up” the gel to about 45 ºC; this temperature was protected throughout electrophoresis, leading to constant heat distribution and good quality fingerprints.

Samples of DNA were denatured by heating the output mixtures for 5min at 95 ºC and after cooling them sharply on ice prior loading the gel. To eliminate urea precipitate or splits of gel, gel wells were entirely rinsed prior to loading by flushing them with buffer using a 20cc syringe, followed by loading 1µL of each denatured sample in the designated lanes, IRDye 700, and IRDye 800 sizing standard to determine band size.

**Experimental design and data analysis**

All treatments in each experiment described above were arranged in a completely randomized design (CRD), and each treatment was replicated four times with five explants (samples) per replicate. The average was calculated for each replicate. The collected data were statistically analyzed using Statistical Package for Social Sciences (SPSS analysis system; License information for SPSS Statistics version 17.0; copy right 1993-2007- polar engineering and consulting: http//: www.winwrap.com), and standard error was calculated.

**RESULTS**

**Vitrification**

The effect of loading solution type on survival and recovery of M. peregrina shoot tips

A significant difference was observed in the survival and recovery (regrowth) rates between cryopreserved and
none-cryopreserved *M. peregrina* STs. In general, survival and recovery rates were better in none-cryopreserved compared to cryopreserved *M. peregrina* STs. The survival rates in non-cryopreserved STs were approximately 90% to 100%. In contrast, in cryopreserved STs, the maximum rates of survival were 50%, 40% and 35% and were obtained at different loading solutions (5% DMSO + 0.5 M sucrose), (10% DMSO + 0.25 M sucrose), and (2 M glycerol + 0.4 M sucrose), respectively. Complete death of STs happened when the other loading solutions were used (Table 1).

On the other hand, recovery (regrowth) rate in none-cryopreserved STs was 100% in both 2 M glycerol + 0.4M sucrose and 5% DMSO + 0.5M sucrose loading solution. The lowest recovery rate was 45% in 10% DMSO + 0.5M sucrose loading solution. For cryopreserved STs, 20% recovery was obtained only at 2M glycerol + 0.4M sucrose. The rest of loading solution types had no viability and recovery of STs after cryopreservation (Table 1).

Table 1: Survival and recovery percentages of vitrified *M. peregrina* as affected by different loading solution types.

<table>
<thead>
<tr>
<th>Loading solution type</th>
<th>Survival %</th>
<th>Recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td>None-Cryopreserved (-LN)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 M sucrose</td>
<td>100.00 ± 0.00*</td>
<td>75.00 ± 9.93</td>
</tr>
<tr>
<td>2 M Glycerol+ 0.4 M sucrose</td>
<td>100.00 ± 0.00</td>
<td>100.00 ± 0.00</td>
</tr>
<tr>
<td>5%DMSO+ 0.25 M sucrose</td>
<td>100.00 ± 0.00</td>
<td>80.00 ± 9.18</td>
</tr>
<tr>
<td>5%DMSO+ 0.5 M sucrose</td>
<td>100.00 ± 0.00</td>
<td>100.00 ± 0.00</td>
</tr>
<tr>
<td>10%DMSO+ 0.25 M sucrose</td>
<td>90.00 ± 6.88</td>
<td>70.00 ± 10.51</td>
</tr>
<tr>
<td>10%DMSO+ 0.5 M sucrose</td>
<td>80.00 ± 9.18</td>
<td>45.00 ± 11.41</td>
</tr>
<tr>
<td>Cryopreserved (+LN)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 M sucrose</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>2 M Glycerol+ 0.4 M sucrose</td>
<td>50.00 ± 10.51</td>
<td>20.00 ± 9.18</td>
</tr>
<tr>
<td>5%DMSO+ 0.25 M sucrose</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>5%DMSO+ 0.5 M sucrose</td>
<td>40.00 ± 11.24</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>10%DMSO+ 0.25 M sucrose</td>
<td>35.00 ± 10.94</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>10%DMSO+ 0.5 M sucrose</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
</tbody>
</table>

* Values represent means ± standard error

Effect of plant vitrification solution types

The results showed that the survival rate recorded in the none cryopreserved *M. peregrina* STs were slightly affected by PVS type after being pretreated with 10% DMSO+0.75M sucrose as a loading solution. The lowest recovery rate (75%) was obtained when PVS3 was used, while 100% recovery of non-cryopreserved STs was obtained when the rest of PVSs was used. On the other hand, for cryopreserved *M. peregrina* STs, the optimum survival was 75% in PVS2 and was 0% in both PVS3 and 15% DMSO. For the recovery rate of cryopreserved STs, the best result (35%) was obtained when PVS2 was used, while no recovery was obtained when both PVS3 and 15% DMSO were used (Table 2).
Table 2: Survival and recovery percentages of *M. peregrina* shoot tips pretreated with 10% DMSO + 0.75M sucrose as affected by different vitrification solution types

<table>
<thead>
<tr>
<th>loading solution type</th>
<th>Vitrification solution type</th>
<th>Survival %</th>
<th>Recovery %</th>
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<tr>
<td>None-Cryopreserved (-LN)</td>
<td>PVS3</td>
<td>95.00 ± 5.00 *</td>
<td>75.00 ± 9.93</td>
</tr>
<tr>
<td>10% DMSO+0.75 M sucrose</td>
<td>15% DMSO+1 M sucrose</td>
<td>100.00 ± 0.00</td>
<td>100.00 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>30% DMSO+1 M sucrose</td>
<td>100.00 ± 0.00</td>
<td>100.00 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>PVS2</td>
<td>100.00 ± 0.00</td>
<td>100.00 ± 0.00</td>
</tr>
<tr>
<td>Cryopreserved (+LN)</td>
<td>PVS3</td>
<td>0.00 ± 0.00</td>
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<td>10% DMSO+0.75 M sucrose</td>
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<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>30% DMSO+1 M sucrose</td>
<td>70.00 ± 10.51</td>
<td>15.00± 8.19</td>
</tr>
<tr>
<td></td>
<td>PVS2</td>
<td>75.00 ± 9.93</td>
<td>35.00 ± 10.94</td>
</tr>
</tbody>
</table>

* Values represent means ± standard error

Meanwhile, the results showed that non-cryopreserved *M. peregrina* STs were not affected by the PVS solution type when 10% DMSO+0.5 M sucrose was used as the loading solution. On the other hand, in the cryopreserved *M. peregrina* the optimum survival rate was 25%, using PVS2 and the minimum was 0% in both PVS3 and 15% DMSO, while the recovery rate was 0% in all PVSs (Table 3).

Table 3: Survival and recovery percentages of *M. peregrina* shoot tips pretreated with (10% DMSO + 0.5 M sucrose) as affected by different vitrification solution types

<table>
<thead>
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<td>100.00 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>30% DMSO+1 M sucrose</td>
<td>100.00 ± 0.00</td>
<td>100.00 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>PVS2</td>
<td>100.00 ± 0.00</td>
<td>100.00 ± 0.00</td>
</tr>
<tr>
<td>Cryopreserved (+LN)</td>
<td>PVS3</td>
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<td>0.00± 0.00</td>
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<td>10% DMSO+0.5 M sucrose</td>
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<td>0.00 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>30% DMSO+1 M sucrose</td>
<td>20.00 ± 9.18</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>PVS2</td>
<td>25.00 ± 9.93</td>
<td>0.00 ± 0.00</td>
</tr>
</tbody>
</table>

* Values represent means ± standard error

When 2M glycerol + 0.4M sucrose was used as the loading solution, the obtained data showed that non-cryopreserved *M. peregrina* STs were not affected by PVS type. For cryopreserved STs of *M. peregrine*, the highest survival and recovery rates were 30%, 20% obtained only for shoot tips exposed to PVS2, while shoot tips pretreated with PVS3 or 15% DMSO died after cryopreservation (Table 4).
Table 4: Survival and recovery percentages of *M. peregrina* shoot tips pretreated with (2 M glycerol+ 0.4 M sucrose) as affected by different vitrification solution types.

<table>
<thead>
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</thead>
<tbody>
<tr>
<td>None-Cryopreserved (-LN)</td>
<td>PVS3</td>
<td>100.00 ± 0.00*</td>
<td>95.00 ± 5.00</td>
</tr>
<tr>
<td>2 M Glycerol +0.4 M sucrose</td>
<td>15% DMSO+1 M sucrose</td>
<td>100.00 ± 0.00</td>
<td>100.00 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>30% DMSO+1 M sucrose</td>
<td>100.00 ± 0.00</td>
<td>100.00 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>PVS2</td>
<td>100.00 ± 0.00</td>
<td>100.00 ± 0.00</td>
</tr>
<tr>
<td>Cryopreserved (+LN)</td>
<td>PVS3</td>
<td>0.00 ± 0.00</td>
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</tr>
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<td>2 M Glycerol +0.4 M sucrose</td>
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<td>0.00 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>30% DMSO+1 M sucrose</td>
<td>20.00 ± 9.18</td>
<td>10.00 ± 6.88</td>
</tr>
<tr>
<td></td>
<td>PVS2</td>
<td>30.00± 10.51</td>
<td>20.00 ± 9.18</td>
</tr>
</tbody>
</table>

* Values represent means ± standard error

**Encapsulation-vitrification**

Non-cryopreserved samples recorded the minimum survival rates (85 and 95%) in 0 and 10 min of exposure to PVS2 solution, while the rest shoot tips which underwent 20, 40, 60, and 80 min exposure duration to PVS2 recorded 100% survival rates (Figure 2). Survival rate in cryopreserved samples had a similar trend, where 0 and 10 min had no survival at all. Survival rates increased gradually until they reached the maximum 75% at 60 min, then declined after that. According to these results, 60 min was the proper exposure duration for *M. peregrina* STs using encapsulation-vitrification that leaded to maximum survival rate (Figure 2).

Recovery rate in non-cryopreserved STs showed the minimum recovery rate (50%) when not pretreated with PVS2, while the recovery rate increased gradually (65%, 75%, and 100%) in PVS2 exposure durations of 10, 20, 40, and 60 min, respectively and declined to 65% in 80 min (Figure 3). For the cryopreserved, the obtained results that showed the best survival or recovery rate were recorded when exposure duration to PVS2 was extended to 60 min, which means that this duration was good enough to keep the balance between freezable water and chemical toxicity in *M. peregrina* tissues or cells.

![Figure 2](image-url)  
Figure 2: Effect of different exposure duration to loading solution on survival rate of *M. peregrina* shoot tips using encapsulation-vitrification technique. Error bars represent ± standard error.
The effect of cryopreservation on genetic stability of *M. peregrine*

The results of AFLP molecular analysis test on the genetic stability of *in vitro M. peregrina* before and after cryopreservation showed that there were no changes in the DNA before and after exposure to +LN (Figure 4 and 5).

Figure 4: DNA banding pattern using AFLP marker for *M. peregrina*. Pre-amplified DNA were selectively amplified using three primer combination (1- E-ACC+M-CTC, 2- E-ACC+M-CTA, 3- E-ACC+M-CAG) using IRDye700, 1 and 2 refer to samples without cryopreservation treatment, 3 and 4 samples with cryopreservation treatment.
DISCUSSION

Vitrification

The obtained results in the vitrification experimental part revealed that using the 0.4M sucrose plus 2M glycerol as a loading solution was found to yield the highest recovery rate (20%) after exposing the shoot tips to cryopreservation. These results were in full agreement with those reported by many researchers, such as Baghdadi, et al. (2011), who worked on wild crocus sp., Hirai and Sakai (2003) who worked on sweet potato, and Kaczmarczyk et al. (2011) who worked on potato. On the other hand, Nishizawa et al. (1992) and Sakai et al. (1991) recommended the loading solution 2M glycerol + 0.4M sucrose was a very effective loading solution to mitigate the dehydration effects of vitrification solutions. However, plant species responded differently to various loading solutions even though they had similar effects as osmotic agents (Kim et al., 2009). For example, the data obtained by Suranthran et al. (2012) contrasted with our results, as they found that 2M glycerol+ 0.4M sucrose was a toxic loading solution for palm polyembryoids.

Moreover, testing the effect of plant vitrification solution types on the survival and recovery rates of the cryopreserved shoot tips of M. peregrina (Forssk.) Fiori showed that the maximum survival and recovery rates of 75% and 35% respectively were obtained in a combination of loading and vitrification solutions that consisted of 10% DMSO + 0.75M sucrose and PVS2. The optimization of using cryopreservation depends on the species type, tissues, and cell sensitivity to the cryoprotectants and LN affects (Heringer et al., 2013). Selecting the optimal PVS requires setting up the proper equilibrium between toxicity and satisfactory cellular dehydration so that vitrification can occur upon rapid
cooling in +LN without build up intracellular crystallization (Fábián et al., 2008). Heringer et al. (2013) found that peach palm (Bactris gasipaes, Kunth) was sensitive to DMSO that is present in PVS2, but they obtained 37% recovery after cryopreservation.

Callus of date palm had effectively responded to PVS2 as the vitrification solution was used (Al-Bahrany and Al-Khayri, 2012). Suranthran et al. (2012) also used PVS2 in the oil palm polyembryoids, and the cell walls were less affected by PVS2. Mukherjee et al. (2009) found that the best PVSs is the PVS2 compared to other PVSs for Dioscorea bulbifera L. Ellis et al. (2006), who studied garlic genotypes, found that five genotypes had good recovery rate when exposed to PVS2 before storage in LN, while another five genotypes had the best recovery rate when exposed to PVS3. On the other hand, many researchers recommended DMSO 10 % + 0.75 as the best combination with PVS2 in the cryopreservation of date palm and oil palm polyembryoids (Al-Bahrany and Al-Khayri, 2012; Suranthran et al., 2012), respectively.

Encapsulation- vitrification

In the encapsulation-vitrification experiment, cryopreserved M. peregrina (Forssk.) Fiori shoot tips recorded the highest survival and recovery rates, i.e. 75% and 20% respectively after 60min of pretreatment with PVS2 vitrification solution before exposure to liquid nitrogen. The key parameters for a successful cryopreservation depend on the exposure period to PVS2, since this stage is important to induce toxicity or not to plant sample towards PVS2 (Lambardi et al., 2008). According to Poobathy et al., (2013), the most common approach to test PVS2 exposure duration on plant sample ranged from 30 to 90min.

Our results are in agreement with Hirano et al. (2009), who found that Phaius tankervilleae gave the best survival rate (79%) when exposed to PVS2 at room temperature for 60min compared to the other period, i.e. 0-90 min. Any increase in exposure time more than 60min. led to a decline in the survival rate. This may be due to an increase in osmotic process and in the chemical toxicity of PVS2 (Sakai et al., 1990).

In contrast, Sharaf et al. (2012), found that 30min of exposure duration to loading solutions was the best exposure time for Artemisia herba-alba Asso shoot tips with 68% and 12% survival and recovery, respectively. Some Monocots like orchids (Cymbidium) and taro had 70%-64.5% recovery rates, respectively, at 40min of exposure time to PVS2 (Thinh et al., 2000). Sinniah and Gantait, (2013) concluded that 90min is the optimum exposure duration of Parkia speciosa shoot tips to PVS2 that provided the highest survival rate up to 60 % and 55.5% for non- and cryopreserved shoot tips, respectively. This might be attributed to the intracellular ice crystals build up by exposure to LN (Gonzalez-Arnao et al., 2008; Al-Ababneh et al., 2002).

Although in our experiments, the temperature of the added PVS2 was 25C, adding PVS2 at 0C was reported in some other studies to provide promising results. For example, Preetha et al. (2013), who worked on Kaempferia galanga L STs cryopreservation, found that the recovery rate could be increased if we used PVS2 at 0°C instead of 25°C. Exposure time to PVS2 for 20min at 0°C gave 36.67% recovery rate in K. galanga, while a short period as 10 min of exposure to PVS2 at 25°C had very low recovery rate 10%-20% (Preetha et al., 2013). It was attributed to the fact that the chemical process of cell plasmolysis was induced by PVS2 dehydration, which may be reduced with low temperature at 0°C. In Dendrobium candidum PLBs, Ching et al. (2012) achieved 76.2% survival rate by 120min at 25°C, while the survival rate could be increased to 89.4% when exposure time to PVS2 increased to 150min at 0°C. These results could indicate another approach towards PVS2 understanding with regard to temperature.

The effect of cryopreservation on genetic stability of M. peregrina

The results of AFLP molecular analysis confirmed that there were no genetic alterations in the in vitro grown M. peregrina before and after cryopreservation (Figure 4 and 5).
These results confirm that cryopreservation is a suitable method for long-term conservation of *M. peregrina* genetic material. The obtained data was similar to the results reported in other previous studies, such as those reported by Merhy et al. (2014), Wang et al. (2014), and Zevallos et al. (2014), as they investigated the effect of cryogenic exposure on the genetic integrity of explants of *Passiflora pohlii*, *Solanum tuberosum* and *Solanum lycopersicum*, respectively.

**CONCLUSIONS**

In this study, the possibility of developing a reliable protocol for cryopreservation of *M. peregrina* (Forssk.) Fiori was investigated using vitrification and vitrification encapsulation-dehydration techniques. In vitrification experiments, the results showed that using 0.4M sucrose plus 2M glycerol as a loading solution resulted in the highest recovery rate compared to the other loading solutions. In addition, the best survival and recovery rates were obtained in a combination of loading and vitrification solutions that consisted of 10% DMSO + 0.75M sucrose and PVS2. *M. peregrina* encapsulated-vitrified shoot tips also recorded the highest survival and recovery rate after 60min exposure to PVS2 before exposure to liquid nitrogen. Moreover, no genetic variation was found between control samples and cryopreserved shoot tips. However, more research is needed to improve the recovery rates of the shoot tips after cryopreservation.

**ACKNOWLEDGMENT**

The authors would like to express their appreciation to the staff of Hamdi Mango Center for Scientific Research (HMCSR) and the Deanship of Scientific Research at University of Jordan (Amman-Jordan) for their generous support.

**REFERENCES**


Ellis, D., Skogerboe, D., Andre, C. Hellier, B. and Volk, G., 2006. Implementation of garlic cryopreservation...


Moringa peregrina (forssk.) Fiori

(vitrification) and Encapsulation vitrification

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