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Cryopreservation of *Thymbra spicata* L. var. *spicata* and Genetic Stability Assessment of the Cryopreserved Shoot Tips after Conservation

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Abstract

The present study aims to introduce endangered Thymbra spicata L. to cryopreservation and to assess genetic stability of shoot tips after cryopreservation using Amplified Fragment Length Polymorphism (AFLP) technique. For the cryopreservation experiments, vitrification and droplet vitrification techniques were examined. In the first vitrification experiment, highest survival (80%) and regrowth (35%) rates were obtained in shoot tips exposed to 2- stepwise application of Plant Vitrification Solution (PVS) technique. Results of the second experiment showed that, 60 min. was the best duration for loading the explants with the loading solution before exposure to PVS2 as it yielded the highest survival and regrowth rates (85 , 45%) after cryopreservation. Moreover, in the third experiment, 2 M glycerol + 0.4 M sucrose had proven to be the best formula as a loading solution compared to the results obtained in the other combinations. Additionally, in the fourth experiment, the highest survival and regrowth percentages (75, 35%) were obtained in explants treated with PVS2 compared to results obtained in tips treated with other PVS solution types. In droplet vitrification experiment, the highest survival and regrowth rates (80, 35%) were recorded in explants exposed to PVS2 for (45 min.). Meanwhile, no genetic differences were detected in the shoot tips before and after cryopreservation.

Keywords: : Cryopreservation, Genetic stability, Droplet- vitrification, Vitrification.

1. Introduction

Cryopreservation protocols were successfully used to conserve many plants for indefinite period of time (Vasanth and Vivier, 2011). Additionally, maintenance of genetic stability of plant material is another achievement of cryopreservation beside long term conservation, and the validity of cryopreservation is only achieved when genetic stability is unchanged after exposure to liquid nitrogen (Zarghami et al., 2008). For decades, it was believed that as the explant is introduced to cryogenic storage, this would cease all forms of metabolic activities, which keeps the plant material true to type after storage (Panis et al., 2002; Kaczmarczyk et al., 2012). Recently, evidence of genetic alterations after cryopreservation of in vitro-derived plant material has been reported in some studies, and, consequently, evaluation of plant genetic uniformity has been carried out to validate newly established cryopreservation protocols (Harding, 2004; Micula et al,, 2011). In many instances, the genetic alteration in the cryopreserved specimens was reported to be accidental, most frequently was carried on a single specimen and was characterized by few markers (Martin and Gonzalez-

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Benito, 2005; Sanchez et al., 2008; Micula et al., 2011). Therefore, there is a growing interest in the assessment of plant genetic uniformity to insure the fidelity of the plants after their exposure to cryopreservation techniques (Harding, 2004; Micula et al., 2011).

Examples on different genotypes that were reported with a genetic variation after cryopreservation are Malus pumila (Hao et al., 2001), Carica papaya (Kaity et al., 2008), Fragaria gracilis (Hao et al., 2002a), Citrus sinensis (Hao et al., 2002b), Ribes sp. (Johnston et al., 2009) and Prunus dulcis (Channuntapipat et al., 2003).

Thymbra spicata L. var. spicata is a medicinal plant that grows wild in the Mediterranean region including Jordan (Akine et al., 2010; Inan et al., 2011; Royal Botanic Garden (RBG) 2012). This plant is used as a spice and as an herbal tea for treating asthma, colic, bronchitis and coughs (Baser, 2002; Baydar et al., 2004). Moreover, Thymbra spicata L. var. spicata has remarkable antifungal, antibacterial and antimicrobial activities that resulted from "carvacrol", the major chemical component of this plant (Akine et al., 2010; Inan et al., 2011). Meanwhile, Thymbra spicata L. var. spicata is facing threats in Jordan due to the uncontrolled collection in addition to the extensive greasing by animals (Taifour and Al-Oqlah, 2014).

In the present study, *Thymbra spicata* L. var. *spicata* was introduced to cryogenic storage using vitrification and droplet vitrification techniques, and genetic stability of the shoot tips was proved to be maintained after cryopreservation.

2. Materials and methods

2.1. In vitro Establishment of Thymbra spicata L. *var. spicata*

The plant material was supplemented by the Royal Botanic Garden (RBG) (Tell Al-Rumman, Jordan) in a form of mature seeds collected on September 2012 from a single mother plant growing solely at RBG located between 32.18772° Latitude and 35.827393° Longitude. Mature seeds of *Thymbra spicata* L. var. *spicata* were surface-sterilized by washing under running tap water for 20 min. Seeds were then immersed in 20% sodium hypochlorite for 10 min before three times rinsing (5min/ time) with sterile distilled water under laminar air-flow. After that, seeds were soaked in 70% ethanol (v/v) for 30 seconds before being rinsed with sterile distilled water for three times (5min. each). Sterilized seeds were then cultured in 100 ml bottles containing Water Media (WM) (water and 0.8 %) supplemented with ($2.9*10^{-6}$ M) gibberellic acid (GA3). Seeds were maintained in growth room under a daily regime of 24±1 °C under a 16/8 (light/dark) photoperiod of 45–50 µmol m−2s−1 irradiance, until germination.

The germinated seedlings were directly subcultured onto the prepared proliferation media consisted of a full strength MS media (Murashige and Skoog, 1962) supplemented with 0.1 M sucrose, $2.7*10^{-6}$ M Benzylaminopurine (BAP) and $9.8*10^{-8}$ M Indolebutric Acid (IBA). The proliferated microshoots were subcultured into the same proliferation media every 4 weeks and cultures were maintained in the growth room under a daily regime of 16- hr light, 8- hrs dark and 24 ± 1°C.

2.2. In vitro Conservation Using Vitrification Technique

All vitrification experiments in section 2.2 were carried out following the protocol stated by Rabba'a et al. (2012).

2.2.1. Effect of Stepwise Application of Plant Vitrification Solution (PVS2)

Shoot-tips of *Thymbra spicata* L. var. *spicata* were disected and precultured aeseptically into a hormone free MS solid medium supplemented with 0.3 M sucrose for 3 days under dark. Then shoot-tips were transferred to sterile cryovials before being loaded with 1.0 ml loading solution (liquid- hormone free MS medium + 2 M glycerol + 0.4 M sucrose) for 20 min at 25 ºC. Next, the loading solution was removed using a sterile micropipette and replaced with 0.8 ml of PVS2 (liquid hormone free-MS medium + 30% glycerol + 15% Ethylene Glycol (EG) + 15% Dimythelsulfoxide (DMSO) + 0.4 M sucrose) for 20 min in three different methods.

1. Shoot-tips were exposed to a full strengh PVS2 for 20 min at 25 ºC, then half of the treated cryovials was plunged directly in LN (Liquid Nitrogen) for 1 hr while the other half was not exposed to LN.
2. Shoot-tips were exposed to 60% PVS2 for 10 min, followed by 100% PVS2 for another 10 min at 25 ºC, and then half of the treated cryovials was plunged directly in LN for 1 hr while the other half was not exposed to LN.
3. Shoot-tips were exposed to 20, 40, 60 and 100% for 20 min (5 min for each concentration) at 25 ºC, then half of the treated cryovials was plunged directly in LN for 1 hr while the other half was not exposed to LN.

After each treatment, PVS2 solution was removed and the non-cryopreserved shoot tips (-LN) were unloaded by unloading solution (liquid hormone free-MS medium + 1.2 M sucrose) washed 3 times (10 min/ time) before being transferred to a recovery solid hormone free-MS media supplemented with 0.1 M sucrose and kept under dark for 4 days, then transferred to dim light (20 µmol $m^{-2}s^{-1}$ irradiance) for another 4 days before being transferred to normal light conditions. After three weeks, shoot-tips were examined under the binocular microscope for any regrowth signs. After cryogenic exposure (+LN), cryovials were thawed in a water bath at 38 ºC for 2-3 min and then treated with the unloading solution (liquid hormone free-MS medium + 1.2 M sucrose) washed 3 times (10 min/ time) before being transferred to a recovery solid hormone free-MS media supplemented with 0.1 M sucrose and then stored under same conditions described above. After three weeks shoot-tips were examined under the binocular microscope for any regrowth signs. Survival percentage was recorded by testing shoot tips from each of +LN and –LN treatments using TTC (2, 3, 5-triphenyl tetrazolium chloride) which was prepared by dissolving TTC salt (0.5% (w/v) in 50 mM K_2HPO_4 at pH 7.0 after 15 h incubation at 25 ºC under dark. Survival percentage was recorded by testing shoot tips from each of treatment using TTC (2, 3, 5-triphenyl tetrazolium chloride) test. Survival percentages were calculated according to the following formula:

Survival percentage = [number of shoots with red color that result due to hydrogen ions released from cells respiration of viable cells) /total number of shoots] × 100%.

2.2.2. Effect of Loading Solution Type

The shoot-tips were precultured, as described in section 2.2.1, and then placed in cryovials before being divided into 3 parts. Each part was loaded with (1.0 ml) of one of the following loading solutions:

1. Hormone- free liquid medium + 1.0 M sucrose for 20 min at 25 ºC.
2. Hormone- free liquid medium + 2.0 M glycerol + 0.4 M sucrose for 20 min at 25 ºC.
3. DMSO (5 or 10%) + sucrose (0. 5 or 0.75 M) for 20 min at 25 ºC.

Next, the vitrification solution PVS2 was removed and the non-cryopreserved (-LN) shoot tips were unloaded and subcultured, as described in section 2.2.1. Then shoot tips were examined after three weeks for any regrowth sign.

After cryopreservation (+LN), shoot tips were thawed, washed and subcultured, as described in section 2.2.1 before being tested after three weeks shoot for any regrowth sign. Survival percentage was recorded by testing shoot tips from each of +LN and –LN treatments using TTC (2, 3, 5-triphenyl tetrazolium chloride) assay as described in section 2.2.1.

2.2.3. Effect of Loading Duration of the Loading Solution

*S*hoot-tips were precultured as described in section 2.2.1 then placed in cryovials before being loaded with 1ml of loading solution (2.0 M glycerol plus 0.4 M sucrose in liquid hormone free-MS medium) for different periods of time (0, 10, 20, 40, 60 or 80 min) at 25 ºC. Next, the loading solution was replaced with 1.0 ml of 100% PVS2 at 25 ºC.

PVS2 solution was then removed and the non-cryopreserved shoot tips (-LN) were unloaded and subcultured as described in section 2.2.1., then regrowth percentage was recorded after three weeks. Shoot tips that were exposed to LN were thawed, washed and subcultured, as described in section 2.2.1 and regrowth data were taken after three weeks.

Survival percentage was recorded by testing shoot tips from each of +LN and –LN treatments using TTC (2, 3, 5-triphenyl tetrazolium chloride) assay, as described in section 2.2.1.

2.2.4. Effect of Plant Vitrification Solution (PVS) Type

Thymbra spicata L. var. *spicata* shoot-tips were precultured, as described in section 2.2.1, then placed in cryovials before being divided into 3 parts and each part was loaded with (1.0 ml) of one of the following loading solutions:

1. Hormone free-liquid medium + 2.0 M glycerol + 0.4 M sucrose for 20 min at 25 ºC.
2. Hormone free-liquid medium + DMSO (10%) + 0. 5 M sucrose for 20 min at 25 ºC.
3. Hormine free-liquid medium + DMSO (10%) + 0.75 M sucrose for 20 min at 25 ºC.

The loading solution was then replaced with 0.8 ml from one of the following vitrification solution:

1. PVS2 (liquid hormone free-MS medium + 30% glycerol + 15% ethylene glycol (EG) + 15% dimythelsulfoxide (DMSO) + 0.4 M sucrose) at 25 ºC for 20 min.
2. Liquid hormone free- MS media + 15% DMSO and 1.0 M sucrose at 25 ºC for 20 min.
3. Liquid hormone free- MS media + 30% DMSO and 1.0 M sucrose at 25 ºC for 20 min.
4. PVS3 (liquid hormone free- MS media + 40% glycerol + 40% sucrose) at 25 ºC for 20 min.

PVS2 solution was removed and the non-cryopreserved shoot tips were unloaded and subcultured as described in section 2.2.1., then after three weeks, regrowth percentage was recorded.

For the cryopreserved shoot tips, the cryovials were thawed, washed and subcultured, as described in section 2.2.1., and regrowth data were taken after three weeks.

Survival percentage was recorded by testing shoot tips from each of +LN and –LN treatments using TTC (2, 3, 5-triphenyl tetrazolium chloride) assay, as described in section 2.2.1.

2.3. In vitro Conservation Using Droplet Vitrification Technique

Shoot tips of *Thymbra spicata* L.var. *spicata* were isolated aseptically then precultured, as described in section 2.2.1. Next, the pretreated shoot tips were loaded with the loading solution at consisted of (hormone free-MS liquid medium + 2 M glycerol + 0.4 M sucrose) at 25 ºC for 20 min then the exposed to chilled PVS2 (hormone free-MS liquid medium + 30% glycerol + 15% Ethylene Glycol (EG) + 15% Dimythelsulfoxide (DMSO) + 0.4 M sucrose) for different periods of time (0, 10, 20, 35 and 45 min) at 0 ºC. The shoot tips were then put on sterilized aluminum foil strips (four shoot tips / strip). A single drop of chilled PVS2 was dripped onto each shoot tip, then half of the treated shoot tips were put into a cryovial and immersed into liquid nitrogen while rest of the shoot tips were directly rinsed with unloading solution (MS hormone free liquid media + 1.2 M sucrose) 3 times (10 min/time) at room temperature. For the cryopreserved shoot tips, after exposure to liquid nitrogen, cryovials were thawed in a water bath at 38 ºC for 2-3 min, and the aluminum foil was taken out from the cryovial, and rinsed with unloading solution (MS hormone free liquid media + 1.2 M sucrose) 3 times (10 min/time) at room temperature.

After unloading, the cryopreseved and non-cryopreserved shoot tips were thawed, washed and subcultured, as described in section 2.2.1 and regrowth data were taken after three weeks.

Survival percentage was recorded by testing shoot tips from each of +LN and –LN treatments using TTC (2, 3, 5-triphenyl tetrazolium chloride) assay, as described in section 2.2.1.

2.4. Assessment of Genetic Stability Using Amplified Fragment Length Polymorphism (AFLP) Technique

To assure the genetic uniformity of tested material, a single microshoot was propagated in the *in vitro* proliferation media described above and then used in all cryopreservation experiments, as described above. Shoot tips recovered before and after cryopreservation were assessed for their genetic stability using Amplified

Fragment Length Polymorphism (AFLP) technique. Also, two samples taken from the wild mother plant and another seed tissue cultured originated microshoot were included in the genetic assessment in order to compare them with the results obtained from the cryopreserved shoot tips.

Extraction of *Thymbra spicata* L. var. *spicata* genomic DNA was performed using the DNeasy Plant Mini Kit (Qiagene, Germany). For AFLP analysis, *Mse*I unlabeled primers, shown in (Table 1), were used following the procedure of Vos *et al.* (1995). The separation of the AFLP products (3µl for each sample) was performed in a 6.5% denaturing polyacrylamide sequencing gel (KBPlus 6.5% gel, LI-COR) with 0.5 × TBE electrophoresis buffer using the LI-COR DNA Analyzer (Model 4300 DNA Analyzer, LI-COR, Lincoln NE, USA) following the following the manufacturer's instructions. The gel was scanned on the LI-COR Odyssey® Infrared Imaging System (LI-COR, Lincoln NE, USA) and the resulted bands were then scored manually for the presence or absence in each sample.

Table 1: *Mse*I unlabeled primers used for AFLP analysis

*Mse*I unlabeled primers
M-CTT + E-ACT (IRDye 700), M-CTA + E-ACT (IRDye 700)
M-CTC + E-ACT (IRDye 700), M-CAG + E-ACT (IRDye 700),
M-CTT + E-AAC (IRDye 800), M-CTA + E-AAC (IRDye 800),
M-CTC + E-AAC (IRDye 800
M-CAG + E-AAC (IRDye 800)

2.5. Experimental Design

All treatments were arranged in a Completely Randomized Design (CRD). Each treatment consisted of five replicates with four explants/ replicate. The collected data were statistically analyzed using Statistical Package for the Social Sciences (SPSS) and standard error was calculated for the cryopreservation treatments.

3. Results

3.1. In vitro Conservation Using Vitrification Technique

3.1.1. Effect of Stepwise Application of Plant Vitrification Solution (PVS2)

The results for none-cryopreserved shoot tips of *Thymbra spicata* L. var. *spicata* showed that stepwise application of PVS2 did not affect shoot tips survival or regrowth after exposure to PVS2 as tetrazollium test had revealed a full survival and regrowth of the treated shoot tips (Table 2). On the other hand, data showed that using 2-step application method of PVS2 (60% PVS2 for 10 min followed by 100% PVS2 for 10 min) had improved survival and regrowth rates of the shoot tips after cryopreservation and recorded the highest survival and regrowth rates (80% and 35%), respectively compared to the results obtained when full strength PVS2 was applied to the shoot tips as one shot (Table 2, Figure 1). Meanwhile, 4-step wise application of PVS2 did improve neither survival nor regrowth in *Thmbra spicata* L. var. *spicata* shoot tips after exposure to LN (Table 2)

Direct exposure of explants to full strength PVS2 was found to reduce recovery in many plants before and after cryopreservation, such as in shoot tips of potato (Kaczemarczyk *et al.*, 2008) and grapevine, which was attributed to the toxic nature of PVS2, as it is composed of highly toxic and concentrated ingredients which are applied to promote cell dehydration before exposure to LN (Markovic *et al*., 2013). Also, many related studies were in full agreement with the obtained results, as they reported two- stepwise application of PVS2 as a proper measure to improve survival and regrowth in some plants such as *Phoenix dactylefera* (Subaih *et al*., 2007), *Artemisia herba- alba* (Sharaf, 2010) and *Achelliae fragrantissima* (Younis, 2012). On the other hand, Mogs *et al*. (2004) reported no significant effect of stepwise application of PVS2 on recovery rates of *Samipoulia ionanth* shoot tips after cryogenic conservation.

Table 2: Survival and regrowth percentages of vitrified shoot tips of *Thymbra spicata* L. var. *spicata* as affected by stepwise application of plant vitrification solution PVS2

Non- cryopreserved (-LN)			Cryopreserved (+LN)		
PVS2 concentration (%)	Survival %	Regrowth %	PVS2 concentration (%)	Survival %	Regrowth %
20-40-60-100	100.0 ± 0.0*	100.0 ± 0.0	20-40-60-100	60.0 ± 6.1	10.0 ± 1.0
60-100	100.0 ± 0.0	100.0 ± 0.0	60-100	80.0 ± 9.3	35.00 ± 9.0
100	100.0 ± 0.0	95.0 ± 5. 0	100	20.0 ± 6.2	5.0 ±0.4

Values represent percentages ± standard error

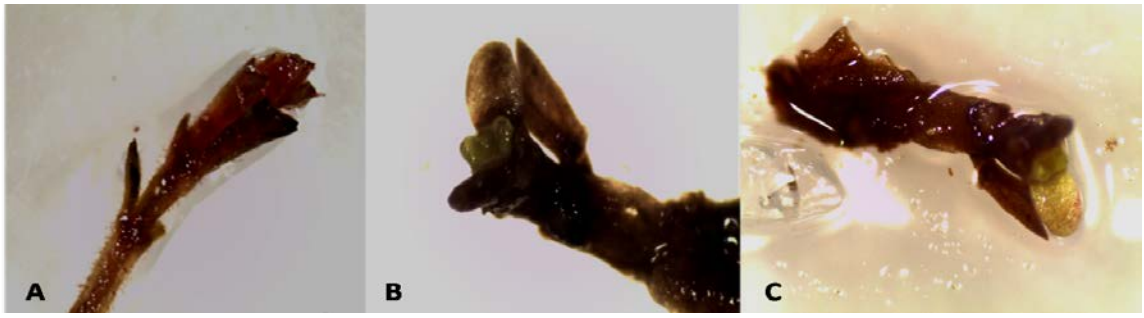

Figure 1. Survival and regrowth of vitrified and cryopreserved shoot tips of *Thymbra spicata* L. var. *spicata* using 2- stepwise application of PVS2 solution method (60% PVS2 for 10 min followed by 100% PVS2 for 10 min). A: Survival using TTC test, B: Start of regrowth after 3 weeks, C: Regrowth after 8 weeks

3.1.2. Effect of Loading Duration

In the second vitrification experiment, data showed that the survival and regrowth rates had decreased slightly when shoot tips of *Thymbra spicata* L. var. *spicata* were not loaded with the loading solution before exposure to PVS2 in the non-cryopreserved shoot tips compared to the other treatments (Table 3). On the other hand, the effect of loading duration of the loading solution had significantly affected both survival and regrowth of after plunging in LN (Table 3). The highest survival (85%) and regrowth (45%) rates were recorded in cryopreserved shoot tips treated with the loading solution for (60 min) (Table 3, Figure 2). Meanwhile, when exposure time to the loading solution exceeded or was less than (60 min), the survival and regrowth rates were adversely affected (Table 3). Sakai and Engelmann (2007) reported that treating the explants before cryopreservation with the Loading Solution (LS) was an effective measure to improve plant cell resistance against the toxic effect of PVS2. However, the survival and regrowth rates in *Thymbra spicata* L. var. *spicata* decreased as exposure durations exceeded or were was less than (60 min). This might indicate that when loading durations to the loading solution were short this could be insufficient for optimum protection of the plant cell against PVS2 toxic hazard and/ or to remove the intracellular water that could have resulted in intracellular ice crystallization after exposure the LN (Al-Ababneh *et al*., 2002; Subaih *et al*., 2007; Kaviani, 2011). Also, the obtained results were in full agreement with Shatnawi *et al.* (2011) who reported a 20%-regrowth in cryopreseved shoot tips of *Caperers spinoza* after 60 min exposure to the loading solution. On the other hand, Mogs *et al*. (2004) reported that exposing African violet for 10-20 min to the loading solution was enough to improve the survival and regrowth after cryopreservation which might indicate that the loading duration is plant-species-dependent.

Table 3: Survival and regrowth percentages of vitrified shoot tips of *Thymbra spicata* L. var. *spicata* as affected by different loading solution exposure durations

Non- cryopreserved (-LN)			Cryopreserved (+LN)		
Duration (min.)	Survival %	Regrowth %	PVS2 concentration (%)	Survival %	Regrowth %
0.0	90.0 ± 6.1*	65.0 ± 12.10	0.0	0.0 ± 0.0	0.0 ± 0.0
10	100.0 ± 0.0	100.0 ± 0.0	10	0.0 ± 0.0	0.0 ± 0.0
20	100.0 ± 0.0	100.0 ± 0.0	20	40.0 ± 0.18	5.0 ± 0.510
40	100.0 ± 0.0	100.0 ± 0.0	40	60.0 ± 10.0	40.0 ± 12.0
60	100.0 ± 0.0	100.0 ± 0.0	60	85.0 ± 10.0	45.0 ± 14.0
80	100.0 ± 0.0	90.0 ± 6.2	80	40.0 ± 0.13	10.0 ± 0.6

*Values represent percentages ± standard error

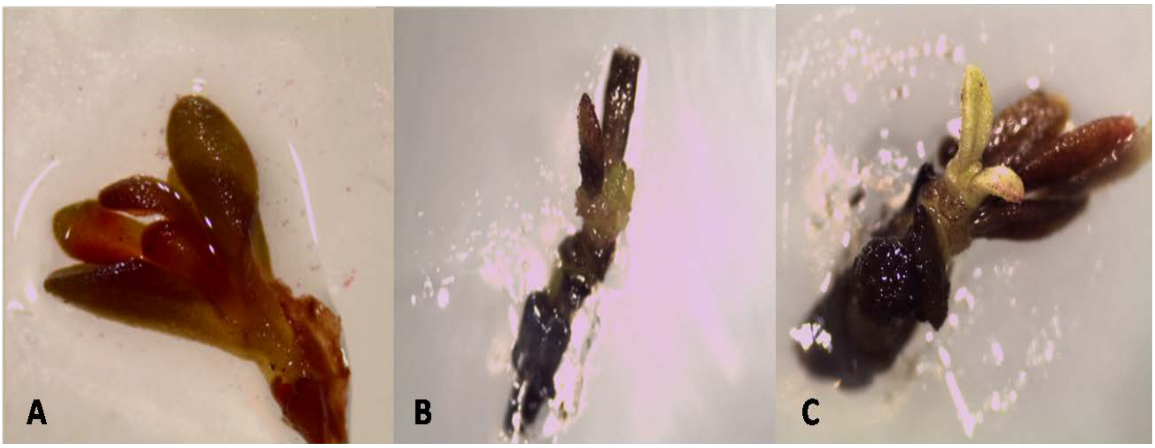

Figure 2: Survival and regrowth of vitrified and cryopreserved shoot tips of *Thymbra spicata* L. var. *spicata* after 60 min loading with the loading solution before exposure to PVS2 and LN. A: Survival using TTC test, B: Start of regrowth after 3 weeks, C: Regrowth after 8 weeks

3.1.3. Effect of Loading Solution Type

The survival and regrowth rates varied with loading solution type in both non cryopreserved and cryopreserved shoot tips of *Thymbra spicata* L. var. *spicata*. The highest survival and regrowth rates were obtained in non-cryopreserved shoot tips loaded with 2 M glycerol + 0.4 M sucrose (Table 4). Also, the highest survival and regrowth rates in the cryopreserved shoot tips of *Thymbra spicata var*. *spicata* were (60%) and(10%), respectively, were recorded only in those treated with 2 M glycerol + 0.4 M sucrose (Table 4). On the other hand, a significant decline in survival rate and/or no survival were recorded by tips treated with the rest types (Table 4). Using 2 M glycerol + 0.4 M sucrose as a loading solution was reported as best combination in many plant species, such as wild crocus (Baghdadi *et al*., 2011), sweet potato (Hirari and Sakai, 2003), potato (Kaczamrczyk *et al*., 2011), thyme (Marcco-Medina *et al.*, 2012), grapevine (Markovic *et al.*, 2013), African violet (Mogs *et al*., 2003), *Artemisia herba alba* (Sharaf *et al.*, 2012), citrus (Sakai *et al.*, 1991) and potato (Zhao *et al.*, 2005) and many others which made it the most popular loading solution described in vitrification protocols (Sakai and Engelmann, 2007). This was attributed to the ability of glycerol to penetrate the cell membrane and yet to reduce the amount of intracellular water unlike the components in the other types which penetration is restricted on cell wall only (Tao and Li, 1986).

Moreover, using 10% DMSO + 0.75 M sucrose combination for cryoprotection had a negative effect on *Thymbra. spicata* L. var. *spicata* shoot tips (Table 4) which might be due to the high toxicity of this combination that yielded a damaging effect on plant cells even before exposure to PVS2 and LN. Meanwhile, the low survival and regrowth rates of cryopreserved shoot tips of *T. spicata* L. var. *spicata* might refer to the short exposure duration (only 20 min) to the loading solution and/or inability of these combinations to increase cell resistance to PVS2 toxicity and cryogenic freezing (Subaih *et al*., 2007; Kaviani, 2011).

3.1.4. Effect of Plant Vitrification Solution (PVS) Type

In the fourth vitrification experiment, survival and regrowth, rates varied according to the type of verification solution PVS used in the three different experiments (Table 4). In the first experiment when (2 M glycerol + 0.4 M sucrose) was used as a loading solution before exposure to LN, the result showed a significant decrease in regrowth (60%) in the non-cryopreserved shoot tips after exposure to PVS3 compared to the other treatments (Table 5).

Markovic *et al*. (2013) reported similar results as the highest recovery rate (50%) was obtained in grapevine exposed to PVS2 compared to complete death resulted in PVS3 treated shoot tips, which was due to the high toxicity of PVS3.

Moreover, after cryopreservation only shoot tips exposed to either PVS2 or (30% DMSO + 1 M sucrose) were able to survive and recover (Table 5) and the maximum survival (75%) and regrowth (35%) rates were recorded in shoot tips exposed to PVS2 before cryopreservation while no survival nor regrowth were obtained in shoot tips treated with (15% DMSO + 1 M sucrose) or PVS3 (Table 4). Meanwhile, the results obtained when (15% DMSO + 1 M sucrose) was used as a vitrification solution were very low (Table 5). This could be due to the fact that this vitrification solution was not a good option as it failed to fulfill proper dehydration requirements needed to prevent ice crystallization and cryogenic injury (Sakai and Engelmann, 2007).

Table 4: Survival and regrowth percentages of vitrified shoot tips of *Thymbra spicata* L. var. *spicata* as affected by different loading solutions

Non- cryopreserved (-LN)			Cryopreserved (+LN)		
Loading solution type	Survival %	Regrowth %	Loading solution type	Survival %	Regrowth %
1M sucrose	100.0 ± 0.0*	65.0 ± 6.10	1M sucrose	0.0 ± 0.0	0.0 ± 0.0
2M glycerol + 0.4M sucrose	100.0 ± 0.0	100.0 ± 0.0	2M glycerol + 0.4M sucrose	60.0 ± 6.1	10.00 ± 2.53
5% DMSO + 0.25M sucrose	95.0 ± 5.0	85.0 ± 10. 0	5% DMSO + 0.25M sucrose	0.0 ± 0.0	0.0 ± 0.0
5% DMSO + 0.5M sucrose	100.0 ± 0.0	95.0 ± 5.0	5% DMSO + 0.5M sucrose	20.0 ± 6.3	0.0 ± 0.0
10% DMSO + 0.25M sucrose	90.0 ± 6.1	70.0 ± 3.51	10% DMSO + 0.25M sucrose	20.0 ± 5.0	0.0 ± 0.0
10% DMSO + 0.5 M sucrose	70.0 ± 9.0	55.0 ± 9.3	10% DMSO + 0.5 M sucrose	0.0 ± 0.0	0.0 ± 0.0

*Values represent percentages ± standard error

In the second treatment when (10% DMSO + 0.5 M sucrose) was used as a loading solution for *Thymbra spicata* L. var. *spicata* shoot tips, PVS2 and (30% DMSO + 1 M sucrose) yielded the higher recovery rates after cryopreservation than those obtained when (10% DMSO + 0.75 M sucrose) was used as a loading solution, as only few shoot tips exposed to either PVS2 or (30% DMSO + 1 M sucrose) survived after cryopreservation (Table 5). This might be a result of double failure of both loading and PVS solutions to minimize the hazards of chemical toxicity that yielded a massive death of shoot tips after cryopreservation (Shatnawi *et al.*, 2011) (Table 5). Moreover, in all experiments data showed that using (2 M glycerol + 0.4 M sucrose) as a loading solution yielded the best results (Table 5).

This was in full agreement with many related research studies in which this loading solution was described as the best formula to be used before exposure to vitrification solution such as results obtained in sweet potato (Hirai and Sakai 2003), thyme (Marcco-Medina *et al.*, 2012), grapevine (Markovic *et al.*, 2013), citrus (Sakai *et al.*, 1991) and potato (Zhao *et al.*, 2005) which made this formula the most widely used in vitrification protocols (Sakai and Engelmann 2007).

However, in a complete contrast to the results obtained in *Thymbra spicata* L. var. *spicata*, Baghdadi *et al*. (2011) reported (15% DMSO + 1 M sucrose) as the best PVS solution for cryopreservation of wild crocus that might lead to a conclusion that the optimum PVS solution needed for successful cryopreservation is species-dependent.

Table 5: Survival and regrowth percentages of vitrified shoot tips of *Thymbra spicata* L. var. *spicata* as affected by vitrification solution type

Loading solution type	Vitrification solution type	Survival %	Regrowth %
Non- cryopreserved (-LN)			
2M glycerol+ 0.4M Sucrose	PVS2	100.0 ± 0.0*	100.0 ± 0.0
	30%DMSO+1M sucrose	100.0 ± 0.0	100.0 ± 0.0
	15%DMSO+1M sucrose	100.0 ± 0.0	100.0 ± 0.0
	PVS3	90.0 ± 6.2	60.0 ± 12.7
Cryopreserved (+LN)			
2M glycerol+ 0.4M Sucrose	PVS2	75.0 ± 11.1	35.00± 3.3
	30%DMSO+1M sucrose	50.0 ± 8.2	15.00± 1.0
	15%DMSO+1M sucrose	0.0 ± 0.0	0.0 ± 0.0
	PVS3	0.0 ± 0.0	0.0 ± 0.0
Non- cryopreserved (-LN)			
10% DMSO+ 0.5M Sucrose	PVS2	100.0 ± 0.0	100.0 ± 0.0
	30%DMSO+1M sucrose	100.0 ± 0.0	100.0 ± 0.0
	15%DMSO+1M sucrose	100.0 ± 0.0	100.0 ± 0.0
	PVS3	95.0 ± 5.0	75.0 ± 11.1
Cryopreserved (+LN)			
10% DMSO+ 0.5M Sucrose	PVS2	45.0 ± 4.0	15. 0 ± 2.07
	30%DMSO+1M sucrose	30.0 ± 7.1	5.0 ± 1.9
	15%DMSO+1M sucrose	0.0 ± 0.0	0.0 ± 0.0
	PVS3	0.0 ± 0.0	0.0 ± 0.0
Non- cryopreserved (-LN)			
10% DMSO+ 0.75M Sucrose	PVS2	100.0 ± 0.0	100.0 ± 0.0
	30%DMSO+1M sucrose	100.0 ± 0.0	100.0 ± 0.0
	15%DMSO+1M sucrose	100.0 ± 0.0	100.0 ± 0.0
	PVS3	100.0 ± 0.0	90.0 ± 6.1
Cryopreserved (+LN)			
10% DMSO+ 0.75M Sucrose	PVS2	25.0 ± 1.8	0.0 ± 0.0
	30%DMSO+1M sucrose	10.0 ± 0.61	0.0 ± 0.0
	15%DMSO+1M sucrose	0.0 ± 0.0	0.0 ± 0.0
	PVS3	0.0 ± 0.0	0.0 ± 0.0

*Values represent percentages ± standard error

3.2. In vitro Conservation Using Droplet Vitrification Technique

The results showed that, in none- cryopreserved shoot tips, a full survival and regrowth rates were recorded in all treatments (Table 6) which could be attributed to the application of PVS2 at 0 °C that optimized the absorption rate of the solution by the cells, and yet eliminated the hazard of chemical toxicity and excessive dehydration during treatment with the PVS2. Similar results were obtained by Matsumoto *et al*. (1998) as they reported very high levels of regrowth in wasabi shoot tips loaded with PVS2 solution at 0°C for up to 60 min without exposure to LN.

Meanwhile, after cryopreservation, survival and regrowth rates of *Thymbra spicata* L. var. *spicata* shoot tips varied with PVS2 exposure duration. The highest survival and regrowth rates were recorded when PVS2 exposure duration was (45 min) as (80% and 35%) survival and regrowth rates were recorded, respectively (Table 6, Figure 3). On the other hand, decreasing PVS2 exposure duration resulted in a decline in both the survival and the regrowth rates or even complete death after cryopresevation (Table 6).

These results agree with Markovic *et al*. (2013), who succeeded in conserving grapevine shoot tips using droplet vitrification technique, where (50 min exposure to PVS2 at 0°C) yielded the highest regrowth rate (Markovic *et al*., 2013). Similarly, a longer exposure period to PVS2 was needed to obtain higher regrowth rates in cryopreserved shoot tips of *Diospyros kaki* where exposure duration of 120 min at 0°C improved significantly the recovery rate (Niu *et al.*, 2010). However, in another related study, cryopreserved wasabi shoot tips had the highest regeneration rate after only (30 min) exposure to full strength PVS2 at 0°C (Matsumoto *et al.*, 1998).

So it can be concluded that the exposure duration to the PVS2 is considered critical for maximizing level of shoot tip recovery after vitrification and cryopreservation and largely affected by the plant species (Sakai and Engelmann 2007). Also, the use of aluminum strips could make it faster and easier to transfer of shoot tips into and out of LN which was described as highly important during the treatment with PVS2 as a slight elongation in exposure durations could be toxic for shoot tips (Kaczmarczyk *et al.*, 2011).

Table 6: Survival and regrowth percentages of vitrified shoot tips of *Thymbra spicata* L. var. *spicata* as affected by different exposure durations to plant vitrification solution PVS2 using droplet vitrification technique

Non-cryopreserved (-LN)			Cryopreserved (+LN)		
Duration (min.)	Survival %	Regrowth %	Duration (min.)	Survival %	Regrowth %
0.0	100.0 ± 0.0*	100.0 ± 0.0	0.0	0.0 ± 0.0	0.0 ± 0.0
10	100.0 ± 0.0	100.0 ± 0.0	10	0.0 ± 0.0	0.0 ± 0.0
20	100.0 ± 0.0	100.0 ± 0.0	20	40.0 ± 10. 0	0.0 ± 0.0
30	100.0 ± 0.0	100.0 ± 0.0	30	55.0 ± 9.0	10.0 ± 0.61
45	100.0 ± 0.0	100.0 ± 0.0	45	80.0 ± 12.2	35.0 ± 1.5

*Values represent percentages ± standard error

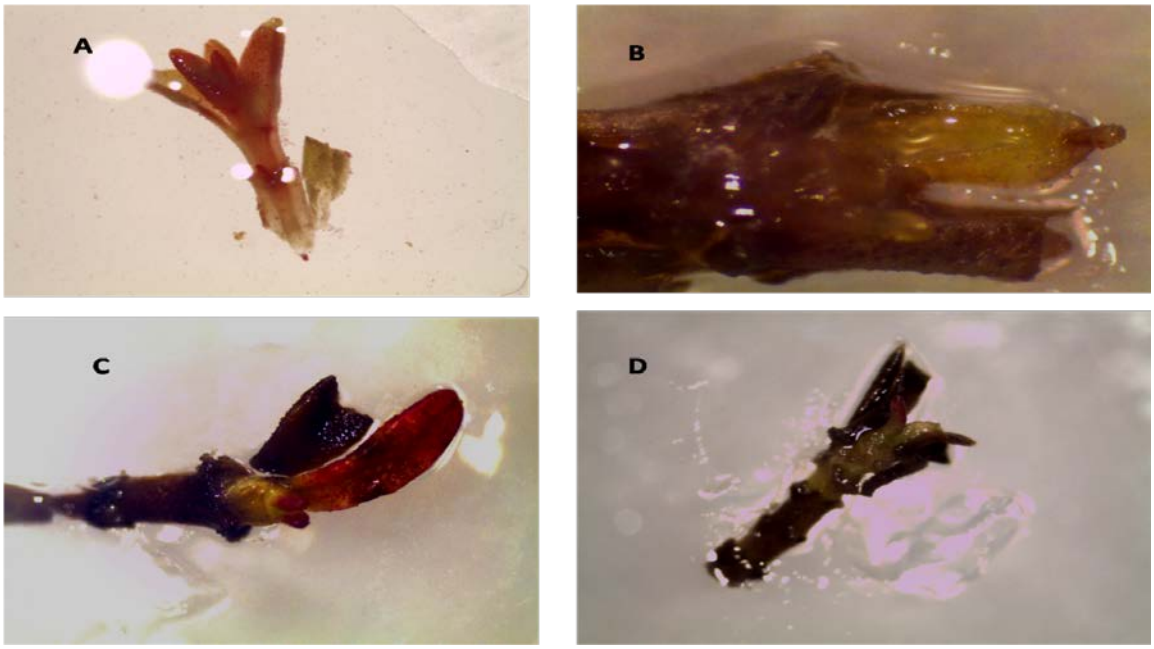

Figure 3. Survival and regrowth of vitrified and cryopreserved shoot tips of *Thymbra spicata* L. var. *spicata* after 45 min exposure to PVS2 at 0 ºC before exposure to LN using droplet vitrification technique. A: Survival using TTC test, B: Start of regrowth after 3 weeks. C: Regrowth after 5 weeks, D: Regrowth after 8 weeks

3.3. Assessment Genetic Stability Using Amplified Fragment Length Polymorphism (AFLP) Technique

No differences between *Thymbra spicata* L. var. *spicata* shoot tips were obtained before and after exposure to liquid nitrogen (Figure 4). Similar results were obtained in other plant species, such as strawberry (Caswell and Kartha, 2009), cork oak (Fernandez *et al.*, 2008), pea (Keller *et al.*, 2006) and *Vanda pumila* (*Orchidaceae*) (Na and Kondo, 1996). Cryopreservation is speculated to maintain the stored material genetically stable and although it might result in cellular injury, there is no clear connection of this injury to any genetic alteration of the stored plants (Kaczmarczyk *et al*., 2012). Furthermore, Harding (2004) reported that no genetic differences were confirmed at morphological, histological and molecular levels in most cryopreserved samples of many species, including Prunus, sugarcane, onion, kiwi, Eucalyptus, coffee, Dendrobium and Cosmos and if any changes happened, this could be attributed to other factors, such as tissue culture, cryoprotection and regeneration process but not only to the cryogenic treatment itself.

Moreover, the results showed genetic differences between the seed-originated cryopreserved shoot tips of *Thymbra spicata* L. var. *spicata* and the wild mother plant sample and another seed-originated sibling tissue cultured plant (Figure 4). The genetic differences between the seed-originated cryopreserved shoot tips of *Thymbra spicata* L. var. *spicata* and the wild mother plant were expected and reflecting the nature of cross pollination existing of this plant in nature (Akine *et al*., 2010).

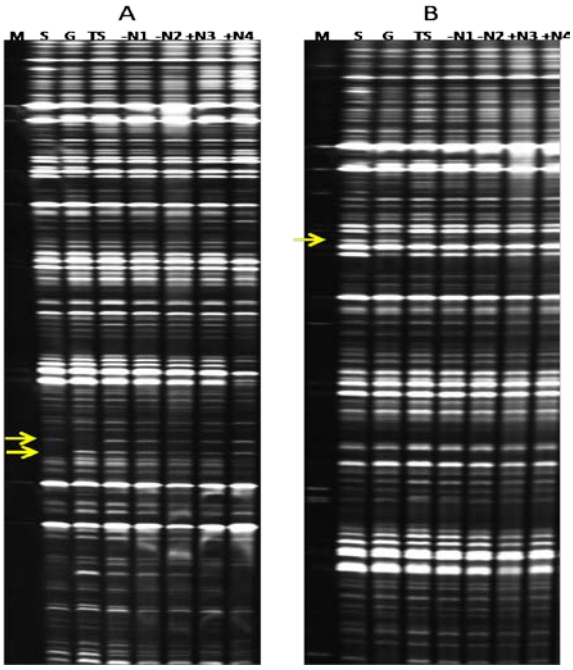

Figure 4. DNA banding patterns using AFLP for *Thymbra spicata* L. var. *spicata*. Pre-amplified DNA products were selectively amplified using two *Eco*RI+*MseI* primer combinations: A= E-ACT IR Dye 700 with M-CTA, B= E-ACT IR Dye 700 with M-CTC. M= DNA Marker, S= Wild mother plant, G= Green house plant, TS= Tissue cultured plant, -N1 = Before cryopreservation, -N2= Before cryopreservation, +N3= After cryopreservation, +N4= After cryopreservation

As a conclusion, endangered *Thymbra spicata* L. var. *spicata* was successfully introduced to cryogenic conservation using vitrification, droplet vitrification and encapsulation- vitrification techniques. Also, cryopreservation proved its ability to maintain genetic fidelity of the stored plants, as AFLP analysis showed no difference between the shoot tips before and after exposure to LN. However, more research on measures to improve recovery rates after cryopreservation is needed, such as preculture treatments, optimizing PVS2 and loading solution exposure duration before exposure to LN, in addition to post thawing and recovery treatments.

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