Vitrification and Droplet-Vitrification Cryopreservation for Wild Artemisia (*Artemisia herba-alba* Asso.) Shoot-tips

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ABSTRACT

This study shows the effect of conserving shoot-tips of *Artemisia herba-alba* via two vitrification based cryopreservation techniques (Vitrification and droplet-vitrification). In vitrification three separate experiments were conducted to study the effect of loading and cryoprotectant solutions and their concentrations on the survival and regrowth of Artemisia shoot-tips, The simplest efficient cryopreservation vitrification dependent technique of *Artemisia herba-alba* shoot tips was by using two-step increased concentration of PVS2 as it gave high survival (89%) and regrowth (81%) percentages after freezing. In droplet vitrification the shoot-tips were vitrified with chilled plant vitrification solution and placed on aluminum foil strips prior to plunge in liquid nitrogen. Droplet-vitrification technique was suited method for conserving *Artemisia herba-alba* shoot tips giving high survival (93.3%) and regrowth (73.3%) after 35 min of exposure to PSV2 and after plunge in liquid nitrogen.

Keywords: *Artemisia*, cryopreservation, droplet, preculture, shoot tips, vitrification.

Abbreviations: MS: Murashige and Skoog Media, PSV2: Plant Vitrification Solution , ST: Shoot tips , BAP: 6-benzylaminopurine, GA3: Gibberrelic acid , DMSO: Dimethylsulfoxide , LN: Liquid Nitrogen , 2ip: 6-(y,y- Dimethylallylamino)purine, TTC: 2,3,5-triphenyltetrazolium chloride

INTRODUCTION

Artemisia is one of the large genera in the family *Asteraceae* and the largest genus in the tribe *Anthemideae*, comprises from 200 to more than 500 taxa at the specific or subspecific level (Al-Eisawi, 1998 & 2003). It is highly represented in Eurasia compared to North America, some of *Artemisia* species have a high economic value for food, medicine, forage, ornamentals and other uses (Abou El-Hamd *et al.,* 2010 ; Abu-Irmaileh and Afifi, 2003; Aburjai *et al*., 2007; Ahmed *et al*., 2004; Al-Momani *et al*., 2007; Al-Mustafa and AL-Thunibat, 2008; Al-Qura'n, 2009; Hedi *et al.*, 2010; Hudaib *et al.,* 2006). *Artemisia* species are conventional plants which are used for the treatment of diseases such as cancer, hepatitis, inflammation and infections by fungi, bacteria, and viruses (Kim *et al*., 2002). *Artemisia herba-alba* is native to Jordan (locally known as Shih) , the plant is used locally as folk medicine due to its high content of essential oils and sesquiterpene lactones and other chemical compounds (Abou El-Hamd *et al.,* 2010; Aburjai *et al*., 2007). Flora of Jordan is rich in *Asteraceae* within its area, as for rest of the countries of the world, the plant commonly found in dry mountains

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and desert regions such as Karak, Tafila, Shaubak, Ra's An-Naqab, Mafraq, Zarka and the Eastern Desert (Al-Eisawi, *et al.,* 1998).

Phytochemical studies of *Artemisia* species exposed that they contain numerous classes of secondary metabolites including: terpenoids, flavonoids, coumarins, glycosides, sterols, and polyacetylenes (Ahmed *et al*., 2004; Saleh *et al*., 1985; Tan *et al.,* 1998). *Artemisia herba-alba* is native to Jordan (locally known as Shih) and it was known for its therapeutic and medicinal properties and was used in both traditional and modern medicine (Abu-Irmaileh & Afifi, 2003). The plant is used locally as folk medicine due to its high content of essential oils and sesquiterpene lactones and other chemical compounds (Abou El-Hamd *et al*., 2010; Aburjai *et al*., 2007). In Jordan, *Artemisia herba-alba* is still underutilized and endangered by extension due to heavy grazing, urbanization and over collection.

Vitrification refers to the physical process in which the aqueous solution solidifies into a meta-stable glass-like in the tissue, at sufficiently low temperatures, with no occurrence of ice crystallization (Sakai & Engelmann 2007). Vitrification techniques depend on exposing explant material to extremely concentrated cryoprotectants at non-freezing temperatures, which results in dehydration of the material (Reed, 2008). The vitrification procedures reduce the need for controlled slow freezing apparatus and enable the cells (meristems) to be cryopreserved by direct immersion in liquid nitrogen (Shibli *et al*., 2004)

Vitrification is one of the main and most widely applied plant cryopreservation methods. It consists of three major phases: the loading phase, dehydration with the highly concentrated vitrification solutions, and unloading phase (Ashmore, 1997). Loading phase involves treatment of samples with cryoprotectants or diluted vitrification solutions (Withers, 1991). By using a highly concentrated vitrification solution, samples were dehydrated (Sakai, *et al*., 1991).

Plant vitrification solution (PVS2): is an aqueous cryoprotectant solution in which living systems can be cooled slowly to below the glass transition temperature without appreciable ice formation either intra or extra-cellularly (Fahy, *et al*., 1987; Kim, *et al*., 2006; Zhao, *et al*., 2005). As a result, water flows out of the cells and dehydration of tissues occurs (Ashmore, 1997). Cryoprotectants must be not toxic at the proper concentration, and with low molecular weight, readily miscible with water, and with ability to penetrate cells rapidly (Reinhoud, *et al*., 1995; Shibli, *et al*., 2006).

Most vitrification protocols apply the plant vitrification solution 2 (PVS2) (Reed, 2008). It is a mixture of cryoprotectants containing 30% w/w glycerol, 15% w/w ethylene glycol, 15% w/w dimethylsulfoxide DMSO in MS medium supplemented with 0.4 M sucrose (Sakai and Engelmann, 2007). Unloading phase; starts after rapid thawing, where vitrification solution is drained out of the cryovials and replaced in most cases with 1.2 M sucrose, or with a lower concentration, for 10-20 min (Shibli, *et al*., 2006; Shatnawi, *et al*., 2011).

Droplet vitrification characterized by optimizing the exposure duration of loading and Plant Vitrification Solution (PVS2) exposure (Grapin *et al*., 2007). This method is the development of a vitrification procedure differs in dealing with cells in order to maintain its vitality during the conservation but it is not widely used. For example, droplet vitrification of banana increased regrowth by 40–50% over standard vitrification procedure (Panis *et al*., 2005). It was also useful for Australian banana (Turner et al., 2001), potato cultivated varieties and wild species (Sarkar et al., 1998), and yam (Leunufna & Keller, 2005).

Kartha *et al.* (1982) developed droplet vitrification in

which the shoot tips of cassava shoot tips were placed in droplets of cryoprotective medium before ultracooling (Sakai and Englemann, 2007). In this technique shoot tips are plated on aluminium foils and dehydrated with cryoprotectants, such as PVS2**(** Kaczmarczyk *et al.*, 2011)**.** The major achievement of this technique is the possibility of obtaining very high cooling/warming rates due to the very small volume of cryoprotective medium in which the explants are placed (Sakai and Englemann, 2007).

Variations in plants and the differential in their nature of growth led to various selections of the cryopreservation methods to suit each plant separately. For example, some plant species were cryopreserved successfully after dehydration without encapsulation (Shibli *et al*., 2004), while droplet vitrification (Grapin *et al*., 2007) and preculture desiccation (Shibli *et al*., 2004) were effectively applied for a number of other species. This study shows the effect of conserving the *in vitro p*roduced shoot tips (ST) of wild Artemisia (*Artemisia herba-alba*) via two vitrification based cryopreservation techniques (vitrification and droplet-vitrification)

MATERIALS AND METHODS

The work was conducted at the Plant Tissue Culture and Plant Biotechnology Laboratory, in the Department of Horticulture and Crop Science, at the University of Jordan, (Amman-Jordan*)*.

In Vitro Culture of Plant Material

In vitro microshoots of *Artemisia herba-alba* was initiated from seeds on Paper-Bridges of filter papers inserted in half MS with 1.0 mg l^{-1} GA3. Shoot tips (ST) used for these experiments were cultured from in vitro seedlings plants on full strength Murashige and Skoog (1962) media (MS) supplemented with 0.5 mg/l 6-benzylaminopurine (BAP) and 1.0 mg/l Gibberrelic acid (GA_3). Subculturing was performed every five weeks using a fresh growth medium to establish sufficient shoot tips stock for experimentations. All cultures were incubated at 25±1 °C under a 16/8 (light/dark) photoperiod of 45 μmol.m^{-2}.s^{-1} irradiance provided by Phillips cool white fluorescent tubes.

Cryopreservation

Vitrification: *Effect of Cryoprotectant Combinations,*

Shoot tips of Artemisia herba-alba were precultured on hormone free solid MS media containing 0.3 M sucrose (Hirai & Sakai 2003) and incubated at 4 ºC for one week. The precultured STs were placed in 2 ml sterile cryovials and loaded for 20 min with 1 ml of a loading solution consisting of hormone free liquid MS medium supplemented either with: 1.0 M sucrose; 2.0 M glycerol plus 0.4 M sucrose; Dimethylsulfoxide (DMSO) 5% and 0.5 M sucrose; or DMSO 10% and 0.5 M sucrose.

Half of the cryovials with the STs (five cryovials for each –LN &+ LN with five shoot tips per replicate/ each trteatment) and cryoprotective solution were plunged in liquid nitrogen (LN) and stored for at least 1 h . The other half were kept without LN. Thawing was performed at 38 ºC for 3 min. The cryoprotective solution for -LN and +LN cryovials were replaced by 1 ml of unloading solution (hormone free MS liquid medium supplemented with 1.2 M sucrose) and this solution was changed 3 times every 10 min. Half of -LN and +LN STs were transferred to recovery conditions and examined under a binocular microscope for recovery signs after two weeks. The other half about 10 shoot tips from each treatment for –LN and 10 shoo tips from each treatment for +LN were used for viability testing using tetrazolium chloride assay (Baghdadi *et al*., 2011; Moges *et al*., 2004).

Effect of the Type of Loading and Vitrification Solutions,

Shoot tips of *Artemisia herba-alba* were precultured on hormone free solid MS medium supplemented with

0.3 M sucrose (Hirai & Sakai 2003) and incubated at 4 ºC for one week. The precultured STs were placed in 2 ml sterile cryovials and loaded with 1 ml of loading solution of hormone free liquid MS medium supplemented either with: 2.0 M glycerol plus 0.4 M sucrose; 5% DMSO and sucrose (0.5 M); or 10% DMSO and sucrose (0.5 M). After 20 min the loading solution was replaced by 1 ml of Plant Vitrification Solution (PVS2) of hormone free liquid medium supplemented either with 15% DMSO and 0.5 M sucrose; or 30% DMSO and 0.5 M sucrose and kept for another 20 min. Then half of the cryovials (5 cryovials with 5 STs and the vitrification solution were directly plunged in liquid nitrogen. Cryovials were thawed in a water bath at 38 ºC for 2-3 min. The vitrification solution in the –LN and +LN cryovials were removed and replaced with unloading solution ((MS media with 1.2 M sucrose), which were changed three times for 10 min. Treated STs were then transferred either to recovery conditions as described earlier and kept for further recovery, or to viability testing.

Effect of PVS2 Concentrations,

Shoot tips of *Artemisia herba-alba* were precultured on hormone free solid MS medium supplemented with 0.3 M sucrose and incubated at 4 ºC for one week. Then precultured STs were placed in 2 ml sterile cryogenic vials and loaded with 1 ml loading solution (MS medium plus 2.0 M glycerol and 0.4 M sucrose) for 20 min (Baghdadi *et al*., 2011). Then the loading solution was removed with a sterile 1 ml micropipette (satnam pipettes) and replaced with 1 ml of vitrification solution (PVS2) containing (w/v) 30% glycerol, 15% dimethylsulfoxide (DMSO) and 15% ethylene glycol (EG) in hormone free liquid medium supplemented with 0.4 M sucrose for 20 min. The cryovials were separated in three groups (each group is consisted of (5 cryovials with 5 shoot tips each) for + LN experiment and the same number was used for – LN experiments). A group was treated with a highly concentrated PVS2 (100%) solution for 20 min. Another third of the STs was treated firstly with 60% PVS2 for 10 min then with 100% PVS2 for another 10 min. The last third of the STs was treated with a step-wise increased concentration (20%, 40%, 60% and 100%) of PVS2 for 20 min; 5 min for each concentration.

Half of the cryovials with the STs and the vitrification solution were plunged in liquid nitrogen (+LN) for at least 1 h and the other half remain without liquid nitrogen (-LN). After exposure to liquid nitrogen; the +LN cryovials were thawed for 3 min at 38 ºC. The vitrification solution for vitrified non-frozen (-LN) and frozen (+LN) STs was removed and replaced with 1 ml unloading solution (liquid MS medium containing 1.2 M sucrose), which was changed three times for 10 min. Half of the STs (-LN and +LN) were then cultured on recovery medium containing 0.1 M sucrose, 1.0 mg/l 2iP and incubated in dark for 3 days then transfer to the growth room as described earlier and kept for further recovery. The examination of survival was done under a binocular microscope directly after each treatment and for recovery signs after two to four weeks. The other half of the -LN and +LN STs were tested for survival using TTC assay under a binocular microscope.

Droplet-Vitrification

Shoot-tips (STs) of *Artemisia herba-alba* were precultured on hormone free MS media supplemented with 0.3 M sucrose and incubated at 4 ºC for one week. The STs were immersed in a small beaker (50 ml) containing 20 ml of sterilized loading solution that contained 2 M glycerol and 0.4 M sucrose dissolved in MS medium (pH 5.8) for 20 min at 25 ºC (Sakai *et al*., 1990). After loading, the solution was replaced with 20 ml of chilled PVS2 solution. The sterilized PVS2 solution consisted of 30% (w/v) glycerol, 15% (w/v) ethylene glycol and 15% (w/v) DMSO in liquid MS medium containing 0.4 M sucrose at pH 5.8 (Sakai *et al*.,

1990). Just before the end of the PVS2 treatment time, STs were sucked up in a sterile disposable micropipette (0.25 ml) and placed on a strip of sterile pieces of aluminum foil (2 cm length × 1.0 cm width × 0.005 cm depth). Five minutes prior to use, the strip was placed on a plastic Petri dish resting on top of a refrigerator to maintain the temperature around 0 ºC for 1 hour.

After the PVS2 treatment (1 hour in a refrigerator at4 ºC), the aluminum strips and the STs with iced PVS2 were plunged directly into cryovial. The cryovials with the strips were immersed directly in LN for at least 1 h. Rapid thawing was executed by removing the aluminum strip from liquid nitrogen with fine forceps and quickly treated with 1 ml of sterilized unloading solution (liquid MS medium with 1.2 M sucrose at pH 5.8) (Sakai *et al*., 1990), for 15 min at 25 ºC. Half of these STs were then plated on solid MS medium supplemented with 0.1 M sucrose and 1.0 mg/l 2iP. These Petri-dishes were transferred to dark conditions in the refrigerator at 4 ºC over night followed by five days in the dark in the growth room at 25 ºC before transfer to normal growth conditions. Regrowth was recorded after four weeks following cryopreservation. The other half of the treated non-cryopreserved (-LN) and cryopreserved (+LN) STs were transferred to test survival using TTC test.

Experimental Design and Statistical Analysis:

Treatments were arranged in a completely randomized design (CRD). Each treatment was replicated five times with five shoot tips per replicate. The collected data were statistically analyzed using Statistical Analysis System (SAS program). Means were separated according to the least significant difference (LSD) at 0.05 probability level.

RESULTS AND DISSCUSSION

In vitro Establishment

Maximum germination of seeds (94%) was obtained at Paper-Bridge inserted in half MS with 1.0 mg l^{-1} GA_3. Highest proliferation of *Artemisia herba-alba* (23.6) microshoots numbers and length were obtained when MS medium was supplemented with 0.5 mg l^{-1} 6-benzylaminopurine (BAP) and 1.0 mg l^{-1} Gibberrelic acid (GA_3), as shown in Figure 1. A bout 0.7 mm shoot tip height were selected and precultured to be used for cryopreservation treatment.

Figure 1: In vitro propagation of *Artemisia herba-alba* on MS media supplemented with 0.5 mg l^{-1} 6-benzylaminopurine (BAP) and 1.0 mg l^{-1} Gibberrelic acid (GA_3)..

Vitrification

Significant variations in survival and regrowth of non-cryopreserved (-LN) and cryopreserved (+LN) *Artemisia herba-alba* shoot tips were obtained within the various cryoprotectants combinations (Table 1). Figure 2 &3 showing the survival and regowth; respectively of shoot tips after being cryoprserved by different cryoprotectant combinations.

Maximum survival (100-98 %) of non-cryopreserved shoot tips was achieved when shoot tips of *Artemisia herba-alba* shoot tips were cryoprotected with 1.0 M

sucrose or with 0.4 M sucrose plus 2 M glycerol for 20 min (Table 1). The survival was experimented with TTC test and a red color was an indication to survival. For high survival percentage dark red was obtained and for less survival perccentges red and light red colors were obtained respectively. High survival (91%) rates for non-cryopreserved *Artemisia herba-alba* shoot tips were also achieved with 5% DMSO plus 0.5 M sucrose. After cryopreservation (+LN), the highest survival percentage (46 %) of shoot tips of *Artemisia herba-alba* was obtained when shoot tips were cryoprotected with 0.4 M glycerol plus 2.0 M sucrose (Table1).

In the current study, minimum (84%) survival of non-cryopreserved (-LN) shoot tips of *Artemisia herba-alba* was obtained, when shoot tips were pretreated with 10% DMSO plus 0.5 M sucrose with 74% regrowth (Table1). The variations obtained in survival (Table 1) for the various cryoprotectant combinations tested might be due to their differences in permeability inside plant tissues of shoot tips and ability to induce osmotic stress and toxicity effect (Al-Ababneh *et al*., 2011; Reed, 2008). DMSO as a cryoprotectant solution was successful in increasing intra-cellular viscosity and thus avoiding formation of ice crystals (Subaih *et al*., 2006). Increasing survival percentage obtained for non-cryopreserved (-LN) *Artemisia herba-alba* shoot tips (Table 1) might be due to the cryoprotectants mixture tested, were probably not toxic or the duration of exposure was not long enough to cause destructive effect on cell wall activity (Subaih *et al*., 2006; Reed, 2008). After cryopreservation (+LN), the highest survival percentage (46 %) of *Artemisia herba-alba* shoot tips (Table 1) was obtained when shoot tips were cryoprotected with 0.4 M glycerol plus 2.0 M sucrose (Table1). Similarly, the highest survival (53.3%) of date palm calli was obtained when calli were cryoprotected with 0.4 M glycerol plus 2.0 M sucrose (Subaih, 2005). Moges *et al.* (2004) found that higher survival (95%) of non-cryopreserved African violet shoot tips was achieved when shoot tips were cryoprotected with 0.4 M sucrose plus 2 M glycerol at 25 ˚C for 20 min. In addition, Al-Ababneh *et al.* (2003) reported that maximum survival (96.7%) of non-cryopreserved sour orange shoot tips were obtained after using 5% DMSO with 1.0 M sucrose for 20 min at 25 ˚C. Moreover, Baghdadi *et al*. (2011) found (44.4-55.6%) survival calli of *Crocus hyemalis* and *Crocus moabiticus* respectively was obtained when calli were cryoprotected with 0.4 M glycerol plus 2.0 M sucrose.

Moreover, survival after freezing occurred for *Artemisia herba-alba* shoot tips was not completely lost, it gave (36%) at the combination of 10% DMSO plus 0.5 M sucrose (Table 1). Moges *et al.* (2004) reported a complete loss of survival of cryopreserved African violet shoot tips was occurred when shoot tips were pretreated with 10% DMSO plus 0.5 M sucrose; this might be attributed to osmotic stress resulting from increased sucrose concentration.

After cryopreservation (+LN), the survival rates decreased (Table 1); this might be due to cryoprotectants mixture, were possibly not able to produce high freezing tolerance (Subaih *et al*., 2006). Variation in color intensity in TTC test was observed for both non-cryopreserved (-LN) and cryopreserved (+LN) *Artemisia herba-alba* shoot tips (Table 1). The variation in red color intensity indicated the level of cell activity. This might indicate that, not all cells or tissues in the shoot tips survived due to cryo-injury (Benson *et al*., 1996).

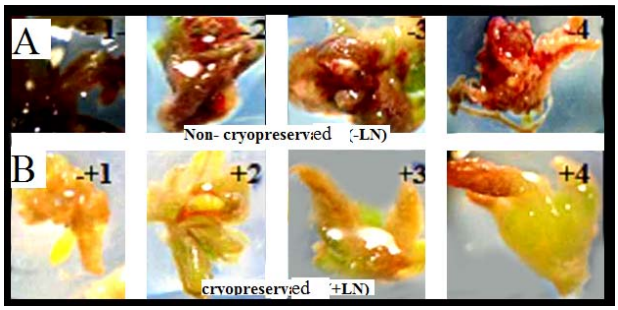

Figure 2: Effect of loading solution on survival and red color of shoot tips of *Artemisia herba-alba* (A): Before cryopreservation (-LN) from(-1 to -4), and (B): After cryopreservation (+LN) from (+1 to +4):

1. **1.0 M sucrose;**
2. **2.0 M glycerol + 0.4 M sucrose;**
3. **5% DMSO + 0.5 M sucrose;**
4. **10% DMSO + 0.5 M sucrose.**

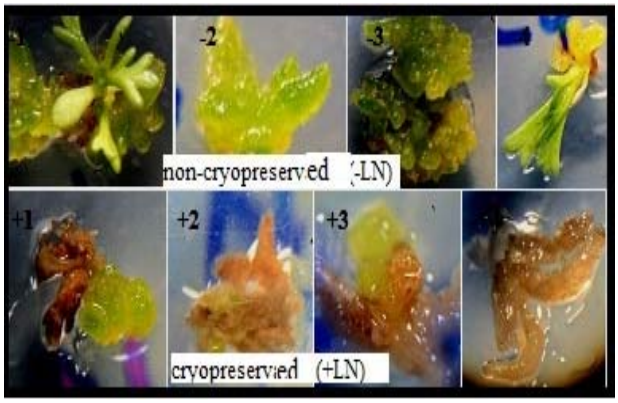

Figure 3: Effect of loading solution on regrowth percentages for shoot tips of *Artemisia herba-alba* : Non- cryopresered shoot tips (-LN) from(-1 to -4), and after cryopresered shoot tips (+LN) from (+1 to +4) with Green callus formation after 40 days of incubation in growth room conditions.

1. **1.0 M sucrose;**
2. **2.0 M glycerol + 0.4 M sucrose;**
3. **5% DMSO + 0.5 M sucrose;**
4. **10% DMSO + 0.5 M sucrose.**

Table 1. Effect of loading solution on survival and regrowth percentages and red color intensity in TTC test of non-cryopreserved (-LN) and cryopreserved (+LN) shoot tips of *Artemisia herba-alba.*

Cryoprotectant	Survival	Regrowt	Red
Non-cryopreserved (-LN)			
1.0 M sucrose	100 a	96 a	Dark
2.0 M glycerol + 0.4 M	98 ab	90 ab	Red
5% DMSO + 0.5 M	91 bc	82 bc	Red
10% DMSO + 0.5 M	84 c	74 c	Red
Cryopreserved (+LN)			
1.0 M sucrose	44 a	14 bc	Light
2.0 M glycerol + 0.4 M	46 a	34 a	Light
5% DMSO + 0.5 M	16 b	4 c	Light
10% DMSO + 0.5 M	36 a	24 bc	Light

* Means within column having different letters are significantly different according to LSD= at $P \leq 0.05$.

Effect of Loading Solution Type and Vitrification Solution, Significant differences in term of survival of non-cryopreserved and cryopreserved *Artemisia herba-alba* shoot tips were obtained for the different loading solutions and vitrification solutions combination (Table 2). A complete survival of non-cryopreserved Artemisia herba-alba shoot tips was obtained with all loading and vitrification solutions except the combination of loading solution (10% DMSO + 0.5 M sucrose) and cryoprotectant (30% DMSO + 0.5 M sucrose) (Table 2).

For most loading and vitrification solutions combinations, low regrowth rate for non-cryopreserved shoots was attained; about 50% of shoot tips with brown leaf color were obtained. Maximum regrowth 33% with green color and green callus in the base was achieved in both loading solution of 10% DMSO plus 0.5 M sucrose and vitrification solution 15% DMSO plus 0.5 M sucrose.

After cryopreservation(+LN), the highest survival of shoot tips (93%) was obtained after loading solution with 2.0 M glycerol plus 0.4 M sucrose and vitrification with cryoprotectant 30% DMSO plus 0.5 M Sucrose (Table 2). Maximum regrowth of cryopreserved shoot tips (23%) was obtained by loading solution (10% DMSO + 0.5 M sucrose) and cryoprotectant (15% DMSO + 0.5 M sucrose).

Variation in leaves color was observed in non-cryopreserved and cryopreserved shoot tips with vitrification solution combinations, increasing the concentration of vitrification solution result in pale-green leaf color. Significant effects were occurred for the different loading solution and vitrification solution combinations (Table 2); Moges *et al*. (2011), also reported significant differences in terms of survival and regrowth of non-cryopreserved and cryopreserved African violet shoot tips for different loading solutions and vitrification solutions combinations tested. Baghdadi *et al.* (2011) found significant differences in survival of cryopreserved crocus calli were obtained for the different loading solutions and vitrification solutions combination tested. Sarkar and Naik (1998) demonstrated that loading phase was necessary to reduce osmotic shock caused by direct exposure of precultured explants to a highly concentrated PVS2.

Survival of unfrozen shoot tips of *A. herba-alba* was higher than survival of frozen shoot tips (Table 2) due to the formation of intracellular ice crystals during freezing or thawing (Al-Ababneh *et al*., 2002) which can be attributed to inadequate dehydration of shoot tips (Reed, 2008). Sakai (2000) reported that complete vitrification of cryopreserved plant tissues would reduce the concerns for the potentially damaging effects of intra- and extra-cellular crystallization and could lead to elevated survival percentages.

There was a significant difference in terms of regrowth among non-cryopreserved or cryopreserved shoot tips (Table 2). Baghdadi *et al*., (2011) reported that the highest survival of cryopreserved calli (27.8-55.6%) was obtained after cryoprotection with 1.0 M sucrose plus 15% DMSO and dehydration with 2.0 M glycerol plus 0.4 M sucrose prior to freezing in *Crocus hyemalis* and *Crocus moabiticus*, respectively. Not all survived shoot tips were able to regrow due to the fact that only a group of cells often localized in primordia of leaf tissues and meristematic dome area remains alive after stress of freezing and thawing (Matsumoto, 1994). This might also be attributed to insufficient, dehydration of shoot tips (Sakai, 2000). Changing color of the *Artemisia herba-alba* shoot-tips (Table 2) may be due to osmotic stress caused by vitrification solution (Subaih *et al*., 2006) and water depletion during dehydration (Shibli et al., 2006).

Table 2. Effect of loading solution type and vitrification solution combinations on survival and regrowth percentages and leaf color of non-cryopreserved (-LN) and cryopreserved (+LN) *Artemisia herba-alba* shoot tips.

Loading Solution	Cryoprotectant Mixture	Conserving	Survival %	Regrowth %	Leaf color
1	**A**	**-LN**	100 a	26 a	Green
		+LN	80 ab	20 ab	Pale-green
	B	**-LN**	100 a	6 b	Pale-green
		+LN	93 a	11 b	Pale-green

Loading Solution	Cryoprotectant Mixture	Conserving	Survival %	Regrowth %	Leaf color
2	**A**	**-LN**	100 a	33 a	Green
		+LN	86 ab	10 bc	Pale-green
	B	**-LN**	100 a	6 b	Green
		+LN	86 ab	8 c	Pale-green
3	**A**	**-LN**	100 a	33 a	Pale-green
		+LN	73 bc	23 a	Pale-green
	B	**-LN**	73 bc	10 b	Pale-green
		+LN	66 c	6 b	Pale-green

* 1) 2.0 M glycerol + 0.4 M sucrose; 2) 5% DMSO + 0.5 M sucrose; 3) 10% DMSO + 0.5 M sucrose

** A) 15% DMSO + 0.5 M sucrose; B) 30% DMSO + 0.5 M sucrose

***Means within column having different letters are significantly different according to LSD at $P \leq 0.05$.

Significant variations were obtained in terms of survival and regrowth of non-cryopreserved and cryopreserved shoot tips among the different concentrations of PVS2 (Table 3). Complete survival (99-100%) was obtained for non-cryopreserved *Artemisia herba-alba* shoot tips irrespective to concentration of the PVS2 after 20 min treatment (Table 3).

After cryopreservation (+LN), high survival (89 %) was obtained when shoot tips were treated with two-step increased concentration of PVS2 for *Artemisia herba-alba* shoot tips (Table 3), whereas, the maximum regrowth (81%) of shoots tips with green color appearance was also obtained when shoot tips were dehydrated with two-step increased concentration of PVS2. It might also be due to synthesis of toxic material resulting from stresses during cryopreservation and/or cellular damage during freezing and thawing (Baghdadi et al., 2011)

The complete survival rates (Table 3) obtained irrespective to PVS2 concentration (Table 3) was also reported by Moges *et al.* (2004). Moreover, Al-Ababneh (2002) reported a complete survival and 97.5% regrowth with green appearance for non-cryopreserved sour orange shoot tips after dehydration with 100% PVS2 at 25 ˚C for 10 min and then at 0 ˚C for 10 min. Furthermore, , Subaih *et al.,* (2006), elucidated high survival (86.7-100%) was obtained for non-cryopreserved date palm (*Phoenix dactylifera*) embryogenic callus treated with a four or two step-wise increased concentration of PVS2 at 25 °C for 20 min. Moreover, Baghdadi *et al*., (2011) reported that complete survival (100%) was obtained for non-cryopreserved crocus calli treated with a four step-wise increased concentration of PVS2 for 20 min.

After cryopreservation, two-step dehydration of *Artemisia herba-alba* shoot tips with PVS2 gave the highest survival and regrowth rates (Table 3). Moges et al., (2004) obtained survival of African violet shoot tips was obtained after cryopreservation, irrespective of concentrations of PVS2. Al-Ababneh, (2003) reported that the changes in color of the cryopreserved shoot tips might be attributed to osmotic shock or unfavorable regrowth conditions.

Table 3. Effect of the concentration of Vitrification Solution PVS2 on survival and regrowth percentages and leaf color of non-cryopreserved (-LN) and cryopreserved (+LN) shoot tips of *Artemisia herba-alba.*

PVS2	Survival	Regrowth	Leaf
Vitrified non-cryopreserved (-LN)			
A*	100**a	99 a	Green
B	100 a	96 a	Green
C	99 a	96 a	Green
Vitrified cryopreserved (+LN)			
A	69 c	51 c	Pale-
B	89 a	81 a	Green
C	75 b	66 b	Green

** Means within column having different letters are significantly different according to LSD at $P \leq 0.05$

A) 100% PVS2 solution for 20 min. B) 60% PVS2 for 10 min then with 100% PVS2 for another 10 min. C) Step-wise increased concentration (20%, 40%, 60% and 100%) of PVS2 for 5 min for each concentration.

Droplet-vitrification

Significant variations in terms of survival and regrowth for vitrified-non cryopreserved and vitrified-cryopreserved shoot tips were observed among the different duration of dehydration with 100% PVS2 at 0 °C (Table 4). Vitrified non-cryopreserved (-LN) *Artemisia herba-alba* shoot tips gave complete survival (100%) irrespective to PVS2 exposure duration except for 45 min (Table 4); while complete regrowth (100%) was obtained when vitrified non-cryopreserved shoot tips were dehydrated with concentrated PVS2 solution (100%) for 0.0 and 10 min (Table 4).

The survival rate of vitrified-cryopreserved (+LN) shoot tips was increased when the PVS2 treatment time was increased from 20 min to 35 min (86.6-93.3%; respectively), but when the duration prolonged to 45 min survival decreased. The trends for regrowth percentage were same as those for survival, and as shown in (Table 4). In this experiment, 35 mins PVS2 duration gave the highest shoot tips regrowth after cryopreservation.

In droplet vitrification prolonging the duration of dehydration resulted in decreasing the regrowth rate (Table 4). This indicated that the shoot tips were injured in prolonged duration due to overexposure to the PVS2 solution during the dehydration, and therefore resulted in reducing regrowth percentage (Subaih et al., 2006).

Table 4. Effect of droplet-vitrification procedure and exposure duration to PVS2 solutions on survival and regrowth percentages and leaf color of non-cryopreserved (-LN) and cryopreserved (+LN) shoot tips of *Artemisia herba-alba*.

PVS2	Survival	Regrowth	Leaf color
Non-cryopreserved (-LN)			
0	100 a*	100 a	Green
10	100 a	100 a	Green
20	100 a	93 a	Green
35	100 a	93 a	Green
45	93 a	86 b	Green
Cryopreserved (+LN)			
0	53.3 c	6.70 c	Yellow-
10	66.7 bc	20.0 c	Pale green
20	86.8 ab	53.3 ab	Pale green
35	93.3 a	73.3 a	Pale green
45	66.8 c	46.7 b	Pale green

* Means within column having different letters are significantly different according to LSD at $P \leq 0.05$.

CONCLUSION

It can be concluded that increasing the duration of exposure to a highly concentrated PVS2 decreased the

viability of shoot tips. The present study described for the first time a simple and efficient cryopreservation vitrification dependent technique for *Artemisia herba-alba* shoot tips by using two-step increased concentration of PVS2 (dehydrated with 60% then by 100%). Droplet-vitrification technique was the most suited method for conserving *in vitro Artemisia herba-alba* shoot tips.

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الحفظ طويل الأمد بالتبريد بواسطة التزجيج و طريقة التزجيج بالقطرات للقمم الطرفية في نبات الشيح البري

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ملخص

تبين هذه الدراسة تأثير الحفظ لقمم النامية نبات الشيح عن طريق اثنين من طرق التزجيج بالحفظ بالتبريد وهي (التزجيج والتزجيج بالقطرات) . في التزجيج أجريت ثلاث تجارب منفصلة لدراسة تأثير محاليل التحميل ومحاليل الحفظ بالتبريد وتركيزاتها على حيوية و إعادة نمو القمم النامية نبات الشيح. وكانت أبسط تقنية و افضل كفاءة في الحفظ بالتبريد عن طريق التزجيج القمم النامية نبات الشيح باستخدام خطوتين من زيادة تركيز محلول التزجيج للحفظ بالتبريد حيث أعطى نسبة بقاء (حيوية) عالية (89 ٪) و إعادة نمو (81 ٪) بعد التجميد. في طريقة التزجيج بالقطرات تم تزجيج السويقات في محلول تزجيج مبرد للنبات وتم وضعها على شرائح من ورق الألومنيوم قبل غمرها في النيتروجين السائل. وأما تقنية التزجيج بالقطرات فكانت اكثر طريقة ملاءمة للحفاظ على نمو القمم النامية لنبات الشيح حيث اعطت نسبة بقاء (حيوية) عالية (93.3 ٪) و إعادة نمو (73.3 ٪) بعد 35 دقيقة من التعرض لمحلول التزجيج و بعد وضعه في النيتروجين السائل.

الكلمات الدالة: نبات الشيح، الحفظ بالتبريد، القطيرات، الزراعة المسبقة، قمم نامية، الحفظ بالتزجيج.

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