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Cryopreservation of *Aerva lanata* (L.) shoot tips

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Abstract
Shoot tips from the established in vitro shoot cultures of Aerva lanata L., *a rare medicinal plant, were successfully cryopreserved at –196˚C using the vitrification procedure. Effect of the concentration of sucrose for preculture, loading solution, vitrification treatment with plant vitrification solution 2 (PVS2), and recovery medium on the cryopreservation of shoot tips has been analyzed. Shoot tips were precultured on hormone-free MS solidified medium supplemented with 0.3 M sucrose for 3 days and then treated with a mixture of 2.0 M glycerol plus 0.4 M sucrose (LS solution) for 20 min at25 ˚C, shoot tips were treated with thePVS2 vitrification solution and plunged directly into liquid nitrogen. Higher viability of shoot tips was obtained when using a step-wise dehydration of the explants rather than direct exposure to 100% (PVS2). The highest value of survival percentage (76.7- 93.3%) and 63.3-86.7 % regrowth was achieved with two or four step-wise dehydration, respectively with PVS2 at 25˚C for 20 min prior to freezing. The effect of different cryoprotectants on survival of shoot tip was also tested. The use of 2.0 M glycerol plus 0.4M sucrose or 10% dimethyl sulfoxide (DMSO) plus 0.5M sucrose as a cryoprotectant resulted in 56.7% survival of shoots. Furthermore, the effect of combinations of different loading and vitrification solutions on viability of shoot tip was tested. The greatest survival (90-83.3%) and maximum regrowth (80- 66.7%) were obtained when shoot tips were cryoprotected with 10% DMSO plus 0.5M sucrose followed by dehydration with 100% PVS2 or 2.0 M glycerol plus 0.4 M sucrose followed by dehydration with15% DMSO plus 1.0 M sucrose, respectively. Shoot tips cryoprotected with 2M glycerol plus 0.4M sucrose for 20 min exhibited the highest survival (100%) and the highest regrowth (56.7%).Subculturing of the recovered shoot tips in MS medium supplemented with 4.44µM6-benzyl adenine (BA) and 2.14µM α-naphthaleneacetic acid (NAA) resulted plantlet production. Regenerated plantlets were cultured in pots and successfully transferred to the greenhouse.*

Key words: *Aerva lanata; shoot tips; cryopreservation; vitrification; cryoprotectant; PVS2.*

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I. Introduction

Aerva lanata belonging to the family Amaranthaceae is a rare perennial shrub or erect herb growing in the Gabal Elba protected southeast corner of the Eastern Desert of Egypt (Bolous 2009; Hassanen2021)the plant has effective secondary compounds that are used in curative applications in traditional and folk medicine in many regions of the world (Bitasta and Madan 2016). These active compounds consist of phytochemicals such as flavonoids, alkaloids, phenolics, steroids, terpenoids, tannin and saponins (Kamalanathan and Natarajan 2014; Akanjiet al.2018; Al-Ansari et al.2019). These compounds are known for their properties, pharmacological activity and use widely such as antiurolithiatic, diuretic, hepatoprotective, anticancer, antimicrobial, antioxidant, antihyperlipidemic, antidiabetic, immunomodulatory, antihelminthic and antiflammatory (Akanjiet al.2018;Musaddiq et al. 2018; Singh et al.2020). *A. lanata* in natural populations is very threatened because it has a narrow germplasm base and continuous exploitation of the plant due to its many precious medicinal properties. Furthermore, traditional seed propagation is a slow process. Also, the fruit set is low, seed viability and germination are poor. Due to the indicated medicinal importance and the limitation of the plant, it is therefore important to preserve it as an important genetic resource.

Cryopreservation is currently considered as a powerful tool and an ideal safe mean for long-term conservation of plant genetic resources (Engelmann 2011; Wang et al. 2014, 2018; Li et al. 2018). Furthermore, cryopreservation has also been reported to offers real hope for the long-term preservation of rare and endangered species., either in the form of seeds or of in vitro cultures. Therefore, the vitrification method is a promising technique for shoot-tip cryopreservation in liquid nitrogen (LN) at -196°C. The ultra-fast cooling/warming rates are crucial to avoid lethal ice crystal formation (Condello et al.2011; Sakai and Engelmann2007).Cryopreservation is used for the long-term storage of biological tissues due to the extremely low temperature of the liquid nitrogen (LN) arresting all chemical and physical reaction sallowing long-term

storage (Engelmann, 1997).These cryopreservation protocols are based on the vitrification of intracellular solutes, thus avoiding the formation of intracellular ice crystals, which destroy cellular components, compromising their survival. Crystallization is avoided by extracting most or all the freezable water from tissues by osmotic and/or physical dehydration before freezing and by using very high cooling rates. The highly concentrated intracellular solutes reach an amorphous glassy stat (i.e. become vitrified) upon rapid cooling of plant samples by direct immersion in liquid nitrogen (Engelmann 2011; Kulus 2019).

Many cryopreservation techniques such as simple freezing, vitrification, encap-sulation/dehydration and encapsulation/vitrification have been reported for successful use for many cells, tissues and organs of plant species (Engelmann, 2000; Grout, 1995). However, for successful cryopre-servation, many factors are involved, such as starting materials, pretreatment conditions, cry procedures and post-thaw treatment (Reinhoud et al., 2000).

Cryopreservation is normally performed using shoot apices, 1-3 mm long, as such explants are composed of actively dividing meristematic cells, which have a small size, a dense cytoplasm, and a low number of vacuoles. Cells displaying such characteristics are more tolerant to desiccation and freezing stress, thus leading to high recovery percentages after rewarming (Ashmore 1997). Studies performed with endangered species producing low percentages of viable seeds (Gonzalez-Benito and Perez 1997; Turner et al. 2001) have demonstrated that cryopreservation of vegetative explants could play an important role in conservation of genetic resources of these species. In this study, cryopreservation of *A. lanata* was investigated employing the vitrification technique. As in classical vitrification protocols, explants are osmo-protected with a loading solution, then dehydrated with a vitrification solution before immersion in LN. Cooling is performed by placing the explants in 1.2mm of VS distributed in cryotube, which are directly plunged in LN (Sakai and Engelmann 2007).Rewarming rapidly is performed by direct plunging the cryotubes in a water bath (45˚C) for 1min then then move to 25˚C water for 1-2 min. The explants are then rinsed with washing solution and placed in a recovery medium.

As far as the researcher knows, there is no study on cryopreserving the *A. lanata* by vitrification shoot tips protocol and investigated the effects of various vitrification solution (VSs), loading solution (LS) and duration of LS on explant survival and recovery. Hence, this study aimed to develop an efficient cryopreserved shoot tips for conserving *A. lanata* as a rare medicinal herb in Egypt.

II. Materials And Methods

Plant Material

Micro- shoot cultures of *Aerva lanata* were maintained by subculturing every four weeks on MS (Murashige and Skoog,1962) medium with 30 g/l sucrose, 100 mg/l meso inositol and solidified with 2.75 g/l (w/v) phytagel (Duchefa, Haarlem, Netherlands)and supplemented with 4.44 µM benzyladenine (BA) and2.14 µM naphthalene acetic acid(NAA)to establish sufficient plant stock. Cultures were maintained at 24±2˚C with 16 light/8 h dark provided by cool-white fluorescent lamps (F140 t9d/ 38, Toshiba) at a photosynthetic photon flux density (PPFD) of approximately40 µmol m^{-2} s^{-1}. Shoot tips (1–3 mm) with two or three non-expanded leaf primordia were aseptically excised from healthy four weeks-old plantlets and used for experimentation.

Cryogenic procedure

Preculture of shoot tips: Excised shoot tips (1.0-3.0 mm in length) containing 2–3 young leaf primordia were precultured on hormone-free solid MS medium supplemented with different concentrations(0.1, 0.3, 0.5nd 0.7 M) of sucrose and incubated for 1,2, and 3 days at 25°C. Precultured shoot tips were then treated with loading solution (LS) for osmo-protected explants before being dehydrated with a highly concentrated vitrification solution (PVSs).

Effect of plant vitrification solution concentration: Precultured shoot tips were placed in a1.8 ml sterile cryotube (Photo 1.A and B) and loaded with 1.8 ml of a loading solution (MS medium containing 2.0M glycerol plus 0.4M sucrose) for 20 min at 25˚C (Nishizawa et al. 1992 ;Matsumoto et al. 1994). The loading solution was removed and replaced with 1.8 ml of PVS2 solution [30% glycerol,15% DMSO, and 15% ethylene glycol (EG; w/v)in a hormone-free liquid medium supplemented with 0.4M sucrose) (PVS2; Sakai et al.1990)]. One-third (N=30) of the cryoprotected shoot tips were treated with 100% PVS2 for 20 min and one third were treated with 60% PVS2 for 10 min followed by 100% PVS2 for another 10 min. The remaining shoot tips were treated with a step-wise and increased PVS2 concentration (20%, 40%, 60%,100%) at 25˚C for 20 min (5 min for each concentration). The cryotubes were then plunged in liquid nitrogen for 1 h, using containers and holders for freezing and storing cryotubes in LN tank (Photo 1.C and D).

Effect of cryoprotectants combination: Precultured shoot tips were placed in sterile cryotubes and loaded with 1.8 ml of a loading solution that consisted of a hormone-free liquid MS medium supplemented with (1) 1.0, 1.2, or 2.0M sucrose alone, (2) 2.0M glycerol plus 0.4M sucrose, or (3) a combination of 5 or 10% DMSO and 0.3, 0.5, 0.7, 1.0, or 2.0M sucrose for 20 min at 25˚C.The cryotubes were then plunged in liquid nitrogen for 1 h.

Effect of loading and vitrification solutions: Precultured shoot tips were placed in sterile cryotubes and loaded with 1.8 ml of a loading solution that consisted of a hormone-free liquid MS medium supplemented with 10%DMSO plus 0.5M sucrose, 5% DMSO plus 0.7M sucrose, or 2.0M glycerol plus 0.4M sucrose for 20 min at 25˚C. The loading solution was replaced with 1.8 ml of a vitrification solution (100% PVS2, 30% DMSO plus 1.0Msucrose, or 15% DMSO plus 1.0M sucrose in a hormone-free liquid MS medium) for 20 min at25˚C. The cryotubes were then plunged in liquid nitrogen for 1 h.

Effect of duration of exposure to the loading solution: Precultured shoot tips were placed in sterile cryotubes and loaded with 1.8 ml of acryoprotectant mixture of 2.0M glycerol plus 0.4M sucrose in a hormone-free liquid MS medium at 25˚C for 10, 20, 30, 60, or 90 min. The loading solution was replaced with 1.8ml of a concentrated vitrification solution [30% glycerol, 15% DMSO, and 15% EG (w/v) in a hormone-free liquid MS medium containing 0.4M sucrose] for 20 min at 25˚C.The cryotubes were then plunged in liquid nitrogen for 1 h.

Rewarming and recovery

After at least one hour of storage, the cryotubes were removed from LN and rewarmed rapidly in a water bath (45˚C) for 1minwith stirring and then move to 25˚Cwater for 1-2 min. The PVS2 was removed from the cryotube and the explants were rinsed with washing solution (WS; liquid MS medium with 1.2 M sucrose) for 30 min. WS was replaced after half-time exposure, in order to dilute the highly concentrated PVS2which have penetrated the cells during osmotic dehydration. Thereafter, the shoot tips were inoculated onto a solidified MS recovery medium supplemented with 0.09 M (30 g) sucrose, 2.22µM BA, and 2.75 g/l phytagel. The cultures were kept for 48 h at 23 ± 1 ˚C in darkness and then transferred to a 16-h photoperiod and kept at a light intensity of approximately 13.5µmol/ m^{-2} $s^{-1}$for 5 days. Then, the cultures were grown in the same conditions as the in vitro stock plants.

In all the above-mentioned experiments, cryopreserved and non- cryopreserved surviving shoot tips were cultured on MS medium supplemented with 4.44 µM BA+ 2.14 µM NAA (the best multiplication medium).For rooting, shoots longer than 1 cm were then rooted onto a gelled MS medium containing 4.9µM IBA. Finally, separated the plantlets and roots washed with sterile distilled water to get rid of medium. Then, the plantlets were transferred to pots containing garden soil mixed with sand and peat moss at a 1:1:1 ratio. The pots were covered with polyethylene bags to maintain high relative humidity and kept in green-house. After two weeks, polyethylene bags were removed gradually. The plantlets were irrigated with half strength medium every four days.

Experimental design and statistical analysis

In all of the above experiments, treatments were arranged in a completely randomized design. Ten shoot tips were tested for each of three replicates for each experiment. Variance analysis of data was carried out using the ANOVA program for statistical analysis. The differences among means for all treatments were tested for significance at 5% level using Duncan's multiple range test (Duncan, 1955) as modified by Snedecor and Cochran (1990).

III. Results And Discussion

To enhance osmo-tolerance shoot tips to vitrification solution (PVS2), a significant interaction effect of sucrose concentration and dehydration period (1, 2 and 3days) was detected on regrowth of non-cryopreserved and cryopreserved shoot tips. The highest regrowth66.7 of shoots was obtained when cryopreserved shoot tips were cultured with 0.3M sucrose for 3days (Table 1).

Table (1): Effect of preculture duration (days) and sucrose concentration on regrowth percentage of non-cryopreserved (-LN) and Cryopreserved (+LN) shoot tips of *Aerva lanata*

Sucrose concentration (M)	Regrowth (%)					
	1day		2day		3day	
	- LN	+ LN	- LN	+ LN	- LN	+ LN
0.1	23.3b	0.0d	40 b	26.7c	76.7a	43.3d
0.3	43.3a	16.7c	56.7a	43.3b	83.3a	66.7b
0.5	13.3c	0.0d	43.3b	16.7d	56.7c	23.3f
0.7	13.3c	0.0d	26.7c	0.0f	36.7e	0.0h

Means having the same letter (s) in column are insignificantly different at 5% level

This result is agreement with these found by Matsumoto and Sakai (2003) who found the highest recovery of cryopreserved grape axillary shoot tips which occurred when shoot tips were pretreated with3.0 M sucrose for 3 days. Also, Sarkar and Naik (1998) reported that sucrose is an important regrowth additive for the acquisition of dehydration tolerance. Nevertheless, the high concentration of sucrose (0.7M) gave negative value for cryopreserved shoot tips regrowth percentage (0.0) when the explants preculture duration at 1,2 and 3

days. While, the concentration of sucrose (0.5 M) gave negative and low values for cryopreserved shoot tips regrowth percentage (0.0,16.7 and 23.3%, respectively) when the explants preculture duration at 1,2 and 3 days (Table 1). The reduction in regrowth may due to the osmotic block at higher sucrose concentrations (Wang et al. 2002). The disaccharide sucrose is more effective than the monosaccharide glucose for vitrification (Sikora et al.2007). Sucrose is used to promote dehydration before and/or during cryopreservation. Sucrose is normally membrane-impermeable and has low toxicity. The concentration of sucrose used in cryopreservation processes varies from 5% (w/v) to 50% (w/v)], but most often 40% sucrose (w/v) (Sopalun et al. 2010) is used. For mint shoot tips, sucrose reduces the toxicity of ethylene glycol and DMSO at 22 ˚C and Glycerol at 0 ˚C (Volk et al.2006).

Effect of plant vitrification solution concentration: Significant variation in survival and regrowth percentage of non-cryopreserved shoot tip were obtained among the different concentrations of PVS2(Table 2).High survival (96.7-100%) was obtained for non-cryopreserved shoot tips treated with a two or four step-wise, respectively, increased concentration of PVS2 at 25 °C for 20 min (5min for each concentration). Similarly, Al-Ababneh (2001) 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 10min.After cryopreservation, a high survival (93.3%) was observed when shoot tips were treated with a four or two stepwise increased concentration of PVS2 (Table 2),whereas, the maximum regrowth (86.6%) of shoot tip was obtained when explants were dehydrated with four step-wise increased concentration ofPVS2.

Table (2): Effect of the plant vitrification solution (PVS2) concentration on survival and regrowth of non-cryopreserved (-LN) and Cryopreserved (+LN) shoot tips of *Aerva lanata*

PVS2 concentration (step-wise)	- LN		+ LN	
	Survival (%)	Regrowth (%)	Survival (%)	Regrowth (%)
(A) One – step	83.3b	66.7c	53.3c	33.3c
(B) Two- step	96.7a	83.3b	76.7b	63.3b
(C) Four -step	100a	100a	93.3a	86.7a

(A) One - step: Direct exposure to 100% PVS, **(B)** Two - step: Treating with 60% PVS2 followed by 100% PVS2, **(C)** Four - step: Loading with 20 % PVS2 followed by 40 %, 60% then 100% PVS2. Means having the same letter (s) in column are insignificantly different at 5% level

Sakai et al. (1991) reported high survival (90%) of cells of naval orange after dehydration with 100% PVS2 solution at 25 °C for 3 min prior to freezing and a brief exposure of shoot tips to full strength of PVS2 minimized the toxicity effect of PVS2.Direct exposure of shoot tips to the vitrification solution reduced survival and regrowth due to osmotic shock induced by the vitrification solution (Al-Ababneh et al., 2003; Moges et al., 2004).Harmful and deadly effect of PVS Concentration may be attributed to both the osmotic stress induced by PVS2 (Grospietsch et al., 1999) and the phytotoxicity effect of PVS2 (Benson et al., 1996).On the other hand, properly dehydrated shoot tips reduce the injurious effects, due to the concentration, over exposure to PVS during dehydration, and increase the ability to be vitrified upon rapid cooling into LN (Matsumoto and Sakai,2003).Although, cell-penetrating constituents of PVS2 replace water as the cells become dehydrated and prevent injurious cell shrinkage caused by dehydration (Volk and Walters,2006).

Effect of cryoprotectant combination: The highest survival (93.3%) of non-cryopreserved shoot tips was achieved when shoot tips were cryoprotected with 2.0M glycerol plus 0.4M sucrose at 258C for 20 min (Table3). High survival percentage (86.7-90%) was also achieved with 5% DMSO plus 0.7M sucrose or10% DMSO plus 0.5M sucrose, respectively. This indicated that such cryoprotectant mixtures were probably not toxic, or the period of exposure was not long enough to cause damaging effects on cell activity.

In this context, Moges et al. (2004) found that the maximum survival (95%) of non-cryopreserved African violet shoot tips was achieved when shoot tips were cryoprotected with 0.4 M sucrose plus 2.0 M glycerol at 25 °C for 20 min. On the contrary, Al-Ababneh (2001) reported that higher survival (96.7%) of non-cryopreserved sour orange shoot tips was obtained after using 5% DMSO with 1.0 M sucrose at 25 °C for 20 min.

Table (3): Effect of loading solution (LS) on survival percentage of non-cryopreserved (-LN) and cryopreserved shoot tips of *Aerva lanata*.

Cryoprotectant concentration			Survival (%)	
Glycrol (M)	DMSO (%)	Sucrose (M)	- LN	+ LN
-	5	0.3	56.7 ed	30 e
-	10	0.3	63.3c	23.3ef
-	5	0.5	80b	33.3e
-	10	0.5	90ab	56.7cd
-	5	0.7	86.7ab	50d
-	10	0.7	80b	50d
-	5	1.0	23.3efg	6.7hi
-	10	1.0	20efg	0i
-	5	2.0	16.7fgh	0i
-	10	2.0	10ghi	0i
-	-	1.0	46.7d	23.3ef
-	-	1.2	63.3c	0.0i
-	-	2.0	0.0c	0.0i
2.0	-	0.4	93.3a	56.7cd

Means having the same letter (s) in column are insignificantly different at 5% level

Data also in Table (3) showed that, using sucrose at 1.0 or 2.0M in combination with 5 or 10% DMSO, resulted in very low survival of non-cryopreserved shoot tips. However, a complete loss of survival of non-cryopreserved shoot tips was observed when shoot tips were pretreated with 2.0M sucrose. This result is agreement with these found by Moges et al. (2004) who reported a complete loss survival of non-cryopreserved African violet shoot tips which occurred when shoot tips were pretreated with 2.0 M sucrose alone.

After cryopreservation, the highest survival (56.7%) was obtained using10% DMSO plus 0.5M sucrose or 2.0M glycerol plus 0.4M sucrose, followed by high value of survival percentage (50%) was also achieved with sucrose at 0.7M in combination with 5 or 10% DMSO (Table 3). Plessis et al. (1993) reported that the best survival of cryopreserved grapevine (*Vitis vinifera* L.) shoot tips was obtained after a 2days stepwise preculture with 1.0M sucrose. While, very low survival (6.7%) cryopreserved shoot tips were obtained when shoot tips were pretreated with 1.0 M sucrose combined with 5% DMSO. Moreover, a complete loss of survival after freezing was exhibited when shoot tips were cryoprotected with a mixture of 1.0 M sucrose with 10% DMSO or 2.0 M sucrose plus 5 or 10% DMSO as well as with 1.2 or 2.0 M sucrose alone (Table 3).This might be attributed to osmotic stress resulting from increased sucrose concentration (Al-Ababneh et al., 2002; Moges et al., 2004) or toxicity effects resulting from increased cryoprotectant concentration in the medium (Reed, 1995). However, Gazeau et al. (1998), reported that the use of DMSO as a cryoprotectant was effective in increasing intracellular viscosity and thus avoiding formation of ice crystals. The relatively low survival percentages after cryopreservation indicate that the cryoprotectant mixtures were probably not able to produce high freezing tolerance. Reduction in survival and regrowth after cryopreservation was shown to be associated with intracellular ice formation due to insufficient dehydration (Mycock et al., 1995).The key changes in the plant tissues that are most important for survival of cry storage are cytoplasm to vacuole ratio, water content, and accumulation of cytoplasmic solutes (GÖldner et al., 1991). The variations observed in survival for the various cryoprotectant combinations tested in the current study might be due to differences in permeability inside the plant tissue and ability to induce osmotic stress and toxicity effect (Al-Ababneh, 2001).

Effect of loading and vitrification solutions: Complete survival of non-cryopreserved shoot tips was achieved for the different loading and vitrification tested (Table 4). After cryo preservation, significant differences in terms of survival of cryopreserved shoot tips were obtained for the different loading solutions and vitrification solutions combination tested (Table4).

Table (4): Effect of loading (LS) and vitrification solution (PVS2) combination on survival regrowth percentage of non-cryopreserved (-LN) and cryopreserved (+LN) shoot tips of *Aerva lanata*.

Loading solution (LS)	Cryoprotectant mixture	Survival (%)		Regrowth (%)	
		- LN	+ LN	- LN	+ LN
1	A	100a	90a	86.7b	80a
	B	100a	76.7b	100a	60c
	C	100a	76.7b	100a	60c
2	A	100a	63.3c	93.3b	40f
	B	100a	80b	73.3d	46.7e
	C	100a	76.7b	80c	66.7b
3	A	100a	73.3b	86.7b	56.7cd
	B	100a	73.3b	90b	53.3d
	C	100a	83.3ab	90b	66.7b

(1) 0.5 M sucrose + 10% DMSO, **(2)**0.7M sucrose + 5% DMSO, **(3)** 2.0 M glycerol+ 0.4 M sucrose and **(A)**100% PVS2, **(B)** 30% DMSO+1.0 M sucrose, and **(C)** 15% DMSO+1.0 Sucrose. Means having the same letter (s) in column are insignificantly different at 5% level

The highest survival was (90-83.3%) when shoot tips were cryoprotected with 0.5 M sucrose +10% DMSO and dehydrated with concentrated PVS2 or 2.0 M glycerol +0.4 M sucrose and dehydrated with 15% DMSO+ 1.0 M sucrose, respectively (Table 4). Sarkar and Naik (1998) demonstrated that the loading phase was necessary to reduce the osmotic shock caused by direct exposure of shoot tips to a highly concentrated PVS2 solution. Furthermore, increasing the concentration of the vitrification solution to an optimal level was shown to increase solute concentration inside the plant tissue (Bachiri et al., 1995). Complete vitrification of cryopreserved plant tissues would eliminate the concern for the potentially damaging effects of intra- and extracellular crystallization (Sakai et al., 1991) and could lead to high survival percentages. The maximum regrowth (80%) after cryopreservation was obtained when shoot tips were cryoprotected with 0.5M sucrose +10% DMSO and vitrified with concentrated PVS2 (Table 4). However, only 40–46.7%regrowth was achieved using 0.7M sucrose + 5% DMSO and dehydrated with 100% PVS2 or 30% DMSO+1.0 M sucrose prior to freezing. Irrespective of the loading and cryoprotectant combination tested, recovered shoot tips developed a yellowish appearance (data not shown), probably due to osmotic stress caused by the vitrification solution (Sakai et al., 1991) and water depletion during dehydration (Shibli et al., 1992). Synthesis of toxic material as are salt of stress during cryopreservation (Engelmann, 1997) and/or cellular damage during freezing and thawing (Lambardi et al., 2000) might also contribute to the color change.

Effect of exposure time to the loading solution: Although survival of non-cryopreserved and cryopreserved shoot tips was high (90–100%), regrowth varied depending on duration of exposure to the loading solution (Table 5). In this respect, Moges et al.(2004) found that a complete survival rate for non-cryopreserved African violet shoot tips irrespective of the duration of exposure to loading solution, while higher regrowth (90 or 100%) rates for non-cryopreserved African violet shoot tips were obtained after 10 or 20 min of exposure to loading solution.

Table (5): Effect of exposure time to the loading solution (2.0Mglycerol+0.4 M sucrose) on survival and regrowth of non-cryopreserved (-LN) and Cryopreserved (+LN) shoot tips of *Aerva lanata.*

Exposure time (min)	- LN		+ LN	
	Survival (%)	Regrowth (%)	Survival (%)	Regrowth (%)
10	100 a	100a	90b	46.7cd
20	100 a	90b	100a	56.7bc
30	100 a	60c	100a	40 d
60	100 a	56.7cd	100 a	46.7 cd
90	100 a	46.7d	90 b	40 d

Means having the same letter (s) in column are insignificantly different at 5% level

Without cryopreservation, regrowth of shoot tips decreased with increasing duration of exposure to the loading solution with the highest (90–100%) being achieved after 10–20 min. The reduction in regrowth with increased duration of exposure to the loading solution might be due to excess dehydration of the cells which would lead to accumulation of solutes that could cause osmotic stress or chemical toxicity to the cells (Sakai et al., 1990). Matsumoto et al. (1994) also demonstrated that regrowth decreased with longer durations of exposure to loading solutions due to chemical toxicity. After cryopreservation, the highest regrowth (56.7%) was obtained with a 20 min exposure to the loading solution. Sufficient time should be available to enhance solute permeation into the cytoplasm (Sarkar and Naik, 1998). Sakai et al. (1990) reported that 20 min of exposure of nucellar cells of navel orange to the loading solution was sufficient to reduce the toxic effect of concentrated PVS2. However, Al-Ababneh (2001) reported that maximum survival and regrowth of non-cryopreserved and cryopreserved shoot tips of sour orange were obtained when shoot tips were exposed to the loading solution for 60 min. In the current study, regrowth of cryopreserved shoot tips was higher than that of non-cryopreserved shoot tips, particularly when exposure to the loading solution was shorter than 60 min, probably due to formation of intracellular ice crystals during freezing and thawing (Gonzalez-Arnao et al., 1998).

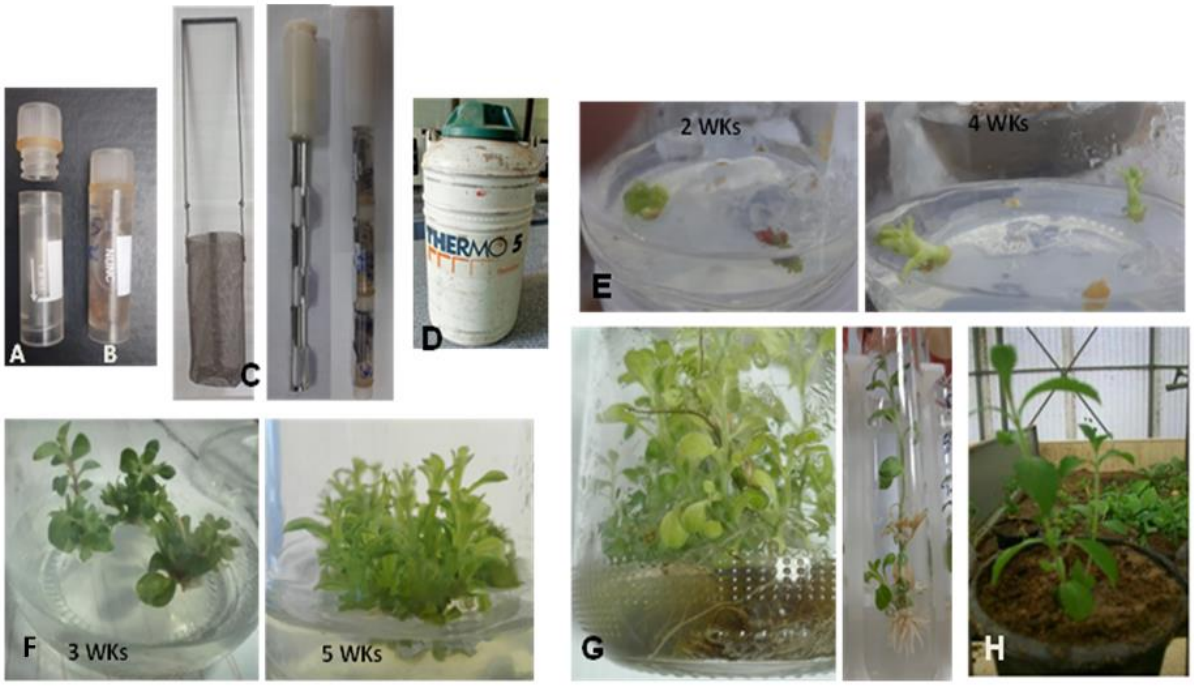

Photo 1. Plantlets regeneration of *A. lanata* from cryopreserved shoot tips. **A.** Sterile 1.8ml cry tubs used for the storge in LN.**B**. cryotube containing shoot tips and loading with vitrification solutions after exposing to LN,**C.** containers and holders for freezing and storing cryotubes, **D.** a Liquid Nitrogen tank (Dewar vessel low form approx. 1 liter volume),**E.** frozen-thawed shoot tips 2 and 4 weeks after culture on MS phytagel medium plus 4.44µM, **F.** multiple shoot formation from cryopreserved shoot tips on MS medium supplemented with 4.44µMand 2.14µM NAA after three weeks and after five weeks, **G.** plantlets with well-developed shoots and roots, and **H.** Acclimatized plantlets in plastic pots after three weeks from transfer to greenhouse.

A successful cryopreservation system is measured by the success of the resulting plants from the protocol. This is a critical issue for the preservation system. Regrowth of surviving shoot tips (photo1.E) and multiplication can be obtained on MS medium containing 4.44µM and 2.14µM NAA.(photo1.F). Well-developed rooted plantlets that are regenerated from cryopreserved shoot tips (photo1.G) and have been successfully acclimatized in greenhouse after storage period with a survival frequency of 80%(Photo1.H).

IV. Conclusions

In conclusion, these results show that the vitrification procedure can be successfully applied to *Aerva lanata* cryopreservation. In short, after vitrification, the shoot tips are directly immersed in LN. For recovery, the shoot tips are rapidly warmed at 45˚C, washed in a liquid MS medium containing 1.2 M sucrose, and recultured on gelled MS medium containing 4.44µM BA for regrowth of surviving shoot tips. The results indicated in detail that pre culturing shoot tips on hormone-free MS medium containing 0.3M sucrose for 3 days dehydration resulted in 66.7% regrowth, after cryopreservation. The highest values of survival percentage (76.7-93.3%) and 63.3-86.7 % regrowth of cryopreserved shoot tips was achieved with two or four step-wise dehydration respectively, with PVS2 at 25˚C for 20 min prior to freezing. Only 56.7% survival was obtained using 10% DMSO plus0.5M sucrose or 2M glycerol plus 0.4M sucrose as a cryoprotectant combination. The highest survival (90 - 83.3%) and maximum regrowth (80 - 66.7%)after cryopreservation was obtained when shoot tips were cryoprotected with 10% DMSO plus 0.5M sucrose followed by dehydration with 100% PVS2 or 2.0 M glycerol plus 0.4 M sucrose followed by dehydration with 15% DMSO plus 1.0 M sucrose, respectively. Shoot tips cryoprotected with 2.0M glycerol plus 0.4M sucrose for 20 min (as exposure time to the loading solution) exhibited the highest survival (100%) but only 56.7% regrowth .Faster regrowth of surviving shoot tips and multiplication can be obtained on medium containing 4.44µM BA plus 2.14µM NAA. Moreover, rooting of elongated shoots can be easily achieved on IBA-containing medium. Following this procedure, almost 80% rooted shoots can be obtained from cryopreserved shoot tips.

In general, cryopreservation of shoot tips by vitrification appeared to be a good (more promising than the other) technique. However, further studies should be initiated to improve regrowth of the surviving shoot tips and to study genetic stability after cryopreservation.

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