Plant Cell Tiss Organ Cult
DOI 10.1007/s11240-011-0054-0

ORIGINAL PAPER

Cryopreservation of wild Shih (*Artemisia herba-alba* Asso.) shoot-tips by encapsulation-dehydration and encapsulation-vitrification

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Received: 23 June 2011 / Accepted: 27 August 2011

Abstract *Artemisia herba-alba,* called Shih is a medicinal herbal plant found in the wilds. The biodiversity of this plant is heavily subjected to loss because of heavy grazing, land cultivation and collection by people to be used in folk medicine. In the current study, two cryopreservation dependent techniques to conserve the shoot-tips of in vitro grown Shih were evaluated: encapsulation- dehydration and encapsulation- vitrification. Shoot-tips of Shih were encapsulated into sodium-alginate beads. In encapsulation-dehydration, the effect of sucrose concentration (0.5, 0.75 or 1.0 M) and dehydration period (0, 2, 4 or 6 h) under sterile air-flow on survival and regrowth of encapsulated shoot tips were studied. Maximum survival (100%) and regrowth (27%) rates were obtained when encapsulated unfrozen *Artemisia herba-alba* shoot tips were pretreated with 0.5 M sucrose for 3 days without further air dehydration. After cryopreservation the highest survival (40%) and regrowth (6%) rates were achieved when *Artemisia herba-alba* shoot tips were pretreated with 1.0 M sucrose for 3 days without further air dehydration. Viability of *Artemisia herba-alba* shoot tips decreased with increased dehydration period. In encapsulation-vitrification, the effect of dehydration of encapsulated *Artemisia herba-alba* shoot tips with 100% PVS2 for various dehydration durations (10, 20, 30, 60 or 90 min) prior to freezing was studied. After cryopreservation the dehydration of encapsulated and vitrified shoot tips with 100% PVS2 for 30 min resulted in 68% survival and 12% regrowth rates. Further conservation techniques must be evaluated to increase both survival and regrowth percentages.

Keywords *Artemisia* · Cryopreservation · Encapsulation · Preculture · Shoot tips · Vitrification

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Introduction

Artemisia herba-alba, called Shih is a medicinal herbal plant found in the wild, it is a greenish-silver perennial dwarf shrub rising and growing in arid and semi-arid climates (Jacob et al. 1977). It is found in the deserts of the Middle East, North Africa and Spain, extending into North Western Himalayas (Haouari and Ferchichi 2009; Hedi et al. 2010). The existence of this plant is heavily subjected to loss because of heavy grazing, land cultivation and collection by people to be used in folk medicine (Abou El-Hamd et al. 2010; Aburjai et al. 2007). Therefore, this germplasm must be conserved and protected from loss to ensure its availability for future use and plant improvement.

In vitro propagation can be seen as an important tool in conservation, as it may provide approving conservation options for plant species with limited reproductive ability (Bunn et al. 2007). Plant tissue culture techniques are well thought-out to produce high number of genetically alike plants that can be cultivated from only one single plant stock (Pierik 1997). Single plant may formulate thousands or even millions of plants depending on the facility of the tissue culture method (Conger 1981; Pierik 1997). Furthermore, micropropagation and conservation of plant

Published online: 28 September 2011

genetic resources via tissue culture has become an important effort by research work in the last three decades (Al-Qudah et al. 2011; Mostafa et al. 2010; Musallam et al. 2011).

Cryopreservation is a safe and cost-effective gene banking strategy for long-term preservation. Cryogenic storage in liquid nitrogen (LN) at −196°C cease cell division, metabolic and biochemical processes (Hopkins 1999; Burritt 2008). Additional advantages also include small storage space requirements, low maintenance costs, and only a modest number of replicates is required to conserve a plant effectively (Shibli and Al-Juboory 2000). According to International Plant Genetic Resources Institute (IPGRI) publications, presently about 5 million accessions for about 20,000 species are reportedly conserved by *ex situ* techniques including in vitro slow growth preservation and cryopreservation in genebanks, worldwide (Anonymous 2010).

This study shows the effect of cryopreservation on the survival and regrowth of in vitro produced shoot tips (ST) of wild Shih (*Artemisia herba-alba*) via two techniques, in particular, encapsulation-dehydration (ED), (Sakai and Engelmann 2007; Engelmann 1997) and encapsulation-vitrification (EV), (Matsumoto et al. 1994).

Materials and methods

Plant material

Seeds of wild *Artemisia* were germinated in vitro on a medium consisting of full strength Murashige and Skoog (1962) [MS] medium supplemented with 0.5 mg/l 6-benzyladenine(BA) and 1.0 mg/l gibberrelic acid (GA_3) as shown in Fig. 1.

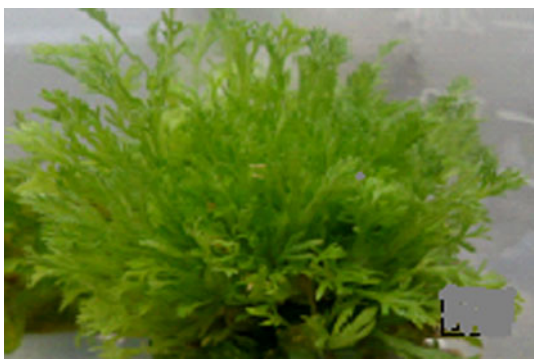

Fig. 1 In vitro established cultures of Shih (*Artemisia herba-alba*) on the surface of MS media supplemented with 0.5 mg/l BA and 1.0 mg/l GA_3

Cryopreservation

Preculture treatments

Preculture on different sucrose concentrations at 25°C for ED

Subculturing of shoot cultures was performed once every 5 weeks to generate sufficient shoot stocks for cryopreservation studies. All cultures were incubated at 25 ± 1°C under a 16/8 (light/dark) photoperiod of 45–50 µmol m^{-2} s^{-1} irradiance provided by Phillips cool white fluorescent tubes.

Shoot-tips [STs] (2–3 mm) were precultured on hormone free MS media with a range of concentrations (0, 0.1, 0.3, 0.5 or 1.0 M) of sucrose. Cultures were incubated for 2, 4, 6 or 8 days at 25°C. After each incubation period, STs were transferred to MS hormone free medium supplemented with 0.1 M sucrose. Cultures were kept in the dark for 1 week and then transferred to normal conditions as described previously.

Data collected were percentages of survival and recovery. Survival percentage of STs was determined after 2 day of transfer to regrowth conditions, using 2, 3, 5-triphenyltetrazolium chloride assay (TTC test) (Lutts et al. 1996; Shibli et al. 2009). Change in color percentages was determined immediately after each incubation period following formula:

$$\text{Color change } \% = \left[\text{No. of changed color STs} / \text{Total number of STs}\right] \times 100$$

Recovery percentages were assessed after 2 weeks of transfer to regrowth conditions.

STs from cultures grown on MS media containing 0.3 M sucrose at 25°C were used in the ED experiments.

Preculture on different sucrose concentrations at 4°C for EV

STs (2–3 mm) were precultured on hormone free MS medium with a range of sucrose concentrations (0.0, 0.1 or 0.3 M). Cultures were incubated for 2, 4, 6 or 12 days at 4°C. After each incubation period, STs were transferred to MS hormone free medium supplemented with 0.1 M sucrose. Cultures were kept in the dark for 1 week and then transferred to normal growth conditions. Data was collected as previously described for survival and regrowth.

STs from cultures grown on MS media containing 0.3 M sucrose at 4°C were used in the EV experiments.

Encapsulation-dehydration: in sterile airflow

STs were precultured on a hormone-free solid MS medium containing 0.3 M sucrose for 3 days at 25°C. Precultured

STs were then suspended in a solution containing 3% sodium alginate in calcium-free and hormone-free in a modified liquid MS minerals and vitamins solution (in which calcium is omitted) supplemented with 0.1 M sucrose. The STs were picked up individually using 1 ml wide mouth sterile pipette with some alginate solution and then dropped into a liquid MS medium containing 100 mM calcium chloride and 0.1 M sucrose to form beads of about 4 mm in diameter (Baghdadi et al. 2010). The beads were kept in the calcium chloride solution for 30 min for polymerization.

Beads, each containing one shoot tip, were transferred to a hormone-free liquid dehydration MS media with various concentrations (0.5, 0.75 and 1.0 M) of sucrose; then incubated on a rotary shaker (100 rpm) at 25°C for 3 days. The dehydration solution was then removed by sterile pipette and beads were subjected to air dehydration on sterilized filter paper in uncovered 9-cm Petri dishes in a laminar airflow cabinet for 0, 2, 4 or 6 h at room temperature.

Half of the beads (LN) were then transferred to a recovery medium for rehydration and growth while the other half of beads were placed in 2-ml sterile cryovials and directly immersed into LN. The beads were kept in LN for at least 1 h. After cryopreservation, cryogenic vials containing beads were thawed in a water bath at 38°C for 2–3 min. Encapsulated non-cryopreserved (−LN) and encapsulated cryopreserved (+LN) beads were inoculated onto a solid MS recovery media containing 1.0 mg/l isopentenyladenin (2iP) and 0.1 M sucrose, then kept in the dark for 3 days. Survival rates were tested for a half of non–frozen and frozen encapsulated STs using TTC test (Lutts et al. 1996). The other half of the STs were transferred to the normal growth conditions as described previously and kept for further recovery. After 4 weeks, STs were checked under a binocular microscope for any recovery signs.

Determination of moisture content (MC)

For moisture content (MC) determination of the beads during dehydration, beads devoid of STs were transferred to a hormone-free liquid dehydration MS medium with various concentrations (0.5, 0.75 or 1.0 M) of sucrose then incubated on a rotary shaker (100 rpm) at 24°C for 3 days. The dehydration solution was then removed by using sterile micropipette and beads were subjected to air dehydration on sterilized filter paper in uncovered 9-cm Petri dishes in a laminar airflow cabinet for 0, 2, 4 or 6 h at room temperature. After each dehydration period, beads were weighed, dried in an oven at 90°C for 16 h and then reweighed (Subaih et al. 2006). The moisture content was determined using the following formula:

$$\text{MC } \% = [(\text{Fresh weight} - \text{Dry weight})/\text{Fresh weight}] \times 100$$

Encapsulation-vitrification

STs of Shih were precultured on hormone free MS media supplemented with 0.3 M sucrose and incubated at 4°C for 1 week. Precultured STs were then suspended in calcium-free and hormone-free liquid MS medium supplemented with 3% (w/v) sodium alginate, 2.0 M glycerol and 0.4 M sucrose (Moges et al. 2004; Shibli et al. 2009). The STs were picked up using 1 ml sterile wide-mouth pipette with some alginate solution and then dispensed into a hormone-free liquid MS medium containing 100 mM calcium chloride and 2.0 M glycerol plus 0.4 M sucrose. The beads were kept in calcium chloride solution for 30 min for polymerization.

Half of beads which were used with (+LN) were transferred to sterile 2 ml cryovials then were dehydrated with a highly concentrated Plant Vitrification Solution (PVS2) (Sakai et al. 1990), [(w/v) 30% glycerol, 15% DMSO and 15% Ethylene glycol (EG)] for 10, 20, 30, 60 and 90 min at 25°C. The cryovials with the beads and the vitrification solution were then immersed directly in LN for at least 1 h. Cryovials were then thawed in a water bath at 38°C for 2–3 min. The PVS2 solution was then removed and replaced with unloading solution (hormone free liquid MS medium supplemented with 1.2 M sucrose), which was changed three times for 10 min.

Half of STs in beads with or without LN storage were transferred to a solid MS recovery medium for regrowth percentages determination. Survival rates were determined using the other half of the STs by TTC test.

Experimental design and statistical analysis

Treatments were arranged in a complete randomized design (CRD). Each treatment was replicated five times with five STs per replicate. The collected data were statistically analyzed using SAS (Statistical Analysis System, Cary, NC, 2001). Means were separated according to the least significant difference (LSD) at 0.05 probability level.

Results

Preculture on different sucrose concentrations at 25°C for ED

Significant variations in survival and regrowth of non-cryopreserved STs were obtained along with the different concentrations of sucrose in the preculture medium and the

Table 1 Effect of preculture duration and sucrose concentration on survival and regrowth percentages of non-cryopreserved (−LN) shoot tips of Shih (*Artemisia herba-alba*) at 25°C

Preculture duration (day)	Sucrose conc. (M)	Survival %	Regrowth %
2	0.0	100 a*	100 a
	0.1	100 a	100 a
	0.3	100 a	100 a
	0.5	84 b	66 c
	1.0	45 c	39 c
4	0.0	100 a	100 a
	0.1	100 a	100 a
	0.3	96 a	84 b
	0.5	55 c	42 c
	1.0	17 d	6 e
6	0.0	100 a	100 a
	0.1	100 a	100 a
	0.3	74 b	65 b
	0.5	52 c	40 c
	1.0	14 d	2 e
8	0.0	100 a	100 a
	0.1	100 a	100 a
	0.3	60 c	42 c
	0.5	30 d	20 d
	1.0	0.0 e	0.0 e

* Means within column, for each preculture period, having different letters are significantly different according to LSD at $P \leq 0.05$

duration of preculture for Shih (Table 1). High survival (96–100%) rates were obtained after preculture of the STs of Shih with 0.0, 0.1 or 0.3 M sucrose at 2 or 4 days. Complete survival and regrowth of STs were obtained along with 0.0 and 0.1 M irrespective of preculture duration.

The minimum survival and regrowth of shoot tips were obtained at 1.0 M sucrose for all preculture duration. By above result, preculture of STs in ED step was decided and performed for 3 days at 25°C on the MS medium with 0.3 M sucrose.

Preculture on different sucrose concentrations at 4°C for EV

Successful cold preculture at 4°C with different sucrose concentrations were obtained for Shih STs. High survival (90–100%) rates were obtained when preculture the STs of Shih with 0.0, 0.1 and 0.3 M sucrose irrespective of duration culture (Table 2). Also, this result lead to the decision of preculture step of STs in EV for 1 week at 4°C on the MS medium with 0.3 M sucrose.

Table 2 Effect of preculture duration and sucrose concentration on survival and regrowth percentages of non-cryopreserved (−LN) shoot tips of Shih (*Artemisia herba-alba*) at 4°C

Preculture duration (day)	Sucrose conc. (M)	Survival %	Regrowth %
2	0.0	100 a*	100 a
	0.1	100 a	100 a
	0.3	100 a	100 a
4	0.0	100 a	100 a
	0.1	100 a	100 a
	0.3	100 a	88 b
6	0.0	100 a	100 a
	0.1	100 a	100 a
	0.3	97 a	85 b
12	0.0	100 a	100 a
	0.1	100 a	98 a
	0.3	90 b	73 b

* Means within column, for each preculture period, having different letters are significantly different according to LSD at $P \leq 0.05$

Table 3 Effect of sucrose concentration and dehydration period on survival and regrowth percentages of encapsulated non-cryopreserved (−LN) shoot tips of Shih (*Artemisia herba-alba*)

Sucrose (M)	Dehydration period (h)	Survival %	Regrowth %	MC %*
0.5	0	100 a**	27 a	42.5*
	2	100 a	18 ab	37.08
	4	66 c	12 bc	4.71
	6	54 d	0 d	2.64
0.75	0	96 a	24 a	47.1
	2	82 b	27 a	24.76
	4	53 d	12 ab	4.54
	6	25 e	0 d	3.31
1.0	0	57 cd	15 abc	32.45
	2	48 d	6 cd	12.56
	4	15 f	0 d	6.26
	6	7 f	0 d	3.75

* MC% = Moisture content percentage for beads

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

Encapsulation-dehydration: in sterile airflow

There was a significant interaction effect of dehydration solution (sucrose concentration) and dehydration duration on survival and regrowth of non cryopreserved STs of Shih (Table 3) and cryopreserved STs (Table 4; Fig. 2) Survival and regrowth of encapsulated non-cryopreserved STs decreased with increasing sucrose concentration and dehydration period. In our current study, minimum survival

Table 4 Effect of sucrose concentration and dehydration period on survival and regrowth percentages of encapsulated cryopreserved (+LN) shoot tips of Shih (*Artemisia herba-alba*)

Sucrose (M)	Dehydration period (h)	Survival %	Regrowth %
0.5	0	15 de*	3 ab*
	2	19 cd	3 ab
	4	37 ab	6 a
	6	45 a	0 b
0.75	0	15 de	0 b
	2	13 de	0 b
	4	21 cd	3 ab
	6	30 bc	6 a
1.0	0	40 ab	6 a
	2	36 ab	6 a
	4	6 e	0 b
	6	6 e	0 b

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

(7%) was observed when non-cryopreserved shoot tips were suspended in 1.0 M sucrose for 3 days with 6 h dehydration in sterile airflow resulting in complete loss of regrowth capacity (Table 3).

The greatest survival (100%) was obtained when non-cryopreserved (−LN)) STs of Shih were pretreated with 0.5 M sucrose for 3 days followed by 0 or 2 h dehydration period (Table 3), where the beads attained 42.5 and 37.08% moisture content. Also high survival (82–96%) was obtained with 0.75 M sucrose dehydration for 3 days followed by 0 or 2 h dehydration period where the beads attained 47.1 and 24.76% moisture content (Table 3).

In addition, the greatest survival (40%) for cryopreserved (+LN) STs of Shih was obtained when shoot tips were pretreated with 1.0 M sucrose for 3 days followed by 0 h dehydration (Table 4) where the beads attained 32.45% moisture content (Table 3). While the highest regrowth (6%) was obtained with 0.5 M sucrose after 4 h of dehydration for Shih STs or with 1.0 M sucrose after 0 or 2 h of dehydration (Table 4).

Encapsulation-vitrification

Significant variations in terms of survival and regrowth for encapsulated non-cryopreserved and cryopreserved shoot tips were observed among the different duration of dehydration with concentrated 100% PVS2 at 25°C (Table 5; Fig. 3).

After cryopreservation the greatest survival (76%) with regrowth (6%) of Shih STs were obtained when encapsulated STs were dehydrated with concentrated PVS2 solution (100%) for 60 min (Table 5). On the hand, highest regrowth (12%) percentage was obtained when encapsulated STs were dehydrated with concentrated PVS2 solution (100%) for 30 min (Table 5).

Discussion

Preconditioning or preculture stage refers to the culturing of plant material on medium supplemented with osmotic agents at different concentrations and at different durations depending on plant species and type of plant material used (Shibli et al. 2006). In preculture step, the osmotic agent (i.e. sucrose, manitol and/or sorbitol) concentrations in the plants raise considerably (Reed et al. 2008) and it increased the efficiency of cryopreservation in many species (Shibli et al. 2006). For example, preculture of innala (*Solemostemon rotundifolius*) buds in 0.3 M sucrose for 2 days at 25°C produced the greatest regrowth of STs (Niino et al. 2000).

The complete survival and regrowth of Shih STs at 0.0, 0.1 or 0.3 M sucrose dehydration solutions (Table 1) was similar to the findings of Al-Ababneh et al. (2003) in which high survival and regrowth were obtained for STs of bitter almond (*Amygdalus communis* L.) when STs were precultured on 0.1 or 0.3 M sucrose. Moges et al. (2004) reported that 95–100% of African violet STs were survived

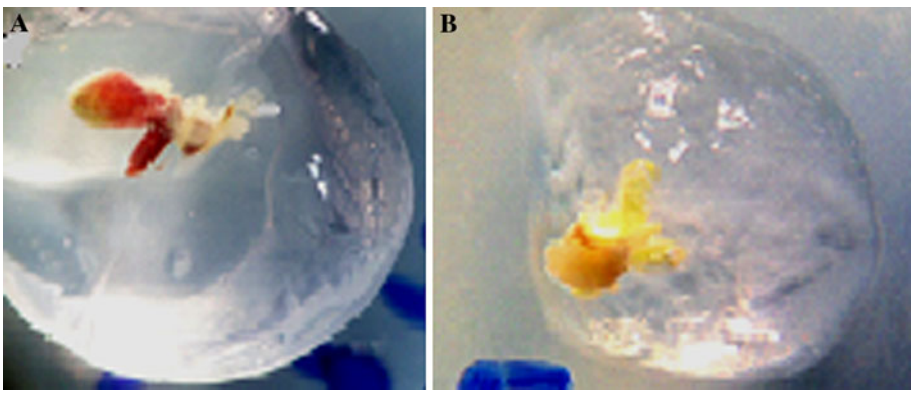

Fig. 2 Beads with shoot tips of Shih (*Artemisia herba-alba*) after Encapsulation—dehydration cryopreservation **a** Survival after +LN, **b** Regrowth initiation after 20 day

Table 5 Effect of the dehydration duration with concentrated PVS2 on survival and regrowth percentages and leaf color of non-cryopreserved (−LN) and cryopreserved (+LN) shoot tips of Shih (*Artemisia herba-alba*)

Duration (min)	Survival %	Regrowth %
Non-cryopreserved (−LN)		
10	100 a	0.00 c
20	100 a	6.00 c
30	98 a	36.0 b
60	92 b	69.0 a
90	78 c	9.00 c
Cryopreserved (+LN)		
10	50 b	0.0 b
20	68 a	0.0 b
30	68 a	12.0 a
60	76 a	6.0 ab
90	42 b	0.0 b

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

after 2 days of preculture with 0.3 M sucrose or with 0.5 M sucrose. Moreover, Baghdadi (2008) reported that maximum survival (100%) rates were obtained after preculture of crocus calli on 0.1 and 0.5 M sucrose irrespective of preculture duration.

The high survival percentages (90–100%) of preculturing Shih STs at 4°C (Table 2) is may be because that tropical and subtropical plants are less tolerant to cold storage than temperate plants (Moges et al. 2003). While *Coffea* spp. was successfully survived after precultured and stored at 20°C (Bertrand et al. 1992); wild pear (*Pyrus syriaca* Bioss.) germplasm has been stored and conserved at 4°C (Tahtamouni and Shibli 1999). Tissues conserved in vitro at low temperature might show some physiological disorders during conservation followed by a dramatic decrease in survival and regrowth ability (Tahtamouni and Shibli 1999). Moreover, maximum successes of *Dioscorea bulbifera* L. calli were observed by using of 0.75 M sucrose at 4°C for 7 days (Ming-Hua and Sen-Rong 2010).

ED is generally widely used because it is simple, inexpensive and provides a high level of genetic stability (Sakai et al. 1990; Khoddamzadeh et al. 2011). This method depends on a successive osmotic and evaporation dehydration steps performed on plants material (Shibli et al. 2001). The ED technique for cryopreservation was initially reported for shoot tips of *Solanum* sp. (Fabre and Dereudre 1990), and additional plant species, including pear, apple, sugarcane, and potato (Sakai and Engelmann 2007). Recently many researches illustrate the protocol of encapsulation-dehydration which is consisted of the encapsulation of plant material in alginate beads followed by a dehydration step in a medium containing a high concentration of sucrose ranging from 0.1 to 1.0 M for at least 1 day (Shibli et al. 2009; Subaih et al. 2006).

The decline in survival and regrowth of encapsulated non-cryopreserved shoot tips with increased dehydration (Table 3) is may be due to the osmotic shock at higher concentrations (Maruyama et al. 1998). As the moisture content inside the beads containing Shih STs decreased (Table 3) the survival rates after cryopreservation increased (Table 4). Similar to our results, Subaih et al. (2006) reported that high (80%) survival was obtained when date palm calli were pretreated with 0.3 sucrose for 2 days followed by 2 h dehydration where the beads attained 55.4% moisture content. While the highest regrowth (33.3–40%) of date palm (Subaih et al. 2006) was obtained with 0.1 M sucrose after 2 or 4 h of dehydration. Moges et al. (2004) reported complete survival (100%) and 75% regrowth of encapsulated cryopreserved African violet STs after 0.3 M sucrose dehydration for 2 days incubation and followed by 2 or 4 h dehydration. In general, cryopreservation of Shih STs by ED with sterile air flow resulted in lower recovery and regrowth of the Shih than EV.

EV was developed (Matsumoto et al. 1994) to treat a huge number of meristems (samples) at the same time. It is

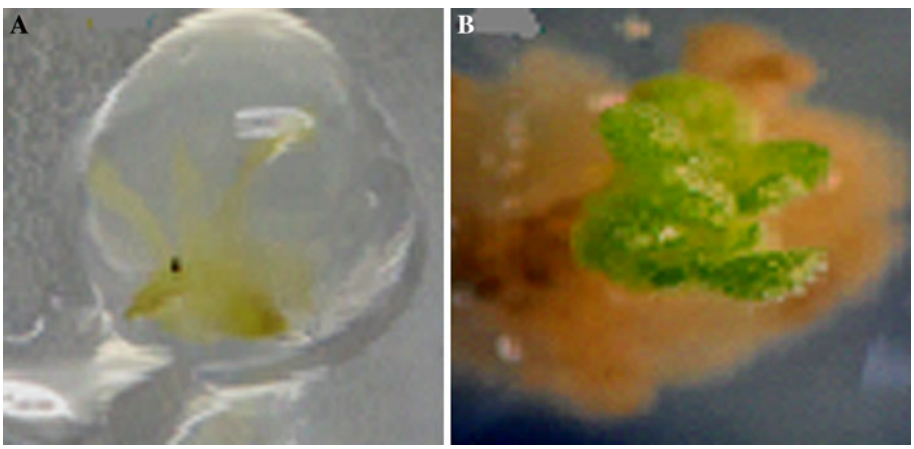

Fig. 3 STs of Shih (*Artemisia herba-alba*): **a** bead with Shih (*Artemisia herba-alba*) shoot tips using Encapsulation-Vitrification method, **b** regrowth of cryopreserved (+LN) shoot tips of *Artemisia herba-alba* after using vitrification solution

a combination of encapsulation and vitrification techniques having the benefits of ease to conduct on plant material and decreases the time needed for dehydration and reducing toxicity of vitrification solution on explants and provides higher and earlier recovery growth than encapsulation-dehydration (Shibli et al. 2006; Baghdadi et al. 2010).

Reduction in regrowth (12%) was obtained when encapsulated Shih SHs were dehydrated with concentrated PVS2 solution (100%) for 30 min., (Table 5). While Moges et al. (2004) found that the greatest survival (80–85%) and regrowth (70–80%) of cryopreserved African violet shoots with pale green or yellow color were obtained when encapsulated shoot tips were dehydrated with concentrated PVS2 at 25°C for 30 min**.** Also, Al-Ababneh et al. (2002) found maximum recovery of sour orange shoot tips after dehydration with concentrated PVS2 at 0°C for 2–3 h. In addition, it has been reported that the greatest survival (55.6 and 75.0%) and regrowth (66.7 and 66.7%) of *Crocus moabiticus* and *Crocus hyemalis* calli, respectively were obtained when encapsulated calli were dehydrated with concentrated PVS2 solution (100%) for 20 min. (Baghdadi et al. 2010). Regarding the cryopreservation of Shih by EV, decreasing the Shih STs exposure duration to a highly concentreated PVS2 vitrification solution increased the viability of shoot tips.

Acknowledgments The authors would like to thank the Deanship of Research at the University of Jordan; Amman, Jordan, for funding this research.

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