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Multiplication and Cryopreservation of Yarrow (*Achillea millefolium* L., Asteraceae)

M. A. Shatnawi[1]

ABSTRACT

Achillea millefolium **belonging to the** ***Astreaceae*** **family is an endangered medicinal plant of Jordan and of its neighboring countries. As an alternative to seed propagation, an efficient micropropagation of** ***A. millefolium*** **and its subsequent rooting were developed as an option for its** ***in vitro*** **conservation. A maximum of 5.9 shoots per microshoot were obtained on Murashige and Skoog agar medium supplemented with 0.9 mg L^{-1} of 6-Benzyl Amino Purine (BAP). The effect of different types and concentrations of auxins were tested, i.e. IBA (Indole-3-Butyric-Acid), IAA (Indole-3-Acetic-Acid) or Naphthalene Acetic Acid (NAA). Maximum root number (20.8 roots ex-plant^{-1}) was obtained from media containing 1.2 mg L^{-1} of IBA. A survival of 70% was obtained when rooted explants were acclimatized** ***in vivo*** **in equal portions of perlite and peat soil.** ***In vitro, A. millefolium*** **shoots were successfully stored for up to 32 weeks on MS medium supplemented with different concentrations of either sucrose, glucose or fructose, at 24±2°C. After 32 weeks past, 88.6% of the shoots survived on the medium supplemented with 3% sucrose. Moreover, 85.3% of the shoots were able to re-grow when stored under light conditions. Cryopreservation through vitrification was successfully achieved (80% re-growth) when shoot tips precultured on a medium supplemented with 0.4M sorbitol and 0.1M sucrose for 1 day, followed by loading shoot tips with concentrated plant vitrification solution 2 (PVS2) for 20 minutes, then being dehydrated with PVS2 for 60 minutes at 0°C prior to storage in Liquid Nitrogen (LN).**

Keywords: *Achillea millefolium*, Liquid nitrogen, Long-term conservation, Shoot tips, Vitrification.

INTRODUCTION

Achillea millefolium L. is a perennial medicinal plant belonging to the *Asteraceae* family. *A. millefolium* has been traditionally used as a remedy for external wounds of skin such as burn, swollen and irritated skin conditions. It has also been used as herbal tea to relieve gastro-intestinal inflammations (Bezic *et al*., 2003; Dokhani *et al*., 2005; Innocenti *et al*., 2007). Recently *A. millefolium* is attracting further attention among contemporary medicinal plant researchers because some human diseases resulting from bacterial antibiotics have gained worldwide concern. As a result of intense harvesting from the wild and as a result of small number of this plant are widely cultivated, there by presenting a considerable risk of decrease its genetic diversity.

Tissue culture has been applied as a potent method for multiplication and conservation of various plant species. As compared to conventional propagation, micropropagation benefits from the advantage of allowing rapid propagation within limited time and space. *A. millefolium* propagates vegetatively in its natural state, but propagation is too slow for its commercial production (Turek *et al*., 2009). To overcome slow propagation rate, micropropagation would be a very useful technique for mass multiplication of *A. millefolium*. Conservation of genetic material in field conditions suffers from such several disadvantages as extensive labour cost and as well the risk of losses due to pathological and

[1] Department of Biotechnology, Faculty of Agricultural Technology, Al-Balqa Applied University, Al-Salt 9117, Jordan. e-mail: mshatnawi1@yahoo.com.au

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environmental threats. The development of a micropropagation system for *A. millefolium* provides a useful means for plant preservation within a short term, and also cryopreservation technique as a means for longer-term preservation. *In vitro* conservation techniques are employed, depending on the storage duration required. For medium-term storage, the aim is to reduce growth and to increase the intervals between subcultures (Engelmann, 2004).

Cryopreservation could be employed as an alternative for the preservation of plant material at ultra-low temperatures (-196°C, i.e., the temperature of liquid nitrogen). At this temperature, all cellular divisions and metabolic processes come to a standstill. During the present study the possibility of using a vitrification technique for cryopreservation of the species was explored. Vitrification involves extended desiccation of the target tissue before freezing through the exposure of samples to highly concentrated cryoprotective solutions and/or to physically drying conditions (Gonzalez-Arnao *et al*., 2008). The key to successful vitrification is the acquisition of osmo-tolerance and the mitigation of any injurious effects during the dehydration process (Shatnawi *et al*., 2007). The use of highly vitrification concentrated solution (PVS2) is recommended (Sakai *et al*., 1990). The concentrated solution dehydrates tissues without causing injury because it creates a stable and live glass when they are stored in liquid nitrogen (González-Benito *et al*., 2004; Shibli *et al*., 2006; Shatnawi *et al*., 2007). Therefore, this study demonstrated the first report of the development of a simple reliable in clonal propagation and cryopreservation of *in vitro* grown *A. millefolium* through vitrification.

MATERIALS AND METHODS

Plant Material and Culture Conditions

Seeds of *A. millefolium* were collected from Jarash, Jordan (Jerash city is located in north of Jordan (latitude and longitude are 32.2806° N and 35.8953° E). Seeds were sterilized by being immersed in 70% ethanol for 60 seconds then shaken for 30 minutes in 3% NaOCl, together with two drops of Tween 20. The sterilized seeds were rinsed three times in sterile deionized water in a laminar flow cabinet. Seeds were initially germinated on water medium, and the shoots of the germinated seeds subcultured onto Murashige and Skoog (MS) medium (1962) supplemented with 0.01 mg L^{-1} 6-Benzyl Amino Purine (BAP) and 3% sucrose. The pH of the medium was adjusted to 5.8, and 7 g L^{-1} of agar added prior to being autoclaved at 121ºC for 20 minutes. Each 60 mL aliquot of the medium was dispensed into a 250 mL flask. Microshoots were incubated at 24±2ºC of a 16 hour photoperiodism and a irradiance of 50 µmol m^{-2} s^{-1} and illuminated by cool white fluorescent lamps. Microshoots produced from these explants were subcultured onto MS medium supplemented with 1.0 mg L^{-1} 6-Benzyl Amino Purine (BAP) and 0.1 mg L^{-1} of Indole Acetic Acid (IAA).

Effect of Cytokinins on Shoot Proliferation

Explants were subcultured on the MS medium with no growth regulators for four weeks to avoid the carryover effect of the cytokinin. Microshoots with apical meristem (15 mm in length) were then subcultured in fresh MS medium. For shoot proliferation, the microshoots were subcultured on MS medium supplemented with BAP, kinetin or zeatin at 0.0, 0.3, 0.6, 0.9, 1.2, 1.5 or 2.0 mg L^{-1}. For each replicate, a 60 mL of the medium aliquot was dispensed into a 250 mL flask. Each treatment consisted of 30 replicates each sample containing 3 microshoots. Culture conditions were maintained as described in the previous section. Data was collected regarding the number of new shoots per initial microshoot as well as maximum shoot height after the passage of 5 weeks.

Effect of Auxins on *In vitro* Root Formation

Microshoots of 15 mm length were subcultured on MS medium containing Indole-3-Butyric Acid (IBA), IAA or Naphthalene Acetic Acid (NAA) at 0.0, 0.3, 0.6, 1.2, 1.5 or 2.0 mg L^{-}

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[1]. For each replicate, 60 mL of the medium was dispensed into a 250 mL culture vessel. Each treatment consisted of 30 replicates with each sample containing 3 microshoots. Culture conditions were identical to those previously described. Number of new shoots, maximum shoot length, number of roots per initial micorshoot, root length, as well as shoot developing root percentages were recorded after the passage of 5 weeks.

Ex vitro Acclimatization

In vitro rooted plantlets were extracted from the medium, washed in a water bath at 25-30 °C and transplanted to 6×6×6 cm plastic pots containing a sterile mixture of 1 peat: 1 perlite. Shoots were grown under intermittent mist. Humidity was reduced gradually by creating a hole on the plastic bag, increasing its size every 3 days over a period of 2-3 weeks. The survival percentage of the acclimatized plants was recorded following 5 weeks.

Medium Term Conservation

Before starting the experiment, the *in vitro* grown shoots were transferred to hormone-free MS medium for 2 weeks. The shoots that were of 15 mm length were transferred to hormone-free MS medium supplemented with a series of concentrations of sucrose, glucose or fructose (0, 3, 6, 9 or 12% w/v). The stored explants were incubated in the growth chamber at 24±2°C under either 16 hours photoperiodism (50 µmol m^{-2} s^{-1}) or dark conditions. The survival rate was reported after 16 and 32 weeks of storage. Re-growth data was collected 4 weeks following subculture on hormone-free fresh MS medium.

Cryogenic Storage Method

Shoot Tip Isolation and Loading with Loading Solution

Shoot tips of 2-3 mm length were excised under microscope from healthy *in vitro* shoots. The meristematic dome appeared as a glossy spot, surrounded by either 2 or 3 leaf primordia. Excised shoot tips were precultured on solid MS medium supplemented with 0.4M sorbitol and 0.1M sucrose for 1 day under dark conditions. Precultured shoot tips were placed in cryotubes (10 tips per cryogenic tube) and loaded for a duration of 20 minutes with 1.0 mL of the loading solution (80% PVS2; Sakai *et al*., 1990) at 0ºC. Subsequently shoot tips were cultured on MS medium containing 3% sucrose and incubated in the dark for 1 week, and then transferred to the growth chamber of 24±2ºC.

Vitrification of Shoot Tips

The vitrification technique as based on Sakai *et al*. (1990) were employed. The loading solution was removed and replaced with PVS2 which contained 30% (w/v) glycerol, 15% (w/v) DMSO, 15% ethylene glycol and 14% (w/v) sucrose dissolved in full strength MS medium. Shoot tips were exposed to the vitrification solution for 0, 20, 40, 60, 80, 100, 120, 160 or 200 minutes at either 25 or 0ºC. The vitrification solution was replaced once at the end of the exposure time, and then the tips transferred to 2 mL cryotubes and suspended in 0.5 mL of fresh PVS2, prior to immersion in liquid nitrogen (LN). The cryogenic vials containing the shoot tips and the vitrification solution were directly plunged into LN at –196ºC for a period of 1-24 hours. Another set of shoot tips undergoing the same treatment was not exposed to LN, being used as control (-LN). Following storage in LN, cryotubes were re-warmed rapidly in a water bath at 45ºC for 2 minutes. Subsequently, the vitrification solution was drained and washed for 15-20 minutes from the cryotubes and replaced with MS liquid medium supplemented with 14% (w/v) sucrose. Consequently, the shoot tips were transferred to solid hormone-free MS medium supplemented with 3% (w/v) sucrose and the cultures incubated for 1 week under dark conditions, and then

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transferred to the growth chamber at 24±2 ºC.

Recovery and Survival Assessment

Re-growth was defined as the percentage of shoot tips resuming growth within 8 weeks after being planted out. Recovery of shoot tips was observed at weekly intervals. The young plantlets were subcultured on the optimally defined multiplication medium. Surviving plantlets were subcultured into a hormone-free MS medium every 3 weeks. Approximately 10 shoots were tested with each experiment repeated for 6 times.

Statistical Analysis

Data were subjected to ANOVA analysis, with means plus standard errors estimated. Mean values were compared according to Fisher's Least Significant Differences test (LSD) at 0.05 probability. Data were analyzed using STATISTICA (StatSoft, 1995).

RESULTS AND DISCUSSION

In vitro Shoot Formation

The presence of BAP, kinetin or zeatin enhanced shoot development after 5 weeks in the culture (Table 1). Plant growth regulator-free medium did not affect shoot proliferation. MS medium supplemented with 0.9 mg L^{-1} BAP produced 5.9 microshoots following a growth period of 5 weeks (Figure 1). This was significantly higher (P=0.05) than the number of

Table 1. The effect of 6-Benzyl Amino Purine (BAP), kinetin or zeatin concentrations on the number of new shoot formation and shoot length of *in vitro* produced *Achillea millefolium* plantlets after 5 weeks being on culture medium.

Growth regulator (mg L^{-1})	Number of new shoot formations	Maximum shoot length (mm)
	BAP	
0.0 (control)	2.5 ef *	39.5 c
0.3	3.7 c	48.5 a
0.6	4.9 b	50.5 a
0.9	5.9 a	44.5 b
1.2	3.1 cd	42.2 bc
1.5	3.2 cd	37.2 c
2.0	2.2 ef	31.5 cd
	Kinetin	
0.3	4.9 b	48.5 a
0.6	5.8 a	44.5 b
0.9	3.1 cde	43.2 b
1.2	3.5 cd	40.3 bc
1.5	3.2 cd	42.2 bc
2.0	3.1 cde	39.9 c
	Zeatin	
0.3	4.8 b	44.5 b
0.6	3.0 de	45.6 b
0.9	2.9 de	43.5 b
1.2	2.8 de	44.5 b
1.5	2.6 ef	35.6 cd
2.0	2.5 ef	34.6 cd

*Means followed by the same superscript(s) within the column are not significantly different according to LSD at $P \leq 0.05$.

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Figure 1. Formation of multiple shoots of *Achillea millefoilum.* Extensive formation of shoots on (MS+0.9 mg L^{-1} BAP) after a passage of 5 weeks. The bar represents 5 mm.

microshoots produced from MS medium supplemented with 0.3 to 0.6 or 1.2 mg L^{-1} BAP. Similarly, BAP was found to produce maximum number of shoots in *Achillea millefolium* (Tuker *et al*., 2009), *Artemesia vulgaris* (Sujatha and Kumari, 2008), *Syzygium francisii* (Shatnawi *et al*., 2004) and *Prunus avium* (Shatnawi *et al*., 2007). The present study indicated that higher concentration of BAP, kinetin or zeatin reduced the number of micropropagated shoots. A similar observation was reported by Sujatha and Kumari (2008), on *Artemesia vulgaris* (Sujatha and Kumari 2008) and on *Prunus avium* (Shatnawi *et al*., 2007). Medium supplemented with 0.3 mg L^{-1} of either kinetin or zeatin increased shoot proliferation up to 4.8-4.9 shoots (Table 1). Callus formation was observed at the shoot bases in the presence of BAP, kinetin or zeatin. BAP at 0.9 mg L^{-1} proved to be useful for micropropagation because of its conspicuous capability to induce a higher number of shoots in comparison with either zeatin or kinetin. Multiplication parameters were significantly increased (P= 0.05) following an addition of 2.0 mg L^{-1} BAP, zeatin or kinetin to the medium. A similar result was reported on *Sesbania rostrata* (Jha *et al*., 2002). The effect of BAP, kinetin and zeatin on shoot length of microshoots cultured on MS medium was varied. Increasing BAP concentrations decreased shoot length (P= 0.05).

In vitro Root Formation

Root initiation was achieved from the bases of shoots after incubation on solid MS medium containing IBA, IAA, or NAA. Maximum rooting (20.8 roots ex-plant^{-1}) with a 41.8 mm average length was obtained on MS medium containing 1.2 mg L^{-1} of IBA. However, the number of roots per explant varied with increase in IBA concentration ranging from 7.2 to 20.8 (P= 0.05, Table 2). Responses of root number and length to hormone types and concentrations were obvious (P= 0.05). NAA applications at 0.3 mg L^{-1} were found to promote rooting, but not as prolifically as the other auxins (Figure 2). Maximum shoot length (71 mm) was recorded at 1.2 mg L^{-1} IBA (Table 2). The rooting percentage was 100 % in MS medium containing 2.0 mg L^{-1} of IBA; whereas hormone-free media produced the lowest rooting percentage. On the other hand, media containing IBA produced higher root number in comparison

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Table 2. Effect of Indole-3-Butyric Acid (IBA), Indole Acetic Acid (IAA) and Naphthalene Acetic Acid (NAA) concentrations on the number of new shoots per explant, maximum shoot length, number of roots per explant and maximum root length of *in vitro* shoots of *Achillea millefolium* after 5 weeks being on culture medium.

Grwoth regulators (mg L^{-1})	Number of new shoots/explants	Shoot length (mm)	Number of roots/explants	Root length (mm)	Shoot developing root (%)
		IBA			
0.0	1.35	38.10 d	7.25 c *	40.8 b	10 c
0.3	1.60	38.10 d	13.14 c	40.5 b	80b
0.6	1.65	57.35 b	14.04 c	40.5 b	95 a
0.9	1.55	58.40 b	18.46 b	39.4 b	95 a
1.2	1.45	71.30 a	20.58 a	41.8 b	95 a
1.5	1.45	58.20 b	19.64 a	35.3 b	90 a
2.0	1.45	65.10 a	13.60 c	34.9 b	100 a
		NAA			
0.3	1.50a	49.10 c	11.14 c	52.5 a	90 a
0.6	1.50	58.20 b	13.25 c	60.9 a	100 a
0.9	1.60	58.56 b	12.76 c	55.7 a	100 a
1.2	1.50	56.21 c	12.00c	55.7 a	100 a
1.5	1.50	56.48 c	13.05 c	55.8 a	100 a
2.0	1.50	62.28 b	10.15 c	40.8 b	100 a
		IAA			
0.3	1.50	49.47 c	12.04 c	39.5 b	60 b
0.6	1.50	61.47 b	11.10 c	40.2 b	95 a
0.9	1.50	68.55 a	9.07 c	54.83 a	95 a
1.2	1.45	67.40 b	12.10 c	53.7 a	100 a
1.5	1.50	64.18 b	10.15 c	41.8 b	100 a
2.0	1.60	36.18 b	11.15 c	40.8 b	100 a

*Means followed by the same superscript(s) within the column are not significantly different according to LSD at $P \leq 0.05$.

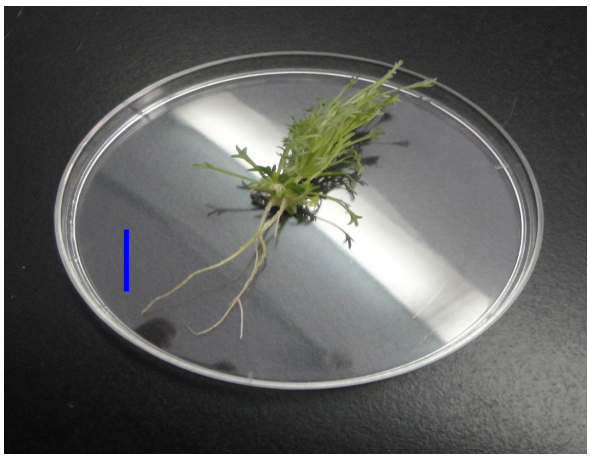

Figure 2: *In vitro* root formation of *Achillea millefolium* on MS + 0.3 mg/L NAA after 5 weeks. The bar represents 5 mm.

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with those containing either NAA or IAA (Table 2). The establishment of plantlets in the soil could be considered as easy because it produced thick roots in comparison with *Prunus avium* (Shatnawi *et al*., 2007). The optimum rooting response while using IBA was reported in *Achillea millefolium* (Tuker *et al*., 2009), *Syzygium alternifolium* (Sha Valli Khan *et al*., 1997), *Azadirachta indica* (Eeswara *et al*., 1998), and *Zyziphus jujuba* (Hossain *et al*., 2003).

Acclimatization

Survival of the rooted plants reached 70% under acclimatization conditions in the greenhouse, and the survived plants were green, healthy, and vigorous. All surviving plants showed uniform growth, had normal leaf development and lacked any detectable morphological variation. Further studies may be required on the *ex vitro* acclimatization to attain higher survival rates.

In vitro Conservation

Storage of microshoots at growth chamber temperature (24±2°C) for 32 weeks could prove proper for medium-term conservation method. Standard culture medium can be used for medium-term conservation. In this study, the culture media have been modified to induce growth reduction. This was achieved by increasing osmotic concentration of the medium using sucrose, glucose or fructose (Table 3). However, carbon source in the culture medium is an essential source of energy for maintaining the osmotic pressure (Shibli *et al*., 2006). In this study, re-growth was suppressed in the absence of the carbon source in the tissue culture medium, this observation being similar to those of the previous studies by Sujatha and Kumari (2008) on *Artemisia vulgaris*. Only 85% of the microshoots stored for 32 weeks on medium containing 3% sucrose were able to develop shoots (Table 3). Similar results were obtained by Shatnawi *et al*. (2011) on *Stevia rebuandia* and Sheyiab *et al*. (2010) on *Antirrhinum majus*. Relative to 3–9 % sucrose, glucose or fructose the 12% concentration decreased the survival after 16 and 32 weeks past of storage (P= 0.05). High sucrose concentration during the storage periods decreased the survival and the re-growth,

Table 3. Effect of different sucrose, glucose or fructose concentrations on survival and re-growth percentage of in vitro plantlets with shoot tips of Achillea millefolium stored under light/dark conditions at 24±2ºC after 16 or 32 weeks.

Osmotic agents % (w/v)	Sucrose			Glucose			Fructose		
	Survival after 16 weeks (%)	Survival after 32 weeks (%)	Survival after 32 weeks (%)	Re-growth after 32 weeks (%)	Survival after 16 weeks (%)	Survival after 32 weeks (%)	Survival after 16 weeks (%)	Survival after 32 weeks (%)	Re-growth after 32 weeks (%)
				Light conditions					
0	0.0 e	0.0f	0.0 d	0.0 d	0.0 c	0.0 b	0.0 d	0.0 c	0.0 e
3	98.6 a	88.6a	85.3 a	66.6 a	55.0 a	40.3 a	48.3 a	38.3 ab	23.3 b
6	60.3 b	85.0bc	40.6 b	63.6 b	56.6 a	31.6 a	48.3 a	38.3 ab	36.6 a
9	50.3 bc	33.6de	27.3 c	67.6 a	0.0 c	0.0 b	42.0 ab	41.0 a	17.0b c
12	40.6 cd	28.0de	23.6 c	40.6 b	0.0 c	0.0 b	28.3 bc	3.6 c	6.7 d
				Dark conditions					
0	0.0 e	0.0a	0.0 d	0.0 d	0.0 c	0.0 c	0.0 d	0.0 c	0.0 e
3	63.3 b	41.6cd	44.6 b	55.6 bc	51.7 a	36.6 a	18.3 c	19.3 b	6.6 c
6	50.0 bc	40.3cd	42.3 b	52.6 bc	50.0 a	30.0 a	41.6 ab	35.3 ab	15.0 bc
9	52.0 bc	42.3cd	38.3 c	76.6 a	25.0 b	12.3 b	31.6 bc	9.2 bc	3.7 de
12	18.0 d	3.6f	3.6 d	44.6 bc	11.6 c	0.0 b	3.5.0 d	0.0 c	0.0 e

Means followed by the same superscript(s) within the column are not significantly different according to LSD at P≤ 0.05.

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since, high sucrose concentration may decrease the moisture content of the tissue, may inhibit the respiration of tissues due to osmo-stress and this may consequently this may result in poor plant development (Danso and Ford-Lioyd, 2003; Shibli *et al*., 2006).

No new shoot was developed or root obtained during storage in treatments containing sucrose, glucose or fructose. This could be due to the absence of the required hormones, as the medium used was hormone-free to avoid any callus formation after storage. Therefore, the need for growth regulators under these conditions is not clear and may depend on the hormonal balance and requirements of either the particular species or genotype. Indexing culture using bacteriological media may be necessary to detect contamination prior to conservation to provide healthy cultures for longer storage periods and healthy material for distribution. However, further investigation is needed to extend the duration of storage before direct transfer to the recovery medium in order to make the technique fully applicable to *Achillea* germplasm storage.

Cryogenic Storage

An efficient and reliable cryopreservation of *A. millefolium* was performed using the vitrification technique. Shoot tips were precultured on solid MS medium supplemented with a mixture of 0.4M sorbitol and 0.1M sucrose for 1 day in the dark. In this study, preculture with those carbon sources have been shown to be very effective in improving the survival of the cryopreserved shoot tips. Similar results have been reported for *Wasabia japonica* (Matsumoto *et al*., 1994), and for *Prunus avium* (Shatnawi *et al*., 2007). Certainly sucrose contributes to glass formation of membranes protecting the functional and structural integrity of membrane and protein upon severe dehydration (Crowe *et al*., 1999).

Survival of the non-cryopreserved (-LN) shoot tips at 0 or 25°C was decreased with a gradual increase in the duration of exposure to PVS2 (Tables 4). The prolonged exposure (more than 80 minutes) to PVS2 may affect shoot tips' growth, because of the high PVS2 toxicity or because of the osmotic stress met by the shoot tips. Re-growth of non frozen shoot tips, following 20-80 minutes of exposure to PVS2 at 0°C, always exceeded 90% (Table 4), whereas, unfrozen shoot tips treated with PVS2 at 25 °C showed lower re-growth rate (77%) after a passage of 80 minutes. The re-growth rate obtained from cryopreserved shoot tips treated with PVS2 at 0°C for 60 to 80 minutes was 76 to 80% (Table 4). On the

Table 4. Effects of exposure time to PVS2 (minute) at 0°C on the re-growth (%) and 25°C on the survival (%) of non frozen (-LN) and frozen (+LN) *Achillea millefolium* shoot tips.

Exposure time (Min)	0°C on the re-growth		25°C on the re-growth	
	Non frozen	Frozen	Non frozen	Frozen
0.0	100 a*	0.0 d	100 a*	0.0 d
20	100 a	0.0 d	100 a	0.0 d
40	90.0 b	13.3 c	92.0 b	9.30 c
60	89.7 b	76.5 b	80.7 b	20.5 b
80	90.7 b	80.6 a	77.7 b	37.6 a
100	50.5 c	40.5 b	66.5 c	35.5 a
120	53.3 c	16.3 c	55.3 d	10.3 c
160	50.5 c	3.3 d	48.5 d	0.0 d
200	10.2 d	0.0 d	9.20 e	0.0 d

*Means followed by the same superscript(s) within the column are not significantly different according to LSD at $P \leq 0.05$.

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other hand, pretreatment with PVS2 at 25°C; permitted a maximum re-growth rate of 37% after 80 minutes of exposure to PVS2. Further increase in the duration of exposure to the vitrification solution PVS2 (more than 80 minutes), significantly decreased the survival of the non-cryopreserved and cryopreserved shoot tips (P= 0.05). Previous studies showed that vitrified cells and meristems with PVS2 at 0°C were expected to be reduced of injurious effects (González-Benito *et al*., 2004; Matsumoto *et al*., 1994). In this study, the maximum survival percentage of cryopreserved shoot tips (80%) was obtained when the shoot tips were exposed to PVS2 for 80 minutes at 0°C. Finally, re-grwoth of cryopreserved shoot tips was completely lost when they were treated with PVS2 for 200 minutes.

Optimizing the time of exposure, or the temperature during exposure to PVS2, is equally important in order to produce a high level of shoot formation after vitrification (Panis and Lambardi, 2005; Shibli *et al*., 2006). Additionally, one of the keys to successful cryopreservation is the careful control of dehydration and the prevention of the injury by chemical toxicity or excess stresses during preculture with PVS2 (Engelmann, 2004; Panis and Lambardi, 2005). Therefore, in order to determine the optimal exposure time to PVS2, shoot tips were treated with PVS2 either at 0 or 25°C, for different periods before being plunged in LN. However, for shoot tips quenched in LN, no survival was obtained prior to dehydration with the vitrification (PVS2) solution. The results showed that the vitrification procedure exerted a strong effect on the survival of shoot tips in *A. millefolium* following cryopreservation. With exposure to PVS2 at 25°C, the re-growth rate of frozen shoot tips was found to decrease when exposure duration to PVS2 increased, and the highest survival rate (37%) after storage in LN was reached after an 80 minutes exposure. Thus the duration of contact between the explants and the vitrification seems to be a critical parameter, in view of high toxicity. Successful vitrified and warmed shoot tips retained green colour and developed microshoots within 3 weeks without intermediate callus formation. Growth regulators were not added to the medium on which the shoot tips are plated after thawing. Plants derived from cryopreserved shoot tips were morphologically uniform. Cryopreservation of *A. millefolium* will allow long term storage of germplasm for *A. milliefolium*, and it would provide the opportunity to create the first *Achillea* collection in the near future. Further studies are necessary to increase the levels of re-growth of *A. millefolium* using other cryopreservation techniques.

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تکثیر و درسرما نگهداری گیاه بومادران (*Achillea mille folium* L. Asteraceae)

م. ا. شاتناوی

چکیده

گیاه بومادران *Achillea millefolium* که متعلق به خانوادهٔ Asteraceae است گیاهی داروئی و در معرض انقراض در اردن و کشورهای همسایه می‌باشد. از ریزتکثیری بذر (micropropagation) و به دنبال آن ریشه دوانی گیاه به عنوان راه حل مؤثر دیگری برای تکثیر بذر و حفاظت آزمایشگاهی *(In vitro)* آن استفاده شد. تعداد ۵/۹ ساقه در هر میکرو شاخه در محیط آگار MS که با ۰/۹ mg/L از (۶- بنزیل آمینو بیورین (BAP) تقویت شده بود به دست آمد. تأثیر انواع علظت‌های مختلف تنظیم کننده‌های رشد شامل ایندل -۳- بوتیریک اسید (IBA)، ایندول -۳- استیک اسید (IAA) یا نفتالین استیک اسید (NAA) بر روی رشد مورد آزمایش قرار گرفتند. بیشترین تعداد ریشه (۲۰/۸ ریشه در گیاه) از محیطی که دارای ۱/۲ mg/L از IBA بود به دست آمد. زنده ماندن گیاه در سطح ۷۰ درصد وقتی اتفاق افتاد که گیاهان ریشه‌دار به محیط خاک آزمایشگاهی شامل مخلوطی از پرلیت و پیت خو گرفته بودند. ریشه‌های A. millefolium به مدت تا ۳۲ هفته در محیط MS (تقویت شده با غلظت‌های متفاوتی از ساکازر، گلوکز یا فروکتوز) در دمای ۲ ± ۲۴ °C نگهداری شدند. پس از گذشت ۳۲ هفته ۸۸/۶ درصد از ریشه‌ها در محیطی که با ۳٪ ساکازر تقویت شده بود ادامه حیات دادند. علاوه بر این ۸۵/۳ درصد ساقه‌ها وقتیکه در معرض نور قرار گرفتند به رشدشان ادامه دادند. حفاظت در سرما از طریق ویتریفیکاسیون (Vitrification) تا رشد مجدد در سطح درصد تحت شرایط زیر مقدور گردید. رئوس شاخه‌ها در محیطی که با ۰/۴M سر بیتل و ۰/۱M ساکارز تقویت شده بود به مدت یک روز قرار گرفتند. به دنبال مرحلهٔ بالا نوک شاخه‌ها به مدت ۲۰ دقیقه در معرض محلول غلیظ ویتریفیکاسیون گیاهی (PVS_2)۲ قرار داده شدند و سپس در برودت °C به مدت ۶۰ دقیقه و قبل از اینکه در نیتروژن مایع قرار گیرند جهت خشک شدن در تماس با PVS_2 قرار داده شدند.

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