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In vitro Conservation and Cryopreservation of *Nandina domestica*, an Outdoor Ornamental Shrub

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Abstract

The study focused on an economically-important ornamental outdoor shrub, *Nandina domestica*, with the aims to (i) optimize an effective *in vitro* conservation method, and (ii) develop a cryopreservation protocol for shoot tips by the PVS2 vitrification and droplet-vitrification techniques. For *in vitro* conservation of shoot cultures, the tested parameters were sucrose content in the storage medium (30, 45, 60 g/L) and storage temperature (4 °C or 8 °C). Cryopreservation was performed by applying the PVS2 vitrification solution, in 2-ml cryovials or in drops over aluminum foil strips, for 15, 30, 60 or 90 min at 0 °C, followed by the direct immersion in liquid nitrogen of shoot tips. Results show that *N. domestica* shoots can be conserved successfully for 6 months at both the temperatures tested, especially when 60 g/L sucrose is used in the storage medium. However, conservation at 4 °C showed to be more appropriate, as hyperhydricity was observed in post-conservation of shoots coming from storage at 8 °C. As for cryopreservation, a daily gradual increase of sucrose concentration (from 0.25 to 1.0 M) produced better protection to the samples that were stored in liquid nitrogen. Indeed, with this sucrose treatment method, a 30-min PVS2 incubation time was enough to produce, 60 days after thawing, the best recovery (47% and 50%) of shoot tips, cryopreserved with PVS2 vitrification and droplet-vitrification, respectively.

Keywords: cold storage, droplet-vitrification, liquid nitrogen , one-step freezing, PVS2-vitrification, slow growth storage

Introduction

Integrated conservation programs combine the maintenance of plants *in situ* (i.e., in their natural habitats) and *ex situ* (i.e., outside their natural habitats) to provide maximum safety of plant germplasm against genetic erosion (Havens *et al*., 2006). *Ex situ* conservation approach may comprise conventional methods, such as seed banks and field collections, or may involve biotechnological laboratory approaches, such as DNA banks, *in vitro* conservation and cryopreservation. The latters utilize *in vitro* tissue culture techniques for the conservation of plant tissues and organs at low (above freezing) or cryogenic temperatures (Lambardi and De Carlo, 2003). *In vitro* conservation (also called 'slow growth storage', 'minimal growth storage' or 'medium-term conservation') provides a unique support for commercial micropropagation laboratories, as they allow the safe and economic maintenance of stock shoot cultures, thus helping the nursery to counteract market competition by broadening the offer of species and cultivars (Lambardi and Ozudogru, 2012). The technique allows the storage of *in vitro* shoot cultures for many months (depending on the species and the method applied), in aseptic conditions, without requiring periodic subculturing. This is achieved through the reduction of plant metabolism (i.e., growth rate) which permits the reduction of subculture frequency, and reduces markedly the hand labor and cost, as well as the risk of contamination. The plant metabolism can be limited in several ways: (i) by reducing temperature and light intensity provided to the cultures (cold storage), (ii) by addition of osmotic compounds (such as mannitol or sucrose) or (iii) by using growth retardants in storage medium (Grout, 1995). Among these, the most commonly used approach so far is cold storage, which reduces the respiration, water loss, wilting, and ethylene production of cultures, which in turn significantly reduces the cell metabolism and the shoot growth (Lambardi *et al.*, 2010).

Cryopreservation ensures the storage and maintenance of plant material for theoretically unlimited periods

of time, allowing long-term conservation of plant germplasm. Initially, cryopreservation protocols were based on 'slow cooling' of specimens, by reducing their temperature of 1 °C/min up to -40 °C, followed by their immersion in liquid nitrogen (LN, -196 °C) (Lambardi *et al*., 2008). Further developments, based on physical (i.e., desiccation) or chemical (i.e., vitrification) dehydration of the samples, allowed the direct immersion of the plant material in LN ('one-step freezing techniques') (Panis and Lambardi, 2006). Among the several approaches of one-step freezing technique, PVS2-based vitrification methods are proved to be highly effective for numerous indoor and outdoor ornamentals [e.g., *Chrysanthemum morifolium* (Halmagyi *et al*., 2004); *Rosa* spp. (Halmagyi and Pinker, 2006); *Thymus cariensis* and *Thymus vulgaris* (Ozudogru and Kaya, 2012); *Sequoia sempervirens* (Ozudogru *et al*., 2011), *Kalopanax septemlobus* (Shin *et al*, 2012)]. However, as PVS2 (30% glycerol (w/v), 15% ethylene glycol (w/v), 15% DMSO (v/v), in MS medium supplemented with 0.4 M sucrose, developed by Sakai *et al*., 1990) can be toxic (due to its DMSO content) for plant tissues, its concentration as well as its application time and temperature should be optimized carefully every time a new species or cultivar is tested. In the previous reports on ornamental plants, PVS2 treatment ranged from 5 min (*Chrysanthemum grandiflora*, Halmagyi *et al*., 2004) up to 3 h (*Dianthus caryophyllus*, Halmagyi and Deliu, 2007). A recently developed PVS2-based method, 'droplet-vitrification', enables the use of minute drops of the solution (4-5 µl) on aluminum strips which, once loaded with the explant, are immersed in chilled 2-ml cryovials, filled with LN (Panis *et al*., 2011).

Present study aimed to establish effective protocols of both *in vitro* cold storage of *Nandina domestica* shoot cultures, and cryopreservation of shoot tips by PVS2-based one-step freezing techniques. *N. domestica* (also known as Heavenly bamboo) is a suckering shrub of Berberidaceae family, originated from eastern Asia. Its evergreen leaves are brightly pink to red colour when they are young, green when they are mature, and they turn into red or purple again before they fall. The flowers are white (Dirr, 1990). It is a species of great economical importance in the Italian market, and it is largely produced by micropropagation. Hence, the development of conservation approaches, is regarded of strategic importance for commercial laboratories, as well as for its germplasm protection.

Materials and methods

Plant material

In vitro shoot cultures of *Nandina domestica* were obtained by Vivai Piante Battistini Società Agricola (Cesena, Italy) and maintained at standard culture conditions (i.e., 23±1 °C, 16 h photoperiod with a light intensity of 36 µmol m^{-2} s^{-1}) by monthly subcultures to fresh semi-solid DKW medium (Driver and Kuniyuki, 1984), supplemented with 0.5 mg/L benzyl adenine (BA) (proliferation medium). Shoot cultures, maintained in multiplication phase for 6 months, were used for *in vitro* conservation and cryopreservation trials.

In vitro conservation by cold storage

Single shoots of *in vitro* shoot cultures were transferred to 500-cc gas-tight glass jars containing 100 mL of semi-solid DKW medium, supplemented with 0.3 mg/L BA and different concentrations of sucrose (30, 45 and 60 g/L). After 15 days at standard culture conditions, the jars were directly transferred to 4 °C or 8 °C in darkness and stored for 6 months (cold storage). Following 3 and 6 months of conservation, 3 jars for each sucrose concentration were recovered from cold storage, healthy shoots were subcultured on fresh proliferation medium at standard culture conditions and monitored for their recovery potential.

Cryopreservation by PVS2-based vitrification methods

Consecutive steps of the cryopreservation protocol were as follows:

1. Cold hardening of in vitro shoot cultures

At the end of a four week subculture period, *in vitro* shoot cultures were directly transferred to 4 °C in darkness where they were maintained for 1 week.

2. Sucrose preculture of shoot tips

Two different approaches were tested for sucrose preculture of shoot tips, excised from cold-hardened shoot cultures. Shoot tips of the first group were transferred to semi-solid DKW medium, supplemented with 0.5 M sucrose for 48 h. The second group was plated on semi-solid DKW medium, supplemented with 0.25 M sucrose, and sucrose concentration was increased gradually by transferring them daily to DKW media containing 0.5, 0.75 and 1.0 M sucrose. Both the treatments were applied at 4 °C in darkness.

3. PVS2 treatment, freezing, thawing and plating of shoot tips

The classical PVS2 vitrification method (developed by Sakai *et al*., 1990) and the droplet-vitrification method (derived from droplet-freezing method; Kartha *et al*., 1982) were compared. For PVS2 vitrification, shoot tips (Fig. 1A) were transferred to 2-ml cryovials (12 explants per cryovial, Fig. 1B) and incubated with loading solution (LS; 2 M glycerol and 0.4 M sucrose - Matsumoto *et al*., 1994) for 30 min at room temperature.

LS was then replaced with PVS2 solution and shoot tips were treated for 15, 30, 60 or 90 min at 0 °C. Following PVS2 treatment, a group of explants was suspended in 0.6 ml of fresh PVS2 and directly immersed in LN. After at least 1 day of storage in LN, the frozen shoot tips were thawed in a water bath at 38 °C for 2 min and rinsed in a washing solution (liquid MS medium supplemented

with 1.2 M sucrose, Sakai *et al*., 1990) for 30 min at room temperature. Shoot tips were then transferred to recovery medium (semi-solid DKW medium supplemented with 0.5 M sucrose, 24 h) and afterwards to proliferation medium (semi-solid DKW medium supplemented with 0.5 mg/L BA). They were then monitored for their post-thaw recovery at standard culture conditions. A second group of samples (non-frozen control group) was immediately rinsed in a washing solution for 30 min at room temperature after PVS2 treatment and transferred to recovery and proliferation media.

For droplet-vitrification method, shoot tips were incubated with LS, contained in a Petri dish, for 30 min at room temperature. Pre-prepared sterile aluminum foil strips (~ 5 x 15 mm) were placed in an open Petri dish, resting on a frozen cooling element (~ 0 °C), and three drops of 4-5 µL PVS2 were dripped on each aluminum foil strip. One shoot tip was placed into each PVS2 drop (thus each aluminum foil strip contained three explants; Fig. 1C) and treated with the solution for 15, 30, 60 or 90 min.

After PVS2 treatment, the aluminum foils were transferred into chilled LN-filled cryovials (one aluminum foil per cryovial) and directly plunged into LN. Thawing was performed at room temperature by removing the aluminum foils from the cryovials and immersing them in the washing solution. When the explants were totally defrosted, they were transferred to recovery medium for 24 h, then on proliferation medium at standard culture conditions for regrowth. A group of control samples, treated with PVS2 but not frozen in LN, was washed and transferred to recovery and proliferation media, accordingly.

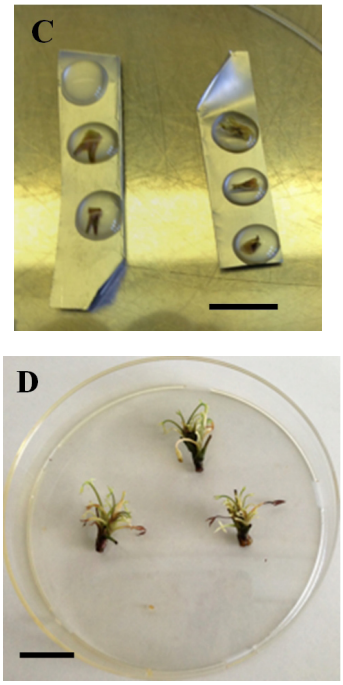
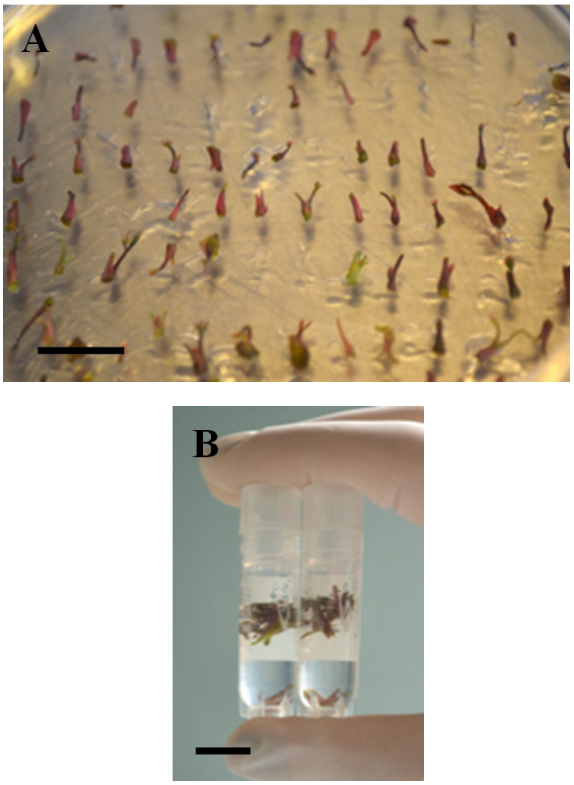


Fig. 1. Cryopreservation of *N. domestica*. (A) Shoot tips used in cryopreservation experiments (bar, 10 mm), (B) Shoot tips transferred to 2-ml cryovials for PVS2 treatment (bar, 5 mm), (C) Shoot tips placed into PVS2 drops on aluminium foils for droplet-vitrification (bar, 5 mm), (D) Shoot tip post-thaw recovery after gradual sucrose preculture, 30-min PVS2 application in droplet-vitrification method and storage in LN (bar, 15 mm)



All semi-solid media used in the study were supplemented with 30 g/L sucrose and gelled with 3 g/L gelrite. The pH was adjusted to 5.8 with 1N NaOH or HCl prior to the inclusion of gelrite and autoclaved for 20 min at 121 °C (1.4 Kg cm^{-3}).

Experimental design, data collection and statistical analysis

Three glass jars, each containing 12 shoots, were used for each slow growth storage trial. Data of shoot survival (%, nº of green or etiolated, shoots / total nº of shoots in conservation x 100), Relative Growth Rate [RGR, calculated as: (ln FW_{FINAL} – ln $FW_{INITIAL}$) x 100 / total days of conservation (where FW is the fresh weight of the plants)] and basal callus proliferation (%, nº of shoots producing callus / total nº of shoots in conservation x 100) were recorded after 3 and 6 months. Afterwards, the healthy shoots were transferred to fresh proliferation medium for the observation of their post-conservation regrowth. Data recorded were: shoot regrowth (%, nº of shoots that elongated or produced new shoots / total nº of shoots x 100), RGR, basal callus proliferation (%) and hyperhydricity (%, nº of hyperhydric shoots / total nº of shoots x 100).

For cryopreservation, 4 replications of 12 shoots tips were used for each treatment and the experiment was repeated at least twice. Following cryopreservation experiments, data of post-thaw recovery were recorded 30 days and 60 days after the transfer to proliferation medium.

Statistical analyses of percentages were subjected to ANOVA (after their arcsin transformation) and averages were tested using the Scott-Knott test ($P \leq 0.05$).

Results and discussion

To date, only limited numbers of reports are available in literature dealing with *in vitro* conservation of ornamental species. All these reports are based on cold storage approach, where shoot cultures are stored few degrees above freezing (usually 4-5 °C) (eg., Hempel and Hempel, 1984; Ballester *et al*., 1997; Reed *et al*., 2003; Previati *et al*., 2008). Storage in darkness is often preferred (eg., Hempel and Hempel, 1984; Previati *et al*., 2008). Accordingly, present study focused on development of an effective cold storage approach for *N. domestica* shoot cultures, testing different sucrose concentration in the storage medium at two different temperatures (4 °C and 8 °C) and in darkness.

Effect of sucrose concentration in storage medium during in vitro conservation

In vitro shoot cultures could be efficiently conserved for 3 months on different sucrose concentrations, exhibiting survival levels of 87-100% (Tab. 1).

Plant storage at 4 °C did not result any significance between the different sucrose concentrations in terms of survival and RGR. When storage was done at 8 °C, survival was always very high, reaching maximum (100%) when 60 g/L sucrose was used. In comparison to 4 °C, the conservation at 8 °C always produced higher RGR indexes. Interestingly, basal callus proliferation was decreased as the sucrose concentration was increased, independently from the storage temperature used.

In post-conservation, after the first subculture at standard culture conditions, regrowth was at the maximum (100%) when 30 and 45 g/L sucrose were used at 4 °C (Tab. 1). In contrast to what was observed after 3 months of conservation, in post-conservation shoots showed higher callus proliferation if they were previously conserved at higher sucrose concentrations. The storage at 8 °C always produced 100% shoot regrowth in post-conservation. However, although hyperhydricity has never been a problem for shoots conserved at 4 °C, it was 30-47% when shoots were conserved at 8 °C on storage medium containing 45 or 60 g/L sucrose, respectively. The highest post-conservation RGR (thus the fastest recovery of the shoot regrowth potential) was achieved when shoots were conserved at 4 °C on medium containing 60 g/L sucrose.

When conservation was prolonged to 6 months, a decrease was observed in the survival of the shoots maintained at 4 °C, and this was significant especially when 30 g/L sucrose was used in the storage medium (Tab. 2).

Differently, conservation at 8 °C did not have any detrimental effect on the survival of the shoots. With the prolonged time of conservation, the shoots showed higher RGR in comparison to the storage at 4 °C. Presumably, this effect was due to the initial stress imposed to the shoots when moving from standard culture conditions to cold storage. The lowest growth potential (RGR = 0.4) was observed in shoots maintained at 4 °C over 30 g/L sucrose containing medium. As for callus proliferation, in comparison to conservation for 3 months, the percent-

Tab. 1. Three-month conservation of *N. domestica* shoot cultures at 4 °C or 8 °C, in darkness. For each conservation temperature, different letters in each column indicate percentages and RGR values significantly different at $P \leq 0.05$

Sucrose Concentration (g/L)	During conservation			Post-conservation			
	Survival (%)	RGR*	Callus (%)	Regrowth (%)	RGR*	Callus (%)	Hyperhydri_ city (%)
At 4 °C, in darkness							
30	94.2[a]	0.00[a]	60.7[a]	100.0[b]	1.46[b]	17.3[c]	0.0[a]
45	93.9[a]	0.32[a]	41.0[a]	100.0[b]	1.56[b]	50.0[b]	0.0[a]
60	86.8[a]	0.44[a]	20.0[b]	92.0[b]	2.19[a]	90.7[a]	0.0[a]
At 8 °C, in darkness							
30	97.2[a]	0.34[b]	69.3[a]	100.0[a]	1.74[a]	39.0[c]	0.0[c]
45	97.2[a]	1.19[a]	65.7[a]	100.0[a]	1.61[a]	53.3[b]	30.3[b]
60	100.0[a]	1.81[a]	42.3[b]	100.0[a]	1.73[a]	96.7[a]	47.3[a]

* RGR= $(\ln_{FW_{FINAL}} - \ln_{FW_{INITIAL}}) \times 100$ / total days of conservation (where FW is the fresh weight of the plants)

Tab. 2. Six-month conservation of *N. domestica* shoot cultures at 4 °C or 8 °C, in darkness. For each conservation temperature, different letters in each column indicate percentages and RGR values significantly different at $P \leq 0.05$

Sucrose Concentration (g/L)	During conservation			Post-conservation			
	Survival (%)	RGR*	Callus (%)	Regrowth (%)	RGR*	Callus (%)	Hyperhydricity (%)
At 4 °C, in darkness							
30	75.0[b]	0.40[b]	16.7[b]	48.0[b]	0.62[b]	10.0[b]	0.0[a]
45	80.6[a]	1.11[a]	77.7[a]	86.0[a]	1.22[a]	83.0[a]	0.0[a]
60	88.3[a]	1.67[a]	37.0[b]	100.0[a]	1.27[a]	100.0[a]	5.6[a]
At 8 °C, in darkness							
30	97.2[a]	2.03[a]	94.3[a]	100.0[a]	0.37[a]	100.0[a]	23.7[b]
45	97.2[a]	1.96[a]	94.6[a]	100.0[a]	0.78[a]	100.0[a]	49.3[a]
60	100.0[a]	2.17[a]	94.6[a]	100.0[a]	1.05[a]	100.0[a]	52.0[a]

* $RGR = (\ln_{FW_{FINAL}} - \ln_{FW_{INITIAL}}) \times 100$ / total days of conservation (where FW is the fresh weight of the plants)

age of shoots showing basal callus proliferation was always higher, with the only exception of when 30 g/L sucrose was used at 4 °C.

After 6 months, regrowth of shoots in post-conservation was only 48% and RGR index 0.6 if they were stored at 4 °C and on the medium containing 30 g/L sucrose (Tab. 2). Such low regrowth level of the surviving shoots confirmed unsuitability of this *in vitro* storage combination (4 °C, 30 g/L sucrose) for *N. domestica* shoot cultures. However shoot regrowth and RGR increased as the sucrose concentration of the storage medium was 45 and 60 g/L (Fig. 2A-C).

Shoots stored at 8 °C showed a maximum regrowth in post-conservation, the fastest regrowth being achieved when the highest sucrose concentration was used in storage medium (Fig. 2D-F). It is important to note that, with the exception of the combination of 4 °C-30 g/L sucrose, the prolongation of conservation time to 6 months produced high percentages of shoots with basal callus proliferation (83-100%). However, basal callus proliferation never affected negatively the shoot regrowth and proliferation. Hyperhydricty in post-conservation was more evident in shoots from the storage at 8 °C in the darkness.

These results demonstrate that *in vitro* shoot cultures of *N. domestica* can be conserved successfully for 6 months at both the temperatures tested, especially when 60 g/L sucrose is used in the storage medium. This confirmed previous experiences with cold storage of fruit rootstocks ('GF677', 'Gisela 5®' and 'Mirabolano 29C'), the kiwifruit (*Actinia deliciosa*) cv 'Hayward' (Roncasaglia *et al.*, 2009) and ornamental species (*Anthurium andreanum* and *Ranunculus asiaticus* - Benelli *et al.*, 2012), where the increased sucrose concentration of the storage medium to 45 or 60 g/L provided better survival and recovery levels and made possible to extend the maximum storage time of fruits to 18-21 months and ornamentals to 8-9 months. As for the 2 tested temperatures, conservation at 4 °C seems to be more appropriate, taking into consideration the lowest incidence of shoot hyperhydricity during their regrowth in post-conservation. Those results are encouraging to test longer conservation periods also for *N. domestica* shoot cultures.

Cryopreservation of N. domestica by PVS2-based vitrification methods

Pre-treatment of explants prior to PVS2 incubation and storage in LN is a common approach which usually enhances their post-thaw recovery. Typical pre-treatments include: (i) the exposure of shoot cultures to temperatures below their active growing conditions (cold hardening, usually at 4 °C for temperate species) which induces freezing tolerance, and (ii) applying osmotic agents (e.g., sucrose preculture) which reduces cell water content, i.e., freezable free water which may damage the tissues during freezing. Indeed, *Photinia fraseri* survived better to cryogenic applications if the shoot cultures were cold hardened at 4 °C in darkness for 2-3 weeks (Ozden-Tokatli *et al.*, 2008). Protocorm-like bodies of *Oncidium*, precultured with high concentrations of sucrose and glycerol, maintained their cell shape and subcellular components and remained intact after storage in LN, while those that were not precultured were severely damaged (Miao *et al.*, 2005). Halmagyi and Pinker (2006) tested sucrose, glucose, mannitol and sorbitol in preculture medium and observed that the tolerance of rose shoot tips to storage in LN was the highest when sucrose was used. In the present study, one week of cold hardening at 4 °C in the dark was applied to *in vitro* shoot cultures of *N. domestica*, and two different approaches of sucrose preculture of excised shoot tips were tested prior to cryopreservation trials.

Unfrozen shoot tips (control group), cold hardened and precultured on 0.5 M sucrose for 2 days prior to PVS2

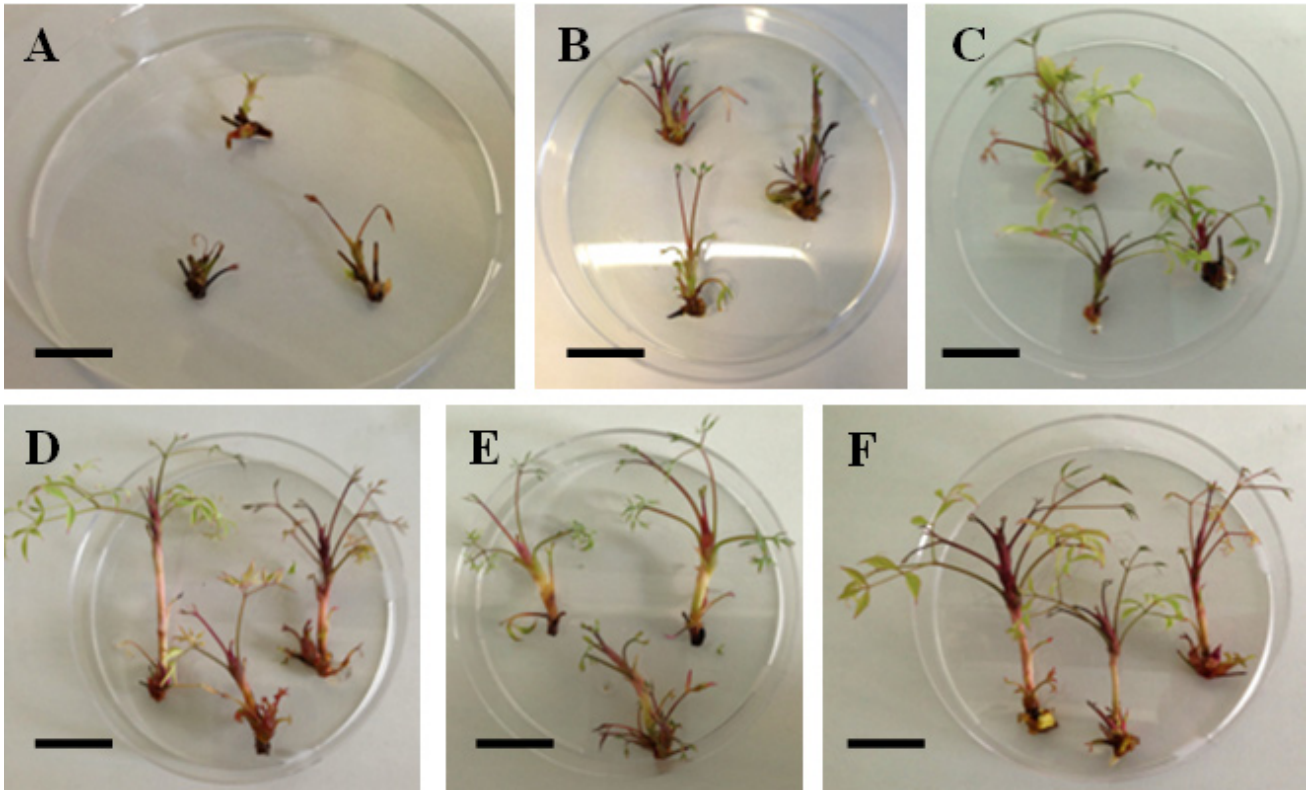


Fig. 2. *In vitro* conservation of *N. domestica* by cold storage. Shoot recovery following 6 months of conservation at 4 °C in darkness on storage medium containing 30 (A), 45 (B) or 60 g/L(C) sucrose, or at 8 °C in darkness on 30 (D), 45 (E) or 60 g/L(F) sucrose. A, bar 15 mm; B-F, bar 20 mm

incubation (PVS2 vitrification), maintained a high degree of vigour after 30 days of incubation, which further increased in time and reached 64-79% recovery after 60 days, being independent of the PVS2 incubation time (Tab. 3). Recovery rate was very high also when PVS2 was applied in drops (droplet-vitrification), best results being achieved after 30 (87%) and 60 (79%) min incubation.

As for the cryopreserved shoot tips, their recovery rates tended to increase as the culture period increased from 30 to 60 days. In both the cryopreservation methods, best results (37% for PVS2 vitrification; 50% for droplet-vitrification) were recorded after 60 min of PVS2 incubation. After 90 min of PVS2 incubation, a decrease in recovery rate of the shoot tips (frozen or not) was observed, indicating a toxic effect of the solution to the tissues when its use is prolonged, as reported elsewhere (e.g., Lambardi, 2002).

Control samples which were subjected to the gradual increase of the sucrose concentration during preculture suffered more and were more sensitive to the PVS2 solution as, for every time of PVS2 treatment, lower survival rates were obtained in comparison to constant treatment with 0.5 M sucrose (Tab. 3, down). However, the gradual sucrose treatment approach seemed to produce better protection to the samples that were stored in LN. Indeed, with both the cryo-methods, a 30-min PVS2 incubation time was enough to produce the best post-thaw recovery of shoot tips (47% and 50% for PVS2 vitrification and droplet-vitrification, respectively) (Fig. 1D).

It is worthy of note that droplet-vitrification method always produced better recovery rates of samples. In recent years, several reports showed the effectiveness of the droplet-vitrification method in comparison with other PVS2-based techniques (Panis *et al*., 2011). Halmagyi *et al.* (2004), for instance, compared different cryopreservation techniques (slow cooling, encapsulation-dehydration, droplet vitrification) for the cryopreservation of *Chrysanthemum morifolium* shoot tips, and reported that the latter was by far the most effective one in terms of post-thaw explant regrowth. Differently from classical PVS vitrification method, where samples are transferred to LN inside 2-ml cryovials, in droplet-vitrification method aluminum strips, which conduct the temperature much faster, are used. In addition, the aluminum strips are transferred to cryovials filled with LN. In this manner, the method provides a direct contact of the sample with LN. The extremely fast reduction of explant temperature from room temperature to -196 °C is believed to be the reason of the success of the droplet-vitrification method, able to prevent the formation of intra-cellular ice crystals more efficiently that the other PVS2-based techniques.

Tab. 3. Recovery (%) of *Nandina domestica* shoot tips after PVS2 incubation (Control) or PVS2 incubation and immersion in liquid nitrogen (LN+) by using two different cryopreservation methods: PVS2 vitrification and droplet-vitrification. For each sucrose treatment, different letters in each column indicate percentages significantly different at $P \leq 0.05$

PVS2 Incubation (min)	PVS2 Vitrification				Droplet-vitrification			
	Control		LN+		Control		LN+	
	Recovery 30 days (%)	Recovery 60 days (%)	Recovery 30 days (%)	Recovery 60 days (%)	Recovery 30 days (%)	Recovery 60 days (%)	Recovery 30 days (%)	Recovery 60 days (%)
Sucrose preculture: 0.5M, 2 days								
15	75.9a	75.9a	16.7a	20.8a	66.7a	66.7b	12.5a	37.5a
30	91.7a	79.2a	20.8a	33.3a	70.8a	87.5a	8.3a	41.7a
60	58.3b	70.8a	25.0a	37.5a	66.7a	79.2a	33.3a	50.0a
90	60.3a	64.4a	20.8a	20.8a	66.7a	68.3b	20.8a	24.4b
Sucrose preculture: 0.25-0.5-0.75-1.0M, 1 day each								
15	57.5a	61.5a	17.4b	39.0a	55.0a	58.3a	25.0b	41.7a
30	45.0a	45.0b	38.2a	47.3a	40.0a	40.0b	45.0a	50.0a
60	55.0a	55.0a	28.6a	38.2a	33.2a	33.2b	40.0a	39.0a
90	41.7a	41.7b	14.5b	28.2a	45.0a	45.0b	20.0b	35.0a

Conclusions

In vitro shoot cultures of *N. domestica* could be successfully conserved in slow growth storage for 6 months at both the temperatures tested. Use of relatively high (60 g/L) sucrose concentration in storage medium was necessary to obtain satisfactory survival levels of shoots at the end of the cold storage period, as well as their regrowth after they were transferred back to standard culture conditions.

Data collection of the cryopreservation trials were performed twice, i.e., 30 and 60 days after shoot tip thawing and plating on proliferation medium. An increase in the recovery rates was observed when monitoring was performed after 60 days, suggesting that evaluating the results just after the first subculture in post-thaw may not be sufficient as cryopreservation may induce a sort of "artificial dormancy" of explants that needs time to be overcome. Thus, a second observation is recommended to have more reliable results.

The study showed also that PVS2 was less toxic to the control samples when they were previously precultured on 0.5 M sucrose for 2 days; this application produced 50% post-thaw recovery only when PVS2 was applied as droplet-vitrification for 60 min. Although control samples suffered relatively more of the gradual increase of sucrose concentration during preculture, this procedured provided better protection to the samples frozen in LN, enabling the use of the toxic PVS2 solution for shorter period (30 min) to reach 47-50% recovery in both the methods. In summary, best cryopreservation protocol developed in this study is composed of (i) one week of cold hardening at 4 °C in the dark, (ii) a daily gradual increase of sucrose concentration (from 0.25 to 1.0 M) during preculture, and (iii) 30 min-PVS2 incubation in droplet-vitrification method.

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