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RESEARCH NOTE

Cryopreservation of white mulberry (*Morus alba* L.) by encapsulation-dehydration and vitrification

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Abstract Shoot apices of in vitro-grown plantlets of white mulberry, *Morus alba* L. cv Florio, were cryopreserved using either encapsulation-dehydration or vitrification. For encapsulation-dehydration, alginate beads containing apices were dehydrated for 1, 3, 5 or 7 days in a liquid medium containing various sucrose concentrations (0.5, 0.75, 1.0 or 1.25 M). Bead desiccation was performed using silica gel for either 0, 4, 6, 8, 9 or 14 h. For vitrification, apices were directly immersed for either 5, 15, 30 or 60 min in a vitrification solution (PVS2). Following encapsulation-dehydration, treatment of alginate beads with 0.75 M sucrose was more effective in promoting re-growth of explants after immersion in liquid nitrogen than in the presence of 0.5 M sucrose for either 1 or 3 days. Re-growth of explants was also observed following vitrification and this reached 47% with increasing duration of the PVS2 treatment from 5 to 30 min. Overall, the highest frequency of explant re-growth was obtained when explants were subjected to encapsulation-dehydration in the presence of 0.75 M along with a 3 day sucrose dehydration pre-treatment and followed by desiccation for 9 h in silica gel.

Keywords Alginate beads · Germplasm conservation · PVS2 · Re-growth · Silica gel · Sucrose

Abbreviations

BA	6-Benzyladenine
DMSO	Dimethylsulphoxide
LN	Liquid nitrogen
LS	Loading solution
MS	Murashige and Skoog basal medium
PVS2	Plant vitrification solution number 2
US	Unloading solution

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Introduction

Plant biodiversity is essential for classical and modern plant breeding programmes and provides a source of compounds to the pharmaceutical, food and crop protection industries. Preservation in field collections is risky, as valuable germplasm can be lost because of pests, diseases, adverse weather conditions and the maintenance of collections is labour-intensive and costly (Panis and Lambardi 2005).

Advances in biotechnology have generated new opportunities for genetic resources conservation and utilization and maintenance of plant materials at cryogenic temperatures (cryopreservation) is now a suitable option for long-term storage (Kameswara Rao 2004; Kong and Von Aderkas 2010; Reed 2008; Reed et al. 2000).

Cryopreservation protocols prevent formation of intracellular ice crystals, which can cause cell death and destruction of cell organelles during the freezing process, by inducing vitrification, an amorphous glassy state of water in cells, by applying dehydration and desiccation treatments and/or cryoprotectant combinations (Benson 2008).

In the encapsulation-dehydration procedure, originally described for cryopreservation of *Solanum* shoot tips (Fabre and Dereuddre 1990), shoot apices are encapsulated in alginate beads, dehydrated in liquid medium enriched with sugar for several hours or days and partially desiccated

prior to immersion in LN. This protocol has been developed for shoot apices of various species including apple, pear, grape and cassava (Engelmann et al. 2008 and references within reported).

Vitrification methods are based on the use of cryoprotective solutions (Sakai et al. 1990) which are viscous and easily supercooled leading to vitrification and avoiding intracellular ice crystal formation (Sakai et al. 2008). In the encapsulation-vitrification, a combination of the above methods, explants are encapsulated in alginate beads and treated with vitrification solutions before immersion in LN (Matsumoto et al. 1995; Sakai and Matsumoto 1996).

White mulberry (*Morus alba* L.) is native of East Asia and is extensively naturalized in eastern North America; its leaves are the sole food source of the silkworm, but mulberry is also cultivated for ornamental, commercial and pharmaceutical uses (Lee et al. 2011). The fruits of some varieties have a pleasant flavour and Stewart et al. (2003) reported that the mature fruit contains significant amounts of resveratrol, a strong antioxidant and putative anti-cancer agent. Mulberry is also a rustic plant with a deep root apparatus. For this reason it can grow at various altitudes, in lime-rich, dry and saline soils, in hot or cold areas and consequently can be planted for the reforestation of marginal zones (Tang et al. 2010). Interest in mulberry cultivation for the production of biomass is also increasing (Lu et al. 2009).

Morus alba was probably introduced in Italy between the ninth and twelfth century and its diffusion was related to the development of the sericulture. In Italy are either present plants derived from spontaneous hybridisation or selected varieties, reproduced by vegetative propagation (Cappellozza 2002).

The conservation of mulberry (*Morus* spp.) biodiversity is a priority and large ex situ in field germplasm collections are present in China, Japan and India (Atmakuri et al. 2009; Sohn 2003). Cryopreservation of *Morus* spp. have been developed using winter-dormant buds (Atmakuri et al. 2009; Niino et al. 1992b) and in vitro-grown shoot apices or buds in *Morus bombicys* (Niino 1995; Niino et al. 1992a; Niino and Sakai 1992; Yakuwa and Oka 1988).

The main aim of this study, was to establish the most suitable protocol for cryopreservation of shoot apices of *Morus alba* L. optimizing various steps involved in the process.

Materials and methods

Plant material and culture conditions

In vitro-grown shoots of mulberry (*Morus alba* L.), cultivar Florio (Fig. 1a), originated from axillary buds excised from one-year-old plant, were cultured on MS (Murashige and Skoog 1962) basal medium supplemented with 4.4 µM benzyladenine (BA), 87.6 mM sucrose (Eridania, Italy) and 5.5 g L^{-1} agar (B & V—Italy) and pH-adjusted to 5.7. Cultures were grown at 24 ± 1°C, under a 8 h photoperiod with a light intensity of 37 µmol m^{-2} s^{-1} (standard light conditions) and were sub-cultured every 21 days. All chemicals were purchased from Sigma-Aldrich, except where differently specified.

Shoot apices (2–3 mm long), excised from the micropropagated plantlets (Fig. 1b) and cold-hardened for 3 days at 5°C in darkness, were used for cryopreservation experiments. After recovery, explants from the optimal cryopreservation treatment were multiplied on the standard culture medium and rooting was performed on an MS basal medium supplied with 4.9 µM indole-3-butyric acid.

Encapsulation-dehydration

Apices were encapsulated in 3% alginate beads (Dereuddre et al. 1990) and treated in liquid MS medium with various sucrose concentrations (0.5 or 0.75 M) for 1, 3 or 5 days or 1.0 M for 1 day. Desiccation was performed by placing the beads in air-tight vessels of 50 ml (5 beads for each) with 8 g silica gel for 0, 4, 6, 8, 9, 14 or 20 h. Afterwards, beads were placed in 2 mL sterile polypropylene cryo-tubes (10 beads/tube) and immersed in LN for 24 h. In order to follow the desiccation process, beads pre-cultured under optimal conditions (0.75 M sucrose for 3 days) were used to measure residual water content.

Re-warming was performed by placing the cryotubes in the current air of a laminar flow cabinet. For each treatment, 10 explants were directly transfer to re-growth conditions for the control. Recovery was performed by transferring beads in Petri dishes containing an MS medium supplied with 1.0 mg L^{-1} BA. Explants were placed in darkness for 1 week then under low light conditions (5 µmol m^{-2} s^{-1}) for other 15 days and finally cultured in standard light condition.

Vitrification

Shoot apices were transferred to 2 mL sterile polypropylene cryotubes and suspended for 20 min in 2 ml of loading solution (LS): 2 M glycerol + 0.4 M sucrose in MS liquid medium at 25°C.

In the standard vitrification protocol the LS was removed and replaced with 2 ml of full strength vitrification solution, PVS2, consisting in 30% glycerol, 15% ethylene glycol, 15% dimethylsulphoxide (DMSO) and 0.4 M sucrose in liquid MS medium (Sakai et al. 1990). According to Matsumoto and Sakai (2000), in the three-step vitrification, LS was replaced with 2 ml of half-strength PVS2 solution for

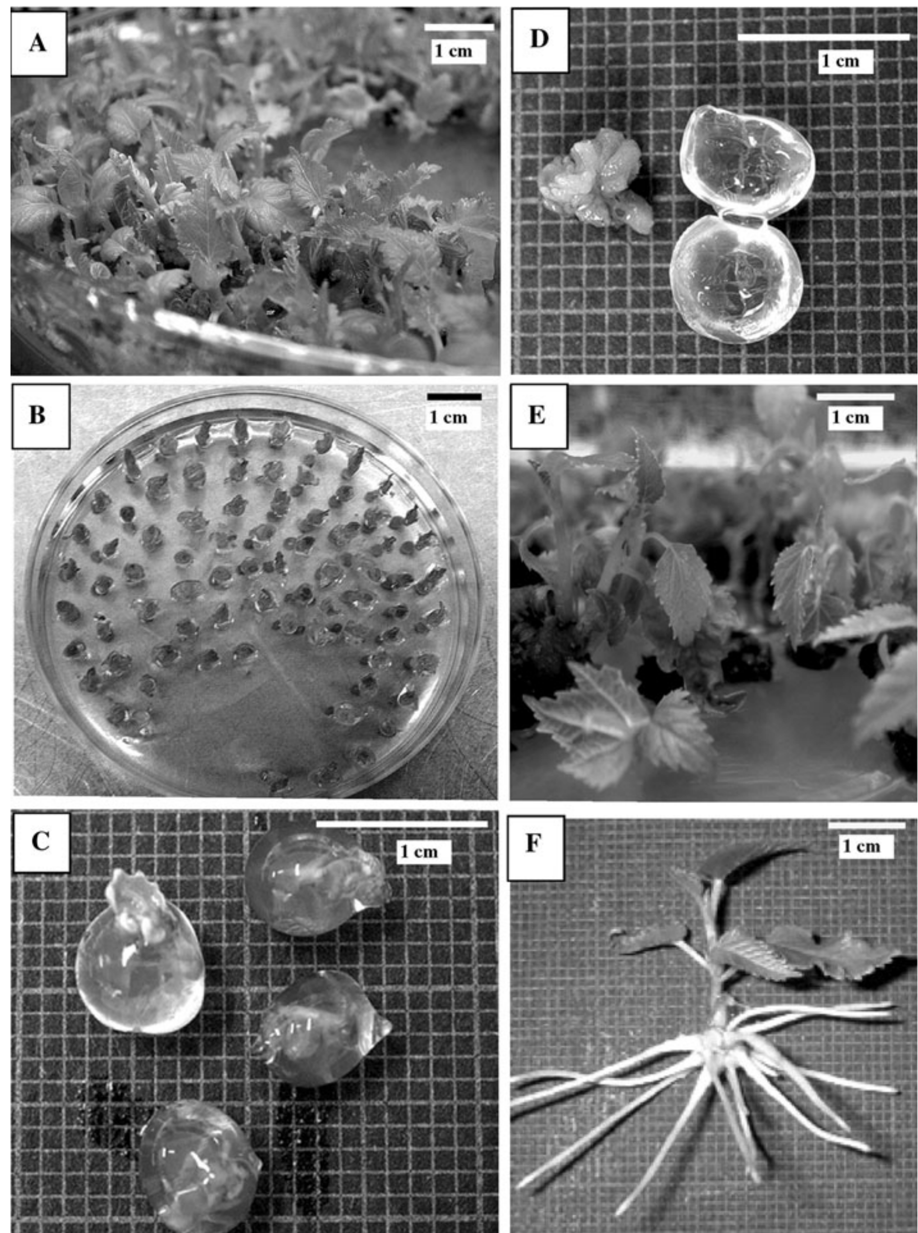

Fig. 1 Mulberry (*Morus alba* L.), cultivar Florio, cryopreservation process by encapsulation-dehydration. **a** In vitro grown shoots of mulberry. **b** Preparation of apices for cryopreservation **c** Re-growth starting 2 weeks after immersion in LN with incapsulation dehydration, 0.75 M sucrose for 3 days and 9 h silica gel. **d** Apices re-growth 4 weeks after cryopreservation. **e** In vitro multiplication and rooting (**f**) of recovered shoots

30 min. and finally with the full strength PVS2. When 5% DMSO pretreatment (Yamada et al. 1991) was applied shoot tips were immersed for 2 days on MS basal medium containing 5% DMSO, 87.6 mM sucrose and 0.3 g L^{-1} gelrite (Scott Laboratories Inc., Carson, CA, USA). Shoot apices were then transferred to the LS for 20 min and to the full strength PVS2.

For encapsulation-vitrification (Matsumoto et al. 1995), shoot apices were encapsulated in alginate beads, immersed for 20 min in LS and finally plunged in PVS2.

All the treatments with full strength PVS2 were performed in 2 ml cryo-tubes at 0°C for 5, 15, 30 or 60 min. The PVS2 solution was renewed 5 min before the time was over. Then cryotubes were directly plunged in LN and kept there for 24 h. For each treatment, 10 explants were directly transfer to re-growth conditions (control). Unloading was performed by immersing the cryotubes in a water bath at 40°C for 80 s. Then PVS2 was removed and apices were washed with the unloading solution (US) consisting in basal culture medium supplemented with 1.2 M sucrose for 15 min. For regrowing apices were placed on growth regulators free MS solid medium containing 0.4 M sucrose in darkness. After 2 days the apices were transferred to the standard culture medium for 1 week in darkness and then cultured in standard light condition.

Table 1 Encapsulation-dehydration in *Morus alba* L.

DES (h)	BM (% FW)	Length and concentrations of sucrose treatment						
		1 day			3 days		5 days	
		0.5 M	0.75 M	1 M	0.5 M	0.75 M	0.5 M	0.75 M
LN								
0		0	0	0	0	0	0	0
4		0	0	0	0	0	0	0
6		12 gh	30 e	0	10 h	30 e	10 h	0
8		25 ef	43 c	25 ef	33 de	60 b	30 e	20 f
9		33 de	55 b	30 e	58 b	67 a	30 e	30 e
14		35 d	43 c	23 ef	35 d	45 c	25 ef	20 f
20		10 h	18 fg	17 g	10 h	13 g	12 gh	10 h
Control								
0	77	100 a	100 a	97 a	97 a	93 a	77 b	73 b
4	33	100 a	97 a	93 a	97 a	93 a	73 b	77 b
6	21	100 a	97 a	97 a	93 a	93 a	70 b	67 b
8	20	97 a	93 a	97 a	97 a	93 a	67 b	67 bc
9	19	93 a	90 a	77 b	83 ab	80 ab	43 c	40 cd
14	18	90 a	87 a	77 b	80 ab	73 bc	47 c	43 c
20	17	47 c	43 c	37 cd	20 d	13 d	3 e	7 e

Effect of duration (days) and concentration (M) of treatment with sucrose and desiccation (DES) with silica on re-growth (%) of apices after cryopreservation (LN) and without immersion in LN (Control)

MC: bead moisture

Data marked by the same letter are not significantly different according to Duncan's test after arcsin transformation ($P < 0.05$); LN: n = 60; control: n = 30

Assessment of re-growth and statistical analysis

Re-growth was evaluated 6 weeks after re-warming. Apices which resumed normal development (production of new leaves and/or expansion of a new shootlet) were considered re-growing. Results of cryopreservation are means of 20 apices for three replications for each experimental condition. Ten apices for three replications were used for controls. A completely randomized design was used for the experiments. Analysis of variance (one way ANOVA) was carried out (Statgraphics Centurion software) and Duncan's multiple range test was applied to determine the significance of differences among means ($P < 0.05$).

Results and discussion

Encapsulation-dehydration

Osmoprotection treatment with 0.75 M sucrose was more effective in allowing re-growth of explants after LN immersion than 0.5 M sucrose either for 1 or 3 days; longer treatments (5 days) did not increase recovery or were even detrimental. Recovery in the control explants was not significantly affected till 3 days-treatment (Table 1).

Our findings showed that the application of a correct exposure time and concentration of sucrose is critical to insure re-growth in *Morus alba* as previously shown in cryopreservation of shoot apices of temperate and tropical species and embryogenic tissue of *Dioscorea bulbifera* and *Pinus nigra* (Engelmann et al. 2008 and references within reported; Ming-Hua and Sen-Rong 2010; Salaj et al. 2011).

Optimal desiccation time is related to the species and to the type of explant; for instance 32% of final moisture content was suitable for protocorm-like bodies of *Phalaenopsis bellina* (Khoddamzadeh et al. 2011) while 18-28% was optimal for *Sabal* embryos (Wen and Wang, 2010). However, water content of about 20% is reported to induce best recovery in several species including *Picea* (Hazubska-Przybyl et al. 2010), *Pyrus* (Condello et al. 2009) and *Morus bombicys* (Niino et al. 1992b). In *Morus alba* no re-growth after cryopreservation was obtained applying a physical desiccation treatment in silica gel for 4 h which reduced bead moisture to 33%. Increasing re-growth frequencies were observed with silica treatments that lowered bead moisture till 19% (9 h), while desication treatments longer than 14 h significantly decreased explant recovery (Table 1).

Table 2 Vitrification (Vitr.), three-step vitrification (TS Vitr.), pre-treatment with 5% DMSO and encapsulation-vitrification (E Vitr.) in *Morus alba* L.

PVS2 (min)	LN				Control			
	Vitr.	TS Vitr.	5% DMSO	E Vit.	Vitr.	TS Vitr.	5% DMSO	E Vit.
0	0	0	0	0	88 a	87 a	85 a	87 a
5	10 e	23 d	17 de	17 de	88 a	87 a	85 a	85 a
15	25 c	23 d	23 d	27 c	85 a	85 a	82 a	82 a
30	47 a	38 b	28 c	38 b	87 a	85 a	83 a	80 a
60	43 a	35 b	27 c	37 b	82 a	83 a	82 a	82 a

Effect of duration of the PVS2 treatment (min.) on re-growth (%) of shoot apices after cryopreservation (LN) and without immersion in LN (Control)

Data marked by the same letter within LN and control data are not significantly different according to Duncan's test after arcsin transformation ($P < 0.05$); LN: n = 60; control: n = 30

The overall highest re-growth after cryopreservation (67%) was obtained with an osmoprotection of shoot apices in 0.75 M sucrose for 3 days, followed by a physical desication in silica gel for 9 h (Table 1, Fig. 1c, d). Recovered shoots were easily multiplied and rooted (Fig. 1e, f).

Vitrification

Effects of the application of the vitrification protocols on cryopreservation of mulberry shoot apices are reported in table 1. Re-growth after immersion in LN increased with the increasing of the duration of PVS2 treatment from 5 to 30 min and explants treated for 30 min with the standard vitrification protocol showed the highest recovery (47%). A PVS2 treatment of 60 min did not further increase re-growth, regardless the method applied. Accurate control of dehydration is critical for prevention of tissue damage by chemical toxicity and strong osmotic stress during PVS2 treatment (Sakai et al. 2008). However, increasing PVS2 exposures did not significatively reduce re-growth in control mulberry shoot apices (Table 1) suggesting that this species is relatively resistant to PVS2, as previously shown for apple (Condello et al. 2011). Modified vitrification protocols did not produce significant improvements with respect to the standard vitrification protocol (Table 2).

Encapsulation-vitrification has been successfully applied to several species including apple (Sakai and Engelmann 2007 and references therein) and grapevine (Wang et al. 2004). However, we found that this protocol was less effective than standard vitrification for cryopreservation of shoot apices of *Morus alba*. This response was also previously found in apple cultivars and it was attributed to the physical protection of the alginate on the explants, reducing damage from manipulation during the cryopreservation process (Paul et al. 2000).

Overall, the most effective protocol was encapsulation-dehydration which gave higher re-growth frequencies than the vitrification-based ones.

Cryopreservation of *Morus alba* L. was previously reported using winter-dormant buds by Atmakuri et al. (2009). Dormant buds successful cryopreservation is related to the cold-hardy state of explants and consequently to the temperature fluctuation of the year (Towill and Ellis 2008). In addition, a slow freezing method is required. The protocol presented in this paper, based on the use of shoot apices excised from in vitro-grown shoots, can represent an improvement for cryopreservation of the species since it is not season-dependent for collection of explants and the direct immersion of explants in LN can facilitate its application.

In conclusion, we believe that this protocol can be an useful option for cryopreservation management of *Morus alba* germplasm.

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