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Research Note

Application of vitrification-derived cryotechniques for long-term storage of poplar and aspen (*Populus* spp.) germplasm

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The application of three different vitrification-based freezing strategies for the cryostorage of white poplar (*Populus alba* L.) and hybrid aspen (*P. tremula* L. × *P. tremuloides* Michx.) have been assessed. The PVS2 vitrification protocol was successfully applied to two white poplar *in vitro* clones stored for more than 6 months in slow-growth conditions (4 °C, in darkness) and showing clear signs of explant etiolation and decay. After 60 min of PVS2 treatment, *P. alba* L. (cv. Villafranca) explants isolated from axillary buds demonstrated significantly better potential for post-freeze regrowth (64%) compared to those obtained from apical buds (17%). Similarly, a high level of survival (78%) of the frozen hybrid aspen shoot tips was recorded following the application of the same technique. Using the 'encapsulation-vitrification' procedure, no toxic effects of the PVS2 treatment were noticed after 120 min exposure, however none of the cryopreserved (poplar and aspen) explants survived after 3 weeks. In contrast, the 'droplet-vitrification' technique appeared to be very efficient in the cryopreservation of white poplar shoot tips, which increases the opportunities for wider application of this method in other woody species.

Key-words: Aspen, cryopreservation, droplet vitrification, encapsulation-vitrification, *Populus* spp., PVS2 vitrification.

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Introduction

The poplar (*Populus* spp.) is rapidly being adopted as a model for studying different aspects of forest tree genetics for a number of reasons: its relatively small genome size, considerable genetic variation (both natural and resulting from breeding activity), fast juvenile growth, ease of *in vitro* clonal propagation and efficient transformation (Taylor 2002). Poplars are common throughout the northern hemisphere and well-adapted to a broad range of environments and climatic conditions. The white poplar (*P. alba* L.) is native to Central and Southern Europe and also found across Western Siberia and Central Asia in the riparian zones, as well as in natural forests. The species is appreciated for its ornamental value and broadly cultivated in urban areas. Among the white poplar cultivars in commercial use for years, the cv. Villafranca is that which has been intensively exploited in the production of timber and as a potential candidate for establishing 'energy biomass' plantations (Confalonieri et al. 2000).

Although the poplar genome has recently been entirely sequenced (Tuscan et al. 2004), the genetic variability within the species would suggest that efforts to identify reliable methodologies for preserving its germplasm should be intensified. In this context, the cryogenic approach can be considered as a highly innovative strategy, offering unrivalled opportunities for cost-effective long-term germplasm preservation (Tsaiand Hubscher 2004).

There have been successful attempts at applying both slow-cooling (i.e. controlled-rate freezing) and 'one-step freezing' procedures for the cryopreservation of various poplar explants, such as twigs, buds, seeds and callus (Lambardi 2002). An efficient system for long-term cryopreservation of white poplar by vitrification was developed by Lambardi et al. (2000) and successfully applied, with some modification, also to the cryostorage of a hybrid (*Populus tremula* L. × *Populus tremuloides* Michx.) aspen (Jokipii et al. 2004). However, the step of experimental protocols tested on one or a few genotypes in a genus may produce variable results with different genotypes/cultivars (Reed 2001). Therefore, additional studies may be required to adapt established procedures so that they are applicable to a wider range of genotypes/cultivars of interest.

The present study aims to:

- test the opportunity to adapt and optimize protocols based on PVS2 vitrification for the cryopreservation of white poplar (*P. alba* L.) in regard to other valuable *Populus* genotypes (clones, hybrids);
- evaluate the possibility of applying cryoprotocols to the preservation of shoot cultures, previously stored at 4°C for 6 months;
- develop and compare alternative one-step cryotechniques (encapsulation-vitrification and droplet-vitrification).

Materials and methods

White poplar (*P. alba* L.) shoot cultures from cv. Villafranca and from two clones (''CSM'' and ''MLF'') of significant interest in terms of germplasm preservation were used in this study. The two clones were established *in vitro* from ancient monumental white poplar trees located on the outskirts of Florence, Italy, representing local genetic resources.. Shoot cultures of these clones were maintained at 4 °C in darkness (slow-growth storage) for over 6 months and showed clear signs of etiolation and decay. In addition, material from a hybrid (*P. tremula* L. × *P. tremuloides* Michx.) aspen *in vitro* clone was also included, in a preliminary experiment.

Three different techniques for cryopreservation (Fig. 1) were used. They were (A) the basic procedure of cryopreservation by PVS2 vitrification (Lambardi et al. 2000) was tested with all the poplar and aspen clones/cultivars; (B) 'encapsulation-vitrification'; and (C) 'droplet-vitrification'. The last two techniques were applied to the ''CSM'' and ''MLF'' clones.

The shoot cultures of both cv. Villafranca and the hybrid aspen were previously cold hardened for two weeks at 4 ºC under cool white fluorescent light (PPFD of 60 μmol m^{-2} s^{-1}, 16 h photoperiod). Shoot tips (1.5–2 mm long) consisting of the api-

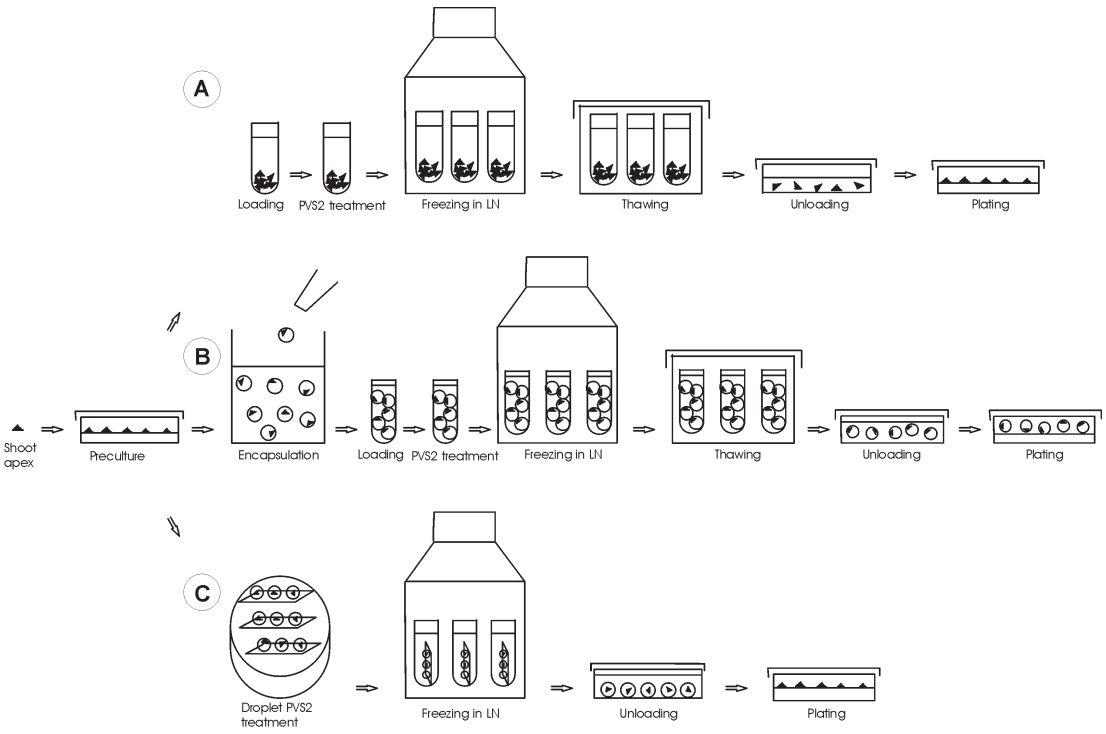

Fig.1. Schematic representation of the different procedures applied for cryopreservation of poplar/aspen shoot tips: A) PVS2 vitrification; B) encapsulation-vitrification; and C) droplet-vitrification.

cal meristem and 4–5 leaflets were excised under a laminar flow hood from apical and axillary buds (cv. Villafranca), or from other apical buds (‘‘CSM’’, ‘‘MLF’’ and aspen). In all experiments, the excised shoot tips were precultured for 48 h on hormone-free MS medium (Murashige and Skoog 1962), under the same conditions described for cold hardening.

The ‘CMS’ and ‘‘MLF’’ clones were used directly for shoot-tip excision. Here, a pool of shoot tips were directly plated on MS medium without NH_4NO_3 and supplemented with 1.5 μM BA, 0.5 μM GA_3 and 0.09M sucrose to test their potential to regrow when transferred to standard culture conditions (23 °C and 16 h photoperiod).

A. PVS2 vitrification

The shoot tips were loaded inside cryovials (2ml, 5–10 tips per cryovial) for 20 min at 25 °C with cryoprotectant solution (CP: 2M glycerol and 0.4M sucrose, Matsumoto et al. 1994). The cryoprotectant was then replaced with PVS2 solution (30% glycerol, 15% ethylene glycol and 15% DMSO, all w/v, in MS medium containing 0.4 M sucrose, Sakai et al. 1990). A 60-min exposure with PVS2 at 0 ºC was applied to the aspen and to both the ‘CSM’ and ‘MLF’ clones. Two different PVS2 exposure times (30 and 60 min) were tested with cv. Villafranca shoot tips. After the PVS2 treatment, the shoot tips were suspended in 0.6 ml of fresh PVS2 and directly plunged into liquid nitrogen (LN) for at least one hour.

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B. Encapsulation-vitrification

For encapsulation, shoot tips were immersed in an MS liquid solution (lacking the calcium component), which contained 3% (w/v) Na-alginate. Using a pipette, the buds were then individually transferred drop-by-drop (each drop containing a bud) to the complexing solution consisting of 100 mM $CaCl_2 \cdot 2H_2O$ and gently stirred to prevent the beads that had formed from sticking. The beads were then allowed to harden in the complexing solution for 20 min and retrieved using an autoclaved strainer. For cryopreservation, the beads were loaded with CP for 1 h at 25 °C, treated with PVS2 for 60 or 120 min at 0 °C, and finally plunged into LN.

C. Droplet-vitrification

With this procedure, droplets of PVS2 solution (10 µl) were put on small aluminium strips (0.5 × 2 cm), which were then placed on frozen tiles. One shoot tip was then introduced into each droplet and kept there for 30 and 60 min, respectively. Afterwards, the strips were placed in cryovials (containing LN) and then plunged in LN (Fig. 2).

Rewarming and plating

With each of the experiments, after at least 24 h storage at –196 °C, the shoot tips were rewarmed in a water bath at 40 ºC for either 1 min (PVS2 vitrification and droplet-vitrification) or 2 min (encapsulation-vitrification). The shoot tips (naked or encapsulated) were washed for 20 min at room temperature with liquid MS medium supplemented with 1.2 M sucrose, and were then plated on MS medium without NH_4NO_3 and supplemented with 1.5 µM BA, 0.5 µM GA_3 and 0.09 M sucrose.

Experimental design and statistical analysis

The experimental design consisted of 20–25 explants per treatment and each experiment was repeated at least twice. According to the specific technique, 5-10 shoot tips or 5 beads or an aluminum strip with 3 explants, were placed in each cryovial. Frequency of survival was defined as a percentage (%) of green shoot tips that clearly demonstrate regrowth (i.e., showing leaf expansion/shoot development) three weeks after plating on regeneration medium. Data were subjected to chi-square (χ^2) test for independence using SPSS statistical software (SPSS Inc.)

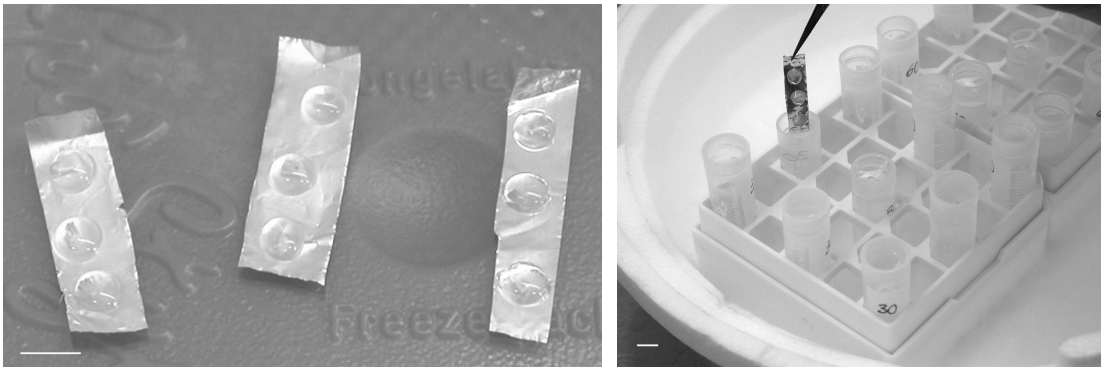

Fig. 2. Shoot tips of white poplar introduced into PSV2 drops on aluminium strips, placed on a frozen tile (left, bar = 5 mm) and then placed in 2.0 ml cryovials (right, bar = 5 mm).

Results

White poplar (*Populus alba* L.)

After 6 months of storage at 4 °C in the dark, the shoot tips excised from the etiolated *in vitro* cultures of the 'CSM' and 'MLF' clones showed regrowth and the *de novo*-formed shoots resumed proliferation in 2–3 weeks. When this material was used for the PVS2 vitrification trial, both 'CSM' and 'MLF' clones showed acceptable levels of survival, 54% and 44% respectively (Table 1). Following cryopreservation, the shoot development was much faster in the clone 'CSM'. The cv. Villafranca recorded a better survival of 64%, with shoot tips excised from axillary buds and treated with PVS2 solution for 60 min. This survival rate was significantly (p<0.001) higher than that of the apical buds (17%), as well as that of the shoot tips from both apical and axillary buds treated with PVS2 for 30 min (25% and 12% respectively).

In our study, the 'droplet-vitrification' technique improved the survival of shoot-tips, when compared to the PVS2 vitrification procedure. This effect was more evident with the clone 'CSM' where significantly (p<0.001) better survival (74%) of explants was observed following longer (60 min) PVS2 treatment (Table 2). To a lesser extent, the same effect was observed with the 'MLF' clone, but as distinct from the 'CSM' clone, peak survival of 55% was achieved after 30 min of PVS2 treatment. It is worth noting that, in general, the shoot tips took about 5 weeks on regeneration medium to convert into well-formed shoots.

No post-freeze survival was achieved after the application of encapsulation-vitrification technique to both the white poplar 'CSM' and 'MLF' clones. The survival of the control shoot tips (encapsulated and unfrozen) was 67%, even after a 120-min PVS2 treatment (Fig. 3).

Table 1. Survival of white poplar shoot tips 3 weeks after cryopreservation by 'PVS2 vitrification'.

White poplar clone/ cultivar	Type of buds	Survival % (–LN)		Survival % (+LN)	
		PVS2 30 min	PVS2 60 min	PVS2 30 min	PVS2 60 min
CSM	Apical	nt**	100.0	nt	53.8
MLF	Apical	nt	44.4	nt	44.2
'Villafranca'*	Apical	100.0 a	100.0 a	25.0 a	16.7 a
	Axillary	100.0 a	100.0 a	12.0 a	64.3 b

*Within the control (–LN) and liquid nitrogen (+LN) treatments, values followed by the same letter are not significantly different (p<0.001, Chi-square test).

**nt – not tested

Table 2. Survival of white poplar (*P. alba* L.) shoot tips 3 weeks after cryopreservation by 'droplet-vitrification' technique.

White poplar clone	Survival % (–LN)		Survival % (+LN)	
	PVS2 30 min	PVS2 60 min	PVS2 30 min	PVS2 60 min
CSM*	100.0 a	80.0 a	34.5 a	74.0 b
MLF	88.9 a	100.0 a	54.5 a	44.0 a

*For each clone and within the control (–LN) and liquid nitrogen (+LN) treatments, values followed by the same letter are not significantly different (p<0.001, Chi-square test)

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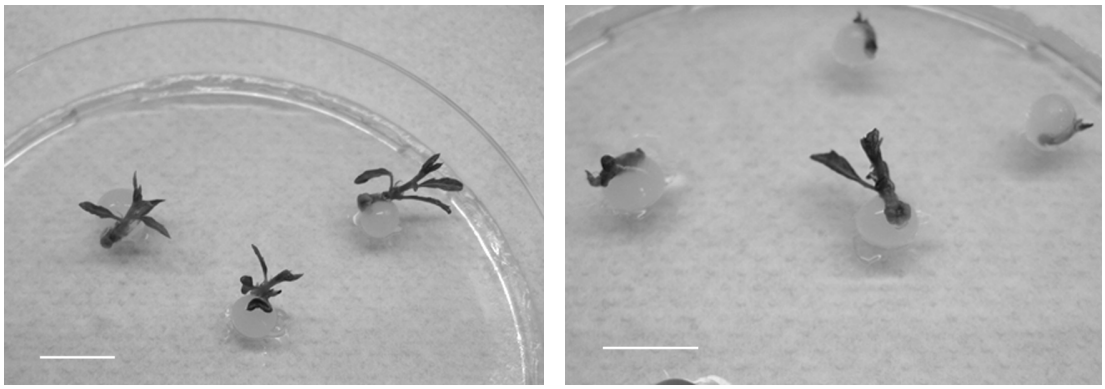

Fig. 3. Regrowth of white poplar encapsulated shoot tips (clone 'MLF') after PSV2 vitrification (non-frozen control). A) 120 min PVS2 treatment (bar = 10 mm); B) 60 min PVS2 treatment (bar = 10 mm).

Hybrid aspen (*Populus tremula* L. × *Populus tremuloides* Michx.)

With the hybrid aspen, 78% survival was achieved after the shoot tips were treated with PVS2 for 60 min, directly immersed in LN, thawed and plated on regeneration medium (data not shown).

Discussion

The results from the cryopreservation of shoot tips excised from white poplar clones kept for longer than 6 months at 'slow-growth storage' revealed new opportunities for the direct cryostorage of this plant germplasm. Despite these explants showing clear symptoms of tissue decay prior to cryopreservation, viable cultures regrew following exposure to LN. The successful cryopreservation results could possibly be explained by the acquisition of 'cold hardiness' during the 'slow-growth storage'. In particular, the rates of survival obtained after PVS2 vitrification/one-step freezing open the door to future opportunities for the reliable use of this technique to cryopreserve plant material with altered physiology. In addition, the level of shoot tip survival from the preliminary study with the hybrid aspen was similar to that obtained by Jokipii et al. (2004) with the same species. These encouraging results obtained with the poplar clones demonstrate the potential of the PVS2 vitrification protocol to be directly used with other *Populus* genotypes of wider or unknown genetic origin. The results also indicate that the normal regrowth of non-frozen control explants after vitrification is not connected to possible PVS2 toxic effects, and so cannot explain the lack of survival following the 'encapsulation-vitrification' procedure. Hence, further experiments are required using longer exposure times to the vitrification solution of the beads. 'Droplet-vitrification' is a relatively new technique, up until now only applied to a restricted number of plant species (Sakai and Engelmann, 2007). Some recent reports however would seem to confirm that it is a promising technique that will improve the tolerance of shoot tips to ultra-rapid freezing in LN (Panis et al. 2005, Sant et al. 2008). The results following the application of 'droplet-vitrification' are quite positive, and raise the possibility of further using this technique with other woody species. To the best of our knowledge, this is the first report describing the successful application of the 'droplet-vitrification' technique for cryostorage of forest species germplasm.

Abbreviations:
BA – benzyladenine,
CP – cryoprotective solution,

DMSO – dimethylsulfoxide,
GA_3 – gibberellic acid,
LN – liquid nitrogen,
MS – Murashige & Skoog,
PPFD – photosynthetic photon flux density,
PVS2 - plant vitrification solution 2

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