Strategies for Fast Multiplication and Conservation of Forest Trees by Somatic Embryogenesis and Cryopreservation: a Case Study with Cypress (Cupressus sempervirens L.)

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Abstract

Common cypress (Cupressus sempervirens L.) is one of the most widespread species in the Mediterranean area. It has been traditionally cultivated for its ornamental value, becoming a typical feature of urban and rural landscapes, and high timber quality. In the last 30 years, cypress has been subjected to important breeding programmes, aimed to select clones tolerant to the widespread canker caused by the pathogenic fungus Seiridium cardinale, leading to various patented varieties today, available on the market, as well as for genotypes producing null or low amount of allergenic pollen. Somatic embryogenesis is a suitable in vitro regeneration method for fast cloning of conifer trees, and the cryopreservation of embryogenic callus is a significant tool for the safe long-term conservation of valuable cell lines. Recently, a complete protocol for the production of cypress plants from somatic embryogenesis was developed for the patented clone ‘Mediterraneo’. Here, the coupling of somatic embryogenesis and cryopreservation may offer a superior tool to propagate and maintain superior genotypes of cypress by overcoming repetitive subculturing of selected embryogenic callus lines. For the above, this study aimed to compare different cryopreservation techniques (PVS2-based vitrification and slow cooling) with the ‘Mediterraneo’ embryogenic callus line. Best results were obtained after the optimization of a slow cooling procedure, based on the 30-min treatment of embryogenic masses with a cryoprotective solution containing 180 g l⁻¹ sucrose and 7.5% DMSO, followed by the reduction of the temperature at a rate of -1 °C min⁻¹ up to -40 °C and the subsequent immersion in liquid nitrogen ("two-step freezing").

Keywords: embryogenic masses, liquid nitrogen, PVS2, slow cooling, vitrification

Introduction

Common cypress (Cupressus sempervirens L.) is a conifer belonging to the Cupressaceae family. The tree is widespread in the Mediterranean region since the Roman Empire for its aesthetic value and the high quality of its timber. Over time, cypress has become, together with olive, the symbol of the Tuscan landscape, where its iconic presence characterizes the hills and the countryside. In reforestation, cypress has also been largely used to protect soils from erosion and as a windbreaker for vegetables and fruit crops. Moreover, since cypress is one of the few forest species that are tolerant to calcareous, clayey and rocky soils, it is particularly suitable for marginal and sub-marginal sites.

A serious bark canker disease, caused by the fungus Seiridium cardinale, threatened the cypress starting from the half of the previous century (Panconesi, 1990; Danti et al., 2013a), producing extensive economic losses and spoiling the landscape both in the Tuscany territory and in other parts of the Mediterranean basin. Moreover, common cypress is known to produce and release a huge amount of allergenic airborne pollen during winter and, due to its wide distribution, the prevalence of winter pollinosis has reached more than 20% in the past decade in central Italy (Sin et al., 2008; Caimmi et al., 2012). As a consequence, large-scale breeding programs started in the past to select for canker-tolerant clones, leading to various patented varieties today available on the market (Panconesi and Raddi, 1991; Danti et al., 2006, 2013b), as well as more recently to select genotypes producing null or low amount of allergenic pollen.
Cypress has been traditionally propagated by grafting, even if the long reproductive cycles makes the breeding programme labour intensive and costly, as field testing and selection of clones may take 5 years or more. On the other hand, as many other conifers, common cypress is not an efficient species for micropropagation by axillary bud proliferation, starting from adult material (Giovannelli and De Carlo, 2007). However, efficient protocols of somatic embryogenesis have been developed in the time for many conifer species (see, e.g., Montalban et al., 2016; Klimaszewska et al., 2016), making this process a suitable tool in clonal forestry for large-scale production of selected genotypes and high-quality trees, as well as for synthetic seed production and for the maintenance of selected superior individuals. Somatic embryogenesis allows the production of bi-polar structures (somatic embryos) from proliferating undifferentiated cells, initially induced from somatic tissues. In conifers, the cells having the highest potential to produce an embryogenic callus culture are the suspensor cells of immature zygotic embryos. The process starts with the initiation of embryogenic callus, often called in conifers ‘embryogenic masses,(EMs), followed by their proliferation, development and maturation of somatic embryos and, finally, the conversion of somatic embryos to plantlets (Filonova et al., 2000; von Arnold et al., 2005; Klimaszewska et al., 2016). One weak point of somatic embryogenesis is due to the fact that the maintenance of each selected embryogenic callus line requires periodical subculturing, during which the EMs are divided and transferred to fresh proliferation medium. In the time (from months to some years depending on the species) a decline in the embryogenic potential of cells is observed; moreover, at each subculture, the callus line runs the risk of being accidentally contaminated during its handling under the laminar flow hood. Hence, the development of efficient cryopreservation procedures are today considered of strategic importance for the safe long-term conservation of such valuable embryogenic callus lines (Ozudogru and Lambardi, 2016). The coupling of somatic embryogenesis and cryopreservation may offer a superior tool to propagate and maintain selected genotypes of conifer species by overcoming repetitive subculturing of embryogenic callus lines.

Cryopreservation is an efficient technique, allowing the storage of plant material (tissues and organs) at ultra-low temperatures, generally the one of liquid nitrogen (LN, -196 °C). At such temperature, as well as at the temperature of the nitrogen vapour (about -160 °C), all the metabolic processes of cells are arrested. Cell viability is maintained when appropriate processes induce the vitrification (i.e., the transformation of the cell cytosol in a glassy state) of intra-cellular water molecules, thus preventing the formation of deleterious ice crystals and allowing the tissue to recover after cryostorage (Morris and Acton, 2013). Various cryogenic procedures have been developed over the past years for the long-term storage of different plant tissues and organs, such as shoot tips, somatic embryos, dormant buds, seeds and embryonic axes (Panis and Lambardi, 2006). The available cryo-techniques can be grouped in those allowing the direct immersion of specimens in LN ("one-step freezing"), and those requiring a "two-step freezing", i.e., an initial slow cooling (generally to -40 °C) before the further reduction of temperature to -196 °C (Lambardi and Shahravi, 2017). The latter is largely the most used cryotechnique for the long-term conservation of embryogenic callus lines from conifer species (Ozudogru and Lambardi, 2016).

A reproducible protocol for clonal reproduction of common cypress through somatic embryogenesis was first described by Lambardi (2000), and afterwards optimized by Barberini et al. (2016). The present study aims to compare different vitrification methods to develop an effective cryopreservation protocol of Cupressus sempervirens embryogenic callus. In particular, the attention was focused on a callus line, selected for its high embryogenic potential and derived from a patented clone of common cypress. Survival rate and regrowth capacity after the storage in LN was reported after treating the embryogenic cell lines with the two different cryopreservation approaches, i.e., the "one-step" and the "two-step freezing".

Materials and Methods

Establishment and proliferation of embryogenic cultures

A specific embryogenic callus line (‘DII1’), induced from the patented clone ‘Mediterraneo’ and selected for its high embryogenic regeneration capacity (Barberini et al., 2016), was used as a starting material for cryopreservation trials. Briefly, EMs developed from the suspensor cells of excised immature embryos after two months of culture on induction medium composed by DCR salts and vitamins (Gupta and Durzan, 1985), supplemented with cascin hydrolysate (500 mg l-1), myo-inositol (200 mg l-1), glutamine (100 mg l-1) and 10 µM 2,4-dichlorophenoxyacetic acid (2,4-D). The same medium without plant growth regulators (PGR-free) was used as proliferation medium, maintaining the selected cell line in the proliferation phase for several months by subculturing in 4-week intervals. Induction and proliferation phases took place in semi-solid media (gelled with 7 g l-1 “Plant agar”), inside 90- mm Petri dishes, maintained in growing chamber in the dark at 23±1 °C.

Before cryopreservation trials, Petri dishes containing EMs during active proliferation phase (i.e., after 10 days of culture on PGR-free medium) were transferred to a 4 °C climate chamber, in the dark, for 7 days (cold hardening).

Cryopreservation by PVS2-vitrification technique ("one-step freezing")

In the PVS2-vitrification technique, all the explants were treated with a loading solution (LS), containing 2M glucose and 0.4M sucrose (Matsumoto et al., 1994) for 30 min at room temperature. After LS removal, explants were treated with PVS2 solution (30% glycerol, 15% ethylene glycol and 15% DMSO, all w/v, prepared in liquid medium with a final concentration of 0.4M sucrose; Sakai et al., 1990) and held for 30, 60 or 90 min at 0 °C in Nalgene tray. After each treatment, the PVS2 solution was replaced with 0.6 ml of fresh PVS2, then cryovials were immediately plunged into LN and stored for at least 2 h. One out of 6 cryovials per treatment was treated as above, but not frozen in LN and used as control. Control explants were immediately washed...
Cryopreservation by slow cooling ("two-step freezing")

The procedure developed by Ozudogru et al. (2010), with some modifications, was followed. In short, 3 different cryoprotective solutions were tested, all having the common base of liquid PGR-free DCR medium and 7.5% (v/v) of DMSO: 120, 150, and 180 g l⁻¹ sucrose (equivalent to 0.35, 0.44 and 0.53M). For each solution, 3 treatment times were compared: 30, 60 and 90 min. Thirty clumps of EMs per treatment were picked and grouped together in the same 15 ml-plastic tube, filled with 10 ml of the appropriate solution. The final amount of 7.5% (v/v) DMSO was gradually reached by the following procedure: 5 ml cryoprotective solution containing the selected amount of sucrose and 15% DMSO were gradually added in three steps (1 ml, 2 ml and 2 ml) to 5 ml of DMSO-free sucrose solution, over a total period of 30 min (i.e. 7.5, 7.5 and 15 min), 60 min (15, 15 and 30 min) or 90 min (22.5, 22.5 and 45 min), to reach the final DMSO concentration of 7.5%. Following DMSO treatment, the callus clumps were mixed thoroughly and distributed to cryovials, each cryovial containing 1 ml of the suspension mixture. Slow cooling was achieved by placing the cryovials in a Mr. Frosty box (Nalgene), containing 250 ml isopropyl alcohol. The device was then kept in a -70 °C freezer until the temperature reached -40 °C at the rate of -1 °C min⁻¹. Afterwards, the cryovials were rapidly plunged into LN and kept therein for at least 2 h. The samples were then thawed in a 40 °C water bath until the DMSO solution had totally melted, poured onto a 50-mg Whatman filter paper, placed on gelled proliferation medium and cultured at 23±1 °C in the dark for 3 days. Subsequently, the clumps of EMs were subcultured(453,422),(879,434) by transferring the filter paper onto gelled fresh medium, and this operation was repeated every 2 weeks until the day 45.

In a further trial, EMs were (i) treated with DMSO and sucrose (180 g l⁻¹ for 30 min) and immediately placed in proliferation medium (control), or (ii) treated as above, slowly cooled to -40 °C in Mr. Frosty and plated, or (iii) treated, slowly cooled, stored in LN for 2 h, thawed and then plated. Following each thesis, a pool of samples was treated with the TTC test (see below), in order to determine EMs viability, and evaluated for survival in terms of percentage of cryovials showing red staining. Regrowth of samples was evaluated after 21 days after plating and expressed in terms of percentage of Petri dishes with proliferating calli.

Optimization of the slow cooling protocol

According to the results obtained from the above slow cooling experiments, a subsequent trial was carried with the aim to test a two-step treatment of callus clumps with high sucrose concentrations, i.e., a first in gelled medium, followed by the best treatment sucrose+DMSO selected in the previous trial. Following cold hardening at 4 °C for 7 days, EMs were cultured for 3 days at 4 °C, in darkness on a gelled DCR medium with (i) a crescent osmolarity (0.3, 0.5 and 1M sucrose, 24 h per each one), or (ii) at a constant concentration of 0.5M for the 3 days. The callus clumps were then treated with 180 g l⁻¹ sucrose + DMSO 7.5% (v/v), at final concentration, for 30 min. The following steps (slow cooling, immersion in LN, thawing and regrowth) were applied as described above. Control samples consisted of only treated, slow cooled and plated samples.

Somatic embryo maturation

After 45 days of culture on proliferation medium, the regrowing EMs from both controls and cryopreserved samples were transferred on a maturation medium, i.e., gelled DCR PGR-free medium, supplemented with 75 g l⁻¹ of polyethylene glycol (PEG). The number of mature somatic embryos was recorded after additional 3 months (equal to 3 subcultures) at 23±1 °C in the dark, as reported by Barberini et al. (2016).

Determination of viability by the TTC (triphenyl tetrazolium chloride) test

After each trial, one additional cryovial containing 3 clumps of EMs was incubated overnight with 1% TTC solution (1% w/v in 0.05 Sørensen’s buffer, pH 7.4) at 30 °C in the dark, and observed for the appearance of the red staining. Samples were scored as alive if the cells stained as a deep red colour, or dead if they remained yellowish.

Data analysis

Five (“one-step freezing”) to 8 (“two-step freezing”) replicates (cryovials) were used per each treatment of cryopreservation, while one cryovial was used as control. Each cryovial contained 3 clumps of EMs (0.25-0.50 mg each). As for the slow cooling experiment, 2 extra cryovials were included as slow cooled but not ultra-frozen in LN. Each experiment was repeated at least 3 times. After each treatment, viability was qualitatively assessed by TTC test. Regrowth of callus clumps was determined by weighting the callus at various times. Survival and regrowth rates were expressed as percentage of Petri dishes with viable regrowing cells, determined 3 and 30 days, respectively, after thawing and plating. All the data were expressed as mean±standard error (SE), and statistically analysed by ANOVA, using the STATISTICA 10 software. Percentage values were analysed after their arcsin square root transformation. Significant differences between means were determined by the Tukey’s post hoc test at P≤0.05.

Results

Cryopreservation of EMs by the PVS2-vitrification technique

In the “one-step freezing” approach, none of the treatments with the PVS2 vitrification solution induced tolerance to ultra-freezing in LN. As an example, Fig. 1 shows the regrowth of callus following 90 min of treatment.
in PVS2. After 10 days from plating, EMs treated with PVS2 but not ultra-frozen in LN started slowly to recover proliferation, and from the 20th day proliferation rate increased constantly, showing that no toxic effect was produced by the prolonged contact of cells with the vitrification solution. On the contrary, no sign of regrowth was observed from the cryopreserved EMs that, after 20 days of regrowth on proliferation medium, started to become necrotic. The same trend of both control and cryopreserved EMs was observed following the treatments with PVS2 for 30 and 60 min (data not shown).

**Cryopreservation of EMs by the slow cooling procedure**

The slow cooling procedure was effective in inducing the survival and regrowth of cryopreserved EMs of common cypress. The TTC showed to be very effective as preliminary test to evaluate embryogenic cells survival, immediately after the recovering from LN and plating (Fig. 2A), allowing the speeding up of the cryo-protocol optimization. Both the treatment time (from 30 to 90 min) and the sucrose concentration (from 120 to 180 g l\(^{-1}\)) showed to influence markedly the tolerance of EMs to the storage in LN by the "two-step freezing" approach (Fig. 3).

The highest callus regrowth after 30 days from LN conservation was obtained with the combination of 30 min of treatment with 180 g l\(^{-1}\) sucrose and 7.5% v/v of DMSO, with a significant difference respective to 120 and 150 g l\(^{-1}\) of sucrose treatment. After a 2-week period of slow regrowing rate (Fig. 2B), the cryopreserved embryogenic cells treated with 180 g l\(^{-1}\) sucrose for 30 min had a sudden rise of proliferation rate (Fig. 2C), which then remained faster for the whole tested period (45 days), in comparison with the 60- and 90-min treated ones (Fig. 4). No statistically significant difference was observed among the 3 levels of sucrose when the EMs were treated for 60 or 90 min. It should be noted that EMs proliferation after

![Figure 1](image1.png)

**Fig. 1.** Regrowing of non frozen (CTRL) and cryopreserved (LN+) embryogenic masses from 0 to 35 days after plating on proliferation medium, following the treatment with the PVS2-vitrification solution for 90 min. As showed by the control curve, callus survival was not affected by the vitrification treatment, but it did not acquire any tolerance to freezing in LN and did not regrow after thawing and plating. The trends were similar for samples treated for 30 and 60 min.

![Figure 2A](image2A.png)

**Fig. 2A.** TTC staining of cryopreserved embryogenic masses (EMs) after 30 min of treatment with 180 g l\(^{-1}\) sucrose and 7.5% DMSO (control, right), or exposed, slow cooled, plunged into LN and thawed (LN+, left). A red color is showed by both the samples.

![Figure 2B](image2B.png)

**Fig. 2B.** Callus regrowth after 3 days of slow cooling and 2 days of swelling (bar, 1 cm).

![Figure 2C](image2C.png)

**Fig. 2C.** The same callus from cryopreservation after 14 days of regrowth (bar, 1 cm).

![Figure 2D](image2D.png)

**Fig. 2D.** Plantlets developed from somatic embryos, formed from cryopreserved EMs (bar, 1 cm).
recovery from LN proceeded slowly if compared with non LN-frozen control and, usually, a gap of 7 to 15 days was observed. Subsequently, callus proliferation rate increased and, after about 4 weeks, became as high as control EMs. No difference was then observed between LN-frozen cells and control cells in the subsequent subcultures.

A further experiment aimed to compare the survival and regrowth capacity of embryogenic cells only treated with sucrose and DMSO, or treated and slow cooled at -40 °C with Mr. Frosty, or treated+slow cooled+stored in LN. Fig. 5 shows that, according to the TTC test, no significant difference was observed in terms of survival rate, which ranged between 75% and 82%. However, not all the cells treated at ultra-low temperature were able to regrow after 21 days. However, the regrowth rate for EMs plated immediately after slow cooling with Mr. Frosty was similar to that showed by EMs slow cooled and ultra-frozen in LN, indicating that a partial damage of embryogenic cells occurred during the slow cooling process, while the following immersion in LN did not produce any further negative effect on the potential of cell regrowth.

The best sucrose treatment (180 g l⁻¹ for 30 min) was then selected for an additional trial, aimed to evaluate the efficacy of a long pre-treatment with sucrose in gelled medium. According to Table 1, the progressive exposure of explants to 3 increasing sugar concentrations (from 0.3 to 1M, each of 24 h) positively affected both the survival and the regrowth of the frozen EM clumps, leading to a significant improvement of the number of explants able to survive and proliferate after 4 weeks of culture on PGR-free medium.

**EMs maturation and somatic embryos production**

After 45 days in the regrowth medium, stereomicroscopical analysis of EMs coming from both control and cryopreservation showed clusters of developing somatic embryos; those clumps were then transferred to the
maturation medium. After other 4 months of culture on PEG-containing maturation medium, mature somatic embryos developed from the LN-treated EMs, with an average of 11.3 somatic embryos per Petri dish. Somatic embryos were then easily converted in plantlets (Fig. 2D), following the procedure reported by Barberini et al. (2016).

Discussion

The possibility to propagate trees vegetatively creates significant advantages both for the deployment of selected genotypes through mass propagation, and for capturing and enhancing genetic gain in breeding programmes. The combination of somatic embryogenesis and cryopreservation is then beneficial in achieving high-value clonal forestry, as cryostored elite material can be preserved as backup to field collections without the need of repeated subculturing, or safely stored during progeny tests in breeding programmes. Somatic embryogenesis is one of the possible systems of propagation of conifers, and some of them have been also tested for cryopreservation with promising results. Although the "two-step freezing" technique has been largely preferred (Ozudogru and Lambardi, 2016), also "one-step freezing" methods, allowing the direct immersion of explants in LN, have been occasionally proposed (e.g., Kong and von Aderkas, 2011; Ozudogru et al., 2011; Touchell et al., 2002). In this study, only the slow cooling technique allowed satisfactory results in terms of survival and regrowth rates of cypress EMs after their storage in LN. The direct exposure to LN after the application of a PVS2-based vitrification protocol caused a very poor regrowth of embryogenic cells, indicating that this treatment was inadequate for the cryopreservation of *Cupressus sempervirens* EMs, regardless of the PVS2 exposure time.

Numerous protocols of cryopreservation by slow cooling underline the necessity to optimize the several protective steps preceding the "two-step freezing" of explants (Lambardi et al., 2008). The choice of best cryoprotectant is extremely important for the highly-hydrated embryogenic cells which undergo to high risks of intracellular-ice nucleation when exposed to ultra-low temperatures (-196 °C). A mixture of cell penetrating and non-penetrating osmotic substances are generally used, such as sugars, sorbitol, glucose and DMSO (Cyr, 1994; Krajáňková et al., 2011; Ozudogru and Lambardi, 2016). Here, the EMs of common cypress were successfully cryopreserved by adapting a protocol firstly developed for a *Pinus nigra* embryogenic cell line (Salaj et al., 2007) and, afterwards, optimized for *Fraxinus excelsior* (Ozudogru et al., 2010). In this procedure, based on the treatment of explants in a cryoprotective solution of sucrose and DMSO, it is fundamental to select the best combination of sucrose concentration and exposure time. For instance, to achieve best regrowth, EMs of common cypress required a 30-min exposure to a cryoprotective solution containing 180 g l⁻¹ sucrose and 7.5% (v/v) DMSO, i.e., a less intensive osmotic treatment in comparison with the one optimized for common ash (210 g l⁻¹ for 60 min; Ozudogru et al., 2010).

For all the EMs successfully recovered from cryopreservation, a slow regrowth was observed during the first 1-2 weeks of post-thaw, in accordance with previous reports on spruce and sawara cypress (Cyr et al., 1994; Maruyama et al., 2002). After this period of time, however, EMs started to proliferate intensely and to develop somatic embryos which matured and were converted to plantlets.

Conclusions

Common cypress is a good case study, showing how a combined strategy of somatic embryogenesis and cryopreservation can produce important results in the fast production of plants and in the long-term conservation of valuable embryogenic cell lines. To the best of our knowledge, this is the first report describing a successful step-wise protocol for the cryopreservation of embryogenic callus of *Cupressus sempervirens*. In short, the cryopreservation step-wise protocol optimized in this study consisted of (i) the cold hardening of the embryogenic callus, cultured on gelled DCR PGR-free medium (proliferation medium), at 4 °C in the dark for 7 days, (ii) the treatment of EMs in proliferation medium, added of gradually increased concentrations of sucrose (0.3, 0.5 and 1.0M, 24 h for eachone), (iii) the exposure of the callus clamps for 30 min to a cryoprotective solution, containing 180 g l⁻¹ sucrose and DMSO at gradually-reached 7.5% v/v concentration, (iv) their slow cooling at -1 °C up to -40 °C, (v) their immersion in LN, (vi) their thawing in water bath at 40 °C, (vii) the pouring of callus samples in Petri dishes, onto filter paper placed on proliferation medium, cultured for 3 days at 23±1 °C in the dark, (viii) the subculture of EMs (still on filter paper) every 2 weeks for the first 45 days, and every 4 weeks afterwards on gelled proliferation medium.

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References


