Available online: www.notulaebotanicae.ro
Print ISSN 0255-965X; Electronic 1842-4309
Not Bot Horti Agrobo, 2018, 46(1):32-38. DOI:10.15835/nbha46111011
Original Article

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

Maurizio LAMBARDI[1]*, Elif Aylin OZUDOGRU[1], Sara BARBERINI[2], Roberto DANTI[2]

[1]*IVALSA/Trees and Timber Institute, National Research Council (CNR), 50019 Florence, Italy; lambardi@ivalsa.cnr.it (*corresponding author); ozudogru@ivalsa.cnr.it*
[2]*IPSP/Institute for Sustainable Plant Protection, National Research Council (CNR), 50019 Florence, Italy; sara-barberini@libero.it; roberto.danti@ipsp.cnr.it*

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.

Received: 15 Apr 2017. Received in revised form: 09 Aug 2017. Accepted: 11 Aug 2017. Published online: 19 Sep 2017.

Cypress has been traditionally propagated by grafting, even if the long reproductive cycles makes the breeding program 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 intracellular 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 Shaarawi, 2017). The latter is largely the most used cryo-technique 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 casein hydrolysate (500 mg l^{-1}), myo-inositol (200 mg l^{-1}), glutammine (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 hold 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

in a washing solution (liquid DCR medium, containing 1.2 M sucrose) for 25 min and plated onto a filter paper, placed in Petri dishes, over gelled proliferation medium.

Cryopreserved cryovials were recovered from LN, then thawed on a water bath at 40 °C for 2 min. PVS2 solution was discarded and the clumps of EMs were washed with the above described washing solution for 25 min, and then regrown by their transfer to proliferation medium and monthly subcultured. Three clumps from each incubation time and from the control were used for TTC viability test (see below).

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-mm 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 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 plated 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-freezed 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 $P \leq 0.05$.

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

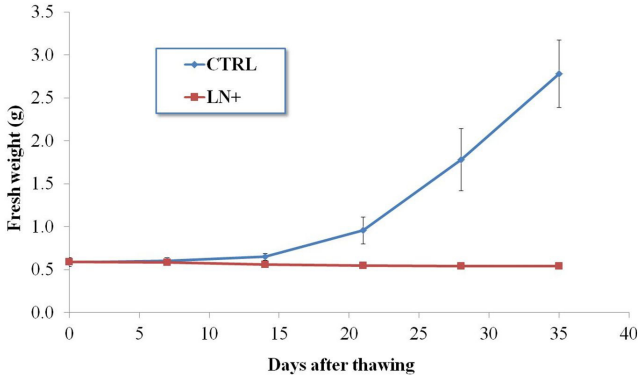

Fig. 1. Regrowing of non frozen (CTRL) and cryopreserved (LN+) embryogenic masses from 0 to 35 days after plating on proliferation medium, following the treatment with the PVS2-vitrification solution for 90 min. As showed by the control curve, callus survival was not affected by the vitrification treatment, 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

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

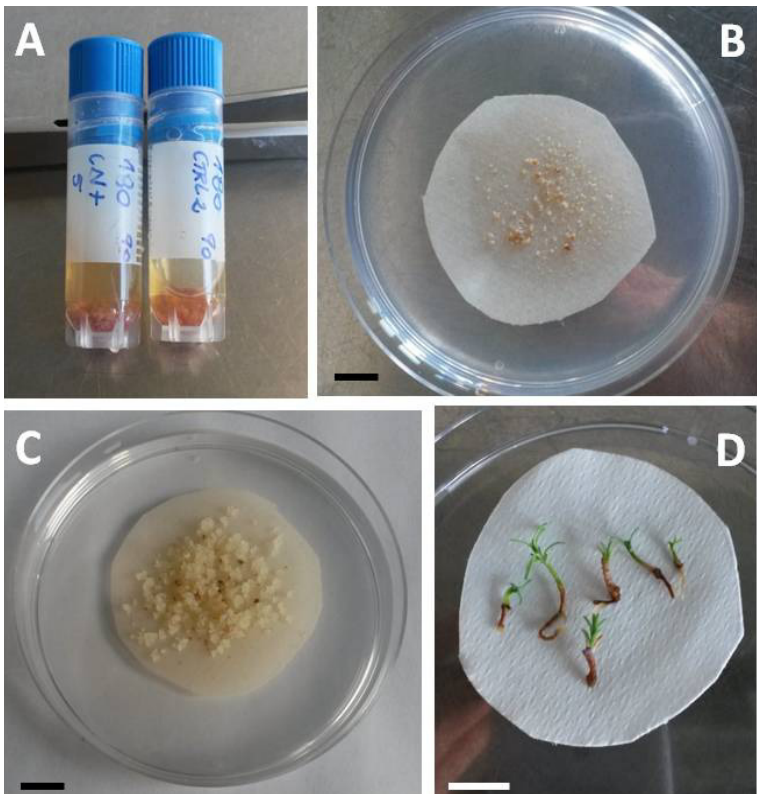

Fig. 2. Cryopreservation of embryogenic masses (EMs) of *Cupressus sempervirens*. A, callus samples treated with TTC after the exposure for 30 min to 180 g l^{-1} sucrose and 7.5% DMSO (control, right), or exposed, slow cooled, plunged into LN and thawed (LN+, left). A red color is showed by both the samples. B, callus from "two-step freezing" cryopreservation (sucrose treatment: 180 g l^{-1} for 30 min), 3 days after thawing and plating on proliferation medium (bar, 1 cm). C, the same callus from cryopreservation after 14 days of regrowth (bar, 1 cm). D, plantlets developed from somatic embryos, formed from cryopreseved EMs (bar, 1 cm)

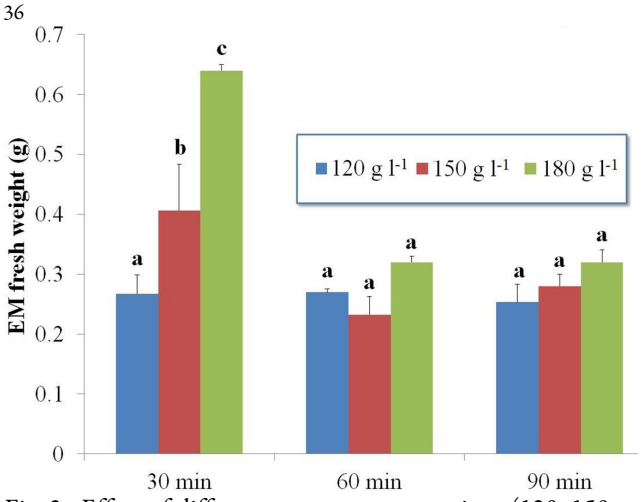

Fig. 3. Effect of different sucrose concentrations (120, 150 or 180 g l^{-1}) and exposure times (30, 60 or 90 min) on embryogenic mass proliferation, following cryopreservation. Data were collected 30 days after thawing and plating on proliferatium medium and expressed as mean±SE. For each treatment time, different letters indicate statistically different values at P≤0.05

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-freezed 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, stereo-microscopical analysis of EMs coming from both control and cryopreservation showed clusters of developing somatic embryos; those clumps were then transferred to the

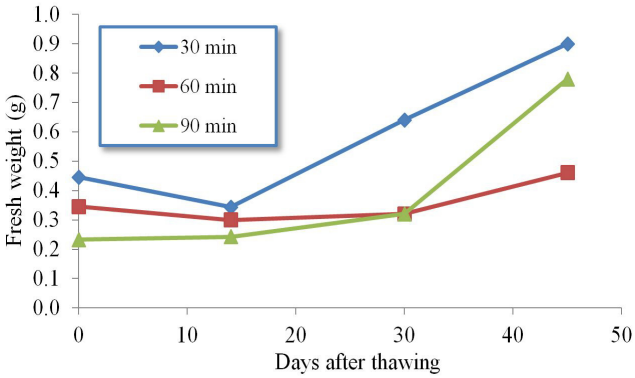

Fig. 4. Regrowth curves of embryogenic masses after cryopreservation by "two-step freezing", thawing and plating on proliferation medium. The curves here refers to callus samples treated with 180 g l^{-1} sucrose and 7.5% (v/v) DMSO for 30, 60 or 90 minutes

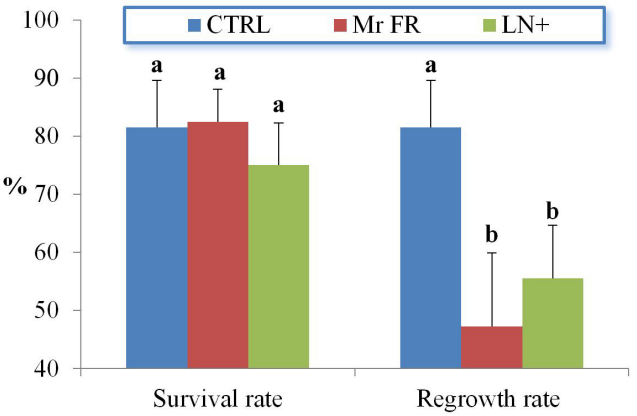

Fig. 5. Survival after 3 days (by TTC test) and regrowth rates (after 21 days on proliferation medium) of embryogenic masses. CTRL, samples treated with DMSO and sucrose (180 g l^{-1} for 30 min), and immediately plated onto proliferation medium; Mr FR, treated as above, slowly cooled to -40 °C in Mr. Frosty®, rewarmed and plated; LN+, treated, slowly cooled, stored in LN for 2 h, thawed and then plated. Bars represent percentages±SE. For each treatment time, different letters indicate statistically different values at P≤0.05

Table 1. Effect of a double sucrose treatment on survival and regrowth rates of *Cupressus sempervirens* embryogenic masses (EMs). CTRL, EMs cultured for 3 days at 4 °C, in darkness, on a gelled DCR medium with different sucrose concentrations, and plated. LN+, EMs treated as above, cryopreserved by "two-step freezing" (see text), thawed and plated. Survival rates determined by TTC test after 3 days of plating; regrowth rates determined after 21 days on proliferation medium. Data are expressed as percentages±SE. Different letters indicate statistically different percentages at P≤0.05

Sucrose concentration	Treatment	Survival rate (%)	Regrowth rate (%)
0.5M (3 days)	CTRL	95.8 ± 2.3 b	75.0 ± 8.1 b
	LN+	56.2 ± 8.8 a	11.4 ± 5.2 a
0.3M; 0.5M; 1M (1 day each)	CTRL	81.5 ± 8.1 b	81.5 ± 8.8 b
	LN+	75.0 ± 7.2 b	55.6 ± 8.2 b

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 conversed 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 élite 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 system 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 underlined 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; Krajňáková *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 conversed to plantlets.

Conclusions

Common cypress is a good case study, showing how a combined strategy of somatic embryogenesis and cryopreservation can produced 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.

Acknowledgements

M. Lambardi and E.A. Ozudogru gratefully acknowledge the financial support from the Italian project RGV-FAO on the conservation of plant genetic resources.

References

Barberini S, Danti R, Lambardi M (2016). Somatic plant regeneration from selected common cypress (*Cupressus sempervirens* L.) clones resistant to the bark canker disease. Plant Cell Tissue and Organ Culture 124:393-403.

Caimmi D, Raschetti R, Pons P, Dhivert-Donnadieu H, Bousquet PJ, Bousquet J, Demoly P (2012). Epidemiology of Cypress Pollen Allergy in Montpellier. Journal of Investigational Allergology and Clinical Immunology 22:280-285.

Cyr DR, Lazaroff WR, Grimes SMA, Quan G, Bethune TD, Dunstan DI, Roberts DR (1994). Cryopreservation of interior spruce (*Picea glauca engelmannii* complex) embryogenic cultures. Plant Cell Reports 13:574-577.

Danti R, Raddi P, Panconesi A, Di Lonardo V, Della Rocca G (2006). 'Italico' and 'Mediterraneo': two *Seiridium cardinale* canker resistant Cypress cultivars of *Cupressus sempervirens*. HortScience 41:1357-1359.

Danti R, Della Rocca G, Panconesi A (2013a). Cypress canker. In: Nicolotti

G, Gonthier P (Eds). Infectious Forest Disease. Oxfordshire, CABI Press, England pp 359-375.

Danti R, Di Lonardo V, Pecchioli A, Della Rocca G (2013b). 'Le Crete 1' and 'Le Crete 2': two newly patented *Seiridium cardinale* canker-resistant cultivars of *Cupressus sempervirens*. Forest Pathology 43(3):204-210.

Filonova L, Bozhkov P, von Arnold S (2000). Developmental pathwayof somatic embryogenesis in *Picea abies* as revealed by timelapse tracking. Journal of Experimental Botany 51(343):249-264.

Giovanelli A, De Carlo A (2007). Micropropagation of mediterranean cypress (*Cupressus sempervirens* L.). In: Mohan Jain S, Haggman H (Eds). Protocols for micropropagation of woody trees and fruits. Springer, The Netherlands pp 93-105.

Gupta PK, Durzan DJ (1985). Shoot multiplication from mature trees of Douglas-fir (*Pseudotsuga menziesii*) and sugar pine (*Pinus lambertiana*). Plant Cell Reports 4:177-179.

Klimaszewska K, Hargreaves C, Lelu-Walter MA, Trontin J (2016). Advances in conifer somatic embryogenesis since year 2000. In: Germanà MA, Lambardi M (Eds). *In vitro* embryogenesis in higher plants. Methods in Molecular Biology n. 1359. Humana Press-Springer, Heidelberg pp 131-166.

Kong L, von Aderkas P (2011). A novel method of cryopreservation without a cryoprotectant for immature somatic embryos of conifer. Plant Cell Tissue and Organ Culture 106:115-125.

Krajňáková J, Sutela S, Aronen T, Gomory D, Vianello A, Hägmann HM (2011). Long-term cryopreservation of Greek fir embryogenic cell lines: recovery, maturation and genetic fidelity. Cryobiology 63:17-25.

Lambardi M (2000). Somatic embryogenesis in cypress (*Cupressus sempervirens* L.). In: Mohan Jain S, Gupta P, Newton R (Eds). Somatic embryogenesis in woody plants, Vol 6. Kluver Academic Publishers (NL), Boston pp 553-567.

Lambardi M, Ozudogru EA, Benelli C (2008). Cryopreservation of embryogenic cultures. In: Reed B (Ed). Plant Cryopreservation: A Practical Guide. Springer, Berlin pp 177-210.

Lambardi M, Shaarawi S (2017). Importance of *in vitro* culture for developing cryopreservation strategies of woody plants. Acta Horticulturae (in press).

Maruyama E, Hosoi Y, Katsuaki I (2002). Somatic embryogenesis in sawara cypress (*Chamaecyparis pisifera* Sieb. et Zucc.) for stable and efficient plant regeneration, propagation and protoplast culture. Journal of Forest Research 7:23-34.

Matsumoto T, Sakai A, Yamada K (1994). Cryopreservation of *in vitro*-grown apical meristems of wasabi (*Wasabia japonica*) by vitrification and subsequent high plant regeneration. Plant Cell Reports 13:442-446.

Montalban IA, Garcia-Mendiguren O, Moncalean P (2016). Somatic embryogenesis in *Pinus* spp. In: Germanà MA, Lambardi M (Eds). *In vitro* embryogenesis in higher plants. Methods in Molecular Biology n. 1359. Humana Press-Springer, Heidelberg pp 405-416.

Morris GJ, Acton E (2013). Controlled ice nucleation in cryopreservation: a review. Cryobiology 66:85-89.

Ozudogru EA, Capuana M, Kaya E, Panis B, Lambardi M (2010). Cryopreservation of *Fraxinus excelsior* L. embryogenic callus by one-step freezing and slow cooling techniques. CryoLetters 31:63-75.

Ozudogru EA, Kirdok E, Kaya E, Capuana M, Benelli C, Engelmann F (2011). Cryopreservation of redwood (*Sequoia sempervirens* (D. Don) Endl.) *in vitro* buds using vitrification-based techniques. CryoLetters 32:99-110.

Ozudogru EA, Lambardi M (2016). Cryotechniques for the long-term conservation of embryogenic cultures from woody plants. In: Germanà MA, Lambardi M (Eds). *In vitro* embryogenesis in higher plants. Methods in Molecular Biology n. 1359. Humana Press-Springer, Heidelberg pp 537-550.

Panconesi A (1990). Pathological disorders in the Mediterranean basin. In: Ponchet J (Ed). Agriculture - AGRIMED Research Programme: Progress in EEC Research on Cypress Diseases, Rep. EUR 12493 EN. Commission of the European Communities, Brussels pp 54-81.

Panconesi A, Raddi P (1991). Agrimed Nr. 1 e Bolgheri. Due nuove selezioni resistenti al cancro del cipresso. Cellulosa e Carta 42(1):47-52.

Panis B, Lambardi M (2006). Status of cryopreservation technologies in plants (crops and forest trees). In: Ruane J, Sonnino A (Eds). The role of biotechnology in exploring and protecting agricultural genetic resources. FAO, Rome pp 61-78.

Sakai A, Kobayashi S, Oiyama I (1990). Cryopreservation of nucellar cells of navel orange (*Citrus sinensis* Osb. var. brasiliensis Tanaka) by vitrification. Plant Cell Reports 9:30-33.

Salaj T, Panis B, Swennen R, Salaj J (2007). Cryopreservation of embryogenic tissues of *Pinus nigra* Arn. by a slow freezing method. CryoLetters 28:69-76.

Sin AZ, Ersoy R, Gulbahar O, Ardeniz O, Gokmen NM, Kokuludag A (2008). Prevalence of cypress pollen sensitization and its clinical importance in Izmir, Turkey, with cypress allergy assessed by nasal provocation. Journal of Investigational Allergology and Clinical Immunology 18:46-51.

Thouchell TH, Chiang VL, Tsai CJ (2002). Cryopreservation of embryogenic cultures of *Picea mariana* (black spruce) using vitrification. Plant Cell Reports 21:118-124.

von Arnold S, Bozhkov P, Clapham D, Dyachok J, Filonova L, Hogberg KA, Wiweger M (2005). Propagation of Norway spruce via somatic embryogenesis. Plant Cell Tissue and Organ Culture 81:323-329.