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Plant regeneration from ploidy-stable cryopreserved embryogenic lines of the hybrid *Pinus elliottii* x *P. caribaea*

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ABSTRACT

Clonal breeding programs of the highly important interspecific hybrid *Pinus elliottii var. elliottii* and *Pinus caribaea var. hondurensis* require the establishment of robust cryopreservation protocols, with no genetic variability. Embryonal masses of this hybrid were successfully cryopreserved by a fast slow-freezing method. The protocol was optimized by testing the effects of dimethyl sulfoxide (DMSO) based cryoprotectant solutions, sugar cryoprotectants (e.g. sucrose or maltose, 0.4 M) and different times of pretreatments and pre-cooling storage. The addition of DMSO in a mixture of polyethylene glycol (PEG) 4000 and sucrose (PSD solution), instead of DMSO alone, was beneficial for recovery of cryopreserved cultures. This parameter, together with the time of pre-cooling storage (before plunging in liquid nitrogen), were the factors that most influenced the survival and regrowth rates of embryonal masses. A pretreatment combination of sucrose (0.4 M) and 5% PSD followed by a pre-cooling storage of 24 h allowed the cryopreservation and regrowth of embryogenic cell lines without major genetic variations (DNA-ploidy) or loss of embryogenic potential. Eight of the nine tested embryogenic cell lines survived to the cryopreservation procedures, however the genotype and treatment conditions clearly influenced the response. Somatic embryos maturation and conversion from recovered embryonal masses (EMs) took place according to our standard somatic embryogenesis protocol for *Pinus*, using modified Litvai (mLV) medium. This is the first report for this hybrid, and the proposed protocol is fast, robust, providing high rates of recovery and working with several genotypes. This protocol's suitability for industrial breeding programmes was also confirmed by allowing the regeneration of plants with no apparent variability from cryopreserved embryonal masses.

1. Introduction

Due to the enormous industrial importance of the hybrid *Pinus elliottii* var. *elliottii x Pinus caribaea* var. *hondurensis*, increasing pressure is put on developing efficient breeding programs allowing the cloning and long term preservation of elite individuals. In fact this hybrid has highly improved characteristics when compared with its parents, mostly derived from a complementary recombination of traits like growth rate, including superior branch quality and more uniform wood from *P. caribaea*, combined with high wood density, stem straightness, wind-firmness and adaptability to wet sites of *P. elliottii* (Nikles, 2000; Dieters and Brawner, 2007). Biotechnological approaches of cloning elite individuals are being developed including the establishment of somatic embryogenesis (SE) protocols for forest species (e.g. Pinto et al., 2002, 2008; Marum et al., 2009). Recently we developed a SE protocol that proved to be highly efficient with different families tested of this hybrid (Marum et al., 2014), using immature zygotic embryos as starting explants. However, the industrial use of SE also requires a full cryopreservation/recovery protocol of embyogenic masses, which has not yet been developed for this interspecific hybrid with high economic

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; %DI, dispersion index; AB, Aabscisic acid; BAP, 6-benzylaminopurine; CPPU, *N*-(2-Chloro-4-pyridyl)-*N*'-phenylurea; CV, coefficient of variation; CSF, classical slow freezing method; DMSO, Dimethyl sulfoxide; ECL, embryogenic cell line; EM, embryonal mass; FCM, flow cytometry; FS, filter-sterilized; FW, fresh weight; FWi, regrowth rate; mDCR, modified DCR medium; mLV, modified Litvay's medium; MVF, multi-varietal forestry; mLV, modified Litvay medium; OP, open pollinated; PEG, polyethylene glycol; Pi, propidium iodide; PGR, plant growth regulators; PPFD, photosynthetic photon flux density; RAPD, randomly amplified polymorphic DNA; SSF, short slow freezing method; Se, somatic embryos; SE, somatic embryogenesis; WPB, woody plant buffer

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importance to the wood and resin industry.

Cryopreservation allows the storage of biological material at ultra-low temperature of liquid nitrogen (−196 °C), and is the only method currently available to ensure the safe and cost-effective long-term conservation of genetic resources of species that have recalcitrant seeds or are vegetatively propagated, including apical or axillary buds, pollen, somatic embryos, and embryogenic tissues (Maeno and Hawtin, 2000; Engelmann, 2004; Fernandes et al., 2008; Martinez-Montero and Harding, 2015). Therefore, SE in conjugation with cryopreservation are two essential tools for the implementation of multi-varietal forestry (MVF). Through the cryo-storage it is possible to preserve a high number of genotypes, preserving also their juvenile characteristics, until getting the progeny tests results. Cryopreservation also prevents the loss of embryogenic capacity and the occurrence of putative somaclonal variations frequently found in long-term subcultures. It also reduces costs associated to the regular subculturing, and prevents the risk of losing plant material due to contaminations, technical or human errors (Ford et al., 2000a; Reinhoud et al., 2000; von Arnold et al., 2002).

From the different procedures that have been used for cryopreservation of plant cells, slow freezing method or two-step freezing, vitrification, encapsulation-dehydration, encapsulation-vitrification, desiccation and droplet-vitrification (Reinhoud et al., 2000; Engelmann, 2011) the first one is the most used to cryopreserve conifer embryogenic cultures. Different cryoprotectant solutions are used to lower the freezing-point in plant cells, resulting in avoidance of crystallization and maintaining a minimal moisture level so allowing cell viability. The most used cryoprotectants are sugars, sugar-alcohols and DMSO (dimethyl sulfoxide) (Reinhoud et al., 2000; Marum et al., 2004). Also, cryo-storage of embryogenic cells requires optimization as excessive dehydration may lead to damaging events associated to e.g., concentration of intracellular salts and changes in the cell membrane permeability (Engelmann, 2000).

The first protocol for cryopreservation of conifer somatic embryogenic cultures was described for *Picea glauca* by Kartha et al. (1988), a slow freezing procedure. Since then several reports successfully used this method in embryogenic cell lines (ECLs) of *P. caribaea* (Laine et al., 1992; David et al., 1995), *P. taeda* (Gupta et al., 1987), *P. patula* (Ford et al., 2000a, 2000b), *P. radiata* (Hargreaves et al., 2002), *P. pinaster* (Marum et al., 2004; Álvarez et al., 2012), *P. sylvestris* (Häggman et al., 1998; Latutrie and Aronen, 2013), *P. roxburghii* (Mathur et al., 2003; Malabadi and Nataraja, 2006) and *P. nigra* (Salaj et al., 2007, 2011). From our knowledge, no cryopreservation protocol (including recovery and plant regeneration) has been described for the economically relevant interspecific hybrid *P. elliottii* var. *elliottii x P. caribaea* var. *hondurensis*, nor its parent *P. elliottii*As described above, cryopreservation exposes plant material to physical, chemical, and physiological stresses that cause cryoinjury (Harding, 2004). These stresses may affect the plant material genetic stability, since the formation of free radicals can cause, among other injuries, mutations in DNA (Dumet and Benson, 2000). So, before cryopreservation can be used as a tool in biotechnology or as a conservation strategy, it is essential to verify that the cryopreservation protocol developed does not induce somaclonal variation in plants regenerated from embryonal masses (EM). Despite its relevance, only few work has been made for the evaluation of plant material genetic stability after cryopreservation, namely for *Abies cephalonica* (Aronen et al., 1999), *Picea glauca* (DeVerno et al., 1999), *Pinus sylvestris* (Häggman et al., 1998) and more recently in *Pinus nigra* (Salaj et al., 2011).

The main objective of this work was to establish a robust and simplified cryopreservation procedure for long-term storing embryogenic cultures of the hybrid *Pinus elliottii* var. *elliottii x Pinus caribaea* var. *hondurensis*. The establishment of an efficient cryopreservation protocol for this hybrid was based on 5 conditions: a) pre-cooling storage; b) pretreatment with DMSO based cryoprotectants; c) carbohydrate pretreatment; d) recovery; e) the ability to generate the mature embryos after cryopreservation, a critical step of somatic embryogenesis. Additionally, this work also addresses for the first time in conifers, the impact of cryopreservation on the whole genome stability of regenerated ECLs, which is extremely important in the industrial application point of view.

2. Materials and methods

2.1. Plant material

Embryogenic cell lines (ECLs) of the five open pollinated (OP) plus mother trees of the hybrid *P. elliotti* var. *elliotti x P. caribaea* var. *hondurensis* were initiated by innoculating megagametophytes on the induction/proliferation medium. Established ECLs were maintained in the dark at 23 ± 2 °C on the same proliferation medium consisting of (1) modified DCR medium (mDCR) based on DCR macro and microelements (Gupta and Durzan, 1985) and MS vitamins (Murashige and Skoog, 1962) supplemented with 1 mg L^{-1} 6-benzylaminopurine (BAP), 2 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), casein hydrolysate (500 mg L^{-1}), L-glutamine (250 mg L^{-1}) and 100 mg L^{-1} inositol (ES2) or (2) modified Litvay's (Litvay et al., 1985) medium (mLV) with half-strength macroelements and full-strength microelements and Fe-EDTA, as described by Klimaszewska et al. (2001) supplemented with the same concentrations of plant growth regulators (PGR), casein hydrolysate (1000 mg L^{-1}) and L-glutamine (500 mg L^{-1}) (ES5). Both media were supplemented with 2% (w/v) sucrose and solidified with 0.4% (w/v) Gelrite (Duchefa). Subculture of the ECLs was performed every 2 weeks, for 4–12 months, before the beginning of the cryopreservation experiments.

2.2. Cryopreservation

2.2.1. Standard protocol

2.2.1.1. Pretreatments before freezing. The standard cryopreservation protocol used in these experiments was based on the method described by Nørgaard et al. (1993) with minor modifications. Embryogenic suspensions were obtained by using 2 g of embryonal masses (EMs), suspended in 20 mL of PGR-free proliferation medium in 50 mL flasks. The EMs were disaggregated with a transfer pipette and the flasks shaken to break up the clumps of tissue into a fine suspension. Samples were pretreated with sucrose as cryoprotective carbohydrate for 48 h, according to Marum et al. (2004). On the third day, the flasks containing the embryogenic suspensions were transferred to ice and, after 15 min, DMSO was carefully added to a final concentration of 5% (v/v). After the addition of the cryoprotectant the suspensions were incubated for one hour on ice, in constant agitation. The cells were allowed to sediment in the flask (~10 min) and part of the supernatant was removed to achieve a final suspension density of 200 mg/mL. Aliquots of 1.8 mL of the pretreated embryogenic suspension were then dispensed into cryovials and were placed on a cell freezer container (Coolcell by BioCision) kept at −80 °C for 24 h before they were plunged directly into liquid nitrogen (−196 °C).

2.2.1.2. Rewarming and regrowth. To regrowth of cryopreserved ECLs, samples were removed from the liquid nitrogen and immediately warmed in a 45 °C water-bath until completely thawed and then transferred onto ice. The cryotubes were then surface sterilized with 70% ethanol and allowed to dry in a laminar flow hood. The content of the vials was then poured on a disk of sterile filter paper (55 mm Whatman No. 2) previously placed on sterile paper towels to drain excess of liquid. Drained filter paper was then placed on proliferation medium. Thereafter, the thawed EMs on filter papers were subcultured every 2 weeks by transfer to fresh proliferation medium, during two months (1st to 4th subculture).