



Ultra-low temperature conservation of Brazilian Pine embryogenic cultures

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ABSTRACT

This study aimed to establish a cryopreservation protocol for embryogenic cultures of *A. angustifolia*, enabling the *ex situ* conservation of the species. Embryogenic cultures were established from immature seeds and treated with variations of the cryoprotectant solutions SuDG, SoD and PVS2 prior to immersion in liquid nitrogen. Cell viability was evaluated after 30, 60 and 90 days of re-growth. The highest re-growth without morphological alterations and with normal biochemical composition was obtained with the PVS2 solution with 40 min immersion in ethanol (-20 °C). This procedure opens new horizons for the *ex situ* conservation of the species genetic.

Key words: cryopreservation, *ex situ* conservation, *in vitro* culture, plant germplasm conservation.

INTRODUCTION

Araucaria angustifolia (Bert.) O. Kuntze is the most important native conifer species in Brazil. It is a key stone species in the forest, providing nesting and feeding areas for animals and supplying many plants with shaded environments for their development. At economical level, its wood has several utilities, the resin is source of chemical compounds of industrial interest and the seeds are source of human food and profits to low-income farmers (Stefenon et al. 2009). Currently,

this species is classified as highly endangered in the IUCN List of Threatened Species, needing special attention concerning actions towards species conservation. Up to now, the application of biotechnological tools aiming the conservation of the species has basically included the use molecular markers for characterizing genetic diversity and the establishment of somatic embryogenesis cultures.

Due to the recalcitrant nature of the *A. angustifolia* seeds, the maintenance of *ex situ* seed banks is a challenge and the use of embryogenic cultures seems to be a feasible alternative for the conservation of species germplasm (Stefenon et al. 2009). However,

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problems related to loss of viability and the accumulation of mutations due to long term sub-culturing could render this process unaffordable (Corredoira et al. 2004). Cryopreservation has been considered an alternative to store large number of genotypes, to avoid loss of embryogenic potential during long-term subculture and to evade possible somaclonal variation (Häggman et al. 1998, Sakai et al. 1991, Verleysen et al. 2004). *Ex situ* conservation approaches based on cryopreservation have been increasingly employed on plant germplasm conservation (Reed 2001), including conifers (Find et al. 1998, Ford et al. 2000, Touchell et al. 2002, Marthur et al. 2003, von Arnold et al. 2005). The present study intended to establish a cryopreservation protocol for embryogenic cultures of *A. angustifolia*, aiming to enable long-term storage.

Embryogenic cultures of *A. angustifolia* were established from immature seeds collected from open-pollinated trees, as described by Santos et al. (2001). Immature zygotic embryos were isolated and inoculated in basal medium (Gupta and Pulmann 1991) supplemented with 0.5 g L⁻¹ casein hydrolysate, 1 g L⁻¹ L-glutamine, 1 g L⁻¹ myo-inositol, 3% sucrose, 2 µM 2,4-dichlorophenoxyacetic acid, 0.5 µM benzylaminopurine and 0.5 µM kinetin (BM₂). The BM₂ medium was autoclaved at 121 °C for 15 min and the pH was adjusted to 5.8 before adding Phytigel® (0.25%). Explants were inoculated in Petri dishes containing 25 mL of BM₂ medium and incubated in the dark at 25±2 °C for 30 days. Suspension cells were established utilizing 0.25 g of embryogenic culture inoculated in 120 mL of liquid BM medium free of growth regulators (BM₀) and incubated in the dark on a shaker at 25±2 °C.

Seventeen cryoprotective treatments were employed (Table I), based on the combination of different osmotic solutions: SuDG [Sucrose: dimethylsulfoxide (DMSO):Glycerol], SoD (Sorbitol:DMSO) and the vitrification solution PVS2 (Sakai et al. 1991). Non-cryopreserved cultures, free of cryoprotective treatments, were used as control.

TABLE I
Composition of the cryoprotective solutions evaluated

Control	
T0	Non-cryopreserved cultures, free of cryoprotective treatments
SuDG	
T1	Sucrose 20% + DMSO 0% + Glycerol 20%
T2	Sucrose 20% + DMSO 10% + Glycerol 20%
T3	Sucrose 0% + DMSO 10% + Glycerol 20%
T4	Sucrose 20% + DMSO 10% + Glycerol 0%
T5	Sucrose 0% + DMSO 0% + Glycerol 20%
T6	Sucrose 0% + DMSO 10% + Glycerol 0%
T7	Sucrose 10% + DMSO 0% + Glycerol 0%
SoD	
T8	Sorbitol 0.3M + DMSO 10%
T9	Sorbitol 0.4M + DMSO 10%
T10	Sorbitol 0.4M + DMSO 0%
T11	Sorbitol 0.3M + DMSO 0%
T12	Sorbitol 0.3M + DMSO 5%
T13	Sorbitol 0.4M + DMSO 5%
PVS2	
T14	Ethylene glycol 15%+Sucrose 0.4M+Glycerol 30%+DMSO 15% (40 min in Ethanol -20°C)
T15	Ethylene glycol 15%+Sucrose 0.4M+Glycerol 30%+DMSO 15% (30 min in Ethanol -20°C)
T16	Ethylene glycol 15%+Sucrose 0.4M+Glycerol 30%+DMSO 15% (20 min in Ethanol -20°C)
T17	Ethylene glycol 15%+Sucrose 0.4M+Glycerol 30%+DMSO 15% (10 min in Ethanol -20°C)

For all treatments, 200 mg of embryogenic cultures were inoculated in cryovials containing BM₀ medium supplemented with one of the cryoprotective solutions. For solutions SuDG and SoD, samples were held in the medium for 30 min at room temperature and directly immersed in liquid nitrogen (LN). For solution PVS2, the samples were held in the vitrification solution at 0°C for 10 min. The cryovials were submerged in ethanol (-20 °C) during 10, 20, 30 or 40 min and subsequently immersed in LN.

After 48 h in LN, the cryovials were re-warmed for 2 min in a 40 °C water bath. These cultures were inoculated in Petri dishes containing 20 mL of BM₀ medium, maintained in darkness at 25±2 °C for re-growth and evaluated by measuring their fresh mass independently after 30, 60 and 90 days of sub-culture. The experiment was conducted in a

complete random block, with four repetitions per block for each treatment. Statistical comparisons were made using ANOVA and the SNK test of means separation ($\alpha=5\%$).

The viability of the cells after freezing was determined by two cytochemical analyses of the embryogenic cultures: (i) staining with 0.5% fluorescein diacetate (FDA) and (ii) staining with acetic carmim/Evans-blue.

Aiming to evaluate the patterns of compounds accumulation considering the dynamic of cellular growth after cryopreservation, cells were collected at days 0, 16 and 32 of sub-culture in BM₀ liquid medium. The contents of proteins and starch were evaluated using three samples: (i) a non-frozen control, (ii) a frozen not re-cultured sample and (iii) a frozen and 60 days re-cultured sample. Embryogenic cultures were cryopreserved using the PVS2 solution with the treatment of 40 min in ethanol -20 °C, since this method presented the best outcomes (see below). The content of total proteins was determined from 300 mg of embryogenic cultures following Read and Northcote (1981), using the bovine serum albumin as standard. Extraction of starch was performed following the method of McCready et al. (1950) using the solid discarded fraction of the material employed for protein analysis. The amount of starch was determined through absorbance with a 620 nm spectrophotometer Shimadzu UV1203, using a standard curve prepared with glucose (0-100 g/mL, diluted in PCA 30%). The experiments were conducted in a complete random block, with three repetitions per block. Statistical comparisons were made using ANOVA and the SNK test ($\alpha=5\%$).

For the cryoprotective solution SuDG, the fresh mass was significantly larger ($p < 0.05$) for the treatments with a combination of two or three cryoprotective compounds in comparison to the control and to treatments using only one cryoprotector (Figure 1). The SoD solution, revealed the best results with the cryoprotective treatments sorbitol (0.3 M) +DMSO (10%), sorbitol (0.4M)+DMSO (10%) and

sorbitol (0.3 M), presenting fresh mass larger ($p < 0.05$) than the control and the other treatments (Fig. 1). The treatment of cultures with PVS2 solution followed by 40 min immersion in ethanol resulted in mean values of fresh mass higher ($p < 0.05$) than those resulting from treatments with 10, 20 or 30 min immersion and higher than those of the control cultures (Fig. 1). Moreover, in comparison to SuDG and SoD solutions, the PVS2 solution revealed a significantly superior performance on the re-growth after cryopreservation (Fig. 1).

Embryogenic cultures are formed by cells in initial stage of growth and present a high amount of water. During the cryogenic process, this high amount of water results in the formation of ice crystals and consequently cell damage. It has been proposed that the cell dehydration may be promoted by increasing the osmolarity of cryoprotective solutions with sugars, DMSO or glycerol (Wang et al. 2002). These substances also inhibit the cell expansion and reduce the cell and vacuole volumes keeping the integrity of plasma membranes and stabilizing proteins in conditions of dehydration and freezing (Gonzales-Arno et al. 2008). In our study, the combination of sorbitol+DMSO or sucrose+DMSO+glycerol was very effective in promoting protection against damages during freezing and storage. Similar effects were reported for the cryopreservation of *Pinus caribaea*, *Pinus pinaster* and *Picea abies* embryogenic cultures (Cyr 1999). Among all osmotic solutions, the best re-growth was obtained using the solution PVS2. This solution penetrates in the cytosol preventing the formation of ice crystals within the cell (Turner et al. 2001).

Regardless of the cryoprotective treatment employed, the morphological characteristics of the responsive cells to re-growth revealed normal aspect in comparison to control cultures (Fig. 2 A-C). The cytochemical analysis using the carmim acetic-Evans blue staining (Fig. 2 D-F) revealed small isodiametric and low vacuolated embryogenic cells

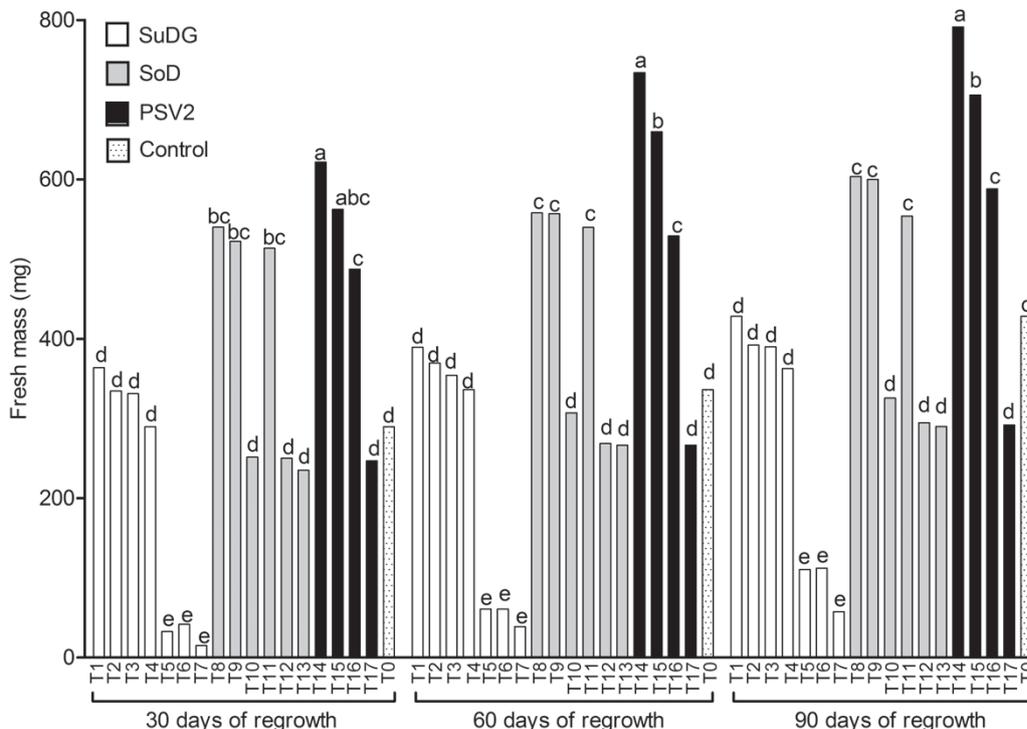


Figure 1 - Fresh mass of the *A. angustifolia* embryogenic cultures after 30, 60 and 90 days of re-growth in BM medium treated with SuDG, SoD and PVS2 cryoprotectant solutions. Columns with different letters in the same sub-culture (30, 60 and 90 days) are statistically different at 5% according to SNK test.

with dense cytoplasm and high nucleus-cytoplasm rate. Larger suspensor cells with relatively bigger vacuoles were also observed, in minor frequency. The FDA staining analysis (Fig. 2 G-I) clearly revealed the presence of viable embryogenic cells in the cryopreserved cultures.

It has been suggested that the elimination of non-embryogenic cells from the cultures during the cryogenic storage, brings a beneficial effect on embryogenic capacity of re-growth (Häggman et al. 1998). Embryogenic cells are relatively small, highly cytoplasmic and have small vacuoles, characteristics that improve their resistance capacity to the freezing process (Zoglauer et al. 2002).

The non-frozen cultures revealed higher ($p < 0.05$) amounts of total protein than the frozen ones at 0 and 32 days (Fig. 3), showing a decrease in the protein amount across the dynamic of cellular suspension growth. The frozen not re-cultured samples revealed a slight decrease in protein amount on the 16th day of

sub-culture, decreasing considerably on the 32nd day. The frozen re-cultured samples presented a decrease in protein amount on the 16th day and a slight increase on the 32nd day (Fig. 3).

Proteins are important in the cryopreservation process since they stabilize the plasmatic membranes during the osmotic treatment (Hughes and Galau 1989). In the dehydrated cell, membrane proteins are incorporated to a vitreous matrix that preserves its activity and structure. However, proteins are also very sensible to the thawing process and can be denatured (Thierry et al. 1999, Dumet and Benson 2000). Thus, the lower amount of total proteins observed in comparison to non-frozen cultures may be a result of denaturation during the defrosting process.

The amount of starch observed was statistically different ($p < 0.05$) for all treatments (Fig 3). The non-frozen cells revealed about the same amount of starch for 0 and 16 days of sub-culture with a significant increase on the 32nd day (Fig. 3). The frozen not re-

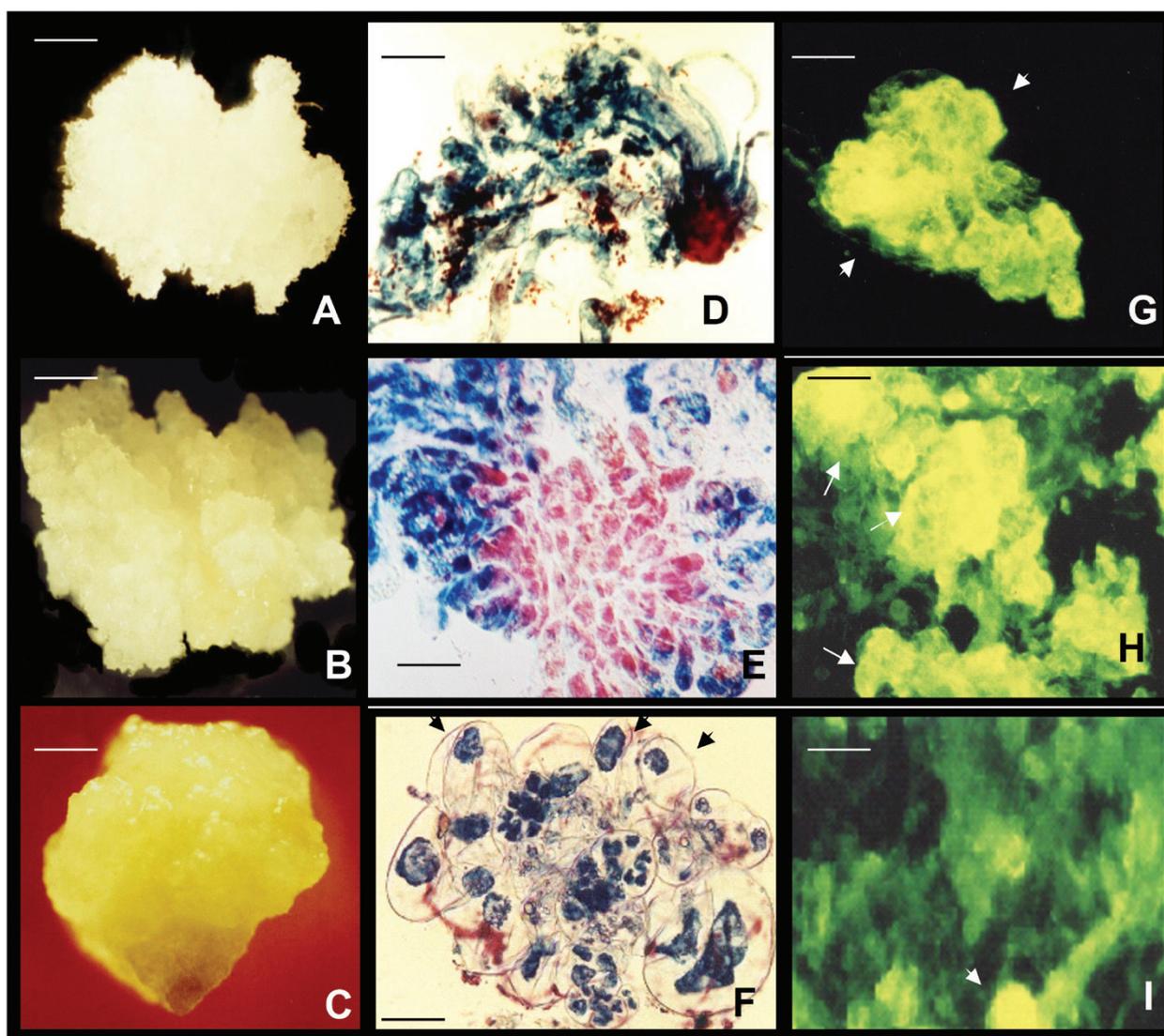


Figure 2 - Cytochemical analysis of the *A. angustifolia* embryonic cultures after 48 h of cryopreservation in liquid nitrogen. A, non-frozen embryonic culture. Bar = 66.7 μ m. B, embryonic culture with responsive cells after cryopreservation. Bar = 1.257 mm. C, embryonic culture without re-growth after 30 days of re-culturing. Bar = 0.587 mm. D, morphological analysis of embryonic cultures stained with acetic carmim and Evans-blue, evidencing the intact cells of a bipolar somatic pro-embryo. Bar = 33.3 μ m. E, morphological appearance of embryonic cultures tolerant to cryopreservation, stained with acetic carmim and Evans-blue. Bar = 33.3 μ m. F, morphological appearance of embryonic cultures not tolerant to cryopreservation, stained with acetic carmim and Evans-blue. Arrows indicate suspensor cells with degraded walls and overflow of cellular content. Bar = 66.7 μ m. G, embryonic culture before freezing, stained with FDA. Bar = 18.84 μ m. H-I, morphological appearance of embryonic cultures tolerant to cryopreservation, stained with FDA. Arrows indicate viable cells. Bar = 18.84 μ m.

cultured sample revealed the lower content at day 16, and the highest amount on the 32nd day, while the frozen re-cultured cells presented basically the same amount of starch for 0, 16 and 32 days of sub-culture (Fig. 3). For samples of day 32, the high content of

starch in the not re-cultured samples evidences the high quantity of sucrose absorbed and converted into starch, while the lower content of starch observed in the frozen re-cultured cells evidences that this compound was metabolized with the cultures re-growth.

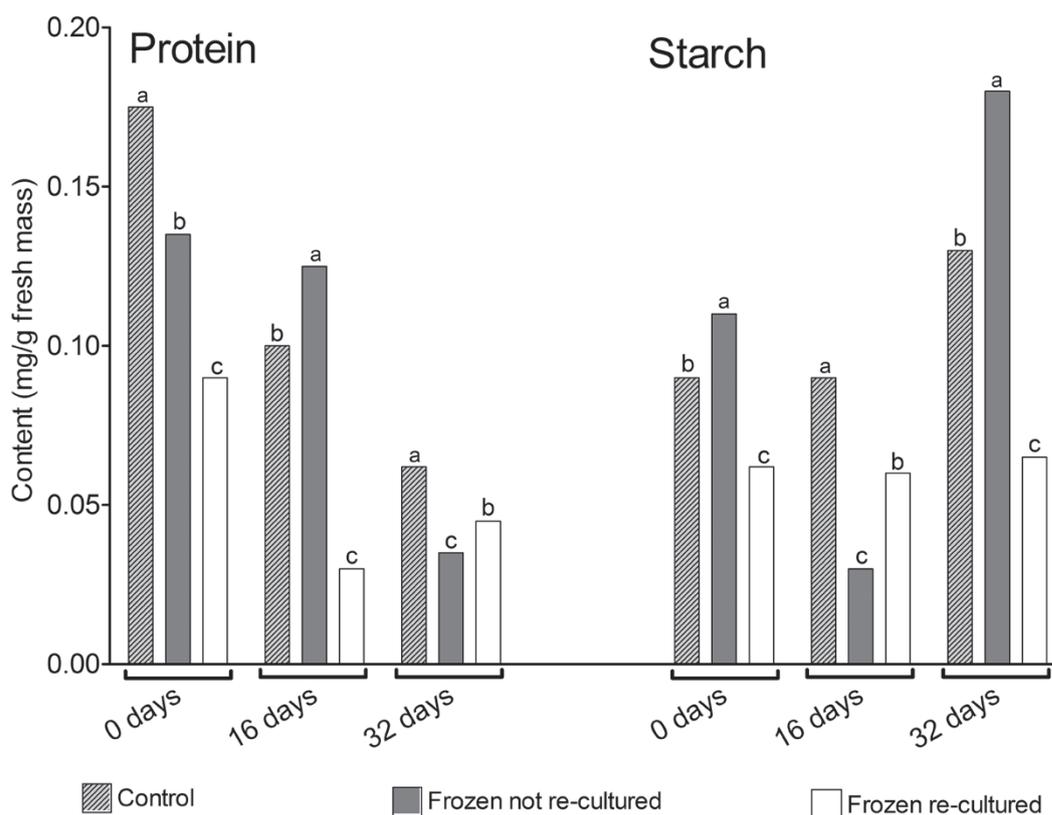


Figure 3 - Content of protein and starch of the *A. angustifolia* embryogenic cultures, cryopreserved using the PVS2 solution with 40 min in ethanol (-20°C). Columns with different letters in the same sub-culture (0, 16 and 32 days) are statistically different at 5% according to SNK test.

Our findings show that the vitrification protocol using the PVS2 solution and 40 minutes of immersion in ethanol -20 °C is a reliable procedure for the cryopreservation of *A. angustifolia* embryogenic cells. This method may be applied for the conservation of embryogenic cultures of the species, opening new horizons for the *ex situ* conservation of its genetic resources.

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RESUMO

Este estudo objetivou estabelecer um protocolo de criopreservação para culturas embriogênicas de *A. angustifolia*, possibilitando a conservação *ex situ* da espécie. Culturas embriogênicas foram estabelecidas a partir de sementes imaturas e tratadas com variações das soluções crioprotetoras SuDG, SoD e PVS2 antes da imersão em nitrogênio líquido. A viabilidade celular foi avaliada após 30, 60 e 90 dias de recrescimento. O maior recrescimento sem alterações morfológicas e composição bioquímica normal foi obtido com a solução PVS2 com 40 min de imersão em etanol (-20 °C). Este procedimento abre novos horizontes para a conservação *ex situ* dos recursos genéticos da espécie.

Palavras-chave: criopreservação, conservação *ex situ*, cultura *in vitro*, conservação de germoplasma vegetal.

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