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Scientific Notes

Cryopreservation of zygotic embryos of the Brazilian Green Dwarf coconut

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Abstract – The objective of this work was to evaluate rewarming procedures and recovery media for Brazilian Green Dwarf coconut (*Cocos nucifera*) embryos cryopreserved by vitrification. The rewarming procedures evaluated were: T1, water bath at 38±2°C; and T2, unloading solution consisting of Y3 medium + 1.2 mol L^{-1} sucrose. The recovery media assessed were: M1, Y3 medium + 45 g L^{-1} sucrose + 1 mg L^{-1} gibberellic acid + 1 g L^{-1} activated charcoal + 2.2 g L^{-1} Gelrite; and M2, Y3 medium + 60 g L^{-1} sucrose + 2.2 g L^{-1} Gelrite. Rewarming procedures showed no significant effect, and the M1 media induced 72.5% regeneration.

Index terms: *Cocos nucifera*, ex situ conservation, PVS3.

Criopreservação de embriões zigóticos de coqueiro Anão Verde do Brasil de Jiqui

Resumo – O objetivo deste trabalho foi avaliar processos de descongelamento e meios de regeneração para embriões zigóticos de coqueiro Anão Verde do Brasil de Jiqui (*Cocos nucifera*) criopreservados por vitrificação. Avaliaram-se os processos de descongelamento: T1, banho-maria a 38±2°C; e T2, solução de descarregamento composta de meio Y3 + 1,2 mol L^{-1} de sacarose. Os meios de regeneração analisados foram: M1, meio Y3 + 45 g L^{-1} de sacarose + 1 mg L^{-1} de ácido giberélico + 1 g L^{-1} de carvão ativado + 2,2 g L^{-1} de Gelrite; e M2, meio Y3 + 60 g L^{-1} de sacarose + 2,2 g L^{-1} de Gelrite. O descongelamento não apresentou efeito significativo, e o meio M1 induziu 72,5% de regeneração.

Termos para indexação: *Cocos nucifera*, conservação ex situ, PVS3.

The conservation of coconut (*Cocos nucifera* L.) genetic resources is usually done in field collections, due to the size and recalcitrance of the seed, which makes its storage difficult (N'nan et al., 2008).

Cryopreservation, however, has been used for the conservation of genetic resources in many species, including coconut. Early studies on the cryopreservation of coconut were carried out by Assy-Bah & Engelmann (1992), who used a vitrification technique on mature zygotic embryos of the PB 121 hybrid coconut (Malayan Yellow Dwarf x West African Tall) and of the Cameroon Red Dwarf, Indian Tall, and Rennel Tall varieties. This technique is based on the exposure of explants to glycerol-based vitrification solutions such as PVS3 (Nishizawa et al., 1993), which has been applied to coconut embryos with varied responses in different accessions including West Coast Tall (Sajini et al., 2011) and Malayan Yellow Dwarf (Cueto et al., 2014).

Promising results have been reported for coconut zygotic embryos, plumules, and pollen using different cryopreservation techniques (Karun & Sajini, 2010; Sajini et al., 2011; Cueto et al., 2014; Machado et al., 2014). However, there are few known studies on the cryopreservation of zygotic embryos of Brazilian coconut accessions. Initial results were published on the viability of coconut zygotic embryos cryopreserved by electrolytic conductivity and potassium leaching,

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as well as on the performance of cryoprotectants, dehydration methods, and tetrazolium tests (Gomes-Copeland et al., 2012, 2015), but without regeneration after cryopreservation. Therefore, in spite of the promising results for other genotypes, further research on procedural modifications according to genotype and explant types is still needed.

Others factors, such as rewarming procedures and recovery medium, should also be considered to adjust cryopreservation protocols. In this context, the use of regular growth medium in the recovery medium may increase regeneration rates. Gibberellic acid (GA_3) has also been shown to promote embryo development and conversion into plantlets, improving coconut embryo culture (Aké et al., 2007; Cueto et al., 2014).

The objective of this work was to evaluate rewarming procedures and recovery media for Brazilian Green Dwarf coconut embryos cryopreserved by vitrification.

The vegetal material used in this experiment came from the coconut germplasm bank of Embrapa Tabuleiros Costeiros, located in the state of Sergipe, Brazil. At the collection site, endosperm cylinders with zygotic embryos were extracted from 150 mature fruits (11 months old) collected from three plants, immersed in a commercial solution of sodium hypochlorite (2.0–2.5% v v^{-1}), and washed in sterile water three times. Embryos were excised from the endosperm cylinders under aseptic conditions, immersed in 70% ethanol for 2 min in a commercial solution of sodium hypochlorite (1.0–1.25% v v^{-1}) stirred for 3 min, washed three times in sterile distilled water, and then placed in sterile Petri dishes.

After disinfection, the embryos were precultured for 72 hours in Y3 medium (Eeuwens, 1976) containing 0.6 mol L^{-1} sucrose + 2.2 g L^{-1} Gelrite as adapted from Sajini et al. (2011), immersed in PVS3 solution (Nishizawa et al., 1993) composed of 2 mol L^{-1} glycerol + 0.4 mol L^{-1} sucrose in liquid Y3 standard culture medium for 16 hours (Sajini et al., 2011) on a shaker at 90 rpm, transferred to sterile polypropylene cryovials, and quickly immersed into liquid nitrogen for 72 hours. After that, the embryos were subjected to two rewarming procedures: T1, water bath at 38±2°C for 2–3 min; and T2, unloading solution consisting of Y3 medium + 1.2 mol L^{-1} sucrose for 90 min. After rewarming, the embryos were cultured on two regeneration media, for two weeks in the absence of light: M1, Y3 medium + 45 g L^{-1} sucrose + 1 mg L^{-1} GA_3 + 1 g L^{-1} activated charcoal + 2.2 g L^{-1} Gelrite; and M2, Y3 medium + 60 g L^{-1} sucrose + 2.2 g L^{-1} Gelrite. Subsequently, the embryos were transferred to a 16/8 hour photoperiod at 25±2°C. For the control, without exposure to liquid nitrogen (-LN), zygotic embryos were extracted, inoculated into the germination medium, and subjected to the PVS3 vitrification procedure (PVS3 -LN).

For the anatomical analysis, the tips were fixed in a formalin-acetic acid-alcohol (faa) solution (40% ethanol, 40% formaldehyde, and 5% glacial acetic acid), dehydrated in ethyl series (80–100%) for 6 hours, infiltrated, and embedded using the hydroxyethyl methacrylate Historesin kit (Leica Biosystems Nussloch GmbH, Heidelberg, Germany). Resin was polymerized at room temperature for 12 hours. Serial histological sections (6 μm) were obtained with the Cut 5062 semi-automatic precision microtome (Slee Medical GmbH, Mainz, Germany), and then placed on slides and stained with toluidine blue 0.05% (w v^{-1}) (Feder & O'Brien 1968). Sections were analyzed and photographed with the Axio Lab. A1 reflected-light microscope equipped with the AxioCam ERc 5 (Carl Zeiss Microscopy GmbH, Jena, Germany). Samples of the embryos, with three replicates of ten embryos each, were used to determine fresh mass both after excision and the PVS3 treatment in a digital scale. Then, the embryos were placed in metal containers and maintained at 105°C for 18 hours to determine dry matter. The moisture content of the embryos was calculated by the formula [(Fresh weight - Dry weight)/Dry weight] x 100.

The experimental design was completely randomized in a 2x2 factorial arrangement (two rewarming procedures x two recovery media) with five replicates per treatment. Survival and regeneration rates were evaluated after 10 and 50 days, respectively. Embryos were considered to have survived if they were intact and showed a white color and/or initial haustorium expansion, and as regenerated, when the apical and radicular meristems were developed. Data were subjected to the analysis of variance, followed by Tukey's post-hoc test, at 5% probability.

No significant differences were observed for the percentage of embryo survival, which was above 80% in all treatments (Figure 1). Similarly, Sajini et al. (2011) reported a regeneration of 75% for the West Coast Tall coconut genotype. It should be noted that,

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in the present study, the rewarming procedures did not have a significant effect on the percentages of survival and regeneration.

The recovery media, however, had a significant effect on the percentage of embryo regeneration. The M1 medium promoted the highest plant regeneration (72.5%) in comparison with M2 (15%) (Figure 1 B). GA_3 probably benefited the development of the cryopreserved embryo. Aké et al. (2007) observed that the percentages of germination and plant uniformity of non-cryopreserved embryos of the Malayan Green Dwarf and Malayan Yellow Dwarf coconuts improved when cultivated in Y3 medium supplemented with 0.46 µmol L^{-1} GA_3.

The moisture content of embryos varied from 76.84% after excision (initial moisture content) to 38.14% after the PVS3 treatment for 16 hours (PVS3 control). The percentages of survival and regeneration observed for the PVS3 control (PVS3 -LN) were 100 and 90%, respectively. The PVS3 solution did not have a toxic effect on zygotic embryos, nor caused osmotic injuries. Histological analyses showed intact epidermal cells, protodermal layer, and procambium of cryopreserved zygotic embryos similar to that of the control embryos (Figure 2).

Cueto et al. (2014) found a lower germination for both the dehydrated control and the cryopreserved (vitrification technique) embryos of the Malayan Yellow Dwarf coconut. According to these authors, these results are attributed to the long period of dehydration with vitrification solution (6 hours). However, in the present study, the PVS3 treatment was applied for 16 hours without negative effects.

This is the first known report on the regeneration of the Brazilian Green Dwarf coconut accession after cryopreservation. The obtained results will contribute to improve the long-term conservation of coconut germplasm in Brazil.

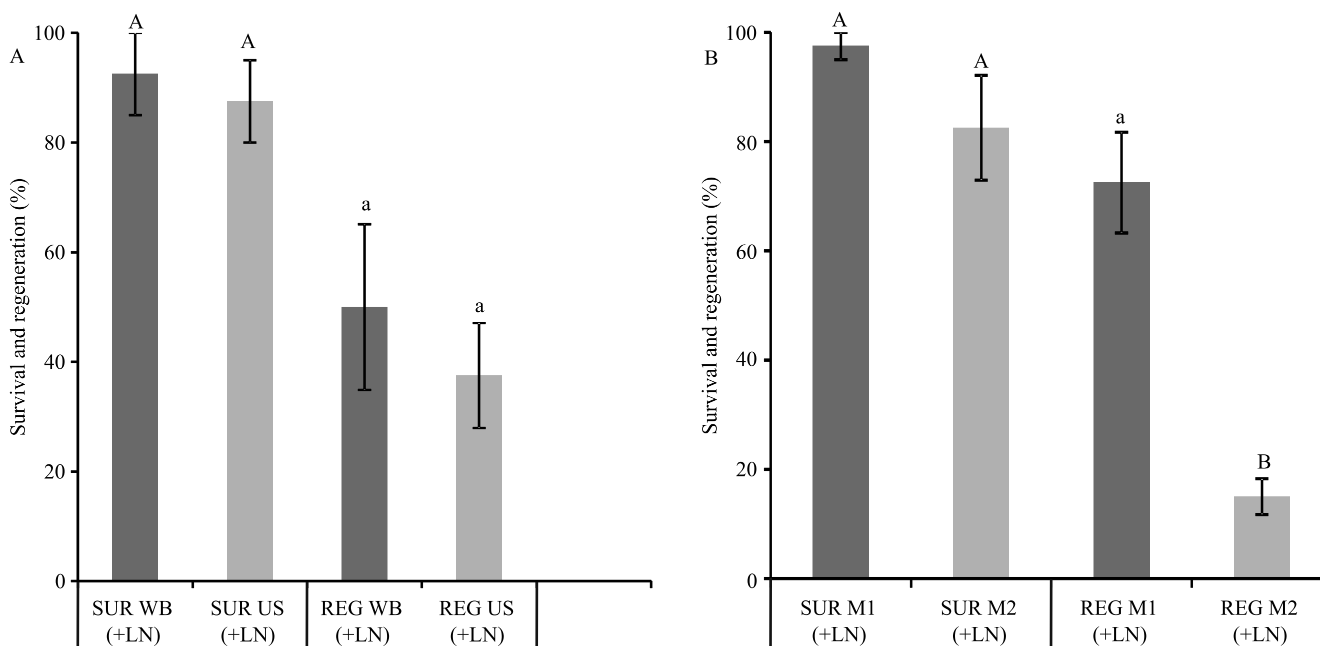

Figure 1. Percentages of survival (SUR) and regeneration (REG) of Brazilian Green Dwarf coconut (*Cocos nucifera*) embryos cryopreserved in liquid nitrogen (+LN). A, rewarming procedures; and B, recovery medium. WB, water bath at 38±2°C for 2 to 3 min; US, unloading solution consisting of Y3 medium + 1.2 mol L^{-1} sucrose for 90 min; M1, Y3 medium + 45 g L^{-1} sucrose + 1 mg L^{-1} gibberellic acid + 1 g L^{-1} activated charcoal + 2.2 g L^{-1} Gelrite; and M2, Y3 medium + 60 g L^{-1} sucrose + 2.2 g L^{-1} Gelrite. Columns followed by equal letters, uppercase for survival and lowercase for regeneration, do not differ by Tuke's test, at 5% probability.

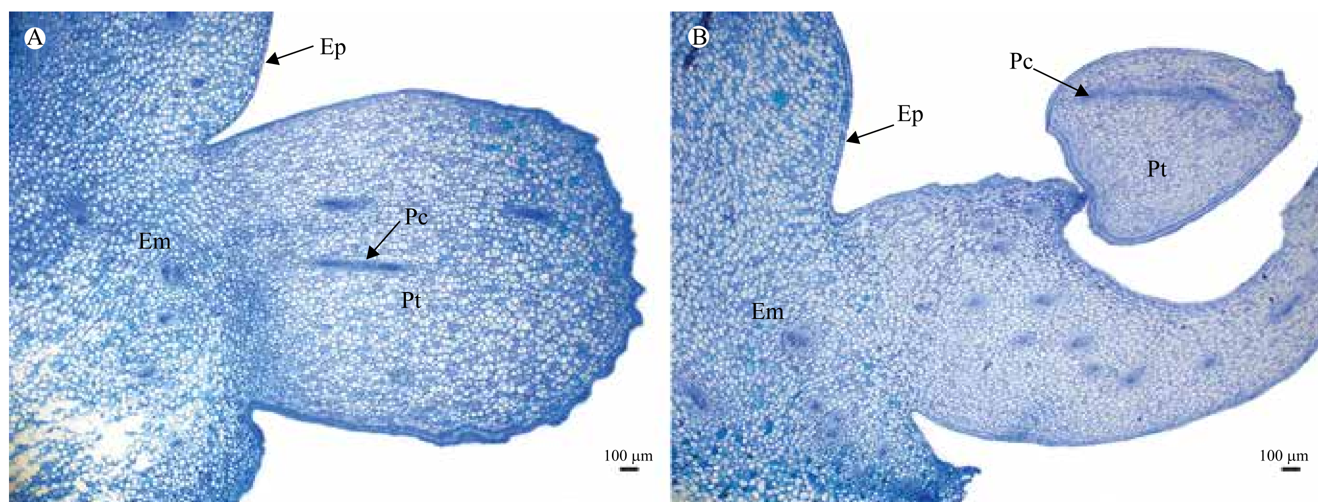

Figure 2. Histological analyses of coconut (*Cocos nucifera*) embryos: A, control treatment without liquid nitrogen; and B, after cryopreservation by vitrification in liquid nitrogen. Em, cryopreserved zygotic embryos; Ep, epidermal cells; Pc, procambium of cryopreserved zygotic embryos; and Pt, protodermal layer.

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