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Performance of cryoprotectants, dehydration methods and tetrazolium test on the zygotic embryos of BGD coconut

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The coconut (*Cocos nucifera* L.) is a palm found in the tropics, but has its origin in Asia. The in vitro germplasm banks offers multiple benefits and the long-term conservation meet those needs, but can also be used to keep recalcitrant and intermediate species. This study aims to test methods for dehydration and cryoprotection in long-term conservation of mature zygotic embryos of Brazil Green Dwarf (BGD), in liquid nitrogen at -196 ºC. Were used fruit zygotic embryos with 10 to 11 months old from a planting of BGD. Were evaluated different times of dehydration, cryoprotectant treatments and drying methods on embryos moisture using three methodologies. The methodology 3 promotes the lowest moisture content in zygotic embryos of BGD coconut accession. The mature zygotic embryos of BGD accession pre-treatment with cryoprotectant with sucrose 1.75 mol L^{-1} + glycerol 15% for 12 and 16 hours presented lower moisture content and larger viability by tetrazolium test.
Palavras-chave: *Cocos nucifera* L.; genetic resources; cryopreservation; tetrazolium test.

Desempenho de crioprotetores, métodos de desidratação e do teste de tetrazólio em embriões zigóticos do coqueiro BGD

O coqueiro (*Cocos nucifera* L.) é uma palmeira encontrada em todos os trópicos, mas tem como origem a Ásia. Os bancos de germoplasma in vitro oferecem vários benefícios, e a conservação em longo prazo supri essas necessidades, como também pode ser utilizado para conservar espécies recalcitrantes e intermediárias. Este trabalho tem como objetivos testar métodos para desidratação e crioproteção na conservação em longo prazo de embriões zigóticos maduros de coqueiro (*Cocos nucifera* L.) anão verde do Brasil de Jiqui, em nitrogênio líquido a -196 ºC. Foram utilizados embriões zigóticos de frutos com 10 a 11 meses de maturação provenientes de um plantio de Coqueiro Anão Verde do Brasil de Jiqui do Brasil (AVeBrJ). Foram avaliados os efeitos de diferentes tempos de desidratação, tratamentos de crioprotetores e tipos de secagem na umidade dos embriões. Após a seleção do método 3, embriões zigóticos de coqueiro AVeBrJ foram criopreservados e após 48 horas foram submetidos a etapa de recuperação em meio de cultura Y3 líquido. A metodologia 3 promove as menores umidades nos embriões zigóticos do acesso AVeBrJ. O pré-tratamento de embriões zigóticos do acesso AVeBrJ com crioprotetor com 1,75 mol L^{-1} de sacarose + 15% de glicerol por 12 e 16 horas promove a menor umidade dos embriões.
Keywords: *Cocos nucifera* L.; recursos genéticos, criopreservação, teste de tetrazólio.

1. INTRODUCTION

Coconut palm (*Cocos nucifera* L.) originates from tropical and subtropical Pacific Ocean islands. Southeast Asia is its main reference point as the center of origin and diversity, and its cultivation has spread to Latin America, Caribbean and Tropical Africa. Currently, coconut palm is grown in over 200 countries and is found in large crop areas between 23°N and 23°S [1]. In Brazil, its culture is developing, mainly along the coast, extending from the state of Para to Espírito Santo. Brazil is currently the fourth largest producer of coconut in the world, being the northeast where it concentrates most of this production.

The genetic resources conservation of coconut are primarily based in field collections, due to the size of its seed and for being a recalcitrant specie [2,3]. Therefore, the genetic resources conservation of coconut has been a priority for the development of conservation programs since

1985 Bioversity International. Thus, the formation of in vitro germplasm collections is of great value due to bring several benefits, such as high rates of multiplication, pathogen free matrix production, reduced demand for space, maintaining of organized structures viability, low cost, among others.

The cryopreservation has been used for the genetic resources preservation in many species, especially recalcitrant species such as coconut [4,2]. Early studies of cryopreservation with coconut were filed by Assy-Bah & Engelmann [5] which used the vitrification technique in mature zygotic embryos of hybrid PB 121 coconut (Malayan Yellow Dwarf x West African Tall), the Cameroon Red Dwarf (CRD), Indian Tall (IT7) and Rennel Tall (RT). Later, researchers such as Karun et al. [6,7], Malaurie et al. [8], Bandupriya et al. [9], N'Nan et al. [2,3], Sisunandar et al. [10], among others, have published promising results with cryopreservation using zygotic embryos, plumule and pollen as explants. However, there remains a need for research aimed at the cryopreservation of coconut, because even with promising results, adjustments, and adequacy of protocols according to genotype and explant types have been demanded. Furthermore, there are no research results on the technique performance for a growing as the Brazil Green Dwarf (BGD), requiring specific protocols for their conservation.

Plant cells have a high amount of water, both intra-and extracellular, and therefore are extremely sensitive to sub-zero temperature due to the formation of ice crystals. Intracellular crystallization causes the rupture of cell membrane system, which can also be caused by extreme dehydration [11]. Cryopreservation techniques have been developed to minimize both types of damage [12].

The dehydration of plant tissue can be obtained using various techniques: dehydration of the material in the presence of silica gel, saline solutions, in airflow, as well as by treatment with concentrated cryoprotectant solutions (vitrification solutions) like as dimethyl sulfoxide (DMSO), ethylene glycol, methanol, glycerol and propylene glycol [11]. However, these cryoprotectants may be toxic or cause osmotic stress, leading to cell death or modifying its morphogenetic response in culture [13] and adds one more step in the process of dehydration.

More recently, sugars are used as cryoprotectant substances due to their low cytotoxicity, even when they accumulate in large quantities in the cytoplasm [11]. Although the action mode of sugars is not very elucidated yet, nevertheless we can say that are used significant amounts of sugars or sugar alcohols in plant tissues, they accumulate endogenously increasing the stability of membranes under conditions of extreme dehydration [14]. The vitrification can be considered as a simple technique and could be used for complex structures such as embryos and shoot tips [15]. This method has been used successfully in cryopreservation of zygotic embryos of several species [16] aiming to develop and adjust germplam banks protocols in the world.

Another aspect to be considered is the absence of viability tests applied to zygotic embryos of coconut. One of the methodologies used is the tetrazolium test due to the speed in determining viability in seeds. The application of the tetrazolium test in seeds of some palm trees is not feasible mainly due to seed size and difficulty to exposure the external tissues, such as coconut, requiring excision of the zygotic embryo. The use of zygotic embryos for viability studies of palm seeds is recent and has shown adequate precision [17,18, 19].

The study aims to evaluate the cryoprotectant solutions, different dehydration times and methods on the zygotic embryo dehydration for future cryopreservation protocols of Brazil Green Dwarf (BGD) accession and the applicability of the tetrazolium test for viability studies of embryos.

2. MATERIAL AND METHODS

Collection and excision of BGD coconut mature zygotic embryos

The mature fruits of Brazil Green Dwarf (BGD) accession were collected in a commercial plantation (Fazenda H. Dantas - Coco Verde de Sergipe), Neópolis, Sergipe, Brazil (10°22’53.30”S; 36°44’00.40”E, 127 meters above sea level). The region presented ‘As’ climate according to Köppen classification, characterized by tropical rainy climate with dry summer.

The plants were under six years of age, planted on a yellow Argisol soil, medium to low fertility, with irrigation system. Endosperm cylinders with zygotic embryos extracted from 750 mature fruits collected in 30 plants were immersed in 2-2.5% commercial sodium hypochlorite and washed in sterile water three times at the collection site. The embryos were then placed in sterile containers and sent to the Plant Tissue Culture laboratory of Embrapa Tabuleiros Costeiros, Aracaju, Sergipe, Brazil. In aseptic conditions, embryos were excised from the endosperm cylinders and immersed in 70% ethyl alcohol for two minutes and in a commercial solution of sodium hypochlorite (2-2.5% NaOCl) for 20 minutes, shaken and then washed three times in sterile distilled water and placed in sterile Petri dishes [20].

Dehydration methodologies of BGD coconut mature zygotic embryos

The effect of dehydration times and cryoprotectants treatments on the embryo moisture content were evaluated. Aseptic embryos were placed in open Petri dishes containing filter paper with porosity of 14 micrometers without culture medium and dehydrated for four hours in the air stream of laminar flow at room temperature (Methodology 1; Assy-Bah & Engelmann [5]) and by four hours in silica gel at room temperature (Methodology 2; Assy-Bah & Engelmann, [5]) before the immersing in cryoprotectant treatments. The Methodology 3 (adapted of Karun & Sajini, [21]), which the dehydration process was reversed by immersing them in cryoprotectant treatments before dehydration by four hours in silica gel. The embryos, partially dehydrated, were inoculated in Y3 liquid culture medium [22] with four different cryoprotectant pre-treatments: T1- 1.75 mol L^{-1} sucrose + 15% glycerol, T2- 1.75 mol L^{-1} glucose + 15% glycerol, T3- 1.75 mol L^{-1} sucrose and T4- 1.75 mol L^{-1} of glucose. The cultures were maintained under orbital shaking under 100 rpm for 12, 16 and 20 hours (times of immersing in cryoprotectants). After this period, the sample of embryo was placed in a Petri dish containing filter paper to remove excess culture medium and then determined the weight of fresh embryo (WF). Then, the embryos were placed in metal containers and kept at 105°C for 18 hours for determination the dry embryo weight (WD). The moisture content (MC) of the embryos was determined using the following formula: $\% MC = (WF-WD)/WF \times 100$ [23]. Each experiment was conducted in a factorial completed randomized design with four cryoprotectant treatments combined with three immersing times for a total of 12 treatments with three replicates, each plot with 10 embryos (triplicates of 10 embryos/treatment), and a total of 360 embryos.

Viability by tetrazolium test of zygotic embryos of BGD accession cryopreserved

To determine the feasibility of cryopreserved coconut zygotic embryos, previously submitted by method of dehydration 3, we used a concentration of 0.25% of tetrazolium solution prepared according to AOAC [23], in 12 cryopreserved embryos treatments. After the cryopreservation, the cryovials were immediately thawed in a water bath at 40°C for five minutes. Then, samples of 10 embryos from each cryoprotection treatment were immersed in a tetrazolium solution (0.25%) and kept in the dark in BOD 40°C for two hours. Subsequently, washed with water and left on filter paper in a Petri dish to remove the water excess. The embryo viability was assessed by the percentage area of the embryo with red color, result of reduction reaction of tetrazolium salts: 0% - embryo with no staining (no reaction), 25% staining the meristematic zone of the embryo; 50% staining in the meristematic zone of the embryo, 75% of fully colored meristematic zone and part of the basal zone of the embryo; 100% of the embryo with staining.

Statistical analysis

The data of all of the experiments were submitted to analysis of variance using the F-test and compared using Tukey's test at 5% significance using the statistical software SISVAR and calculated the standard deviation by EXCEL.

3. RESULTS AND DISCUSSION

Dehydration methodologies of zygotic embryos of BGD coconut accession

The results showed that treatment with 1.75 mol L^{-1} sucrose + 15% glycerol (T1) and dehydration of embryos for four hours in a laminar flow chamber, resulting in the lowest moisture content, independent of immersion time for the methodology 1 (Table 1). This result also was observed for the same cryoprotectant treatment and dehydration in silica gel (methodology 2) as well as to the methodology 3. Similar results were obtained by Martinez-Montero et al. [24] for sugarcane somatic embryos dehydrated with 1.5 M glycerol and 0.3 M sucrose.

Table 1: Moisture content of zygotic embryos of BGD coconut accession in four cryoprotectant treatments combined with 12, 16 and 20 hours incubation times in three dehydration methodologies. Embrapa Tabuleiros Costeiros, Aracaju, Sergipe, Brazil

Time (hours)	Moisture Content (%)			
	T1	T2	T3	T4
	Methodology 1 (Assy-Bah and Engelmann [5], laminar flow)			
12	34.50 ± 0.63aD	49.30 ± 0.88bB	45.08 ± 1.03aC	61.68 ± 3.09aA
16	35.73 ± 0.53aD	51.25 ± 0.22abB	45.01 ± 0.41aC	62.41 ± 0.80aA
20	35.58 ± 0.15aD	52.51 ± 1.54aB	46.10 ± 0.17aC	61.06 ± 0.30aA
VC (%) 2.26				
	Methodology 2 (Assy-Bah and Engelmann [5], silica gel)			
12	42.69 ± 6.07aA	50.63 ± 0.46aA	48.98 ± 0.45aA	61.83 ± 0.25aA
16	36.53 ± 0.74bC	50.91 ± 1.18aAB	45.72 ±0.38aAB	62.78 ± 0.03aA
20	35.35 ± 0.07bC	50.54 ± 0.20aAB	44.82 ±0.20aAB	62.00 ± 0.17aA
VC (%)18.05				
	Methodology 3 (Adapted of Karun and Sajini [21], silica gel)			
12	28.63 ± 1.29abC	34.23 ± 1.25bB	30.70 ±6.21aBC	45.45 ± 0.68aA
16	23.48 ± 3.37bC	31.07 ± 1.10bB	28.00 ±0.63aBC	43.17 ± 3.08aA
20	31.87 ± 6.86aB	42.89 ± 0.23aA	32.60 ± 1.34aB	41.38 ± 2.89aA
VC (%) 9.25				

Values represent the mean of three replicates ± standard deviation of mean. The means followed by capital letter in a row and small letter in a column do not differ by the Tukey test at the 5% significance level. T1- 1.75 mol L^{-1} sucrose + 15% glycerol, T2- 1.75 mol L^{-1} glucose + 15% glycerol, T3- 1.75 mol L^{-1} sucrose and T4- 1.75 mol L^{-1} glucose. VC= Variation Coefficient

The embryos submitted to methodology 3 presented in 16hT1 23.48 ± 3.37% moisture. This low moisture content, compared to other methods, occurred probably because the embryos were first immersed in the cryoprotectant solution that provided a pre-dehydration and then were dried in silica gel. The silica gel adsorbed more water compared to drying in a laminar flow chamber. Intracellular ice crystal formation causes disruption of cell membranes system, resulting in loss of cell permeability and partitioning, consequently, the cells collapse and die [11]. However, Assy-Bah & Engelmann [5] had, on average, during the first 15 hours of pretreatment, a decrease of moisture of 79.3 ± 6.7% to 5.7 ± 4.2% in the time from 0 to 24 hours. The embryos with larger dimensions (RT and IT7 accessions) dehydrated more slowly

than smaller (CRD accession). These results disagree with those obtained in this work that had, on average, higher humidity for the BGD coconut. Probably the humidity of the embryos vary according to genotype.

Regarding the immersion time of embryos in the cryoprotectant solution, the period of 12 hours promoted the lowest moisture (34.50 ± 0.63%) in laminar flow. For the silica gel, the lower humidity was obtained by immersion for 16 and 20 hours (36.53 ± 0.74 and 35.35 ± 0.07%). In methodology 3, showed the lowest moisture content of the embryos immersed for 16 hours (23.48 ± 3.37%). However, the period of immersion of 20 hours in the methodology 3 caused an increase in humidity, contradicting the hypothesis that the longer the time, greater the dehydration. Assy-Bah & Engelmann [5] showed that moisture content decreased with the passage of time (zero up to 24 hours) with four varieties of coconut mature embryos. Probably within 20 hours, there was a saturation of hydrogen bonds that formed during the dehydration process does not occur over the replacement of water molecules bound to phospholipids in the hydrated state. In studies conducted by Assy-Bah & Engelmann [5] were observed that the initial water content of embryos of coconut is usually between 50 and 60% and maximum survival rate was 10 to 16% in just 2 to 4 hours of dehydration. Therefore obtaining methodologies that provide lower moisture content in the fragments is of great importance for this species.

The T1(1.75 mol L^{-1} sucrose + 15% glycerol) may have favored a greater dehydration of coconut zygotic embryos due to the solution mixture of sucrose and glycerol, because as these are disaccharide of glucose and fructose and polyol, respectively, present in its structure several hydroxyl groups, compared to other treatments, and these groups have the ability to form hydrogen bonds with the polar "heads" of membrane phospholipids and thus replacing the water molecules bound to phospholipids in the hydrated state [25]. According to Dumet et al. [26] sugars can act as outside osmotic agents, removing the intracellular water excess via an osmotic gradient. Hao et al. [27] obtained satisfactory results when added to the culture medium sucrose or polyethylene glycol (PEG) 1500-6000 on the stability of lysosomes, suggesting that the ability to form hydrogen bonds performs an important role in osmotic protection of the solute to the lysosome. The authors report that, especially in solutions containing mixtures of sucrose and polyvinyl alcohol, the hydrogen bonds between water and solute are so strong and numerous that they become thermodynamically stable, promoting greater stability in the cell membrane.

The methodology 3 showed higher moisture on embryos, probably because the initial drying was in laminar flow and/or silica gel after treatment with cryoprotectants, which may have compromised the ideal dehydration of the embryos, as they may have absorbed water extender solution. Low moisture content in the explant is important, since the smaller the amount of water in plant tissue, greater will be the survival after immersion in liquid nitrogen by reducing the possibility of formation of ice crystals.

Viability by tetrazolium test of zygotic embryos of BGD coconut accession

In cryopreserved embryos pretreated with 1.75 mol L^{-1} sucrose + 15% glycerol (T1) by 12, 16 and 20 hours showed the highest percentage of viability compared to other treatments in all periods of immersion. Embryos pretreated with T4 had the lowest percentage of viability (Table 2).

The tetrazolium test was able to indicate more percentage of viability, indicated by the higher percentage of colored meristematic zone and part of the basal zone of the embryo, on the embryos were immersed in cryoprotectant solution that promoted the greatest reduction in moisture content.

Table 2: Viability of zygotic embryos of BGD accession by tetrazolium test in different cryoprotectants and times of immersing. Embrapa Tabuleiros Costeiros, Aracaju, Sergipe, Brazil

Time (hours)	**Viability (%)**			
	T1	**T2**	**T3**	**T4**
12	80.00 ± 11.18aA	75.00 ± 20.41aA	68.75 ± 12.50aB	45.00 ± 11.18aB
16	80.00 ± 11.18aA	56.25 ± 12.50bB	62.50 ± 14.43aAB	55.00 ± 11.18aB
20	75.00 ± 17.67aA	65.00 ± 13.69abB	68.75 ± 12.50aB	55.00 ± 11.18aC
VC (%)	11.67			

Values represent the mean of five replicates ± standard deviation of mean. The means followed by capital letter in a row and small letter in a column do not differ by the Tukey test at the 5% significance level. T1- 1.75 mol L^{-1} sucrose + 15% glycerol, T2- 1.75 mol L^{-1} glucose + 15% glycerol, T3- 1.75 mol L^{-1} sucrose and T4- 1.75 mol L^{-1} glucose. VC= Variation Coefficient

There are few reports in the literature for the application of tests for rapid responses to assess the viability of embryo cryopreserved. The use of zygotic embryos for viability studies of palm seeds is recent and has shown adequate precision [17,18,19]. The results indicated that the tetrazolium test for analysis of viability of cryopreserved zygotic embryos of coconut is very promising.

4. CONCLUSIONS

This study concluded that the methodology 3: immersion of zygotic embryos in cryoprotectant solution (1.75 mol L^{-1} sucrose + 15% glycerol) by 12 and 16 hours and subsequent drying in silica gel by four hours promotes the lowest moisture contents in mature zygotic embryos and presented high potential use in future cryopreservation protocols. The tetrazolium test has been used for viability analysis of embryo cryopreserved of BGD accession.

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