Ciência Rural, Santa Maria, v.50:1, e20190020, 2020 http://dx.doi.org/10.1590/0103-8478cr20190020
ISSNe 1678-4596
BIOLOGY

Cryopreservation of Brazilian green dwarf coconut plumules by droplet-vitrification

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ABSTRACT: *This study evaluated the effect of vitrification solutions and exposure time on the cryopreservation of Brazilian green dwarf coconut plumules (BGD) using the droplet vitrification technique. Explants were excised from BGD mature fruits from the Active Germplasm Bank of Embrapa Tabuleiros Costeiros, Sergipe, Brazil. Firstly, embryos were disinfected, and after excision, plumules were pre-cultivated for 72 hours in Y3 + 0.6 M sucrose + 2.2 g L^{-1} Gelrite® culture medium. Plumules were exposed to PVS2 and PVS3 solutions for 15 and 30 minutes and rapidly immersed in liquid nitrogen (-196 ºC). After cryopreservation, they were thawed in culture medium solution (Y3 + 1.2 M sucrose) and cultured in regeneration medium. The experimental design was completely randomized in a 2x2 factorial scheme (vitrification solutions per exposure times), with five replicates per treatment. Data were compared by the Tukey's test at 5% probability. Significant differences were observed in the callogenesis percentage for the solutions x exposure time interaction for non-cryopreserved cultures (-NL) and for exposure time after cryopreservation (+NL). PVS2 and PVS3 combined with 15 minutes of exposure promoted the highest callus formation (70 and 100%, respectively) in control cultures. The exposure time of 30 min, regardless of vitrification solution, resulted in 30% embryogenic callus formation after cryopreservation. These results contributed to the long-term conservation of coconut palm.*
Key words: *Cocos nucifera L., cryoprotection, PVS2, PVS3.*

Criopreservação de coqueiro anão verde do Brasil de Jiqui por vitrificação em gotas

RESUMO: *O objetivo desse estudo foi avaliar o efeito das soluções de vitrificação e do tempo de exposição na criopreservação de plúmulas de coqueiro anão verde do Brasil de Jiqui (BGD), pela técnica de vitrificação em gotas. Os explantes foram excisados de frutos maduros oriundos do Banco de Germoplasma Ativo de Embrapa Tabuleiros Costeiros, Sergipe, Brasil. Os embriões foram desinfestados e as plúmulas, após a excisão, pré-cultivadas durante 72 horas em meio de cultura Y3 suplementado com sacarose 0,6 e 2,2 g L^{-1} Gelrite®. As plúmulas foram expostas em soluções de PVS2 e PVS3 durante 15 e 30 minutos, e rapidamente imersas em nitrogênio líquido (-196 ºC). Após a criopreservação, foram descongeladas na solução de meio de cultura Y3 com 1,2 M de sacarose, e cultivadas em meio de regeneração. O delineamento experimental foi inteiramente casualizado em esquema fatorial 2x2 (soluções de vitrificação x tempos de exposição), com cinco repetições por tratamento. Os dados foram comparados pelo teste de Tukey à probabilidade de 5%. Observaram-se diferenças significativas na porcentagem de calogênese para a interação entre soluções e tempo de exposição para as culturas não criopreservadas (-NL), e para o tempo de exposição após a criopreservação (+NL). O PVS2 e o PVS3 combinados com 15 minutos promoveram a maior formação de calo (70 e 100%, respectivamente) nas culturas de controle. O tempo de exposição de 30 min, independente da solução de vitrificação, promoveu 30% da formação de calos embriogênicos após a criopreservação. Estes resultados contribuem para a conservação em longo prazo do coqueiro.*
Palavras-chave: *Cocos nucifera L., crioproteção, PVS2, PVS3.*

INTRODUCTION

Coconut (*Cocos nucifera* L.) is also known as the "tree of life", because each component of the palm tree can be used for fresh consumption or transformed by industrialization. It is a tropical plant that presents a range of products and by-products for use in human nutrition, construction industry, cosmetics, pharmaceuticals and in production of biodiesel. Since it is adapted to soils of low natural fertility, it is cultivated in more than 86 countries. In 2017, Brazil ranked sixth in the world, with production around 2,342,942 tons (FAO, 2019).

Coconut farming is significant to the Northeastern Brazil economy, where the most production is located (71.36%), producing 684,501,049 thousand fruits. The largest producers in this region are the states of Bahia, Ceará, Pernambuco and Sergipe. The Northern region, with 13.54%, and the Southeastern region, with 14.20% of production, also contributed to the national supplying (IBGE, 2017).

The conservation of coconut genetic resources is mainly accomplished through field collections due to the seed size and recalcitrance, making its storage difficult (N'NAN et al., 2008). Cryopreservation comprises the conservation of plant material at ultra low temperature provided by liquid nitrogen at -196 ° C, or with its vapor phase at -150 °C. Thus, the technique becomes a viable

Received 01.09.19 Approved 10.25.19 Returned by the author xx.xx.xx
CR-2019-0020.R1

procedure for conservation of biological material for long periods of time, requiring little space and maintenance (BENSON, 2008).

Cryopreservation has been used for genetic resources conservation of many species, especially recalcitrant ones. First studies with coconut were carried out by ASSY-BAH and ENGELMANN (1992), using vitrification technique in mature PB 121 zygotic hybrid coconut embryos (Malayan yellow dwarf x African tall), Cameroon red dwarf, Indian tall and Rennel tall coconut genotypes. Several researchers have published promising results with different techniques using zygotic embryos, plumules, inflorescence and pollen as explants (SAJINI et al., 2011; CUETO et al., 2014; MACHADO et al., 2014; WELEWANNI et al., 2017).

There is scarce literature about the cryopreservation of plumules and zygotic embryos from accessions collected in Brazil such as Brazilian green dwarf (BGD accession) and Brazilian tall genotypes. Preliminary studies have been published to evaluate the viability of cryopreserved BGD accession by means of electrolytic conductivity and potassium leaching tests (COPELAND-GOMES et al., 2012, 2015); however, without regeneration after cryopreservation. Recent studies have shown the feasibility of the vitrification technique for the cryopreservation of BGD zygotic embryos (LEDO et al., 2018). Moreover, regeneration techniques by somatic embryogenesis from plumules are more interesting from the point of view of breeding and conservation of genetic resources because they allow obtaining higher number of plants (NGUYEN et al., 2015).

Studies with cryopreserved of Malayan yellow dwarf coconut plumules by encapsulation-dehydration reached only 20% survival (N'NAN et al., 2008). The vitrification technique that proposes the cryoprotection of explants was reported for other coconut genotypes, with good performance of vitrification solution 3 (PVS3), according to NISHIZAWA et al. (1993). According SAJINI et al. (2011) PVS1, PVS2 and PVS4 vitrification solutions were harmful to zygotic embryos. This study evaluated the effect of vitrification solutions and exposure time on the cryopreservation of BGD accession plumules using the droplet vitrification technique.

MATERIALS AND METHODS

Collection, disinfect and isolation of plant material

Mature BGD coconut fruits (10-11 months of age) originated from three mother plants of the Coconut Active Germplasm Bank of Embrapa Tabuleiros Costeiros, Sergipe, Brazil, were used. The endosperm cylinders containing the zygotic embryos were removed and sterilized by immersion in 2-2.5% commercial sodium hypochlorite solution for 30 minutes, followed by triple wash in water. Subsequently, the material was packed in sterile plastic bags and sent to Plant Tissue Culture Laboratory.

In laminar flow chamber, embryos were excised from the endosperm cylinders. Then, they were disinfected by immersion in 70% ethyl alcohol for 30 seconds, in sodium hypochlorite solution (2-2.5% v / v) for five minutes, followed by triple washing in distilled and sterile water and in sealed vials.

Pre-culture, cryoprotection, cryopreservation, thawing and regeneration

After disinfecting, embryos were pre-cultured in sterile 140 mm x 15 mm polystyrene Petri dishes containing 4 mL of Y3 medium (EEUWENS, 1976) containing 0.6 M sucrose and 2.2 g L^{-1} Gelrite® (adapted from SAJINI et al., 2011). Before that, the culture medium pH was adjusted to 5.8 ± 0.1 and autoclaved for 15 minutes at temperature of 121 ± 1 °C and pressure of 1.05 atm.

Cultures were maintained in growth room for 72 hours at controlled temperature of 25 ± 2 ºC, relative humidity of about 70% in the absence of light. After this period, plumules were excised and exposed to Plant Vitrification Solution 2 - PVS2 (SAKAI et al., 1990): (30% (v/v) glycerol; 15% (v/v) ethylene glycol and 15% (v/v) dimethyl sulfoxide-DMSO and Plant Vitrification Solution 3- PVS3 (NISHIZAWA et al., 1993; KIM et al., 2009): 50% glycerol (w/v) and 50% sucrose (w/v) solutions at 0 °C for 15 and 30 minutes and rapidly immersed in liquid nitrogen (-196 °C). To this end, drops containing 0.25 mL of cryoprotective solutions were added with the aid of a Pauster pipette in aluminum foil strips (~ 5 mm x 15 mm, 05 drops / aluminum strip), and plumules were immersed in the drops and kept for 15 and 30 minutes. After each exposure time, strips were immersed in liquid nitrogen and inserted into sterile polystyrene cryotubes of 2 mL capacity and quickly transferred to liquid nitrogen for 24 hours (SAKAI & ENGELMANN, 2007).

After cryopreservation, plumules were thawed in solution composed of Y3 culture medium supplemented with 1.2 M sucrose at temperature of 25 ± 2 °C for 15 to 20 minutes. Then, they were cultured in 140 mm x 15 mm sterile polystyrene Petri dishes containing regeneration medium composed of salts

and vitamins from the Y3 culture medium with 50 g L^{-1} sucrose; 100 mg L^{-1} of 2,4-Dichlorophenoxyacetic acid (2,4-D); 3 g L^{-1} activated charcoal and 2.2 g L^{-1} Gelrite®. Cultures were maintained in growth room with controlled temperature of 25 ± 2 °C, relative humidity of about 70% in absence of light until formation of the first embryogenic structures, which were transferred to indirect light (26 µmol m^{-2} s^{-1}).

In order to evaluate the effects of cryoprotectant solutions on plumules exposed (+NL) and non-exposed (-NL) to liquid nitrogen, the survival percentage and percentage of embryogenic callus formation were observed at 45 days of *in vitro* culture.

Histological analyses

Samples were fixed in FAA solution composed of formaldehyde; 70% alcohol and glacial acetic acid (JOHANSEN, 1940). Dehydration was performed in increasing ethylic series (80%, 90% and 100%) at intervals of 1 hour each. Subsequently, samples were placed in pre-infiltration solution for 2 hours and then in infiltration solution for 24 hours in the refrigerator. The inclusion solution was prepared following proportions indicated by the historesin kit (Leica Microsystems, Heidelberg, Germany), with the addition of activated resin hardener. Plant fragments were infiltrated in histomolds and polymerized at room temperature. After material inclusion, microtomy was performed using semiautomatic rotating microtome (SLEE, Mainz, Germany). By defining thickness of 8 µm, sections were performed and placed on blade containing a small amount of water. After drying at room temperature, callus samples were stained in toluidine blue at pH 4.8 and then washed with acetic water. After drying, blades were fixed with stained glass and then observed under optical microscope (Nikon Eclipse E100 coupled to Infinity 1 camera), where photomicrographs were performed.

Experimental design and statistical analyses

The experimental design was completely randomized in a 2 x 2 factorial scheme (2 vitrification solutions x 2 exposure times) with five replicates per treatment, each plot consisting of ten plumules. Data were transformed into arcsine of $\sqrt{X + 0.5}$, according to analysis of variance prerequisites. Averages were compared by the Tukey test at 5% significance. The SAS® statistical software (SAS version 9.2) was used.

RESULTS AND DISCUSSION

Effect of the cryoprotectant solution and exposure time on survival and callus induction percentage of non-cryopreserved (-NL) and cryopreserved (+ NL) BGD plumules

There was a significant effect of cryoprotectant solution, exposure time and interaction of factors on the survival and callogenesis percentages of non-cryopreserved and cryopreserved plumules (Table 1).

High survival percentage of non-cryopreserved and cryopreserved plumules was observed in different solutions and exposure times; however, same response was not observed for callogenesis percentage in cryopreserved plumules (Table 2). Coconut is considered one of the most recalcitrant species for *in vitro* regeneration (PEREZ-NUNEZ et al., 2006) and probably the process of exposure to liquid nitrogen has contributed to this behavior.

Initial induction (30 days) of calluses with coloration ranging from whitish to creamy-yellowish

Table 1 - Analysis of variance of survival (% SOB) and callogenesis percentage (% CALO) of non-cryopreserved (-NL) and cryopreserved (+ NL) Brazilian green dwarf coconut plumules as a function of the cryoprotectant solution and exposure time[1].

Source of variation	DF[2]	AS[3] % SOB (-NL)	AS % CALO (-NL)	AS % SOB (+NL)	AS % CALO (+NL)
Cryoprotectant (C)	1	20.8267**	90.0841**	2.3681 ns	0.8420 ns
Time (T)	1	47.8551**	23.8469**	0.3767 ns	145.2670**
C * T	1	30.3080**	23.8469**	0.0169 ns	0.8420 ns
Error	16	3.9601	43.1823	0.7545	0.4863
VC (%)		6.02	20.79	9.27	25.88

[1]data transformed into arcsine $\sqrt{x + 0.5}$; DF- Degrees of freedom, AS- Average square.
ns - not significant at 5% probability; ** significant at 1% probability.

Table 2 - Mean [1] survival percentage at 30 days and callogenesis at 60 days in non-cryopreserved (-NL) and cryopreserved (+ NL) Brazilian green dwarf coconut plumules as a function of cryoprotectant solution [2,3] and exposure time.

	----------% Survival – NL----------		Mean	----------% Callogenesis –NL----------		Mean
	15 minutes	30 minutes		15 minutes	30 minutes	
PVS2	92aA	80aB	86	18bB	66bA	42a
PVS3	100aA	20bB	60	100aA	100aA	100a
Mean	96	50		59	83	
	----------% Survival +NL----------		Mean	----------% Callogenesis +NL----------		Mean
	15 minutes	30 minutes		15 minutes	30 minutes	
PVS2	96aA	92aA	94	0aA	30 aA	17.50a
PVS3	84aA	80aA	82	0aA	25 aA	12.50a
Mean	90	86		0B	30A	

[1]Means followed by the same uppercase letter, in the line, and lower case, in the column do not differ by the Tukey test at 5% significance.

[2]PVS2-Vitrification solution 2 composed of 30% glycerol (v / v), 15% ethylene glycol (v / v), 15% dimethylsulfoxide- DMSO (v / v) (SAKAI et al., 1990).

[3]PVS3- Vitrification solution 3 composed of 50% glycerol (v / v) and 50% sucrose (v / v) (NISHIZAWA et al., 1993).

(Figure 1A) was observed on the entire surface of non-cryopreserved plumules of non-friable consistency. The same morphogenetic pattern was observed by AZPEITIA et al. (2003) and Pérez-Nunez et al. (2006). Later, at 45-60 days of callogenesis, the formation of translucent "ear" structures was observed (Figure 1B) and then the development of globular structures (Figure 1C). This pattern was also observed by Pérez-Nunez et al. (2006) in cryopreserved Malayan green dwarf coconut. Cryopreserved Brazilian Green Dwarf coconut plumules presented the same development of non-cryopreserved plumules; however, with later onset of the process around 45 to 60 days.

Callogenesis percentage in plumules submitted to PVS2 and PVS3 for 15 minutes was 92% and 100%, respectively, higher than the time of 30 minutes, 80 and 20%, respectively, in non-cryopreserved (-NL) plumules (Table 2). This result indicated that BGD accession plumules may present less tolerance to longer exposure time in cryoprotectant solutions prior to cryopreservation. However, SAJINI et al. (2011) did not observe variations in the survival of West Coast tall coconut zygotic embryos at different times of exposure to PVS3.

The immersion in PVS3 solution for 15 minutes, in non-cryopreserved (-NL) plumules promoted 100% survival and 100% callogenesis. This result can be explained by absence of dimethylsulfoxide (DMSO) in PVS3 solution and the shortest exposure time.

Considering that cryopreservation involves cryoprotection, ultra-low temperature conservation and thawing, the ideal cryoprotectant solution should biologically protect cells during these steps (KARTHA & ENGELMANN, 1994). However, several authors have reported cytotoxicity and / or osmotic stress induced by exposure time and by DMSO, ethylene glycol and propylene glycol (SAJINI et al., 2011). These authors, studying various compositions of vitrification solutions, obtained 75% survival and 20% regeneration in West Coast tall coconut zygotic embryos treated with PVS3 for 16 hours. However, in the present study, no toxic effects of DMSO were observed on non-cryopreserved plumules.

The longer exposure time of 30 minutes promoted, on average, higher callus induction (30%) in cryopreserved plumules when compared to 15 minutes (0%) (Table 2).

Histological analyses

Histological analyses revealed the embryogenic callus section, showing globular somatic embryo (GSE), in which the tissue forms a ring around the embryo containing meristematic cells. Most of these cells were observed in peripheral zone of callus, with denser staining in cytoplasm (Figure 2A). The embryogenic callus section

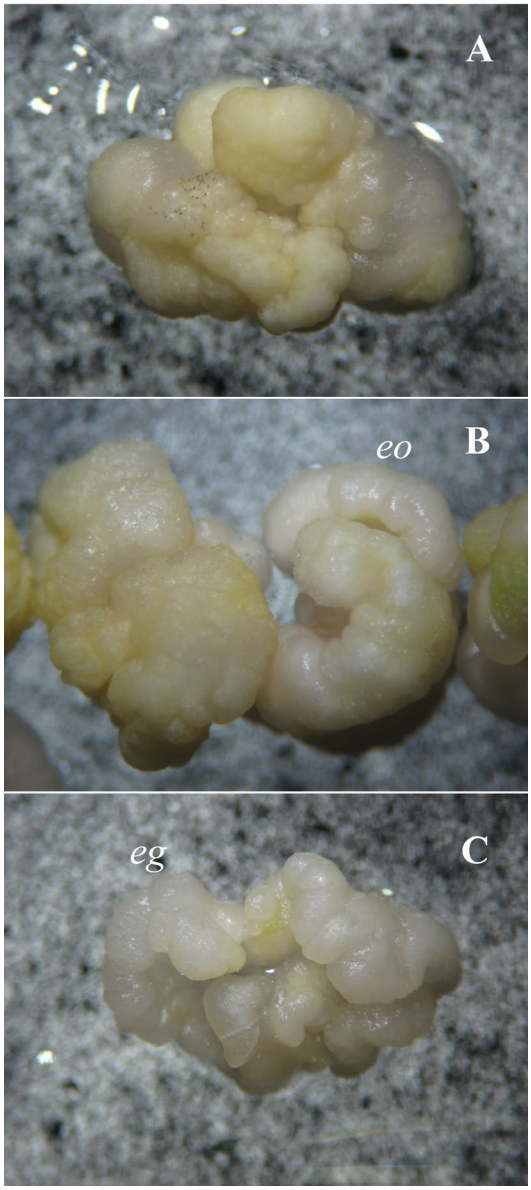

Figure 1 - Development of embryogenic calluses in non-cryopreserved and cryopreserved Brazilian green dwarf coconut plumules. A - calluses with whitish to creamy-yellowish coloration; B - "ear" type structure (eo); C- globular structures (egs). Photos: Leila Albuquerque Oliveira.

showing the formation of meristematic nodules (MN) and protodermis (PD) indicated by presence of small and densely stained meristematic cells developed in meristematic nodules, located along the peripheral zone of embryogenic structures, but below a layer of stained cells that form protodermis (PD) (Figure 2B). Histological analysis after cryopreservation showed embryogenic callus section with presence of meristematic cells (MC), where the embryo did not reveal a well-defined meristem, but some layers of meristematic cells with dense cytoplasm staining in peripheral tissues (Figure 2C). Embryogenic callus section showing formation of meristematic nodules (MN) and protodermis (PD) also after cryopreservation, where there was multiplication of embryogenic

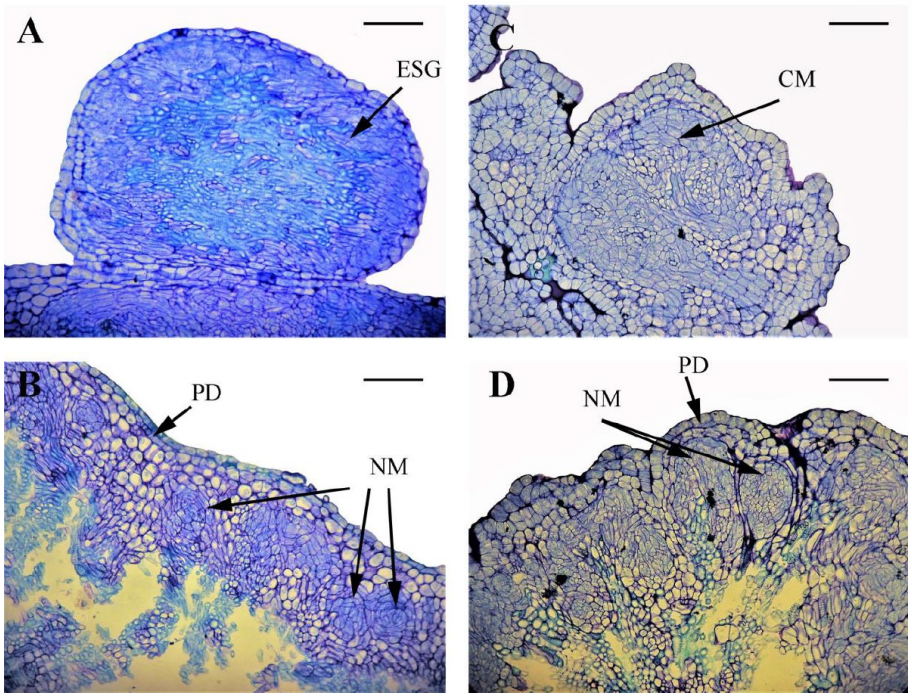

Figure 2 - Histological sections of coconut culture callus formed before (A and B) and after cryopreservation (C and D) using PVS2. A- Embryogenic callus section showing globular somatic embryo (GSE). B- Embryogenic callus section showing the formation of meristematic nodules (MN) and Protodermis (PD). C- Embryogenic callus section showing the presence of meristematic cells (CM). D- Embryogenic callus section showing the formation of meristematic nodules (MN) and Protodermis (PD). Scale bar = 100 µm.

callus, with formation of meristematic nodules, with large and agglomerated cells with a small nucleus, and presence of protodermis (Figure 2D).

The use of coconut plumules as source of explants promotes possibilities for germplasm conservation, allowing the maintenance of genetic diversity through cryobanking (N'NAN et al., 2008). Due to small size and phloem-free nature of plumule tissues, it could be expected that this material would be disease-free, thus facilitating the germplasm exchange. According to histological analysis, it was inferred that even after e cryopreservation of plumules, there was callogenesis with formation of embryogenic structures, which indicates that multiplication can be efficiently obtained.

The presence of meristematic cells, meristematic nodules and protodermis after cryopreservation of coconut plumules was also observed by PÉREZ-NÚÑEZ et al. (2006). The authors emphasized that these embryogenic structures produced by the embryogenic callus are able to form somatic embryos. The formation of structures occurred within a few days of culture and the induction of embryogenic calluses and somatic embryos was faster from primary somatic embryogenesis using explants from plumules.

Thus, cryopreserved plumules presenting pattern of embryogenic callus development at 45-60 days submitted to PVS2 for 15 minutes presented 92% callogenesis, maintaining all the structures necessary for the development of globular somatic embryos.

CONCLUSION

PVS2 and PVS3 vitrification solutions and exposure time of 30 minutes induced greater amount of embryogenic calluses in cryopreserved BGD coconut plumules.

After cryopreservation of plumules, no changes in the cell structures that compromise the development of embryogenic structures were observed.

ACKNOWLEDGEMENTS

Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), for financial support and fellowship.

DECLARATION OF CONFLICT OF INTERESTS

The authors declare no conflict of interest. The founding sponsors had no role in design of the study; in collection, analyses, or interpretation of data; in writing of the manuscript, and in decision to publish the results.

AUTHORS' CONTRIBUTIONS

All authors contributed equally for the conception and writing of the manuscript. All authors critically revised the manuscript and approved of the final version.

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