



Long term conservation of embryonic axes of genipap accessions

Conservação a longo prazo de eixos embrionários de acessos de jenipapeiro

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The *ex situ* conservation of seeds in cryobanks demands previous studies of dehydration procedures for the adjustment of the adequate moisture that makes long-term conservation possible. The objective of this work was to evaluate the effect of dehydration time on the regenerative capacity of embryonic axes of two genipap accessions (Umbaúba and Núcleo Bandeirante). Seeds were submitted to desiccation in polycarbonate box containing silica gel for 0, 12, 16 and 20 h at room temperature and after each period, seed moisture content was determined. Seeds were inoculated in germination medium and samples were inserted into cryotubes and immersed in liquid nitrogen (-196°C). Desiccation in silica gel was efficient in reducing the moisture content in both accessions. Umbaúba accession presented 100% germination at all desiccation treatments before cryopreservation and Núcleo Bandeirante was more sensitive to desiccation with 10% germination in 20 h. Umbaúba accession showed a reduction of growth variables with increased desiccation time. The treatments tested did not affect the growth variables of Núcleo Bandeirante accession. The desiccation, although changes observed in the ultra-structures of embryonic axis, did not influence the regeneration percentage of cryopreserved accessions.

Keywords: *Genipa americana* L., tissue culture, cryopreservation.

A conservação *ex situ* de sementes em bancos criogênicos exige estudos prévios de procedimentos de desidratação para o ajuste da umidade adequada que possibilita a conservação em longo prazo. O objetivo deste trabalho foi avaliar o efeito do tempo de desidratação sobre a capacidade regenerativa de eixos embrionários de dois acessos de jenipapo, Umbaúba e Núcleo Bandeirante. As sementes foram submetidas à dessecação em caixas de policarbonato contendo sílica gel por 0, 12, 16 e 20 h em temperatura ambiente e, após cada período, o teor de umidade da semente foi determinado. As sementes foram inoculadas no meio de germinação e amostras para cada período foram inseridas em criotubos e imersas em nitrogênio líquido (-196°C). A dessecação em sílica gel foi eficiente na redução do teor de umidade (% mc) de ambos os acessos. O acesso Umbaúba apresentou 100% de germinação em todos os tempos de dessecação antes da criopreservação e o Núcleo Bandeirante foi mais sensível à dessecação com 10% de germinação em 20 h. O acesso Umbaúba apresentou uma redução das variáveis de crescimento com o aumento do tempo de dessecação. Os tempos testados não afetaram as variáveis de crescimento do acesso Núcleo Bandeirante. O tempo de dessecação, apesar das alterações nas ultraestruturas do eixo embrionário, não influenciou a porcentagem de regeneração dos acessos criopreservados.

Palavras-chave: *Genipa americana* L., cultura de tecidos, criopreservação.

1. INTRODUCTION

Genipap (*Genipa americana* L.), a native species belonging to Rubiaceae family, is found in northern South America and Central America. Genipap, as it is popularly known, has potential for socioeconomic use and is used for various purposes, among them for food and textile industrials, wood exploitation and medicines. For presenting resistance to soaked soils, it arouses great interest for the recovery of degraded areas, permanent preservation areas, among others [7, 23].

The species has fruits widely appreciated in the Northeastern region of Brazil, and despite the great demand in the region, it is still exploited in an extractive way [30]. The conservation of its

genetic resources is mainly based on field collections, due to the physiology of its seeds, which present intermediate behavior in storage, not tolerating desiccation at extremely low levels [5, 18]. In order to ensure the ecological and economic sustainability of the species, several research institutions have conserved its genetic diversity in the field [29].

The advancement of biotechnological techniques presents the cryopreservation as a strategy complementary to conservation. Cryopreservation consists of preserving the biological material at ultra low temperatures using liquid nitrogen at -196°C , or at its vapor phase at -150°C for long periods [3]. Cryopreservation becomes a viable procedure for the preservation of biological material for long periods of time, requiring reduced space and little maintenance [2, 26].

There are different cryopreservation methods, especially dehydration, encapsulation - dehydration, droplet-vitrification and vitrification [11]. These techniques have been successfully used for different explants in *Citrus* sp. [13]; *Coffea arabica* L. [9], *Hancornia speciosa* Gomes [20, 25, 26], *Genipa americana* L. [23, 24], *Pistacia vera* L. [1] and *Ananas comosus* L. Merr. [31]. Different explants can be cryopreserved as embryonic axes, apical meristems and calluses [25].

The intracellular water content is a limiting factor for the survival of the cryopreserved explant, thus dehydration is essential, which can be physical through evaporation promoted by the exposure of the material to silica gel or laminar flow cabinet or by the use of cryoprotectants that cause osmotic dehydration of the material [14, 22]. The reduction of water from explants to extremely low levels to avoid the formation of ice crystals is the most critical step in obtaining a successful cryopreservation protocol [12].

Many damages can be caused to frozen samples with inadequate water content such as: mechanical disorganization of plant structures, mechanical damage resulting from cell volume reduction and cell membrane injury [24]. Tolerance to dehydration varies from species to species and among tissues of the same plant, and appropriate humidity must be reached to ensure adequate survival of the material [22]. Besides that, desiccation tolerance is directly related to changes in cellular components, to the activation of antioxidant enzyme systems, to oligosaccharides and to heat-resistant proteins [3, 8, 28].

The *ex situ* conservation of seeds in cryobanks demands previous studies of dehydration procedures for the adjustment of the adequate moisture that makes long-term conservation possible. Several classical methods of evaluating seed viability have been applied; however, the *in vitro* culture of embryos or embryonic axes also allows the evaluation of the physiological integrity of cryopreservation [24].

The present work had as objective to evaluate the effect of different dehydration times in silica gel on the regenerative capacity of cryopreserved embryonic axes of different genipap accessions.

2. MATERIAL AND METHODS

2.1. Plant Material

Genipap (*Genipa americana* L.) seeds from mature fruits, collected in 2017 from plants of natural populations of Núcleo Bandeirante, Federal District ($15^{\circ}51'55.72''\text{S}$; $47^{\circ}57'34.59''\text{W}$) and Umbaúba, Sergipe accessions ($11^{\circ}1'56.1''\text{S}$; $37^{\circ}41'32.8''\text{W}$), were used as source of explants. Seeds were manually extracted and washed with detergent until the pulp was completely removed and immersed in ethyl alcohol ($\text{C}_2\text{H}_5\text{OH}$) at 70% for 3 min and in sodium hypochlorite (NaOCl) solution at 2 to 2.5% for 15 min and then rinsed three times and dried for 24 h at room temperature ($24^{\circ}\text{C} \pm 2^{\circ}\text{C}$).

2.2. Effect of desiccation time on the moisture content and viability of genipap accessions before cryopreservation

To obtain the germination percentage (control 1), seeds were inoculated in MS culture medium [17] with 30 g/L sucrose and 3.5 g/L Phytigel® (germination medium).

For studies desiccation effects on the germination of embryonic axes, seeds were submitted to desiccation in magenta boxes containing 50 g of silica gel (SiO_2) for 0, 12, 16 and 20 h at room

temperature. Seed moisture (five samples of 10 seeds per genotype) was determined for each desiccation time. After that, seeds were inoculated in germination medium, as described above, and two samples of 10 seeds for each treatment were immediately inserted into cryotubes and immersed in liquid nitrogen (-196°C).

To determine the initial moisture content of seeds and after desiccation, three replicates of five seeds were weighed to obtain the wet weight (W_w) and transferred to laboratory oven for 72 h at $105^\circ\text{C} \pm 3^\circ\text{C}$ to obtain the dry weight (W_d). The moisture content, on wet basis (% mc_{wb}) was determined by the following equation: $\% mc_{wb} = [(W_w - W_d) / W_w] \times 100$.

To evaluate the germination percentage after cryopreservation, cryotubes were thawed in water bath at $40^\circ\text{C} \pm 2^\circ\text{C}$ for 3 min and seeds were immersed in sterile water for 24 h, according to Santos & Salomão (2016) [24]. After this period, the embryonic axes were excised and inoculated in MS germination medium.

Variables percentage germination, shoot and root length and number of leaves were measured after each germination test at 60 days. The design was completely randomized in a 2×4 factorial scheme (two genotypes and four desiccation times), with five replicates, with plot being represented by 50 seeds.

2.3. Effect of desiccation time on the regeneration and growth of genipap accessions after cryopreservation

To obtain the germination percentage (control 1), fresh seeds were inoculated in previously described MS culture medium. For studies on the effect of desiccation on the germination of embryonic axes, seeds were submitted to desiccation in magenta boxes containing 50 g of silica gel for 0, 12, 16 and 20 h at room temperature. Seed moisture (five samples of 10 seeds per genotype) was determined for each desiccation period. After that, seeds were inoculated in the germination medium (control 2) and two samples of 10 seeds for each desiccation time were immediately placed into cryotubes and immersed in liquid nitrogen (-196°C). To evaluate the germination percentage, cryotubes were thawed in water bath at $40^\circ\text{C} \pm 2^\circ\text{C}$ for 3 min and seeds were immersed in sterile water for 24 h, after which the embryonic axes were excised and immediately inoculated in MS culture medium with 30 g/L sucrose and 3.5 g/L Phytigel®.

The regeneration percentage, shoot and root lengths and number of leaves were evaluated after each germination test at 60 days. The design was completely randomized in a 2×4 factorial scheme (two genotypes and four desiccation times), with five replicates, with plot being represented by 50 seeds.

2.4. Histological analyses

Analyses were carried out in the laboratories of Plant Anatomy and Laboratory of Electron Microscopy and Ultrastructure Analysis of the Federal University of Lavras. Embryonic axes were excised from seeds and fixed in formaldehyde FAA (formalin, acetic acid and ethyl alcohol) [6]. Samples were then submitted to 70%, 80%, 90% and 100% alcohol gradient at 2 h intervals for dehydration. Subsequently, samples were infiltrated by 100% ethanol + liquid resin (1: 1) by 2 h. Activated resin = infiltration solution (resin + activator powder, as indicated by the manufacturer) by 24 h in refrigerator. Subsequently, the inclusion solution was prepared by adding the hardener to the infiltration resin or activated resin (following the proportions indicated by the manufacturer).

Plant fragments were infiltrated into 6 mm x 8 mm Leica® histomolds (plastic forms suitable for inclusion). At the time of inclusion, fragments were adequately positioned and polymerized in a laboratory oven at 37°C . Blocks were kept with low humidity, stored in containers with good sealing and content silica gel. After material inclusion, microtomy was performed using semi-automatic rotating microtome that was standardized at cut thickness ranging from 5 to 8 μm . Then, they were spread over the slide containing a small amount of water. After drying at room temperature, slides were stained using the Toluidine Blue dye prepared in acetate buffer (pH 4.7). After 3 min, slides were washed in acetic water until excess of dye was removed. After drying, slides were fixed with Etterlan®, and later observed under a Nikon Eclipse E100 microscope coupled to Infinity 1 camera, where they were photomicrographed.

2.5. Statistical Analyses

All data were subjected to one-way ANOVA and means compared by the Tukey's Test and differences at $P \leq 0.05$ were considered to be significant. For studies of qualitative effects and regression equations for quantitative effects were estimated. Statistical analyses were performed with the SAS-9.4 program [27].

3. RESULTS

3.1. Effect of desiccation time on the moisture content, germination and growth of genipap accessions before cryopreservation

There was significant effect of interaction between accession and desiccation time on seed moisture content (mc) and germination percentage $P < 0.05$ (Table 1).

Table 1. Analysis of variance of seed moisture content (MC) and germination percentage (GE) of genipap accession seeds submitted on different desiccation times.

Source	DF	MS MC	MS GE
Accession (A)	1	92.1592**	27562.5000**
Desiccation Time (T)	3	195.5522**	2229.1667**
A * T	3	57.5130**	2229.1667**
error	16	1.6965	437.5000
VC		9.64	24.37

** Significantly different at $p = 0.01$ by F 's Test. DF- Degrees of freedom; MS- means of Sum of squares; VC- Variation Coefficient.

As show in Figure 1A, the Núcleo Bandeirante accession presented linear behaviour. At time T0, the mc was 15.48%, with the exposure to silica gel at different periods, the mc was reduced with minimum of 9.86% in 20 h. For the Umbaúba accession, the seed mc presented quadratic behaviour. At T0 treatment, the mc was 28.46 %, with reduction of the water content, the minimum mc reached was 9.0% in 20 h of exposure to silica gel. Umbaúba accession obtained 100% germination at all desiccation time tested. However, Núcleo Bandeirante accession after T0 treatment presented a drastic reduction of germination showed linear behaviour (Figure 1B).

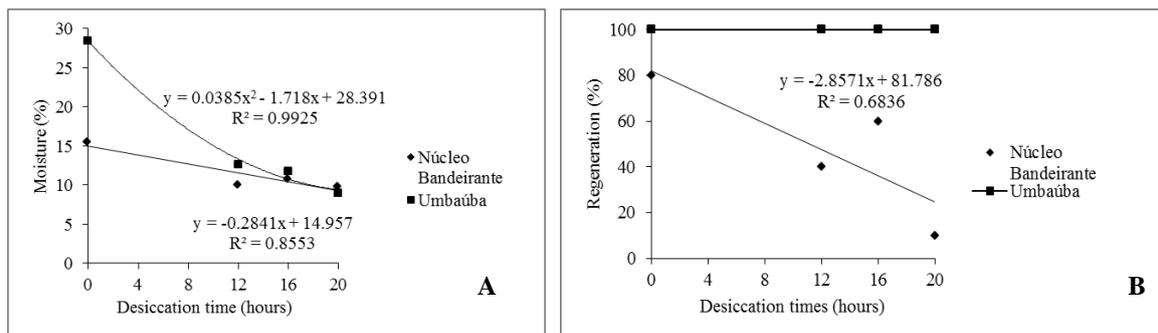


Figure 1. A- Effect of desiccation time on moisture content and regeneration of genipap accessions; B- Effect of desiccation time of seeds on the regeneration of genipap accessions.

Regarding the growth variables for Umbaúba accession, there was a significant effect of desiccation time for shoot length and number of leaves ($P < 0.05$) and no effect on root length at 60 days of *in vitro* culture (Table 2). Shoot length and number of leaves presented linear behaviour (Figures 2A-B).

For shoot length, time T0 (control) was higher with mean of 4.28 cm, with linear reduction at the different desiccation times, reaching minimum value of 1.34 cm in 20 h. For number of leaves, the same behaviour was observed with higher T0, with mean of 2.0 leaves. Considering the root length, exposure to silica gel negatively influenced the speed at which radicle protrusion occurred,

root length exhibits linear regression. At time T0, it presented mean of 5.64 cm, reaching minimum value of 2.92 cm in 20 h (Figure 2C).

Table 2. Analysis of variance of shoot length (SL), root length (RL) and number of leaves (NL) of Umbaúba accession plantlets submitted on different desiccation times before cryopreservation.

Source	DF	MS SL	MS RL	MS NL
Desiccation Time (T)	3	7.2751*	6.2613 ^{ns}	3.7833**
error	16	1.8425	3.3967	0.5250
VC		13.14	24.06	8.56

** Significantly different at $p = 0.01$ by F's Test, * Significantly different at $p = 0.05$ by F's Test; ns -not significantly; DF- Degrees of freedom; MS- Means of sum of squares; VC- Variation Coefficient

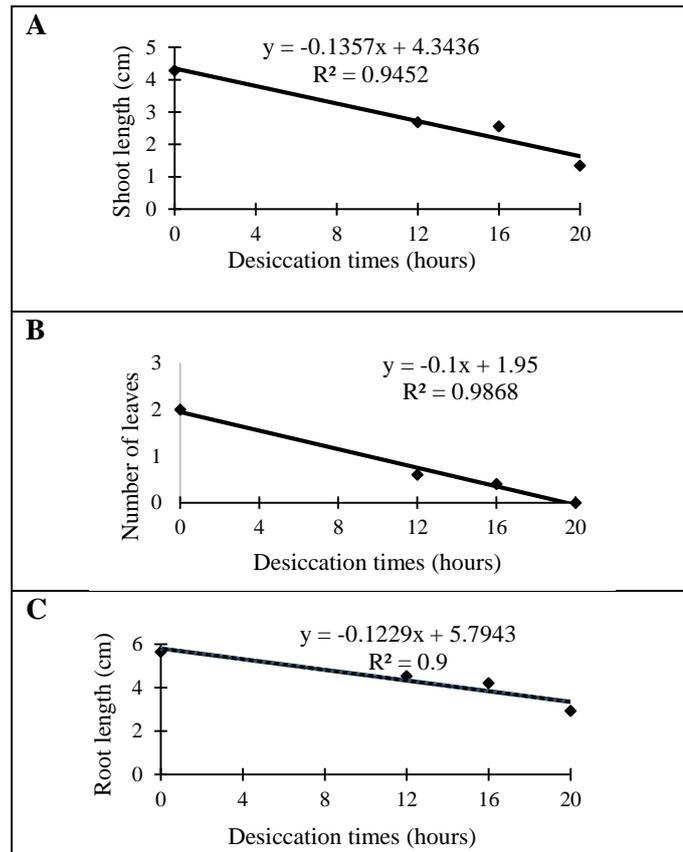


Figure 2. Effect of desiccation time on silica gel of Umbaúba accession plantlets at 60 days of *in vitro* before cryopreservation: A- shoot length; B- number of leaves and C- root length.

As show in Table 3, for Núcleo Bandeirante accession there was no significant effect of desiccation time on shoot length, root length and number of leaves at 60 days of *in vitro* culture ($P > 0.05$). Due to drastic reduction of germination percentage at time T20 (20 h) of desiccation the growth variables were not evaluated.

Table 3. Effect of desiccation time on the shoot length (SL), root length (RL) and number of leaves (NL) of Núcleo Bandeirante accession plantlets at 60 days of *in vitro* culture.

Desiccation times (hour)	SL (cm)	RL (cm)	NL
0	4.36a	4.00a	2.00a
12	4.25a	3.62a	1.75a
16	3.80a	3.34a	1.40a
CV (%)	8.05	8.03	10.14

Means in a column followed by a same letter were not significantly different at $p = 0.05$ by Tukey's Test.

3.2. Effect of desiccation time on the regeneration and growth of genipap accessions after cryopreservation

There was no significant effect of desiccation time on the regeneration percentage of cryopreserved embryonic axes at 60 days of *in vitro* culture ($P > 0.05$) for both accessions, according Table 4. The results for Umbaúba accession showed high regeneration of embryogenic axes percentage from 90 % to 100 % and for Núcleo Bandeirante accession from 80 % to 100 %.

Table 4. Effect of desiccation time on the regeneration percentage of embryonic axes of genipap accessions at 60 days of *in vitro* culture after cryopreservation.

Desiccation times (hour)	Umbaúba	Núcleo Bandeirante
0	90a	100a
12	95a	85a
16	100a	90a
20	95a	80a
CV (%) 9.11		

Means in a column followed by a same letter were not significantly different at $p = 0.05$ by Tukey's Test.

Regarding *in vitro* growth after cryopreservation (Table 5), there was a significant effect of desiccation time for shoot and root lengths and number of leaves of seedlings regenerated from embryonic axes ($P < 0.05$).

Table 5. Analysis of variance of shoot length (SL), root length (RL) and number of leaves (NL) of Umbaúba accession plantlets submitted on different desiccation times after cryopreservation.

Source	DF	MS SL	MS RL	MS NL
Accession (A)	1	37.3456**	48.1803**	34.2250**
Desiccation Time (T)	3	25.0810**	17.8175**	3.4250*
A * T	3	0.3600 ^{ns}	2.3283 ^{ns}	4.7583 ^{ns}
error	32	0.3823	1.0711	0.8125
VC		21.79	24.94	23.81

** Significantly different at $p = 0.01$ by F's Test, * Significantly different at $p = 0.05$ by F's Test; ns -not significantly; DF- Degrees of freedom; MS- Means of sum of squares; VC- Variation Coefficient

With increasing desiccation time, there was a drastic linear reduction of shoot and root lengths (Figures 3A-3C). The number of leaves varied according to a quadratic equation, with reduction after 8 hours of desiccation (Figure 3B).

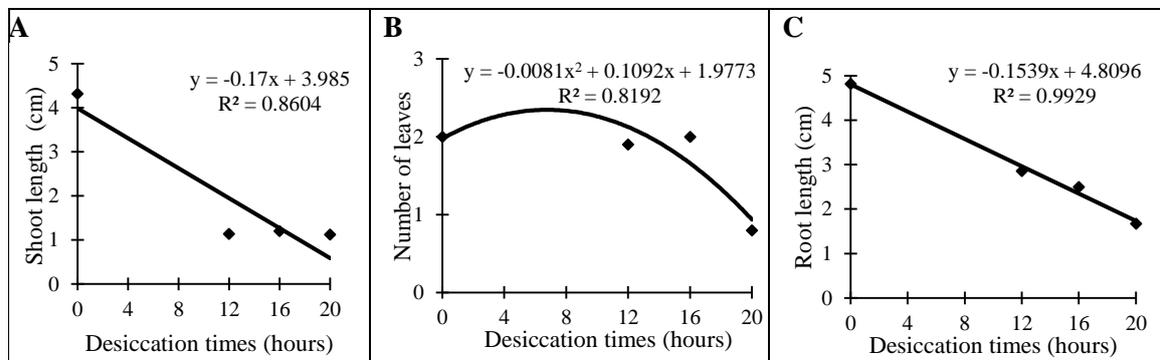


Figure 3. Effect of desiccation time on silica gel of Umbaúba accession plantlets at 60 days of *in vitro* after cryopreservation: A- shoot length; B- number of leaves and C- root length.

There were significant effects of accessions for growth variables ($P < 0.05$). Umbaúba accession showed seedlings with higher root and shoot growth and number of leaves compared to Núcleo Bandeirante accession (Table 6).

Table 6. Effect of accession on shoot (SL), root length (RL) and number of leaves (NL) of genipap embryonic axes at 60 days of *in vitro* culture after cryopreservation.

Accession	SL (cm)	RL (cm)	NL
Umbaúba	2.95a	4.06a	2.70a
Núcleo Bandeirante	1.95b	1.87b	0.75b
CV (%)	31.79	34.94	22.10

Means in a column followed by a same letter were not significantly different at $p = 0.05$ by Tukey's Test.

As complementary analysis to the effect of desiccation and cryopreservation on the embryonic axes of Núcleo Bandeirante accession, the ultrastructural analysis was performed with Electron Microscopy and Optical Microscope. The external morphology of the non-cryopreserved zygotic embryo presented highly organized cells, with intact cell wall. The embryonic axis presents cylindrical shape and no apparent lesions and rounded cotyledons with no visual damage in its structure (Figure 4).

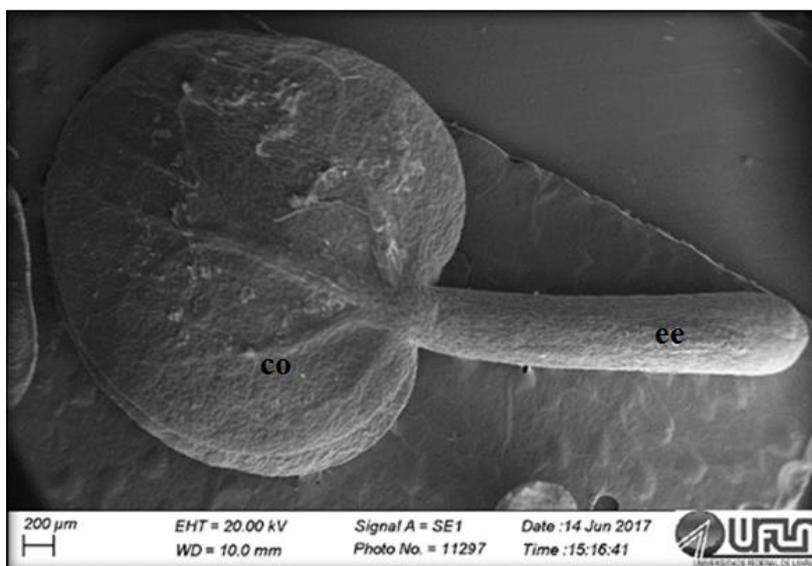


Figure 4. Zygotic embryonic structures of non-cryopreserved genipap. ryo: co-cotyledons; ee - embryonic axis in Scanning Electron Microscope.

Umbaúba accession seeds submitted to 12h of desiccation with moisture from 10% to 13% presented wilted cell structure, observing a reduction of the cell wall and an increase of the intracellular space in seeds before immersion in LN (Figures 5A-B). Cells apparently with less intracellular space may have occurred due to tissue rehydration for a period of 24 h after thawing (Figures 5C-D). In spite of twice the desiccation time in relation to the previous treatment, the moisture content reached values close to 10%, presenting the same damages shown above.

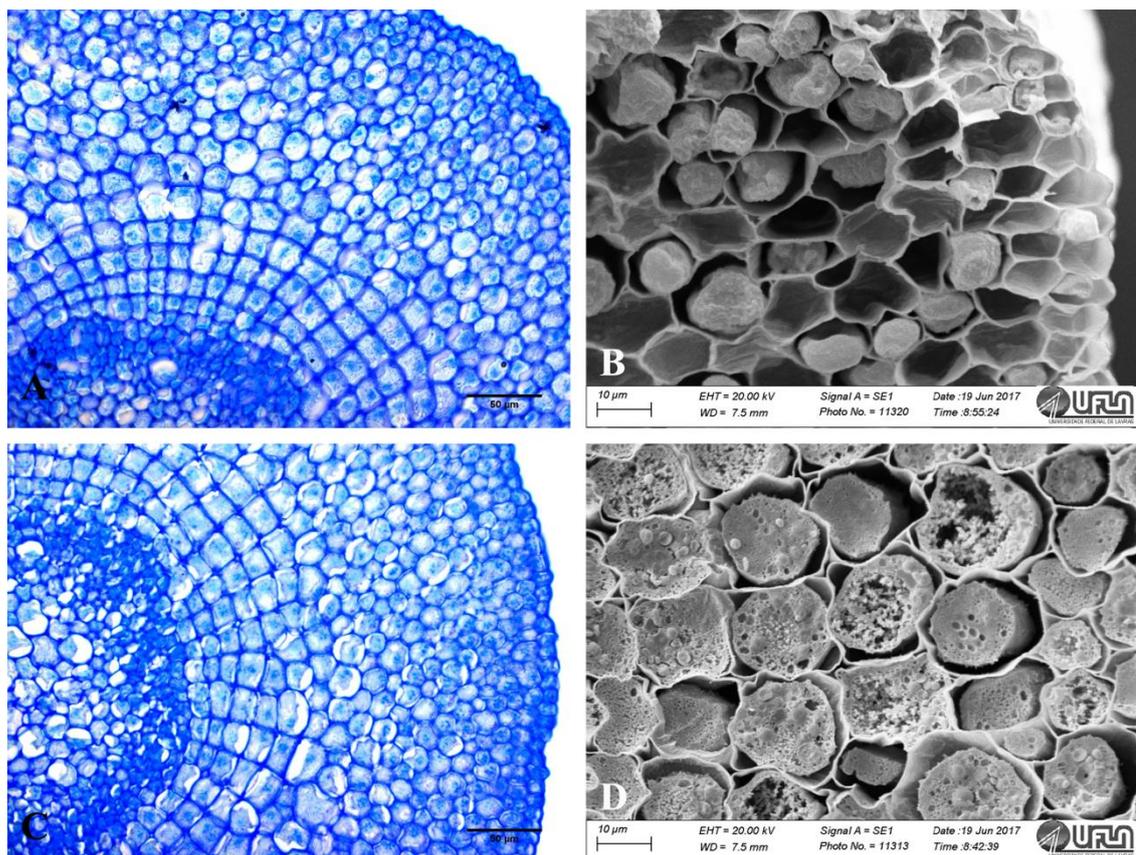


Figure 5: Cross-sectional section and scanning electron micrographs of the embryonic axis before and after cryopreservation (NL), with 12 h desiccation. A and B- embryonic axis cells before NL, C and D - embryonic axis cells after NL.

4. DISCUSSION

The desiccation in silica gel reduced the mc to close values in both accessions. Similar results were observed by Santos & Salomão (2016) [24] in genipap seeds from the Brazilian Cerrado. The initial mc of 43.89% decreased gradually, reaching minimum value of 6.79% in 36 h at different desiccation times in silica gel [24]. Silica gel provides fast drying in aseptic environment by adsorption of water in the pores of its surface [10]. The lower germination presented by Núcleo Bandeirante accession after the desiccation is probably due to the fact of its greater sensitivity to desiccation. According Spanó et al. (2011) [28] different protective mechanisms seem to act in combination but their relative importance changes as maturation progresses and in response to the drying method. There was formation of normal seedlings, with well-developed root and shoots and with no apparent damage at 60 days of *in vitro* culture in both accessions. Genipap has classification of intermediate seeds and cannot suffer drastic desiccation, tolerating approximately 10% of moisture content [5]. According to Bewley & Black (1994) [4], the mechanisms of seed desiccation tolerance are acquired after histodifferentiation and are initiated by maternal tissues and not by environmental signals.

Recently harvested and germinated of genipap seeds present high germination percentage [18; 21], losing their viability as they are dried out. The loss of water associated with the speed with which it is removed may influence the viability of genipap seeds [16], which can impair processes related to recovery, and longer time is required for rehydration [19]. Carvalho & Nascimento (2000) [5] studied the ability of genipap seeds submitted to different rapid drying procedures to tolerate desiccation, obtaining germination of 96% in fresh seeds (39.8% mc) and values higher than 50 % with moisture reduction after 24 h (10.3 % mc). However, under continuous exposure up to 72 h, seeds had mc of 6.2 % and total loss of viability. Germination rates of 98%; 91%; 87%; 68%; 35% and 10% and mc of 46.9 %; 30.5 %; 20.8%; 15.6%; 10.2% and 5.4%, respectively, were observed

by Magistrali et al. (2013) [16] when studying the effect of the drying rate on desiccation tolerance and storage of genipap seeds.

Regarding the growth variables for Umbaúba accession, there was a significant effect of desiccation time for shoot length and number of leaves. There was no effect on root length at 60 days of *in vitro* culture.

For Núcleo Bandeirante accession, there was no significant effect of desiccation time on shoot length, root length and number of leaves at 60 days of *in vitro* culture. Due to the greater sensitivity of Núcleo Bandeirante accession to moisture reduction in comparison to Umbaúba accession, seedlings have lower growth development probably due to intrinsic mechanisms of tolerance to low moisture content.

The similar regeneration percentage presented by both accessions after cryopreservation. In relation to the accessions under study, there was no significant effect of the desiccation time on the regeneration percentage of cryopreserved embryonic axes at 60 days of *in vitro* culture.

There are few published studies on genipap in long-term conservation conditions. Recently results published by Santos & Salomão (2016) [24] show after cryopreservation, embryonic axes of genipap reached high germination percentages, 93%; 96% and 93%, in seeds with 13.26%; 9.57% and 6.79% mc, respectively).

The growth responses after cryopreservation as to root length and number of leaves can be explained by greater rehydration capacity of Umbaúba accession cells after dehydration. According to Walters et al. (2001) [32], the normal functions of cells are altered when it is submitted to contents intermediate to those of total hydration. Several factors can cause damages and modifications in the physiological behavior of sensitive seeds during the desiccation process, among them maturation stage and temperature [14]. Benmahioul et al. (2015) [1] report the interaction between the water content of embryonic axes during freezing and seed viability.

Genetic-dependent responses after cryopreservation have been observed for other species. Differences in the regeneration capacity and development of embryonic axes of *Gossypium hirsutum* were reported by Lopes et al. (2013) [15]. Kaya & Souza (2017) [12] evaluated three *Saccharum officinarum* genotypes using different cryopreservation techniques of apical meristems observed variation in regeneration and growth. High genotype-dependence was also reported by Souza et al. (2016) [31] among *Ananas comosus* accessions.

Ultrastructural changes of cells were observed with decrease of mc as reduction of storage cell volume and compaction in the internal reserve content and increase the intracellular space in the endosperm. Seeds with 5.4% moisture presented uncharacterized cell morphology with loss of turgidity of cell walls. Similar results were obtained by Magistrali et al. (2013) [16] with scanning electromicrographs of genipap tissues rapidly dried up to 10.2% moisture.

5. CONCLUSION

The dehydration in silica gel can be applied to genipap accessions, considering that exposure to NL did not compromise the survival and regeneration of embryonic axes from Umbaúba and Núcleo Bandeirante seed accessions.

Desiccation in silica gel is efficient in reducing the moisture content (% mc) of Umbaúba and Núcleo Bandeirante seed accessions.

Umbaúba accession presents 100% germination at all desiccation times on silica gel before cryopreservation and Núcleo Bandeirante is more sensitive to desiccation with 10% germination in 20 h. Umbaúba accession presents a reduction of growth variables with increased desiccation time and the times tested do not affect the growth variables of Núcleo Bandeirante accession.

The desiccation time does not influence the regeneration percentage of embryonic axes of cryopreserved Umbaúba and Núcleo Bandeirante accessions.

The changes observed in the ultrastructures of the embryonic axis of genipap caused by desiccation do not affect the germination rate of cryopreserved seeds.

6. ACKNOWLEDGMENTS

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