Revista Ciência Agronômica, v. 54, e20228531, 2023
Centro de Ciências Agrárias - Universidade Federal do Ceará, Fortaleza, CE
www.ccarevista.ufc.br

Scientific Article
ISSN 1806-6690

Cryopreservation of *Genipa americana* seeds[1]

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ABSTRACT - *Genipa americana* L. is a forest species of high socioeconomic potential. However, predatory extractivism actions threaten its existence, making it necessary to adopt conservationist practices. *G. americana* seeds show sensitivity to desiccation and cooling, making it unfeasible for conservation by conventional methods. Thus, cryopreservation is an promising alternative for the long-term conservation of species that produce unorthodox seeds, such as the genipap. In this sense, the objective was to cryopreservation of seeds of *G. americana* and to evaluate the effects of desiccation and freezing on germination and establishment of seedlings. Initially, seeds were dehydrated in silica gel 0, 16, 18, 20, 22 and 24 h, and then were cryopreserved in liquid nitrogen (LN) (-196 °C) during 24 h. After thawing, the viability and germination were analyzed. Dehydrated and non-cryopreserved seeds were also analyzed. The silica gel desiccation caused a reduction in viability and germination of the seeds of *G. americana*. The initial seed water content was so high (47%) that storage in LN (+LN) without prior dehydration treatment resulted in seed mortality. It was verified that, the dehydration in silica gel for the minimum time 20 h (corresponding to 14% water content) provides greater freezing tolerance, allowing the successful cryopreservation. Silica gel dehydration followed by immersion in LN was shown to be highly efficient for cryopreservation of seeds of *G. americana*, besides germinatio after thawing, high survival rates (100%) were obtained, with growth and normal establishment of the seedlings after acclimatization.

Key words: Conservation. Dehydration. Long-term storage. Rubiaceae.

DOI: 10.5935/1806-6690.20230045
Editor-in-Chief: Prof. Salvador Barros Torres - sbtorres@ufersa.edu.br
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Received for publication 08/07/2022; approved on 16/12/2022
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INTRODUCTION

Genipa americana L. is a forest species native to South and Central America, belonging to the Rubiaceae family and shows high socioeconomic potential due to its medicinal, ornamental, wood and food attributes (NASCIMENTO *et al*., 2020; SOUZA *et al*., 2016, 2019). Furthermore, was selected by the World Bank, Global Environment Facility and Ministry of the Environment (MMA) among the highest priority species in the "Plants of the Future Program", and with the greatest potential for immediate use among native fruit trees (BRASIL, 2016; FAO, 2017). However, predatory extractivism associated with actions such as deforestation, burning and mining compromise the existence of this species, thus stimulating the adoption of conservationist practices (SOUZA *et al*., 2019).

G. americana is classified as intermediate, therefore, its seeds tolerate desiccation between 7% and 10% of water content, and cannot withstand low temperatures. In addition, they lose viability in a short period (60 days), precluding their conservation using "conventional" storage practices, such as seed banks (NASCIMENTO *et al*., 2020; OLIVEIRA *et al*., 2011; SALLA; JOSÉ; FARIA, 2016). Alternatively, cryopreservation of biological material at ultra-low temperatures (-196 ºC) using liquid nitrogen (LN) is a promising alternative for the long-term conservation of unorthodox germplasm (REN *et al*., 2022). In addition to storage without damage for an indefinite period, it allows the maintenance of genetic stability, low space requirements, absence of contaminants and low maintenance need (FOLGADO *et al*., 2014).

The use of plant material in the form of zygotic embryos and seeds is preferred and has been successfully used for cryopreservation of several species that show seeds with unorthodox behavior (FIGUEIREDO *et al*., 2021; FREITAS *et al*., 2016; PINTO *et al*., 2016). The preference for the use of these structures is attributed to the fact that it constitutes a more organized system and allows forming an entire plant, dispensing more complex stages of *in vitro* cultivation (BALLESTEROS *et al*., 2014). However, large seed size, irregular geometry and high moisture content hinder to prevent intracellular ice formation that is the main challenge of cryopreservation (WESLEY-SMITH *et al*., 2015).

Success in cryopreservation depends on the prevention of lethal damage to cell membranes and organelles. Typically, these damages are associated with water content and expansion characteristics during freezing and formation of ice crystals inside cells (REN *et al*., 2022). Thus, the adjustment of the water content inside cells, triggered by the explant dehydration before freezing, is fundamental (COELHO *et al*., 2018; PAULA *et al*., 2018).

The dehydration conferred by the material exposure to silica gel is advantageous both because it is an easy and low-cost technique and because it allows preserving the dried material directly in LN without the use of cryoprotectants that can prevent toxicity to the cells (PRADA *et al*., 2015; STEGANI *et al*., 2017). Furthermore, it is a technique that has been shown to be effective in cryopreservation of several seed species (NASCIMENTO *et al*., 2020; PINTO *et al*., 2016; SILVA *et al*., 2018). However, there is little information on the tolerance of genipap seeds to freezing (SANTOS; SALOMÃO, 2016) and there is no well-established cryopreservation protocol for this species. In this context, the aim was to establish a protocol for cryopreservation of *Genipa americana* L. seeds by dehydration in silica gel and the evaluation of the effects of desiccation and freezing on germination and establishment of seedlings.

MATERIAL AND METHODS

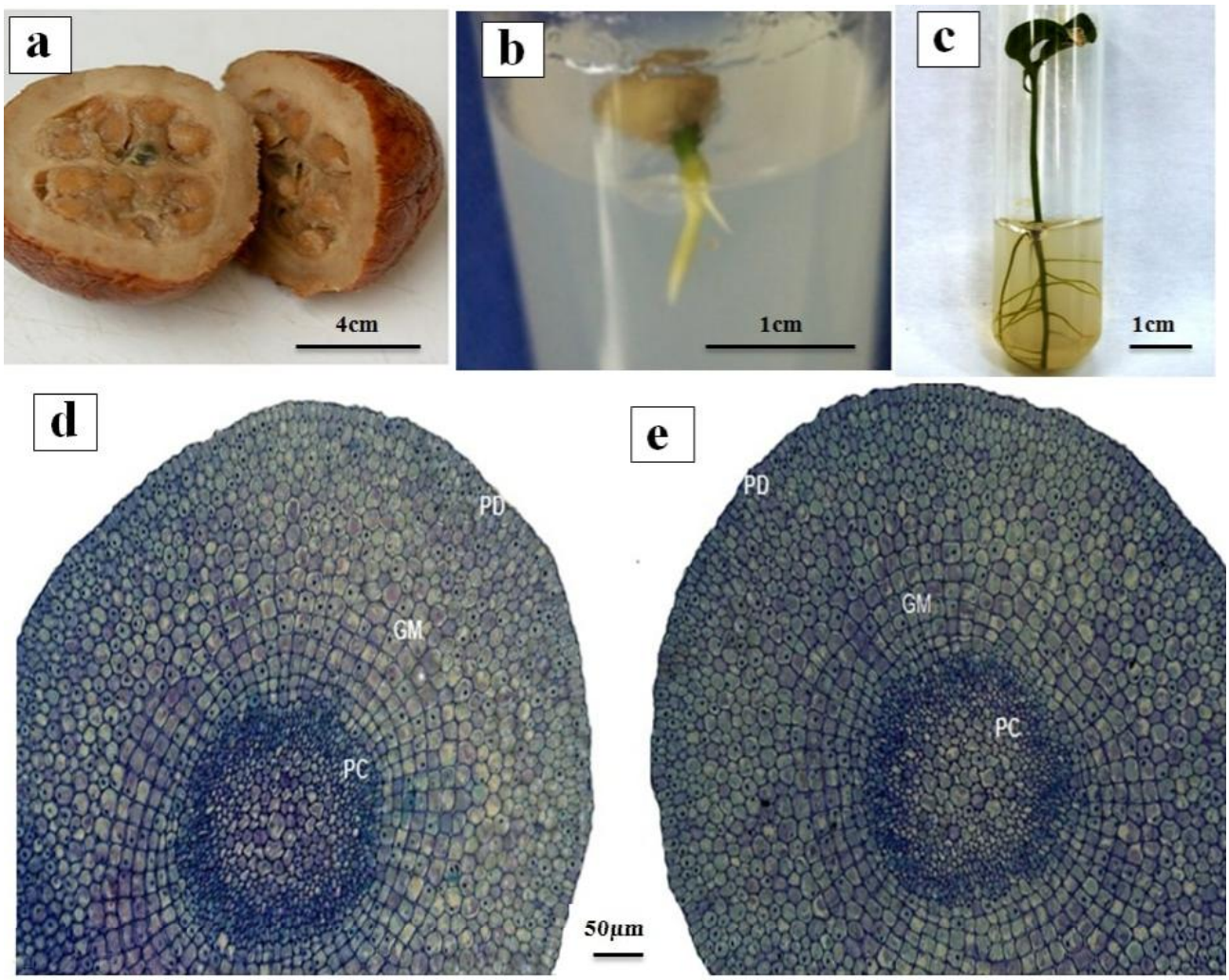
Ripe fruits of *G. americana* (Figure 1A) were pulped and the seeds were rubbed onto sieve for complete mucilage removal. For the quantification of the initial water content, three replicates consisting of 25 seeds were used, and the determination was performed by the oven drying method at 105 °C for 24 h.

After the determination of the initial water content, seeds were dried in a closed container (10.5 x 10.5 x 10.5 cm) containing 80 g of silica gel for 16, 18, 20, 22, and 24 h. Monitoring of water loss was performed at each dehydration time using the formula: MC% (Moisture content) = [(wet weight - dry weight) x 100)]/wet weight. In total, 150 seeds were used per treatment, 75 for control treatments (dehydrated at different periods and non-cryopreserved) and 75 for cryopreservation treatments. Seeds dehydrated in each period (Control, 16, 18, 20, 22, and 24 h) were separated, placed in cryotubes (2 mL) and immersed in LN for 24 h. Subsequently, they were subjected to thawing, placing the cryotubes in a water bath at 40 ºC for 2 min. After thawing, the seeds were subjected to viability and germination analyses. Dehydrated and non-cryopreserved seeds were also analyzed.

Viability after dehydration and freezing

Embryo excision and submergence in 0.5% solution of 2,3,5-Triphenyl tetrazolium chloride for 2 h in the absence of light in incubator at 30 °C (CLEMENTE; CARVALHO; GUIMARÃES, 2012) were performed. They were then washed in running water, individually observed and classified as viable or non-viable according to the formed staining. The data were transformed into $\sqrt{x}$ and expressed as a percentage of viable embryos.

Figure 1 - *Genipa americana* L.; (a) ripe fruits, (b) seed germination after 13 days of cultivation, (c) seedlings showing a normal development appearance at 45 days after cultivation, (d) histological section of embryos excised from non-cryopreservation seeds (control) medium radicle (20x magnification), (e) histological section of embryos excided from cryopreserved seeds (20x magnification). PD –protoderm; GM ground meristem; PC – Procambium



Germination

The dehydrated (-LN) and cryopreserved (+LN) seeds were disinfested in 2.5% (v/v) NaOCl solution for 20 min, inoculated in germination medium [½ MS (MURASHIGE; SKOOG, 1962) salts + 15 g L^{-1} sucrose and 0.7% agar] and stratified in the dark for 16 days. Afterwards, they were transferred to the growth room under controlled conditions of temperature (25 °C), relative humidity (70%), and photoperiod (16 h) (SOUZA *et al*., 2016). At 45 days after cultivation, germination and normal seedling percentages, shoot length (cm), and root length (cm) were evaluated. Germination was considered as the radicle protrusion accompanied by geotropic curvature, and the well-developed and morphologically perfect seedlings were considered as normal.

Histological analysis

Light microscopy was performed on histological slides of excised embryos from non-cryopreserved seeds with initial water content (Control) and cryopreserved in the best treatment. After thawing, the cryopreserved seed and control treatment embryos were excised, fixed in 70% ethanol and dehydrated in an ethanol gradient (70%, 80%, 90%, and 100%), remaining at each concentration for 1h. After dehydration, the infiltration with hydroxyethylmethacrylate plastic resin (Leica Historesin; Heraeus Kulzer, Hanau, Germany) was performed according to the manufacturer's instructions. Cross sections of 5 µm thickness were cut with a rotary microtome (Leica RM 2045) and subsequently stained in 0.1% toluidine blue (SRIDHARAN; SHANKAR, 2012). Sections were

observed at 20x magnifications and images were captured digitally using a microscope with a video camera coupled to a computer executing the software IM50 (Leica microsystem).

Acclimatization

At 60 days after cultivation, seedlings regenerated from non-dehydrated (control) and cryopreserved seeds (best treatment) (+LN) were removed from the test tubes and their roots washed in running water. They were then transferred to polypropylene containers (300 ml) filled with commercial substrate (Tropstrato hp®) and covered with clear plastic in order to avoid excessive moisture loss from the seedlings after transfer from *in vitro* to *ex vitro* environment. Cuts were made every five days in each plastic bag until complete withdrawal at 15 days. The seedlings were kept for 14 days in a growth room with controlled temperature of 25 ± 2 °C and photon irradiance of 67 μm m^{-2} s^{-1}. They were then transferred to greenhouse with a 30% shading screen. At 30 days of acclimatization, the percentage of surviving plants, shoot and root length (cm), and number of leaves were evaluated.

Experimental design and statistical analyses

The experimental design was completely randomized with treatments distributed in a 2x6 factorial design related to storage in LN (non-cryopreserved (-LN) and cryopreserved in LN (+LN)) and dehydration periods in silica gel (0, 16, 18, 20, 22, and 24 h). The obtained data were submitted to analysis of variance (ANOVA) and and, when significant, to an "F" test ($P < 0.05$) and then averages were compared by Skott-Knott test ($p > 0.05$).

RESULTS AND DISCUSSION

The time of exposure of the seeds in silica gel caused a gradual reduction in the moisture content of the seeds of *G. americana*, resulting in reduction from 47% (0 h of desiccations) to 11% of water content at 24 h of desiccation (Table 1).

The time of exposure of the seeds to silica gel, besides causing a decreased in the water content, also caused a reduction in the viability and germination potential of seeds. This resulted in a reduction of approximately 23% in the viability and germination of the seeds when desiccated for 24 h (Table 1). Thus, the longer the desiccation time on silica the lower the viability and germination of *G. americana* seeds.

Similarly, evaluated the behavior of dehydrated *G. americana* seeds in saturated solution of sodium chloride and silica gel, Salla, José and Faria (2016) verified that desiccation at a water content of approximately 15% resulted in a reduction in viability and germination. However, the available information regarding the tolerance to desiccation of *G. americana* seeds is conflicting. For some authors (CARVALHO; NASCIMENTO, 2000; SANTOS; SALOMÃO, 2016), seeds tolerate desiccation at low water content (approximately 5%) without loss of viability and germination potential.

The variation of responses in the different studies may be related to several factors that directly interfere in the behavior and degree of tolerance to desiccation, such as: maturity stage of the seeds, employed method, intensity and duration of drying (NASCIMENTO *et al*., 2020). Furthermore, the seed variability and its complex structure with very heterogeneous cell composition can result in sensitivity and unequal drying rates within the same seed batch (BALLESTEROS *et al*., 2014; SAHU *et al*., 2017).

Despite the reduction in the water content caused decreased in seed viability and seed germination, it is noted that the dehydration process was essential for survival after thawing (Table 1). Whereas, the initial water content (47%) of *G. americana* seeds was so high that storage in LN (+LN) without previous

Table 1 - Effect of dehydration in silica gel on water content (%), viability (% ± SE) and germination (% ± SE) of *Genipa americana* L. seeds cryopreserved (+LN) or not (-LN)

Desiccation period	Seed water content (% ± SE)	Viability (% ± SE)		Germination (% ± SE)	
		-LN	+LN	-LN	+LN
Control	47 ± 0.57 a	97.6 ± 2.2 aA	0.0 ± 0.0cB	83.3 ± 8.3 aA	0.0±0.0 cB
16 h	17 ± 1.66 b	69 ± 2.7 bA	19.3 ± 10 bB	71.7 ± 4.6 bA	0.4 ± 0.3 cB
18 h	15 ± 1.66 b	60.6 ± 5.5 bA	30.3 ± 2.7 aB	64.3 ± 5.6 bA	0.8 ± 0.1 cB
20 h	14 ± 1.15 b	57 ± 1.6 bA	38.6 ± 5.5 aB	60.1 ± 2.3 bA	35 ± 1.3 aB
22 h	13 ± 0.88 b	57 ± 1.4 bA	30.3 ± 2.7 aB	72.2 ± 2.7 bA	24.0 ± 0.9 bB
24 h	11.3 ± 1.33 b	58 ± 9.6 bA	30.3 ± 2.7 aB	68.0 ± 3.6 bA	21.9 ± 0.3 bB

Data were expressed as average ± standard errors (SE). Averages followed by the same capital letter on the column and lowercase on the line do not differ among themselves by Scott-Knott test ($p < 0.05$)

dehydration treatment resulted in total seed mortality. The high water content in the cells during freezing favors the formation of ice crystals, causing lethal damage to the membranes and hence leading to cell death (LIMA; DUTRA; CAMILO, 2014; REN *et al*., 2022). Therefore, to avoid damage caused by freezing and desiccation, the adjustment of the amount of water present in explants before immersion in LN is fundamental (FIGUEIREDO *et al*., 2021; NINAGAWA *et al*., 2016).

The maximum desiccation period estimated to obtain maximum percentages of viability and germination after thawing (+LN) was 36 h (Table 1). The results also demonstrate that the drying of *G. americana* seeds to a maximum content of 10% and a minimum of 14% is essential to obtaining better rates of viability, germination and formation of normal seedlings after storage in LN (Table 1 and 2). Since the drying provided by the exposure of seeds to silica contributed to the occurrence of the vitreous state, resulting from the increase in cytoplasmic viscosity and low cell mobility, conferring better tolerance to freezing (BALLESTEROS *et al*., 2014; REN *et al*., 2022).

The percentage of normal seedlings obtained from cryopreserved seeds (+LN) showed a linear behavior as a fuction of the desiccation period (Table 2). Aditionally, the desiccation of seeds in silica gel for 22 h before immersion in LN allowed the successful cryopreservation of *G. americana* seeds, contributing to obtain normal seedlings with similar rates among cryopreserved and non-cryopreserved seeds (Table 2).

Seedlings obtained from cryopreserved seeds (+LN) compared to non-cryopreserved seeds (-LN) showed reduced growth with lower height and root length (Tabela 2). Freezing induces a series of stresses on the plant material, making it susceptible to modifications in the ultrastructural organization of cells and subsequent growth (GALDIANO *et al*., 2012). However, it is important to note that the pretreatment of dehydration allowed an increase in growth parameters on seedlings obtained from cryopreserved seeds (Tabela 2).

Although the seedling obtained from cryopreserved seeds showed slow and reduced growth was observed that after acclimatization the seedlings showed 100% survival in and no differences were observed in relation to the growth between regenerated seedlings of cryopreserved and non-cryopreserved (Figure 2).

In studies on plant cryopreservation, only the evaluation of cell survival after freezing is performed. However, in order to obtain an efficient cryopreservation protocol, the viability of the material subjected to freezing in LN should be referred as the recoverability of the largest amount of living cells that provide the regeneration and recovery of individual's normal growth. Consequently, higher survival rates of seedlings and the establishment in greenhouses and field are expected, besides maintaining genetic integrity (FIGUEIREDO *et al*., 2021; LI *et al*., 2016).

The observation of histological sections of embryos excised from cryopreserved seeds revealed intact protoderm cell contents and without the presence of large intercellular spaces, with characteristics similar to embryos excised from non-cryopreserved seeds (Figure 1D-E), indicating high cell recovery capacity after cryopreservation of *G. americana* seeds.

Dehydration in silica gel followed by immersion in LN is a simple, easy and low cost technique that proved to be efficient for cryopreservation of *G. americana* seeds, besides allowing germination after thawing, high survival rates, with growth and normal establishment of seedlings after acclimatization.

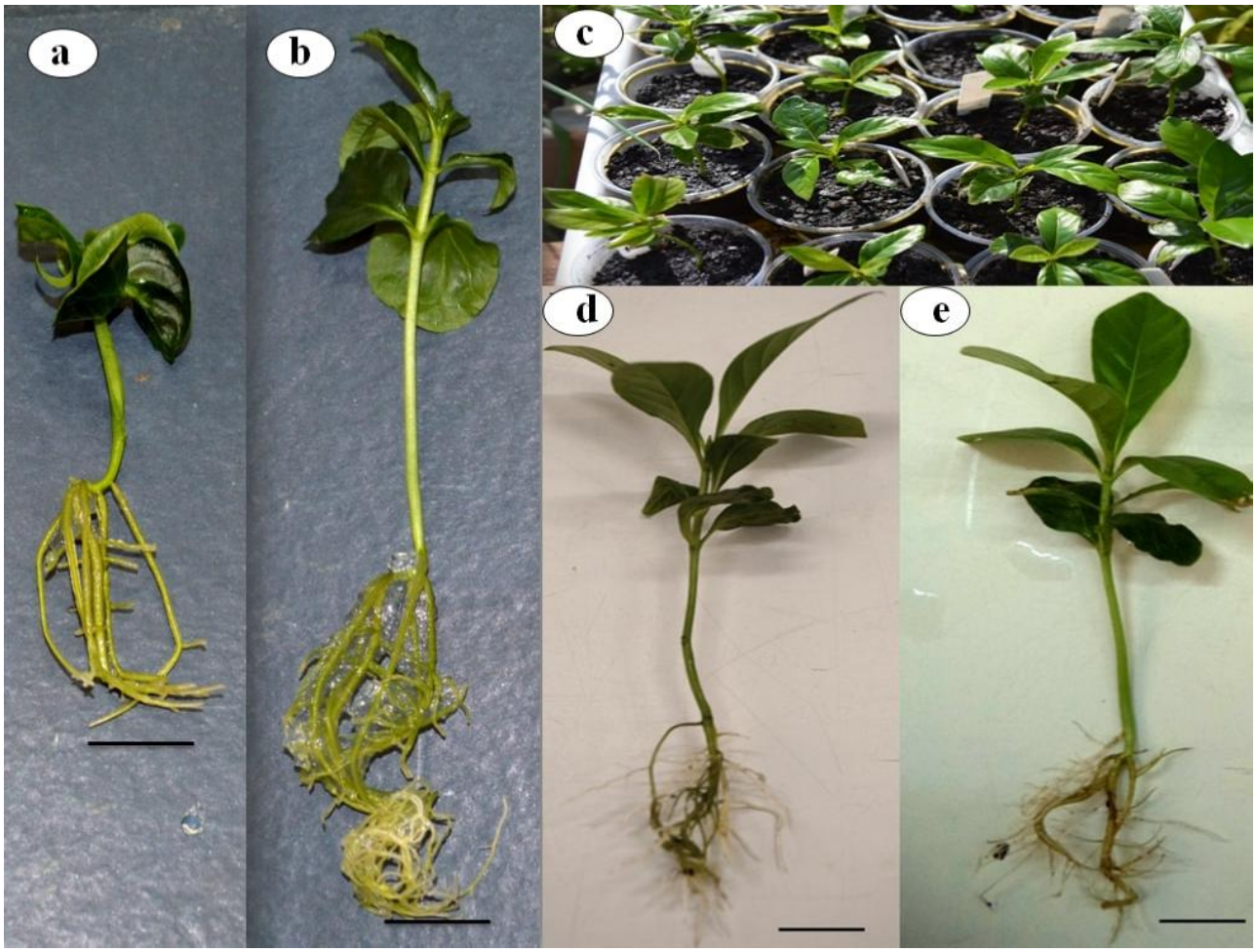
The results demonstrate that the cryopreservation of *G. americana* seed emerges with an important species conservation strategy, due to the small space required for the technique, low maintenance cost, low labor costs, long-term storage and high survival rates.

Table 2 - Effect of dehydration in silica gel on the percentage of normal seedlings (% ± SE), height (cm ± SE), and root length (cm ± SE) of regenerated seedlings of *Genipa americana* L. cryopreserved (+ LN) or not (-LN)

Desiccation period	Normal seedlings (% ± SE)		Height of seedlings (cm ± SE)		Root length (cm ± SE)	
	-LN	+LN	-LN	+LN	-LN	+LN
Control	100 ± 0.0 aA	0.0 ± 0.0 dB	6.2 ± 0.0 aA	0.0 ± 0.0 cB	4.14 ± 0.005 dA	0.0 ± 0.0 eB
16 h	100 ± 0.0 aA	0.0 ± 0.0 dB	5.4 ± 0.13 bA	0.0 ± 0.0 cB	4.68 ± 0.03 bA	0.0 ± 0.0 eB
18 h	100 ± 0.0 aA	100 ± 0.0 aA	5.1 ± 0.12 bA	2.7 ± 0.04 bB	4.61 ± 0.04 bA	3.97 ± 0.01 cB
20 h	92.3 ± 0.6 bA	94.7 ± 0.5 aA	4.9 ± 0.13 cA	3.0 ± 0.05 aB	4.95 ± 0.01 aA	4.51 ± 0.06 aB
22 h	91.6 ± 3.9 bA	75.0 ± 1.5 bB	5.1 ± 0.10 bA	3.1 ± 0.06 aB	4.96 ± 0.02 aA	3.70 ± 0.02 dB
24 h	87.5 ± 0.5 cA	60 ± 0.7 cB	4.9 ± 0.10 cA	3.1 ± 0.09 aB	4.50 ± 0.02 cA	4.16 ± 0.08 bB

Data are expressed as average ± standard errors (SE). Averages followed by the same capital letter on the column and lowercase on the line do not differ among themselves by Scott-Knott test ($p < 0.05$)

Figure 2 - *Genipa americana* L. seedlings after 60 days of cultivation from cryopreserved (a), non cryopreserved seeds (b), *ex vitro* acclimatization (c), seedlings after acclimatization, showing normal appearance and without phenotypic changes from cryopreserved (d) and nono-cryopreserved (e) seeds. Bar: 1cm



CONCLUSION

The Dehydration in silica gel for the minimum time 20 h (corresponding to 14% water content) and a maximum of 36 h (content of 10%) allows *G. americana* seeds being successfully cryopreserved, allowing germination after thawing high survival rates (100%), with growth and normal establishment of seedlings after acclimatization.

ACKNOWLEDGMENTS

The authors kindly acknowledge the National Council for Scientific and Technological Development (CNPq), the Coordination for the Improvement of Higher Education Personnel (CAPES), and the Research Support Foundation of the State of Minas Gerais (FAPEMIG) for the financial support of this study

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