

## Research Article

# Viability Assessment of *Genipa americana* L. (Rubiaceae) Embryonic Axes after Cryopreservation Using *In Vitro* Culture

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Embryonic axes excised from seeds of *Genipa americana* L. desiccated to different water contents were successfully cryopreserved by rapidly plunging seed samples directly into liquid nitrogen. Control and cryopreserved embryonic axes were excised and grown in WPM culture medium for viability assessment. All control embryonic axes ( $-LN_2$ ) excised from fully hydrated seeds (43.89% moisture content) germinated after 21 days of culture *in vitro*. These high germination percentages persisted even after the water content of the seeds was as low as 6.79%. After freezing in liquid nitrogen high germination percentages, 93%, 96%, and 93%, were observed for embryonic axes excised from seeds dehydrated to 13.26%, 9.57%, and 6.79% moisture content, respectively. The cryopreservation technique described here is recommended for long term conservation of *G. americana* germplasm.

## 1. Introduction

*Genipa americana* L., Rubiaceae family, is a large tree species that occurs throughout Brazil in different vegetation types, especially in humid ecosystems. It is traditionally used for many purposes. The pulp of mature fruits contains expressive amounts of proteins (12%), carbohydrates (14%), fibers (6.3%), vitamin C (26 mg/100 g), and phenolic compounds (176.3 mg EAG/100 g) and is used to prepare juice, ice cream, preserves, and liqueur, as well as a syrup used for the treatment of bronchitis and asthma [1]. A dark blue pigment and tannins are extracted from green fruits for industrial application [2, 3]. The blue pigment is used in body painting by indigenous peoples. The wood (density 0.68 g/cm<sup>3</sup>) is used for naval and civil construction and carpentry works and in making of containers for storage and transport of wine. The tree is widely used in reforestation programs and for urban arborization of plazas and parks throughout the country [4].

This species is not yet domesticated and is undergoing genetic erosion due to human activities, such as elimination of native populations due to expansion of agricultural and urban frontiers [4] and predatory harvest of fruits and wood which compromise the natural regeneration of its native

populations. As a result, it is vital to promote research efforts towards the establishment of conservation strategies for *G. americana* germplasm to ensure maintenance of the existing genetic diversity, sustainable crop production, and utilization of germplasm for crop improvement.

Desiccation of seeds (5–7% moisture content) and storage in cold rooms at  $-20^{\circ}C$  are usually the conventional storage approach for *ex situ* long term conservation of plant germplasm, since seeds are the natural propagation unit for the majority of plant species [5]. However, seeds of numerous species are damaged and lose viability if stored under these conditions. Such seeds are classified as recalcitrant or intermediate, depending on the level of susceptibility to desiccation or exposure to subzero temperatures, or both [6, 7]. *G. americana* seeds present intermediate behavior under conventional storage conditions; that is, they tolerate reduction of their water content (10% range) without significant decrease in germination percentages but lose viability gradually when dry seeds are stored at  $-20^{\circ}C$  for extended periods of time [3, 8]. Therefore, conservation of *G. americana* seeds using the conventional seed conservation technique, routinely used in genebanks around the world, is not recommended and alternative methods must be attempted.

Progress in biotechnology has provided alternative techniques for conservation of genetic resources of plant species that have recalcitrant or intermediate seeds or that are vegetatively propagated. The possibility of obtaining whole plants from isolated cells, organs, and plant tissues using *in vitro* culture techniques has led to the establishment of *in vitro* genebanks that ensure conservation of active plant cultures of elite genotypes and breeding lines under slow growth conditions or of cryobanks, in which samples are stored in liquid nitrogen at  $-196^{\circ}\text{C}$  [9]. Freezing and storage of plant germplasm at the temperature of liquid nitrogen allow the conservation of biological material for many decades ensuring high viability and genetic stability, having been considered the most promising method for the long term conservation of cells, tissues, and plant structures, from which whole plants can be obtained [10]. Over the last few decades reports of successful cryopreservation of numerous tropical species have been published [5, 11]. Different techniques have been used in each case and a variety of plant cells and tissues have been used, including protoplasts, cell suspensions, callus, lateral buds, shoot tips, meristems, whole seeds, somatic and zygotic embryos, and biotechnological products [12]. However, to the best of our knowledge there has been no previous report of cryopreservation of *G. americana* seeds in the literature and the present work was undertaken with the main objective of evaluating the effect of dehydration and storage in liquid nitrogen ( $-196^{\circ}\text{C}$ ) on the viability of zygotic embryonic axes of *G. americana*, as a step towards the development of a protocol for long term conservation of this species.

## 2. Materials and Methods

**2.1. Plant Material.** Mature fruits of *Genipa americana* L. were collected from trees growing in Brasília, Federal District, Brazil, and taken to the Cryobiology Laboratory at Embrapa Genetic Resources and Biotechnology, where they were manually processed to extract the seeds (Figures 1(a) and 1(b)). Seeds were rinsed thoroughly under running tap water, soaked in water containing a few drops of commercial grade detergent, rinsed a couple of times and then soaked in a commercial solution of sodium hypochlorite (2.0% available chlorine) for 5 minutes, rubbed against a metal sieve until all pulp residue was removed, and then rinsed thoroughly under running tap water. Seeds were then spread out over a single layer on germination paper sheets and allowed to dry at room temperature ( $25 \pm 2^{\circ}\text{C}$ ) for 24 hours. Dried seeds were kept in brown paper bags, at room temperature ( $25 \pm 2^{\circ}\text{C}$ ) until use.

**2.2. Determination of Moisture Content of Seeds.** For the determination of the moisture content (mc) of the seeds, three samples of 15 seeds were weighed, their initial weight was recorded, and they were transferred and maintained overnight (18 hours) in a dry heat oven at  $105 \pm 3^{\circ}\text{C}$ . Subsequently they were weighed again to obtain the dry weight of the sample. The mc of seeds was calculated using the initial and dry weight values obtained and expressed on a fresh weight basis (FWB).

**2.3. Excision of Embryonic Axes and In Vitro Culture.** Batches of seeds were transferred to a glass jar and washed with commercial grade detergent for five minutes followed by thorough washing in running tap water. Superficial decontamination of the seeds was carried out by submerging them in 200 mL of a commercial sodium hypochlorite solution (2.0% available chlorine, v/v) plus 4-5 drops of Tween 20, under agitation, using an orbital horizontal shaker, for 15 minutes. In a laminar flow hood seeds were rinsed three times with distilled sterilized water and then kept in 5.0 mL of sterilized water for 48 hours to soften the integument and facilitate embryo excision (according to [13]). After 48 hours of soaking, seeds were cut open and embryonic axes were excised aseptically from seeds and transferred to glass culture tubes containing 10.0 mL of WPM culture medium [14] for the evaluation of their viability. Test tubes containing one embryonic axis each were cultured in a growth room at  $25 \pm 2^{\circ}\text{C}$ , with a 16-hour photoperiod and a photosynthetic photon flux (PPF) of  $62 \mu\text{mol}/\text{m}^2/\text{s}$ . This technique was used to evaluate viability of embryos excised from seeds subjected to desiccation treatment alone or desiccation treatment followed by freezing in liquid nitrogen, which is described below. Three batches of fifteen embryonic axes were used in all germination tests conducted after the desiccation and freezing conditions tested in this study.

**2.4. Desiccation Treatment and Germination Test.** In order to assess the desiccation tolerance of the embryos, seeds were subjected to desiccation over silica gel by placing them over the metal screen of germination boxes containing 50.0 g of blue silica gel for 0 (control), 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 18, 24, and 36 hours, to obtain seed lots with fourteen different mc. The boxes were sealed and kept at room temperature ( $25 \pm 2^{\circ}\text{C}$ ) throughout the desiccation treatment. The mc of seeds was determined using the oven method, described previously. The moisture content values obtained were used to build the desiccation curve. After each desiccation period three samples of fifteen seeds were taken to conduct germination tests. Superficial decontamination of the seeds, embryonic axes excision, and *in vitro* culture were carried out as described above.

**2.5. Cryopreservation and Thawing Treatments.** Three samples of fifteen seeds desiccated to different mc (1–36 hours over silica gel) or fresh control (nondesiccated) were transferred to trifoliate envelopes and heat sealed using electric heat sealer. Seeds were cooled rapidly by direct immersion of the packets in liquid nitrogen ( $\text{LN}_2$ ), at  $-196^{\circ}\text{C}$ , at a cooling rate of approximately  $-263^{\circ}\text{C}/\text{min}$ . Samples were stored in  $\text{LN}_2$  overnight (18 hours). For thawing, envelopes were retrieved from  $\text{LN}_2$  and rapidly submerged in a water bath at  $40 \pm 2^{\circ}\text{C}$ , under agitation, for three minutes.

After warming, three samples of fifteen seeds were taken to conduct germination tests. Superficial decontamination of the seeds and embryonic axes excision were carried out exactly as described above. Embryonic axes *in vitro* culture was essentially the same as described earlier, except that cryopreserved embryonic axes were kept in darkness for 48 h

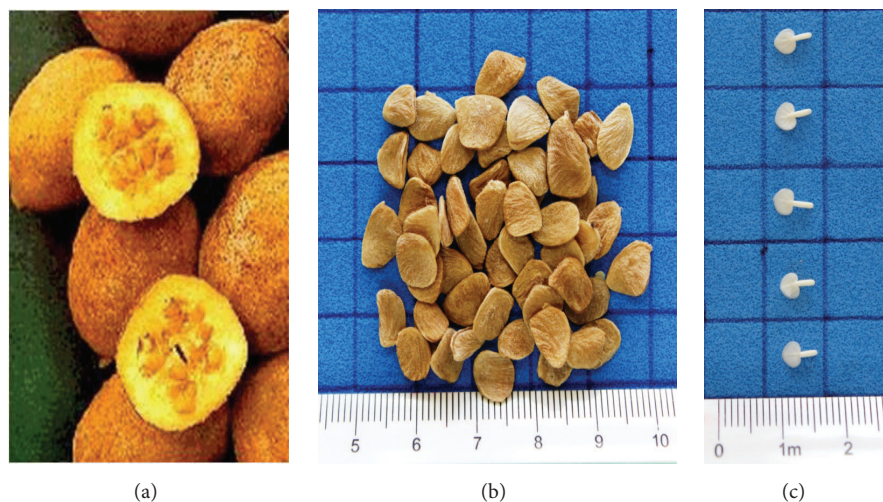


FIGURE 1: Fruits, seeds, and embryonic axes of *Genipa americana* L.: (a) mature fruits (6–8 cm in diameter); (b) sample of whole seeds ready for use, after they were extracted from mature fruits and dried overnight at room temperature (average size:  $8.5 \times 6.4 \times 2.0$  mm; average weight: 0.096 g); and (c) embryonic axes ready for inoculation *in vitro* after they were excised from seeds in aseptic conditions in the laminar flow cabinet (average size: 3.0–5.0 mm, on average).

prior to exposure to the regular light conditions specified above. Embryonic axes excised from seeds that were not exposed to  $LN_2$  were used as fresh controls.

**2.6. Survival Assessment.** Germination of embryonic axes, plantlet recovery, and presence of contaminants were evaluated daily up to one week and then weekly for up to three months. Only the embryonic axes that produced normal seedlings with well-developed apical shoots and root systems formed by a central pivoting root and 3-4 secondary lateral roots were considered as viable and scored. The cultures were kept under the growth conditions described above for at least three months before being discarded.

**2.7. Statistical Analysis.** Experiments were carried out on a completely randomized design. Means of percentages of embryonic axes germination data were subjected to analysis of variance (Two-Way ANOVA) followed by Bonferroni posttest using GraphPad Prism statistical software package. All statistical analysis was performed at the 0.05% probability. Moisture content data were subjected to second-order polynomial regression.

### 3. Results and Discussion

Seeds of *G. americana* are irregularly obovate to round, with rounded edges, a thin and horny seed coat, measuring on average  $8.5 \times 6.4 \times 2.0$  mm and weighing on average 0.096 g (Figure 1(b)). The embryonic axes are shaped like a spatula (Figure 1(c)) and the cotyledons are juxtaposed and oval shaped in frontal view (Figure 1(c)). Normal seedlings show an axial and robust taproot with a few well-developed secondary roots, a reduced and initially indistinguishable plumule, and oblong membranous cotyledons with short petioles, which are bright green with a conspicuous midrib, as shown in Figure 4(b).

Under the conditions described in this research 100% of embryonic axes excised from untreated seeds and cultivated *in vitro* on WPM medium germinated and germination were completed within a week after planting (Figure 3,  $-LN_2$ ). After three days of culture *in vitro* embryonic axes which were completely white showed evidence of growth, greening, and expansion of the cotyledons. After fourteen days of culture seedlings obtained were fully green and were approximately 3.0 cm tall, showing vigorous growth of the root system and full expansion of cotyledons (Figure 4(a)). In contrast, under greenhouse conditions 85% of *G. americana* seeds germinated and onset of germination, evidenced by the rupture of the seed coat and protrusion of the radicle and the hypocotyls, started 8 to 13 days after planting in soil or vermiculite and was maintained at 25°C, 30°C, or 35°C, constant temperature [15]. However, under these conditions germination was asynchronous and slow, taking up to 70 days to be completed [15]. Germination of *G. americana* seeds was significantly improved using *in vitro* techniques by synchronizing germination of the embryonic axes and reducing the time required to complete the process. Viability results were scored considering as a normal seedling only those that presented a robust root system consisting of a central root and a few lateral roots and a well-developed plumule (Figure 4(b)). Fully grown seedlings with 4–6 pairs of leaves were obtained after four weeks of *in vitro* culture (Figure 4(d)).

**3.1. Effect of Dehydration on Control Embryonic Axes Viability ( $-LN_2$ ).** Moisture content of the seeds was determined by the oven method in which seed samples were weighed and then transferred to a drying oven at  $105 \pm 2^\circ C$ , for 24 hours. The mc of fully hydrated seeds (control) was 43.89% (Figure 2), and 100% germination was observed when embryonic axes excised from these seeds were grown *in vitro* on WPM culture medium (Figure 3).

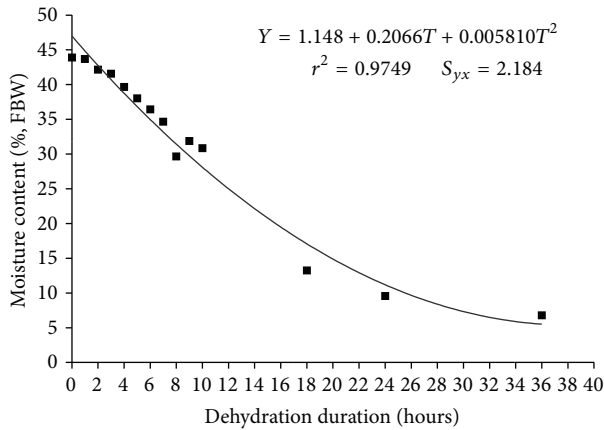


FIGURE 2: Desiccation curve for *Genipa americana* L. seeds. Initial moisture content of seeds was 43.89%. Moisture content data were subjected to second-order polynomial regression using GraphPad Prism statistical software package.

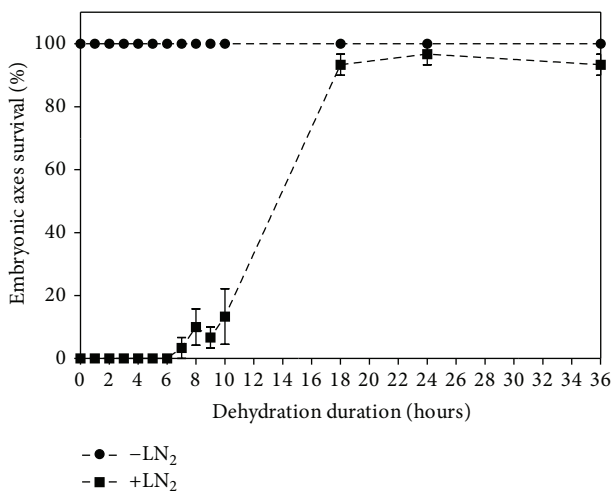


FIGURE 3: Viability of *Genipa americana* L. embryonic axes after desiccation and/or cryopreservation. Embryos were excised from seeds desiccated to different water contents (●, -LN<sub>2</sub>) or seeds that were desiccated and subsequently cryopreserved overnight (918 hours) in liquid nitrogen (■, +LN<sub>2</sub>). Germination data was analyzed using analysis of variance (Two-Way ANOVA); mean comparison was carried out using Bonferroni test. *P* value < 0.0001. Bars represent SEM.

Seeds exposed to desiccation treatments over silica gel for different periods of time lost water gradually and after 36 hours of desiccation their mc dropped to 6.79% (Figure 2). Compared to control seedlings, it was observed that desiccation of whole seeds to 9% or higher mc generally did not compromise germination and vigor of embryonic axes (Figure 4(a), -LN<sub>2</sub>). However, when the mc reached 6.79% (36 hours of dehydration) embryonic axes developed into seedlings with abnormal root systems (Figure 4(d)). In native vegetation *G. americana* usually occurs in temporarily or permanently flooded areas which could account for the observed sensitivity of the root system to desiccation [15].

Progressive loss of vigor and viability of whole seeds of *G. americana* desiccated to moisture contents ranging from 42.0 to 4.0% and stored at various temperatures above (5°C, 10°C, and 15°C) and below zero (-18°C and -20°C) for up to one year was reported previously [3, 8, 16]. According to de Carvalho and do Nascimento [3] drying seeds up to 10.3% moisture content had no negative effect on germination percentage after storage at subzero temperatures, but upon further drying to 8.4% or below, the germination decreased steeply and drying them to 4.2% killed the seeds and no germination was obtained. These results reported confirm that seeds of this species show intermediate behavior when they are dehydrated and stored at low or subzero temperatures. Similar deleterious dehydration effect was previously reported for intermediate seeds of *Magnolia ovate* [17]. However, for *Coffea canephora* seeds, which are also intermediate, desiccation had no negative effect on the physical quality of seeds and on radicle protrusion rate and morphology [18].

Therefore, to ensure long term conservation of seeds of this species it is necessary to apply different conservation methodologies. One of the most reliable methods for long term conservation of plant germplasm currently available is cryopreservation in liquid nitrogen, at -196°C.

**3.2. Effect of Cryopreservation on Embryonic Axes Viability (+LN<sub>2</sub>).** In the present work viability of embryonic axes of *Genipa americana* excised from seeds desiccated to different moisture contents and subsequently immersed in liquid nitrogen was evaluated. Embryonic axes isolated from fully hydrated seeds (43.89% mc) that were immersed in LN<sub>2</sub> lost viability completely and did not germinate, so desiccation of seeds before freezing was essential for allowing embryonic axes survival after freezing (Figure 3, +LN<sub>2</sub>). Embryonic axes isolated from seeds desiccated to mc between 29.67 and 6.79% germinated after storage at -196°C, and as the mc decreased, a gradual increase in germination percentage was observed (Figure 3, +LN<sub>2</sub>). Highest germination percentages, ranging from 93% to 96%, were obtained for embryonic axes isolated from seeds dried to 13.26% and 9.57% mc, respectively (Figure 3, +LN<sub>2</sub>). However, some differences in seedlings growth rate, vigor, and morphological aspect were observed and apparently were correlated to the mc of the seeds. Embryonic axes development was slower for cryopreserved seeds with lower mc, in comparison with development of seedlings originated from embryonic axes excised from seeds that were dehydrated but that were not exposed to liquid nitrogen (Figure 4(a)). Additionally, some of the seedlings showed abnormal development of the plumule and this malformation was probably caused by desiccation and freezing, Figures 4(c) and 4(d), respectively.

Cryopreservation of whole seeds and embryonic axes rescue using *in vitro* culture had been reported previously for plant species whose seeds exhibit intermediate storage behavior, such as coffee, citrus, and *Nothapodytes nimmoniana* [13, 19, 20]. The success of a cryopreservation protocol depends on many factors which can affect cell viability following exposure to LN<sub>2</sub>. The amount of freezable water present in the cells is a key factor influencing cell survival. Typically plant cells are highly hydrated and contain high amounts of water and

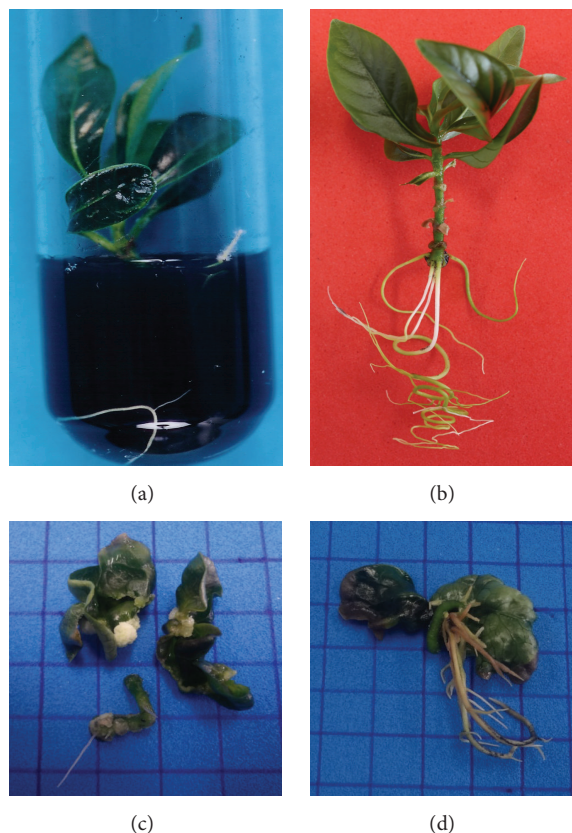


FIGURE 4: Seedlings obtained from *G. americana* L. embryonic axes after 30 days of growth *in vitro*. Normal seedlings obtained from embryonic axis that was desiccated to approximately 13% of the initial mc (a) or embryonic axis that was desiccated and cryopreserved in liquid nitrogen (b). Abnormal seedlings showing desiccation damage to the root system (c) and freezing damage to the apical meristem (d).

if they are exposed to liquid nitrogen at such high water contents the water present in the cells crystallizes forming harmful ice crystals that cause mechanical rupture and consequentially death of the cells [21]. Therefore, adjusting water content in the cells before immersion in liquid nitrogen is essential for avoiding cell destruction as a consequence of freezing. However, water has many biological functions in the cells of living organisms and water removal might also cause damage to the plant cells [5]. Therefore adjusting water content and identifying the water content that allows plant cells to survive freezing in liquid nitrogen without causing desiccation injury are essential for the development of a successful cryopreservation protocol.

Embryonic axes of *G. americana* were successfully cryopreserved by adjusting seed moisture content. High germination percentages (93%, 96%, and 93% germination) were obtained for embryonic axes isolated from seeds dried to moisture contents of 13.26%, 9.57%, and 6.79%, respectively.

In conclusion, the methodology described here warranted high germination rate of embryonic axes of *G. americana* after liquid nitrogen cryopreservation and the recovery of seedlings with normal growth rate and morphology and is recommended for the long term conservation of germplasm of this tropical species.

## Disclosure

Antonieta Nassif Salomão is coauthor.

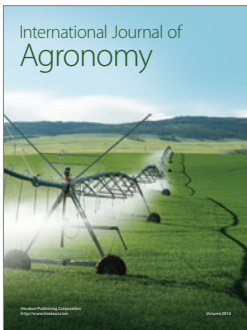
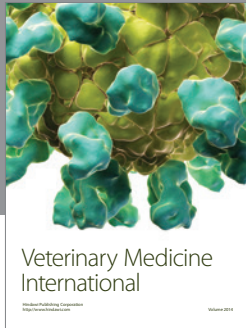
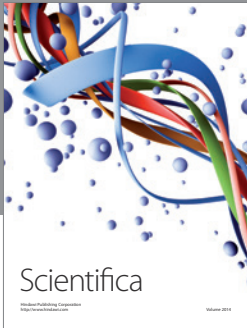
## Competing Interests

The authors declare that they have no competing interests.

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