



## Cryopreservation of *Byrsonima intermedia* embryos followed by room temperature thawing

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**ABSTRACT.** *Byrsonima intermedia* is a shrub from the Brazilian Cerrado with medicinal properties. The storage of biological material at ultra-low temperatures (-196°C) is termed cryopreservation and represents a promising technique for preserving plant diversity. Thawing is a crucial step that follows cryopreservation. The aim of this work was to cryopreserve *B. intermedia* zygotic embryos and subsequently thaw them at room temperature in a solution rich in sucrose. The embryos were decontaminated and desiccated in a laminar airflow hood for 0-4 hours prior to plunging into liquid nitrogen. The embryo moisture content (% MC) during dehydration was assessed. Cryopreserved embryos were thawed in a solution rich in sucrose at room temperature, inoculated in a germination medium and maintained in a growth chamber. After 30 days, the embryo germination was evaluated. No significant differences were observed between the different embryo dehydration times, where they were dehydrated for at least one hour. Embryos with a MC between 34.3 and 20.3% were germinated after cryopreservation. In the absence of dehydration, all embryos died following cryopreservation. We conclude that *B. intermedia* zygotic embryos can be successfully cryopreserved and thawed at room temperature after at least one hour of dehydration in a laminar airflow bench.

**Keywords:** long-term storage, native plant, zygotic embryos, rapid freezing, desiccation.

## Criopreservação de embriões de *Byrsonima intermedia* seguido de descongelamento em temperatura ambiente

**RESUMO.** *Byrsonima intermedia* é um arbusto do Cerrado brasileiro com propriedades medicinais. A conservação de material biológico em temperatura ultrabaixa (-196°C) é conhecida como criopreservação e representa uma técnica promissora para a preservação da diversidade vegetal. O descongelamento é uma etapa crucial durante a criopreservação. O objetivo deste trabalho foi criopreservar embriões zigóticos de *B. intermedia* e realizar o descongelamento utilizando uma solução rica em sacarose à temperatura ambiente. Os embriões foram submetidos à desinfestação e dessecação em uma câmara de fluxo laminar por 0-4 horas antes da imersão em nitrogênio líquido. O teor de umidade (%) dos embriões foi avaliado. Embriões criopreservados foram descongelados em uma solução rica em sacarose à temperatura ambiente antes da inoculação em meio de germinação e mantidos em sala de crescimento. Após 30 dias, a germinação dos embriões foi avaliada. Após uma hora de desidratação não foram observadas diferenças estatísticas entre os diferentes tempos testados. Embriões com teor de umidade entre 34,3 e 20,3% germinaram após a criopreservação. Embriões não desidratados não sobreviveram ao processo de criopreservação. Embriões zigóticos de *B. intermedia* podem ser criopreservados com sucesso e descongelados à temperatura ambiente quando desidratados por pelo menos uma hora em câmara de fluxo laminar.

**Palavras-chave:** armazenamento por tempo prolongado, planta nativa, embrião zigótico, congelamento rápido, desidratação.

### Introduction

The Brazilian Cerrado shelters numerous plant species with high fruitful and/or medicinal potentials. The Cerrado harbours the largest savannah plant biodiversity on the planet (KLINK; MACHADO, 2005), with more than 10,000 plant species (RATTER et al., 1997), which is persistently threatened by the intensive agricultural use of the

land (PEREIRA; GAMA 2010). Studies on the propagation of native species are scarce, and numerous species that represent an ecological and/or pharmaceutical interest could become extinct before being properly studied.

*Byrsonima intermedia* A. Juss. is a medicinally valuable species that belongs to the Malpighiaceae family from the Cerrado, and, such as numerous species of this genus, it presents seeds with a coat

dormancy that are difficult to propagate (NOGUEIRA et al., 2004). A wide variety of medicinal properties are attributed to this plant, including anti-inflammatory (MOREIRA et al., 2011; ORLANDI et al., 2011), anti-ulcer (SANNOMIYA et al., 2007; SANTOS et al., 2012), gastroprotective (SANTOS et al., 2009, 2012), healing (SANTOS et al., 2009), duodenal antimicrobial and antidiarrheal effects (SANTOS et al., 2012).

Plant biodiversity conservation can be performed by protecting the natural habitats (*in situ*) or by growing the plants in field collection (GONZÁLEZ-BENITO et al., 2003; ENGELMANN, 2011; WATANAWIKKIT et al., 2012). These two types of germplasm conservation tactics are costly and prevent the exchange of material due to the risk of disease and pathogen spreading (ENGELMANN, 1997) in addition to the risk of plague attacks and natural disasters (GONZÁLEZ-BENITO et al., 2003). Germplasm conservation using *in vitro* cultures is a technique that effectively conserves plant biodiversity; however, it is also extremely laborious and costly to maintain a large collection. Conversely, seed cryopreservation is an accessible, efficient and one of the most promising techniques for long-term and safe storage (ENGELMANN, 2004; JOHNSON et al., 2012; N'NAN et al., 2012).

Zygotic embryos or embryonic axes from a wide variety of plant species can be desiccated (ENGELMANN, 1992). Water removal is essential for preventing injury during freezing as well as for maintaining post-thaw viability (PANIS et al., 2001). During cooling or thawing, intracellular water may form lethal ice crystals that lead to cell death through a crystallisation or recrystallization process, respectively (MAZUR, 1984). For example, crystallisation is typically avoided by a rapid cooling by directly plunging the embryos into liquid nitrogen (LN) (GONZALEZ-ARNAO et al., 2008). However, during rewarming, living cells may suffer damage by recrystallization (FKI et al., 2012), a phenomenon that may occur if the thawing is not performed properly (GONZALEZ-ARNAO et al., 2008; HOPKINS et al., 2012). To avoid recrystallization, thawing must be rapid (HOPKINS et al., 2012; MAZUR, 1984).

The classical thawing method involves plunging an enclosed cryovial tube containing explants into a sterile water bath (~ 40°C) for a short period of time (JOHNSON et al., 2012; N'NAN et al., 2012; WEN; WANG, 2010). However, this procedure requires specific equipment and precise time control. Conversely, using a small volume of a

sterile solution to thaw the embryos at room temperature may be advantageous during embryo cryopreservation. In this context, we aimed to establish a cryopreservation protocol for *B. intermedia* zygotic embryos by rapidly freezing desiccated embryos and subsequently thawing them at room temperature.

## Material and methods

Ripe *B. intermedia* fruits were collected from a natural population in southern Minas Gerais State, Brazil, located at 918.0 m altitude, 21°14'S and 44.9°00'W GRW. After harvesting, the pulp was removed, and the seeds were soaked in 0.1 M sodium hydroxide (NaOH) for five minutes and washed in unsterile running water for 10 minutes. Endocarps were dried for three days at room temperature over filter paper and stored at 4°C prior to decontamination.

Embryos were decontaminated by two methods: (i) Method 1 - Endocarps were opened in non-aseptic conditions using forceps, and the embryos were extracted. In a laminar airflow hood (LAF), the embryos were plunged into 70% alcohol v/v for 30 seconds and subsequently into a NaOCl solution (1% of active chlorine), pH 8.5, plus one drop of Tween for five minutes; and (ii) Method 2 - In a LAF (aseptic conditions), the seeds were subjected to 95% alcohol for two minutes and to NaOCl (2% active chlorine) plus one drop of Tween for 20 minutes. They were washed three times in sterile water, and the endocarps were opened using forceps. The embryos were extracted and plunged into 70% alcohol v/v for 30 seconds and then into a NaOCl solution (1% active chlorine), pH 8.5, plus one drop of Tween for five minutes. For both treatments (Method 1 and 2), after the NaOCl immersion, the embryos were washed three times in sterile water, the integuments (endotegmen and exotesta) were removed and the embryos were inoculated on MS medium (MURASHIGE; SKOOG, 1962) with 0.09 M sucrose, 3 g L<sup>-1</sup> Phytigel® and with pH-adjusted to 5.8. The cultures were maintained in a growth room at 25 ± 2°C at a 16h photoperiod. For each treatment, 50 seeds were inoculated with one seed per test tube, where each tube represented one replicate. After 30 days, the number of decontaminated embryos and germination events, which are characterised by the presence of normal seedlings (NS) that displayed both roots and shoots, were evaluated.

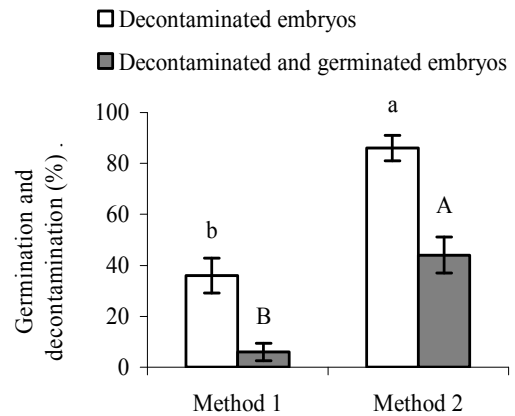
For embryo desiccation, germination and cryopreservation, the seeds were decontaminated and the embryos were extracted using Method 2.

For the control germination, the embryos were desiccated in a LAF over a filter paper disc ( $\varnothing$  60 mm) in an uncovered Petri dish (90 × 15 mm) for 0–4 hours at 25°C and inoculated in MS medium (as described above). Ten embryos were inoculated per each desiccation time. To obtain an accurate MC, three replicates of 10 embryos each were used. After desiccation, the embryos were completely dried in an oven at 70°C for 72 hours, and the MC was calculated using the following equation: % MC (FWb) = [(FW – DW)/FW]\*100, where: % MC (FWb) represents the percentage of moisture on a fresh weight basis, FW represents the fresh weight (mg) and DW represents the dry weight (mg). For cryopreservation, dehydrated embryos (24 per desiccation time) were placed in a 2.0 cm<sup>3</sup> cryotube (6 embryos per cryotube) and plunged into and maintained in LN for 60 minutes. The cryotubes were only closed after the LN plunge. We used open cryotubes to allow the embryos to directly contact the LN, and thus an ultra-rapid cooling rate of approximately 130°C min.<sup>-1</sup> was obtained (GONZÁLEZ-BENITO et al., 2003).

Before thawing in a LAF, the cryotubes were opened, the LN was withdrawn and the embryos were thawed at room temperature for 15 minutes in a low volume (5 cm<sup>3</sup>) of filter-sterilised unloading solution (US) (SAKAI et al., 1991) in a sterile Petri dish (60 × 15 mm). Next, the embryos were inoculated in MS medium with 0.3 M sucrose, maintained for 24h in the dark, transferred to MS basal medium and maintained in the dark for six days. After seven days, the control and cryopreserved embryos were placed in light conditions at a photoperiod of 16h at 25 ± 2°C. After 30 days, the number of embryos displaying roots, shoots or normal seedlings (NS) were evaluated. The statistic tool R (R DEVELOPMENT CORE TEAM, 2012) was used for analysis, and the data were subjected to ANOVA, Chi-squared and Scott-Knot tests ( $p \leq 0.05$ ).

## Results and discussion

A significant difference between the two decontamination methods was observed ( $p < 0.0001$ ). Decontamination Method 2 was more efficient, resulting in a higher percentage of contaminant-free explants (86%) vs. Method 1 (36%). The percentage of germinated embryos was also significantly higher ( $p < 0.0001$ ). Approximately half (44%) of the contamination-free embryos processed using Method 2 germinated vs. 4% using Method 1 (Figure 1).



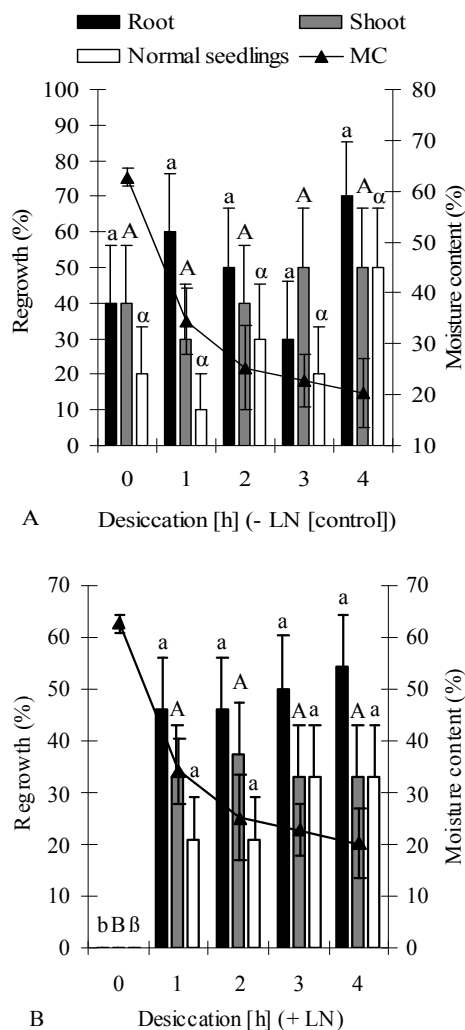
**Figure 1.** Germination and decontamination efficiencies of *Byrsonima intermedia* embryos. The bars represent the means ± SE of 50 seeds per treatment. The bars displaying the same letters for the same treatments are not significantly different according to the Chi-squared test ( $p \leq 0.05$ ).

Factors that may account for this low germination are: (i) the *Byrsonima* genus displays a high embryonic dormancy (LORENZI, 2002), (ii) the embryo extraction procedure is harmful due to an indehiscent and highly lignified endocarp layer (LORENZI, 2002) and (iii) the embryo cotyledons harbour phenolic compounds (SOUTO; OLIVEIRA, 2005). The inhibitory effect of phenolic compounds on seed germination has been well documented (TESIO et al., 2011; TOKUHISA et al., 2007; VICENTE; PLASENCIA 2011). However, we hypothesise that the low percentage of growing NS after decontamination is due to the procedure used to extract embryos, which involves the use of forceps. When the extremely hard endocarp is broken, the sensitive embryonic axis may be damaged. Moreover, integuments are extremely difficult to remove without harm.

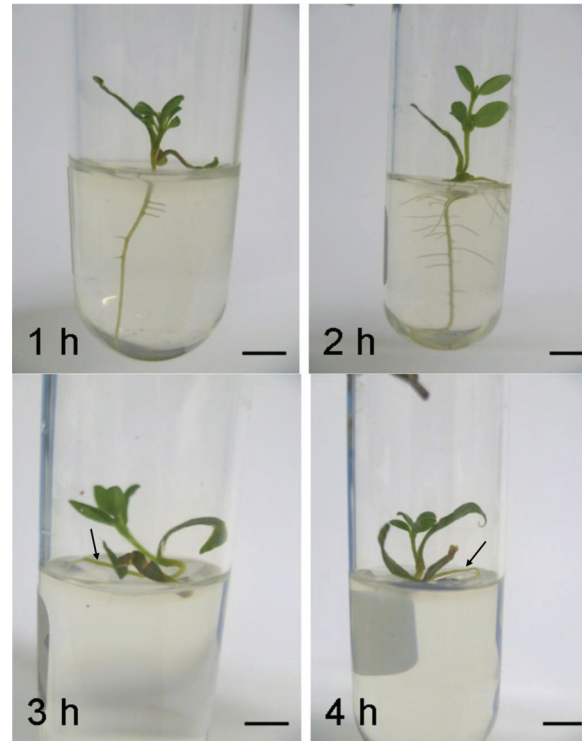
Embryos that were not plunged into LN (control) did not statistically differ in the incidence of roots ( $p = 0.7839$ ), shoots ( $p = 0.8991$ ) and NS ( $p = 0.3283$ ), which averaged 56, 42 and 26%, respectively. A desiccation of up to four hours did not decrease the germination percentages of the control embryos (Figure 2A). The reduction in the % MC (FWb) after four hours of desiccation was significant ( $p < 0.0001$ ). We observed a high initial MC (62.7%) just after the embryos were decontaminated (Figure 2A and B).

We observed a significant difference in the number of embryos displaying roots ( $p = 0.0003$ ), shoots ( $p = 0.0195$ ), and NS ( $p = 0.0311$ ) with averages of 39, 27 and 22%, respectively, between desiccated and non-desiccated embryos after cryopreservation. Non-desiccated embryos did not survive exposure to LN (Figure 2B). This result was

expected when the MC was too high (62.7%), which allowed for lethal ice crystals to form. No differences were observed between the desiccated and cryopreserved embryos for all parameters evaluated. Compared to the controls (Figure 2A), desiccated and cryopreserved embryos (Figure 2B) displayed similar regeneration rates. Nogueira et al. (2011) previously reported a 10% germination incidence, only characterised by root protrusion, for *B. intermedia* zygotic embryos with 15 or 25% MC after being plunged into LN and thawed in a water bath at 37°C for 5 minutes. Compared to those results, we observed a four-fold higher root protrusion (Fig. 2B). NS after 30 days of cryopreservation are illustrated in Figure 3.



**Figure 2.** Moisture content ( $\pm$ SE,  $n = 30$ ) and regrowth (%) of the roots, shoots or normal seedlings ( $\pm$ SE,  $n = 10$ ) after embryo desiccation for different periods of time (hours), cryopreservation and thawing at room temperature. Embryos were not plunged (A) or plunged (B) into liquid nitrogen ( $-196^{\circ}\text{C}$ ). The bars followed by the same letter are not significantly different according to the Skott-Knott test ( $p \leq 0.05$ ). MC (moisture content); -LN (no LN plunge); + LN (LN plunge).



**Figure 3.** Normal seedlings after 30 days of cryopreservation were obtained from embryos desiccated for one to four hours, plunged into liquid nitrogen ( $-196^{\circ}\text{C}$ ), thawed at  $25^{\circ}\text{C}$  and inoculated in MS basal medium. The roots are indicated by an arrow (3-4h). Bars = 0.5 cm.

Zygotic embryos are large and complex structures with a heterogeneous cellular composition (ENGELMANN, 1992) and can display differential sensitivities to desiccation and cryopreservation. The embryonic root poles appear to be more resistant than the shoot poles (ENGELMANN, 2004), which may explain the high percentage of root protrusion compared to NS regeneration (Figure 2).

Given that excised and non-decontaminated embryos displayed a MC of approximately 6.6% (data not shown), the high initial MC percentage observed may be due to water imbibition. The seeds were in contact with water for varying periods: five minutes during decontamination in NaOCl, three fast washes in sterile water, and, for extracting embryo integuments (endotegmen and exotesta), they were placed over moistened sterile filter paper for several minutes. Integument extraction is laborious and time-consuming, and thus, from the first until the last embryo collected for dehydration, at least four hours were required, and imbibition was thus unavoidable during this period. Imbibition occurs during the phase I (PI) of germination, which is characterised by a fast water influx (BEWLEY, 1997; WEITBRECHT

et al., 2011), where even dead seeds can soak (KRISHNAN et al., 2004). Therefore, the PI duration is variable (4-24h for most species). In soybeans, viable and non-viable seeds reached the end of PI after 12h of imbibition (KRISHNAN et al., 2004). Schopfer and Plachy (1984) demonstrated a PI of 6 – 9 for *Brassica napus* germination. For *Arabidopsis thaliana*, the PI terminates at ~ 4h (WEITBRECHT et al., 2011). The PI of tobacco seeds is ~12h (MANZ et al., 2005) and 24h for *Tabebuia impetiginosa* (SILVA et al., 2004). According to the behaviour of the species cited above, we believe this period was sufficient for the embryos to absorb a substantial amount of water. At the end of the desiccation period (4h), the embryos displayed a 20.3% MC (FWb) (Figure 2).

We did not follow the classical cryopreservation procedure for thawing zygotic embryos, where the embryos enclosed in a cryotube are typically rewarmed by a short exposure to a sterile water bath (~ 40°C), e.g., *Sabal spp* at 40°C / 1 min. (WEN; WANG, 2010), *Cocos nucifera* L. at 40°C / 2 min. (N'NAN et al., 2012) and *Xyris tennesseensis* Kral. at 37°C / 1-2 min. (JOHNSON et al., 2012). Our thawing approach was successfully performed at room temperature using a filter-sterilised US composed of 1.2 M sucrose dissolved in MS medium, pH 5.8. Performing the thawing procedure without a water bath may reduce the risk of contamination. Despite this, this solution is widely used for thawing, in room temperature, PVS2 (plant vitrification solution 2 [SAKAI et al., 1991]) treated and cryopreserved meristems (CONDELLO et al., 2011; PANIS et al., 2005; SANT et al., 2008). This study was the first to successfully apply this technique for thawing zygotic embryos.

## Conclusion

*Byrsonima intermedia* zygotic embryos can be successfully cryopreserved using a rapid freezing method after at least one hour of desiccation in a laminar airflow hood.

These embryos can be successfully thawed using a filter-sterilized unloading solution at room temperature.

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