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Cryopreservation of the mangaba tree (*Hancornia speciosa* Gomes): a protocol for long-term storage

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ABSTRACT. The aim of this study was to evaluate the efficiency of vitrification and droplet vitrification for the cryopreservation of *Hancornia speciosa* shoot tips. The shoot tips were subjected to four different periods of exposure (15, 30, 45 and 60 min.) to plant vitrification solution 2 (PVS2) before plunging into liquid nitrogen. We evaluated the regrowth of *H. speciosa* shoot tips that were cryopreserved by the classical vitrification technique and by the droplet vitrification technique. Shoot tips were submitted to different periods of pre-culture (absence, 24 or 48h) in a medium containing 0.3 M sucrose prior to cryopreservation. With a PVS2 exposure period of 60 min., significant differences were observed between the two techniques used in this study with respect to regrowth rates; however, both cryopreservation methods allowed for a shoot tip regrowth rate of over 70%. Use of a preculture step increased the regrowth rate of cryopreserved shoot tips. Shoot tip cryopreservation techniques employing vitrification and droplet vitrification proved to be viable for the long-term storage of mangaba tree samples.

Keywords: native species, *in vitro* conservation, recalcitrant seeds, plant genetic diversity conservation, vitrification, droplet vitrification.

Criopreservação de mangabeira (*Hancornia speciosa* Gomes): um protocolo para armazenamento a longo prazo

RESUMO. O objetivo deste trabalho foi avaliar a eficiência da *droplet vitrification* e vitrificação na criopreservação de ápices caulinares de mangabeira. Foi avaliada a retomada de crescimento de ápices de mangabeira criopreservados conforme a técnica de *droplet vitrification* utilizando diferentes tempos de tratamento com a solução de vitrificação (15, 30, 45 e 60 min.) e submetidos a diferentes períodos de pré-cultivo (ausência, 24 ou 48h) em meio com 0,3 M de sacarose antes da criopreservação. Além desta técnica, foi avaliado o efeito da técnica clássica de vitrificação utilizando quatro tempos de exposição à solução de vitrificação (15, 30, 45 e 60 min.). Foram observadas diferenças significativas no tempo de exposição para a técnica de vitrificação e *droplet vitrification*. Para ambas as técnicas o tempo de imersão de 60 minutos promoveu uma retomada de crescimento superior a 70% nos ápices criopreservados. O pré-cultivo promoveu o aumento na sobrevivência dos explantes criopreservados. As técnicas de criopreservação de ápices caulinares por *droplet vitrification* e vitrificação mostraram-se viáveis para a conservação em tempo prolongado de mangabeira.

Palavras-chave: espécie nativa, conservação *in vitro*, sementes recalcitrantes, conservação da diversidade vegetal, vitrificação, *droplet vitrification*.

Introduction

Mangaba tree (*Hancornia speciosa* Gomes) is a native Brazilian fruit tree occurring in the Atlantic Forest and Cerrado. This species has economic potential due to its fruits, which are consumed fresh and also used to produce juices, sweets and ice creams (MOURA et al., 2011). Recent studies have shown that the leaf extract has anti-hypertensive and vessel dilator effects (PEREIRA et al., 2012), whereas the latex has anti-inflammatory properties (MARINHO et al., 2011). These recent findings also indicate the medicinal potential of this species.

H. speciosa produces recalcitrant seeds and is difficult to propagate asexually. Moreover, the species has undergone a process of genetic erosion in several natural populations due to agricultural activity and urban sprawl into natural conservation areas (SÁ et al., 2011). Thus, micropropagation studies are needed to allow the proliferation of genotypes (SÁ et al., 2012) and to provide new

means of ensuring the *in vitro* conservation of species diversity (SANTOS et al., 2011).

Currently, conservation of *H. speciosa* populations is accomplished through field gene banks; however, this type of conservation is expensive (LI; PRITCHARD, 2009), and plants growing in fields are susceptible to pests, diseases and plague attacks (BARRACO et al., 2011). Another strategy is to develop protocols for *in vitro* storage that involve slowing the growth of the plant by treatment with either sorbitol (SANTOS et al., 2011) or mannitol plus abscisic acid (SÁ et al., 2011) or (most commonly) by decreasing the temperature of the culture environment (ENGELMANN, 1991; LATA et al., 2010). This *in vitro* conservation strategy may facilitate the exchange of plant material between research institutions (PATEÑA; BARBA, 2011); however, this is a medium-term storage strategy and requires constant subculture for maintenance, thus increasing the costs and the inherent risk of contamination (BURRITT, 2008).

Cryopreservation, which is the conservation of biological material at an ultra-low temperature (-196°C) in liquid nitrogen (ENGELMANN, 2011), is a useful technique for *ex situ* conservation, especially for species that either do not produce seeds or produce seeds that are recalcitrant (KACZMARCZYK et al., 2011) and therefore cannot be preserved by seed bank conservation (PILATTI et al., 2011). Storage in liquid nitrogen is safe and economically viable (SANT et al., 2008) and allows for long-term *in vitro* conservation (CASTILLO et al., 2010; REED et al., 2011; CEJAS et al., 2012).

Because there is no established protocol for long-term conservation of *H. speciosa*, the aim of this study was to evaluate the efficiency of two cryopreservation techniques—the classical vitrification method and droplet vitrification—for *H. speciosa* shoot tip preservation in liquid nitrogen.

Material and methods

Plant material

Fruits of the mangaba tree were collected in Pirambu city (Sergipe state, Brazil), which is an area where mangaba trees naturally occur. In the tissue culture laboratory, the fruit was pulped in a laminar flow hood. The seeds were extracted and decontaminated with 70% alcohol (v/v) for 30 seconds followed by sodium hypochlorite treatment with 2% active chlorine for 15 minutes. Subsequently, the seeds were washed three times with sterile water before the explants were inoculated in WPM (LLOYD; MCCOWN, 1980) supplemented with 0.09 M sucrose and 7 g L^{-1} agar, and the pH was adjusted to 5.8 before sterilization (SOARES et al., 2009). The explants were maintained in a growth chamber with a temperature of 25 ± 2°C, an irradiance of 36 µmol m^{-2} s^{-1} and a 16-hour photoperiod. The seedlings were used for shoot multiplication 30 days after seed inoculation.

Shoots were established *in vitro* and multiplied according to the protocol described by Soares et al. (2011) using WPM medium supplemented with 2.0 mg L^{-1} BAP (6-benzylaminopurine), 0.09 M sucrose and 7 g L^{-1} agar. The pH was adjusted to 5.8 before autoclaving. Five shoot subcultures (30 days each) were performed prior shoot tip cryopreservation.

Droplet vitrification

Cryopreservation of shoot tips by the droplet vitrification method was performed as described by Panis et al. (2005). Shoot tips (approximately 1.0 mm^2) were excised and loaded for 20 minutes at room temperature in filter sterilized loading solution (LS) (2 M glycerol and 0.4 M sucrose dissolved in Murashige and Skoog [1962] liquid medium). After loading, the shoot tips were treated with filter sterilized PVS2 (3.26 M glycerol, 2.42 M ethylene glycol, 1.9 M dimethylsulfoxide and 0.4 M sucrose dissolved in MS liquid medium [SAKAI et al., 1990]) at 0°C for different time periods (15, 30, 45 and 60 min.). PVS2-treated shoot tips were placed on an aluminum foil strip (1.5 × 0.5 cm) over a cooled surface with a drop of PVS2 prior to plunging into liquid nitrogen (LN), where they were maintained for 30 minutes. After this period, thawing was carried out at room temperature in filter sterilized unloading solution (US) (1.2 M sucrose dissolved in MS liquid medium) for 15 min.

Preculture at a high sucrose concentration

The effect of preculturing shoot tips in WPM medium with a high sucrose concentration was evaluated. The shoot tips were either (1) precultured for 24 hours on 0.3 M sucrose prior to treatment with LS for 20 minutes before exposure to PVS2 (0°C) for 15, 30, 45 and 60 minutes or (2) precultured for 48 hours on 0.3 M sucrose and treated with LS for 20 minutes before exposure to PVS2 (0°C) for 15 and 30 minutes. After PVS2 treatment, the shoot tips were plunged into LN and maintained for 30 minutes prior to thawing/unloading in US at room temperature for 15 minutes.

Vitrification method

Cryopreservation of shoot tips by the vitrification method was performed as described by

Charoensub et al. (2003). Shoot tips were excised and precultured for 24 hours in WPM medium with 0.3 M sucrose. After preculture, the explants were treated with LS for 20 minutes before transfer to 2.0 mL cryotubes with 1.0 mL PVS2. The cryotubes were closed, and the shoot tips were exposed to PVS2 for 15, 30, 45 and 60 minutes at 0°C prior to plunging into LN. After 30 minutes in LN, the samples were thawed rapidly in a water bath at 39 ± 2°C for two minutes. The PVS2 was immediately removed after thawing and replaced with 1.0 mL US at room temperature. The unloading in US was carried out for 15 minutes.

Post-thawing

After thawing and unloading of samples processed by droplet vitrification or classical vitrification, the shoot tips were inoculated in WPM medium with 0.3 M sucrose, 20 ppm ascorbic acid, 7 g L^{-1} agar and no growth regulators at a pH of 5.8 and maintained in darkness for 24h. After this period, the explants were transferred to WPM medium with 0.09 M sucrose, 2 mg L^{-1} BAP, 20 ppm ascorbic acid and 7 g L^{-1} agar at a pH of 5.8 and maintained in darkness for six additional days prior to transfer to a 16-h photoperiod. After 30 days of cultivation, we evaluated shoot tip regrowth and callus formation. Regrowth was defined as an increase in the size and leaf expansion of shoot tips. This procedure was performed with samples from the three cryopreservation experiments described above.

Shoot tips that were successfully cryopreserved were transferred to tubes (25 × 150 mm) containing 15 mL of the same medium described above and maintained under the same growing conditions.

Experimental design and statistical analysis

The experimental design was completely randomized with five independent replications of each PVS2 exposure time. Each replication was performed using 10 shoot tips: Seven were cryopreserved, and three were not plunged into LN (controls). Prior to statistical analysis, the original data were submitted to arcsine transformation (y' = arcsine $y^{1/2}$, y = original percentage/100). The transformed data were subjected to ANOVA and analyzed by regression or Tukey test ($p \leq 0.05$). The statistical software SISVAR (FERREIRA, 2011) was used for all statistical analyses.

Results and discussion

Regarding the regrowth of non-precultured meristems in WPM medium with a high sucrose concentration, the interaction *time of exposure to PVS2 × explant type (control and cryopreserved shoot tips)* was significant (p = 0.0269). Non-cryopreserved shoot tips exhibited 100% regrowth after 15 and 30 minutes. After PVS2 treatment, a reduction in the regrowth rate was observed. According to the regression test applied, the maximum estimated regrowth for cryopreserved shoot tips (81%) occurred at 51 minutes. This is very similar to the real regrowth average (82%) observed at 60 minutes of PVS2 treatment. On the other hand, after 15 minutes of exposure to PVS2, only 45% of the cryopreserved shoot tips exhibited regrowth (Figure 1). The callus formation was lower (4.6%), and no significant differences (p = 0.77) were observed between the different PVS2 exposure periods.

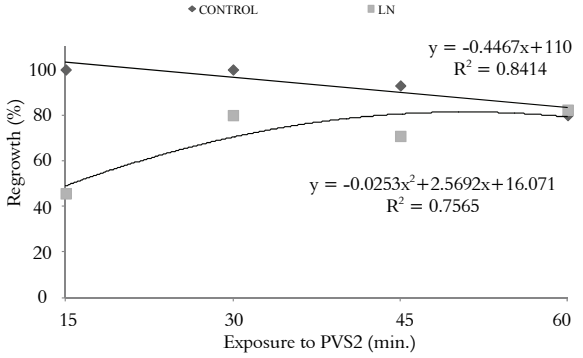

Figure 1. Percentage regrowth of non-precultured *Hancornia speciosa* shoot tips after cryopreservation by droplet vitrification.

The 15-min. PVS2 treatment was not sufficient to promote efficient vitrification of cryopreserved shoot tips when compared with the 15-min. control treatment (Figure 1); thus, the shoot tip cells may have had a high water content upon plunging into LN, which resulted in irreversible damage to the membranes due to water crystallization (MAZUR, 1984; SURANTHRAN et al., 2012). PVS2 is not used solely for dehydration; it is essential for avoiding water crystallization but also has a colligative effect during cryopreservation, reducing molecule mobility and allowing cell vitrification during immersion into LN (VOLK; WALTERS, 2006).

Preculture in 0.3 M sucrose promoted an increase in the regrowth of cryopreserved explants at all PVS2 treatment periods tested (Figure 2) when compared with non-precultured cryopreserved shoot tips (Figure 1). The interaction *time of exposure to PVS2 × explant type (control and cryopreserved shoot tip)* was significant (p = 0.0079). According to the regression curve, cryopreserved shoot tips achieved 90% regrowth after 49 minutes of exposure to PVS2 (Figure 2).

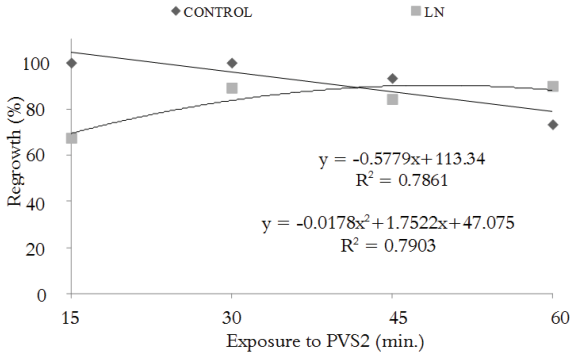

Figure 2. Percentage regrowth of *Hancornia speciosa* shoot tips cryopreserved by droplet vitrification after preculture in 0.3 M sucrose for 24h.

Although the lowest precultured shoot tip regrowth percentage was observed for explants treated with PVS2 for 15 min. (67%; Figure 2), this percentage represents an increase of 22% over the regrowth percentage of non-precultured shoot tips that were exposed to the vitrification solution for the same time period (Figure 1).

The increase in the regrowth of explants may be attributed to the beneficial effect of sucrose absorption during preculture, which promotes both a reduction in explant water content by dehydration (osmotic effect) and a reduction in the freezing point of water within the tissue (PANIS et al., 1996). Moreover, sucrose protects cell membranes during cooling (CARPENTIER et al., 2010; QUAIN et al., 2009). According to Burritt (2008), soluble sugars such as glucose and fructose play important roles in stabilizing phospholipid bilayers and proteins in biological systems exposed to low water potential. Callus formation in the precultured explants reached an average of 16%. Although this value is higher than that obtained for non-precultured explants (4.6%), no significant differences were observed between samples exposed to PVS2 for different time periods ($p = 0.56$).

Cryopreservation of shoot tips by vitrification requires the use of vitrification solution, which contains substances that promote osmoprotection. Moreover, the high osmolarity of these solutions promotes explant dehydration. Thus, the success of cryopreservation is linked with the water balance of the cells and the use of cryoprotectant substances (VOLK; WALTERS, 2006).

Cryoprotectants may be cytotoxic (FULLER, 2004), and the substances that compose PVS2, mainly DMSO, may lead to cell death, especially if PVS2 treatment is performed at temperatures higher than 0°C or if the exposure period is too long (PANIS et al., 2005; FULLER, 2004). Due to these issues, we attempted to increase the survival percentage while reducing the PVS2 exposure period at the same time. To accomplish this, shoot tips were precultured in a medium containing 0.3 M sucrose for 48h and subsequently treated for 15 or 30 minutes with PVS2 at 0°C prior to cryopreservation. After preculturing the shoot tips for 48h, we observed no significant differences in regrowth between the control and cryopreserved explants exposed to PVS2 for 15 or 30 minutes (Figure 3). Cryopreserved explants treated with PVS2 for 15 min. and precultured for 48h with 0.3 M sucrose (Figure 3) showed the same regrowth percentage as non-precultured shoot tips (Figure 1), while the regrowth rate of shoot tips treated with PVS2 for 30 minutes (Figure 3) was approximately 30% lower than that of non-precultured shoot tips (Figure 1). Explants that were precultured for 48 hours in medium containing 0.3 M sucrose exhibited approximately 4% callus formation with no significant difference between the PVS2 treatments.

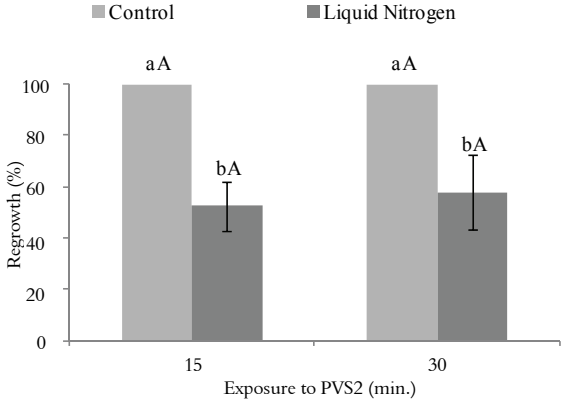

Figure 3. Percent regrowth of *Hancornia speciosa* shoot tips cryopreserved by droplet vitrification after preculture in 0.3 M sucrose for 48 hours. Lowercase letters are comparisons between control and liquid nitrogen within each PVS2 exposure time. Capital letters are comparisons between different PVS2 exposure times for either control or liquid nitrogen. For each comparison, bars with the same letters are not significantly different according to the Tukey test ($p \leq 0.05$). The bars represent means ± SE.

The osmotic shock caused by the cryopreservation solutions (LS, PVS2 and US) added to the effect of preculture in a medium with a high sucrose concentration (0.3 M for 48h) may promote deleterious effects on the explants (PINKER; HALMAGYI, 2006; RABA'A et al., 2012). A reduction in explant survival after longer preculture periods was also reported by other researchers (COSTE et al., 2012; SHATNAWI;

JOHNSON, 2004). Matsumoto et al. (1994) observed that wasabi apical meristems precultured for three days in 0.3-0.7 M sucrose also showed a decrease in survival following cryopreservation.

When the vitrification method was applied, we observed a significant difference ($p = 0.0001$) in the regrowth of cryopreserved mangaba tree shoot tips depending on exposure time to PVS2. Using the regression curve, it was possible to estimate the highest regrowth rate, which was 74% at 60 minutes of PVS2 treatment for cryopreserved shoot tips (Figure 4). No significant difference was observed for the control treatment ($p = 0.0665$). The average regrowth of non-cryopreserved shoot tips was 85%. Only 7% of the shoot tips exhibited regrowth after thawing when treated with PVS2 for 15 minutes using the vitrification method. Generally, the regrowth rate using this method (Figure 4) is lower than that achieved with the droplet vitrification technique (Figure 2). When explants were treated no longer than 30 min. in PVS2, a low regrowth percentage was noted when using the vitrification method; this was also observed previously in cryopreserved *Carica papaya* L. shoot tips (TSAI et al., 2009). Chen et al. (2011) reported that the droplet vitrification method was more efficient for cryopreservation of *Lilium* apical meristems compared with the vitrification method. The same pattern was observed for mangaba tree shoot tips.

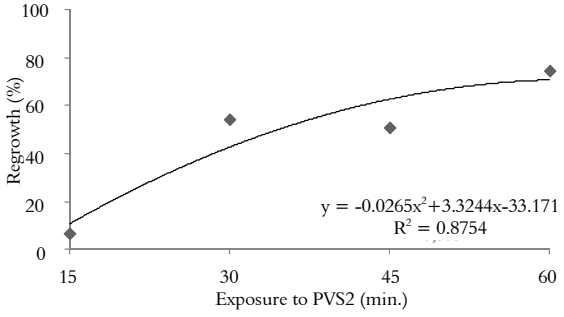

Figure 4. Percentage regrowth of *Hancornia speciosa* shoot tips cryopreserved by the vitrification method after preculture in 0.3 M sucrose for 24 hours.

By comparing the two techniques that were applied in this work, it is clear that the main difference occurs in the thawing process. Droplet vitrification and classical vitrification involve ultra-rapid (PANIS et al., 2011; SAKAI; ENGELMAN, 2007) and rapid cooling rates (PANIS; LAMBARDI, 2005; MAZUR, 1984), respectively, thus avoiding water crystallization (MAZUR, 1984). Droplet vitrification thawing is carried out in US at room temperature, which ensures an ultra-fast rewarming rate (CONDELLO et al., 2011; PANIS et al., 2011; SAKAI; ENGELMAN, 2007; PANIS et al., 2005), whereas the vitrification method involves fast thawing in a water bath at 40°C. Thawing/rewarming is a critical step in cryopreservation due to the recrystallization phenomenon (HOPKINS et al., 2012). Recrystallization may take place when the rewarming rate is not fast enough to prevent the aggregation of small ice crystals, leading to ice crystal growth. The growth of such crystals causes irreversible cellular damage and consequent cell death (HOPKINS et al., 2012; GONZALEZ-ARNÃO et al., 2008; MAZUR, 1984).

The increase regrowth rate occurred when the shoot tips were treated with PVS2 for only 15 min. which is directly related to preculture in 0.3 M sucrose for 24h (Figures 1 and 2). However, the explants precultured with the same sucrose concentration and PVS2 period of treatment in the vitrification method did not displayed the same behavior (Figure 4). Crystallization may not have occurred owing to the fast cooling rate for both methods (PANIS; LAMBARDI, 2005; MAZUR, 1984), and the control (no plunge into LN) exhibited a higher regrowth rate. The low regrowth rate of cryopreserved mangaba tree shoot tips may be dependent on the thawing procedure and consequently on recrystallization.

The successful use of the droplet vitrification technique for cryopreservation of mangaba tree shoot tips demonstrates the potential of this technique for the cryopreservation of a variety of plant species, as observed previously for chrysanthemum (LEE et al., 2011), lily (CHEN et al., 2011), papaya (KAITY et al., 2008), *Byrsonima intermedia* (SILVA et al., 2013) and banana (PANIS et al., 2005), among others. There are also efficient vitrification protocols for species such as cassava (CHAROENSUB et al., 2003) and citrus (AL-ABABNEH et al., 2002).

Most reports examining Brazilian native species describe the cryopreservation of orthodox seeds (PILATTI et al., 2011; NOGUEIRA et al., 2011). The development of protocols for shoot tip cryopreservation using vitrification and droplet vitrification may significantly contribute to the long-term storage of other Brazilian native species, particularly those with recalcitrant seeds. As shown in our study, shoot tips undergoing cryopreservation exhibited high post-thaw regrowth percentages and normal development with the production of new leaves and increases in height (Figure 5).

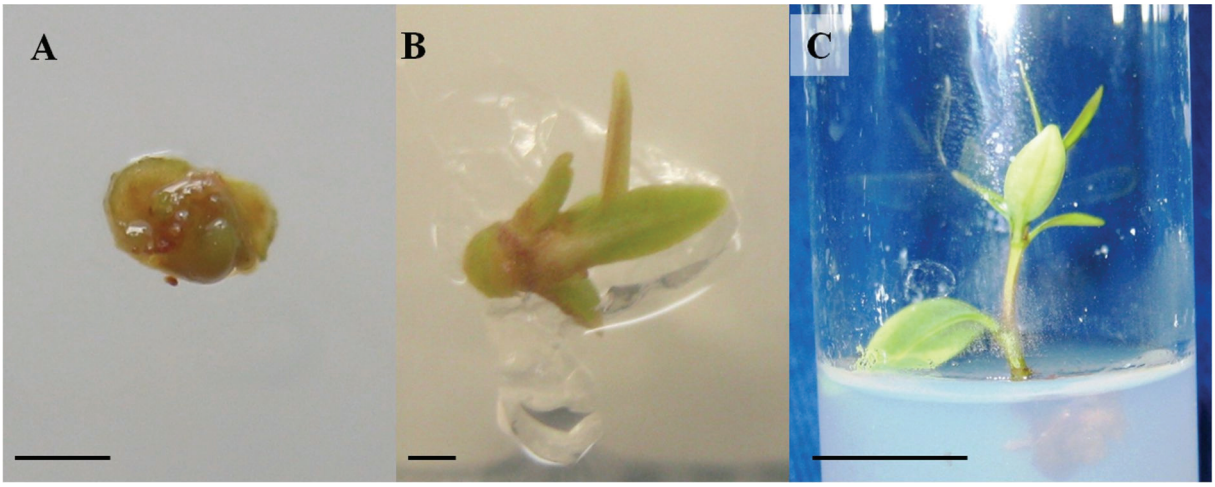

Figure 5. *Hancornia speciosa* shoot tips after cryopreservation by the droplet vitrification technique. Shoot tip at one (A), 30 (B) and shoot at 90 (C) days after cryopreservation. Bars = 0.5 mm (A), 1 mm (B) and 10 mm (C).

Conclusion

Mangaba tree shoot tip cryopreservation through droplet vitrification and vitrification proved to be viable and could be used for the long-term storage of this species.

The preculture of mangaba tree shoot tips for 24h in a medium with 0.3 M sucrose increases the regrowth of explants cryopreserved by droplet vitrification.

Acknowledgements

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