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Cryopreservation as an alternative for conservation of *Anacardium humile* achene (Monkey nut)

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Abstract

The present study aimed to evaluate the possibility of using cryopreservation method for conservation of cajuzinho-do-cerrado achene (*Anacardium humile*). To accomplish this aim, the physiological quality of cryopreserved achenes was compared with the quality of samples stored under other conditions. The quality of cryopreserved achenes with different water contents was also evaluated to establish a water-content limit for seed-viability maintenance. Achenes with physiological maturity were stored in liquid nitrogen at -196 °C with the following water contents: 28 (initial content) 14, 12, 8 and 5% moisture content (m_c). Subsequently, the achenes were subjected to 3 thawing methods: slow/gradual, fast and microwave thawing. Achenes were also subjected to different six conservation methods: cryopreservation in liquid nitrogen (LN) at -196 °C, cryopreservation using 10% cryoprotectants (Dimethyl sulfoxide (DMSO) or Glycerol), 10 °C biochemical oxygen demand (BOD) incubator, -80 °C ultra-low freezer and room temperature (25 °C ±3), with testing after storage in these environments for 5 and 10 months. The treatment effect on achene, physiological quality was assessed by seed germination (percentage) and vigour (germination speed index, electrical conductivity and root and shoot length). The results showed that reduction in the water content considerably reduced the germinating potential of *Anacardium humile* achenes for cryopreservation. Conversely, the thawing methods had no effect on the physiological quality of cryopreserved achenes; thereby, allowing the choice of the most practical thawing method (microwave). We also concluded that cryopreservation in liquid nitrogen may be used as a viable alternative for conservation of *Anacardium humile* achene.

Keywords: storage; cajuzinho-do-cerrado; desiccation; water content limit; vigour.

Introduction

Brazilian forest species have different seed longevity patterns (Wetzel et al., 2003), and some species native to the Cerrado (Brazilian savannah) have short longevity, which reduces the viability and; therefore, the use of seeds (Martins and Pinto, 2014).

Anacardium humile St. Hil., commonly known in Brazil as 'cajuzinho-do-cerrado', 'cajuzinho-rasteiro' and 'cajuí', is a shrub with height ranging from 0.60 to 0.80 m and a reddish yellow pseudo-fruit (Silva et al., 2001). The pseudo-fruit is edible and may be consumed fresh or used as a source of raw material by small, traditional candy factories and in therapeutic recipes for home remedies due to its antifungal, antibacterial and anti-diarrhoeal activity, playing a key role in the culture and economy of the Brazilian West-Central population (Soares, et al., 2013).

Cryopreservation is a technique that uses extremely low temperatures for reducing or completely inhibiting cellular metabolism (Batista, 2000) and is widely used for long-term storage of genetic material (Henshaw et al., 1980), enabling the preservation of genetic diversity (Marcos Filho, 2005). Reducing the metabolism and biochemical activities of seeds precludes biological deterioration (Kartha, 1985), enabling the preservation of biological material for an indefinite period (Harding, 2004; Benson, 2008).

Cryopreservation is the only safe and economical option for long-term germplasm conservation of recalcitrant seed species, vegetatively propagated species and biotechnol-ogical products (Engelmann, 2004). In the context of cryopreservation, seed storage is undoubtedly the most effective method for the *ex situ* preservation of plant genetic resources. The low storage costs, combined with seed collection facilitate the whole-plant regeneration from genetically diverse material, offer distinct advantages for seed cryopreservation compared with other plant tissue types, such as meristems and pollen (Pritchard, 1995).

Establishing the optimal water content is a critical factor for seed cryopreservation. Seeds with high water content, including recalcitrant seeds, are unable to tolerate cryogenic conditions at -196 °C. In orthodox seeds, the decrease in water content to a specific levels, before transfer to liquid nitrogen, has positive effects on post-thawing seed survival and germination. However, the decrease in seed water content or desiccation below the optimal level may adversely affect seed survival and germination (Naderi Shahab et al., 2017). Several studies have observed the limitations created by seed water content for cryopreservation success (Coelho and Cavalcanti Mata, 2008) because a marked loss of physiological quality may occur if the seed water content reaches inappropriate levels (Goldfarb et al., 2008).

Thus, developing a cryopreservation protocol requires knowledge of biochemical and biophysical mechanisms associated with tissue response to dehydration and freezing (Stushnoff and Seufferheld, 1995). Therefore, this study aimed to evaluate the possibility of using cryopreservation for cajuzinho-do-cerrado achenes, since in *Anacardium humile* St. Hil., the preservation of the achenes means automatic conservation of the seeds. To accomplish this aim, the physiological quality of cryopreserved achenes was compared with the physiological quality of samples stored under other conditions. The physiological quality of cryopreserved achenes with different water contents was also evaluated to establish suitable storage conditions and therefore seed viability maintenance.

Results

Cryopreservation experiment: determination of the water content limits for cryopreservation (WCLC)

A positive and significant correlation was observed between the decrease in water content and the decrease in the germination percentage of cryopreserved seeds (Fig. 2). Conversely, the high germination percentage (96%) maintained by achenes with initial water content (28%), regardless of the thawing method used, indicating that *Anacardium humile* is a cooling-resistant plant. However, this cooling must occur while maintaining the high-water content in the seeds.

Maintaining the water content at 5% m_c affected the root and shoot length of normal cajuzinho-do-cerrado seedlings, regardless of the thawing method (Fig. 3). On average, those variables decreased approximately 5 cm between achenes with 5 and 8% m_c water contents, showing that reducing the water content to lower than 8% m_c impairs seedling growth.

Storage experiment: determination of the suitable conditions for cryopreservation

The percentage of seed germination (Fig. 4) was decreased sharply in achenes stored at room temperature (25 °C±3), when compared with achenes cryopreserved without the presence of cryoprotectant or cryopreserved in 10% DMSO, showing that cryopreservation may be a good alternative method for *ex situ Anacardium humile* achene conservation. The use of cryoprotectants protected the achene membranes, which resulted in a high germination percentage (65%). The membranes remain intact because the cryoprotectant causes a vitreous effect. Thus, achenes cryopreserved without cryoprotectant were not adversely affected, demonstrating the efficiency of the cryopreservation technique for *Anacardium humile* achene storage.

A lower germination percentage was also observed in seeds stored for 10 months at room temperature, in a -80 °C ultra-low freezer or even in liquid nitrogen with glycerol, showing that long storage times may reduce cajuzinho-do-cerrado seed viability, regardless of using low temperatures and cryoprotectants.

The findings for percentage of emergence (Fig. 5) show that achenes cryopreserved in liquid nitrogen reduced the long-term percentage of emergence, regardless of the storage conditions.

Differences in mean achene electrical conductivity were observed between the two study times, and achenes stored for 10 months showed higher electrolyte leakage (22, 34). The conditions -80 °C ultra-freezer and liquid nitrogen with the glycerol cryoprotectant increased the release of exudates, characterising the deterioration of seed membranes throughout storage, which corroborates the data from the physiological quality assessments (Fig. 6).

Hypocotyl length was only significantly different for the room temperature condition at 25 °C, confirming that this condition adversely affects the physiological quality of seeds (Fig. 7). Furthermore, the storage conditions ultra-low freezer and -10 °C BOD incubator shortened seedling length throughout the storage period.

The root length (Fig. 8) also shortened with the cajuzinho-do-cerrado achene storage time, corroborating the hypothesis of a decrease in seed viability throughout storage.

Discussion

On average, germination decreased approximately 50 percentage points (pp) between achenes with 14% and 5% m_c water (Fig. 2). These findings are apparently contrary to the general rules of storage, which predict that water contents higher than 13% are undesirable (Labbé, 2003) and that maintaining low water contents in orthodox seeds may increase the germination percentage and seed vigour (Souza et al., 2016). Similar results were recently reported for *Calamus shendurunii* (Jacob et al., 2016), whose seeds had 35% initial water content and 97% germination.

Calamus shendurunii seed desiccation to 28% reduced the germination to 77%, and seed desiccation to lower than 14% m_c caused the complete loss of seed germinability. In *Anacardium humile*, the mean germination was only 29% when the water content was reduced to 5%, considering the three thawing methods tested. However, most plants behave differently. For example, *Prunus padus* L. seeds tolerate a desiccation range from 3.5 to 15.0% m_c and show percentages of germination similar to those of non-cryopreserved seeds (Popova et al., 2016). In *Anethum graveolens* (dill) cryopreservation, the seed water content limit ranges from 9 to 11% m_c (Almeida et al., 2007).

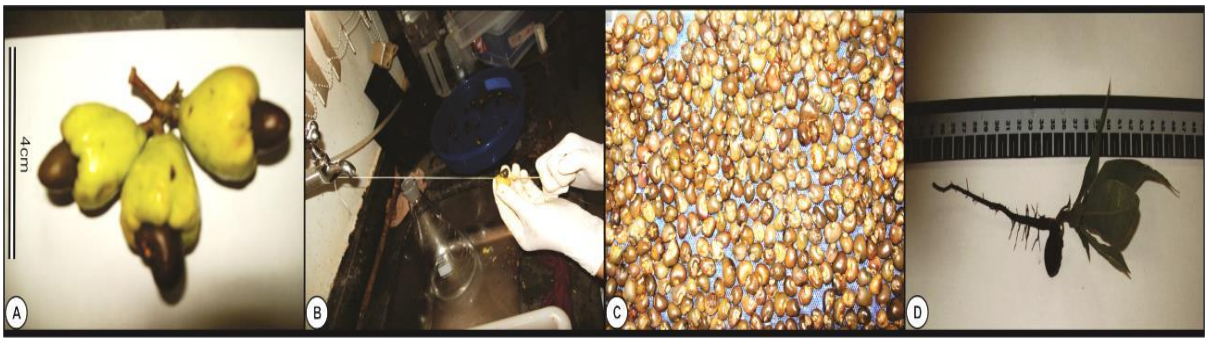

Fig 1. Different parts of *Anacardium humile* (Monkey nut) plant. (A) pseudo-fruit; (B) pulping and achene separation; (C) achenes on silica gel for drying; (D) seedling length.

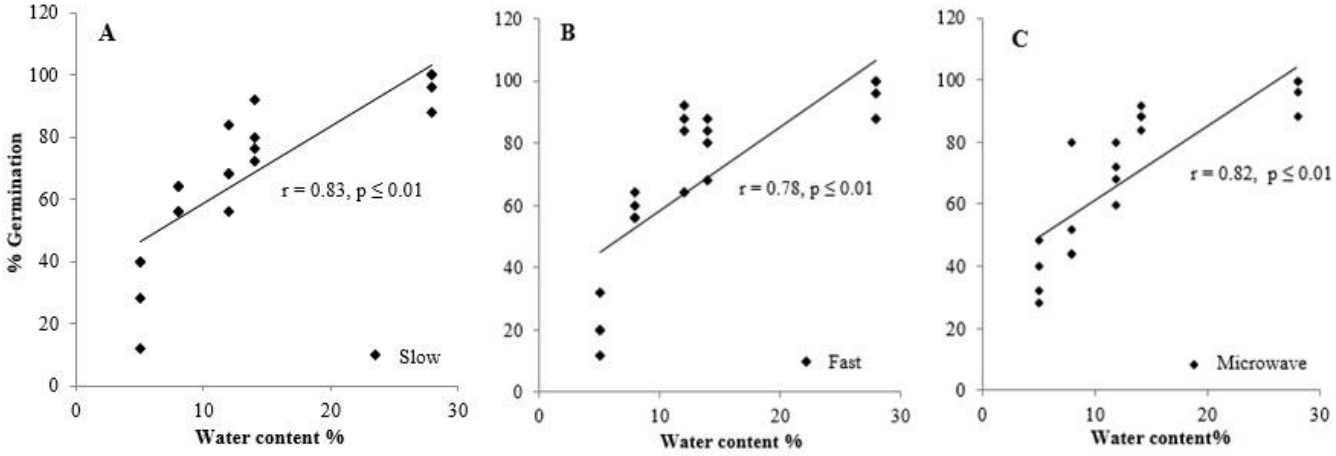

Fig 2. Germination of Cajuzinho-do-cerrado (*Anacardium humile* St. Hil.) achenes cryopreserved with different water contents and subjected to different thawing methods (A: slow; B: fast; C: microwave).

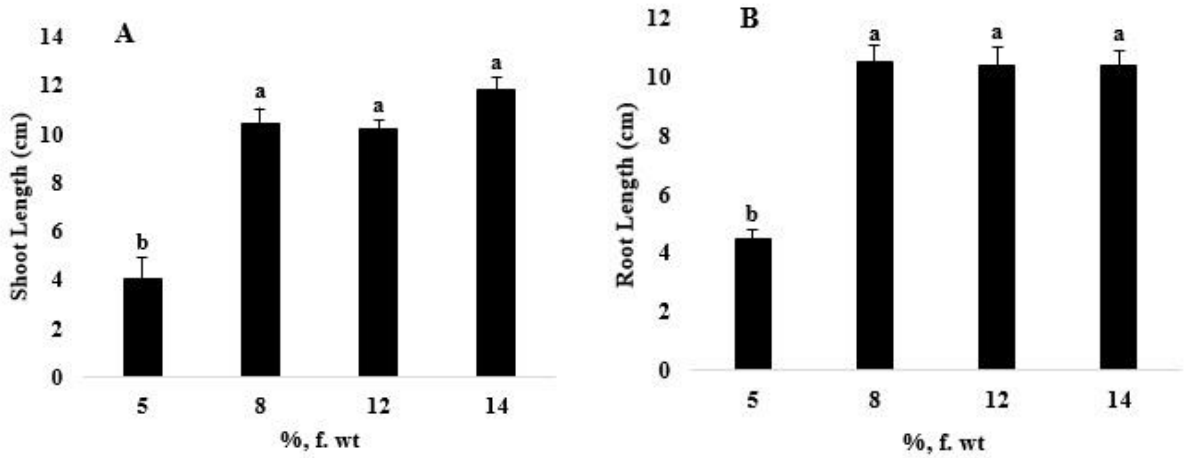

Fig 3. Shoot (A) and root (B) length of normal cajuzinho-do-cerrado (*Anacardium humile* St. Hil.) seedlings originating from cryopreserved achenes with different water contents (5%, 8%, 12%, and 14% m_c). Letters compare different water contents. The same letters indicate no difference according to the Tukey's test at 5% significance.

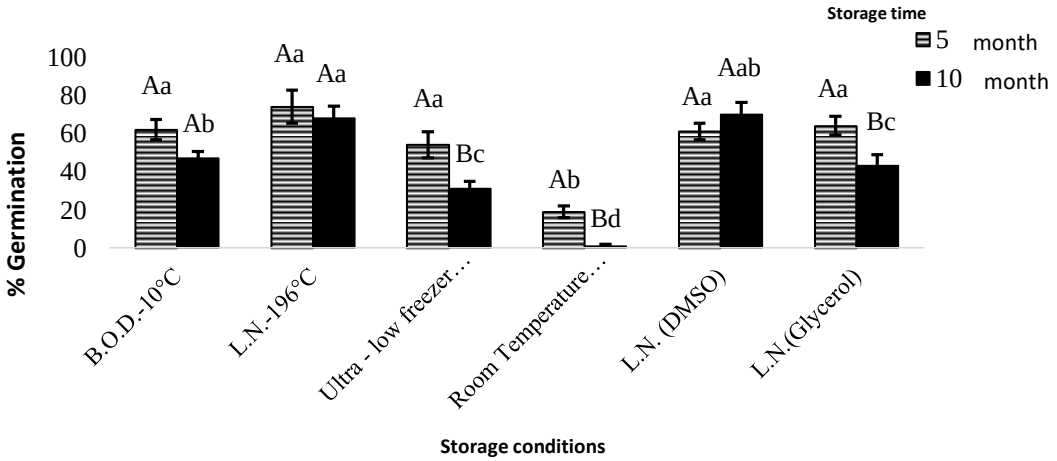

Fig 4. Germination of cajuzinho-do-cerrado (*Anacardium humile* St. Hil.) seeds subjected to different storage conditions and times. Uppercase letters compare storage times and lowercase letters compare storage conditions. The same letters indicate no difference according to the Tukey's test at 5% significance.

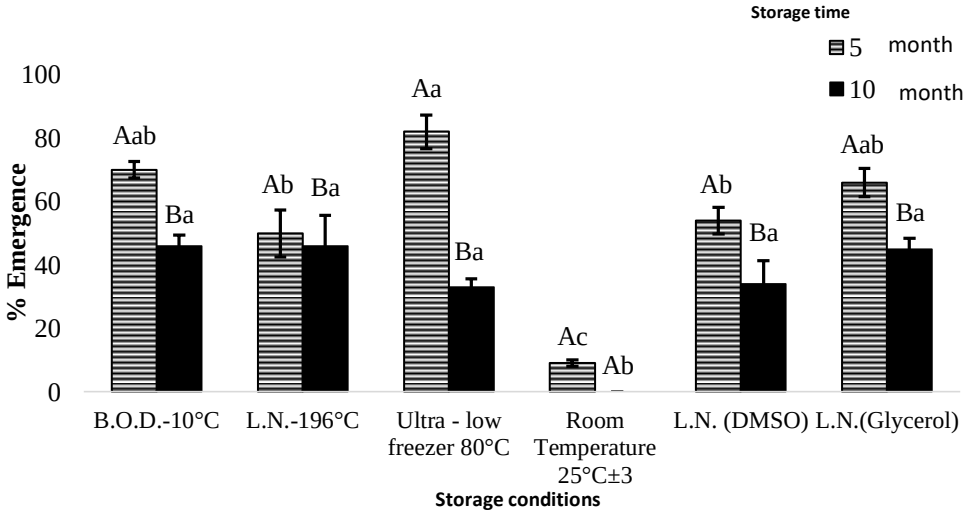

Fig 5. Emergence of cajuzinho-do-cerrado (*Anacardium humile* St. Hil.) plants originating from cryopreserved achenes in different storage conditions and times. Uppercase letters compare storage times and lowercase letters compare storage conditions. The same letters indicate no differences according to the Tukey's test at 5% significance.

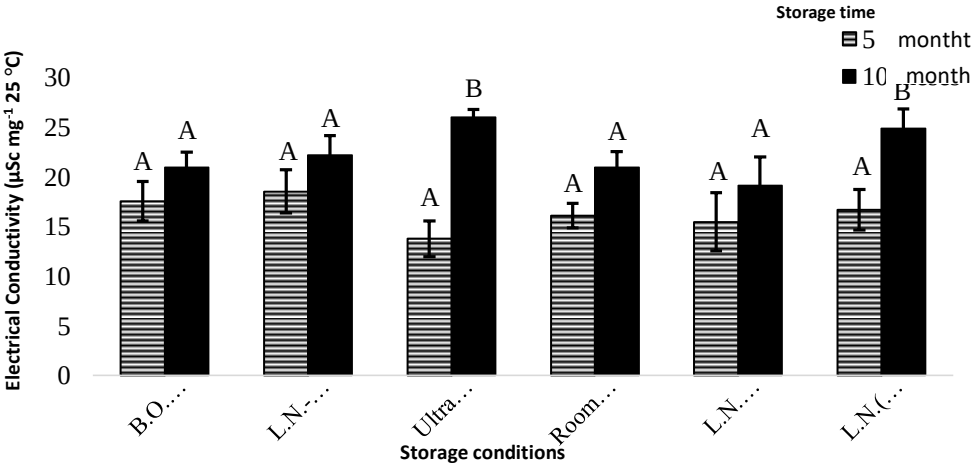

Fig 6. Electrical conductivity of cajuzinho-do-cerrado (*Anacardium humile* St. Hil.) achenes subjected to different storage conditions and times. Letters compare storage times within each condition. The same letters indicate no differences according to the Tukey's test at 5% significance.

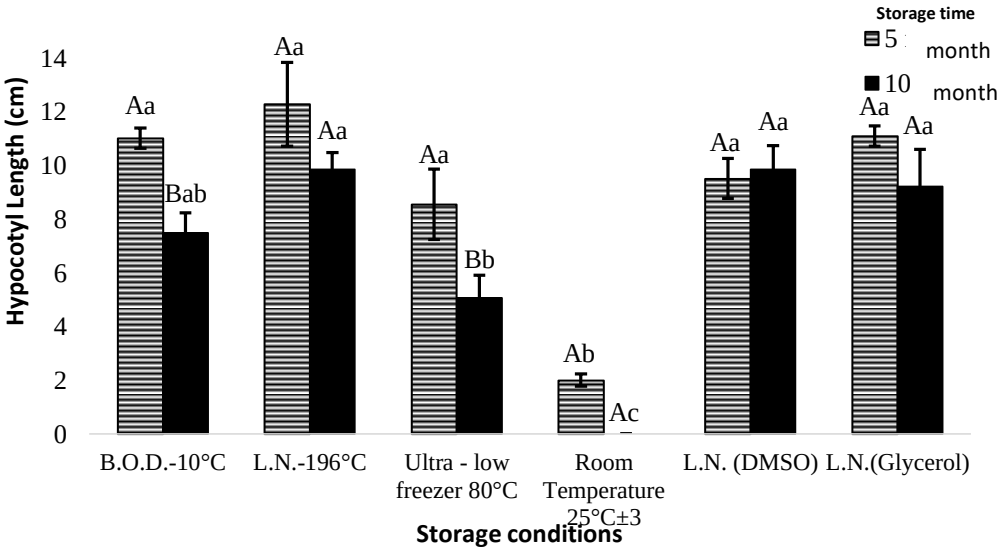

Fig 7. Hypocotyl length of cajuzinho-do-cerrado (*Anacardium humile* St. Hil.) seedlings originating from cryopreserved achenes in different storage conditions and times. Uppercase letters compare storage times and lowercase letters compare storage conditions. The same letters indicate no differences according to the Tukey's test at 5% significance.

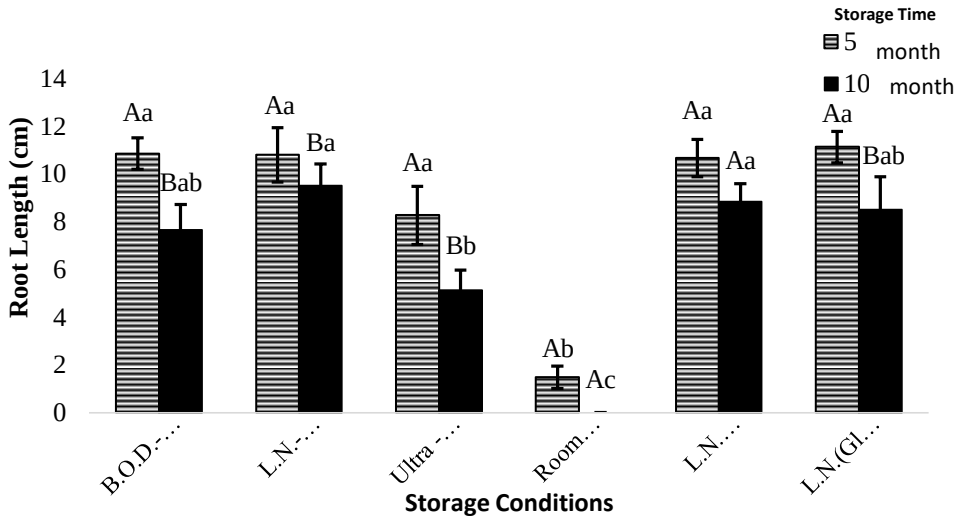

Fig 8. Root length of Cajuzinho-do-cerrado (*Anacardium humile* St. Hil.) seedling originating from cryopreserved achenes in different storage conditions and times. Uppercase letters compare storage times and lowercase letters compare storage conditions. The same letters indicate no differences according to the Tukey's test at 5% significance.

Conversely, *Stryphnodendron adstringens* (barbatimão) seed cryopreservation may be performed with water contents ranging from 6 to 9% m_c (Porto et al., 2014). Similarly, the *Physalis angulata* seeds may also be subjected to long-term storage without their germination potential being decreased by cryopreservation (Farias, et al., 2016).

Among desiccated achenes, those with water content higher than or equal to 12% m_c had a higher mean germination percentage and were more viable than achenes with high water content reduction. Conversely, Castro et al. (2001) observed no changes in germination for water contents ranging from 6 to 12% m_c when studying the water content limit for cryopreservation of *Bixa orellana* L. (urucum) seeds. Accordingly, *Astronium urundeuva* (urunday) seeds maintained their viability when cryopreserved with 6% m_c water content (Medeiros et al., 1992). Similarly, *Jatropha curcas* L. (Barbados nut) seeds maintained their vigour index when stored in liquid nitrogen or in vapour (Goldfarb et al., 2010) and their high viability when cryopreserved with 8% m_c water content (Silva et al., 2011). *Tabebuia chrysotricha* (golden trumpet tree) seeds showed higher germination when cryopreserved with 4% m_c water content (Tresena et al., 2010). Similar results were also observed for *Pinus elliottii* (slash pine) seeds (Fonseca et al., 2012).

In addition to profoundly affecting germination, *Anacardium humile* achene cryopreservation with 5% m_c water content also affected the vigour (GSI), regardless of the thawing method. Thus, this characteristic was less sensitive to low water contents than the germination percentage. Conversely, the storage in liquid nitrogen (-196 °C) of *Gossypium hirsutum* (cotton) seeds with water contents higher than 10% m_c reduced seed viability (Rocha et al.,

20009). These findings indicate that plants have specific requirements regarding the optimal water content for cryopreservation and are able to respond differently to desiccation followed by cryopreservation (Rocha et al., 2009).

Conversely, microwave thawing is more suitable for cryopreserved *Allium cepa* (onion) seeds than room temperature or water bath (Molina, et al. 2006), and the thawing method had no effect on the vigour of cajuzinho-do-cerrado. Thus, the microwave method may be chosen because it is practical.

Thus far, no such behaviour was observed in other oleaginous species. For example, water contents ranging from 4 to 10% m_c are the most appropriate for *Gossypium hirsutum* (cotton) seed cryopreservation (Rocha et al., 2009). The suitable water contents for *Sesamum indicum* (sesame; Batista, 2000) and *Ricinus communis* (castor bean) (Almeida et al., 2002) seed cryopreservation are low, ranging from 6 to 8% m_c.

In contrast to the other study characteristics, no difference was found between the electrical conductivity values of achenes with different water contents and thawing methods. This result confirms the findings of Mendes et al. (2010), who stated that the electrical conductivity test is inadequate for vigour assessment when assessing the physiological potential of castor bean seeds.

Vargas et al. (2004) noted the need for using cryoprotectant for the cryopreservation of *Origanum vulgare* (oregano) seeds. This was not the case for *Anacardium humile* because the means were not different from those found when cryopreserving the achenes in LN without cryoprotectant (71%), despite the high percentages of germination found when using cryoprotectant (LN–DMSO).

Different results were reported for *Passiflora mucronata, P. suberosa* and *P. edulis* seeds, which may be preserved using long-term cryopreservation without cryoprotectants (Araujo et al., 2016).

Notably, no decrease in physiological potential was observed in achenes cryopreserved without cryoprotectant, when compared with achenes cryopreserved with cryoprotectant. Thus, *Anacardium humile* achene cryopreservation is recommended over room temperature storage, but cryoprotection is not required. Under room temperature conditions, the high temperature combined with microbial attack quickly precludes seed germination. However, the relation between increased storage time and decreased seed physiological quality should always be considered.

The purpose of the electrical conductivity test is to measure the amount of electrolytes the seeds release into the water in which they are immersed (Fig. 6). This amount is proportional to the degree of plasma membrane disruption and seed permeability (Vieira and Krzyzanowski, 1999).

Materials and methods

Plant material collection

Cajuzinho-do-cerrado fruits (Fig. 1A) were collected from the Parque Nacional das Emas in the municipality of Mineiros-Goiás state (GO) (18° 6′ 23″ South, 52° 55′ 40″ West) at 820 m altitude. After collection, the fruits were processed at the Seed Laboratory of the Goiás Federal Institute of Education, Science and Technology (Instituto Federal de Educação, Ciência e Tecnologia Goiano – IFG) - Rio Verde Campus, and the voucher specimen was deposited in the herbarium of the Universidade Federal de Goiás – Campus Jataí, under record HJ7275/ Federal University of Goiás (Universidade Federal de Goiás – UFG) - Jataí.

The fruits were de-pulped and the achenes were separated using Nylon thread (Fi. 1B). The achenes were washed in 2% sodium hypochlorite solution for 5 min to determine the initial water content (28% m_c). Subsequently, the achenes were dried in silica gel (Fig. 1C) at a temperature of 25±2 °C until reaching 14, 12, 8 and 5% m_c water contents. Water content was determined using the oven method at 105±3 °C, for 24 h, according to the Seed Analysis Rules (Regras para Análise de Sementes – RAS; Brasil, 2009), in four 7-g subsamples.

Treatments

For cryopreservation, achenes with 14, 12, 8 or 5% mc water content were wrapped in aluminium foil and packaged in cylindrical aluminium tubes (canisters). Subsequently, the tubes were placed in cryogenic cylinders, insulated with partial vacuum and held at -196 °C for 10 days. After this period, the achenes were subjected to a slow and gradual thawing treatment, according to the following sequence of steps: -80 °C freezer, -26±2 °C freezer, 10 °C biochemical oxygen demand (BOD) incubator and 25 °C room temperature, for 1 h at each step. Achenes were also subjected to fast thawing treatments in a water bath, at a temperature of 60 °C for 8 min and in a microwave, at a power of 15,000 w for 3 min. After being thawed, the achenes were washed in distilled water and subjected to tests to assess the physiological quality.

In the storage experiment for the determination of conditions suitable for cryopreservation, *Anacardium humile* achenes were subjected to the following environments to assess the storage environment effect on their physiological quality: liquid nitrogen without cryoprotectant (-196 °C), liquid nitrogen with the cryoprotectant glycerol (10%; -196 °C), liquid nitrogen with the cryoprotectant dimethyl sulfoxide (DMSO; 10%; -196 °C), -80 °C ultra-freezer, 10 °C BOD incubator and room temperature. The achenes remained in these environments for 5 and 10 months, always wrapped in aluminium foil.

Traits measured

The physiological quality was assessed by seed germination (percentage) and vigour (germination speed index, electrical conductivity and root and shoot length).

The germination percentage was assessed using the germination test and four replicates of 25 achenes each. Seeding was performed between three sheets of germitest paper moistened with distilled water in approximately 2.5 times the dry mass of substrate. Then, the achenes were transferred to a Mangelsdorf germinator and kept at 30 °C for 32 days.

The Germination Speed Index (GSI) was determined by daily counts, beginning on day 8 after sowing, assessing the number of germinated seed. Seeds with a radicle longer than or 1 cm were considered germinated. Maguire's equation (1962) was used to calculate the GSI.

Achenes were sown in sand at 2 cm of depth and kept in the greenhouse to determine the root and shoot length (Fig. 1D). A total of 100 experimental units were sown, divided into four replicates of 25 seeds each. After total seedling emergence (50 days), 10 normal seedlings were measured in each replicate, using a millimetre ruler.

The electrical conductivity test was performed in a disposable cup with 75 mL of deionised water, at 25 °C in a BOD incubator, with readings performed after 24 h of soaking (Vieira and Krzyzanowski, 1999) with four replicates, each consisting of 10 achenes, which were weighed.

In the storage experiment for the determination of conditions suitable for cryopreservation, the achenes submitted to storage in liquid nitrogen were thawed according to the best method determined in the cryopreservation experiment. After the seeds were submitted to different storage conditions, the following parameters were evaluated: water content, germination and vigour (electrical conductivity, root length and shoot length), according to the above methods.

Statistical design

The water content limit was tested using a completely randomised 4 x 3 factorial experimental design (water contents x thawing methods) CDR, with four replicates. Each replicate consisted of 240 seeds, totalling 2880 experimental units. A correlation model was fitted to explain the relation between the different water contents tested and the percentages of germination assessed. Analysis of variance and a mean comparison test (Tukey test, at 5% significance) was chosen for biometric assessments (shoot length and root length). The tests were performed using the statistical package R (R Core Team, 2016).

The experiment storage of suitable conditions for cryopreservation was performed in a 6 x 2 factorial experimental design (six environments and two storage times), and the variance effect was tested by analysis of variance followed by the Tukey test for comparison of means, at 5% significance. The tests were performed using the statistical package R (R Core Team, 2016).

Conclusion

Reducing the water content of *Anacardium humile* seeds reduced their germination potential for cryopreservation. These seeds are tolerant to cooling but not drastic desiccation, which was indicated by a positive correlation between decreased m_c and reduced germination. Conversely, the thawing methods have no effect on the physiological quality of cryopreserved cajuzinho-do-cerrado achenes; thereby, enabling choice of the most practical thawing method (microwave). We also concluded that cryopreservation in liquid nitrogen may be used as a viable alternative for *Anacardium humile* achene conservation, despite confirming the relation between storage time and loss of achene physiological quality.

Acknowledgements

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