Cryopreservation of seeds of *Melocactus zehntneri* Braun ex Ritter f. and *Cereus gounellei* Luetzelb ex Schum k. by the vitrification method

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ABSTRACT

The Brazilian Caatinga houses a number of cacti species, which are considered endangered due to human influence. Two of them, *Melocactus zehntneri* Braun ex Ritter f. and *Cereus gounellei* Luetzelb ex Schum k., are endemic of the Brazilian Caatinga. Cryopreservation can provide a means for long-term preservation of endangered plant material. The aim of this study was to evaluate the efficiency of cryoprotectants for the cryopreservation of seeds from these two species. The treatments consisted of immersing seeds in different cryoprotectant solutions and vitrification solutions before storage in liquid nitrogen (NL) at -196 °C, as follows: T1- Control: no cryoprotectants; T2- PVS2 (10 min), T3- PVS2 + phloroglucinol 1% (10 min), T4- PVS2 (10 min) + Supercool 1%; T5- PVS2 + phloroglucinol 1% (10 min) + Supercool 1%. Seed germination was evaluated after cryopreservation. The experimental design was completely randomized with five treatments and five replicates per treatment. Seed moisture at the beginning of the experiment was 6.2% for *M. zehntneri* and 7.8% for *C. gounellei*. There were no statistically significant differences for the seed germination percentage among the different treatments. Therefore, we concluded that seed of these two cacti species can be cryopreserved directly without the need for cryoprotectant solutions.

Keywords: Conservation, germination, liquid nitrogen, liquid nutrient, organs, tissues.

INTRODUCTION

In Brazil, the Caatinga has a rich diversity of cacti species. The most endemic cacti catalogued include species in the genera *Cereus*, *Pilosocereus* and *Melocactus* (Taylor & Zappi, 2004; Queiroz, 2006). However, the Caatinga biome is in danger due to human action, which have placed many of the existing plant species in this environment under vulnerable and endangered conditions.

Cryopreservation is a technique that allows the long-term storage and conservation of plant material, including seeds, pollen, and tissues, and can assist with the maintenance, preservation and the development of studies of the plant species that occur in endangered habitats. Cryopreservation allows the preservation of biological material in liquid nitrogen at extremely low temperatures, in the liquid (-196 °C) or vapor phase (-150 °C), while maintaining the original characteristics of the material and allowing recovery after thawing (Sakai & Engelmann, 2007; Vendrame & Faria, 2011). Cryopreservation allows the long-term preservation of different plant materials, in contrast to short-term storage when using other techniques (Bajaj, 1995; Galdiano, Lemos, Faria, & Vendrame, 2012; Lopes, Almeida, Carvalho, & Bruno, 2013). However, due to ultra-low temperature of liquid nutrient, tissues could be damaged due to the formation of intracellular ice crystals. Therefore, the use of cryoprotectant solutions is essential for the successful recovery of organs and tissues and regeneration of plants after cryopreservation.

Vitrification is the most popular technique used in cryopreservation protocols, being easy and efficient, not requiring the use of sophisticated equipment, and with a high recovery percentage and survival of the biological material cryopreserved. Hirano, Godo, Mii and Ishikawa (2005) utilized the vitrification method in seeds of *Bletilla striata* and observed a high survival rate in the seeds, with no reduction in germination.

Thammasiri (2000) also found satisfactory results, with the recovery of 62% germination in seeds of *Doritis pulcherrima* treated with PVS2 before immersion in liquid nitrogen. Seeds of these species directly immersed in liquid nitrogen without cryoprotectants and vitrification treatments did not germinate or survive. Vitrification can be an efficient method for cryopreservation as it utilizes cryoprotectant solutions that are highly concentrated and pre-cooled at very low temperatures, thus resulting in a cooled viscous and stable solutions, which prevents the formation of ice crystals inside the cells and the injuries that can cause cell and tissue death. Vitrification also restricts the diffusion of products within the cell such as water and other substrates, leading to a state of metabolic quiescence, and resulting in the prevention of chemical reaction that are dependent on the diffusion process. Due to the characteristics of the 'vitreous state' of the vitrification solutions, the deterioration of biological system can be suppressed (Santos, 2000).

Hirano, Yukawa, Miyoshi and Mii (2011) reported successful cryopreservation of seeds in seven species of *Cymbidium* using vitrification treatments. The most common vitrification solution used in cryopreservation protocols is the -pre-vitrification solution number 2 (PVS2) developed by Sakai, Kobayashi and Oiyama (1990). The solution is composed of 30% glycerol, 15% ethylene glycol, 15% dimethyl sulfoxide (DMSO) and 0.15 mol L^{-1} sucrose (Sakai, Kobayashi, & Oiyama, 1990). Sucrose at 0.4 mol L^{-1} is sometimes added to the PVS2 as a cryoprotectant additive (Nishizawa, Sakai, Amano, & Matsuzawa, 1993). Variations of the PVS2 solution are also used in cryopreservation protocols, such as PVS3 and PVS4.

The objective of this study was to verify the germinability in seeds of these two species of cacti: *Melocactus zehntneri* Braun ex Ritter f. and *Cereus gounellei* Luetzelb ex Schum k. that were submitted to cryopreservation by the vitrification method.

MATERIALS AND METHODS

Fruits of two cacti species, *Melocactus zehntneri* Braun ex Ritter F. and *Cereus gounellei* Luetzelb ex Schum K. were collected from plants in the caatinga biome, in the Tejuçuoca city (3°59'20''S, 39°34'51''W), at an altitude of 140 meters, in the state of Ceará, Brazil on February, 25th, 2014. The fruits were collected upon opening in morning to prevent birds from eating them. The collected fruits were packed in styrofoam boxes and taken to the floriculture laboratory in the Department of Plant Sciences of Federal University of Ceará, where the seeds were washed, pulped, dried in ambient temperature (25 °C) environment, and then stored in plastic containers under refrigeration (8 °C).

The seeds were manually extracted, dried and placed in paper bags for storage and transportation. Cryopreservation experiments were conducted in the Ornamental Horticulture and Biotechnology Laboratory at the Tropical Research and Education Center, Institute of Food and Agricultural Science, University of Florida, located in Homestead, Florida, USA. Seeds were maintained in a desiccator for 10 days until constant weight.

Seeds were submitted to a germination test prior to cryopreservation, whereby they were placed in polystyrene Petri dishes, containing seed germination paper moistened with distilled water at a rate of 2.5 times the weight of the dry paper (Ministério da Agricultura, Pecuária e Abastecimento [MAPA], 2009). Twenty-five seeds were used per Petri dish.

For cryopreservation treatments, seeds were placed into 2-ml cryotubes containing 1 mL of 2M glycerol solution for 20 minutes at 25 °C, as described by Nishizawa et al. (1993). After 20 minutes, the glycerol solution was removed and PVS2 was added. This PVS2 pre-vitrification solution is composed of 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) dimethyl sulfoxide (DMSO) and 0.4M sucrose (Sakai et al., 1990). Treatments prior to cryopreservation consisted of the addition of either 1% phloroglucinol for 10 minutes at 0 °C or 1% Supercool for 10 min at 0 °C. Afterwards, seeds were immersed in liquid nitrogen (-196°C) for 10 days. The following cryopreservation treatments were used:

T1- Control: no cryoprotectants
T2- PVS2 (10 min)
T3- PVS2 + phloroglucinol 1% (10 min)
T4- PVS2 + Supercool 1% (10 min)
T5- PVS2 + phloroglucinol 1% + Supercool 1% (10 min)

After 10 days, the cryotubes were removed and rapidly thawed in warm water (40 °C) for 1.5 min. Seeds were removed from cryotubes and rinsed with distilled water three times, under vacuum to facilitate

removal of the cryoprotectant solution. Seeds were then placed into Petri dishes inside a germination chamber with a 16/8 h photoperiod and temperature of 25 °C ± 2 °C. After 15 days, the percentage of germinated seeds was calculated for each treatment.

The experiment was completely randomized, with five treatments and five replications of 20 seeds per treatment. The data were subjected to analysis of variance (ANOVA) and means were compared by Tukey's test at 5% probability.

RESULTS AND DISCUSSION

The germination tests for both cacti species were 83% for *M. zehntneri* and 94% for *C. gounellei.* Initial seed moisture was 13.3% initially for both species, but after 7 days in desiccator, moisture dropped to 6.2% for *M. zehntneri* Braun and 6.0% for *C. gounellei*. Stanwood (1985) reported that seed moisture varying from 10 to 30% can lead to a reduction in germination after cryopreservation due to the formation of intracellular ice crystals during the freezing process, causing cell rupture and death. Therefore, low moisture content is critical for achieving success in cryopreservation of plant material (Benson, 2008; Engelmann, 2011). Seeds from several cacti species are considered orthodox and optimum moisture content for storage should be between 10% and 12% (Marcos Filho, 2005). Bewley and Black (1986) recommend lower moisture content, ranging from 8 to 9%. In this study, the reduction of moisture content in the cacti species evaluated was carefully addressed to reduce moisture content in the range of 6.0% and therefore within the recommended range to prevent cell damage.

After removal from cryopreservation, seeds from both species were placed in germination for 15 days, when maximum germination percentages were observed. For *M. zehntneri,* germination ranged from 77 to 89%. For *C. gounellei,* germination ranged from 79 to 95%. However, no significant differences were observed among treatments for both species (Figure 1, 2,3 and 4). All treatments had germination higher than 77% for *M. zehntneri* and higher than 79% for *C. gounellei,* and they were not significantly different from the controls (83% and 94%, respectively) whereby seeds were directly immersed in LN with no pre-vitrification solutions or cryoprotectants (Figures 1, 2). Seed germination was achieved, and seedlings had healthy appearance with no abnormalities for the two different species (Figures 3 and 4).

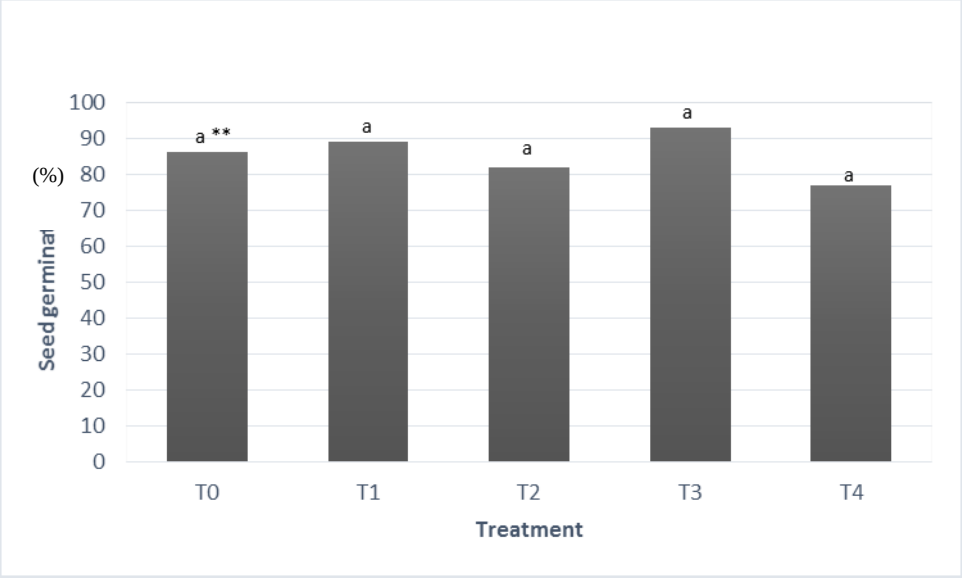

Figure 1. Germination (%) of cryopreserved seeds of *Melocactus zehntneri* Braun ex Ritter f. pretreated with cryoprotectant solutions, after 15 days recovery.
**T0- Control: no cryoprotectants (no glycerol, no PVS2, no Phloroglucinol); T1- PVS2 (10 min), T2- PVS2 + phloroglucinol 1% (10 min); T3- PVS2 + Supercool 1% (10 min); T4- PVS2 + phloroglucinol 1% + Supercool 1% (10 min).

These results can be explained by the behavior of orthodox seeds, which have the capacity to tolerate drying to low moisture content without damage to their metabolism, as well as preserving their integrity during long-term cold storage (Marcos Filho, 2005). Orthodox seeds allow the potential for conservation within certain limits of temperature. Marcchi et al. (2013) reported successful cryopreservation and

subsequent recovery and germination of seeds of three cacti species from Caatinga; *Dioscocactus zehntneri*, *Pilosocereus gounellei* and *Stephanocereus luetzelburgii,* adding that the germination percentages for the three species were not reduced by exposure to liquid nitrogen. Similarly, germination of *Cereus jamacaru* and *Microchanthus flaviflorus* were not affected by cryopreservation (Veiga-Barbosa, González-Benito, Assis, & Pérez-García, 2010).

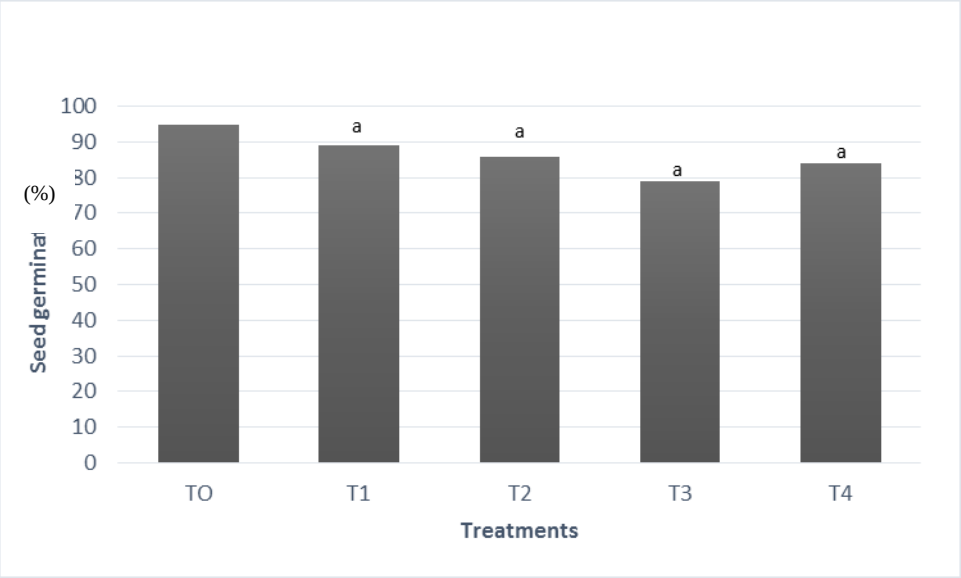

Figure 2. Germination (%) of cryopreserved seeds of *Cereus gounellei* Luetzelb ex Schum k. pretreated with cryoprotectant solutions, after 15 days recovery.
**T0- Control: no cryoprotectants (no glycerol, no PVS2, no Phloroglucinol); T1- PVS2 (10 min), T2- PVS2 + phloroglucinol 1% (10 min); T3- PVS2 + Supercool 1% (10 min); T4- PVS2 + phloroglucinol 1% + Supercool 1% (10 min).

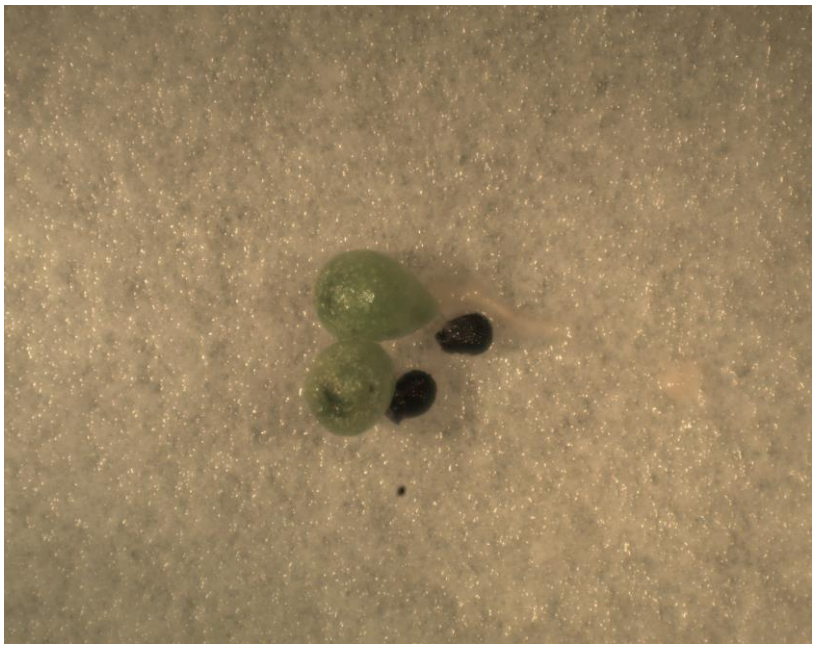

Figure 3. Detail of germination of cryopreserved seeds of *Melocactus zehntneri* Braun ex Ritter f. after 15 days recovery.

In contrast, exposure to liquid nitrogen has been reported to increase germination percentage in leguminous seeds (Stanwood, 1980). Similarly, Rocha (2009) also found that seed germination and vigor increased in cotton with an increase in exposure time to liquid nitrogen. These results may be related to the degradation of abscisic acid at low temperature, which inhibits germination (Lee & Looney, 1978; Pinfield, Stutchburry, & Bazaid, 1987; Powell, 1987). It is possible that the exposure to liquid nitrogen for 10 days contributed to the germination of cryopreserved seeds of the cacti species in this study.

Figure 4. Detail of germination of cryopreserved seeds of *Cereus gounellei* Luetzelb ex Schum k. after 15 days recovery. The figure shows seeds germinating after cryopreservation under treatment 3, PVS2 + Supercool 1% (10 min).

CONCLUSIONS

Based on the results observed, it is evident that the cryoprotectant solution is not necessary for the cryopreservation of seeds for both *M. zehntneri* and *C. gounellei*. Seeds of these two species can be directly immersed in liquid nitrogen for long-term storage without negative effects on germination after recovery and removal from LN. Germinating seedlings had normal growth and development and no abnormalities were observed

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