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Cryopreservation of seeds of *Encholirium spectabile* Martius ex Schultes f. by the vitrification method[1]

Criopreservação de sementes de *Encholirium spectabile* Martius ex Schultes f. pelo método da vitrificação

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ABSTRACT - The bromeliad *Encholirium spectabile* Martius ex Schultes f. is a endemic species of the Caatinga. The aim of this study was to evaluate the efficiencies of cryoprotectant in bromeliad seeds. The treatments consisted of immersing seeds in different cryoprotectant solutions and vitrification solutions before immersion in liquid nitrogen (NL) at -196 °C, as the following treatments: T1- Control: without cryoprotectants; T2- glycerol 2M (20 min) + PVS2 (10 min), T3- glycerol 2M (20 min) + PVS2 with phloroglucinol 1% (10 min), T4- sucrose 0.4M (20 min) + PVS2 (10 min); T5- sucrose 0.4M (20 min) + PVS2 with phloroglucinol 1% (10 min); T6- glycerol 2M (20 min) + sucrose 0.4M (20 min) + PVS2 (10 min), T7- glycerol 2M (20 min) + sucrose 0.4M (20 min) + PVS2 with phloroglucinol 1% (10 min). The experimental design was a completely randomized design with seven treatments and five replicates per treatment. Data were subjected to analysis of variance (ANOVA) and means were compared by the Tukey test at 5%. The seed moisture at the beginning of the experiment was 8.4%. There were statistically significant for the variables percentage of seeds germinated and seedling length, but for the variable seedling dry weight, T3 treatment was presented a value statistically high to T1, T4, T5 and T6, but did not differ from T2 and T7 treatments. It is concluded that for the cryopreservation of seeds of *Encholirium spectabile* is not necessary to use cryoprotectant solutions.

Key words: Bromeliaceae. Conservation. Germination. Liquid nitrogen.

RESUMO - A bromélia *Encholirium spectabile* Martius ex Schultes f. é uma espécie endêmica da Caatinga. O objetivo deste trabalho foi avaliar a eficiência de soluções crioprotetoras em sementes de bromélia. Os tratamentos consistiram da imersão das sementes em soluções crioprotetoras e de vitrificação, antes da imersão em nitrogênio líquido (NL) (-196 ºC), conforme os tratamentos a seguir: T1 - controle: sem crioprotetores; T2 - glicerol 2M (20 min) + PVS2 (10 min); T3 - glicerol 2M (20 min) + PVS2 com floroglucinol a 1% (10 min); T4 - sacarose 0,4M (20 min) + PVS2 (10 min); T5 - sacarose 0,4M (20 min) + PVS2 com floroglucinol a 1% (10 min); T6 - glicerol 2M (20 min) + sacarose 0,4M (20 min) + PVS2 (10 min); T7 - glicerol 2M (20 min) + sacarose 0,4M (20 min) + PVS2 com 1% de floroglucinol (10 min). O delineamento experimental foi o inteiramente casualizado com sete tratamentos e cinco repetições. Os dados foram submetidos à análise de variância (ANOVA) e as médias comparadas pelo teste de Tukey a 5%. O grau de umidade das sementes no início do experimento foi de 8,4%. Não apresentaram diferença estatística significativa para as variáveis porcentagem de sementes germinadas e comprimento de plântula, porém, para a variável massa seca de plântula, o tratamento T3 apresentou valor estatisticamente superior aos tratamentos T1, T4, T5 e T6, mas não diferiu dos demais tratamentos. Para a criopreservação de sementes da bromélia *Encholirium spectabile* não é necessário a utilização de soluções crioprotetoras.

Palavras-chave: Bromeliaceae. Conservação. Germinabilidade. Nitrogênio líquido.

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INTRODUCTION

The family *Bromeliaceae* comprises 3140 epiphytic, terricolous and saxicolous species, grouped into 58 genera; its distribution is neotropical, occurring in humid forest habitats and xerophytic environments (GIVNISH *et al*., 2011). The species *Encholirium spectabile* is a saxicolous, xerophylic bromeliad, endemic to the Caatinga, and occurring in the Cerrado, Rupestral and Atlantic Forest regions of Brazil. It is found in the Brazilian states of Piauí, Rio Grande do Norte, Ceará, Paraíba, Pernambuco, Sergipe and Bahia (FORZZA, 2005). Due to human action, the animal and plant species existing in these biomes have become vulnerable and endangered.

The cryopreservation of seeds can therefore assist in the maintenance, preservation and later study of the plant species that occur in these environments. Cryopreservation is a method of preserving biological material at extremely low temperatures of down to -196 °C in liquid or vapour phase nitrogen (-150 °C), maintaining the original characteristics of the material after thawing (SAKAI; ENGELMANN, 2007; VENDRAME; FARIA, 2011), as verified for some species of bromeliads (PEREIRA; CUQUEL; PANOBIANCO, 2010; TARRÉ *et al*., 2007).

It is therefore possible to preserve different plant materials for long periods, which by other methods would not be possible (BAJAJ, 1995; GALDIANO JUNIOR *et al*., 2012; LOPES *et al*., 2013). However, to prevent damage to the membranes of these materials due to the formation of ice crystals during the freezing process, cryoprotectant techniques can be used.

Vitrification is the most widely used technique for the cryopreservation of explants, being easy to carry out, not requiring the use of programmable equipment, and above all, having a high recovery percentage. Hirano *et al*. (2005), studying the vitrification method in seeds of *Bletilla striata*, observed a high survival rate in the seeds, with no reduction in germination rate after thawing. Satisfactory results were also found by Thammasiri (2000), with the recovery of 62% germination in seeds of *Doritis pulcherrima* treated with PVS2 before being frozen in liquid nitrogen, while seeds placed into liquid nitrogen with no pre-treatment did not germinate.

Vitrification is an efficient method against freezing, as the cryoprotectant solutions used are highly concentrated and cool at very low temperatures, thus causing solidification of the structures in such a way that they remain viscous and stable, besides preventing the formation of ice crystals inside the cells and the injuries they may cause. Vitrification restricts the diffusion of substrates and products within the cell, leading to a state of metabolic quiescence, and resulting in the prevention of chemical reactions which are dependent on the diffusion process. Due to these characteristics of the vitreous state, the deterioration of biological systems is suppressed (SANTOS, 2000). According to Hirano *et al*. (2011), the use of this method was efficient in the cryopreservation of seeds in seven species of *Cymbidium*, indicating that it may also be effective for other species.

PVS2 is one of the most used solutions in the vitrification process. Originally composed of 30% glycerol, 15% ethylene glycol, 15% dimethyl sulfoxide (DMSO) and 0.15 mol L^{-1} sucrose (SAKAI; KOBAYASHI; OIYAMA, 1990), later a concentration of 0.4 mol L^{-1} sucrose was used (NISHIZAWA *et al*., 1993).

The aim of this study was to evaluate germinability in seeds of the bromeliad *Encholirium spectabile*, submitted to cryopreservation by the vitrification method.

MATERIAL AND METHODS

The experiment was conducted in the Cryopreservation Department of the Laboratory for Plant Science, in the Centre for Agricultural Sciences of the State University of Londrina (UEL), in Pernambuco, Brazil.

Fruits of the bromeliad *Encholirium spectabile* Martius ex Schultes f. were harvested from plants in the semi-arid region of the State of Ceará, in the municipality of Tejuçuoca (3°59'20" S, 39°34'51" W, at an altitude of 140 m), at the start of spontaneous aperture, and left to finish drying in the shade. The seeds were extracted manually and stored in paper bags for 5 days at 25 $\pm$2 °C, until the experiment was set up. The seeds were evaluated at the start of the experiment for moisture content, and for germinability using the germination test.

The germination test was carried out in crystal polystyrene boxes (Gerbox®), on Germitest® paper moistened with an amount of distilled water 2.5 times the weight of the dry paper (BRASIL, 2009). Twenty seeds were used per box with five boxes per treatment, which were kept in a growth chamber at 25 ± 2 °C, under a photoperiod of 16 hours at an intensity of 25 μmol m^{-2} s^{-1}.

The moisture content of the seeds was obtained by the gravimetric method using an oven at 105 $\pm$ 3 °C, based on the weight of water removed from the seeds after remaining in the oven for 24 hours (BRASIL, 2009).

The seeds were placed into cryotubes containing either 1 mL of 2M glycerol solution, 0.4M sucrose or a mixture of 2M glycerol and 0.4M sucrose, for 20 min at 25 °C, as described by Nishizawa *et al*. (1993).

After the solutions were removed, PVS2 plant vitrification solution was added, composed of 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) dimethyl sulfoxide (DMSO) and 0.4M sucrose (SAKAI; KOBAYASHI; OIYAMA, 1990). The seeds were immersed in this solution, with or without the addition of 1% phloroglucinol, for 10 minutes at a temperature of 0 °C, for subsequent immersion in liquid nitrogen (LN) at -196 °C.

The following treatments were used:

T1 - control: no glycerol, no PVS2, no phloroglucinol;

T2 - 2M glycerol (20 min) + PVS2 (10 min);

T3 - 2M glycerol (20 min) + PVS2 with 1% phloroglucinol (10 min);

T4 - 0.4M sucrose (20 min) + PVS2 (10 min);

T5 - 0.4M sucrose (20 min) + PVS2 with 1% phloroglucinol (10 min);

T6 - 2M glycerol (20 min) + 0.4M sucrose (20 min) + PVS2 (10 min);

T7 - 2M glycerol (20 min) + 0.4M sucrose (20 min) + PVS2 with 1% phloroglucinol (10 min).

After 24 hours with the seeds immersed in LN, the cryotubes were removed and rapidly reheated to a temperature of 40 °C in a water bath for 1.5 minutes. Removal of the cryoprotectant solutions was carried out with the help of a sterile, disposable plastic transfer pipette. The seeds were then rinsed with autoclaved distilled water and submitted to the germination test, following the above methodology.

Seed survival was determined by the percentage of germinated seeds at twelve days after sowing, the period at which the maximum percentage of seed germination was achieved.

After 60 days, the length (cm) and dry weight (g) of the seedlings were evaluated. Seedling length was determined with a graduated rule, using as reference the distance from the radicle apex to the apex of the last expanded leaf. To determine seedling dry weight, a forced air circulation oven was used at 60 °C until constant weight, reached after 48 hours; for this purpose, an electronic scale with a precision of 0.001g was used.

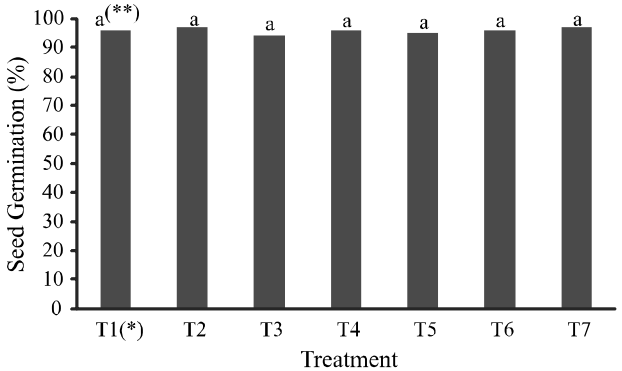
The experimental design was completely randomised, with seven treatments and five replications made up of 20 seeds. The data were subjected to variance analysis (ANOVA) and the means compared by Tukey's test at 5%.

RESULTS AND DISCUSSION

The germination tests were carried out under 16 h of light, as it was found by Tarré *et al*. (2007) that for six species of this genus the process of seed germination only takes place in the presence of light. In the evaluation to characterise the batch of seeds performed after harvesting, germination of 98% and a moisture content of 8.44% were found. A seed moisture content between 10 and 30% leads to a reduction in germination rate after cryopreservation, due to the formation of intracellular ice during the freezing process (STANWOOD, 1985) and the consequent rupture of the cell membranes. A low moisture content in the tissue is therefore critical to achieving success in the cryopreservation of plant materials (BENSON, 2008; ENGELMANN, 2011).

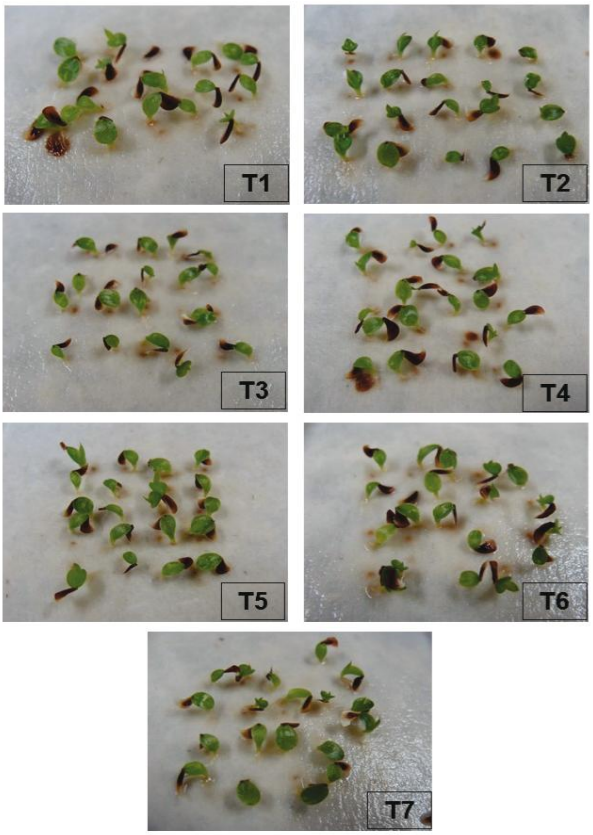
For the germination test carried out after the seeds were frozen in liquid nitrogen and thawed out in a water bath, the percentage of seed germination remained high, ranging from 94 to 97%, with no statistically significant difference between treatments (Figures 1 and 2) or between seed germinability after harvest (98%). The maximum percentage of seed germination was at 12 days after sowing. However, Tarré *et al*. (2007) found maximum germination for six species of the genus *Encholirium* at 16 days after sowing and at temperatures of from 20 to 30 °C.

Figure 1 - Percentage of germination in cryopreserved seeds of the bromeliad *Encholirium spectabile* Martius ex Schultes f., pretreated with cryoprotectant solutions, at twelve days after sowing on sheets of moistened Germitest® paper



(*)T1 - control: no glycerol, no PVS2, no phloroglucinol; T2 - 2M glycerol (20 min) + PVS2 (10 min); T3 - 2M glycerol (20 min) + PVS2 with 1% phloroglucinol (10 min); T4 - 0.4M sucrose (20 min) + PVS2 (10 min); T5 - 0.4M sucrose (20 min) + PVS2 with 1% phloroglucinol (10 min); T6 - 2M glycerol (20 min) + 0.4M sucrose (20 min) + PVS2 (10 min); T7 - 2M glycerol (20 min) + 0.4M sucrose (20 min) + PVS2 with 1% phloroglucinol (10 min). (**) Mean values followed by the same letter do not differ by Tukey's test at $p \leq 0.05$

Figure 2 - Germination in cryopreserved bromeliad seeds (*Encholirium spectabile* Martius ex Schultes f.), pretreated with cryoprotectant solutions



T1 - control: no glycerol, no PVS2, no phloroglucinol; T2 - 2M glycerol (20 min) + PVS2 (10 min); T3 - 2M glycerol (20 min) + PVS2 with 1% phloroglucinol (10 min); T4 - 0.4M sucrose (20 min) + PVS2 (10 min); T5 - 0.4M sucrose (20 min) + PVS2 with 1% phloroglucinol (10 min); T6 - 2M glycerol (20 min) + 0.4M sucrose (20 min) + PVS2 (10 min); T7 - 2M glycerol (20 min) + 0.4M sucrose (20 min) + PVS2 with 1% phloroglucinol (10 min)

It possible to infer from the above that these seeds display the behaviour of orthodox seeds, as they tolerate drying to a low moisture content without damage to their metabolism, and can be preserved during storage for long periods. Theoretically, the potential for conservation in orthodox seeds is inversely related to seed moisture content and room temperature, within certain limits (MARCOS FILHO, 2005).

Further, according to the aforementioned author, for the storage of orthodox seeds, the moisture content should be between 10 and 12%; while Bewley and Black (1986) recommend percentages of between 8 and 9%. Therefore as a general rule, the cryopreservation of orthodox seeds can be successfully carried out when the seeds have a naturally low moisture content.

However, for the species *Encholirium heloisae*, *E. magalhaesii*, *E.reflexum*, *E. subsecundum*, *E. scrutor*, *Dyckia sordida* and *D. usina*, Tarré *et al.* (2007) found that there was no negative effect from the cryopreservation of seeds on germination percentage, when the seed moisture content in these species ranged from 11.2 to 28.2%. Lower temperatures may therefore be important for maintaining seed viability in some species of bromeliads, if the conservation of germplasm is to be considered, as per Tarré *et al.* (2007) and Pereira, Cuquel and Panobianco (2010).

Testing seed tolerance in various leguminous species at ultra-low temperatures, Stanwood (1980) noted that for most, increasing the exposure time to liquid nitrogen increased the percentage of germination. Agreeing with the same author, Rocha (2009) found that with an increase in the time cotton seeds remain in liquid nitrogen, seed germination and vigour also increase. This may be related to the degradation at low temperatures of abscisic acid, a germination inhibitor (LEE; LOONEY, 1978; PINFIELD; STUTCHBURRY; BAZAID, 1987; POWELL, 1987).

For all treatments, the germinated seeds and seedlings displayed normal growth and development, i.e. morphologically similar to the control seedlings, which were not subject to cryopreservation (Figure 3).

Figure 3 - A cryopreserved bromeliad seedling (*Encholirium spectabile* Martius ex Schultes f.) pre-treated with cryoprotectant solution, at sixty days after germination

For the variable of seedling length, which ranged from 1.95 to 2.17 cm, the treatments under test displayed no significant average effect. However, for seedling dry weight, treatment T3 (2M glycerol + PVS2 with 1% phloroglucinol) had a statistically higher average than treatments T1 (control), T4 (0.4M sucrose + PVS2), T5 (0.4M sucrose + PVS2 with 1% phloroglucinol) and T6 (2M glycerol + 0.4M sucrose + PVS2), but was no different to treatments T2 (2M glycerol + PVS2) and T7 (2M glycerol + 0.4M sucrose + PVS2 with 1% phloroglucinol) (Table 1). Such treatments may have had an influence on seed vigour and consequently on the seedlings, since these cryoprotectants can cause cytotoxicity and osmotic stress, leading to cell death or modifying the morphogenetic response (KARTHA, 1985; SAKAI, 1995). Vendrame and Faria (2011) noted that the use of PVS2 vitrification solution plus 1% phloroglucinol resulted in a high recovery rate of cryopreserved protocorms in *Dendrobium nobile*. The same was observed by Galdiano Junior *et al*. (2012) in seeds of a *Dendrobium* hybrid.

Table 1 - Seedling length (SL) and seedling dry weight (SDW) in the bromeliad, *Encholirium spectabile* Martius ex Schultes f., at 60 days after germination

Treatment	SL (cm)	SDW (g)
T1 - Control	2.17(ns)	0.0240 bc(*)
T2 - 2M Glycerol (20 min) + PVS2 (10 min)	2.14	0.0263 abc
T3 - 2M Glycerol (20 min) + PVS2 (10 min) + 1% Phloroglucinol	2.03	0.0308 a
T4 - 0.4M Sucrose (20 min) + PVS2 (10 min)	2.03	0.0228 c
T5 - 0.4M Sucrose (20 min) + PVS2 (10 min) + 1% Phloroglucinol	1.95	0.0235 bc
T6 - 2M Glycerol + 0.4M Sucrose (20 min) + PVS2 (10 min)	2.07	0.0235 bc
T7 - 2M Glycerol + 0.4M Sucrose (20 min) + PVS2 (10 min) + 1% Phloroglucinol	2.07	0.0280 ab
F	1.263	6.875
CV (%)	27.76	8.81

(ns) There was no statistically significant difference between treatments; (*) Mean values followed by the same letter do not differ by Tukey's test at $p \leq 0.05$

CONCLUSIONS

1. For the cryopreservation and subsequent recovery of bromeliad seeds, the use of cryoprotectant solutions is not necessary;
2. Bromeliad seeds can be stored in liquid nitrogen without harming germination.

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