

Cryopreservation of *Adenium obesum* seeds in liquid nitrogen







Abstract – The objective of this work was to evaluate cryoprotectants on the physiological quality of *Adenium obesum* seeds preserved in liquid nitrogen. The following treatments were used: T1, control, without cryoprotectant and without immersion in liquid nitrogen; T2, with immersion in liquid nitrogen and without cryoprotectant; T3, with 0.4 mol L⁻¹ sucrose; T4, with 0.8 mol L⁻¹ sucrose; T5, with 1.0 mol L⁻¹ glycerol; T6, with 2.0 mol L⁻¹ glycerol with PVS1; T7, with PVS1; T8, with PVS2; T9, with PVS2 + phloroglucinol (modified PVS2); and T10, with PVS3. *Adenium obesum* seeds show tolerance to immersion and storage in liquid nitrogen and do not need cryoprotectants to maintain their physiological quality when stored in a water content of 7.5%.

Index terms: *Adenium obesum*, cryoprotectants, freezing tolerance, seed germination.


Criopreservação de sementes de *Adenium obesum* em nitrogênio líquido

Resumo – O objetivo deste trabalho foi avaliar crioprotetores na qualidade fisiológica de sementes de *Adenium obesum* preservadas em nitrogênio líquido. Foram utilizados os seguintes tratamentos: T1, controle, sem crioprotetor e sem imersão em nitrogênio líquido; T2, com imersão em nitrogênio líquido e sem crioprotetor; T3, com 0,4 mol L⁻¹ de sacarose; T4, com 0,8 mol L⁻¹ de sacarose; T5, com 1,0 mol L⁻¹ de glicerol; T6, com 2,0 mol L⁻¹ de glicerol com PVS1; T7, com PVS1; T8, com PVS2; T9, com PVS2 + floroglucinol (PVS2 modificado) e T10, com PVS3. As sementes de *Adenium obesum* apresentam tolerância à imersão e ao armazenamento em nitrogênio líquido, e não necessitam de crioprotetores para manter a qualidade fisiológica quando conservadas em água a 7,5%.

Termos para indexação: *Adenium obesum*, crioprotetores, tolerância ao congelamento, germinação de sementes.

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The promising genus *Adenium*, Apocynaceae family, native to the semi-arid regions of Africa and the Arabian Peninsula, stands out among the ornamental plants with high commercial value (Limonas Briones et al., 2018). *Adenium obesum* (Forssk.) Roem. and Schult., the best-known species of the genus, is a succulent plant with a distinctive stem, sculptural appearance, and flowers with variations in color and shape (Colombo et al., 2018). It is also of interest for its pharmaceutical properties, with important biological activities, such as antiviral, antibacterial, antitumor, and antioxidant ones (Gurung et al., 2020).

The economic, pharmaceutical, and ornamental importance of this succulent reinforces its priority for ex situ conservation.

Adenium obesum presents seeds with reduced viability in the medium and long term (Colombo et al., 2018), consequently, cryopreservation is a safe and inexpensive alternative for the conservation of biological material of the species (Gurung et al., 2020). In cryopreservation, the vitrification of the cell is induced by freezing, which guarantees the reduction of the metabolism of the biological material for long periods, maintaining its integrity and survival after thawing (De Paula et al., 2022)

Cryoprotectants may have two mechanisms of action: extracellular ones, which act outside the cells, such as sugars like trehalose and glucose; and intracellular cryoprotectants, which act inside the cells, such as glycerol, dimethylsulfoxide and ethylene glycol. However, some of these compounds, such as glycerol and dimethyl sulfoxide, can be toxic to the seeds of some species, causing irreversible damage, making it essential to know their mechanisms of action during freezing (De Paula et al., 2022).

A success cryopreservation in seeds depends on low water content in the seeds, in order to promote dehydration and prevent the formation of ice crystals, which might lead to mechanical ruptures in the cell wall, in the membrane system, and consequent loss of germination capacity. The method of cooling and thawing also needs to be fast to improve the preservation of physiological characteristics.

The objective of this work was to evaluate cryoprotectants on the physiological quality of *Adenium obesum* seeds cryopreserved in liquid nitrogen.

The work was carried out in the Laboratory of Phytotechnology, Department of Agricultural Sciences, of Universidade Estadual de Londrina (UEL), in the municipality of Londrina, in the state of Paraná, Brazil. Seeds of *Adenium obesum* were obtained from parent plants of a farmer, who lives in the municipality of Warta, in the state of Paraná, Brazil. Mature pods were harvested 90 days after pollination.

To obtain the water content, 0.5 g of seeds were placed in an oven at $105\pm 3^{\circ}\text{C}$ for 17 hours (Brasil, 2009). For each treatment, four replicates of 25 seeds were placed in cryotubes with 2 mL. The treatments carried out were: T1, control, with no cryoprotectant and not immersed in liquid nitrogen; T2, with

immersion in liquid nitrogen without cryoprotectant; T3, with 0.4 mol L^{-1} sucrose; T4, with 0.8 mol L^{-1} sucrose; T5, with 1 mol L^{-1} glycerol; T6, with 2 mol L^{-1} glycerol and plant vitrification solutions PVS1, which consisted of 19% glycerol (v/v), 13% ethylene glycol (v/v), 6% dimethyl sulfoxide (v/v); T7, with PVS1; T8, with PVS2, which consisted of 30% glycerol (v/v), 15% ethylene glycol (v/v), 15% dimethyl sulfoxide (v/v); T9, with PVS2 and floroglucinol (modified PVS2); and T10, with PVS3, which consisted of 50% glycerol (v/v) and 50% sucrose (v/v) diluted in distilled water (Sakai et al., 1990; Teixeira et al., 2014).

Germination was performed in polypropylene plastic trays of 200 cells each, filled with commercial substrate, maintained in a climate-controlled greenhouse, covered with transparent polycarbonate panels and diffuser, with a controlled temperature of $28\pm 3^{\circ}\text{C}$ and daily irrigation. The germination test lasted 15 days. Only normal seedlings with part of the surface exposed above the substrate and with potential to continue their development were quantified (Brasil, 2009).

Simultaneously with the germination test, the germination velocity index (GVI), the mean velocity time (MVT), aerial part length (APL), root length (RL), aerial part dry mass (APDM), and root dry mass (RM) were evaluated.

The length of ten seedlings per replicate was randomly selected and measured with a pachymeter and expressed in millimeters. The dry mass was determined in an oven with forced air circulation at 65°C until constant mass was reached and weighed on a 0.0001 g precision balance, with the result expressed in milligrams.

The design was completely randomized, with ten treatments and four replicates. The data obtained were subjected to analysis of variance (ANOVA), and the means were compared by Scott-Knott's test at 5% significance using R software (R Core Team, 2023).

In the evaluation carried out to characterize the seed lot of *Adenium obesum*, it was observed that they presented $80\pm 2.72\%$ of viability and a water content of 7.5% before the cryopreservation process, according to Ferrari et al. (2020), who recommend a content below 10%, with the intention of acquiring vitreous characteristics and suppressing metabolic reactions during freezing.

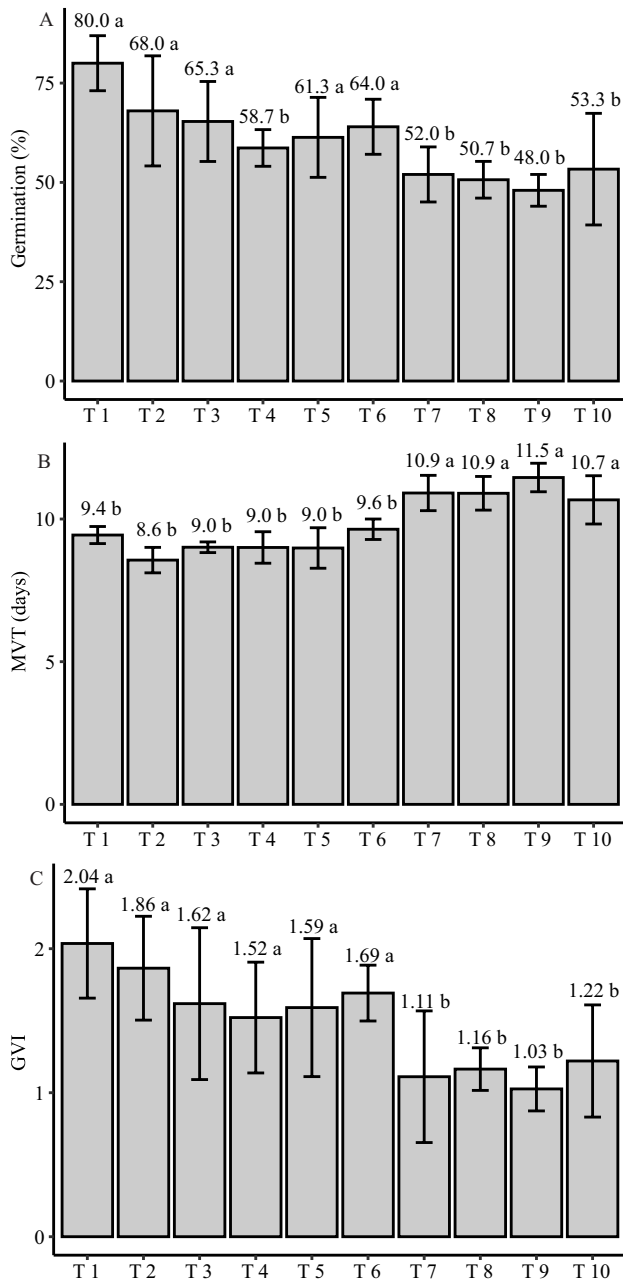


Figure 1. Germination (A), mean velocity time (MVT) (B), germination velocity index (GVI) (C) of *Adenium obesum* seeds under the following treatments: T1, free from cryoprotectors and not immersed in liquid nitrogen; T2, free from cryoprotective substances; T3, sucrose 0.4 mol L⁻¹; T4, sucrose 0.8 mol L⁻¹; T5, glycerol 1 mol L⁻¹; T6, glycerol 2 mol L⁻¹; T7, PVS1 [19% glycerol (v/v), 13% ethylene glycol (v/v), 6% dimethylsulfanoxide (v/v)]; T8, PVS2 [30% glycerol (v/v), 15% ethylene glycol (v/v), 15% dimethylsulfanoxide (v/v)]; T9, PVS2 + 1% floroglucinol; T10, PVS3 [50% glycerol (v/v) and 50% sucrose (v/v)]. Averages followed by the same letter do not differ from each other by Scott-Knott's test at 5% probability.

The germination ranged from 48 to 80% (Figure 1 A), the MVT, from 8.6 to 11.5 days (Figure 1 B) and the GVI, from 1.03 to 2.04 (Figure 1 C). The best results were obtained for GVI and MVT for T1, T2, T3, T4, T5, and T6 PVS solutions, while T7, T8, T9, and T10 showed the least significant indices for GVI and MVT.

The seeds immersed in liquid nitrogen without cryoprotectant treatment (T2) presented a germination of 68% after cryopreservation, with no statistical significance when compared to the control treatment (T1) with 80% germination, consequently better GVI and lower MVT.

Therefore, the treatments T3, with extracellular action, and T5, with intracellular action, did not show any statistical difference when compared to T2, in which the seeds were immersed in liquid nitrogen without cryoprotectants (Figure 1).

Similar results, in which seeds without cryoprotectants showed good germination after exposure to liquid nitrogen, were observed for *Encholirium spectabile* Martius ex Schultes (Ferrari et al., 2020), *Dyckia* spp. (De Paula et al., 2022), and orchids (Ferrari, 2020).

In this study, it was possible to observe that the treatments T7, T8, T9, and T10, when compared to the other treatments, diverged negatively in the results, with reduced physiological potential and viability of the seeds, presenting GVI of 1.11 and MVT of 10.91.

For APL, only treatments T1, T3, and T4 did not differ from each other and presented the highest values (Figure 2 A). The length and dry mass of the seedlings were also evaluated to assess their growth and vigor. For the RL, treatments T1 and T3 presented the highest means and did not show significant differences when compared. The other treatments showed no difference between them and treatments T6, T9, and T10 had the lowest means (Figure 2 B).

For APDM, treatment T1 presented the highest mass, and treatments T7, T8, T9, and T10 had the lowest values (Figure 2 C). For RM, treatments T1 and T5 presented the highest means and were not different from each other (Figure 2 D).

Adenium obesum seeds show tolerance to immersion and storage in liquid nitrogen and cryoprotectants are not necessary to maintain their physiological quality when stored in a water content of 7.5%.

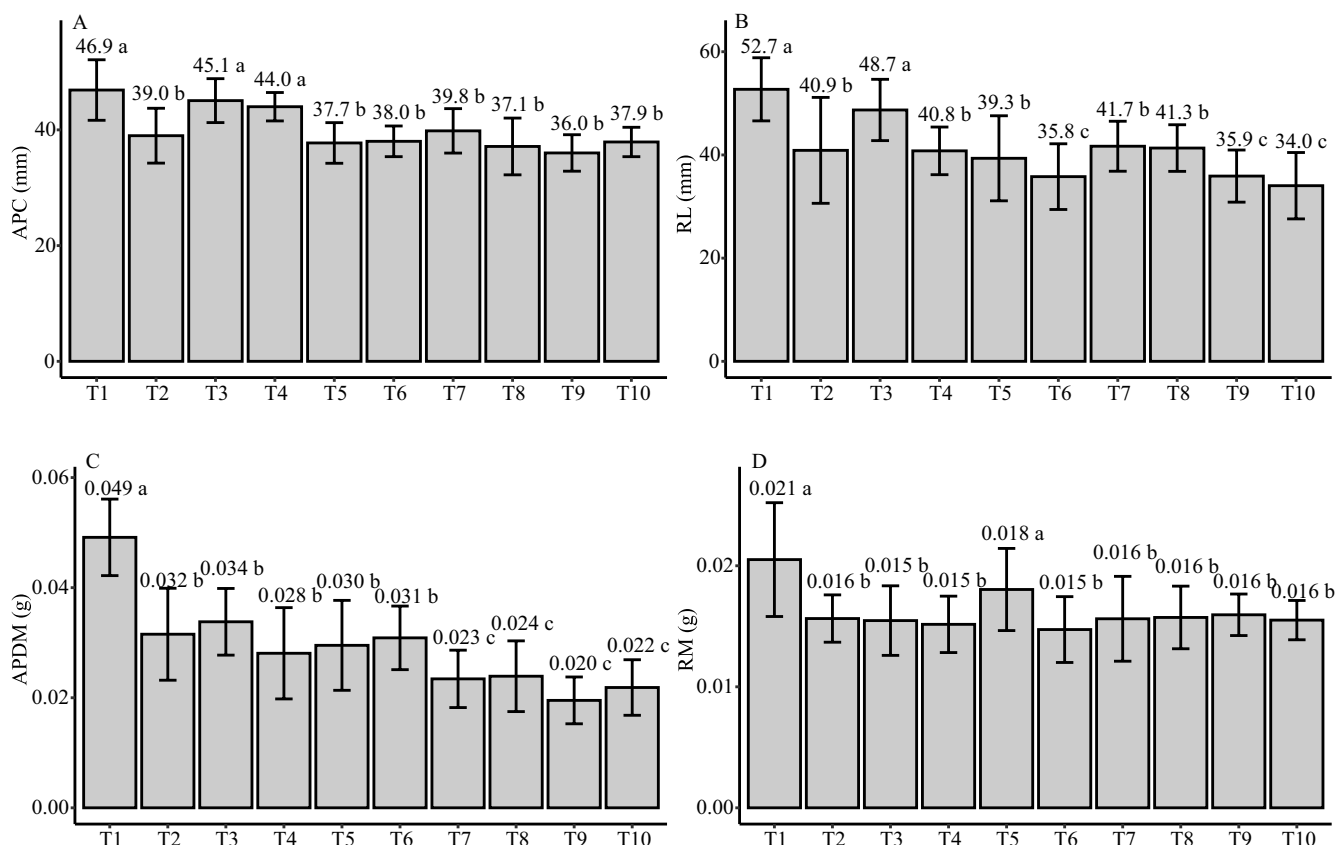


Figure 2. Aerial part length (APL) (A), root length (RL) (B), aerial part dry mass (APDM) (C), and root dry mass (RM) (D) of *Adenium obesum* seeds under the following treatments: T1, free from cryoprotectors and not immersed in liquid nitrogen; T2, free from cryoprotective substances; T3, sucrose 0.4 mol L⁻¹; T4, sucrose 0.8 mol L⁻¹; T5, glycerol 1 mol L⁻¹; T6, glycerol 2 mol L⁻¹; T7, PVS1 [19% glycerol (v/v), 13% ethylene glycol (v/v), 6% dimethylsulfanoxide (v/v)]; T8, PVS2 [30% glycerol (v/v), 15% ethylene glycol (v/v), 15% dimethylsulfanoxide (v/v)]; T9, PVS2 + 1% floroglucinol; T10, PVS3 [50% glycerol (v/v) and 50% sucrose (v/v)]. Note: Except for T1, in all other treatments, the seeds were immersed in liquid nitrogen. Averages followed by the same letter do not differ from each other by the Scott-Knott's test at 5% probability.

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