Research, Society and Development, v. 13, n. 7, e10413746010, 2024
(CC BY 4.0) | ISSN 2525-3409 | DOI: http://dx.doi.org/10.33448/rsd-v13i7.46010

Cryopreservation of seeds' threatened species - Bignoniaceae

Criopreservação de sementes de espécies ameaçadas – Bignoniaceae

Crioconservacón de semillas de espécies amenazdas – Bignoniaceae

Received: 05/27/2024 | Revised: 07/08/2024 | Accepted: 07/18/2024 | Published: 07/21/2024

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Abstract
Bignoniaceae family includes plant species that produce large amounts of seeds, stenospermic, thin, with two membranous wings, high lipid content and short lifespan under natural and artificial storage conditions, even though they are classified as orthodox seeds for the purposes of conservation. Such is the case of the Brazilian threatened native species *Handroanthus impetiginosus*, (Mart. ex DC.) Mattos, *H. serratifolius* (Vahl) S. Grose, *Tabebuia aurea* (Silva Manso) Benth. & Hook. f ex S. Moore and *T. roseoalba* (Ridl.) Sandwith, which have seeds with high lipid content named oleaginous or aleuro-oleaginous. Due to the characteristics of seed chemical components, storage behavior and vulnerability, an appropriate technique for conserving their genetic variability is a priority. Thus the objective of this study was to establish a cryopreservation technique for seeds of these species. In this study, the effect of exposure to liquid nitrogen at -196° C, on germinability of seeds, adopting rapid freezing (-263 °C.min.$^{-1}$) and slow thawing (5 °C.min.$^{-1}$), was tested for the seeds of these four species. High germination percentages were obtained after exposure to liquid nitrogen, 89% for *H. impetiginosus*, 93% for *H. serratifolius*, 100% for *T. aurea* and 94% *T. roseoalba*. These results suggest that cryopreservation in liquid nitrogen is a safe approach and can be used to ensure long-term storage of these four plant species in cryogenic banks.
Keywords: Trumpet species; Cryopreservation; Seed; Germinability.

Resumo
A família Bignoniaceae inclui espécies vegetais que produzem grandes quantidades de sementes, estenospérmicas, delgadas, com duas asas membranosas, alto teor lipídico e curta viabilidade em condições de armazenamento natural e artificial, embora sejam classificadas como sementes ortodoxas para fins de conservação. Este é o caso das espécies nativas brasileiras ameaçadas *Handroanthus impetiginosus*, (Mart. ex DC.) Mattos, *H. serratifolius* (Vahl) S. Grose, *Tabebuia aurea* (Silva Manso) Benth. & Gancho. f ex S. Moore e *T. roseoalba* (Ridl.) Sandwith, que possuem sementes com alto teor lipídico denominadas oleaginosas ou aleuro-oleaginosas. Devido às características dos compostos químicos das sementes, de seu comportamento durante a conservação e de sua vulnerabilidade, uma técnica adequada para conservar sua variabilidade genética é prioritária. Assim o objetivo deste estudo foi determinar uma técnica de criopreservação para sementes destas espécies. Neste estudo, o efeito da exposição ao nitrogênio líquido, a -196° C, na germinabilidade de sementes, adotando-se congelamento rápido (-263 °C.min. $^{-1}$) e descongelamento lento (5 °C.min. $^{-1}$), foi testado para as sementes destas quatro espécies. Altas porcentagens de germinação foram obtidas após exposição ao nitrogênio líquido, 89% para *H. impetiginosus*, 93% para *H. serratifolius*, 100% para *T. aurea* e 94% *T. roseoalba*. Esses resultados sugerem que a criopreservação em nitrogênio líquido é uma abordagem segura e pode ser usada para garantir o armazenamento em longo prazo dessas quatro espécies de plantas em bancos criogênicos.
Palavras-chave: Espécies de ipês; Criopreservação; Semente; Germinabilidade.

Resumen
La familia Bignoniaceae incluye especies vegetales que producen grandes cantidades de semillas, estenospérmicas, delgadas, con dos alas membranosas, alto contenido en lípidos y corta viabilidad en condiciones de almacenamiento natural y artificial, aunque se clasifican como semillas ortodoxas para fines de conservación. Este es el caso de las especies nativas brasileñas amenazadas *Handroanthus impetiginosus*, (Mart. ex DC.) Mattos, *H. serratifolius* (Vahl) S. Grose, *Tabebuia aurea* (Silva Manso) Benth. & Gancho. f ex S. Moore y *T. roseoalba* (Ridl.) Sandwith, que tienen semillas con alto contenido en lípidos llamadas oleaginosas o aleuro-oleaginosas. Debido a las características de los

compuestos químicos de las semillas, su comportamiento durante la conservación y su vulnerabilidad, una técnica adecuada para conservar su variabilidad genética es una prioridad. El objectivo de este estudio fue determinar una técnica de crioconservación para las semillas de estas especies. En este estudio se analizó el efecto de la exposición al nitrógeno líquido, a -196° C, sobre la germinabilidad de las semillas, adoptando congelación rápida (-263 °C.min. $^{-1}$) y descongelación lenta (5 °C.min. $^{-1}$), para las semillas de estas cuarto especies. Se obtuvieron altos porcentajes de germinación tras la exposición al nitrógeno líquido, 89% para *H. impetiginosus*, 93% para *H. serratifolius*, 100% para *T. aurea* y 94% para *T. roseoalba*. Estos resultados sugieren que la criopreservación en nitrógeno líquido es un enfoque seguro y puede usarse para garantizar el almacenamiento a largo plazo de estas cuatro especies de plantas en bancos criogénicos.
Palabras clave: Especies de lapachos; Crioconservación; Semilla; Germinabilidade.

1. Introduction

For many decades now, the agrobiodiversity of selected crop species, namely specific genotypes of crop species that present tolerance to biotic and abiotic stresses, crop's wild relatives, landraces and local varieties have been preserved by *ex situ* conservation in conventional seed germplasm banks (Engels & Ebert 2021). In the most diverse tropical phytophysiognomies natural habitat destruction has escalated due to intense anthropogenic activities, such as predatory logging, deforestation for agricultural purposes, urban growth and construction of road systems. These negative impacts have led to fragmentation of plant formations, changes in the functioning and self-regulation of vegetation populations and, above all, the loss of genetic diversity requiring *in situ* and *ex situ* actions and polities for the preservation of plant biodiversity (Oliveira et al. 2019; Santos et al. 2020).

In an ideal scenario, *in situ* and *ex situ* conservation actions and polities would be conducted simultaneously. Given the difficulties of these actions being complementary, in the vast majority of situations, seed conservation becomes a relevant and valuable alternative to guarantee the availability of biological material (Salomão et al. 2015).

Currently, conservation of seed germplasm of non-domesticated species traditionally used as timber, medicinal, melliferous, ornamental, artisanal, ecological, pharmacological and other purposes, is a major priority in order to safeguard the genetic variability of these species to meet future demands of research and breeding programs, recovery of degraded areas, environmental restoration, carbon sequestration and mitigation of greenhouse gas emissions (Prakash & Verma 2022; Pimenta et al. 2023).

The storage procedures adopted by conventional seed germplasm banks (-18 °C or -20 °C) were designed for orthodox seeds, which, however, do not meet the specificities of plant species' seeds component of the biodiversity, regardless of them showing orthodox, intermediate or recalcitrant behavior (Wyse et al. 2018). It is noted that orthodox seeds preserved in gene banks under conventional conditions are subject to senescence processes over time, as the storage conditions of the germplasm banks do not halt the basal metabolism of these seeds (van Treuren et al. 2013). Seed deterioration is a continuous process that begins when they reach physiological maturity. In theory, the interdependence between genetic and physiological characteristics has been attributed to the low storability and longevity of seeds in conservation conditions (Walters & Pence 2021; Zinsmerster et al. 2020). During and after conservation, some signs of seed deterioration are identified, such a gradual or total loss of germinability, uneven seedling emergence, an increase in the number of abnormal seedlings and a reduction in seedling vigor. In this sense, there is evidence that the loss of viability during conservation occurs more quickly in lipidic seeds, followed by aleuro-oleaginous, aleuro-starchy seeds and with less intensity in starchy seeds. Lipidic seeds are more susceptible to loss of germinability, viability and vigor possibly due to the occurrence of hydrolytic and oxidative rancidity of fatty acids (Balešević-Tubic et al. 2010) and other molecular, biochemical, physiological and cellular events culminating in seed death (Sano et al. 2016; Fu et al. 2015). Conservation using cryogenic methods, at -196 °C, is recommended for seeds of threatened species that have a high lipid content and are not long-lived (Normah et al. 2019; Civatti et al. 2014). Regarding the

effect of storage conditions on seed metabolism and longevity, it is clear that suppression of basal metabolism can only be achieved at cryogenic temperatures, using liquid nitrogen (-196 ºC), and under such conditions it is possible to preserve orthodox, intermediate and recalcitrant seeds for much longer periods of time (Santos & Salomão 2017; Michalak et al. 2013).

Generally, Bignoniaceae species produce large amounts of seeds, which are stenospermic, thin, with two membranous wings, high lipid content and short lifespan under natural and artificial storage conditions, even though they have orthodox behavior for the purposes of conservation. Such is the case of the Brazilian native species *Handroanthus impetiginosus*, (Mart. ex DC.) Mattos, *H. serratifolius* (Vahl) S. Grose, *Tabebuia aurea* (Silva Manso) Benth. & Hook. f ex S. Moore and *T. roseoalba* (Ridl.) Sandwith, which have high lipid content seeds or aleuro-oleaginous types (Apóstolo et al. 2016; Abbade & Takaki 2014; Silva et al. 2011; Duarte et al. 2010).

These species have been targeted by *in situ* and *ex situ* conservation actions, due to the growing demand for their timber production, as well as their habitat reduction and population fragmentation. According to the IUCN Red List of threatened species *Tabebuia roseoalba* is listed as Near Threatened under criteria A2cd+3cd, *H. impetiginosus* and *H. serratifolius* are listed as Near Threatened under criteria A3cd+4cd (IUCN 2023) and *T. aurea* occurs in biomes under strong human and agricultural pressure.

Thus, due to the characteristics of seed chemical components, storage behavior and vulnerability, an appropriate technique for conserving their genetic variability is a priority. In this study, the effect of exposure to liquid nitrogen at -196° C, on germinability of seeds, adopting rapid freezing at -263 °C.min. $^{-1}$ and slow thawing at 5 °C.min. $^{-1}$ was tested for the seeds of these four species to establish a seed cryopreservation technique for safe long-term conservation for these species germplasm.

2. Methodology

2.1 Experiment Laboratories

In this experiment, three laboratories at Embrapa Genetic Resources and Biotechnology (Embrapa – Cenargen), Brasília, Federal District - Brazil were used: Geoprocessing Laboratory, Seed Laboratory and Plant Cryobiology Laboratory.

2.2 Geographic occurrence of species

Data on the specific occurrence of the four species in Brazilian territory (Figure 1) were obtained using information on the geographic coordinates indicated on germplasm collection vouchers, literature data (ca. 200 scientific articles) and exsiccates deposited in 28 herbaria. The species' occurrence coordinates were plotted on the biomes maps of Brazil using the Geographic Information System (GIS), at the Geoprocessing Laboratory (Embrapa Cenargen).

2.3 Reproductive plant material

Semi-open fruits from different individuals of the four species were collected in Brasília (Federal District) localities: *H. impetiginosus* (15°44'10" S and 47°55'40" W) and *H. serratifolius* (15°44'07" S and 47°56'01" W) were collected in the Brasília National Park, *T. aurea* were collected on the North Lake peninsula (15°43'11" S and 47°51'06" W) and of *T. roseoalba* in the IBGE Ecological Reserve (15°56'59" S and 47°52' 44"W).

2.4 Seeds processing

The fruits remained in Seeds Laboratory conditions at 21 ±2 °C until they completely opened. The seeds were manually removed from the fruits, then they were cleaned, selected and those damaged were discarded. The seeds were kept at

room temperature (25 ± 2°C) for two days. After that, seed samples of each species were homogenized and divided into subsamples to determine moisture content and germinability of control and after seed exposure to liquid nitrogen (LN). Control samples were packed in trifoliated aluminized bags and maintained in the Laboratory of Seeds bench, at room temperature (25 ± 2 °C) for one week. The subsamples to be cryopreserved were transferred to Plant Cryobiology Laboratory (Embrapa Cenargen).

2.5 Seed moisture content determination

Seed moisture contents (mc) were determined by the gravity convection method at 103 ± 2°C/24 hrs. (Brasil, 2009). Five repetitions of 20 seeds were weighed and they were transferred and maintained overnight (24 hrs.) in a dry heat oven at temperature described above. After 24 hrs. seeds were weighed again to obtain the dry weight of the sample, and the mc results were expressed as average percentages of seed fresh weight (FWB).

2.6 Seeds cryopreservation

The technique adopted for seed cryopreservation was based on protocols established for seeds of tropical species (Santos et al. 2013; Salomão 2002). This technique consists of rapid freezing and slow thawing, adjusting these rates according to the species. At the Plant Cryobiology Laboratory seeds were packed in trifoliated aluminized bag, sealed with parafilm and identified with the name of respective species. The bags were immersed directly into LN at -196 °C, at a cooling rate -263 °C.min. $^{-1}$. After one week, the material was removed from the LN and kept on the laboratory bench to thaw slowly at room temperature (25 ± 2 °C), at a thawing rate of approximately 5 °C.min^{-1}. After thawing, the seeds were taken to the Seed Laboratory.

2.7 Seed germination

Frozen and control seeds were immersed in a commercial neutral detergent solution at 2% (v/v) concentration for 5 min, followed by rinsing under running tap water until complete removal of the product. After surface disinfection, seeds were pre-soaked in distilled water for two hours before sowing.

Germination tests were conducted with four replications of 50 seeds on paper roll substrate (Germitest®) moistened with distilled water (volume equivalent to 2.5 times the mass of the dry paper) [Brasil, 2009]. Paper rolls were placed into plastic bags, incubated in a germination chamber set at constant temperature of 25 °C, photoperiod 16 hrs. light provided by eight 40 w fluorescent bulbs and 8 hrs. dark. Daily counts of normal seedlings were done for 20 days. Seedlings were considered normal if they presented firm and healthy essential structures, such as the first pair of expanded eophils and the main root. The results were expressed as percentage of normal seedlings.

2.8 Statistical analysis

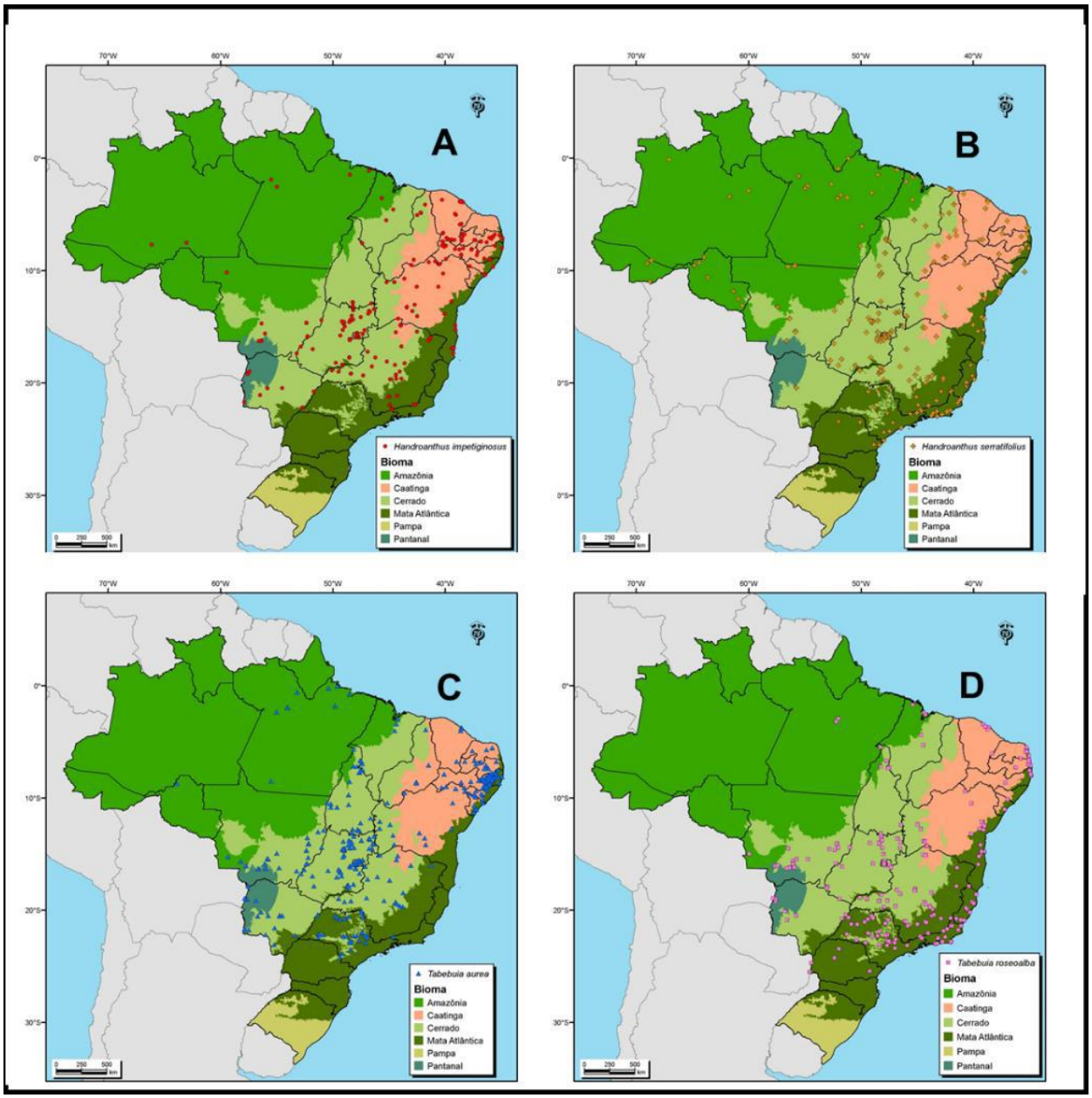
The experimental design was completely randomized with two treatments (without and with exposure to LN) and four replications of 50 seeds per treatment and species. Germination results were subjected to analysis of variance (ANOVA), followed by comparison of means using the Bonferroni posttest ($P<0.05$). The program used for statistical analysis was GraphPad Prism (@2017 Graph Pad Software Inc).

3. Results and Discussion

In tropical vegetation, interaction between *in situ* and *ex situ* conservation policies and actions for plant diversity is

recommended. However, *ex situ* conservation plays a preponderant role on an emergency basis, as it allows safeguarding the genetic diversity of plant fragmented populations. The original geographic distribution of *H. impetiginosus*, *H. serratifolius*, *T. aurea* and *T. roseoalba* in Brazilian territory is illustrated in Figure 1. The limitation in the geographic distribution of these species can be attributed to urban expansion and the increase in areas designated for agroforestry activities, as well as their economic importance.

Figure 1 - Original geographic distribution of (A) *Handroanthus impetiginosus* (Mart. ex DC.) Matto, (B) *Handroanthus serratifolius* (Vahl) S. Grose, (C) *Tabebuia aurea* (Silva Manso) Benth. & Hook. f ex S. Moore and (D) *Tabebuia roseoalba* (Ridl.) Sandwith in different Brazilian biomes.



Source: Authors.

The occurrence of these species is found in vulnerable phytophysiognomies of the Amazon, Caatinga, Cerrado, Atlantic Forest and Pantanal biomes (Figure 1). In these areas concentrate the majority of the Brazilian population resulting in

intense and constant anthropogenic pressures. All those practices reinforce the need to promote conservation strategies for the intra and inter specific genetic diversity of those species.

The four species are target of predatory exploitation in their habitats (Table 1). The main commercial use of these species is for timber production; the wood obtained from these plants is a very resistant hardwood used in everything from civil construction to furniture making. Studies have demonstrated that these species produce antioxidant compounds and active metabolites, being the most important secondary metabolites the 1,4-naphthoquinones (Silva et al. 2012). Their derivatives have been reported as antimicrobial and antitumoral compounds (Navarro-Tovar et al. 2023). In traditional medicine, different parts of these plants are used as decoction, infusion, maceration, liniment and poultice.

Table 1 - Multiutilization of *Handroanthus impetiginosus (*Mart. ex DC.) Mattos, *Handroanthus serratifolius* (Vahl) S. Grose, *Tabebuia aurea* (Silva Manso) Benth. & Hook. f ex S. Moore and *Tabebuia roseoalba* (Ridl.) Sandwith species.

Species	Multi-purpose wood	Pharmacologically compounds	Ornamental and landscaping	Folk medicine	Animal feed	Environmental goods and services
Handroanthus impetiginosus	X	X	X	X		X
Handroanthus serratifolius	X	X	X	X		X
Tabebuia aurea	X	X	X	X	X	X
Tabebuia roseoalba	X	X	X	X		X

Source: Authors (based on Santosa et al. 2024; Moraes Neto 2023; Leandro et al. 2019; Chaves et al. 2018; Felix et al. 2018; Santos 2017; Zuntini & Lohmann 2016; Cabral et al. 2003; Gentry 1992).

As observed for other multipurpose species, they are more vulnerable to extinction (Wyk & Prinsloo 2019). In this case, the most effective way to mitigate this situation is to prevent the loss of existing genes through conservation.

Figure 2 - Flowers of (A) *Handroanthus impetiginosus* (Mart. ex DC.) Matto, (B) *Handroanthus serratifolius* (Vahl) S. Grose, (C) *Tabebuia aurea* (Silva Manso) Benth. & Hook. f ex S. Moore and (D) *Tabebuia roseoalba* (Ridl.) Sandwith.

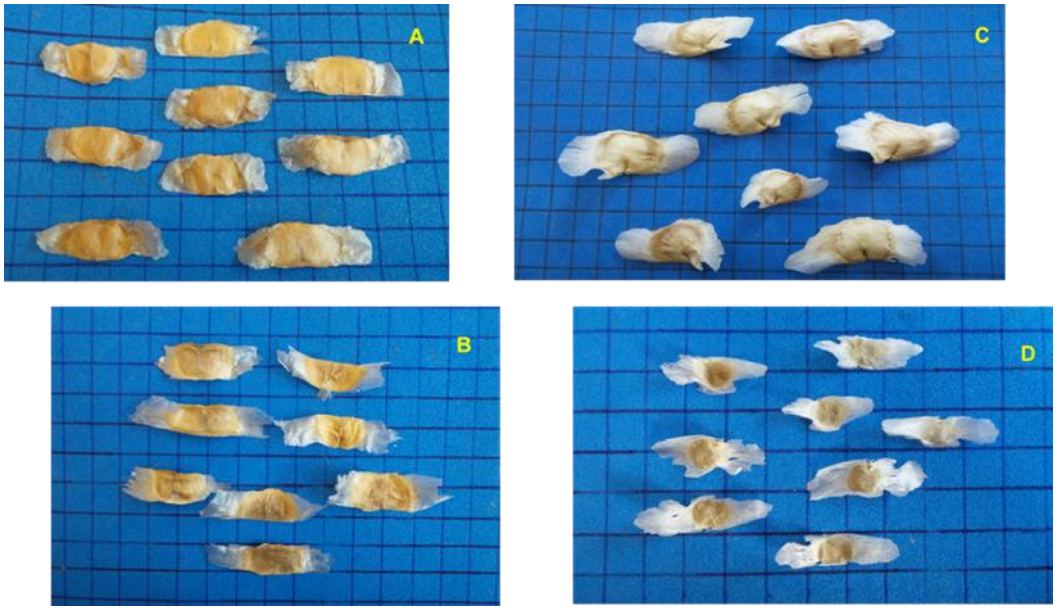
Source: Authors.

The beauty and colors flowers pink to purple (*H. impetiginosus* - trumpet pink), yellow (*H. serratifolius* and *T. aurea* - trumpet golden) and white (*T. roseoalba* – trumpet white) arranged in terminal paniculate inflorescences are widely used as ornamentals and in landscaping, as evidenced in Figure 2.

Regardless of whether the seeds are orthodox, intermediate or recalcitrant, they are the most used biological structures for plant conservation, enabling the regeneration of species via seeds, their parts or vegetative structures (Santos & Salomão 2016). Cryopreservation is proposed as the most appropriate technique for species under constant anthropogenic pressure and intense predatory exploitation, thus guaranteeing vegetation restoration and its future uses (Walters & Pence 2021; Civatti et al. 2014; Pijut et al. 2012; Salomão 2002). The storage of biological material in LN, at -196 °C, preserves the integrity and preventing aging of stored samples for unlimited periods (Panis 2019; Kalaiselvi et al. 2017). In addition to the germplasm of threatened species in their habitats, cryopreservation should be adopted for seeds with lipid reserves or aleuro-lipidics that present viability loss when stored in conventional gene banks. This is because in cryogenic conditions there is a delay in molecular movement, stability of the cellular structure and chemical limitation of the cytoplasm when solidified (Michalak et al. 2013; Benson 2008). However, a complex interaction of factors is responsible for maintaining biological material integrity, as seeds from the same species may respond differently depending on their origin or the way they are managed (Souza et al. 2023; Santos et al. 2013).

In this study, drying under ambient conditions (25 ±2°C/ 2 days) allowed the mc of the seeds to reach values compatible with exposure to LN. The chemical and structural characteristics of the genera *Handroanthus* and *Tabebuia* seeds' favour rapid desiccation under environmental conditions (Figure 3).

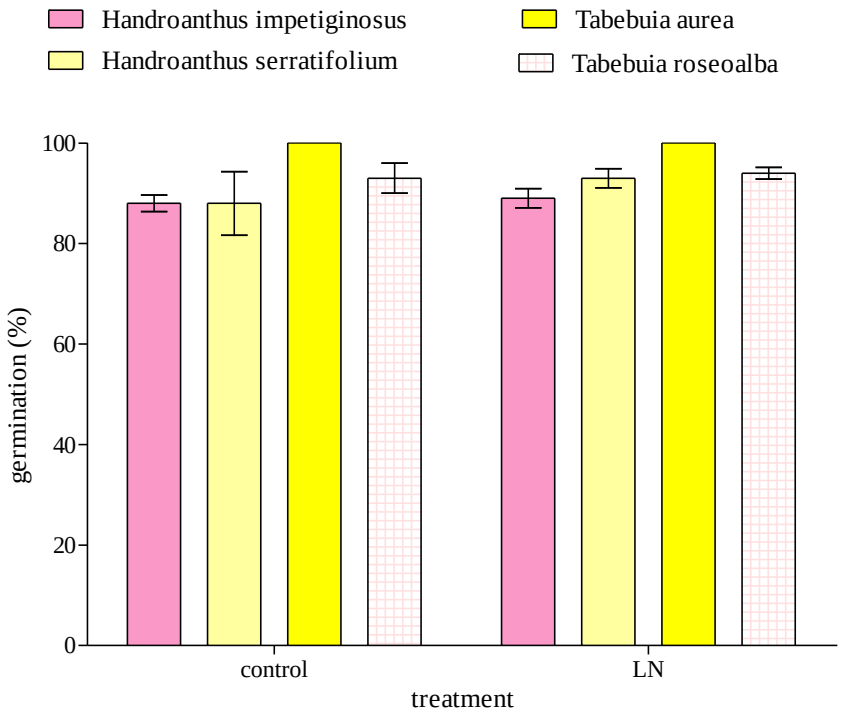
Figure 3 - Seeds of (A) *Handroanthus impetiginosus* (Mart. Ex DC.) Matto, (B) *Handroanthus serratifolius* (Vahl) S. Grose, (C) *Tabebuia aurea* (Silva Manso) Benth. & Hook. F ex S. Moore and (D) *Tabebuia roseoalba* (Ridl.) Sandwith.



Source: Authors.

The mc hit by the seeds were 5.8% (H. impetiginosus), 5.4% (H. serratifolius), 6.9% (T. aurea) and 6.2% (T. roseoalba). The protocol used for the cryopreservation of seeds from those species was adjusted according to results obtained for several tropical tree, shrub and herbaceous species (Salomão et al. 2020). The rapid freezing (-263 °C.min^{-1}) and slow thawing (5 °C.min.$^{-1}$) adopted in this study were favourable to maintaining the physical integrity of the seeds. The germination percentages before and after LN exposure were 88% - 89% (*H. impetiginosus*) 88% -93% (*H. serratifolius*), 100% - 100% (*T. aurea*) and 93% - 94% (*T. roseoalba)*, respectively. There were no significant ($P< 0.05$) loss of seed germinability, uneven seedling emergence or an increase in the number of abnormal seedlings of each species tested after exposure to LN (Figure 4).

Figure 4 - Germination percentages (control and after liquid Nitrogen exposure) of *Handroanthus impetiginosus* (Mart. ex DC.) Mattos, *Handroanthus serratifolius* (Vahl) S. Grose, *Tabebuia aurea* (Silva Manso) Benth. & Hook. f ex S. Moore and *Tabebuia roseoalba* (Ridl.) Sandwith seeds (vertical error bars represent SE of means of four replications, $P < 0.05$).



Source: Authors.

The combination of factors, initial physiological quality, mc, freezing and thawing rates did not affect the germination performance of the seeds (Figure 4). Cryopreservation protocols are established knowing the storability of seeds and their responses desiccation and cryogenic temperature (Kalemba et al. 2023; De Vitis et al 2020). The functional and structural integrity of seeds, during and after cryopreservation, depends on the appropriate adjustment of mc and freezing and thawing rates. In this way, critical or lethal damage to biological material is avoided (Santos & Salomão 2017; Pereira et al. 2014; González-Arnao et al. 2014). As evidenced for seeds of several tropical species, some criteria are common to orthodox seeds, such as tolerance to rapid freezing and slow thawing and the lack of need to use cryoprotectant on whole seeds. Thus, the success of cryopreservation is conditioned on methods that allow rapid freezing by direct immersion in LN and slow thawing at room temperature (Salomão et al. 2015; Salomão 2002).

Rapid freezing has been shown to be beneficial as it favours the formation of small ice crystals, thus preventing cell dehydration and membrane rupture. Depending on the speed of thawing, chemical and biophysical events may occur, resulting in the loss of material. It has been observed that slow thawing is one of the cryopreservation steps that maintain the integrity of tropical seeds (Salomão et al, 2018; González-Arnao et al. 2014). Seeds of some species cultivated in the tropics and which have a high lipid content responded favourably to cryopreservation, even though the regeneration of the material is carried out via the embryonic axis. As example there are, sunflower, cotton, soybean, peanut, castor, jatropha and coffee (Souza et al. 2024; Salomão et al. 2016; Lopes et al. 2013; Almeida et al. 2010; José et al. 2010).

The technical approach adopted in this study, rapid freezing at -263 °C.min^{-1} followed by slow thawing at 5 °C.min^{-1}, was effective for the seeds of the four species of the Bignoniaceae family. The germination percentages before and after LN exposure were, *H. impetiginosus* 88% - 89%, *H. serraifolius* 88% - 93%, *Tabebuia aurea* 100% - 100% and *Tabebuia*

roseoalba 93% - 94%, respectively (Figure 4). These results are compatible with those evidenced in seeds of others species of the Bignoniaceae before and after LN exposure, such as *Handroanthus spongiosus* (Rizzini) S. rose (90% - 91.5%); *Handroanthus chrysotrichus* (Mart. Ex Dc.) Mattos (87% - 82%); *Tabebuia pentaphylla* Helmsl (89% - 88%), *Cybistax antisyphilitica* (Mart.) Mart. (20% - 62%) and *Pyrostegia venusta* (Ker Gawl.) Miers (88% - 98%) [Silva et al. 2022; Salomão et al. 2020; Higa et al. 2011; Tresena et al. 2010].

The interaction between freezing and thawing rates in LN and mc seed guaranteed the survival and germination performance of the four species' seeds. It is proposed that the appropriate mc allows biophysical and biochemical morphophysiological stability of lipids and proteins (Reed 2017; Almeida et al. 2010). Biochemical and physiological changes were observed in seeds of *H. impetiginosus*, *H. serratifoliun* and *Tabebuia roseoalba* after storage in non-cryogenic conditions (Santos et al. 2020; Leandro et al. 2019; Abbade & Takaki 2014). These changes were expressed in decline in germinability or loss of viability. In seeds of *Handroanthus* spp. and *Tabebuia* spp. rapid freezing (-263 °C.min^{-1}) and slow thawing (5 °C.min^{-1}) at room temperature (25 °C ± 2 ºC) did not cause apparent damage such as morphological changes and reduced germinability (Figure 4).

4. Conclusions

Seeds of the four Bignoniaceae species with mc < 7.0% are tolerant to cryopreservation at liquid nitrogen temperature, -196° C, adopting rapid cooling and slow thawing as a protocol. In agreement with the results obtained in this study, cryopreservation presents itself as an effective tool that can be used on a routine basis for long storage of seed germplasm of *H. impetiginosus*, *H. serratifolius*, *T. aurea* and *T. roseoalba*.

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