eISSN 2236-5257
10.46551/ruc.v27n2a5

In Vitro Germination of *Nicotiana glauca*: Unlocking Its Biotechnological Potential

Germinação In Vitro *de* Nicotiana glauca*: Desvendando Seu Potencial Biotecnológico*

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ABSTRACT

Objectives: The aim was to optimize temperature storage protocols and in vitro culture conditions to improve germination rates and plant growth. **Method:** Seeds were stored at three temperatures (room temperature, 6°C, and -4°C) and germinated with different concentrations of sorbitol. Germination rates, plant height, and biomass were assessed. **Results:** showed that low-temperature storage at 6°C, combined with sorbitol treatment, significantly enhanced seed viability and germination rates. This suggests that sorbitol can mitigate osmotic stress. In contrast, storage at -4°C decreased viability, highlighting the importance of temperature control. **Conclusions:** This study provides valuable knowledge into the factors affecting N. glauca seed storage and germination. The findings contribute to developing efficient propagation protocols and the potential for biotechnological applications of this species.

Keywords: Temperature, Sorbitol, Wild tobacco, Storage, Anabasine.

RESUMO

Objetivo: O trabalho teve como objetivo otimizar protocolos de armazenamento e condições de cultivo *in vitro* para melhorar as taxas de germinação e o crescimento das plân-

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Recebido em	Aceito em	Publicado em
28-12-2024	09-06-2025	18-08-2025

tulas. **Método:** As sementes foram armazenadas em três temperaturas diferentes (temperatura ambiente, 6 °C e -4 °C) e submetidas à germinação em meio de cultivo contendo diferentes concentrações de sorbitol. Foram avaliadas as taxas de germinação, altura das plantas e biomassa. **Resultados:** O armazenamento a 6 °C, associado ao uso de sorbitol, aumentou significativamente a viabilidade das sementes e as taxas de germinação. Isso indica que o sorbitol pode atenuar o estresse osmótico. Por outro lado, o armazenamento a -4 °C reduziu a viabilidade das sementes, evidenciando a importância do controle da temperatura. **Conclusão:** Este estudo oferece informações importantes sobre os fatores que afetam o armazenamento e a germinação das sementes de *N. glauca*. Os resultados contribuem para o desenvolvimento de protocolos eficientes de propagação, ampliando o potencial de uso biotecnológico dessa espécie.

Palavras-chave: Temperatura, Sorbitol, Tabaco-bravo, Armazenamento, Anabasina.

INTRODUCTION

Nicotiana glauca Graham (Solanaceae) is commonly known as the wild tobacco tree or tobacco bush. This species is native to South America, specifically Argentina, Bolivia, Paraguay, and Uruguay[1], but has become naturalized in regions such as Australia, California, Africa, and the Mediterranean[2]. *Nicotina glauca* (*N. glauca*) exhibits significant biotechnological potential, primarily due to its production of anabasine, a pyridine alkaloid that is structurally analogous to nicotine[2,3,4], which exhibits notable properties.

Anabasine derivatives are used as medicaments to treat asthma, chest allergies, and atopic dermatitis due to their full agonistic action on nicotinic acetylcholine receptors[5]. Anabasine has shown promise in addressing neurodegenerative diseases by enhancing cholinergic transmission and acting as ligands for specific nicotinic receptors[6]. Anabasine also improves sensory inhibition in Alzheimer's disease[7].

In addition to anabasine, *N. glauca* produces other compounds with medicinal properties, which have been widely used to treat various illnesses. Its methanolic extract is rich in chlorogenic acid and rutin[8], which are linked to notable anti-inflammatory, anti-aging, and anticancer properties[9]. Traditionally, warmed leaves have been applied to the head to relieve headaches, placed on the throat to soothe sore throats, and even put in shoes to alleviate foot pain[10]. Furthermore, *N. glauca* extracts have demonstrated antimicrobial activity

against *Escherichia coli* and *Staphylococcus aureus*, due to the highest concentrations of these phytochemicals[11].

Beyond its medicinal applications, anabasine from *N. glauca* has also proven to be an effective pesticide, outperforming nicotine against certain pests[12]. This pyridine alkaloid's insecticidal properties arise from its interaction with nicotinic acetylcholine receptors, the exact mechanism underlying its therapeutic effects[13]. Toxicity studies have highlighted anabasine as a more efficient agent against citrus and red mites than nicotine, reinforcing its dual role as a therapeutic agent and a biotechnological resource for pest control[12]. These overlapping mechanisms of action emphasize the diverse applications of anabasine and its potential for further innovation in medicine and agriculture.

N. glauca also has a high sprouting capacity, with up to 100% of the plant capable of sprouting, producing large quantities of above-ground biomass[14]. Phytochemical analysis of *N. glauc*a growing in Egypt revealed that environmental conditions significantly influenced the flavonoid content and antioxidant activity of the plant[15]. This finding shows the importance of considering regional variations in the composition of active or potentially toxic metabolites when using medicinal plants for research or therapeutic purposes. Therefore, research should account for the use of medicinal plants relative to their composition of active and/or toxic metabolites collected from different regions.

In this context, *in vitro* propagation techniques are essential for advancing research and practical applications in plant sciences, particularly for species of medicinal, agricultural, and biotechnological importance. These techniques help optimize metabolite production, and increase biomass in a short period, and ensure pathogen-free material[16].

Plant tissue culture is the leading core of biotechnology in medicinal plants[17, 18], enabling rapid multiplication and large-scale plant production[19]. Strategies such as media optimization, elicitation, and *Agrobacterium*-mediated transformation rely on plant tissue culture to enhance the *in vitro* production of valuable plant compounds[20]. Plant tissue culture also enhances the production of their active secondary metabolites[20].

Despite its biotechnological promise, the successful propagation and utilization of *N. glauca* face significant challenges, particularly in seed germination and storage. Seed dormancy and sensitivity to storage conditions often limit germination rates, making propagation and research applications difficult. Therefore, optimizing storage protocols and *in*

vitro culture systems is important to the species' full potential. The establishment of *in vitro* propagation of *N. glauca* for biotechnological proposes is influenced by factors such as seed dormancy and viability and the composition of the culture medium, which affect seed germination and subsequent plant development[21, 22].

Seed dormancy plays a critical role in the propagation strategies of *Nicotiana* species, posing a threat to their conservation and biotechnological development. Due to high dormancy levels23, germination rates are often below 40%, overcoming this limitation is essential for maximizing the species' potential. Research into specific protocols for established *in vitro* plants is crucial for enhancing germination success, improving economic efficiency, and ensuring the biotechnological applications of *N. glauca*.

Therefore, plant biotechnology has developed a technique to improve seed germination and seedling length by exposing it to a stress medium, which activates physiological changes in the seed[24,25,26]. For this, the culture medium should have a high osmotic level so that the plant tissue does not absorb excessive amounts of water, which can cause cell rupture. Sorbitol, a sugar alcohol, is commonly used to reduce the osmotic level of the culture medium without interfering with metabolic activities, thus positively affecting germination and plant growth[27, 28].

This study evaluates the effects of storage conditions and osmotic regulation, specifically sorbitol supplementation, on the *in vitro* germination and development of *N. glauca* seeds. Sorbitol, commonly used as an osmotic regulator in tissue culture, provides a controlled environment that mitigates stress during germination. By investigating the interplay between storage temperatures (room temperature, 6°C, and -4°C) and sorbitol concentrations, this research seeks to refine propagation protocols, enhance germination success, and ensure the sustainable use of biotechnology in *N. glauca*. The findings contribute to advancing biotechnological applications of *N. glauca*, from its use in pharmaceutical research to sustainable agricultural practices.

MATERIALS AND METHODS

Plant samples were collected on April 20th, 2017 in Divinopolis located at 20º.8’42.45” S, 44º53’29.92” W, and stored at room temperature for six months.

To evaluate the cold treatment, the seeds were separately stored in sealed Petri dishes at room temperature (25±1°C), 6 °C, and -4°C for up to 180 days.

Seeds stored and in vitro establishment

The seeds, stored at room temperature, -6°C, and 4°C for different periods (34, 54, 107, 159, and 180 days), were disinfected by exposed gas formed from the reaction between sodium hypochlorite and hydrochloric acid in a 5:1 ratio and held for 3 hours in a glass desiccator.

After disinfestation, the seeds were sown in a half-strength MS medium[29] containing sorbitol as a carbon source and solidified with 6 $g.L^{-1}$ agar. The culture media's pH was adjusted to 5,8 ± 0,1 before autoclaving at 121 °C at 1 atm for 20 min. The culture tubes were maintained in a controlled environment at a temperature of 27 ± 1 °C under a 16-hour light/8-hour dark photoperiod.

IN total, three temperatures (room temperature, 6°C, and -4°C), five sorbitol concentrations (0.04, 0.08, 0.17, 0.25, 0.29 M), and five storage periods (34, 54, 107, 159, and 180 days) were tested, delineated by Rotational Central Composite Design (RCCD). RCCD optimizes experimental conditions by reducing the number of required experiments, enhancing predictive capability, ensuring rotatability, and providing flexibility across different fields of study. The experiment was carried out using an RCCD with ten replicates. Means differing significantly were compared using the Newman-Keuls multiple range test at the 5% probability level, with statistical analysis performed using Statistica software version 7.0.

Germination rate was analyzed after 7 days in all treatments. Plant height and dry and fresh weight were inspected after 30 days (Figure 1).

RESULTS AND DISCUSSION

Storage at room temperature

The results of Table 1 show a significant decline in the germination percentage of *N. glauca* seeds with increasing storage time at room temperature *In vitro* germination rate was 100% after 34 days of storage at room temperature, however, seed viability decreased,

showing a germination rate of 80% after 54 days of storage, and ultimately dropped to zero (0%) at 107 days (Table 1). This data suggests that *Nicotiana glauca* seeds have a relatively short viability at room temperature, with significant germination decline occurring within the first few months of storage.

Table 1 - Germination percentage of *Nicotiana glauca* seeds stored at room temperature for up to 180 days.

Storage time before inoculation (days)	Germination Rate (%)
34	100a
54	80b
107	0c
159	0c
180	0c

*Letters indicate statistically significant difference at $p < 0.05$

Sorbitol concentration did not significantly affect germination rate, root and shoot size, or fresh and dry weight. It likely serves as an osmotic regulator in tissue culture rather than a carbon source, as it neither promotes *in vitro* shoot growth nor is metabolized by higher plants[30, 31,32].

Seed degeneration, encompassing morphological, physiological, and biochemical changes, is unavoidable during extended storage periods. This degeneration eventually results in a loss of seed viability[33, 34], as evidenced by germination failure after 107 days of storage. The seeds examined in this study also exhibited characteristics indicating desiccation sensibility[35] and could only be stored at room temperature for short periods without losing viability. Tobacco seeds can be successfully preserved under controlled temperature and relative humidity (50%) for up to ten years. However, these seeds lost their ability to germinate after 11 years when ambient conditions and humidity were no longer regulated[35].

Without the ability to control humidity, seeds likely experienced dehydration, resulting in irreversible damage to their membrane systems[36]. Prolonged storage exacerbates degeneration and ultimately leads to a complete loss of germination capacity, particularly after 107 days, regardless of the osmotic conditions in the storage media.

Sorbitol's function in *tissue culture* is primarily associated with its capacity to regulate osmotic pressure, rather than serving as a carbon source for growth. Despite being present in

the media, sorbitol had little to no impact on the morphological parameters of the plants, such as shoot length and biomass accumulation. This finding aligns with previous research[30, 31] that highlights sorbitol's limited role in promoting *in vitro* growth.

The germination dynamics observed under different storage conditions indicate that degenerative processes linked to long-term storage are the leading cause of reduced seed viability. This study shows that the germination rate significantly decreased from 100% at 34 days of storage to 0% at 107 days. These results highlight the critical need to optimize storage conditions, particularly relative humidity, to extend seed viability.

Ultimately, the findings highlight the necessity of integrating advanced seed conservation techniques with biotechnological initiatives to facilitate the sustainable use of *N. glauca* in research and commercial settings. Controlling environmental factors like temperature and humidity during seed storage is crucial to prevent seed degeneration and ensure the long-term survival of plant populations. Understanding how storage conditions and media composition interact with seed physiology provides valuable insights into unlocking the full potential of *N. glauc*a as a versatile and valuable biotechnological resource.

Storage at 6°C

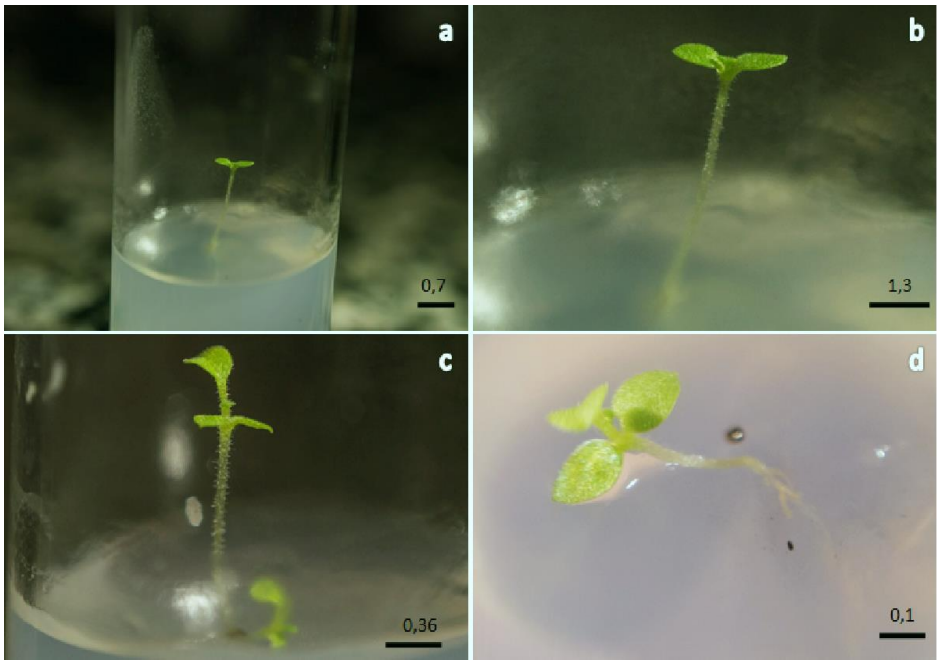

Figure 1. *Nicotiana glauca* seedlings with 15 days developed from seeds stored for up to 180 days at a temperature of 6°C. Photographer: Valerio, D. (2018).

At a storage temperature of 6°C, germination is influenced by the seed storage duration and sorbitol concentration in the medium. After 180 days of storage, seeds treated with 0.17 M sorbitol (-1.14 MPa osmotic potential) achieved a 100% germination rate (Figure 1), indicating practical preservation of seed viability over time (Table 2).

This finding emphasizes the critical role of an osmotic agent like sorbitol[37]. By lowering the medium's water potential[28, 38], sorbitol restricts water availability to the seeds, allowing gradual hydration during early germination stages, before radicle emergence[39]. This controlled water uptake prevents seed rupture by ensuring slow water entry into the seed cells. Additionally, many plant defense mechanisms against cold stress are like those activated by other abiotic stresses, such as salinity or drought[40].

Table 2. Germination percentage of *Nicotiana glauca* seeds stored for up to 180 days at a temperature of 6°C, with different treatments of sorbitol in the media.

Storage time before inoculation (days)	**Sorbitol concentration (M)**	**Osmotic potential of the media (MPa)**	**Germination rate (%)**
34	0,17	-1,14	80 b
54	0,08	- 0,54	66 c
54	0,25	-1,41	0 h
107	0,04	-0,4	20 f
107	0,17	-1,14	8 g
107	0,29	-1,64	10 g
159	0,08	- 0,54	53 d
159	0,25	-1,41	49 e
180	0,17	-1,14	100 a

*Letters indicate statistically significant differences at $p < 0.05$

The seed osmotic agent strongly influences the lag phase of seed germination, as most metabolic changes occur during this phase. Consequently, an osmotic agent can reduce the time between seed sowing and seedling emergence[41]. Different osmotic agents, such as sorbitol and distilled water, have been shown to improve seed germination[42].

Considering this, it is important to know the optimal combination of temperature and sorbitol to maintain the seed viability for future use. The data indicate that a temperature of 6°C, combined with a low sorbitol (0,17M), effectively protects plant cells and enhances seed

viability. Due to their small molecular size and nature as compatible solutes, sorbitol plays a significant role in cellular osmotic regulation[43]. Plants can leverage the beneficial properties of sorbitol, adjusting their content, allocation, and metabolism to mitigate abiotic stresses[44], such as storage temperature, utilized in this study.

Moreover, seeds stored at 6°C for 180 days may be considered a vernalization period that can break dormancy[45, 46]. Winter plants can perceive low temperatures and measure the cold dosage during this sensitive period of vernalization[47]. The plants' reaction to vernalization is influenced by two main factors: the duration and the temperature of the vernalization period[48, 49].

Low temperatures reduce water availability in plant cells, causing osmotic stress and stimulating ABA production and biosynthesis. A high concentration of ABA can enhance cold-temperature tolerance by acting as a chemical signal that activates the synthesis of proteins during cold stress[40]. If physiological dormancy is not strongly pronounced, it can be broken by storing the seeds under dry conditions for weeks or months. Another effective method is subjecting the seeds to cold stratification, where they are exposed to temperatures between 4 °C and 10 °C for 2 to 4 days[50].

N. glauca traits include high seed production (a fully grown plant can produce 10,000–1,000,000 seeds), a substantial soil seed bank, resilience to prolonged waterlogged and drought conditions, the capability of resprouting after small amounts of rainfall, and high seed germination rates across a range of temperatures. Studies found that a large quantity of seeds are stored in the soil, although the dynamics and longevity of the small seed remain unknown[51].

The high soil seed bank density of *N. glauca* suggests that many viable seeds in the soil bank may germinate after rainfall. However, in the absence of data on seed viability, such as persistence of seeds in the soil after their release from the capsule, it is misleading to suggest that seeds from soil banks will continue to germinate after rainfall[51]. Monitoring seed viability is essential for planning, control, and regeneration programs[35] Morphological characteristics of plants from the seeds stored at 6°C were evaluated, showing that storage time affected root and shoot size and dry and fresh weight (Table 3) (Figure 2).

Table 3. Root and shoot growth, dry and fresh weight of *Nicotiana glauca* seedlings developed from seeds stored for up to 180 days at a temperature of 6°C.

Storage time before inoculation (days)	Root (cm)	Shoot (cm)	Dry weight (g)	Fresh weight (g)
34	10,00 a	7,50 a	0,04 a	0,84 a
54	3,09 b	7,00 a	0,01 b	0,33 b
107	0,60 c	0,85 c	0,01 b	0,03 c
159	2,71 b	3,43 b	0,01 b	0,13 c
180	2,13 b	3,17 b	0,00 b	0,04 c

*Letters indicate statistically significant difference at $p < 0.05$

The speed of germination plays an important role in the plant growth cycle[52]. Before the primary root or the radicle emerges, a physiological phase occurs in which an important metabolic process absorbs water to repair the cellular components damaged during the maturation drying period[38]. Besides that, plantlets are very sensitive to biotic and abiotic stresses[52]. Although sorbitol is used as an osmotic agent to reduce water availability and promote the slow growth of plants, it can have a phytotoxic effect when used for conservation as observed with *P. pyramidalis* [28]. Therefore, quick germination can therefore be advantageous. The germination typically declines with an increase in seed storage time[52].

The findings on seed storage at 6°C underscore the significant impact of sorbitol on enhancing seed viability over extended periods. The ability of seeds to germinate for up to 180 days emphasizes sorbitol's effectiveness in reducing osmotic stress, thereby facilitating germination. The potential vernalization effect - where low temperatures induce physiological changes - also opens possibilities for improving seed germination and plant productivity. This mechanism is particularly crucial for the *in vitro* propagation of biotechnologically important species like *N. glauca*, where precise control of osmotic potential is essential for optimizing germination and plantlet development. These findings suggest that low-temperature storage, combined with sorbitol supplementation, can effectively maintain seed viability and enhance subsequent plant growth, making *N. glauca* an attractive candidate for bioengineering and sustainable agriculture.

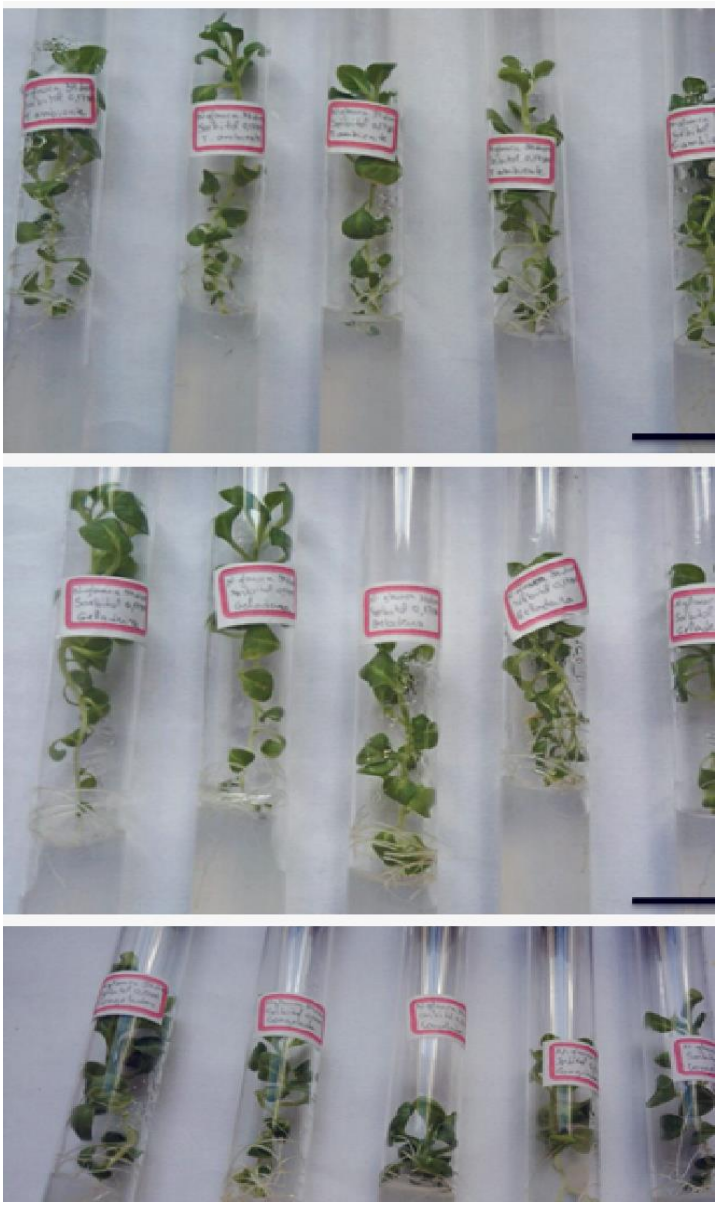

Figure 2. *Nicotiana glauca* seedlings developed from seeds stored up to 34 days at room temperature (a); 6°C (b); and -4°C (c).

Storage at -4°C

At -4°C, the germination rate remained comparable to room temperature after 34 days of storage (90%). However, the loss of viability was more pronounced, with some germination after 180 days due to the low sorbitol concentration in the media composition, with no statistically significant difference. Cold stress leads to the freezing and expansion of cellular water, which damages plant tissues and triggers physiological modifications that plant

cells undergo to prevent injury in subzero temperatures[40]. Research reported that low temperatures delay specific metabolic processes and disrupt the functionality of the cell membrane by reducing its fluidity and limiting its permeability [53].

Investigations of genebank accessions of *N. tabacum* L. and *N. rustica* L. stored at different temperatures have demonstrated that reducing the temperature to − 15/− 18°C can extend seed viability to over 50 years[35, 54]. Seed survival is essential for any plant's life cycle and is crucial for managing germplasm collections and preservation[35]. Understanding the biotic and abiotic factors that influence the seed germination of invasive species such as *N. glauca* is essential. The lack of information regarding environmental factors affecting *N. glauca* seed germination complicates the development of systematic control strategies for this invasive species[51,55,56].

The study of storage conditions at -4°C revealed that, after 34 days, germination rates were like those at room temperature, both at 90%. However, a significant decline in viability over time was observed at -4°C, especially after 180 days, partly due to the low concentration of sorbitol in the growth medium. This highlights the detrimental effects of cold stress on seed viability, and the complex physiological adaptations plants undergo to protect against cellular damage in subzero temperatures[40,53]. Research also emphasizes the potential of low temperatures to enhance seed storability, providing crucial insights for germplasm management and conservation[35, 54].

The challenge of managing invasive species like *N. glauca* underscores the need to understand the biotic and abiotic factors profoundly influencing seed germination. This study emphasizes the importance of research in uncovering environmental nuances that are essential for developing effective control strategies[51,55,56]. Ultimately, the findings not only advance our understanding of seed biology but also highlight the need for interdisciplinary approaches to address the ecological challenges posed by invasive species.

CONCLUSION

This study demonstrated that storage temperature and sorbitol concentration significantly affect the *in vitro* germination and viability of N. glauca seeds. Seeds stored at

6°C with sorbitol-maintained viability for up to 180 days, while room temperature storage led to a complete loss of viability after 107 days. Storage at -4°C initially preserved germination but caused viability loss over time due to cold stress. These findings highlight the importance of temperature control and osmotic regulation in seed conservation. The results support the development of improved *in vitro* propagation protocols and advance the biotechnological use of *N. glauca*. Future studies should evaluate the role of relative humidity in further enhancing storage strategies.

ACKNOWLEDGEMENTS

The authors would like to thank Daniel Teixeira Valerio Caetano for the photographs and acknowledge the research support agencies FAPEMIG (Research Supporting Foundation of Minas Gerais State) and CAPES (Coordination for the Improvement of Higher Education Personnel) for student grants.

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