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DISENTANGLING THE EFFECTS OF LIQUID NITROGEN ON IMPERMEABLE SEED-COATED SPECIES

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Abstract

Nelumbo nucifera, commonly known as the sacred lotus, is a plant of deep cultural, nutritional, and environmental importance, especially in Asian countries. It is widely used for its edible seeds and roots, and it also has ceremonial significance in many cultural traditions. In addition to its use in cuisine and rituals, Nelumbo nucifera plays a crucial role in aquatic ecosystems, oxygenating the water and providing habitat for various species of wildlife. However, the wider use of Nelumbo nucifera in nature management and agriculture is hindered by the physical dormancy of its seeds. This dormant trait prevents seeds from germinating under normal conditions, thereby limiting their natural dispersal and use in controlled cultivation and recovery projects. Liquid nitrogen (LN) treatment is investigated to disturb the dormancy of impermeable Nelumbo nucifera seeds by physically damaging the seed shell and facilitating water and oxygen penetration. Extremely low temperatures during LN treatment can weaken the seed coat, improving water absorption and promoting germination. This research investigates how LN treatment affects seed germination and seedling growth compared to untreated seeds. Various factors, such as seed quality, treatment duration, and germination conditions, are considered when evaluating the effectiveness of LN treatment. Conduct thorough experiments and optimize LN treatment protocols to maximize the benefits of Nelumbo nucifera seed germination.

Introduction

Nelumbo nucifera, commonly known as the sacred lotus, is a plant known for its various medicinal properties and biological activities. One crucial aspect related to Nelumbo nucifera is the physical dormancy of its seeds. Seeds of Nelumbo nucifera exhibit impermeability to water, leading to physical dormancy (Jaganathan et al., 2017). Nelumbo nucifera is a plant with diverse biological activities and medicinal properties. Breaking the physical dormancy of its seeds is crucial for its propagation and growth. The antioxidant, anticancer, and neuroprotective properties of Nelumbo nucifera seeds make them valuable in combating various diseases. Additionally, genetic studies enhance our understanding of the plant's molecular mechanisms, paving the way for further research and applications in medicine and agriculture. Nelumbo nucifera seeds have antioxidant potential, emphasizing their hepatoprotective properties and their use in treating tissue inflammation and cancer (Rai et al., 2006). The presence of phytochemicals with strong antioxidant potential in Nelumbo nucifera seeds underscores their importance in combating oxidative stress-related diseases. Compounds like neferine from Nelumbo nucifera seeds have been shown to have pro-oxidant anticancer mechanisms, indicating the diverse bioactive nature of the plant(Dasari et al., 2020). Moreover, Nelumbo nucifera seeds have been found to possess phenolic antioxidants with extraction optimization techniques enhancing their antioxidant properties (Nawaz et al., 2017). These antioxidants protect cells from damage caused by free radicals and oxidative stress. The plant's extracts have shown protective effects against beta-amyloid protein-induced apoptosis, suggesting a potential role in combating neurodegenerative diseases like Alzheimer's (Kumaran et al., 2018). The genetic and genomic aspects of Nelumbo nucifera have also been explored, with studies focusing on genome assemblies, genetic maps, and gene expression profiles (Gui et al., 2018). Understanding the molecular evolution and functional characteristics of genes in Nelumbo nucifera provides insights into its biological significance and potential applications in various fields. The wider use of Nelumbo nucifera is hindered by the physical dormancy of its seeds. This dormant trait

prevents seeds from germinating under normal conditions, thereby limiting their natural dispersal and use in controlled cultivation and recovery projects.

Physical dormancy in seeds, also known as hard seededness, is a mechanism where seeds have impermeable seed coats that prevent germination until specific conditions are met (Paulsen et al., 2013) . This dormancy type serves as a protective barrier against microbial attacks and predators, aiding in the maintenance of seed banks in soils (Dalling et al., 2011) . Seeds with physical dormancy rely on physical defenses to exclude predators and pathogens, ensuring their survival (Vandelook & Van Assche, 2010). The impermeable seed coat acts as a mechanism imposing dormancy on the seeds by preventing imbibition and germination (Hu et al., 2009). Ecologically, physical dormancy is crucial in seed dispersal and survival strategies. Seeds with physical dormancy can disperse over long distances, as the impermeable seed coat increases their chances of survival during dispersal (Rosbakh et al., 2023). Additionally, physical dormancy enables plants in harsh environments to survive by ensuring long-term seed viability (Bolingue et al., 2010). The release of physical dormancy during imbibition is influenced by environmental factors such as initial water content, with drier seeds requiring longer imbibition times(Jaganathan, 2021). Furthermore, physical dormancy allows seeds to synchronize germination with favorable growing seasons, maximizing seed survival after dispersal (Ibrahim et al., 2021). Understanding the mechanisms behind physical dormancy breaking is essential for seed germination. Environmental signals such as temperature fluctuations, wet heat, dry heat, or alternate wet-dry conditions, can trigger dormancy breaking in physically dormant seeds. The ecological significance of physical dormancy lies in its role in seed dispersal, survival in harsh environments, and synchronization of germination with optimal conditions, highlighting its importance in plant ecology and conservation efforts.

Seed dormancy and impermeable seed coats pose significant challenges to successful germination in plant species. Understanding the effects of various treatments, such as liquid nitrogen exposure, on impermeable seed-coated species is crucial for enhancing germination rates and promoting plant conservation efforts (Coelho et al., 2018); (Cejas et al., 2016). Liquid nitrogen has been explored as a potential method to break seed dormancy and improve germination in impermeable

seed-coated species. A valuable tool for maintaining genetic diversity and preserving endangered plant species (Castillo et al., 2010). LN provides a means to store plant germplasm efficiently, allowing for the conservation of plant biodiversity (Engelmann, 2011). However, the responses of different plant species to liquid nitrogen treatments vary, highlighting the need for a comprehensive understanding of the underlying mechanisms Liquid nitrogen can change the mineral composition of plant tissues, affecting nutrient absorption and physiological processes. Studies have shown that liquid nitrogen exposure may not significantly affect the germinability of specific seed species (Salomão, 2002). This underscores the importance of considering species-specific responses when utilizing cryogenic treatments for breaking seed dormancy. Additionally, research has demonstrated the complexities of impermeable seed coats and the role of scarification in promoting germination in species like Astragalus adsurgens (Jaganathan et al., 2019). Understanding the mechanisms underlying seed coat impermeability and dormancy break is essential for developing effective strategies to enhance germination in impermeable seed-coated species. Using of liquid nitrogen in cryopreservation is associated with changes in antioxidant levels and lipid peroxidation, indicating potential effects on plant stress responses (Uchendu et al., 2010).Furthermore, the variability in responses of different plant species to liquid nitrogen treatments has been highlighted in studies on the cryopreservation of native species (Touchell & Dixon, 1993). While some species exhibit enhanced germination post-treatment, others may experience decreased germination rates. This variability emphasizes the importance of tailoring conservation strategies to the specific needs of different plant species to ensure successful germination and preservation of genetic diversity.

The effects of liquid nitrogen on impermeable Nelumbo nucifera are of interest due to their potential impact on plant physiology and conservation efforts. In addition to liquid nitrogen treatments, other factors such as temperature, desiccation, and storage conditions can influence seed viability and germination. When evaluating the effects of liquid nitrogen on impermeable seed-coated species, the interplay between environmental factors and seed characteristics must be considered. By synthesizing findings from diverse studies, researchers can advance

knowledge in this area and develop targeted approaches to improve germination outcomes in impermeable seed-coated species.

This paper aims to disentangle the effects of liquid nitrogen on impermeable seed-coated species by examining the responses of different plant species to cryogenic treatments. By exploring the mechanisms underlying seed dormancy, germination ecology, and the impact of liquid nitrogen exposure, this study seeks to understand how liquid nitrogen treatments can effectively enhance germination in impermeable seed-coated species.

Materials and method

Seed Collection and Preparation

Lotus (Nelumbo nucifera) seeds were collected from Suqian, Jiangsu Province, China, during the autumn season and subsequently stored at 25 degrees Celsius. Careful attention was paid to seed maturity, size, and integrity to maintain uniformity across experimental batches. The collected seeds underwent rigorous cleaning and sterilization procedures to eliminate external contaminants that could influence experimental outcomes. After being collected, the seeds were sent to the University of Shanghai for Science and Technology in Shanghai, China. They were kept in jute bags at room temperature (around 20°C and 50-60% relative humidity) until they were utilized in the lab tests. All experiments commenced within three days of seed collection.

Seeds moisture content (%, H_2O mass: fresh mass)

The moisture content of seeds were measured using three replicates of 15 seeds each, which were dried in an oven at 103°C for 17 hours. The difference between the fresh and dry weights is calculated as a percentage of the fresh weight. The formula for determining the moisture content based on fresh weight is

$$Moisture\ content\ of\ the\ seeds\ (\%) = \frac{(Fresh\ sample\ weight) - (Dry\ sample\ weight)}{Dry\ sample\ weight} X100 \quad (1)$$

where: Fresh sample weight: Mass of the seeds before placing in the oven. Dry sample weight: Mass of the seeds after drying in the oven.

Separation of permeable and impermeable seeds:

The collected material comprised both permeable and impermeable seeds, as identified in initial imbibition tests. To distinguish the permeable seeds from the impermeable ones, they were placed in sandwich boxes on damp filter paper and kept in a laboratory environment at approximately 20-22°C. Within 12 hours, the permeable seeds started to swell, and these were manually selected and left to dry on a laboratory bench. After 24 hours of bench drying, the moisture content of the seeds was assessed as previously described.

Scarification by immersion in LN

The impermeable seeds with their original mc without cryoprotectant treatments were placed in aluminium foil paper and immersed directly in liquid nitrogen (LN). After 1 week exposure the samples were removed and thawed at room temperature (25 ± 2 C) for 1-2 h.

Germination

Three replicates, each consisting of 15 seeds, were selected to assess moisture content from a batch of 100 seeds that had undergone scarification with liquid nitrogen (LN). Additionally, three replicates of 15 seeds each were arranged in Petri dishes lined with felt paper, which was pre-moistened with 5 ml of distilled water to evaluate germination and imbibition rates. These Petri dishes were then placed in a germination chamber set at 24°C and 80% relative humidity with a photoperiod of 14 hours of light and 10 hours of darkness provided by fluorescent light tubes, where they were kept for a duration of 21 days.

Imbibition Testing Methodology

To determine whether the seeds are permeable or impermeable at the time of dispersal, an imbibition test will be conducted. 800 lotus seeds were used for identifying specific moisture content inducing impermeability. Seeds that absorb water during this test will be identified as non-dormant and will subsequently undergo further drying experiments to elucidate the influence of moisture content on the initiation of dormancy. For the imbibition test, seeds from various species will be arranged on moistened tissue within sizable plastic containers (dimensions: 60 cm long × 50 cm wide × 12 cm high) that are equipped with perforated lids. These lids are designed to reduce water evaporation while still permitting gas exchange. Water will be added as needed to maintain adequate moisture. Seeds that exhibit

swelling within the first 24 hours will be classified as permeable.

Identifying Absolute and Shallow PY

Portions of permeable seeds were subjected to drying using a silica gel with a 4:1 ratio of gel to seed within airtight containers, with the silica gel refreshed every 12 hours. On the 3rd, 7th, and 10th days of each cycle, 100 seeds were removed to assess their moisture content and for imbibition tests, using three replicates of 15 seeds each. Additionally, 10 seeds were set aside for scanning electron microscopy and stored in zip bags. Concurrently, impermeable seeds were allocated to two separate experiments to differentiate between Absolute Physical Dormancy (Absolute PY) and Shallow Physical Dormancy (Shallow PY). Impermeable seeds were placed above water to assess Absolute PY, whereas for Shallow PY, impermeable seeds continued the drying process over silica gel. Similar to the treatment of permeable seeds, every 3rd, 7th, and 10th day of the cycle, another set of 100 seeds was taken to check moisture content, to perform imbibition tests with three replicates of 15 seeds each, and to collect 10 seeds for scanning microscopy, stored in zip bags.

The experimental design aligns with previous research on seed impermeability and dormancy mechanisms. Studies have shown a strong relationship between seed moisture content and the onset of impermeability in various plant species (Jaganathan et al., 2019) ; (Jaganathan et al., 2017). The process of impermeability induction due to reduced moisture content has been well-documented, highlighting the importance of moisture levels in seed coat impermeability Additionally, the separation of impermeable seeds for different experiments to indicate Absolute PY and Shallow PY is a novel approach that can provide insights into the mechanisms underlying seed impermeability (Jaganathan, 2018).

2.8 Wet – dry heat

Seeds of Nelumbo nucifera were exposed to alternating wet and dry heat by immersing them in boiling water for 5 minutes followed by drying in an incubator at 40°C for 24 hours, a process that was repeated ten times (Gama-Arachchige et al., 2013).At intervals of every 3rd, 7th, and 10th day, 100 seeds were sampled for moisture content analysis in triplicates of 15 seeds each. Additionally, for the imbibition test, 3 replicates of 15 seeds were collected, and 10 seeds were reserved

for scanning electron microscopy in zip bags.

Statistical analysis

Each treatment was replicated three times in a Completely Randomized Design. Data were analyzed using the Microsoft Excel software. Standard error (S.E.) was calculated to show differences among developmental means.

Results

The 800 seeds utilized in the experiment had the following outcomes: 200 seeds became impermeable, 569 seeds remained permeable, and 31 seeds rendered unusable due to mold contamination.

MC(%)	Permeable seeds (%)	Impermeable seeds (Absolute PY + Shallow PY)(%)	Shallow PY(%)	Absolute PY(%)
12.11 ±0.77	74	26	=	=
15.09±0.81	100	0	=	=
8.43±0.09	0	100	=	=
5.37±0.12	0	0	0	100

Table.1 Relationship between seed coat penetration and corresponding MC of Nelumbo nucifera

Table .1 demonstrates that 74% of lotus seeds with an initial moisture content of 12.11% were permeable to water, increasing to 100% permeability when the moisture content reached 15.09%. However, after drying these permeable seeds for 48 hours, their moisture content reduced to 8.43%, rendering the seed coats completely impermeable. These findings suggest that a decrease in the moisture content of lotus seeds is associated with a transition from a permeable to an impermeable seed coat and, consequently, from a non-dormant to a dormant state. This indicates that the impermeability of the seed coat is a key factor contributing to the physical dormancy of lotus seeds. To further investigate the specific moisture content related to different types of physical dormancy, some seeds (PY seeds) were subjected to drying while others were placed above water. Drying Nelumbo

nucifera seeds to 8.4% moisture content resulted in 100% impermeable seeds. Further desiccation of impermeable seeds to 5.3% moisture content indicated Absolute Physical Dormancy (PY), as no germination or water imbibition occurred over the 10-day period. Seeds with 8.4% moisture content exhibited an increase in moisture content to 9.44±0.36% (3 days), 9.62±0.67% (7 days), and 9.68±0.31% (10 days), potentially indicating Shallow Physical Dormancy (PY).

Method	100 seeds weight(g)	MC(%)	Max length at 21 days (cm)	Germination at 7 days (%)
Seeds not exposed to LN	114.56 ±0.27	12.11 ±0.77	23	73±0.33
Seeds exposed to LN	109.66±0.31	12.33 ±0.25	42	74.7±0.41
Wet-dry heat	112.33±0.22	14.66±0.63	56,5	84.6±0.23

Table.2 Comparison of effective methods for better germination of Nelumbo nucifer

The effect of exposure to ultralow temperature (liquid nitrogen, LN) on germination of the seeds was investigated in Nelumbo nucifera. The results show that the seeds can survive cryostorage. The wet-dry heat treatment shows the highest germination rate (84.6%), significantly better than the non-treated seeds (73%) and those exposed to LN (74.7%). While the wet-dry heat treatment showed the highest effectiveness in germination and growth among the methods tested, the use of liquid nitrogen (LN) to treat lotus seeds (Nelumbo nucifera) also presents distinct advantages that are worth considering: LN can effectively break physical dormancy to some extent, facilitating the initiation of germination processes. Liquid nitrogen treatment is relatively quick and can be easily applied to large batches of seeds, making it a practical option for large-scale seed treatment operations. This rapid exposure minimizes the handling time and reduces the risk of seed damage during processing

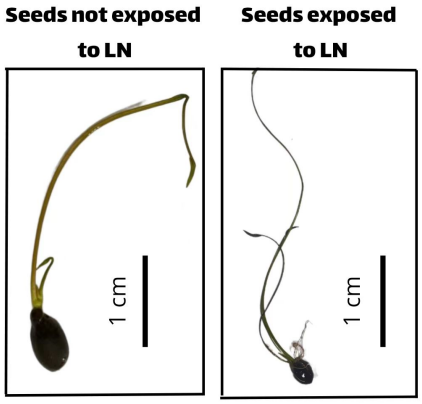

Fig. 1 Nelumbo nucifera seedling morphology at 21 days after LN exposure

Seed exposure to LN improved seed imbibition rate (after 24 h) and germination (21 days; Fig. 1) significantly relative to control seeds/seedlings (Table 1). The seeds treated with liquid nitrogen showed a substantial increase in growth, with the maximum length at 21 days reaching 42 cm, nearly double that of untreated seeds (23 cm). This demonstrates that liquid nitrogen treatment not only aids in breaking dormancy but also promotes vigorous early growth, which is crucial for the successful establishment of seedlings in their natural habitat or cultivation areas.

4. Discussion and Conclusion

Cryopreservation in liquid nitrogen is a well-established method for preserving the viability and genetic integrity of biological materials, including plant seeds. The process involves storing plant material at ultra-low temperatures, typically in liquid nitrogen at -196 ºC, to maintain their viability over extended periods(Coelho et al., 2020). Lotus seeds, known for their resilience to extreme conditions, could benefit from cryopreservation in liquid nitrogen to ensure their long-term storage and transportation (Paudel & Panth, 2015). This method has successfully conserved the genetic diversity of various plant species, including recalcitrant seeds like Acer saccharinum, by storing them at temperatures below -180 ºC using liquid nitrogen (Wesley-Smith et al., 2015). Research has shown that cryopreservation in liquid nitrogen can enhance seed longevity and stress tolerance in plants by preserving

their genetic material effectively (Chen et al., 2016). The use of cryoprotectants and specific cooling procedures before cryopreservation can influence the viability of seeds post-treatment. Additionally, maintaining an optimal moisture content is crucial for the successful cryopreservation of seeds, as demonstrated in studies on Coffea arabica L. seeds (Figueiredo et al., 2021).

The exposure of seeds to liquid nitrogen (LN) has been found to have a significant impact on the alleviation of physical dormancy in legume seeds with a similar seed anatomy to T. labialis. (Geisler et al., 2017); (Janská et al., 2019). This improvement does not stem from a reduction in the thickness of external seed layers due to LN treatment. Instead, exposure to LN appears to induce permeability in the hilar region and compromise the integrity of the testa, thereby facilitating water absorption. (Acosta et al., 2019) ; (Acosta et al., 2020).

Scarification by immersion in LN Nelumbonaceae seeds represents a promising approach to overcome physical dormancy and enhance germination rates. Changes in permeability caused by LN treatment in the root region and disruption of seed integrity play a vital role in enhancing water uptake and germination, highlighting the effectiveness of LN treatment in facilitating seed dormancy.

In conclusion, impermeable seed coats in species with physical dormancy play a crucial role in seed germination ecology. Understanding the mechanisms underlying impermeability and dormancy breakage is essential for conservation and propagation efforts of species with such seed characteristics. Treatments like cryopreservation, dry heat, boiling water, and scarification have been identified as effective methods to break dormancy in seeds with impermeable coats. The adaptive significance of impermeable seed coats in synchronizing germination with environmental conditions underscores the importance of further research in this area. Future studies should focus on elucidating the genetic and physiological basis of impermeable seed coats to enhance our understanding of seed dormancy and germination in impermeable seed-coated species.

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