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Cryopreservation of Terap (*Artocarpus elasticus* Reinw. ex. Blume) Seeds: Viability and Structural Characterization

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Abstract

Terap (*Artocarpus elasticus* Reinw. ex Blume) is an underutilized fruit tree native to Indonesia, valued both for its edible fruit and its diverse applications in traditional medicine. Terap seeds are recalcitrant, so cryopreservation is the most effective method for their long-term preservation. This study aimed to determine the optimal loading and vitrification times for cryopreservation to maintain seed viability. A factorial, completely randomized design was employed with two factors: loading time (0 and 20 min) and vitrification time (0, 30, and 60 min). Seed viability after loading and vitrification treatment with or without freezing in liquid nitrogen was evaluated using tetrazolium chloride (TTZ) and germination tests. Results indicated that the loading and vitrification treatments did not yet improve the viability of terap seeds after cryopreservation, as determined by the TTZ and germination tests. The TTZ test showed that the viability of all treated seeds decreased, regardless of whether they were frozen in liquid nitrogen. Germination tests revealed that all treated seeds without freezing had reduced germination, while none of the seeds treated with freezing germinated. Structural analysis indicated that freezing alters cell degradation, likely due to the formation of ice crystals and mechanical stress. To improve the success of terap seed cryopreservation, future efforts should focus on optimizing exposure times, reducing PVS2 toxicity, and enhancing cryoprotectant penetration by removing the seed coat or using the embryo axis as an explant.

Keywords: germination test, loading, tetrazolium test, vitrification

Introduction

Terap (*Artocarpus elasticus*) is an underutilized fruit from Indonesia with nutritional and medicinal benefits. Recent studies have revealed that the fruit, seed, bark, and wood of this plant contain various beneficial compounds with antioxidant, cytotoxic, anticancer, and antimalarial effects (Bailly, 2021). Terap seeds are classified as recalcitrant (Wardani et al., 2024), making cryopreservation the most effective method for conserving them (Lah et al., 2023). Plant cryopreservation involves cooling and storing plant specimens such as cells, tissues, or organs in liquid nitrogen (LN; -196 °C) or LN vapor (-160 °C) to preserve their viability after thawing, enabling indefinite storage (Benelli, 2021).

Many plant cryopreservation protocols have been developed, and vitrification is widely used because of its ease of application, low cost, and faster process, which involves direct immersion of plant specimens in LN (Benelli, 2021; O'Brien et al., 2020). Vitrification is a physical process that prevents intracellular ice crystallization during ultra-freezing by turning the cytosolic water into an amorphous, glass-like state, thus protecting plant tissues from damage and maintaining their viability during long-term

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storage at -196 °C (Benelli, 2021). Vitrification usually involves a two-step cryoprotection process: loading (incubation in the loading solution (LS)) and dehydration (incubation in the vitrification solution (VS)) (Nagel et al., 2024). The purpose of LS is to biophysically prepare the plant specimens for osmotic stress by exposing them to a lower concentration of osmoticum before severe dehydration with a highly concentrated VS (Roque-Borda et al., 2021). It also helps induce tolerance to the dehydration caused by VS. A common loading solution contains 2 M glycerol and 0.4 M sucrose (Bettoni et al., 2021). The VS acts as a cryoprotective agent (CPA), not only during dehydration but also by modifying the solution's viscosity to prevent nucleation and the formation of ice crystals at low temperatures (Roque-Borda et al., 2021). The VS contains high-concentration chemicals such as ethylene glycol, glycerol, and dimethyl sulfoxide (DMSO), which have been reported to be toxic to plant tissue (Whaley et al., 2021). Therefore, it is important to determine a minimum exposure time to vitrification solutions to effectively dehydrate plant tissue for cryopreservation while preventing damage (Faltus et al., 2021). The most commonly used VS for plant cells is plant vitrification solution-2 (PVS2), which contains 30% (w/v) glycerol, 15% (w/v) DMSO, 15% (w/v) ethylene glycol, and 0.4 M sucrose (Roque-Boeda et al., 2021).

Cryopreservation of terap seeds has not yet been attempted, but some researchers have successfully cryopreserved other fruits from the *Artocarpus* genus, such as jackfruit. Krishnapillay (1989) used an embryo as an explant and treated it with cryoprotectants (10% DMSO + 0.5% proline) for 12 hr, achieving 29%-33% desiccation. They employed a slow-freezing method, and the results showed that 60% of the embryos survived and regrew. Thammasiri (1999) also used an embryo, employing the vitrification method with plant vitrification solution-2 (PVS2) as a cryoprotectant. The results showed that 50% of the embryos could regrow into plantlets. Okunade (2018) employed vitrification with PVS4 as a cryoprotectant and used shoot tips as explants. The results showed that 37.9% of shoot tips regrew into plantlets.

These studies examined the optimal loading and vitrification times for terap seed cryopreservation. We used a 20-min loading time because it achieved a 60% germination rate on papaya 'Caliso' seeds (Wardani et al., 2019). Adu-Gyamfi and Wetten (2012) also used a 20-min loading method, resulting in 100% survival, compared with 55%-90% survival for cacao embryos exposed to PVS2 for longer periods. We tested 0, 30, and 60 min of cryopreservation time, based on Thammasiri (1999), who exposed jackfruit embryos to 50 min of cryopreservation and reported a 50% survival rate. Therefore, this study aimed to identify optimal loading and vitrification times for cryopreserving terap seeds to maintain their vigor and viability using the vitrification method.

Materials and Methods

Plant Materials

Terap seeds were collected from Bogor Botanic Gardens, West Java, Indonesia. They were planted in Block XIX.F.126 (106° 48' 16" E and 6° 36' 4" S). The orange fruits (yellow-orange group 17A) (Figure 1) were harvested and handled following Wardani et al. (2024).

Cryopreservation Procedures

This study employed a completely randomized factorial design to assess the effects of loading time and vitrification on terap seed viability. The experiment consisted of two loading times (0 and 20 min) and three vitrification durations (0, 30, and 60 min), resulting in six treatment combinations, each replicated three times, for a total of 18 experimental units. Each unit contained 20 seeds, resulting in 360 seeds per treatment condition: one without freezing in liquid nitrogen (no-LN) and one with freezing in liquid nitrogen (with-LN), for a total of 720 seeds.

To prepare the seeds, their moisture content was reduced by air-drying them indoors under continuous light at 18 ± 2 °C and 55%–65% relative humidity for 2 days, resulting in an

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average moisture content of 36 ± 2%. Seeds assigned to the loading treatment were soaked in a solution of 2 M glycerol and 0.4 M sucrose in liquid MS medium for 20 min, while control seeds were immersed in sterile water for the same duration. Afterward, the seeds were filtered and immersed in plant vitrification solution-2 (PVS2), which contains 30% (w/v) glycerol, 15% (w/v) dimethyl sulfoxide, 15% (w/v) ethylene glycol, and 0.4 M sucrose (Sakai et al., 1991), at 25 °C for the designated vitrification times.

Seeds were transferred to 5 ml cryotubes for cryopreservation and stored in liquid nitrogen overnight. The seeds in the with-LN treatments were thawed at 40 °C for 90–120 sec (Wardani et al., 2019) and then washed in liquid MS medium for 30 min. Meanwhile, the seeds in the no-LN treatments skipped the thawing process and were directly washed in liquid MS medium.

Viability Test

After cryopreservation, the viability of terap seeds was evaluated using tetrazolium and germination tests. The tetrazolium test used a 1% solution of 2,3,4-triphenyltetrazolium chloride as an indicator to monitor the reduction of tetrazolium chloride during respiration in living cells (Franca-Neto et al., 2022). After cryopreservation, terap seeds were cut with a sharp blade at the cotyledonary area opposite the embryo. They were completely immersed in the staining solution in the dark for 24 hr at 35 ± 2 °C (Franca-Neto et al., 2022). Afterwards, they were dissected along the middle of the cotyledons and the hypocotyl axis with a sharp blade for evaluation. Seed viability was indicated by red colorization in the seeds (Figure 2).

Figure 1

a) A Terap Fruit, b) the Longitudinal Cut of Terap Fruit, and c) Terap Seed

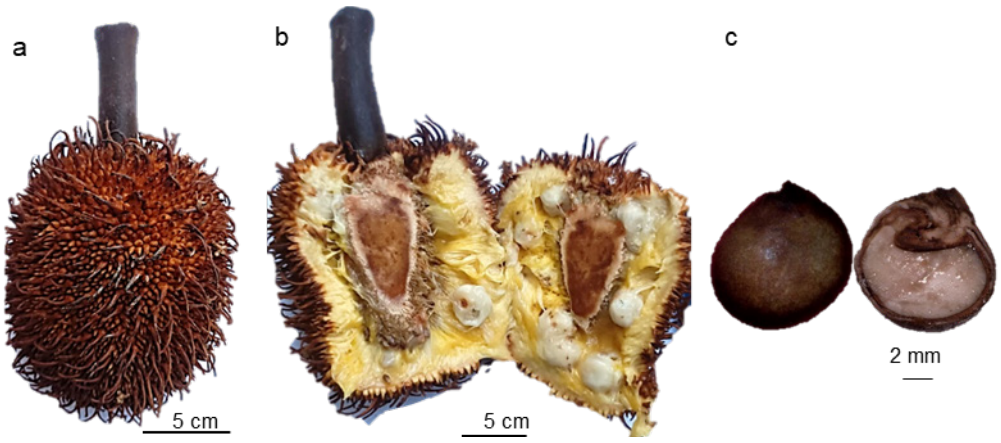
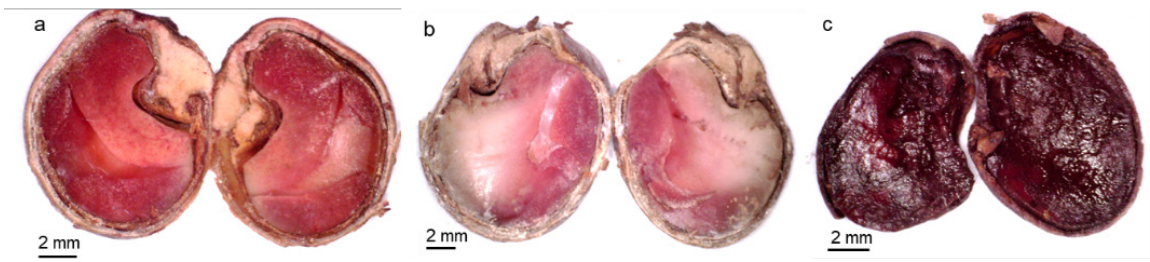


Figure 2

Classification of Terap Seed Quality using the Tetrazolium Test (TTZ): (a) High Viability, (b) Low Viability, and (c) Non-viable



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The germination test was conducted based on Wardani et al. (2024), which used several variables, including germination percentage (GP), vigour index (VI), germination rate (GR), germination uniformity (GU), and radicle emergence ability (EA). All data were processed in Microsoft Excel 2019 and analyzed using the *F*-test (α = 0.05) in the Statistical Tool for Agricultural Research (STAR) Nebula. Data showing significant differences were further evaluated using Duncan's multiple-range test (DMRT).

Structural Analysis

Structural analysis was conducted using a light microscope and a transmission electron microscope (TEM). The materials examined were cotyledons from terap seeds. Observations were performed using a binocular microscope (Olympus BX53 with Digital Camera DP58) at 400X magnification. Transverse sections of the seeds were cut with a scalpel and placed on a microscope slide. A drop of distilled water was put on it and covered with a cover glass. Observations aimed to examine the shape and size of terap seed cells under each treatment. Three sections from three seeds from five treatments were examined under a binocular microscope, i.e., control seeds, 20-min loading seeds, 30-min vitrification, and 20-min loading and 30-min vitrification (both no-LN and with-LN).

TEM observation began by preparing microtome sections using an Ultramicrotome PowerTome PC RMC Boeckeler, based on a modification of the method described by Wikantyoso et al. (2022). Three sections from three seeds in three treatments were examined under TEM: control seeds, 20-min loading, and 30-min vitrification (both with and without LN). Seeds from each treatment were cut into contact-shaped sections measuring 1 mm × 1 mm. The first fixation involved immersing the specimens in a 2.5% solution of glutaraldehyde and paraformaldehyde dissolved in 0.1 M phosphate-buffered saline (PBS), pH 7.4, for 12 hr, followed by three washes with PBS (0.1 M, pH 7.4). The second fixation was performed by immersing the specimens in 1% osmium tetroxide dissolved in PBS (0.1 M, pH 7.4) for 12 hr, followed by three rinses with distilled water. Pre-staining involved immersing the specimens in 1% uranyl acetate dissolved in distilled water for 2 hr, followed by three washes with distilled water. The specimens were then dehydrated through a graded ethanol series, consisting of 50%, 70%, 90%, and 100% ethanol, each for 15 min.

Next, the specimens were gradually infiltrated with ethanol: resin mixtures at ratios of 2:1, 1:1, and pure resin, each for 30 min. The resin used was EMbed-812: DDSA: NMA: DMP-30 in a volume ratio of 5 ml: 2.25 ml: 3 ml: 0.2 ml (resin type = hard), embedded in a Pyramid mold (LADD brand). Ultra-thin sections were cut with a Single Crystal Diamond Knife SYM2045, length 2.0 mm, 45° angle, and 6° inclination, in a cross-section orientation, with a thickness of 80 nm. The grids used were 100 mesh and 200 mesh. The microtome sections were then post-stained with 2% uranyl acetate dissolved in distilled water. Sections were observed using a Thermo Scientific Tundra Cryo-Transmission Electron Microscope (Cryo-TEM) at 100 kV.

Results and Discussion

Seed Viability Test

This study evaluated the viability of terap seeds after loading and vitrification treatment in both with-LN and no-LN treatments using tetrazolium (TTZ) and germination tests. The results from both tests are shown to compare seed viability under different conditions. The TTZ test indicated that the viability of terap seeds was not affected by the loading treatment in the no-LN treatments, but it decreased in the with-LN treatments (Table 1). Moreover, seed viability declined across all vitrification treatments in both the no-LN and with-LN conditions (Table 1). Although the TTZ test detected viable cells in some seeds, it is known to overestimate viability because enzyme activity in non-viable cells can produce false positives (Pradhan et al., 2022). The germination test further supported these

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findings, showing that seed viability decreased across all treatments in the no-LN group, while all seeds in the with-LN group failed to germinate (Table 2). The failure of seeds in the with-LN group was due to insufficient cryoprotection, which induced physiological stress and depleted the energy reserves necessary for germination. In vitro culture might potentially improve germination by providing external nutrients and energy. Aziz et al. (2023) also reported a similar correlation between TTZ and germination tests, indicating that the TTZ test is not always a reliable indicator of seed viability without the germination test. This result aligns with the viability tests conducted on cryopreserved *Phalaenopsis gigantea* seeds (Aziz et al., 2023), palm oil seeds (Tabi et al., 2017), and seeds of *Pisum sativum, Quercus robur,* and *Trichilia dregeana* (Ntuli et al., 2015).

Statistical analysis using the *F* test (α = 5%) revealed significant interactions between loading and vitrification times across all observed germination variables in the no-LN treatments. This interaction indicated that the 20-min loading and 30-min vitrification (L20P30) treatment was the most effective, followed by the no-LN treatment after loading and vitrification. However, the germination test showed that all loading and vitrification treatments decreased all observed variables in the no-LN treatment, and none of the seeds germinated in the with-LN treatments (Table 2). The reduction in germination and the inability to germinate were likely due to the chemical toxicity of the loading and vitrification solutions (Aziz et al., 2023).

In the present study, the loading solution comprised 2 M glycerol dissolved in a liquid MS medium supplemented with 0.4 M sucrose. Glycerol generally shows low toxicity because it has a limited ability to penetrate cell membranes, thus preventing osmotic shock at high concentrations. In the meantime, sucrose is usually impermeable to the membrane and serves as an energy source (Zamecnik et al., 2021). However, in this study, the 20-min exposure to the loading solution was harmful to the terap seed. This may have been too long, causing membrane damage, lowering germination rates, and preventing germination (Streczynski et al., 2019). Popova et al. (2023) highlighted that loading time is crucial for successful cryopreservation. The ideal duration varies among species, as different plant materials show different sensitivities and tolerances to loading solutions (Zamecnik et al., 2021). Usually, loading involves incubating tissues for 10-20 min in the loading solution (O'Brien et al., 2020). For example, *Phalaenopsis gigantea* seeds reached maximum germination after freezing with a 10-min loading time (Aziz et al., 2023), while somatic embryos of cocoa showed optimal results after freezing with a 20-min loading time (Adu-Gyamfi

Table 1

Viability of Terap Seeds Based on the Percentage of Red-colored Seeds on the Tetrazolium Test (TTZ) After Loading and Vitrification Treatments without (no-LN) and with Freezing in Liquid Nitrogen (with-LN)

Treatments	Viability (%)	
	no-LN	with-LN
Control seeds	90.0a	0.0c
30 min vitrification	57.5ab	50.0a
60 min vitrification	45.0b	20.0bc
20 min loading	90.0a	32.5ab
20 min loading and 30 min vitrification	52.5b	35.0ab
20 min loading and 60 min vitrification	57.5ab	45.0a

Notes. The same letter within the same column indicates that the treatment has a nonsignificant effect according to the DMRT (α = 0.05%). LN = liquid nitrogen.

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and & Wetten, 2012).

Similar to the loading solution, PVS2 contains glycerol and sucrose, which generally show low toxicity (Zamecnik et al., 2021). Ethylene glycol has two roles: it acts as a dehydration agent and prevents the formation of ice crystals. However, it can be toxic when broken down into oxalic acid (Zamecnik et al., 2021). DMSO offers cryoprotection by stopping the buildup of electrolytes and other substances during cooling (Roque-Borda et al., 2021). Its low molecular weight allows it to quickly pass through cell membranes and helps stabilize the phospholipid bilayer (Zamecnik et al., 2021).

The exposure time is also a key factor in successful vitrification, as it reduces potential cell toxicity and enhances recovery after storage (de Araújo et al., 2016). Insufficient exposure to PVS2 can cause ice crystal formation, which damages cells, while prolonged exposure may lead to osmotic stress and increased PVS2 toxicity (Streczynski et al., 2019). In this study, exposure to PVS2 for 30–60 min immediately decreased germination of terap seeds before cryopreservation. These exposure durations were harmful and toxic to terap seeds due to acute osmotic shock, which could compromise cell membrane integrity, impair enzyme function, disrupt cell development, and damage DNA, proteins, or other macromolecules (Streczynski et al., 2019).

Benelli (2021) stated that the temperature during PVS2 exposure is also crucial for effective cryoprotection. In this study, the ambient temperature (25 °C) increased PVS2 toxicity because its diffusion through the cell membrane was higher at elevated temperatures (Padilla et al., 2009). While this theoretically allows for more thorough cryoprotection, it also raises the risk of toxicity from cryoprotectants. Best

Table 2

Effect of Loading and Vitrification Times for Germination of Terap Seeds without (no-LN) and with Freezing in Liquid Nitrogen (with-LN)

Loading times (min)	Vitrification times (min) in PVS2					
	0		30		60	
	no-LN	with-LN	no-LN	with-LN	no-LN	with-LN
	Germination percentage (%)					
0	75.6a	0.0b	0.0b	0.0b	0.0b	0.0b
20	26.7b	0.0b	26.7b	0.0b	6.7b	0.0b
	Vigour index (%)					
0	28.9a	0.0b	0.0b	0.0b	0.0b	0.0b
20	0.0b	0.0b	0.0b	0.0b	0.0b	0.0b
	Germination rate (%)					
0	0.1a	0.0a	0.0a	0.0a	0.0a	0.0a
20	0.0a	0.0a	0.1a	0.0a	0.0a	0.0a
	Germination uniformity (%)					
0	64.4a	0.0b	0.0b	0.0b	0.0b	0.0b
20	6.7b	0.0b	40.0a	0.0b	0.0b	0.0b
	Emergence ability (%)					
0	82.2a	0.0c	0.0c	0.0c	13.3bc	0.0c
20	33.3bc	0.0c	46.7ab	0.0c	6.7c	0.0c

Notes. The same letter within the same column indicates that the treatment has a nonsignificant effect according to the DMRT (α = 0.05%). LN = liquid nitrogen.

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(2015) and Popova et al. (2015) found that the toxicity of cryoprotective agents (CPA/PVS2) decreased when exposed to cooling. At cooling temperatures, the mobility of water molecules, which prevents them from forming ice crystals, may be restricted (Ibrahim & Normah, 2013). Padilla et al. (2009) reported that the regrowth of pro-embryonic *Litchi chinensis* after PVS2 exposure at 0 °C was greater than at 25 °C, and it had the same growth rate as the non-cryoprotected pro-embryonic. Similarly, shoot tips of *Garcinia mangostana* and *Artocarpus heterophyllus* exhibited better survival after PVS2 exposure at 0 °C, followed by storage in liquid nitrogen (Ibrahim & Normah, 2013; Okunade, 2018).

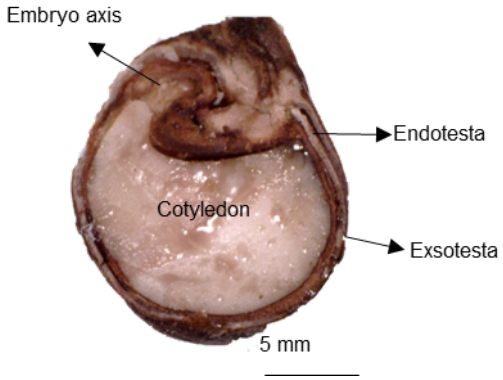
The structure of seeds is also a key factor in achieving effective cryoprotection, especially the seed coat. The terap seed coat consists of two layers: the exotesta and the endotesta (Figure 3). It is thin but quite hard compared to the jackfruit seed coat. As a result, the terap seed coat may contain high concentrations of lignin and cellulose. These high levels in the seed coats primarily serve a structural role, providing mechanical strength and impermeability (Smýkal et al., 2014). This seed coat may have prevented PVS2 from penetrating cells, leading to inadequate cryoprotection (Hervani, 2019). Therefore, removing the seed coat (testa) is recommended to improve the effectiveness of PVS2, as demonstrated in *Coffea* spp. (Dussert et al., 2001), *Citrus* spp. (Hor et al., 2005), and papaya (Hervani, 2019). Additionally, embryo axes tend to be more effective than whole seeds, as shown in jackfruit cryopreservation by Chandel et al. (1995) and Wong (2001).

Structural Analysis

Structural damage to plant cells induced by different treatment protocols can be examined under a microscope. This analysis provides valuable insights into how each stage of the cryopreservation process affects cellular integrity and tissue organization in treated materials, allowing for the assessment and improvement of the most critical steps in the procedure (Popova et al., 2021). Figure 4 shows the effect of cryopreservation treatments on terap seed cells. Figure 4a demonstrates the typical circular shape of terap seed cells (5–7 µm in diameter). After loading treatment, some cells displayed irregular shapes (Figure 4b, marked by a red arrow), indicating they had undergone plasmolysis. Cryopreservation of the root culture of *Tarenaya rosea* and somatic embryo of guinea (*Petivera alliacea*) also reported that loading exposure can cause the cells to become plasmolyzed (da Silva Cordeiro et al., 2020; Pettinelli et al., 2020). After vitrification treatments, the cells retained irregular shapes and expanded to 8–10 µm (Figure 4c). The swelling occurred due to insufficient cryoprotection, allowing cryoprotective agents (e.g., ethylene glycol and DMSO in PVS2) to enter cells and disrupt osmotic balance, potentially leading to excessive swelling (Popova

Figure 3

The Structure of Terap Seed (Longitudinal Cut)



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et al., 2021). Loading and vitrification treatments have the same effect on terap seed cells (Figure 4d). Figures 4e and 4f illustrate that many cells were plasmolyzed after being immersed in the LN, as indicated by their irregular shape and cell wall rupture (red arrow). The cell wall rupture was caused by intracellular ice formation and mechanical damage. It was previously explained that insufficient cryoprotectant protection, or inadequate distribution or penetration into the seed tissues, made some cells susceptible to freezing damage, resulting in degradation in certain regions (Faltus et al., 2021).

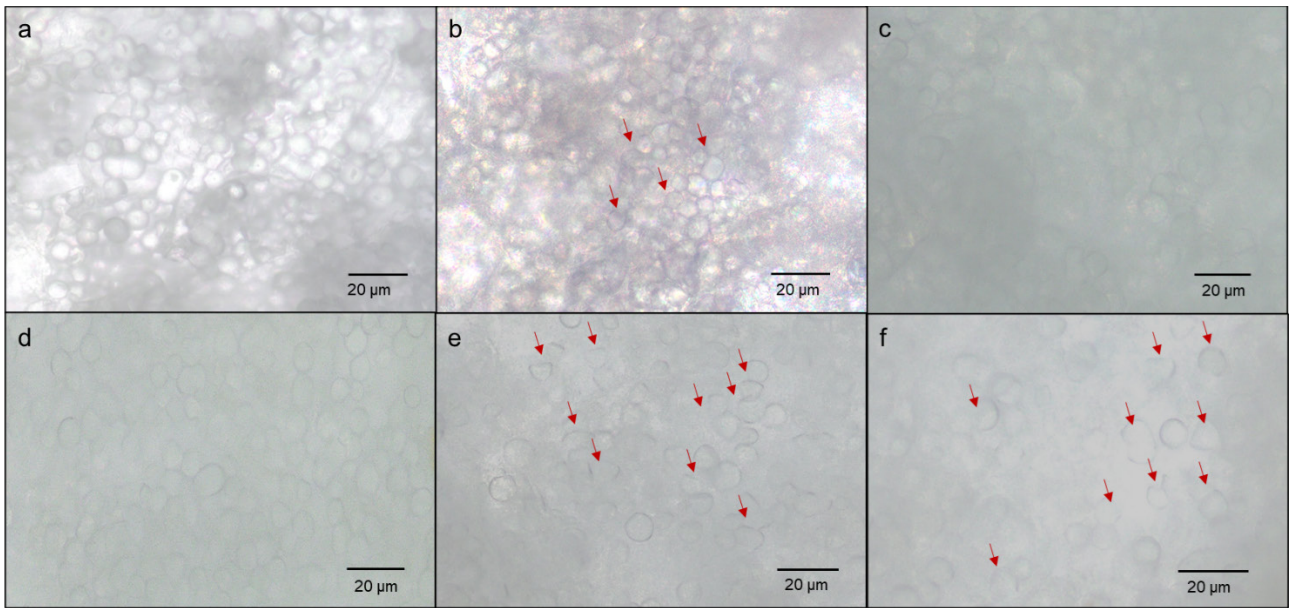
The structural analysis of terap seed cells using TEM showed that control seeds were completely filled with normal cell walls (CW) and clear boundaries (Figure 5 a–c). The cells in the control seeds were mainly filled with amyloplasts (A) containing starch grains. A small number of vacuoles (V) and oleosomes (lipid bodies/ LBs) were also found, primarily located near the plasma membrane. He et al. (2024) stated that oleosomes are usually more distinct than other organelles, indicating active metabolic processes within the cell (the utilization of stored reserves). Most organelles in terap seed cells serve as energy reserves, either as starch in amyloplasts or lipids in oleosomes. These energy reserves are essential during the start of the germination process (Ali & Elozeiri, 2017).

Loading and vitrification treatments did not show significant differences in terap seed cells compared with the control (Figure 5d–f). The organelles found remained the same, namely abundant amyloplasts, vacuoles, oleosomes, and additionally mitochondria (M) in seeds subjected to loading and vitrification treatments. A slight difference from the control seeds was the accumulation of a greater number of smaller oleosomes, which remained near the plasma membrane. Similar findings were reported in root meristem cells grown from *Araucaria angustifolia* seeds (Gasparin et al., 2017). This accumulation may result from active metabolic processes occurring during the loading and vitrification treatments.

Structural analysis results also showed that the loading and vitrification treatments were not performed optimally. This is because the cell walls of terap seeds did not thicken after

Figure 4

The Cells of Terap Seed from Control (a), 20-min Loading (b), 30-min Vitrification (c), 20-min Loading and 30-min Vitrification in the no-LN Treatment (d), and 20-min Loading and 30-min Vitrification in the with-LN Treatment (e and f)



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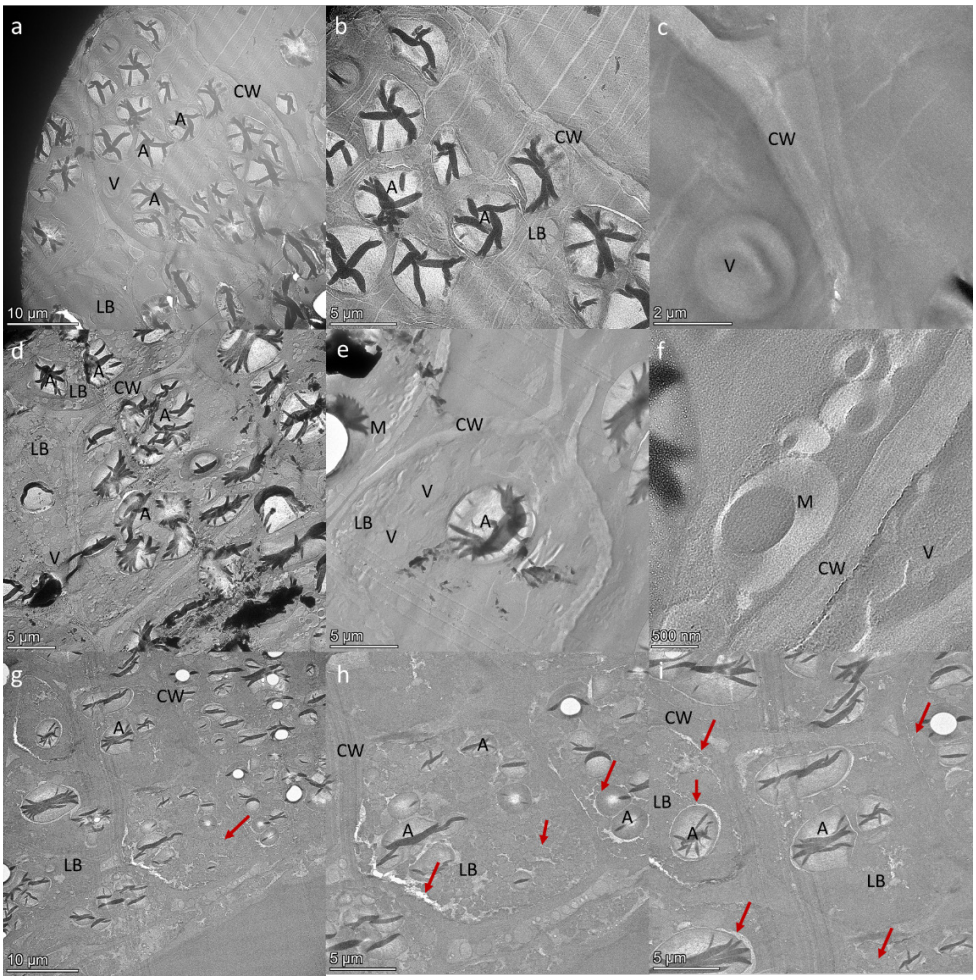
vitrification (Figure 5d–f). Fraga et al. (2016) reported that the cell walls of *A. angustifolia* embryo cultures thicken and show increased cytoplasmic electronegativity after treatment with a cryoprotectant solution (2 M sucrose, 1 M glycerol, 1 M DMSO, and 1% (w/v) L-proline) for 60 min. The thickening of cell walls occurs due to the accumulation of unsaturated fats in the cell membrane, which increases the cells' tolerance to ultra-low temperatures during storage in liquid nitrogen. The lack of cell wall thickening in this study indicates that the loading and vitrification treatments had not yet reached optimal conditions.

Freezing in liquid nitrogen caused damage and disintegration of the cytoplasm in terap seed cells (Figure 5 g–i). This damage was also shown by the dispersion of oleosomes (lipid bodies) throughout the cell (marked with red arrows), leading to a disorganized cell structure. Disintegration of the cell wall was also seen, indicated by the detachment of the cytoplasm from the cell wall (red arrows). Similar phenomena have been reported in embryo cultures derived from *A. angustifolia* seeds (Fragaet al., 2016), zygotic embryos of oil palm seeds (Suranthran et al., 2023), and cotyledons of *Phoebe chekiangensis* seeds (He et al., 2024).

Figure 5

Structural Analysis of Terap Seeds using TEM 100 kV (Tundra): (a–c) Control Seeds, (d–f) Seeds Treated with 20 min of Loading and 30 minof Vitrification (LNN), (g–i) Seeds Treated with 20 min of Loading, 30 min of Vitrification, and Freezing in Liquid Nitrogen (LNP)



Note. CW = cell wall, LB = lipid body/oleosome, V = vacuole, A = amyloplast, M = mitochondria.

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This structural analysis explains why terap seeds fail to germinate after freezing in liquid nitrogen.

In cryopreservation protocols, the degree of plasmolysis indicates the level of dehydration in plant cells undergoing hypertonic treatments, such as cryoprotectant solutions (Popova et al., 2021). Each step in the cryopreservation process gradually increases plasmolysis, reaching its maximum during vitrification, then decreases during unloading and recovery (Popova et al., 2021). In this study, some cells showed swelling, suggesting they were not sufficiently dehydrated, leading to ice crystal formation and mechanical damage. These results imply that the seeds are prone to dehydration-induced damage due to their high moisture content. Therefore, future research may benefit from using zygotic or somatic embryos for cryopreservation to reduce cellular sensitivity.

Conclusions

Loading, vitrification, and freezing treatments significantly affected seed viability. The freezing treatment caused the greatest damage to the seeds. The tetrazolium chloride test indicated that some terap seed cells remained alive (indicated by bright red staining) after all three treatments. However, germination tests showed that terap seeds were unable to germinate after freezing in liquid nitrogen. A combination of osmotic stress, chemical toxicity, and inadequate cryoprotection led to loss of viability. These factors were due to prolonged vitrification treatment and the impermeability of the seed coat (testa). Structural analysis revealed that freezing caused damage due to ice crystal formation, mechanical stress, and inadequate cryoprotection. To improve the success of terap seed cryopreservation, future efforts should focus on optimizing exposure times, reducing PVS2 toxicity, and enhancing cryoprotectant penetration by removing the seed coat or using the embryo axis as an explant.

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