Heliyon 10 (2024) e36970

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Ice crystals and oxidative stress affect the viability of *Areca catechu* seeds following cryopreservation

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A B S T R A C T

This study aimed to examine the effects of ice crystals and oxidative stress on seed viability in the context of cryopreservation, with the ultimate goal of identifying potential solutions to address the persistently low regeneration rate observed in recalcitrant medicinal plant seeds following cryopreservation. Using differential scanning calorimetry technology alongside seeds germination at different moisture levels after cryopreservation helped determine the best moisture content and freezing process for *Areca catechu* seeds. Seeds with 17–21 % moisture content and treated with PVS2 vitrification solution showed higher survival rates after cryopreservation. The oxidative markers of *A. catechu* seed embryos exhibited alterations due to vitrification freezing. However, there was no substantial association between seed viability and oxidative markers, such as reactive oxygen species (ROS), suggesting that oxidative damage mediated by ROS is not the primary factor contributing to the diminished viability of *A. catechu* seeds following cryopreservation. The inclusion of vitamin E, reduced glutathione (GSH), and 2-nitrobenzoic acid in the vitrification cryoprotectant achieved some improvement in seed viability of approximately 10 times, with GSH exhibiting the most pronounced optimizing effect.

1. Introduction

Germplasm resources of medicinal plants are regarded as crucial national assets; thus preservation through both in situ and ex situ conservation methods is required. Among the various ex situ conservation approaches, cryopreservation is widely acknowledged as the optimal technique for ensuring the long-term preservation of plant germplasm [1]. This biotechnological method entails subjecting plant materials to preculture or dehydration treatments and securely storing them in liquid nitrogen at −196 °C. Subsequently, when required, these materials can be returned to ambient temperature and can resume their regular growth and development through specific techniques [2]. The exceedingly low temperatures in cryopreservation effectively halt cellular activities, thereby facilitating the prolonged storage of plant materials [3]. Cryopreservation has proven effective in safeguarding numerous species derived from agriculture, horticulture, forestry, and medicinal plants [4,5,6]. As crucial repositories of plant germplasm resources, seeds are a significant mode of preservation. Seeds are vital for preserving plant germplasm resources due to their efficient reproduction and ability to capture diverse genes, making them widely used for protecting genetic resources [7,8]. Nevertheless, empirical evidence suggests that the viability of plant seeds following cryopreservation may be diverse, with a notable decrease in seed regeneration rates,

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https://doi.org/10.1016/j.heliyon.2024.e36970

Received 22 May 2024; Received in revised form 23 August 2024; Accepted 26 August 2024

Available online 27 August 2024

particularly for recalcitrant seeds [9]. Tropical seeds of 66 plant species from Brazil were tested for their ability to tolerate ultra-low temperatures (−196 °C); liquid nitrogen did not affect the germination of 51 species, but significantly reduced the germination of 6 species [10]. The regeneration rate of oil palm germplasm after freezing was only 34 % [11,12].

Plant cryopreservation at ultra-low temperatures exposes the cells to various stressors, such as osmotic stress, extreme dehydration, and extremely low temperatures. These stressors can lead to cellular damage, thereby affecting the survival rate and regeneration mode of the corresponding plant material [13]. The freezing and thawing processes in cryopreservation induce a glass transition in plant cells, which may result in the formation of intracellular ice crystals. The presence of these crystals can cause significant cellular damage and even lead to cell death [14]. Wesley et al. [9] observed that the cryopreservation of recalcitrant *Acer saccharinum* seeds at ultra-low temperatures led to the formation of a substantial amount of ice crystals within the meristematic cells. The cryopreservation tolerance of the meristematic cells was adversely affected by this phenomenon. The dendritic or spicular morphology of ice crystals, along with the mechanical stress induced by ice fronts, has the potential to disrupt cell membranes and impair cell structures, ultimately resulting in cell death. The mechanical damage caused by ice crystals can be mitigated through strategies such as material dehydration, cryoprotectants, and rapid cooling, thereby enhancing the cell survival rate [15].

The presence of reactive oxygen species (ROS) has been observed in various stages of the plant cryopreservation process [16]. The effects of ROS are contingent on their concentration; that is low ROS levels function as signaling molecules that regulate cellular processes, whereas high ROS levels lead to oxidative stress [17], ultimately resulting in cell damage. Research has indicated that oxidative damage is a contributing factor to the reduced or absent plant regeneration rates observed after ultra-low temperature preservation of diverse plant materials [18,19,20]. During cryopreservation of recalcitrant seeds of tropical and subtropical plants, the occurrence of oxidative damage frequently triggers necrosis of the meristem, thereby impeding the regeneration process [21]. In certain recalcitrant seeds of dicotyledonous plants characterized by fleshy cotyledons, the recovery after ultra-low temperature freezing may lead to the development of roots but the absence of shoot formation [21]. This phenomenon occurs due to the necessity of removing and drying the seed embryo prior to freezing, which in turn leads to a damage reaction upon cutting the cotyledons, resulting in increased ROS. The proximity of the resection site to the shoot meristem may lead to oxidative damage to the meristem, ultimately impeding shoot development [22].

Although damage caused by ultra-low temperature significantly affects the survival rate of plant materials, optimized treatments can effectively mitigate this damage [23]. The risk of crystallization damage in chestnut embryonic axes diminishes when the water content is within 20–60 %, thereby enhancing the feasibility of seed cryopreservation [24]. The inclusion of vitamin C in cryoprotectants results in a twofold increase in the survival rate of *Arabidopsis* seedlings following cryopreservation [25]. The inclusion of vitamin E during various stages of the pre-culture, dehydration, vitrification solution treatment, or recovery culture in media mitigates lipid peroxidation damage in blackberry shoot apical cells and enhances regeneration rates [22]. Therefore, minimizing oxidative damage may be a viable strategy to augment recovery following ultra-low temperature cryopreservation.

Southern medicinal plants play a significant role in traditional Chinese medicine, and their seeds are often classified as recalcitrant. Recalcitrant seeds are characterized by their ability to retain a substantial amount of moisture, even when fully mature, which renders them unsuitable for preservation in conventional seed banks [26]. Cryopreservation is widely recognized as the sole viable approach for the long-term storage of such seeds [3]. The primary obstacle to the preservation of recalcitrant seeds is their vulnerability to desiccation. In contrast to orthodox seeds, recalcitrant seeds cannot tolerate dehydration, and the viability and storage lifespan of recalcitrant seeds are determined by the seed moisture content [24]. In the context of cryopreservation, the probability of intracellular crystallization is generally increased by an elevated seed moisture content, thereby increasing the risk of ice crystal damage. Although, a higher moisture content is linked to freezing damage, a reduced seed moisture content is associated with dehydration damage. Consequently, achieving an appropriate moisture content and integrating enhanced preservation techniques are imperative for the effective storage of recalcitrant seeds.

Areca catechu L. is a prominent medicinal plant in southern China. Its seeds are recognized as the primary medicinal component and are renowned for their efficacy in mitigating qi stagnation, treating malaria and other parasitic diseases, and facilitating diuresis. Previous investigations by our research team have revealed that *A. catechu* seeds with a moisture content above 30 % exhibit more than 75 % viability following cryopreservation in liquid nitrogen [27]. Nevertheless, these seeds were unable to successfully germinate during the regeneration process. The current study aimed to investigate the thermodynamic characteristics of heat flow during the cryopreservation and thawing processes of *A. catechu* seeds at various moisture contents using differential scanning calorimetry (DSC). Additionally, the effect of oxidative stress markers on seed viability following cryopreservation was analyzed, and preservation techniques were enhanced by incorporating antioxidants into the glass-forming cryoprotectants. The objective of this study was to enhance the viability of *A. catechu* seeds following cryopreservation and to offer dependable assistance in mitigating the inadequate regrowth rates of recalcitrant seeds of medicinal plants.

2. Materials and methods

Mature *A. catechu* fruits were collected from the wild in Wanning City and Tunchang County, Hainan Province, China. The fibrous pulp was removed, seeds extracted, and then stored in a sealed container at 4 °C for later use, and the storage time did not exceed 1 week.

2.1. Determination of moisture content

Place the seeds in a nylon mesh bag and dry them in a desiccator with color-changing silica gel at room temperature (25 ± 2 °C) for

0.5–48 h to reduce their moisture content from 36 % to 32 %, 25 %, 21 %, 17 %, 13 %, and 9 %. Regularly measure the moisture content during drying.The high constant temperature drying method was employed to ascertain the seeds' moisture content (*w*). Specifically, selected three seeds from refrigeration, recorded their weight(*m1*), and then dehydrated them in a drying oven set at 130 °C for 1 h before reweighing them (*m2*). The water content calculation formula was as follows: w=(m1-*m2*)/*m1* × 100 %.

2.2. Determination of viability

In accordance with the manufacturer's instructions, the PDHA kit (Beijing Box Biological Technology Co., Ltd, Catalog Number: AKPL010M) was employed to determine the seed dehydrogenase content, which served as an indicator of seed vitality. Weigh 0.2 g of seeds, add 1 mL of extract solution, and chill in an ice bath for 5 min. Dry the seeds with filter paper, grind them into a homogenate in a mortar, transfer to a centrifuge tube, add ethyl acetate to reach 2 mL, and mix thoroughly. Centrifuge at 4 °C and 8000 r/min for 5 min, collect the supernatant, measure absorbance at 485 nm, and determine PDHA content per the manufacturer's guidelines.

2.3. Determination of germination rate

Seeds were sown in a covered germination box with sterile filter paper and cultured at 25°C–30 °C and 70%–85 % humidity for 2–3 weeks. Germination was observed, recorded, and the germination rate calculated.

2.4. Seed cryopreservation

A. catechu seeds were frozen using either the direct freezing method or the vitrification method [27]. The seeds were cut into 1 cm^3 pieces with the embryo at the center and distributed into 50 mL cryovials, with 10 pieces in each vial. In the direct freezing method, the seeds to be preserved were placed in cryovials and immediately immersed in liquid nitrogen for storage.

In the vitrification method, the seeds were placed in cryovials containing loading solution (2 mol glycerol and 0.4 M sucrose per liter of MS solution, pH 5.8) (MS: Murashige & Skoog Basal Medium with Vitamins, M519 PhytoTech, USA) and kept at 25 °C for 20 min. Then, the liquid in the vials was replaced with PVS2 vitrification solution (with all seeds soaked in the PVS2 solution) and kept at 0 °C for 30 min. Finally, the solution was rapidly replaced with pre-cooled fresh PVS2, and the cryovial containing the seeds was immediately transferred to liquid nitrogen. The PVS2 vitrification solution was comprised of 30 % (w/v) glycerol, 0.4 M sucrose, 15 % (w/v) ethylene glycol, and 15 % (w/v) dimethyl sulfoxide per liter of MS solution (pH 5.8) [15,23].

After 48 h of cryopreservation in liquid nitrogen, the seeds were removed and thawed in a 37 °C water bath for 2 min. The vitrified seeds were washed three times with washing solution, followed by two rinses with sterile water. The washing solution consisted of liquid MS medium containing 1.2 M sucrose (pH 5.8) [23]. Subsequently, the seeds were placed in an MS solid culture medium (composed of 7.5 g/L agar, 30 g/L sucrose, and 4.43 g/L MS, pH 5.8) and incubated at 25 °C for recovery cultivation for 24 h.

2.5. Thermodynamic analysis

A Mettler-Toledo D3 series differential scanning calorimeter (Switzerland) was used to perform ice crystal detection in seeds with different moisture contents (9, 13, 17, 21, 25, 32 %) after various treatments. For untreated seeds, 10 mg of embryos from seeds with different moisture contents was placed in an aluminum pan and sealed. For the vitrification solution treatment, a 1 cm^3block was cut from the embryo of the seeds with different moisture contents, and after treatment with PVS2 following the vitrification method, the surface solution was dried with filter paper. Then, 10 mg of embryos was placed in the aluminum pan and sealed. DCS analysis was conducted under the following conditions: the temperature was held at 30 °C for 1 min, then cooled at a rate of 10 °C/min to −80 °C and held for 2 min, followed by heating at a rate of 10 °C/min to 30 °C. DCS software was used to monitor the thermodynamic data of different moisture contents of the seeds during the cooling crystallization and heating melting phases. This included analyzing the crystallization onset temperature, peak temperature, enthalpy, and crystallization of the seeds during the freezing–thawing process. Each moisture content gradient treatment was repeated three times.

2.6. Determination of seed oxidative stress-related markers

Preparation of Enzyme Solution: Weigh 0.1 g of seed embryos and add 1 mL of the provided extraction solution. Place the mixture in a −20 °C freezer for 10 min to pre-cool. Subsequently, grind the sample intermittently for 2 min at a grinding frequency of 60 Hz, with 5-s intervals every minute. Centrifuge the homogenate at 4 °C and 8000 rpm for 10 min. Collect the supernatant and store it at 4 °C for subsequent use.

The peroxidase (POD) activity was determined using a POD activity assay kit (Beijing Box Biological Technology Co., Ltd, Catalog Number: AKAO005M). Take the enzyme solution to be tested and measure the absorbance of the reaction solution at 470 nm according to the manufacturer's instructions. The difference between the measurements taken at 90 s and 30 s was expressed as POD activity.

The catalase (CAT) activity was determined using a CAT activity assay kit (Beijing Box Biological Technology Co., Ltd, Catalog Number: AKAO003-1M). Take the enzyme solution to be tested and measure the absorbance of the reaction solution at 240 nm according to the instructions of the kit. The difference between the measurements taken at 5 s and 65 s was expressed as CAT activity.

The superoxide dismutase (SOD) activity was determined using a SOD activity assay kit (Beijing Box Biological Technology Co., Ltd, Catalog Number: AKAO001M). Take the enzyme solution to be tested and measure the absorbance of the reaction solution at 560

nm according to the manufacturer's instructions. The difference between the sample and the control (distilled water) was expressed as SOD activity.

The malondialdehyde (MDA) content was determined using a MDA Content assay kit (Beijing Box Biological Technology Co., Ltd, Catalog Number: AKFA013M). Take the enzyme solution to be tested and measure the absorbance of the reaction solution at 600 nm、532 nm and 450 nm according to the instructions of the kit. Calculate the difference between the sample and the blank (distilled water) and convert it into MDA content.

The hydroxyl radical (OH·) levels was determined using a Hydroxyl Free Radical Scavenging Capacity Assay Kit (Beijing Box Biological Technology Co., Ltd, Catalog Number: AKAO013M). Take the enzyme solution to be tested and measure the absorbance of the reaction solution at 536 nm according to the instructions of the kit. The difference between the test and the control and between the blank and the control was calculated, and the ratio of the test to the blank was expressed as the hydroxyl radical scavenging ability.

The hydrogen peroxide (H_2O_2) content was determined using a H_2O_2 Content Assay Kit (Beijing Box Biological Technology Co., Ltd, Catalog Number: AKAO009M). Take the enzyme solution to be tested and measure the absorbance of the reaction solution at 415 nm according to the instructions of the kit. Calculate the difference between the sample and the blank, and between the standard and the blank,and convert it into H_2O_2 content value.

The inhibitory and generative superoxide anion free radical detection kit (colorimetric method) from Nanjing Jiancheng Company (China, Catalog Number: A052-1-1) was used to determine the content of superoxide anion radical (O_2·-) in the ROS component. The absorbance of the reaction solution was measured at 550 nm, and the difference between the sample and the standard (0.15 mg/mL vitamin C) was expressed as O_2·- inhibition activity.

2.7. Addition of exogenous antioxidants

Based on the DSC thermodynamic data and the germination results of the frozen seeds following cryopreservation, the vitrification method was the most suitable ultra-low temperature freezing program for *A. catechu* seeds. This study evaluated the effects of vitamin E at 150 mg/L, 2-nitrobenzoic acid at 2 mM, and reduced glutathione (GSH) at 100 mg/L. These compounds were incorporated into the PVS2 solution and recovery medium during the cryopreservation process. Each experiment was repeated three times.

2.8. Data analysis

One-way analysis of variance (ANOVA) was conducted using SPSS Statistics 29.0, while correlation analysis was performed using R

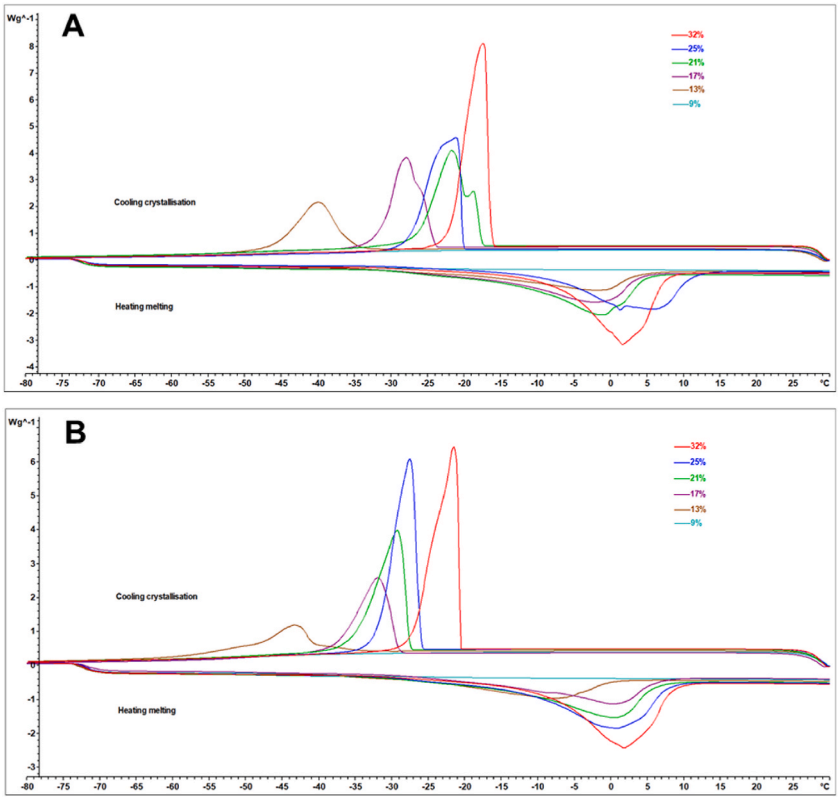

Fig. 1. Thermograms of untreated (**A**) and vitrified (**B**) of *A. catechu* seeds with moisture content of 9, 13, 17, 21, 25 and 32 %.

language. DSC STARe V17.00 software was used to analyze the thermodynamic curve data. For all quantitative data, a one-way ANOVA was used followed by an LSD multiple range test when significant differences were detected ($P < 0.05$).

3. Results

3.1. Thermodynamic analysis

The DSC thermal analysis for both treatments revealed a consistent decrease in the crystallization onset temperature, peak temperature, and enthalpy with a decrease in the seed moisture content (Fig. 1A and B). The maximum temperature reached during the cooling crystallization phase was comparatively lower than that reached during the heating melting phase. This suggests that the temperature required for crystallization to take place during the cooling process was lower than the temperature at which crystals melted during the heating process. Consequently, there was a temperature disparity in the crystallization process between the exothermic and endothermic reactions of the seeds.

As shown in Table 1, during the cooling crystallization stage, the untreated seeds exhibited an embryo crystallization onset temperature of −17.44 °C, a peak temperature of −18.67 °C, and an enthalpy of 155.83 J/g at a moisture content of 32 %. Subsequently, as the moisture content decreased to 13 %, the crystallization onset temperature decreased to −32.93 °C, the peak temperature decreased to −38.22 °C, and the enthalpy decreased to 76.02 J/g. These findings suggest that the dehydration process leads to a rapid decline in crystalline water content, which results in a significant decrease in the crystallization temperature. Hence, maintaining a lower seed moisture content within a specific range reduces the likelihood of crystallization damage and enhances viability following low-temperature preservation. Notably, when the seed moisture content reached 9 %, no crystallization peak was detected. This trend was further corroborated by the observed thermodynamic characteristics during the heating melting stage.

3.2. Cryopreservation and freeze-thaw process heat flow monitoring of seeds with various moisture contents

The seeds with different moisture contents were subjected to cryopreservation using direct freezing and vitrification freezing methods, and their germination rates were determined (Table 2). When the moisture content was 25 % or more, there was no germination of the seeds preserved at ultra-low temperatures using any of the methods. However, when the moisture content was below 25 %, germination occurred in the preserved seeds. Notably, seeds with a moisture content of 17 and 21 % exhibited germination rates of 12.65 and 20.95 %, respectively, after undergoing vitrification freezing, while the remaining seeds had germination rates below 10 %. The germination rates of seeds after direct freezing were all less than 10 %. These findings suggest that the use of vitrification solution treatment can enhance the survival rate of seed embryos following cryopreservation.

In the present study, we monitored ice crystals during the freezing and thawing processes of the seed embryos treated with direct freezing and vitrification freezing methods at various moisture contents (Table 1). When considering the same moisture content, the direct freezing method exhibited higher values of the onset temperature, peak temperature, and enthalpy than the vitrification freezing

Table 1
The thermal properties of *A. catechu* seeds with moisture content of 9, 13, 17, 21, 25and 32 %.

Freezing methods	Seeds moisture content(%)	Cooling crystallization			Heating melting		
		Onset temperature/°C	Peak temperature/°C	Enthalpy/ ($J \cdot g^{-1}$)	Onset temperature/°C	Peak temperature/°C	Enthalpy/ ($J \cdot g^{-1}$)
direct freezing	32	−16.35 ± 1.45a	−18.67 ± 1.6a	155.83 ± 9.25a	−5.06 ± 3.21a	1.56 ± 0.2a	158.66 ± 7.45a
	25	−23.40 ± 1.77b	−24.89 ± 1.87bc	139.06 ± 10.74 ab	−14.63 ± 11.54b	0.33 ± 0.92a	140.57 ± 9.05 ab
	21	−18.53 ± 0.62 ab	−20.72 ± 0.74 ab	132.37 ± 5.72 ab	−13.23 ± 0.37b	−0.50 ± 0.54 ab	136.77 ± 4.92b
	17	−22.01 ± 1.03 ab	−25.61 ± 1.11c	110.27 ± 4.75c	−15.83 ± 0.45b	0.06 ± 1.11a	114.58 ± 4.94c
	13	−32.93 ± 2.72c	−38.22 ± 1.78d	76.02 ± 1.99d	−21.45 ± 1.27c	−2.72 ± 0.72b	80.95 ± 2.84d
	9	No peak	No peak	No peak	No peak	No peak	No peak
vitrification freezing	32	−22.16 ± 1.86a	−23.28 ± 1.95a	148.21 ± 4.75a	−5.12 ± 2.01a	2.67 ± 0.75a	156.84 ± 4.17a
	25	−25.21 ± 2.12 ab	−26.61 ± 1.79a	124.42 ± 8.38 ab	−15.22 ± 2.3b	1.11 ± 0.24 ab	132.58 ± 3.44b
	21	−24.61 ± 1.47 ab	−26.50 ± 1.45a	107.92 ± 6.09b	−16.00 ± 1.06b	−0.89 ± 1.91 ab	113.44 ± 3.74
	17	−28.44 ± 1.03b	−31.28 ± 1.37 ab	76.51 ± 12.66c	−20.69 ± 3.63b	−1.50 ± 2.01 ab	86.36 ± 10.22d
	13	−29.22 ± 5.43b	−35.22 ± 4.06b	60.72 ± 10.71c	−27.80 ± 1.86c	−3.94 ± 2.19b	67.85 ± 5.99e
	9	No peak	No peak	No peak	No peak	No peak	No peak

Different lowercase letters indicate significant differences in the same thermal properties of seeds ($P < 0.05$).

Table 2
Germination rate of *A. catechu* seeds after cryopreservation with moisture content of 9, 13, 17, 21, 25 and 32 %.

Seeds moisture content(%)	Germination rate(%)		
	Control	Direct freezing	Vitrification freezing
36	80.00 ± 1.33a	0.00±0b	0.00±0b
32	70.00 ± 1.76a	0.00±0b	0.00±0b
25	63.33 ± 1.79a	0.00±0b	0.00±0b
21	39.67 ± 1.78a	9.55 ± 0.75c	20.95 ± 0.98b
17	21.63 ± 1.16a	3.53 ± 0.15c	12.65 ± 0.35b
13	19.33 ± 1.27a	4.94 ± 0.44b	6.92 ± 0.52b
9	9.33 ± 0.39a	3.33 ± 0.84b	4.51 ± 0.48b

Different lowercase letters indicate significant differences in seed germination rates among different treatments with the same water content ($P < 0.05$).

method. In the seed embryos with a moisture content of 21 % that were subjected to vitrification solution treatment, there was a noticeable release of heat (−24.61 °C) during the cooling process. This temperature was significantly lower than that of the untreated seeds, suggesting that the vitrification solution effectively reduced the freezing point of the seed embryo cells. During the heating process, the ice crystals started to melt at −16 °C, and a substantial quantity of heat was assimilated, whereby the alteration in heat was notably inferior compared with that in the seeds that had been directly subjected to freezing. This suggests that the vitrification solution diminishes the amount of ice crystals formed in seeds during freezing, thereby minimizing ice crystal-induced damage to seed embryo cells. This finding supports the outcomes of the germination experiments.

3.3. Changes in seed oxidative stress-related markers during cryopreservation

The oxidative stress-related markers were measured and analyzed at crucial stages of vitrification freezing preservation of the seeds with a moisture content of 21 %. The PDHA serves as a quantitative measure of seed viability, reflecting the survival rate of seeds objectively. It is positively associated with the germination rate(Fig. 2). The relative seed viability, as measured by PDHA, exhibited a consistent decline across all steps and recovery phases. The lowest PDHA value was observed 24 h after recovery culture, representing a mere 3 % of the control group (Fig. 3). MDA, a byproduct of membrane lipid peroxidation and a widely used marker for oxidative stress, exhibited notable alterations in seed embryonic cells throughout the freezing preservation procedure. Notably, the highest MDA content was observed after the thawing treatment, where it reached a level 1.26 times higher than that of the control group. Conversely, the H_2O_2 content and O_2^- inhibitory activity remained relatively constant during the vitrification freezing preservation process, experiencing only a marginal decline during the washing and dehydration stages. The scavenging capacity of OH· exhibited a declining pattern, reaching its minimum point after 24 h of recovery culture while remaining consistent with the control group during the thawing phase.

We showed that the antioxidant system demonstrated substantial alterations throughout freezing preservation (Fig. 3). After an initial slight increase, the SOD activity decreased and then sharply increased, ultimately reaching its highest value after 24 h of recovery culture. The highest value was 1.5 times higher than that of the control group. The lowest SOD activity was observed during the thawing stage (228.89 U/g). Furthermore, a negative correlation was observed between the changes in the SOD activity and MDA content. Specifically, as the SOD activity decreased, there was a significant accumulation of MDA. These findings suggest that the thawing process plays a critical role in inducing oxidative stress during the ultra-low temperature preservation of *A. catechu* seeds. The CAT activity was highest during the thawing stage, reaching a value of 108.71 U/g, which was 2.62 times greater than that of the control group. However, the CAT activity showed a significant decline following the washing process, and after 24 h of recovery culture, it further decreased to a level only 0.17 times that of the control group. Conversely, the POD activity exhibited a general decreasing trend throughout the freezing preservation period.

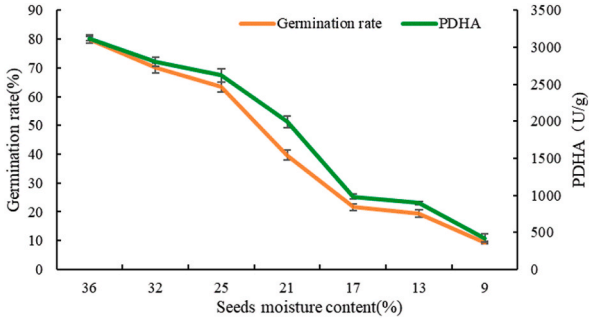

Fig. 2. There was a positive correlation between PDHA and germination rate. Germination rate and PDHA of *A. catechu* seeds after cryopreservation with moisture content of 9, 13, 17, 21, 25 and 32 %.

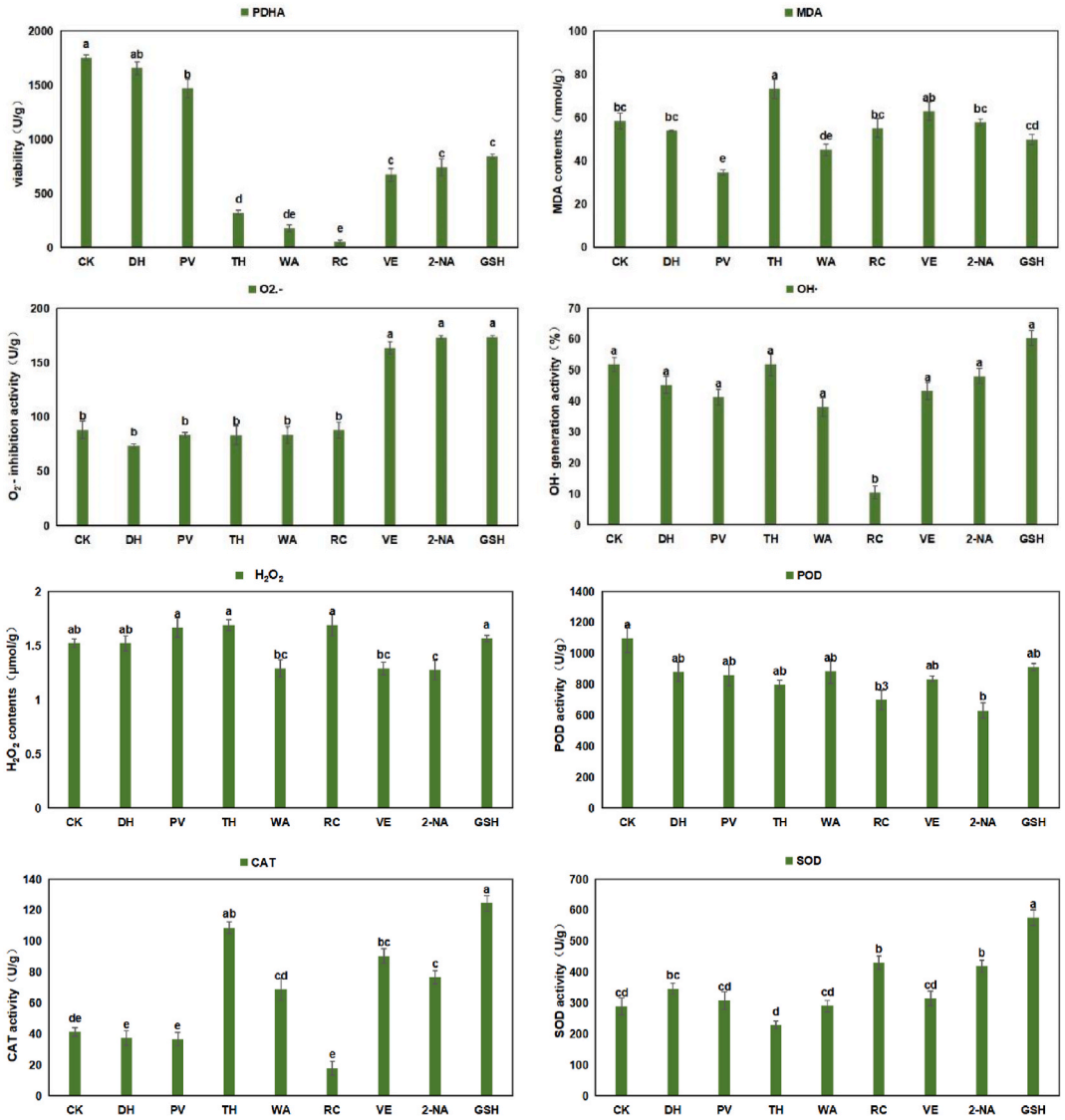

Fig. 3. Quantitative analysis of oxidative stress indicators at key steps during vitrification cryopreservation of *A. catechu* seeds with 21 % water content. *Bars* represent means and standard errors over triplicate detections, and *columns* with *different lowercase letters* were significantly different ($P < 0.05$, least ignificant difference test). *CK* control, *DH* loading solution, *PV* PVS2 vitrification, *TH* thaw, *WA* washing, *RC* recovery, *VE* vitamin E into the PVS2 vitrification solution and recovery medium, 2-NA 2-nitrobenzoic acid into the PVS2 vitrification solution and recovery medium, *GSH* glutathione into the PVS2 vitrification solution and recovery medium.

3.4. Correlation of seed viability and oxidative markers during cryopreservation

The correlation analysis (Fig. 4) revealed no significant relationship between the viability of *A. catechu* seeds and various oxidative markers, including ROS. However, there was a significant positive correlation between the CAT activity and the capacity for eliminating OH·. Nonetheless, there was no significant correlation among the remaining markers. These findings suggest that oxidative damage mediated by ROS is not the primary factor contributing to the diminished viability of *A. catechu* seeds following cryopreservation.

3.5. Effects of exogenous antioxidants on seed viability, ROS, and oxidative markers

After incorporating 150 mg/L vitamin E, 2 mmol/L 2-nitrobenzoic acid, and 100 mg/L reduced GSH into the PVS2 vitrification solution and recovery medium, we observed that GSH achieved a pronounced enhancement in seed viability following cryopreservation (Fig. 3). Consequently, GSH can be regarded as the most effective exogenous additive for future investigations.

The inclusion of the three exogenous antioxidants led to a substantial decrease in ROS levels within the seed embryos. In particular,

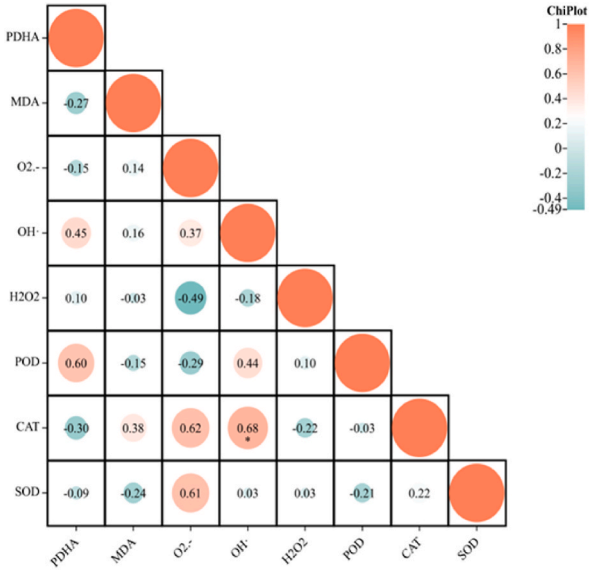

Fig. 4. Correlation analysis of oxidation indexes during vitrification cryopreservation revealed that there wass no significant correlation between *A. catechu* seed embryo viability and oxidation indexes. All the data were correlation coefficients, and significant levels are indicated at * $P < 0.05$.

GSH demonstrated a significant inhibitory effect on O_2^- and a remarkable capacity to eliminate OH·. Furthermore, the addition of antioxidants significantly influenced the antioxidant system, resulting in a notable increase in the activities of POD and CAT in the seed. Among the antioxidants, GSH exhibited the most pronounced inhibition of MDA. The SOD activity showed a negative correlation with MDA content. These findings suggest that the inclusion of exogenous antioxidants, particularly GSH, can ameliorate oxidative stress responses and partially mitigate oxidative harm during the seeds cryopreservation.

4. Discussion

Recalcitrant seeds are challenging for storage due to their susceptibility to dehydration, as they lack the ability to withstand dehydration like conventional seeds. The moisture content of the seed (embryo) plays a crucial role in determining the viability and duration of storage for recalcitrant seeds [28]. DSC is an effective analytical tool for examining thermodynamic alterations in seed or embryo moisture content. Throughout the process of seed or embryo dehydration, three significant factors come into play: the temperature at which crystallization commences, the peak of crystallization, and the enthalpy. The occurrence of the crystallization peak signifies the detrimental effect of ice crystals on the seed embryo [29]. Analysis of the DSC thermal properties of *A. catechu* seed embryos revealed that a reduction in water content led to a notable decrease in the crystallization temperature, crystallization peak area, and enthalpy of the seed embryo. Furthermore, the seed embryos treated with vitrification solution exhibited a lower start temperature, peak temperature, and enthalpy and a higher germination rate than the untreated embryos. Consequently, the incorporation of PVS2 vitrification protectant within a specific range of seed water content can effectively mitigate ice crystal damage to seed embryos and enhance their survival rate.

In the cryopreservation process of plant materials using liquid nitrogen, it is imperative to prevent or minimize intracellular ice crystal damage to preserve cell viability [30]. The vitrification cryopreservation method using PVS2 solution is characterized by a high concentration of cryoprotectants, notably glycerol, resulting in a highly viscous solution [31]. This method involves supercooling the solution with liquid nitrogen to achieve an extremely low temperature, allowing it to solidify directly into an amorphous glassy state without crystallization [15]. The glassy state effectively inhibits the formation of ice crystals within intercellular spaces and cells, thereby safeguarding cells from the potentially lethal damage caused by ice crystal formation. For plant materials to achieve a glassy state, two essential conditions must be met: a rapid cooling rate and elevated cell viscosity [15,20]. PVS2 is commonly used to enhance the adhesive properties of plant cells [32], while drying and dehydration processes can also contribute to increased cell viscosity [33]. Theoretically, prior to immersion in liquid nitrogen, plant materials can be partially desiccated and dehydrated or treated with ultra-low temperature protective agents, in order to achieve a vitrified state [32]. Although the PVS2 cryoprotectant was used when droplet vitrification was used to freeze potato shoot tips, the formation of ice crystals was still observed [13]. Similarly, vitrification freezing of *A. catechu* seed embryos resulted in the formation of ice crystals, and the amount of ice crystals increased in proportion to the seed water content. These findings suggest that further optimization of the ultra-low temperature freezing process for potato shoot tips and *A. catechu* seed embryos is necessary, including adjustments to the treatment procedure of the vitrification solution and control of the moisture content in the materials.

The phenomenon of recrystallization damage during the thawing process necessitates careful consideration [34]. In the case of cryogenically frozen *Ribes ciliatum* shoot tips, 25 % of the samples exhibited impaired regenerative capacity because of such damage

[34]. In comparison to control (untreated explants) potato shoot tips, the application of PVS2 for 40 min at 0 °C resulted in a notable reduction in thermal changes during the thawing process, specifically from 250 to 17 J/g [23]. Our findings indicate that following PVS2 treatment, the enthalpy during the thawing of *A. catechu* seed embryos with 21 % water content decreased from 132.37 to 107.92 J/g, which is potentially attributable to variations in plant material.

The occurrence of adverse stress during cryopreservation, characterized by dehydration and low temperature, has been shown to induce the formation and accumulation of ROS within cells. Excessive ROS production can subsequently result in oxidative damage to various cellular components, such as lipids, proteins, and nucleic acids, among others [35]. This phenomenon stands is a significant factor contributing to the low rates of cell survival and the challenges encountered in the process of regeneration during cryopreservation [35]. According to Berjak, oxidative damage frequently causes necrosis of the embryonic apical meristem in recalcitrant seeds originating from tropical and subtropical regions, thereby leading to challenges in regeneration. Ren et al. [25] and Uchendu et al. [18] concur with this perspective, asserting that oxidative damage plays a significant role in the decline of the recovery rate following cryopreservation. However, we found no significant correlation between seed viability and various oxidative markers, including ROS. This suggests that the low viability of *A. catechu* seed embryos after cryopreservation cannot be attributed to oxidative damage mediated by ROS, which contradicts the results of previous studies.

Interestingly, although oxidative damage was not the main factor contributing to the reduced viability observed in *A. catechu* seed embryos after cryopreservation, the addition of exogenous antioxidant compounds, particularly GHS, showed promise for improving embryo viability. Further research is necessary to investigate the underlying mechanisms. The incorporation of antioxidant compounds to mitigate the production of ROS during cryopreservation has demonstrated efficacy in diminishing oxidative damage and enhancing regrowth in select plant species post-cryopreservation [36]. Wang conducted experiments involving the addition of reduced GSH and ascorbic acid throughout the cryopreservation and regeneration culture processes in potato shoot tips [14]. The results showed that the inclusion of GSH significantly increased the regeneration rate of potato shoot tips, whereas the addition of ascorbic acid did not have a discernible effect on the regeneration rate [23]. Uchendu et al. [37] reported that the inclusion of GSH in the vitrification solution resulted in a decrease in MDA formation following cryopreservation by liquid nitrogen, consequently enhancing the viability of *Rubus* shoot tips. When GSH and acetylsalicylic acid were concurrently introduced into the preculture medium for cryopreservation of grapevine shoot tips, a recovery rate of up to 92 % was achieved [38,39].

Specifically, ultra-low temperature freezing induces significant separation between the plasma membrane and the cell wall, membrane impairment, and nuclear condensation in the recalcitrant embryo cells of *Livistona chinensis* [40]. In the cryopreservation of recalcitrant *Acer saccharinum* seeds, Wesley-Smith [9] observed that ice crystals did not induce mechanical harm to the cell membrane. However, the meristematic cells exhibited a higher formation of ice crystals than the embryo axis cells, which led to inadequate tolerance toward liquid nitrogen freezing in the meristematic cells. Further investigation will be undertaken to examine the histological and ultrastructural attributes of *A. catechu* seeds during ultra-low temperature preservation. The aim will be to elucidate the underlying mechanisms of damage and enhance cryopreservation protocols, thereby addressing the issue of diminished regeneration rates after cryopreservation.

Data availability statement

All data accessed and analyzed in this study are available in the article.

CRediT authorship contribution statement

Lin Zeng: Writing – review & editing, Writing – original draft, Project administration, Funding acquisition, Data curation, Conceptualization. **Sumei Yuan:** Methodology, Investigation. **Liu He:** Methodology, Investigation. **Zheng Sun:** Methodology. **Jianhe Wei:** Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This study was supported by the Hainan Provincial Natural Science Foundation of China (No. 322RC797), and the CAMS Innovation Fund for Medical Sciences(CIFMS) (No. 2021-I2M-1–032).

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