

CRYOPRESERVATION-INDUCED METABOLITE CHANGES IN TERAP (*Artocarpus elasticus* Reinw. ex Blume) SEEDS: IMPLICATIONS FOR SEED VIABILITY

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Abstract

Terap (*Artocarpus elasticus* Reinw. ex Blume) seeds are recalcitrant, making cryopreservation the most effective method for long-term storage. This preservation process could change metabolite profiles, potentially influencing seed viability and germination. Therefore, this study aimed to identify metabolite changes in cryopreserved terap seeds that could affect viability, using chemometric analysis. A randomized factorial design evaluated the effects of loading time (0 and 20 minutes), vitrification time (0, 30, and 60 minutes), and freezing methods (no-LN₂ and with-LN₂). Metabolite profiling using GC-MS detected 247 compounds across all samples, grouped into 42 classes. These compounds were categorized by the influence on germination, namely promoting (15.11%), inhibiting (44.63%), both promoting and/or inhibiting (38.75%), and unidentified (0.50%). Chemometric analysis, including Principal Component Analysis (PCA) and Partial Least Squares Discriminant Analysis (PLS-DA), verified metabolite changes post-cryopreservation. The Variable Importance in Projection (VIP > 1.6) score from PLS-DA showed 10 key metabolites as potential biomarkers for viability loss, namely Benzeneethanol; Hexanal; Supraene; Octane 2,4,6-trimethyl; Nonanal; Elemadienonic acid, methyl ester; 2,4-Decadienal; Hexanoic acid; Butylamine, 3-methyl-1-[5-tetrazolyl]-; and Linoelaidic acid. The results of this study provided new insight into the biochemical mechanisms underlying the decline in seed viability post-cryopreservation and showed key metabolites as indicators of seed deterioration.

Keywords: Cryoprotection; GC-MS analysis; Germination; PCA; PLS-DA; VIP score

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Introduction

Metabolites are small organic compounds with primary and secondary classes based on functions and biosynthetic pathways (Surya *et al.*, 2023). Primary metabolites are essential for plant growth and development, such as glucose, amino acids, fatty acids, and nucleotides. Meanwhile, the secondary metabolites are not directly included in plant growth and development but contribute to the species adaptability for survival, such as terpenoids, phenolics, and alkaloids (Dhaniaputri *et al.*, 2022).

Terap is an underutilized Indonesian fruit with the potential to be a valuable functional food due to its rich nutritional and bioactive composition. A previous study has reported that the flesh and seeds of the fruit contain nutrients, such as protein, carbohydrates, and lipids (Susiarti *et al.*, 2020). The wood and bark contain beneficial compounds, such as rifampicin, isoniazid, kanamycin sulfate, oxepinoflavon (Artoindonesianin E1), and artelastin (Bailey, 2021). Effective use of terap requires successful cultivation, which depends on a comprehensive knowledge of seed storage. The seeds of terap are recalcitrant (Wardani *et al.*, 2024) and classified as an exceptional plant factor 2 (Pence *et al.*, 2022). These seeds are dispersed from the tree after maturation with high moisture content and elevated metabolic activity, posing challenges for long-term storage (Umarani *et al.*, 2015). Currently, cryopreservation is the most effective method for long-term storage of recalcitrant seeds (Barbedo *et al.*, 2013). This method includes preserving plant tissues, such as pollen, seeds, shoot tips, dormant buds, cell suspensions, embryonic cultures, somatic and zygotic embryos, as well as callus tissue, at ultra-low temperatures of -196°C , while maintaining viability (O'Brien *et al.*, 2020).

The vitrification method is widely used in plant cryopreservation protocols (O'Brien *et al.*, 2020). This method typically has two cryoprotection steps, namely (1) loading, where tissues are incubated in a loading solution (LS), and (2) dehydration, where tissues are treated with a vitrification solution (VS) (Sakai and Engelmann, 2007). According to a previous study, a standard LS contains 2 M glycerol and 0.4 M sucrose (Sakai and Engelmann, 2007). The most widely used VS, plant vitrification solution-2 (PVS2), consists of 30% (w/v) glycerol, 15% (w/v) DMSO, 15% (w/v) EG, and 0.4 M sucrose (Sakai *et al.*, 1991).

Cryopreservation can alter seed metabolism (Zevallos *et al.*, 2016), and some studies report no significant metabolic changes following cryopreservation, such as in *Arabidopsis* (Ogawa

et al., 2012), *Chrysanthemum morifolium* 'Hangju' (Bi *et al.*, 2016), and strawberry (Bae *et al.*, 2022). However, there are reports that cryopreservation does influence metabolic stability, such as in tomatoes (Zevallos *et al.*, 2016), *Dendrobium nobile* (Di *et al.*, 2018), *Teramnus labialis* (Acosta *et al.*, 2020), *Swietenia mahagonii* (Entensa *et al.*, 2022), and *Pogostemon yatabeanus* (Lee *et al.*, 2023). A cause of metabolic change is oxidative stress in plant cells during cryopreservation procedures (Ren *et al.*, 2021).

Metabolic changes in seeds can influence germination (Rahmawan, 2023), which is a physiological process. Germination is divided into three phases, namely (1) rapid water uptake, (2) metabolic reactivation, and (3) radicle protrusion (Ali and Elozeiri, 2017; Bareke, 2018). The second phase is critical, as key physiological and biochemical processes-hydrolysis, macromolecule biosynthesis, respiration, subcellular structure activation, and cell elongation- are reactivated, leading to the initiation of germination (Ali and Elozeiri, 2017). Rahmawan (2023) reported that germination promoters and inhibitors were changed after storage in *Rubus fraxinifolius* seeds. Phenol and terpenoids, which act as germination inhibitors, increased after storage in the low-temperature room. The germination promoters, including amino acids, phytosterols, and tocopherols, decreased after storage.

The metabolite changes can be examined using chemometry based on Principal Component Analysis (PCA) and Partial Least Squares Discriminant Analysis (PLS-DA) (Jia *et al.*, 2021). PCA is an unsupervised chemometric method that visualizes clustering and trends in multivariate data through a score plot. The plot shows pattern formations in a two-dimensional space, as well as reveals similarities and dissimilarities among samples (Jia *et al.*, 2021). PLS-DA is a supervised chemometric method that uses multiple linear regression to maximize the covariance between class membership (Y) and the dataset (X), enabling the identification of group differences within the data (Jia *et al.*, 2021). Previous studies examined changes in the metabolic profile of cryopreserved terap seeds. In most cases, terap seeds were treated with loading and vitrification solution before freezing in liquid nitrogen (LN_2). Therefore, this study aimed to identify metabolite changes in cryopreserved terap seeds that affect viability, particularly germination, using chemometric analysis.

Materials and Methods

Plant Materials

Terap seeds were collected from the Bogor Botanic Gardens (BBG), Bogor, West Java, Indonesia, and planted in Block XIX.F.126. Mature orange fruits were harvested and handled based on Wardani *et al.* (2024), as shown in Figure 1. Each terap seed consisted of an exotesta, an endotesta, a cotyledon, and an embryo. The exotesta was yellowish-white, measuring 0.42-0.53 mm in thickness. The endotesta was light brown, directly adhered to the endosperm, and was milky white and sticky, while the embryo was centrally within the cotyledon.

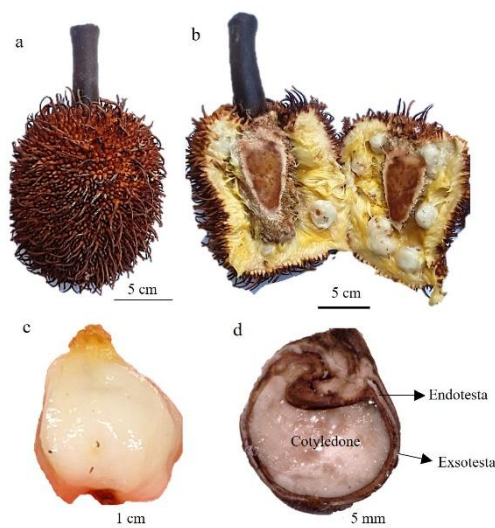


Figure 1. Fruit and seeds of terap, a. compound fruit, b. the longitudinal cut of compound fruit, c. single fruits, and d. the longitudinal cut of seeds

Experimental Design

A completely randomized factorial design was used to evaluate the effects of loading time, vitrification time, and freezing on terap seeds. Two loading (0 and 20 minutes) and three vitrification times (0, 30, and 60 minutes), as well as two freezing treatments (no-LN₂ and with-LN₂) were tested, leading to a total of twelve. Each treatment was replicated three times, for a total of 36 experimental units. There were three seeds in each unit for metabolite profiling, totaling 108 samples.

Cryopreservation Procedures

The terap seed moisture content was reduced by drying under continuous light at $18 \pm 2^\circ\text{C}$ and 55-65% relative humidity for two days, achieving an average moisture content of $36 \pm 2\%$. The seeds were then immersed in the loading solutions (2 M

glycerol in liquid MS medium with 0.4 M sucrose) for 20 minutes. Seeds assigned to the no-loading treatment were immersed in sterile water for 20 minutes to ensure the same conditions as those loaded. After loading, the seeds were filtered and immersed in plant vitrification solution-2 (PVS2) at 25°C for the designated vitrification times. Subsequently, the samples were placed in a 5 mL cryotube and stored in liquid nitrogen overnight. Thawing was performed at 40°C for 90 - 120 sec (Wardani *et al.*, 2019) for seeds with freezing treatment (with-LN₂). After thawing, the seeds were washed in a liquid MS medium for 30 minutes (Wardani *et al.*, 2019). Those without freezing (no-LN₂) skipped the thawing step and were washed directly in liquid MS medium.

Metabolite Profile Analysis

The metabolite profiles of terap seeds were analyzed using gas chromatography-mass spectrometry (GC-MS). In the case of pre-extraction preparation, all the treated seeds were dried at 40°C until a constant weight was achieved, then ground into a fine powder using a mortar and pestle (Azwanida, 2015). Metabolite extraction was carried out using the maceration method following Azwanida (2015). One gram of powdered seeds was macerated with 10 mL of absolute ethanol (1:10) for 48 hours. The resulting filtrate was filtered through Whatman No.1 filter paper and evaporated at room temperature over several days until a paste-like consistency was obtained. The resulting paste was prepared for GC-MS analysis, which was carried out using an Agilent 7890B Gas Chromatograph (GC) coupled with a 5977A Mass Selective Detector (MSD). Separation was achieved using an Agilent HP-5MS UI column (type 19091S-433UI: 5% Phenyl Methyl Siloxane, $30\text{ m} \times 250\text{ }\mu\text{m} \times 0.25\text{ }\mu\text{m}$). The chromatographic conditions included an initial temperature of 40°C for 1 minute, ramp up to 300°C at a rate of 10°C per minute, and a hold at 300°C for 4 minutes. Furthermore, the injector and detector temperatures were set to 250°C , with helium as the carrier gas at a flow rate of 3 mL min^{-1} . The paste sample was dissolved in hexane, and $1\text{ }\mu\text{L}$ of the extract was injected in splitless mode.

Metabolite identification was carried out by interpreting mass spectra using MassHunter Workstation Software (version B.07.03.2129) with NIST20. The mass spectral library was provided by the National Institute of Standards and Technology, Gaithersburg, MD, USA. After identification, all metabolites were divided into compound classes to easily predict the effects on seed germination. The 10 most prominent

compound classes were selected for more in-depth analysis of changes.

All metabolites were analyzed using MetaboAnalyst 6.0 with PCA and PLS-DA for chemometric analysis. The PCA score plot visualizes similarities and differences among samples, showing groupings and patterns in two-dimensional space for all treated seeds (Klau *et al.*, 2023). PLS-DA was used to analyze metabolite data and classify samples into different treatment stages (Jia *et al.*, 2021). Variable Importance in Projection (VIP) scores were identified from the PLS-DA results. VIP scores were discriminative indicators that differentiated samples, with higher values reflecting stronger discriminatory power (Jia *et al.*, 2021; Klau *et al.*, 2023). Metabolites with a VIP score above 1.6 were considered to contribute significantly to the germination of terap seeds following cryopreservation.

Results and Discussion

Compounds Classification

This study used untargeted metabolomics to identify compounds in terap seed extract subjected to different loading and vitrification treatments with (with-LN₂) or without (no-LN₂) freezing in liquid nitrogen. Chromatograms from various samples exhibited distinct patterns and peak intensities, showing variability in metabolite distribution. Metabolite profiling identified 247 compounds across all samples, categorized into 42 distinct compound classes. These classes were further divided based on the effect on germination, namely promoter, inhibitor, either promoter or inhibitor, and unidentified effect for germination (Table 1) based on literature references (Vokou *et al.*, 2003; Villedieu-Percheron *et al.*, 2014; Amin *et al.*, 2016; Fincheira *et al.*, 2017; Whelehan *et al.*, 2022).

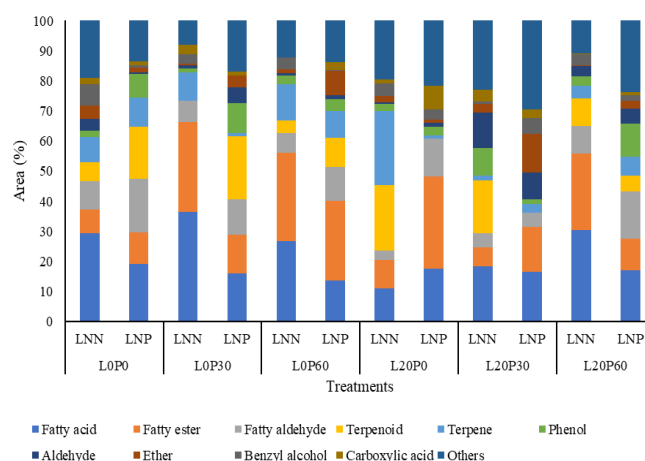
Table 1. Recapitulation of compound class area (%) in ethanol absolute extract of terap seed under different loading and vitrification treatments at no-LN₂ and with-LN₂ treatment, and their effect on germination

Compound group	Total compound	Area (%)												
		L0P0		L0P30		L0P60		L20P0		L20P30		L20P60		
		No-LN ₂	With-LN ₂	No-LN ₂	With-LN ₂	No-LN ₂	With-LN ₂	No-LN ₂	With-LN ₂	No-LN ₂	With-LN ₂	No-LN ₂	With-LN ₂	
Germination promoter														
Alkyl alcohol	2	=	=	=	=	=	=	=	=	=	=	=	=	1.71
Amide	5	=	0.31	0.14	=	0.30	=	0.77	=	1.03	0.99	=	=	=
Amine	11	0.85	0.96	2.50	0.76	1.86	0.68	0.64	1.04	4.85	1.36	=	=	0.51
Amino acid	3	=	=	=	1.56	=	=	0.58	=	1.07	=	=	=	=
Ether	9	4.31	1.58	1.39	3.84	1.48	8.14	2.01	1.07	2.79	12.88	0.48	=	2.69
Formamide	2	=	0.48	=	0.64	0.18	=	1.08	0.37	=	=	0.22	=	0.43
Glycol	4	=	=	0.74	=	=	2.13	0.52	7.40	=	=	2.59	=	4.97
Glyceride	1	=	=	=	0.49	=	=	=	=	=	=	=	=	=
Ketone	9	5.28	4.18	=	0.50	2.92	0.99	0.74	0.77	0.62	4.94	1.44	=	5.47
Nitrile	2	=	=	=	=	=	=	=	=	3.52	=	=	=	1.73
Organosiloxane	6	1.00	=	=	6.44	=	=	5.86	=	0.48	3.19	=	=	=
Palmitic acid	1	=	0.15	0.22	=	=	=	=	7.85	=	0.41	=	=	=
Protein	2	=	=	=	=	=	=	=	=	0.45	6.08	=	=	=
(unclassified)	2	=	=	=	=	=	=	=	=	=	=	=	=	=
Steroid	3	1.17	1.99	=	1.79	1.48	1.56	6.68	=	1.60	=	0.79	=	0.81
Sugar	1	=	=	=	=	=	=	=	0.53	=	=	=	=	=
Sugar alcohol	3	=	=	=	1.89	0.55	3.18	=	=	=	=	0.37	=	1.08
Triazole	1	1.10	=	=	=	=	=	=	=	=	=	=	=	=
Germination inhibitor														
Adamantane	1	=	=	=	=	=	=	=	0.41	=	=	=	=	=
Alcohol	9	2.65	0.76	1.18	0.43	=	=	0.21	=	0.54	0.65	=	=	=
Aldehyde	13	4.09	0.70	0.90	5.23	0.59	1.18	0.67	1.29	11.75	9.09	3.28	=	4.99
Alkane	10	1.73	1.37	0.68	1.06	1.59	1.21	0.66	=	=	2.35	0.82	=	3.05
Alkene	12	=	0.36	0.83	=	=	1.46	0.41	0.68	6.53	3.75	0.30	=	1.27
Benzyl alcohol	3	7.29	0.73	3.12	0.12	3.82	0.29	4.05	3.23	0.73	5.01	3.54	=	1.77
Bicyclic compound	1	=	0.40	=	=	=	=	=	=	=	=	=	=	=
Carboxylic acid	12	2.08	1.36	3.13	1.15	=	2.50	1.42	7.97	4.08	3.04	0.40	=	1.07
Fatty alcohol	9	1.97	0.26	0.28	0.34	0.48	=	=	0.75	1.25	0.60	2.91	=	1.56
Fatty aldehyde	13	9.36	17.88	7.11	11.91	6.36	11.31	3.22	12.77	4.85	4.73	8.98	=	15.75
Hydrazine	3	1.53	0.32	0.30	=	0.47	0.41	=	=	=	=	=	=	0.99
Imidazole	1	=	0.21	=	=	=	=	=	=	=	=	=	=	=
Phenol	4	2.09	7.77	1.46	10.10	2.99	3.98	2.46	2.95	9.15	1.60	3.17	=	10.76
Piperidine	1	=	=	=	=	=	=	=	=	=	=	1.02	=	=
Sesquiterpene	1	=	=	=	=	=	=	=	1.16	=	=	=	=	=
Sesquiterpene alcohol	1	=	=	=	=	=	=	=	=	=	0.33	=	=	=
Terpene	5	8.18	9.83	9.34	1.00	11.90	9.07	24.51	1.00	1.72	2.70	4.16	=	6.46
Terpenoid	5	6.45	17.17	=	20.94	4.41	9.63	21.7	=	17.50	=	9.32	=	5.3

Table 1. Recapitulation of compound class area (%) in ethanol absolute extract of terap seed under different loading and vitrification treatments at no-LN₂ and with-LN₂ treatment, and their effect on germination (Continued)

Compound group	Total compound	Area (%)											
		L0P0		L0P30		L0P60		L20P0		L20P30		L20P60	
		No-LN ₂	With-LN ₂	No-LN ₂	With-LN ₂	No-LN ₂	With-LN ₂	No-LN ₂	With-LN ₂	No-LN ₂	With-LN ₂	No-LN ₂	With-LN ₂
Germination promoter and/or inhibitor													
Fatty acid	34	29.37	19.21	36.51	16.07	26.65	13.50	10.97	17.45	18.43	16.56	30.46	17.08
Fatty ester	32	7.87	10.36	29.81	12.66	29.54	26.54	9.43	30.69	6.09	14.94	25.43	10.31
Lactone	5	0.61	=	0.36	=	2.42	1.19	=	0.63	=	1.53	=	0.23
Organosulfur	1	=	=	=	1.13	=	=	=	=	=	=	=	=
Sulfur compound	3	1.01	0.21	=	=	=	=	0.65	=	=	0.70	=	=
Unidentified effect for germination													
Propane	1	=	=	=	=	=	=	=	=	0.97	=	=	=
Pyrrrolidine	2	=	1.45	=	=	=	=	0.73	=	=	2.57	0.33	=

Note: = = identical to zero, L0P0 = control seeds, L0P30 = 30 minutes of vitrification, L0P60 = 60 minutes of vitrification, L20P0 = 20 minutes of loading, L20P30 = 20 minutes of loading and 30 minutes of vitrification, and L20P60 = 20 minutes of loading and 60 minutes of vitrification.

**Figure 2. Area (%) of compound classes of terap seed extract after loading and vitrification treatment with-LN₂ and no-LN₂, L0P0 (without loading and vitrification), L0P30 (30 minutes vitrification), L0P60 (60 minutes vitrification), L20P0 (20 minutes loading), L20P30 (20 minutes loading and 30 minutes vitrification), and L20P60 (20 minutes loading and 60 minutes vitrification)**

Several compound classes were identified as germination promoters, including alkyl alcohol, amide, amine, amino acid, formamide, glyceride, glycol, ketone, nitrile, organosiloxane, palmitic acid, unclassified protein, steroid, sugar, sugar alcohol, and triazole, accounting for 15.11% of the total area. Similarly, adamantanes, alcohols, aldehydes, alkanes, alkenes, benzyl alcohols, bicyclic compounds, carboxylic acids, fatty alcohols, fatty aldehydes, hydrazines, imidazoles, phenols, piperidines, sesquiterpenes, sesquiterpene alcohols, terpenes, and terpenoids were classified as germination inhibitors, comprising 44.63% of the total area. Fatty acids, fatty esters, lactones, organosulfur compounds, and sulfur compounds had the potential to promote and inhibit germination, contributing 38.75% of the total area. Meanwhile, propane and pyrrolidine, representing 0.50% of the total area, had unidentified effects on germination.

Table 1 showed the compounds affected by each treatment. Control samples without stress contained triazole, which acted as a germination promoter. Freezing treatment or cold stress produced bicyclic compounds and imidazole, which served as germination inhibitors. Loading and dehydration treatment without cold stress produced alkyl and sesquiterpene alcohol, a germination promoter and inhibitor, respectively. Loading combined with freezing treatment produced sugar, a germination promoter, along with adamantane and sesquiterpene, germination inhibitors. Dehydration combined with freezing treatment produced glyceride and organosulfur compounds.

Figure 2 showed the relative abundance (%) of the 10 most prominent compound classes in terap seeds extract after exposure to different loading and vitrification treatments with or without freezing in liquid nitrogen. The relative abundance of these classes varied, with some increasing and others

decreasing. In seed without loading, vitrification, and no-LN₂ treatments, the dominant compound class included fatty acid (29.37%), fatty esters (7.87%), fatty aldehydes (9.36%), terpenoids (6.45%), terpenes (8.18%), phenols (2.09%), aldehydes (4.09%), ethers (4.31%), benzyl alcohols (7.29%), and carboxylic acids (2.08%). Some classes experienced reduction after freezing (with-LN₂), namely fatty acids (19.21%), aldehydes (0.70%), ethers (1.58%), and benzyl alcohols (0.73%). However, others experienced an increase, namely fatty esters (10.36%), fatty aldehydes (17.88%), terpenes (9.83%), terpenoids (17.17%), and phenols (7.77%), showing potential metabolic alterations induced by cryopreservation.

Two dominant factors significantly influenced metabolite changes after freezing without loading or vitrification treatment, namely physical or mechanical cell damage and thermal instability (Aslam *et al.*, 2022). Direct freezing in liquid nitrogen caused rapid intra- and extracellular ice formation, which damaged cell membranes (Rihan *et al.*, 2017). This membrane damage was a primary cause of metabolite alteration, enabling metabolites to leak out or redistribute within the cell. Furthermore, certain metabolites, such as fatty acids and benzyl alcohols, could exhibit instability at cryogenic temperatures. Extreme temperature fluctuations could induce protein denaturation or conformational changes that altered enzyme activity and metabolic pathways (Rihan *et al.*, 2017). Some unstable metabolites underwent degradation or structural modifications.

Fatty acids, esters, and aldehydes were metabolically interconnected compounds in plant lipid pathways, as shown in Figure 3. In this study, fatty acids decreased in all treated seeds after freezing, except seeds with a 20-minute loading treatment (L20P0) (Figure 2). For fatty esters, the loading, vitrification, and freezing treatments did not show a consistent pattern in the metabolite changes. The pattern varied depending on the specific treatment applied, as shown in Figure 2. Fatty esters were unchanged in seeds subjected to a 20-minute loading treatment regardless of whether a 30-minute vitrification treatment was applied, but there was an increase after freezing. There was also an increase during 30-minute vitrification without loading and during 60-minute vitrification without or with 20-minute loading treatments, but decreased after freezing. Fatty aldehydes and aldehydes followed a similar trend across all treatments, as both experienced a reduction after loading and vitrification and increased with freezing.

In this study, the reduction of fatty acids in all treated seeds was due to oxidative stress and physical changes in lipid phases. According to Lin *et al.* (2021), metabolic adjustment after thawing aimed to repair damage and restore normal function, using stored fatty acids, which contributed to the decline. Reactive oxygen species (ROS) generated during freezing and thawing could trigger oxidative stress, leading to lipid peroxidation. This process produced fatty alcohols and aldehydes that induced programmed cell death (PCD) (Iuchi *et al.*, 2021). Fatty aldehydes were formed either as intermediates

or end-products of lipid peroxidation (Valgimigli, 2023) and could also originate from fatty acids through acyl-CoA intermediates (Duan *et al.*, 2011) (Figure 3). Additionally, the conversion of fatty acids to fatty acyl-CoA and to esters exemplified the physical changes in lipid phases (Duan *et al.*, 2011). Fatty esters were synthesized through the esterification of fatty acids and alcohols, a process facilitated by glycerol in the loading and vitrification solution (Ali and Elozeiri, 2017).

Terpenes and terpenoids were compounds in the mevalonic acid or isoprenoid pathway, as shown in Figure 3. Triterpenes and triterpenoids were part of the terpenoids. Furthermore, triterpenoids were triterpenes chemically modified by adding functional groups, such as hydroxyl, carbonyl, and others. Loading, vitrification, and freezing treatments increased all terpenoid classes and followed a consistent pattern. Triterpene levels decreased, and triterpenoid levels subsequently increased in all treated samples. The high terpenoid content observed after loading, vitrification, and freezing was an interesting phenomenon connected to mechanisms of plant stress response. Cryopreservation exposed seeds to extreme stress conditions, triggering the synthesis of secondary metabolites, such as terpenoids, which played a significant role in cell protection against damage (Mittler, 2017). However, despite the protective role, terpenoids inhibited seed germination. The inhibitory effect was due to suppressing cell elongation and interfering with cell wall formation, leading to failed root development (Rahmawan, 2023).

Phenol and benzyl alcohol were synthesized through shikimic acid pathways, as shown in Figure 3. Benzyl alcohols were formed by the phenylpropanoid pathway and acted as intermediate products in the biosynthesis of various aromatic compounds, including phenols (Dhaniaputri *et al.*, 2022). Metabolite profiling showed a distinct pattern, with phenol levels increasing, while benzyl alcohol decreased across all treated samples. This result suggested phenol biosynthesis depended on benzyl alcohol as a precursor, reinforcing the sequential relationship between the compounds.

Ether compounds were connected specifically to the vitrification treatment. Ethylene glycol, a key component of the vitrification solution, could interact with lipids to form ether lipids such as plasmalogen (Dean and Lodhi, 2018). Metabolite analysis showed a decrease in ether content after all loading and vitrification treatments, followed by an increase after freezing. These changes suggested that ether formation played a protective role in cellular stabilization during freezing, thereby facilitating the maintenance of seed viability.

Metabolite Profile Analysis

The PCA result (Figure 4(a)) showed that cryopreservation slightly affected the metabolite profiles of the terap seed. This result was based on the partial overlap between the no-LN₂ and the with-LN₂ treatment clusters in the plot. The first two principal components (PC1 and PC2) explained 25.9% of the total variance (PC1: 13.4% and PC2: 12.5%). PCA showed a small effect, suggesting that metabolites were affected by freezing in LN₂.

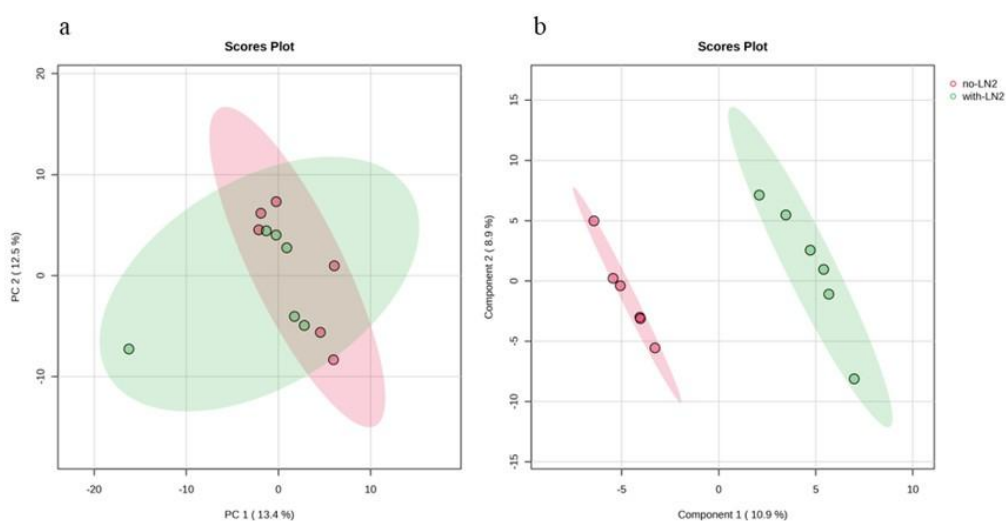


Figure 4. The score plots of Principal Component Analysis (PCA) (a) and Partial Least Squares Discriminant Analysis (PLS-DA) (b) of the metabolite profiles of terap seeds extract

However, this effect was not strong enough to cause clear cluster separation, as shown in Figure 4(a). PLS-DA showed a significant effect of cryopreservation on the metabolite profiles of terap seed. Different from PCA, the no-LN₂ and with-LN₂ treatment clusters were clearly separate, showing distinct metabolic changes following loading, vitrification, and freezing. Component I explained 10.9% of the total variance, while II accounted for 8.9%. The observed metabolite changes contributed to a decrease in terap seed viability after cryopreservation.

Figure 5 shows the compounds that contributed to the decline in terap seed viability. A total of 10 compounds had VIP > 1.6, consisting of six from no-LN₂ treatments (Benzeneethanol; Supraene; Elemadienonic acid, methyl ester; Hexanoic acid; Butylamine, 3-methyl-1-[5-tetrazolyl]-; and Linoelaidic acid) and four from with-LN₂ treatments (2,4-Decadienal; Octane, 2,4,6-trimethyl-; Nonanal; and Hexanal) (Figure 5). These compounds fell into classes that inhibited seed germination (Table 1), namely benzyl alcohols, fatty aldehydes, esters, and free fatty acids.

Benzeneethanol belonged to the benzyl alcohol class, which generally functioned as an herbicide (Kotera *et al.*, 2024) and inhibited seed germination. Hexanal, nonanal, and 2,4-Decadienal were classified as fatty aldehydes. As previously explained, fatty aldehydes (and aldehydes in general) were intermediates or end-products in lipid peroxidation, which could trigger PCD (Valgimigli, 2023). Therefore, the presence of fatty aldehydes in

the terap seed played a key role in the damage. Supraene belonged to the triterpene class, which also inhibited germination by interfering with auxin and ethylene signaling in the cell elongation process (Rahmawan, 2023). Elemadienonic acid, methyl ester, and hexanoic acid were classified as fatty esters. The presence of fatty ester was an example of the physical changes in lipid phases, which could inhibit seed germination (Duan *et al.*, 2011). Butylamine, 3-methyl-1-[5-tetrazolyl]- belonged to the amine classes, which were products of protein anabolism stored in seeds as an energy source for germination (Bareke, 2018). However, Peng *et al.* (2023) reported that amines could inhibit germination. Linoelaidic acids were free fatty acids, showing the occurrence of oxidation reactions in the seed, thereby contributing to germination inhibition (Wang *et al.*, 2021).

The 10 identified compounds differentiated the samples and served as potential markers for detecting reduced terap seed viability after cryopreservation. This decline was caused by several factors, including freezing and thawing processes, cryoprotectants, osmotic stress, temperature fluctuations, ice crystal formation, and oxidative stress (Roque-Borda *et al.*, 2021). Rapid freezing and thawing caused cellular damage, leading to metabolite release or alterations (Streczynski *et al.*, 2019). Cryoprotectants, such as DMSO and glycerol, interacted with cellular metabolites during freezing (Prudente *et al.*, 2019). Osmotic pressure change during freezing could disrupt cellular integrity and metabolite

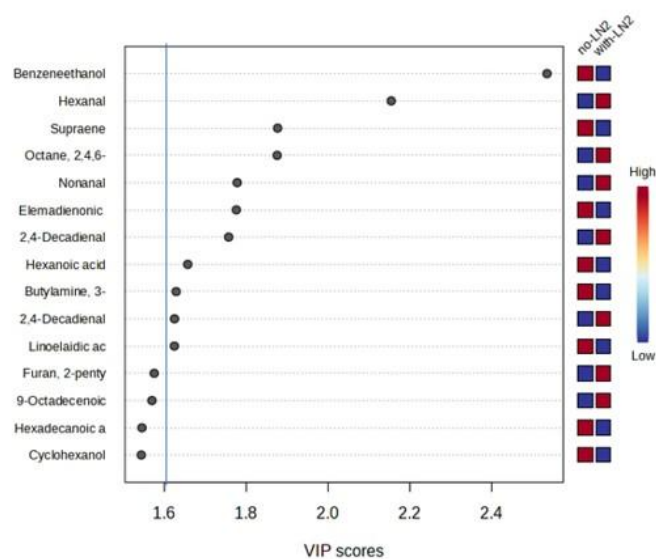


Figure 5. Variable Importance in Projection (VIP) score of the metabolite profiles of the terap seeds extract generated by the PLS-DA module

composition (Zamecnik *et al.*, 2021). Ice crystal formation physically damaged cell structures, leading to metabolite leakage or degradation (Wesley-Smith *et al.*, 2001). Additionally, cryopreservation increased ROS production, which could alter metabolic pathways (Wang *et al.*, 2021). These factors collectively contributed to significant changes in metabolite concentration and composition, thereby affecting seed viability.

Conclusions

In conclusion, metabolite profiling shows significant metabolite changes in cryopreserved terap seeds. These changes are attributed to physical and mechanical cell damage, as evidenced by a decrease in fatty acids, as well as an increase in fatty esters and aldehydes across all samples post-freezing. VIP score (>1.6) shows 10 compounds as potential biomarkers for assessing terap seed viability after cryopreservation, namely Benzeneethanol; Hexanal; Supraene; Octane, 2,4,6-trimethyl-; Nonanal; Elemadienonic acid, methyl ester; 2,4-Decadienal; Hexanoic acid; Butylamine, 3-methyl-1-[5-tetrazolyl]-; and Linoelaidic acid. These results provide valuable insights into the biochemical mechanisms underlying post-cryopreservation loss of seed viability and show key metabolites that reflect seed deterioration.

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Author Contribution

DE contributed to funding acquisition. DE and FFW conceptualized the study and designed the experiments. DE, MRS, BSP, and DL supervised the experiments. FFW executed the experiments, observations, data acquisition, data analysis, and wrote the original manuscript. All authors reviewed and edited the manuscript.

Ethic Approval and Consent to Participate

Ethical approval and consent to participate were not applicable because this study involved only plant materials and no human participants or animals.

Declaration of Generative AI Use

Generative AI was used solely for language editing and grammar improvement. The authors reviewed and edited all AI-generated content and take full responsibility for the final manuscript.

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