

EFFECT OF CRYO-STORAGE ON STORABILITY OF PAPAYA SEEDS

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ABSTRACT

A fruit crop of significant economic importance, papayas are often propagated from seeds. Based on their freezing injury even at low moisture content, papaya seeds are classified as "intermediate seeds"; and rapid loss of viability in storage is assumed to be the primary cause of poor germination; thus, the development of appropriate technology is necessary to prolong the storability of papaya seeds. One such effective technology for prolonged seed storability is cryo-storage. The purpose of the current study was to investigate how cryostorage affects papaya seed storability. For this investigation, TNAU Papaya variety CO 8 seeds were utilised. Five distinct treatments were applied to the seeds, which were then kept for approximately six months at liquid nitrogen's vapour phase (-140°C). The biochemical parameters such as protein content, free amino acid, total carbohydrates, free sugars, total lipids, and dehydrogenase activity were measured on a monthly basis along with seed germination and seedling vigour in the cryopreserved papaya seeds. Results indicated that papaya seeds that had been vitrified (seeds with a 10% moisture content that had been soaked in loading solution for 20 minutes, then immersed in Plant Vitrification Solution 2 (PVS2) for another 20 minutes, packed in cryovials and kept in cryo-storage at -140°C) compared to papaya seeds that were packed in cloth bags and stored in ambient conditions had higher seed germination, dry matter production and vigour index at six months after storage. In comparison to fresh seeds, the results also showed that, after six months of cryo-storage, biochemical proximate, protein content, total carbohydrate content, total lipid content, and dehydrogenase activity were maintained in the aforementioned treatment with minimum reduction, while electrical conductivity, free amino acid content, and free sugar content gradually increased with the storage period at minimum rate. In crux, vitrified papaya seeds packaged in cryovials and kept in cryo-storage at -140°C preserved their quality for six months, meeting Indian Minimum Seed Certification Standards. This could be useful for storing papaya seeds for longer periods of time.

Keywords: Papaya, vitrification, differential scanning calorimetry, liquid nitrogen, cryo-storage

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INTRODUCTION

The papaya, or *Carica papaya* Linn, is a well-liked and significant fruit tree in tropical and subtropical regions. Due to its high nutritional and therapeutic value, papaya is a crop that is commonly farmed for commercial purposes. Whether in its raw form as a fruit or vegetable or processed, the fruit is consumed worldwide. The biological activity and therapeutic uses of papaya have advanced significantly over the past few decades, and it is now recognised as a valuable fruit plant with nutraceutical properties. The growing human population

and increasing awareness of the benefits of consuming high-nutrient, healthful foods are driving up demand for and cultivation of papaya. However, the cultivation of low yielding varieties and the untimely availability of high yielding variety seeds are the main reasons for India's inferior production. Papayas are difficult to produce from seed because they quickly deteriorate after harvest. Nevertheless because of the nature of the seed, germination and obtaining healthy seedlings are challenging with this species. Based on their ability to store physiologically, seeds are classified as orthodox, recalcitrant (non-orthodox), and intermediate seeds

(Ballesteros *et al.*, 2021). Dry seeds that keep for a long time are known as conventional seeds. When preserved in an ex-situ setting, it can withstand extremely low temperatures or dry conditions. Conventional seeds can be kept viable at cold temperatures by reducing the moisture content to 5%. As a result, they live longer than ordinary seeds. This includes guava, cashew, sapota, and most grains. Seeds that are resistant to drying and freezing during ex situ conservation are known as recalcitrant seeds. Recalcitrant seeds start to become sensitive at 5–8% moisture content, and their viability quickly decreases when stored at low temperatures. For this reason, their ex-situ conservation is relatively challenging. Such seeds may experience oxidative damage or cell degeneration upon desiccation. Large seeds like those found in mangos, avocados, cocoa, lychees, jackfruit, etc. are considered recalcitrant. In terms of survival, intermediate seeds fall between conventional and recalcitrant seeds. Certain crop species can be dried to a far lower moisture content than conventional seeds, but when they are stored for an extended period of time, they become susceptible to low humidity and temperatures; for this reason, they are appropriately classified as "intermediate" seed types. Papaya and coffee are two examples.

When seed moisture falls below 10%, germination is considerably decreased, suggesting that papaya seeds exhibit a moderate behaviour pattern as opposed to traditional or resistant seeds. Papaya seeds should not be stored at zero or below zero degrees Celsius since they are susceptible to chilling temperatures. As a result, the seeds are regarded as having an in-between orthodox and recalcitrant quality. Papaya seeds could be preserved for a long time using ex-situ conservation methods (Ogwu *et al.*, 2014). Therefore, in order to strengthen the preservation technologies, appropriate storage procedures must be developed for long-term conservation without reducing seed vigour and viability in a sustainable manner. The next viable method that seems to be suggested for the long-term preservation of papaya seeds is cryo-conservation.

Any kind of plant material can be kept for an extended period of time by cryopreservation by being kept in liquid nitrogen (LN) at incredibly low temperatures of -196°C (liquid phase) and -140°C to -150°C (vapour phase) (Engelmann and Engels, 2002). Throughout the cryo-conservation procedure, all physiological and biochemical processes are significantly reduced, and biological deterioration is stopped. As a result, issues like genetic instability and the potential for accessions to be lost due to contamination can be minimised, and tissues or cells can be kept for incredibly extended periods of time. Typically, the vitrification method with a safe moisture level of 8–10% is utilised to maintain the viability of seeds during cryo-conservation (Engelmann, 2004). The process of vitrification involves

changing liquid water into an amorphous or glassy phase in order to stop the production of ice crystals (Elliot *et al.*, 2017). This can be accomplished by treating tissues with cryo-protectants (Vendrame *et al.*, 2014) like plant vitrification solutions. Nevertheless, there aren't many studies on papaya that use the vitrification method for cryopreserved seeds.

According to Nadarajan and Pritchard (2014), the vacuum-infiltration vitrification approach can be an effective cryopreserve technique for zygotic embryos of papaya species that display distinct seed preservation characteristics. Wardani *et al.* (2019) reported on the cryo-conservation of papaya seeds from the three cultivars Sukma, Callina, and Caliso using basic vitrification procedures. Their investigation's conclusions, however, demonstrated that in terms of preserving seed viability, different papaya genotypes reacted differently to different vitrification treatments. Thus, it is thought that vitrification followed by cryo-conservation would be a workable way to preserve papaya seed and embryos without losing viability at a safe moisture level of 8–10% provided suitable vitrification processes are optimised for routine usage. Keeping these in mind, the current study on papaya seed cryoconservation was started with the goal of examining the physical, physiological, and biochemical changes that occur in the seeds during cryo-storage in order to create an appropriate cryo-storage method.

MATERIALS AND METHODS

The current study was conducted from September 2021 to August 2022 at Tamil Nadu Agricultural University (TNAU), Coimbatore, at the Department of Plant Genetic Resources and the Department of Seed Science and Technology. For the experiment, seeds of the TNAU papaya variety CO 8 were obtained from the Horticulture College and Research Institute, Department of Fruits Science, Tamil Nadu Agricultural University, Coimbatore. The TNAU Papaya variety CO 8 seeds' moisture content was lowered to 8%, 6%, 5%, and 4%. The seeds were then packed in cloth bags or cryo vials and kept at room temperature or cryo-storage at -140°C for a duration of six months. Every month, seed samples were extracted from each treatment and subjected to quality parameter analysis for a maximum of six months following storage (MAS). Papaya seeds were vitrified by reducing their moisture content to 10%, soaking them in loading solution for 20 minutes, and then immersing them in PVS2 for another 20 minutes.

Treatment details: Five treatments were used in the tests. Papaya seed moisture was reduced to 8%, packed in a cloth bag, and stored in an ambient environment (control) (T1). Vitrified papaya seeds were packed in cryovials and

cryostored at -140°C (T5). The remaining papaya seed moisture was reduced to 6%, packed in cryovials, and stored at -140°C (T2). The remaining papaya seed moisture was reduced to 5%, packed in cryovials, and stored at -140°C (T3). Experiments were laid out using Factorial Completely Randomized Design (FCRD) with four replicates.

Seed dehydration: The silica gel was used to dehydrate the seeds. For each treatment, six grammes, or roughly 6000 TNAU papaya variety CO 8 seeds, were placed in fabric bags. The seeds were then incubated for 24 to 36 hours in fresh blue silica gel within an airtight plastic container. A digital seed moisture metre was used to measure the moisture content until the target moisture levels of 8%, 6%, 5%, and 4% were reached.

Pre-cooling of Dewar flask: For one week, LN was used to pre-cool the Dewar flask in order to keep it at the necessary temperature of -140°C . Using a measuring scale, the liquid nitrogen level in the Dewar flask was periodically measured and kept at a maximum of 7 cm.

Cryo-storage of seeds: The cryovials were loaded with seeds from each treatment and marked. The vials were put in freezer holders after being seed packed. Following that, the cryo holders floated inside the flask of Dewar's. In order to maintain the liquid nitrogen level at 7 cm, the Dewar flask was filled every three days during the six months that the cryo vials were incubated.

Thawing of cryo-stored seeds: Every month, the cryopreserved seeds were taken out of the Dewar flask and allowed to defrost at -20°C . They were then frozen for a day, incubated for a week at 5°C , and then allowed to sit at room temperature for a day. Papaya seeds were vitrified by reducing their moisture content to 10%, soaking them in loading solution for 20 minutes, and then immersing them in PVS2 for another 20 minutes. Physical, physiological, and biochemical characteristics of seed were examined prior to storage. For six months of storage, seed samples were taken from each treatment once a month and examined for the following physical, physiological, and biochemical characteristics.

Germination Percentage (%): A germination test was performed using sixteen replicates of twenty-five seeds each for the control and cryostored seeds using paper (between paper) medium kept at $25 \pm 2^{\circ}\text{C}$ and $95 \pm 3\%$ RH in a germination room. The initial germination count was noted on day twelve, and the last count was noted on day twenty-eight. The International Seed Testing Association (ISTA) established standards that stipulated that the daily germination count was used to determine the initial and final germination counts. In accordance with ISTA protocols, aberrant seedlings, fresh ungerminated seeds, and dead seeds were recognised in

addition to normal seedlings. Mean value was calculated and expressed in percentage.

$$\text{Germination (\%)} = \frac{\text{Number of seeds germinated}}{\text{Total number of seeds sown}} \times 100$$

Dry matter (g/10 seedlings): Ten normal seedlings were selected at random during the final count to measure the seedlings' roots and shoots. These particular seedlings were placed in a paper cover, dried for a full day in the shade, and then dried for an additional day at $85 \pm 2^{\circ}\text{C}$ in a hot air oven. Their dry weight was measured in grammes. The desiccator was used for 30 minutes to chill the dried seedlings. An electronic weighing scale was used to calculate the dry weight of the

The seedling vigour index was calculated using the method proposed by (Abdul-Baki and Anderson, 1973) and expressed as a whole number.

$$\text{Seedling Vigour index} = \text{Germination percentage} \times \text{Seedling length in cm}$$

Electrical Conductivity ($\mu\text{S/m}$) seedlings. Grammes per ten seedlings is the expressed mean value, which is computed.

Vigour Index: Samples containing roughly 20 seeds per replication were surface sterilised with HgCl_2 (1%) and then washed three to four times with distilled water. After that, the seeds were steeped for 24 hours in 20 millilitres of distilled water. After the leachate was transferred to a different container, its volume was raised to 25 millilitres using distilled water. Using an EC metre, the electrical conductivity of the seed leachate was determined and reported in $\mu\text{S/m}$.

Protein content (mg/g): To determine the protein content, Lowry *et al.* (1951) colorimetric method was applied. To summarise, 5 ml of 0.1N phosphate buffer was used to homogenise 50 mg of finely powdered seed samples. The supernatant was then collected by centrifuging the mixture at 10,000 rpm for 10 minutes. Before adding 0.5 ml of Folin-Ciocalteu reagent, 5.0 ml of Alkaline copper reagent was added to 1.0 ml of supernatant and the mixture was incubated at room temperature for 10 minutes. This spent thirty minutes in the dark. The solution's optical density was measured in a spectrophotometer at 620 nm in comparison to a blank. Bovine serum albumin fraction V (BSA) served as the benchmark. The samples' protein content was given in milligrammes per kilogramme of weight.

Free amino acid (mg/g): Seeds were homogenised at 55°C and extracted in 10 millilitres of 80% ethanol. The supernatant was collected after the contents were centrifuged for five minutes at 10,000 rpm. One millilitre of distilled water was combined with one millilitre of the supernatant. Ninhydrin reagent (4 ml) was added and well mixed. The extract in the tubes containing the Ninhydrin reagent was cooked for ten minutes in a

boiling water bath in order to acquire colour. Within an hour, the heated tubes were allowed to cool to room temperature, and the absorbance at 570 nm was measured using a spectrophotometer in comparison to the reagent blank. Leucine was used to generate a standard curve. The mg/g value corresponded to the total free amino acids.

Total carbohydrates: Before being cooled to room temperature, a 100 mg seed sample was homogenised and hydrolyzed in 5.0 ml of 2.5 N HCl for three hours in a boiling water bath. After that, the volume was raised to 15 ml and the supernatant was collected. After adding 4.0 millilitres of freshly made anthrone reagent to 1.0 millilitre of supernatant, the mixture was cooked for eight minutes in a boiling water bath. Using a spectrophotometer, the colour shift from green to dark green at 630 nm was measured. The standard utilised was glucose. The amount of carbohydrates in total was stated as mg/g.

Free sugars (mg/g): A seed sample of 100 mg and 5.0 ml of heated 80% ethanol were combined twice in order to extract the sugars. The supernatant was collected and evaporated through incubation over a water bath heated to 80°C. Precipitated sugars were dissolved in ten millilitres of distilled water. Moreover, 0.1 and 0.2 ml pipette aliquots into different test tubes; the volume was then raised to 2.0 ml with distilled water. Pure distilled water was utilised as a blank. One millilitre of alkaline copper tartrate reagent was added to each tube, and it was heated in a water bath for ten minutes. Following cooling, 1.0 millilitre of arseno-molybdc acid reagent was added to each tube. 10 millilitres of distilled water were added to the volume. A spectrophotometer was used to test the blue color's absorption at 620 nm after 10 minutes.

Total lipids (%): Using the gravimetric approach, the samples' total lipid content was ascertained (Bligh and Dyer, 1959). A single gramme of seeds was diced and dried in a hot air oven at 60°C for four hours. Using a pestle and mortar, the dried tissues were homogenised using cooled diethyl ether. The supernatant was collected in china dishes that had been pre-weighed after the homogenates had been centrifuged. The pooled supernatant in the china plates was maintained at 60°C in a hot air oven after the residue was extracted five times using cold diethyl ether. Before being weighed, the porcelain plates holding the dried samples were brought to room temperature by placing them in desiccators. Lipids in one gramme of dry tissue were calculated using the weight difference between the samples before and after drying. A percentage was used to express the total lipids.

Dehydrogenase (OD Value): Dehydrogenase was quantified in accordance with Kittock and Law (1968) recommendations. Each replication's 25 seeds were first

pre-soaked in paper towels for two hours. Next, 25 millilitres of 1% 2,3,5-triphenyltetrazolium chloride were added, and the mixture was incubated for six hours at 40°C. Following the staining period, the solution is decanted, and distilled water is used to wash the seeds. To elute formazon, the discoloured seeds were submerged in 25 millilitres of 2-methoxy ethanol for an entire night. Using 2-methoxy ethanol as a blank, the optical density value was determined at 470 nm using a spectrophotometer.

Tetrazolium viability test (%): The approach of Carvalho *et al.* (2018) was employed to determine the viability of the seeds. From every replication, 25 seeds were extracted. After being pre-moistened in paper towels for two hours, the seeds were chopped lengthwise with blades such that their portions remained together. The seeds were then combined with 25 millilitres of 1% 2,3,5-tetrazolium chloride solution and placed in an incubator set up for dark conditions and 40°C for six hours.

Following the staining period, the seeds were cleaned in distilled water and the tetrazolium solution was emptied. Seeds were examined under a magnifying glass, and those that met the requirement for light pink (healthy tissue) or colourless (dead tissue) were deemed viable or unviable. Percentage of viable seeds was used to express the results.

Statistical analysis: The "F" test was used to assess the significance of data from different experiments, as explained by Panse and Sukhatme (1985). Prior to statistical analysis, the percentage values were converted, if needed, to angular (arc-sine) values. For the purpose of comparing treatment differences at ($P \leq 0.05$), the Standard Error Deviation (SEd) and Critical Difference (CD) were calculated.

RESULTS

The findings showed that during the course of storage, a rise in seed moisture content was seen in all treatments. The percentage rise was greater (15.2%) when papaya seeds were packaged in fabric bags, their moisture reduced to 8%, and they were kept in ambient conditions as opposed to vitrified papaya seeds packed in cryovials and kept under cryo-storage at -140°C at 6 MAS. The minimal rise in moisture content seen in the cryo-storage technique of vitrified papaya seeds packed in cryovials at -140°C could perhaps be attributed to the seeds' resistance to moisture ingress through the cryovial and their inhibition of metabolic activity while stored in liquid nitrogen (Fig 1).

This study found that after six months of cryo-storage, the germination percentage of TNAU Papaya variety CO 8 seeds decreased. The papaya seeds that were vitrified, packed in cryovials, and stored at -140°C showed a greater average germination rate of 75%. These

seeds were then followed by papaya seeds with moisture reduced to 6%, packed in cryovials, and stored at -140°C (71%)—a 17% and 10% increase, respectively, over papaya seeds with moisture reduced to 8%, packed in cloth bags, and stored under ambient conditions.

When comparing the initial germination percentage with the germination percentage after 6 MAS, the percentage drop in seed germination was larger (43 %) in papaya seeds that were packaged in cloth bags, had their moisture reduced to 8%, and were kept in ambient conditions as opposed to other treatments (Table 1).

When papaya seeds were packed in fabric bags and kept at room temperature, their mean electrical conductivity was higher (73.83 μ S/m) than when they were vitrified, packed in cryovials, and kept at cryo-storage temperature (-140 °C). All storage techniques showed an increase in electrical conductivity over the course of storage. On the other hand, the percentage rise was 2.0 % in vitrified papaya seeds packed in cryovials kept in cryo-storage at -140°C, and it was greater (19.0%) in papaya seeds moisture reduced to 8%, packed in cloth bags, and stored under ambient conditions (Table 1).

When papaya seeds were vitrified, packed in cryovials, and cryostored at -140°C (0.462 g/10 seedlings), the mean dry matter production increased (17%) compared to when the moisture content of the seeds was reduced to 8%, packaged in cloth bags, and stored at room temperature (0.395 g/10 seedlings). When papaya seeds were packed in cloth bags and kept in ambient conditions, their moisture content decreased to 8% over the course of storage. This resulted in a dry matter production of 0.312 g/10 seedlings at 6 MAS, which was lower than the initial dry matter production of 0.480 g/10 seedlings at the time of storage. When compared to their original dry matter output of 0.480 g/10 seedlings, vitrified papaya seeds packed in cryovials and stored under cryo-storage at -140°C showed the lowest decline in dry matter production (0.432 g/10 seedlings) at 6 MAS (Table 2). This is an 11.1% decrease.

The calculated vigour index of cryopreserved papaya seeds in the current study revealed a substantial variation depending on the storage method, duration, and combination of these factors. The papaya seed moisture reduced to 8%, packed in a cloth bag and stored under ambient conditions had a lower vigour index of 1393 than vitrified papaya seeds packed in cryovials and stored under cryo-storage at -140°C. This was in contrast to the higher mean seedling vigour index (1717) in vitrified papaya seeds packed in cryovials and stored under cryo-storage at -140°C and papaya seed moisture reduced to 6%, packed in cryovials and stored under cryo-storage at -140°C (1600). Regardless of the storage technique, a progressive decline in the vigour index was noted over the course of storage. At 6 MAS, the mean vigour index was 1394, compared to a higher initial value of 1744.

When vitrified papaya seeds were packed in cryovials and kept in cryo-storage for six months, their vigour index was 1651, which was 53.4% higher than when papaya seeds were packed in cloth bags and kept in ambient conditions, where their vigour index was 1076 (Table 2).

The protein content of seeds kept in ambient conditions versus seeds kept in cryostorage differed significantly. The papaya seeds that were packed in cryovials and stored at -140°C (39.47 mg/g) had a higher mean protein content than the papaya seeds that were packed in cryovials and stored at -140°C (39.24 mg/g). The papaya seeds that were packed in cloth bags and stored at room temperature had a lower protein content of 36.92 mg/g, which was 6.9% and 6.2% lower than the papaya seeds that were packed in cryovials and stored at -140°C.

When papaya seeds were packaged in cotton bags and kept at room temperature at 6 MAS, the protein content decreased to 33.41 mg/g during storage, which was 22.2% less than when the seeds' initial protein level of 40.83 mg/g was reached. When compared to the initial protein content of 40.83 mg/g, the storage method T5, which involves vitrified papaya seeds packed in cryovials and stored under cryo-storage at -140°C, recorded the lowest reduction in protein content (37.81 mg/g) at 6 MAS (Fig 2).

The vitrified papaya seeds stored in cryovials at -140°C had a lower mean amino acid content of 49.53 mg/g. This was followed by the papaya seeds that were moistened to 6%, packed in cryovials and stored at -140°C (50.26 mg/g). On the other hand, the papaya seeds that were moistened to 8%, packed in a cloth bag and stored in ambient conditions had a higher free amino acid content of 51.47 mg/g, which was 3.9% and 2.4% higher than the vitrified papaya seeds packed in cryovials stored at -140°C and papaya seeds that were moistened to 6%, packed in cryovials and stored at -140°C, respectively. When compared to their initial free amino acid content (48.35 mg/g) at the time of storage, the vitrified papaya seeds packed in cryovials and cryopreserved at -140°C for six months showed a lower free amino acid content of 50.91 mg/g, a 5.2% increase. On the other hand, papaya seed moisture decreased to 8% when it was placed in a cotton bag and kept in an ambient environment. At 6 MAS, the seed's free amino acid content increased to 54.64 mg/g, a 13.0% increase over its original 48.35 mg/g (Table 3).

Vitrified papaya seeds packed in cryovials stored under cryo-storage at -140°C (76.17 mg/g) had a higher mean carbohydrate content than papaya seeds reduced to 6%, packed in cryovials and stored under cryo-storage at -140°C (75.25 mg/g). Papaya seeds reduced to 8%, packed in a cloth bag and stored under ambient conditions had a lower mean carbohydrate content of 73.28 mg/g, which was 3.9% and 2.6% lower

than vitrified papaya seeds packed in cryovials stored under cryo-storage at -140°C and papaya seed moisture reduced to 8%, packed in a cloth bag and stored under ambient conditions at -140°C, respectively (Table 3).

Over the course of storage, the amount of lipids in seeds kept in both ambient conditions and cryopreserved conditions significantly decreased. In terms of storage techniques, vitrified papaya seeds packed in cryovials stored under cryo-storage at -140°C (T5) had the highest mean lipid content (2.82%), followed by papaya seeds with moisture reduced to 6%, packed in cryovials and stored under cryo-storage at -140°C (2.79%), and papaya seeds with moisture reduced to 8%, packed in cloth bags and stored under ambient conditions, which recorded a lower mean lipid content value of 2.56%, which was 10.1% and 8.9% lower than vitrified papaya seeds packed in cryovials stored under cryo-storage at -140°C and papaya seeds with moisture reduced to 6%, packed in cryovials and stored under cryo-storage at -140°C, respectively. The papaya seeds packed in cryovials and stored at -140°C under the T5 storage method showed the lowest reduction in lipid content from 2.86% at the beginning to 2.74% at 6 MAS. The papaya seeds packed in cloth bags and stored under ambient conditions showed the highest reduction in lipid content from 2.86 at the beginning to 2.24% at 6 MAS (Fig 3).

Vitrified papaya seeds packed in cryovials and stored at -140°C had a higher dehydrogenase activity (0.507 OD value) than papaya seeds with moisture reduced to 6%, packed in cryovials and stored at -140°C (0.500 OD value). On the other hand, papaya seeds with

moisture reduced to 8%, packed in a cloth bag and stored at ambient temperature had a lower dehydrogenase activity (0.442 OD value), which was 14.7% and 13.1% lower than vitrified papaya seeds packed in cryovials and stored at -140°C and papaya seed moisture reduced to 6%, packed in cryovials and stored at -140°C, respectively. Dehydrogenase activity gradually decreased over the course of storage in all storage methods. Regardless of the storage method, the mean dehydrogenase activity was higher (0.534 OD value) at the beginning of the storage period and decreased to 0.435 OD value at 6 MAS. Of the interaction effect, papaya seeds packed in vitrodials and kept at -140°C under cryo-storage showed a higher dehydrogenase activity after six months of storage (0.476 OD value), which was 47.8% higher than papaya seeds with moisture reduced to 8%, packed in cloth bags, and kept at room temperature (0.322 OD value) (Table 4).

Vitrified papaya seeds packed in cryovials and cryostored at -140°C had the highest mean viability of all the storage methods, with an 83%. This was followed by papaya seeds with moisture reduced to 6%, packed in cryovials and cryostored at -140°C (78%)—an increase of 18.5% and 11.4% over papaya seeds with moisture reduced to 8%, packed in cloth bags and stored under ambient conditions (76%). When compared to alternative storage options, the percentage decrease in seed viability was higher for papaya seeds packed in cloth bags with moisture reduced to 8% and held at room temperature. The interaction effect revealed that the smallest loss in seed viability, which registered at 82% at 6 MAS, was observed in vitrified papaya seeds packed in cryovials and stored under cryo-storage at -140°C (Table 4).

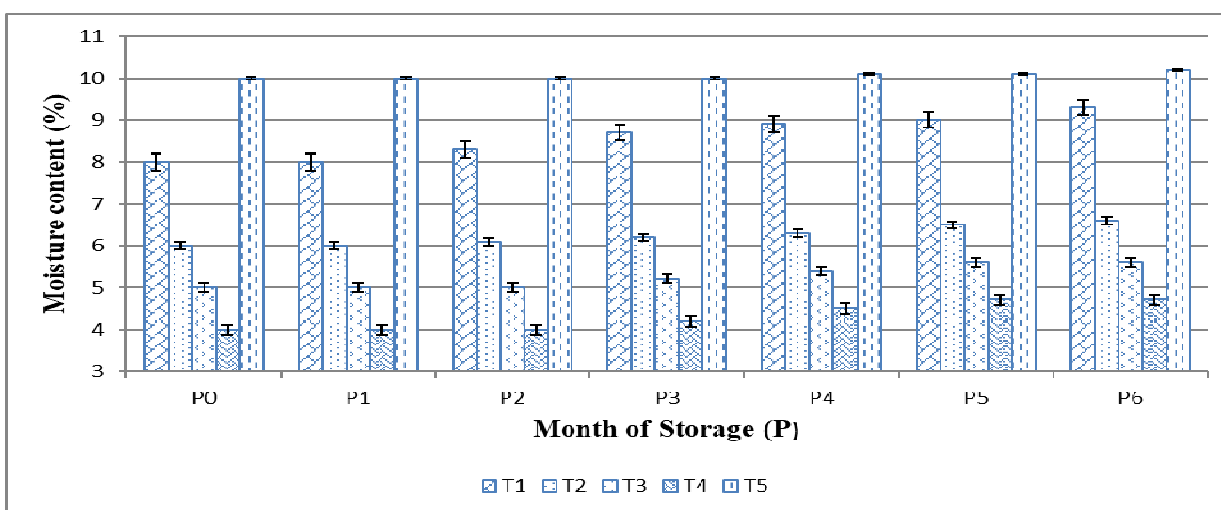


Fig 1. Influence of cryo-storage on moisture content (%) of TNAU Papaya variety CO 8
 T1 - Papaya seed moisture reduced to 8%, packed in cloth bag and stored under ambient condition (control)
 T2- Papaya seed moisture reduced to 6%, packed in cryovials and stored under cryo-storage at -140°C
 T3- Papaya seed moisture reduced to 5%, packed in cryovials and stored under cryo-storage at -140°C
 T4- Papaya seed moisture reduced to 4%, packed in cryovials and stored under cryo-storage at -140°C
 T5 - Vitrified papaya seeds packed in cryovials and stored under cryo-storage at -140°C
 P0 - Control
 P1 - One MAS
 P2 - Two MAS
 P3 - Three MAS
 P4 - Four MAS
 P5 - Five MAS
 P6 - Six MAS

Table 1. Influence of cryo-storage on germination (%) and electrical conductivity ($\mu\text{S/m}$) of TNAU Papaya variety CO 8.

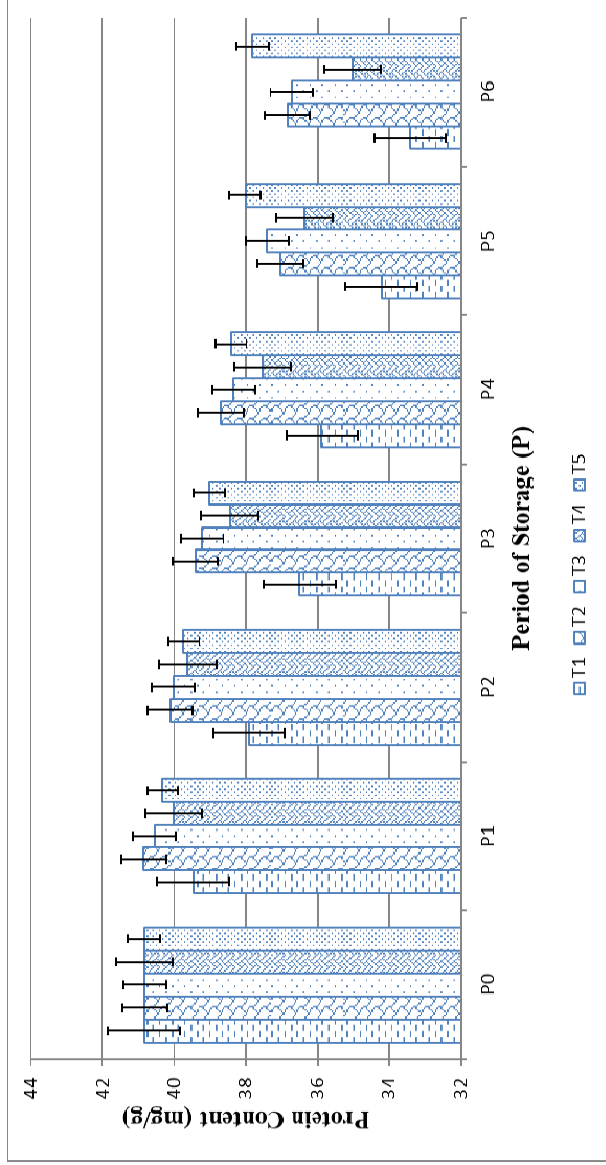
Treatments (T)	Germination (%)										Electrical Conductivity ($\mu\text{S/m}$)					
	Period of storage (P)										Period of storage (P)					
	P ₀	P ₁	P ₂	P ₃	P ₄	P ₅	P ₆	P ₀	P ₁	P ₂	P ₃	P ₄	P ₅	P ₆		
T ₁	76 (60.66)	76 (60.66)	66 (54.33)	61 (51.35)	59 (50.18)	57 (49.02)	53 (46.72)	69.42	69.90	70.73	71.90	74.35	77.06	82.67		
T ₂	76 (60.66)	72 (58.05)	72 (58.05)	72 (58.05)	70 (56.79)	70 (56.79)	70 (56.79)	69.42	69.71	69.72	70.33	70.54	70.82	71.01		
T ₃	76 (60.66)	68 (55.55)	68 (55.55)	68 (55.55)	66 (54.33)	66 (54.33)	66 (54.33)	69.42	69.52	69.90	70.31	70.84	71.21	71.64		
T ₄	76 (60.66)	66 (54.33)	66 (54.33)	66 (54.33)	64 (53.13)	64 (53.13)	64 (53.13)	69.42	69.61	70.02	70.24	70.73	71.01	71.47		
T ₅	76 (60.66)	76 (60.66)	76 (60.66)	76 (60.66)	76 (60.66)	74 (59.34)	74 (59.34)	69.42	69.54	69.84	70.02	70.43	70.75	70.81		
Sed		0.97**		1.15**		2.57**		0.30**		0.36**				NS		
		1.94**		2.29**		5.13**		0.61**		0.72**				NS		
CD (P = 0.05)		0.97**		1.15**		2.57**		0.30**		0.36**				NS		

T₁ - Papaya seed moisture reduced to 8%, packed in cloth bag and stored under ambient Condition (control)T₂ - Papaya seed moisture reduced to 6%, packed in cryovials and stored under cryo-storage at -140°CT₃ - Papaya seed moisture reduced to 5%, packed in cryovials and stored under cryo-storage at -140°CT₄ - Papaya seed moisture reduced to 4%, packed in cryovials and stored under cryo-storage at -140°CT₅ - Vitrified papaya seeds packed in cryovials and stored under cryo-storage at -140°C

TNAU - Tamil Nadu Agricultural University; % - Percent; NS - Non-significant; SEd - Standard Error of difference; P - Probability; MAS -

Months After Storage; °C - Degree Celsius

P₀ - InitialP₁ - One MASP₂ - Two MASP₃ - Three MASP₄ - Four MASP₅ - Five MASP₆ - Six MAS



T₁ - Papaya seed moisture reduced to 8%, packed in cloth bag and stored under ambient Condition (control) P₀- Initial P₄ - Four MAS
 T₂- Papaya seed moisture reduced to 6%, packed in cryovials and stored under cryo-storage at -140°C P₁ - One MAS P₅ - Five MAS
 T₃- Papaya seed moisture reduced to 5%, packed in cryovials and stored under cryo-storage at -140°C P₂ - Two MAS P₆ - Six MAS
 T₄- Papaya seed moisture reduced to 4%, packed in cryovials and stored under cryo-storage at -140°C P₃ - Three MAS
 T₅ - Vitrified papaya seeds packed in cryovials and stored under cryo-storage at -140°C

Fig 2. Influence of cryo-storage on protein content (mg/g) in TNAU Papaya variety CO 8

Table 2. Influence of cryo-storage on dry matter production (g/10 seedlings) and vigour index of TNAU Papaya variety CO 8

Treatments (T)	Dry matter production (g/10 seedlings)										Vigour index																			
	Period of storage (P)					Period of storage (P)					Period of storage (P)					Period of storage (P)														
	P ₀	P ₁	P ₂	P ₃	P ₄	P ₅	P ₆	P ₀	P ₁	P ₂	P ₃	P ₄	P ₅	P ₆	P ₀	P ₁	P ₂	P ₃	P ₄	P ₅	P ₆									
T ₁	0.480	0.472	0.419	0.399	0.357	0.332	0.312	1744	1726	1461	1320	1247	1177	1076	T	0.07**	0.15**	0.09**	0.18**	NS	0.72**	0.86**	1.91**	3.82**	P ₄ - Four MAS	P ₅ - Five MAS	P ₆ - Six MAS			
T ₂	0.480	0.478	0.460	0.460	0.452	0.434	0.421	1744	1619	1613	1604	1552	1542	1529	T x P	NS	NS	1.44**	1.71**											
T ₃	0.480	0.421	0.411	0.400	0.400	0.395	0.386	1744	1506	1498	1481	1429	1405	1388																
T ₄	0.480	0.404	0.406	0.397	0.383	0.376	0.374	1744	1444	1440	1430	1376	1360	1328																
T ₅	0.480	0.480	0.476	0.468	0.459	0.444	0.432	1744	1744	1744	1739	1731	1672	1651																

MAS - Months After Storage; °C - Degree Celsius

Table 3. Influence of cryo-storage on free amino acid content (mg/g) and total carbohydrates (mg/g) in TNAU Papaya variety CO 8

Treatments (T)	Free amino acid (mg/g)										Total Carbohydrates (mg/g)										
	Period of storage (P)					Period of storage (P)					Period of storage (P)					Period of storage (P)					
	P ₀	P ₁	P ₂	P ₃	P ₄	P ₅	P ₆	P ₀	P ₁	P ₂	P ₃	P ₄	P ₅	P ₆	P ₀ - Initial	P ₁ - One MAS	P ₂ - Two MAS	P ₃ - Three MAS	P ₄	P ₅	P ₆
T ₁	48.35	49.88	50.18	51.54	52.31	53.44	54.64	78.14	77.40	76.03	73.97	71.54	69.21	65.83	P ₀ - Initial	P ₁ - One MAS	P ₂ - Two MAS	P ₃ - Three MAS	71.54	72.84	71.54
T ₂	48.35	48.87	49.43	50.37	50.87	51.51	52.42	78.14	77.60	76.75	75.54	74.33	72.84	71.54					74.00	72.82	70.40
T ₃	48.35	49.18	49.67	50.46	51.29	51.90	52.73	78.14	77.50	76.41	75.31	74.00	72.82	70.40					73.10	72.57	71.06
T ₄	48.35	49.52	49.75	50.79	51.63	52.43	52.92	78.14	77.20	76.03	74.54	73.10	72.57	71.06					74.00	74.00	73.45
T ₅	48.35	48.55	49.04	49.50	49.94	50.45	50.91	78.14	77.60	77.28	76.54	75.30	74.00	73.45					74.00	74.00	73.45
Sed		0.18**		0.22**		0.49**		0.16**		0.20**		0.44**		0.89**					0.44**	0.44**	0.89**
CD (P = 0.05)		0.37**		0.44**		0.98**		0.33**		0.40**		0.40**		0.89**					0.44**	0.44**	0.89**

T₁ - Papaya seed moisture reduced to 8%, packed in cloth bag and stored under ambient Condition (control)

T₂ - Papaya seed moisture reduced to 6%, packed in cryovials and stored under cryo-storage at -140°C

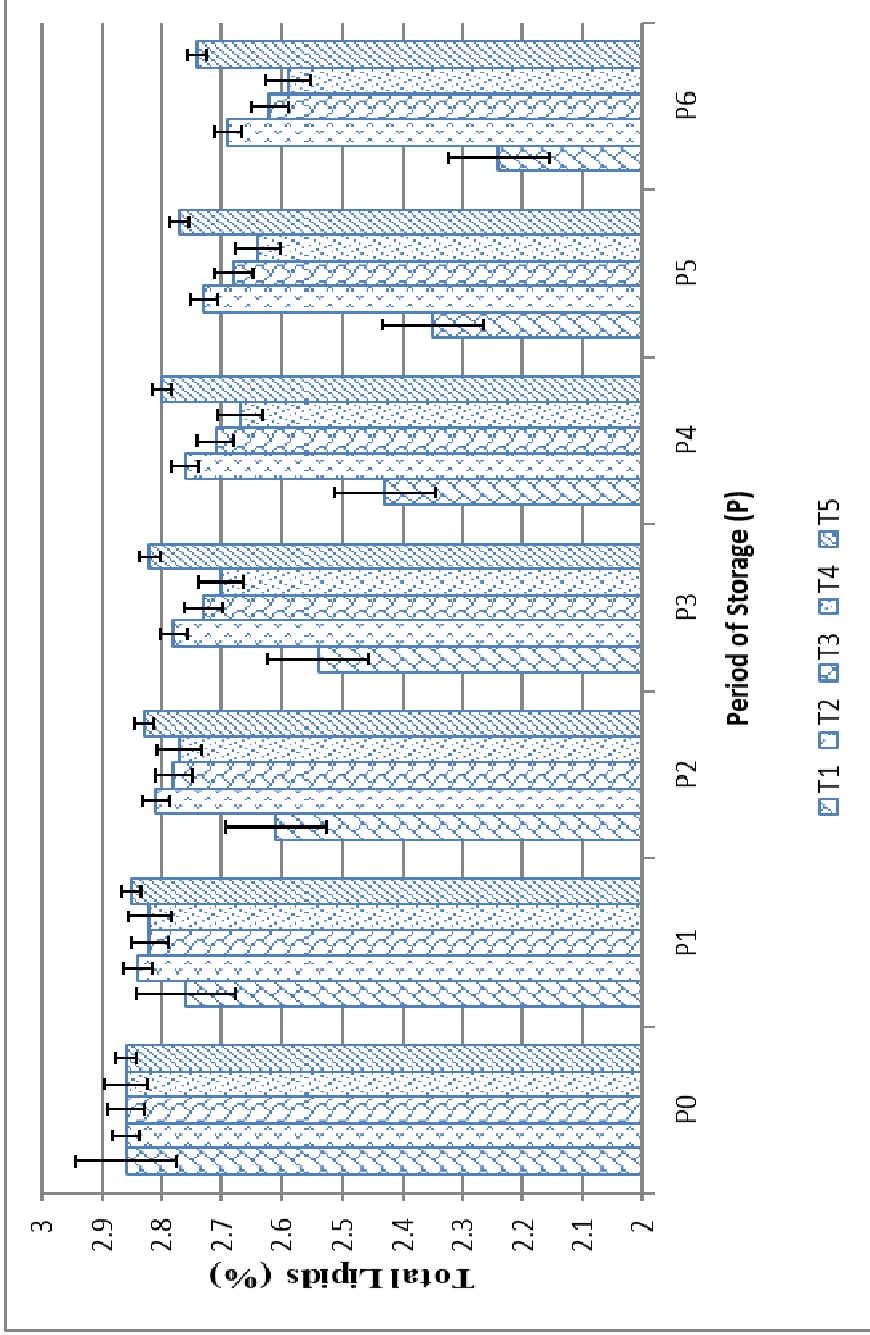
T₃ - Papaya seed moisture reduced to 5%, packed in cryovials and stored under cryo-storage at -140°C

T₄ - Papaya seed moisture reduced to 4%, packed in cryovials and stored under cryo-storage at -140°C

T₅ - Vitirified papaya seeds packed in cryovials and stored under cryo-storage at -140°C

TNAU - Tamil Nadu Agricultural University; % - Percent; NS - Non-significant; SED - Standard Error of difference; CD - Critical Difference; P - Probability;

MAS - Months After Storage; °C - Degree Celsius



T₁ - Papaya seed moisture reduced to 8%, packed in cloth bag and stored under ambient Condition (control)
 T₂ - Papaya seed moisture reduced to 6%, packed in cryovials and stored under cryo-storage at -140°C
 T₃ - Papaya seed moisture reduced to 5%, packed in cryovials and stored under cryo-storage at -140°C
 T₄ - Papaya seed moisture reduced to 4%, packed in cryovials and stored under cryo-storage at -140°C
 T₅ - Vitrified papaya seeds packed in cryovials and stored under cryo-storage at -140°C

P₀ - Initial
 P₁ - One MAS
 P₂ - Two MAS
 P₃ - Three MAS
 P₄ - Four MAS
 P₅ - Five MAS
 P₆ - Six MAS

Fig 3. Influence of cryo-storage on total lipids content (%) in TNAU Papaya variety CO 8

Table 4. Influence of cryo-storage on Dehydrogenase (OD value) and seed viability (%) in TNAU Papaya variety CO 8

Treatments (T)	Dehydrogenase (OD value)												Seed viability (%)									
	Period of storage (P)						Period of storage (P)						P			T × P						
	P ₀	P ₁	P ₂	P ₃	P ₄	P ₆	P ₀	P ₁	P ₂	P ₃	P ₄	P ₆	P ₀	P ₁	P ₂	P ₃	P ₄	P ₆	P ₅	P ₆		
T ₁	0.534	0.496	0.472	0.450	0.427	0.322	(66.42)	(63.43)	(60.66)	(56.79)	(53.13)	(49.60)	84	80	76	70	64	60	(50.76)	58	(49.60)	
T ₂	0.534	0.520	0.509	0.497	0.487	0.469	(66.42)	(62.72)	(62.72)	(62.02)	(62.02)	(60.66)	84	79	79	78	78	76	(60.66)	76	(60.66)	
T ₃	0.534	0.516	0.503	0.490	0.478	0.455	(66.42)	(60.66)	(60.66)	(60.66)	(59.34)	(58.69)	84	76	76	76	74	74	(59.34)	73	(58.69)	
T ₄	0.534	0.510	0.491	0.480	0.471	0.450	(66.42)	(59.34)	(58.05)	(58.05)	(58.05)	(56.79)	84	74	72	72	70	70	(56.79)	70	(56.79)	
T ₅	0.534	0.529	0.519	0.508	0.494	0.476	(66.42)	(66.42)	(66.42)	(66.42)	(66.42)	(64.89)	84	84	84	84	84	84	(66.42)	82	(64.89)	
SED	0.004**	0.009**	0.007**	0.007**	0.013**	0.026**	T	T	T	T	T	T	P	T × P	T × P	P	P	T × P	T × P	T × P	T × P	
CD (P = 0.05)	0.004**	0.009**	0.007**	0.007**	0.013**	0.026**	0.75**	1.50**	0.89**	1.78**	0.89**	1.78**	0.89**	1.78**	0.89**	1.78**	0.89**	1.78**	1.99**	3.98**	1.99**	
T ₁ - Papaya seed moisture reduced to 8%, packed in cloth bag and stored under ambient Condition (control)																						
T ₂ - Papaya seed moisture reduced to 6%, packed in cryovials and stored under cryo-storage at -140°C																						
T ₃ - Papaya seed moisture reduced to 5%, packed in cryovials and stored under cryo-storage at -140°C																						
T ₄ - Papaya seed moisture reduced to 4%, packed in cryovials and stored under cryo-storage at -140°C																						
T ₅ - Vitirified papaya seeds packed in cryovials and stored under cryo-storage at -140°C																						

TNAU - Tamil Nadu Agricultural University; % - Percent; NS - Non-significant; SED - Standard Error of difference; CD - Critical Difference; P - Probability; MAS - Months After Storage; °C - Degree Celsius

DISCUSSION

As it is simpler to apply and produces positive outcomes for a variety of species, the new cryopreservation technology, also known as vitrification, is extensively employed (Wang *et al.*, 2008). According to Nadarajan and Pritchard (2014) findings, papaya seeds remained viable even after being dehydrated to 4–10% moisture content and cryopreserved for a whole day. Additionally, he said that coffee seeds could withstand dehydration up to 8% moisture content, but that they could not withstand exposure to liquid nitrogen. After being exposed to liquid nitrogen for three to eighty days, cotton seeds with a moisture level of between four and twelve percent fared the best in the germination test. Cryo treatment was found by Azimi *et al.* (2005) to inhibit germination by as much as 20% at a moisture level of 5%. The moisture content of *Coffea arabica* seeds and embryos suitable for cryopreservation was examined by Figueiredo *et al.* (2017). Prior to being submerged in LN, the seeds were dried to 5, 10, 15, 20, 30, and 40% fresh wet basis. Zygotic embryos exhibited the best survival rate on a 20% wet basis.

Over the course of storage, germination may lead to a higher rate of metabolic activity in papaya seeds. Papaya seeds that had been vitrified, sealed in cryovials, and kept in cryo-storage at -140°C (T5) demonstrated a minimum loss in germination of seeds that registered at 6 MAS of 74% (Fig 2). Papaya seeds that were dehydrated to equilibrium relative humidity levels of 5, 15, and 30% after being stored in liquid nitrogen for three months demonstrated more than 70% germination, according to Ashmore *et al.* (2009). Without negatively affecting germination or growth, cryo-storage can be successfully used to conserve native species (Kholina Alla and Nina, 2012). According to studies by Sharma *et al.* (2007) and Ashmore *et al.* (2009), soybeans stored at regulated temperatures showed a greater vigour index than those stored at ambient conditions.

When papaya seeds are packaged in fabric bags and kept at room temperature, their greater electrical conductivity may be caused by electrolyte seeping out of the seeds and a breakdown in membrane integrity. The papaya seed moisture content was lowered to 8%. The preservation of membrane integrity and prevention of electrolyte leakage may be the cause of the seeds' reduced electrical conductivity value in cryopreserved stock. After 12 months of cryo-storage, the leachate conductivity was found to be at its highest. The electrical conductivity of ingested neem seeds was lower during the first month of storage but rose as the storage went on (Varghese and Naithani, 2008). According to Acosta *et al.* (2020), there is a notable rise in electrolyte leakage in *Teramnus labialis* seeds that have been cryopreserved. According to Villalobos *et al.* (2019), the cryopreserved sorghum seeds had higher electrolyte leakage. Seed

viability was lost as a result of the seeds' irreversible loss of some important proteins during low-temperature storage. The viability of rye and pigeon pea seeds was lost due to irregular protein production (Bray and Dasgupta, 1976).

Reduced protein solubility and increased free amino acid leaching may be the cause of the decline in protein content (Ahuja *et al.*, 2002). Given that the free amino acid level rose during the storage period, these data corroborate our findings. During the course of storage, the amount of carbohydrates in both the ambient and cryo-stored seeds significantly decreased. Over the course of storage, the amount of carbohydrates in each technique of storage decreased. This process may be brought on by the breakdown of carbohydrates, which can occur at freezing temperatures below zero. If this happens, seeds may lose their viability after being cryopreserved for an extended length of time (Table 2).

It was shown that exposure to a cryogenic temperature of -196°C decreased seed viability while enhancing electrical conductivity and lipid peroxidation in refractory seeds that had undergone dehydration and freezing responses. Lipid peroxidation may lead to soluble sugar accumulation and damage to cell membranes, which may be the result of starch degradation. Lipids broke down because of the formation of free radicals (ROS) brought on by the freezing process. Usually, the membrane is harmed by these lipids in their unstable state, which leads to seed degradation.

The drop in dehydrogenase activity seen in papaya seeds held in both ambient and cryopreserved conditions may be attributed to a correlation between dehydrogenase activity and viability. As viability declines during storage, so does dehydrogenase activity. In this investigation, TNAU Papaya CO 8 seeds that had been cryostored for six months showed a decrease in viability percentage. This could be brought on by a faster rate of metabolic activity and a higher moisture content in the seed. Despite having a low physiological germination percentage, Coelho *et al.* (2017) found that Coffee cultivar Arara seeds dried in silica gel and cryopreserved for 24 hours had a greater percentage of viable embryos. The shelf life of lettuce seeds was extended by cryogenic preservation (Christina Walters, 2004).

Although papaya seeds exhibited resistance to desiccation, their shelf life was comparatively brief in contrast to other conventional seeds. Thus, to extend the shelf life of papaya seeds, appropriate seed storage techniques are required. Conventional seed storage practices in seed banks, which range from 5°C to -20°C, are unreliable due to their inability to guarantee long-term seed storage at low temperatures above zero. Papaya seeds can be cryopreserved in liquid nitrogen at liquid temperature (-196°C) or vapour phase (-140°C to -150°C) for long-term preservation at ultralow temperatures. Benefits of these methods include high

rates of regeneration following cryopreservation and ease of application. After the material has been maintained and the storage process is finished, there is no change or bacterial or fungal contamination. Cryopreservation requires a minimum amount of effort and space.

Conclusion: According to the current study, vitrified papaya seeds packed in cryovials and kept at -140°C for extended periods of time can retain seed viability. This may be because biochemical characteristics that are crucial to seed physiology and biochemistry are preserved. As a result, it can be said that vitrified papaya seeds packaged in cryovials and kept in cryo-storage at -140°C preserved their quality for six months, meeting Indian Minimum Seed Certification Standards. This may be useful for storing papaya seeds for longer periods of time.

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