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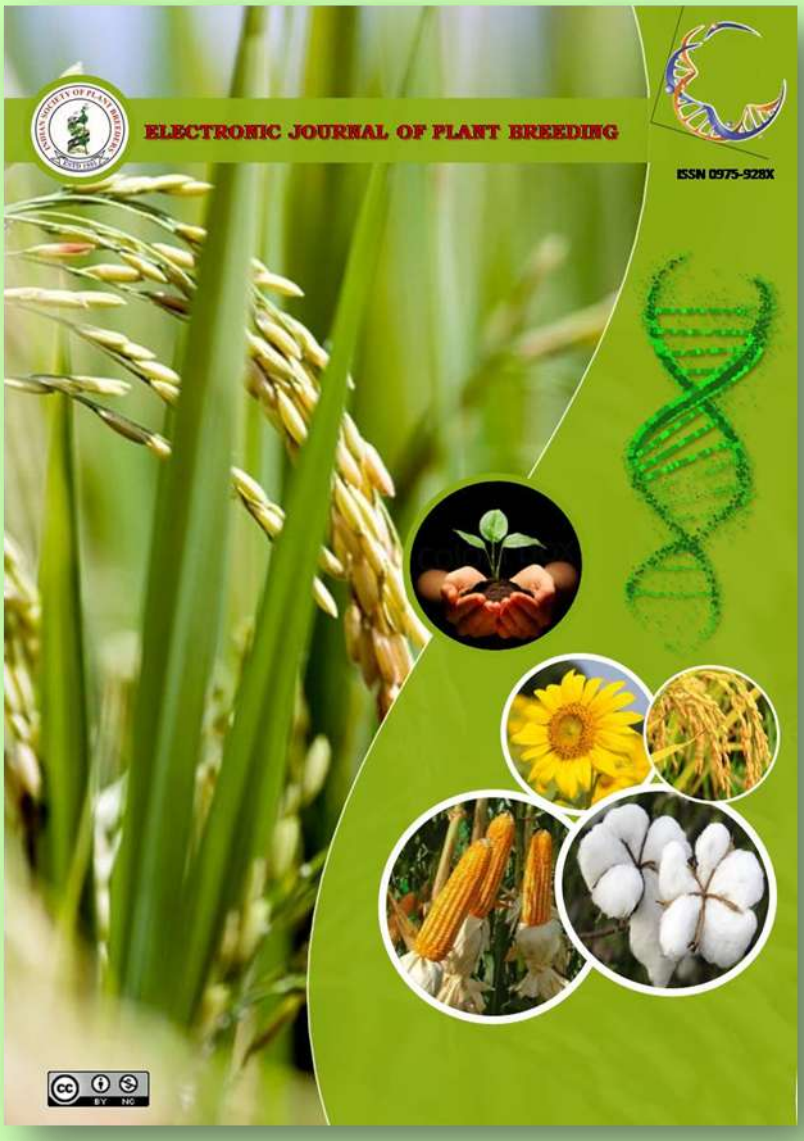

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Research Article

Effect of cryo-storage on germinability and biochemical changes in papaya (*Carica papaya* L.) seeds

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Abstract
The seeds of papaya, based on seed storage behaviour, are classified under "intermediate seed" category as they exhibit freezing injury even at low moisture content. Hence, the present study was initiated to investigate the germinability and biochemical changes of papaya seeds stored at vapour phase (-140°C) of liquid nitrogen for about six months. At monthly intervals, seed viability and biochemical parameters like total carbohydrate, total free sugars, total protein content and total free amino acids were assessed in the cryo-stored papaya seeds. The results showed that the germination percentage decreased from 90 % to 7 % in cryo-stored CO 2 variety of papaya as compared to fresh seeds. Similar response was also observed in TNAU Papaya CO 8 wherein the germination was reduced from 85 % to 23 % after cryo storage. The results also revealed that biochemical proximates, carbohydrate and protein content decreased after upon six months of cryo-storage in comparison to fresh seeds of both papaya genotypes. The level of free sugars and free amino acids increased gradually with the storage period. From the results of the present study, it is evident that seed viability loss could occur in papaya seeds upon long period of storage at extremely low temperature conditions which might possibly be accounted for the drastic reduction in biochemical components such as carbohydrates and proteins, key factors activately participating in seed physiology and biochemistry.

Keywords
Papaya, liquid nitrogen, seed viability, biochemical proximates, cryo-conservation

Introduction

Papaya (*Carica papaya L.*) is an economically important fruit crop of the Caricaceae family and widely grown in all tropical and subtropical regions. It is cultivated mainly for its nutritive value and low calorific value. Unripe papaya can be used for cooking and latex obtained from the fruit contains a proteolytic enzyme papain, which helps in tenderizing meat. It has medicinal uses like antibiotics and antihelminthic property. Leaf extracts are used to prepare anti-malarial drugs. It also increases the industrial value due to the presence of pectin compound which is suitable for the preparation of jam and jelly (Rajasekhar, 2017). Of the world's total papaya production, 75% of the production is contributed by countries like India, Brazil, Indonesia, Nigeria, and Mexico. Among these countries, India ranks first with a production of 5 million tonnes in 1,34,000 ha and the estimated production is around 6 million tonnes under 1,46,000 ha (NHB,2018).

To meet future demand arising due to increasing human population and need for high nutritive food and industrial products from papaya, crop improvement activities should be strengthened in a sustainable manner. This could be achieved by collecting and conserving the genetic resources of papaya to broaden the availability of genetic diversity that could be exploited to improve both production and productivity of the crop. Among the *ex-situ* approaches recommended for germplasm conservation, storage of seeds in gene banks under medium (5°C) and long term (-20°C) conditions is regarded as easiest and popular method. Generally, seed longevity is influenced by storage temperature and seed moisture content. Based on this principle, seeds of many species which can be dried to about 5% moisture content and will be stored with viability under sub-zero freezing temperatures are classified as 'orthodox seed', while 'recalcitrant' seeds become sensitive to desiccation and lose viability rapidly. Interestingly, papaya seeds showed desiccation tolerance but resulted in poor viability when stored under the freezing temperature of -20°C as was reported by Ellis and co-workers and classified under 'intermediate seed'category. Under such conditions, it is not feasible to conserve papaya seeds on long term basis in gene banks (Ellis *et al*., 1991).

Long term conservation of papaya seeds at ultra-low temperatures could be achieved through cryo-

conservation in liquid nitrogen under liquid (-196°C) or vapour phase (-140°C to -150°C) (Engelmann *et al*., 2000).The cryopreservation technique is based on the removal of all freezable water from tissues by physical or osmotic dehydration, followed by rapid freezing. During the process of cryopreservation, all biochemical and physiological activities are significantly reduced and biological deterioration is arrested. So far, only very few studies on the cryo-conservation of papaya seeds are reported in the literature. An earlier study indicated that the storage of papaya seeds at 5-6% moisture content in liquid nitrogen did not affect the germination up-to a month (Ashmore *et al*., 2009). However, another study reported 20% of germination in papaya seeds at 5% moisture content after three months of cryo exposure (Azimi *et al*., 2005). Due to these contradicting reports, it becomes necessary to first test the cryo-fitness of papaya seeds by storing them in liquid nitrogen, preferably for longer duration before cryo-conservation procedures to be established for this crop. Therefore, the present investigation was undertaken to evaluate the effect of liquid nitrogen storage of papaya seeds at vapour phase (-140°C) in terms of seed viability and possible biochemical changes that could be attributed due to exposure to freezing temperature for long periods.

Materials and Methods

Two papaya varieties *viz*., CO 2 and TNAU Papaya CO 8 were selected for the present investigation and the seeds of these varieties were collected from the Department of Fruit Science, Horticultural College and Research Institute, Tamil Nadu Agricultural University, Coimbatore. Seed desiccation and cryo-experiments were performed in the Ramiah gene bank at the Department of Plant Genetic Resources, Centre for Plant Breeding and Genetics, Tamil Nadu Agricultural University, Coimbatore during 2018-2019. The initial moisture content of the seeds was about 10-12% which was determined using a digital seed moisture meter. By using silica gel drying method, seeds were dried overnight by placing seeds in cloth bag over silica gel and moisture content was reduced to 5%. The dehydrated seeds were transferred in to 5 ml polypropylene cryovials at the rate of 100 seeds per vial per replication. Three replications were maintained for each genotype. The vials were then transferred to small experimental cryo vessels of 50 liters capacity and exposed to vapour phase of liquid nitrogen (LN) at -140°C for 6 months. To maintain the temperature, regular topping of liquid nitrogen was done during the entire period of experimentation. Upon retrieval of seeds after cryo-storage, thawing was carried out by incubating the seeds at -20°C for 24 hours followed by exposure at 5°C for a week at room temperature. Seed germination percentage was recorded at monthly intervals in both control (fresh seed) and cryo stored seeds. The seeds were evaluated for germination at ambient temperature following a "between paper method" at the rate of 25 seeds per replication. Three biological replicates were maintained per treatment. The first count was taken on the 12^{th} day and the final count on the 28^{th} day. Germination percentage was worked out as follows:

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

Following biochemical proximates such as total carbohydrate, free sugar, total protein, and free amino acids were determined in both control (fresh seeds) and cryo-stored seeds.

Carbohydrate was determined by the method of Hedge and Hofreiter, (1962). Briefly, the seed samples were homogenized and hydrolyzed using 5.0 ml of 2.5N HCL in boiling water bath for three hours and cooled at room temperature. To the supernatant, 4.0 ml of freshly prepared anthrone reagent was added and heated for eight minutes in boiling water bath. The change in colour from green to dark green was read at 630 nm by a spectrophotometer. The average amount of total carbohydrate was expressed as mg/g of the sample on dry weight basis. Protein content was estimated as suggested by Lowry *et al.,* (1951). Seeds were homogenized with 5 ml of 0.1N phosphate buffer. To the 1.0 ml of supernatant, 5.0 ml of alkaline copper reagent was added and incubated at room temperature for 10 minutes. Then 0.5 ml of Folin-Ciocalteu reagent was added and kept undisturbed in dark for 30 minutes. The optical density of the solution was measured at 620 nm against blank using a spectrophotometer. The average amount of total protein was expressed as mg/g of the sample on dry weight basis. Free sugars were estimated by the method of (Somogyi, (1952). Supernatant was extracted using hot 80% ethanol by evaporating it over a water bath at 80^0 C. After dissolving the precipitated sugars, 1.0 ml of supernatant was taken in a tube to which 1.0 ml of alkaline copper tartrate reagent was added and boiled in a water bath for 10 minutes. After cooling, 1.0 ml of arseno-molybdic acid reagent was added and the volume was made to 10 ml using distilled water. The absorbance of blue colour was read at 620 nm by a spectrophotometer after 10 minutes. The average amount of total free sugars was expressed as mg/g of the sample on dry weight basis. Free amino acids were estimated by the method of Misra *et al*.,

(1972). Seed materials were homogenized and extracted in 10 ml of 80% ethanol at 55°C. Contents were centrifuged at 10,000 rpm for 5 minutes and the supernatant was collected. To the 1.0 ml supernatant, 4.0 ml of ninhydrin reagent was added and mixed well. The extract with ninhydrin reagent was heated in a boiling water bath for 10 minutes for colour development. The heated tubes were allowed to cool at room temperature and the absorbance was measured at 570 nm by a spectrophotometer within one hour, against the reagent blank. The average amount of total free amino acids was expressed as mg/g of the sample on dry weight basis. Data was analyzed statistically using the analysis of variance at a 5% significant level with means separated by paired t-test using SPSS 16.0 version statistical package and significant differences among means of control (fresh seeds) and cryo-treatments were tested using least significant difference.

Results and Discussion

The results revealed that initial germination percentages were 90% and 85% in the fresh seeds of CO 2 and TNAU papaya CO 8, respectively indicating no significant difference for seed viability the two genotypes studied (Fig.1). Drastic reduction in germination percentage was clearly evident when the seeds of both genotypes were stored in liquid nitrogen for about six months. The germination percentages were 50%, 35% and 7% after one, three and six months of cryo-storage of CO 2 genotype. Significantly 90% of decline in seed viability was observed in the cryo stored seeds of CO 2 in comparison with fresh seeds. Similar response was also noticed in TNAU Papaya CO 8 seeds upon six months of cryo-storage. The germination percentages were 84%, 28% and 16% after one, three and six months of cryo storage. The results showed that through the cryo- storage did not significantly affect the seed viability of TNAU Papaya CO 8 papaya seeds during first two months of storage, 80% of reduction in seed viability was found in the cryo-stored seeds after six months as compared to fresh seeds.

The results obtained in the present study suggested that papaya seeds lose viability gradually when stored at extremely low temperatures for longer time period irrespective of genotypes examined and this corrobates with the earlier findings of Ellis *et al*., (1991).The loss of viability of papaya seeds could be attributed due to freezing injury alone as we only used seeds at 5% moisture level, thus the damaging effects of ice-nucleation could be negated. Previous studies in papaya reported no loss in viability, at the low moisture content of 4-10% (Becwar *et al*., 1983; Nadarajan *et al*., 2104; Obisesan *et al*., 2005; Misra *et al*., 1977) which might be substantiated by the fact that in all those studies, seeds were cryo-stored only for a brief period of time, either in hours or days. Therefore, further investigation was conducted to understand the causative factors, particularly biochemical parameters, if any, for the loss in seed viability.

The results on the analysis of various biochemical components of papaya seeds of two genotypes upon exposure to liquid nitrogen (vapour phase) temperatures are presented in Table.1. The data indicated that total carbohydrates and total protein content of cryo-stored papaya seeds decreased gradually as compared to fresh papaya seeds, irrespective of the genotypes studied. The total carbohydrate content of fresh seeds was 20.64 mg/g and 63.01 mg/g on dry weight basis in CO 2 and TNAU Papaya CO 8 genotypes, respectively while it was 6.90 (CO 2) and 25.19 mg/g on dry weight basis (TNAU Papaya CO 8) after six months of cryo-storage. The significant low carbohydrate levels in the cryo-treated seeds noticed in the present study agrees with the findings of Zanotti *et al*., (2012) which might be attributed to the fact that catabolism of carbohydrates may occur at sub-zero freezing temperatures and might contribute to the loss of seed viability upon long term cryo-storage .The results also showed that the total protein content decreased progressively with the storage period. The total protein content of the fresh seeds was found to be 28.44 mg/g (CO 2) and 19.37 (TNAU Papaya CO 8) mg/g on dry weight basis. After six months of storage under vapour phase of liquid nitrogen, the protein content was reduced to 3.21 mg/g and 3.12 mg/g on dry weight basis in the CO 2 and TNAU Papaya CO 8 genotype, respectively. Cherry and Skedsen (1986) hypothesized that the irreversible loss of some essential proteins in the exposed seeds to low temperature conditions led to loss of seed viability. The decline in the total protein content due to impaired protein biosynthetic activity with the gradual loss of seed viability have been reported in seeds of rye and pigeonpea (Hallam *et al*.,1973; Bray *et al*., 1976; Powell *et al*.,1977).

Conversely, no significant difference was observed for both free sugars content and free amino acid content in the fresh seeds and cryo-treated seeds for six months in both the genotypes. The results showed a gradual increase in free sugar content with increase in the storage period under liquid nitrogen in both the genotypes. Similar trend in increase of free amino acid content was also noticed upto six months of storage of papaya seeds

under liquid nitrogen. These results obtained in the present study may indicate a fact that seed viability loss upon long term storage of " intermediate" class of seeds, as in the case of papaya, under freezing conditions may also be attributed to the accumulation of free sugars and free amino acids in the seed tissues. No previous studies have so far documented the influence of free sugars and free amino acids in the seeds exposed to sub-zero temperatures at extended periods. Therefore, understanding the biochemical mechanisms underlying the loss of seed viability in recalcitrant or intermediate seeds crop species would help in devising an appropriate storage conditions for long term conservation of crop germplasm.

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Table 1. Effect of cryo-storage on the biochemical proximates of CO 2 and TNAU papaya CO 8 seeds

Storage period (months)	Total carbohydrates (mg/g)		Free sugars (mg/g)		Total protein (mg/g)		Free amino acids (mg/g)	
	CO 2	TNAU Papaya CO 8	CO 2	TNAU Papaya CO 8	CO 2	TNAU Papaya CO 8	CO 2	TNAU Papaya CO 8
Control (Fresh seeds)	20.64±0.00	63.01±0.57	219.56±0.52	299.74±4.23	28.44±1.33	19.37±0.30	11.32±0.51	35.84±1.58
1	14.73±0.03	45.61±2.74*	195.85±1.00*	217.99±13.47*	9.31±1.28	14.27±2.88*	8.71±0.37*	16.07±0.16
2	10.07±0.18	43.44±2.02*	203.91± 2.54*	252.86±5.94*	7.64±0.78	13.28±3.09*	11.91±0.37*	17.72±0.33
3	9.77±0.09	37.06±1.90	213.51±1.74*	286.45±12.24*	6.82±0.01	12.62±3.14*	12.19±0.61*	18.70±0.30
4	8.43±1.08	33.98±3.56	255.70±18.02*	296.62±13.12*	6.28±0.09	8.42±1.41*	12.77±0.78*	20.15±1.08
5	7.52±0.872	31.90±2.48	274.3 ±6.20*	311.10±17.96*	5.50±0.49	5.54±1.35	12.89±0.75*	22.12±0.22
6	6.90±0.423	25.19±1.95	282.60±6.29*	336.16±21.77*	3.21±0.06	3.12±0.18	13.15±0.97*	22.29±0.20

Data are the mean of three replicates with ± SE. * Significant at 5%

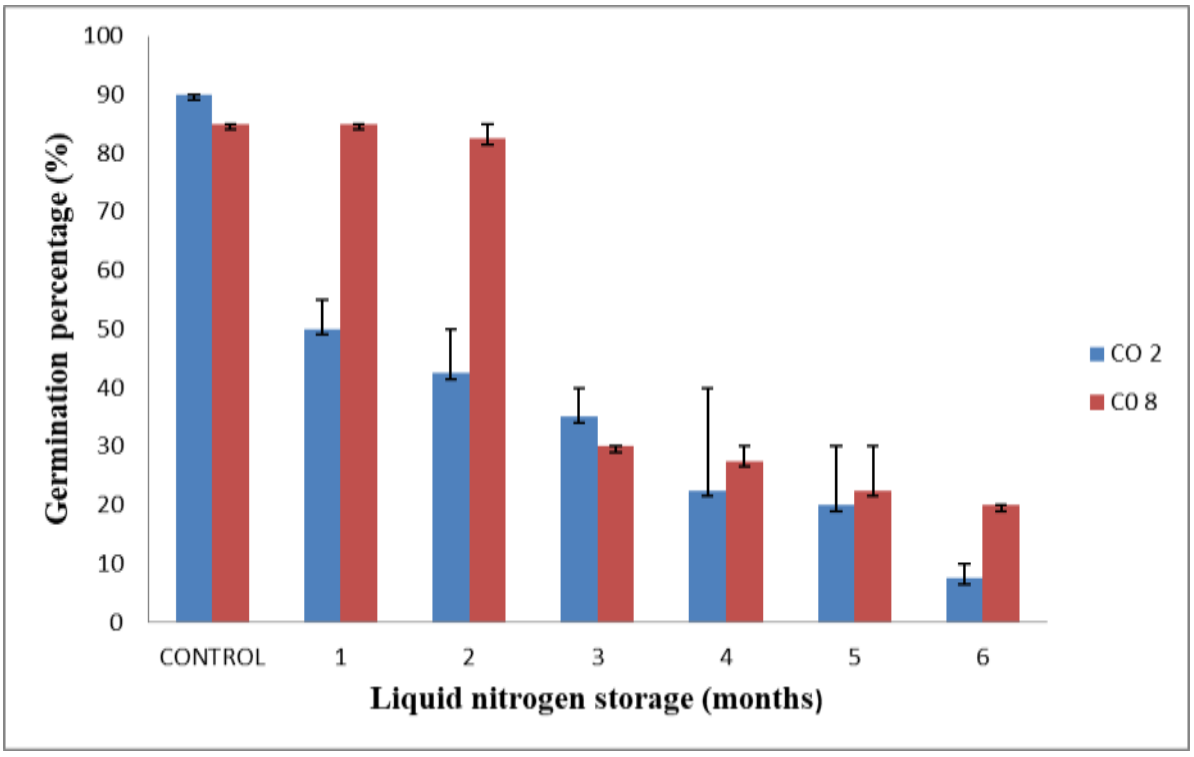

Fig. 1. Seed viability response of two papaya genotypes after six months of cryo-storage. Bars represent the standard error of mean.

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