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Standardization of vitrification treatment for safe cryo-conservation of papaya cultivar (*Carica papaya* L.) using Differential Scanning Calorimetry

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Abstract
Cryo-conservation is the most effective strategy for the long-term preservation of papaya seeds. Before the creation of a secure cryo-conservation method for papaya seeds suitable vitrification treatments involving loading solution in combination with plant vitrification solution 2 (PVS2) have to be standardized. In this study the optimal vitrification treatment was determined using the Differential Scanning Calorimetry (DSC). The results showed that the formation of ice nucleation was observed in the DSC thermogram at a temperature of (-36.64 ºC-Onset, -41.62 ºC-Midpoint and -46.64 ºC-End point) for 9% moisture content control seeds, the temperature of (-34.76 ºC-Onset, -40.81 ºC -Midpoint and -45.43 ºC-End point) for 10% moisture content control seeds and the temperatures of (-37.65 ºC-Onset, -40.36 ºC-midpoint and End point -45.77 ºC) for 11% moisture content control seeds. Similarly the ice nucleation for various vitrification treatment combinations was observed at the temperature range of (-31.25 ºC to -41.21 ºC-Onset, -35.32 ºC to -46.54 ºC-Midpoint and -39.49 ºC to -53.34ºC-End point) for 9% moisture content seeds, (-29.16 ºC to -40.43 ºC-Onset, -35.67 ºC to -45.43 ºC-Midpoint and -37.41 ºC to -52.87 ºC -Endpoint) for 10% moisture content seeds and (-31.43ºC to -40.83 ºC-Onset, -32.61 ºC to -46.46 ºC-Midpoint and -36.67 ºC to -54.36 ºC-Endpoint) for 11% moisture content seeds. Among the twenty-seven treatment combinations tested the treatment LP5 of 9% moisture content and LP5 and LP6 of 10% moisture content was discovered as best treatments for cryo-conservation of papaya seeds due to the formation of glass transition events in the vitrified seed tissues.

Keywords: Papaya, seeds, cryo-conservation, vitrification, differential scanning calorimetry, thermogram

1. Introduction
Papaya (*Carica papaya* L.) is the economically important fruit crop of the Caricaceae family. It is native to Mexico and found almost in all tropical and sub tropical region. Papaya is rich in antioxidants and phytonutrients which fight free radicals and it is very effective against various cancers. The fruit has high industrial value due to the presence of pectin which is used in the preparation of jam and jelly. Papaya seeds are rich in flavonoids that have chemotherapeutic effects which can reduce the risk of cancer by arresting the development of cancerous cells (Neelam *et al*., 2014) [7].

Now-a-days papaya has been grown as a commercial crop due to its high nutritive value. Increase in awareness among people towards various health benefits of papaya promoted its cultivation. But the productivity is lower in India due to the cultivation of low yielding varieties. Papaya seeds are basically a poor storer, there is a need for good storage technique to prolong its storability. Papaya was described as intermediate seed crop as it showed reduced viability below 10% moisture content with a storage temperature of -20ºC (Ellis *et al.*, 1991) [2]. This indicates the fact that papaya seeds are not suitable for long term conservation under sub-zero temperature conditions. Therefore cryo-conservation is the next alternative approach for long term conservation of papaya seeds. To cryo-conserve the seeds with the moisture level of 8-10% the vitrification technique is used to maintain the seed viability during cryo-conservation (Engelmann, 2004) [3]. Vitrification is the transition of cellular water directly from the liquid phase into the amorphous phase to avoid lethal ice-crystal formation which could be achieved by treatment of tissues with cryo-protectants such as plant vitrification solutions (Sakai *et al.*, 1990) [8]. Low water content which minimizes the ice crystallization in seed tissues is vital for successful vitrification (Sherlock *et al.*, 2005) [11]. Therefore, the important objective is to reduce water content in the target plant tissue before storage in liquid nitrogen.

Cryo-preservation procedures must be standardized for all crop species as it is species and genotype specific. To do this it is important to determine the optimal water content during dehydration and freezing in liquid nitrogen and thawing to avoid ice crystallization. In addition to this the amount of freezable water and verifying the glass transition including the analysis of thermal events during cooling and thawing cycles are the key factors that would be helpful in development of an efficient procedure for cryo-conservation. DSC is a thermal analysis technique in which the heat flow in and out of a sample is measured as function of temperature or time while the sample is exposed to controlled temperature program. It characterizes thermal phase transitions (e.g., melting, crystallization, Tg measuring heat of fusion and heat of crystallization. This technique is applied particularly to monitor the changes of phase transition in tissues which is kept for cryo-conservation (Van Holde *et al.*, 2006) [13] In addition to this most important thermal characteristic such as glass transition, ice nucleation, melting and re-crystallization are also obtained which was used for optimization of vitrification treatments (Zamecnik *et al.*, 2019).

Therefore, the present study was carried out with the goal of determining the thermal characteristics of papaya seed particularly the seed tissues using the DSC technique to find out the best vitrification treatments for safe cryo-conservation of papaya seeds.

2. Materials and methods

The seeds of papaya variety CO 8 collected from the Department of Fruits Science, Horticulture College and Research Institute, TNAU, Coimbatore were used for the experiment

Then were subjected to three different moisture content of 9%, 10% and 11%. About 10 mg of seed tissues was excised from the seed and treated with different vitrification combinations and then tested for different thermal properties. Differential Scanning Calorimetry was used to determine the properties. Study of thermal behaviour was carried out in both vitrified and control seeds using DSC 6000 with pyris 13 software. Nitrogen was used as a purge gas. (Fig.1)

Control seeds were subjected to thermal examinations to confirm whether ice nucleation occurs owing to phase change. 10 mg of seed tissue were hermetically sealed in the aluminium pan with small lid on its top using Perkin-Elmer crimper. Then the thermal properties of samples were analyzed using DSC 6000. During the scan the samples were first held at 25ºC for one min and then cooled down from 25ºC to - 150ºC at the rate of 10ºC/min. After then the samples were held at - 150ºC for one minute and then re-warmed from - 150ºC to 25ºC at a rate of 10ºC for one min.

For optimization of suitable vitrification treatment that could be used in the creation of cryo-conservation technique DSC was performed with the seeds of three moisture content. First the seeds were treated with loading solution after that it was treated with Plant Vitrification Solution 2 in various combinations and time duration. The loading solution (LS) is made up of 2M glycerol and 0.4M sucrose and the Plant Vitrification Solution 2 (PVS) composed of 30 % ethylene glycol, 15% Dimethyl Sulfoxide, 15% of glycerol and 0.4M sucrose. For different vitrification treatment the samples were analyzed for their thermal behaviour using DSC with the cooling and warming cycles.

Table 1: Different loading solutions and plant vitrification solution 2 treatment combinations for thermal analysis

9% M.C.	LP1	LS-10 min + PVS2 10 min
	LP2	LS-10 min + PVS2 20 min
	LP3	LS-10 min + PVS2 30 min
	LP4	LS-20 min + PVS2 10 min
	LP5	LS- 20 min + PVS2 20 min
	LP6	LS-20 min + PVS2 30 min
	LP7	LS-30 min + PVS2 10 min
	LP8	LS- 30 min + PVS2 20 min
	LP9	LS-30 min + PVS2 30 min
10% M.C.	LP10	LS-10 min + PVS2 10 min
	LP11	LS-10 min + PVS2 20 min
	LP12	LS-10 min + PVS2 30 min
	LP13	LS-20 min + PVS2 10 min
	LP14	LS-20 min + PVS2 20 min
	LP15	LS-20 min + PVS2 30 min
	LP16	LS-30 min + PVS2 10 min
	LP17	LS-30 min + PVS2 20 min
	LP18	LS-30 min + PVS2 30 min
11% M.C.	LP19	LS-10 min + PVS2 10 min
	LP20	LS-10 min + PVS2 20 min
	LP21	LS-10 min + PVS2 30 min
	LP22	LS-20 min + PVS2 10 min
	LP23	LS-20 min + PVS2 20 min
	LP24	LS-20 min + PVS2 30 min
	LP25	LS-30 min + PVS2 10 min
	LP26	LS-30 min + PVS2 20 min
	LP27	LS-30 min + PVS2 30 min

Cryo-fill Tank Nitrogen gas Cylinder DSC Instrument

Fig 1: Differential Scanning Calorimetry with liquid nitrogen cooling system

3. Results and Discussion

Cryo-conservation is the best alternative for long term conservation of intermediate crops such as papaya. Differential Scanning Calorimetry is a technology that can be used to observe phase transitions in the seed tissues without being invasive (Williams *et al.*, 1993) [14] The cellular metabolism is severely slowed and biological degradation is halted during cryo-conservation. The Cryo-conservation procedure depends mainly on desiccation tolerance and ability of tissue to rejuvenate after the ice nucleation during cooling (Martinez *et al.*, 2000) [4]. Even though it has numerous advantages cryo-conservation techniques developed for many crops cannot be reproducible. This could be due to the action of phase transition which is described by the transformation of cellular water into amorphous glassy state has not been investigated thoroughly. Thermal analysis is the technique commonly used to estimate the phase of biological tissues based on the presence of freezable water in the seeds from

liquid to amorphous glass state using DSC. This is predicated on the fact that anytime a material goes through a physical transformation heat is either released or absorbed as a result of change of condition. DSC exactly allows detecting heat flow in the samples when it is exposed to thermal gradients. By continuous adjustment of the instruments thermal regulator the temperature of sample holder and reference holder will be kept the same. A single proportional to the difference between the heat input to the sample and that of reference holder will be fed to the recorder which produces the thermogram. With the help of the thermogram it is possible to measure the heat changes associated with freezing and melting of water of biological tissues and the information obtained from this is used to obtain a suitable cryo-conservation techniques (Benson *et al*., 2005) [1]. Therefore, the present study was tried in the papaya cultivar CO 8 to understand the thermal parameters associated with phase transition mechanisms using seeds to identify suitable vitrification treatments appropriate for the development of suitable cryo-conservation procedure.

Table 2: Thermal Characteristics as revealed by DSC in the seed tissues of papaya cultivar CO 8 with 9% moisture content during cooling and warming cycles

Vitrification Treatments	Thermal Cycle	Thermal Event	Onset (ºC)	Mid-point (ºC)	End point (ºC)
Control	Cooling	Ice nucleation	-36.64	-41.62	-46.64
	Warming	Ice melt	-14.23	2.74	8.56
LP1	Cooling	Ice nucleation	-32.45	-36.32	-40.19
	Warming	Ice melt	-12.64	4.92	9.55
LP2	Cooling	Ice nucleation	-37.07	-43.85	-50.39
	Warming	Ice melt	-10.68	3.10	8.55
LP3	Cooling	Ice nucleation	-38.21	-44.63	-50.03
	Warming	Ice melt	-6.68	2.51	9.36
LP4	Cooling	Ice nucleation	-31.25	-35.32	-39.49
	Warming	Ice melt	-7.04	3.60	10.13
LP5	Cooling	Ice nucleation	-41.21	-46.54	-53.34
		Tg	-121.8	-122.0	-122.21
	Warming	Ice melt	-13.08	7.54	17.92
LP6	Cooling	Ice nucleation	-37.11	-42.67	-46.51
	Warming	Ice melt	-10.33	5.32	15.02
LP7	Cooling	Ice nucleation	-36.97	-43.65	-52.32
	Warming	Ice melt	-15.86	4.22	17.31
LP8	Cooling	Ice nucleation	-37.07	-44.94	-52.66
	Warming	Ice melt	-5.52	4.14	15.30
LP9	Cooling	Ice nucleation	-35.74	-43.16	-51.18
	Warming	Ice melt	-9.88	2.50	15.81

Table 3: Thermal Characteristics as revealed by DSC in the seed tissues of papaya cultivar CO 8 with 10% moisture content during cooling and warming cycles

Verification treatments	Thermal Cycle	Thermal Event	Onset (ºC)	Midpoint (ºC)	End point (ºC)
Control	Cooling	Ice nucleation	-34.76	-40.81	-45.43
	Warming	Ice melt	-10.24	3.65	7.93
LP1	Cooling	Ice nucleation	-30.21	-35.67	-41.92
	Warming	Ice melt	4.77	5.82	10.76
LP2	Cooling	Ice nucleation	-37.63	-44.40	-51.13
	Warming	Ice melt	-3.11	4.91	10.28
LP3	Cooling	Ice nucleation	-36.47	-43.38	-51.70
	Warming	Ice melt	-5.61	3.63	10.87
LP4	Cooling	Ice nucleation	-29.16	-33.65	-37.41
	Warming	Ice melt	-3.34	3.54	8.12
LP5	Cooling	Ice nucleation	-40.43	-45.43	-48.65
		Tg	-122.6	-125.44	-128.2
	Warming	Ice melt	-10.60	1.77	6.46
LP6	Cooling	Ice nucleation	-39.21	-45.34	-51.43
		Tg	-94.75	-111.59	-119.76
	Warming	Ice melt	-3.13	4.67	10.38
LP7	Cooling	Ice nucleation	-38.48	-43.50	-50.00
	Warming	Ice melt	3.08	4.05	8.47
LP8	Cooling	Ice nucleation	-37.63	-43.41	-52.87
	Warming	Ice melt	-3.71	3.28	7.92
LP9	Cooling	Ice nucleation	-33.05	-39.40	-45.35
	Warming	Ice melt	-3.81	6.47	11.56

Table 4: Thermal Characteristics as revealed by DSC in the seed tissues of papaya cultivar CO 8 with 11% moisture content during cooling and warming cycles

Verification treatments	Thermal cycle	Thermal event	Onset (ºC)	Mid point (ºC)	End point (ºC)
Control	Cooling	Ice nucleation	-37.65	-40.36	-45.77
	Warming	Ice melt	-3.16	5.74	10.99
LP1	Cooling	Ice nucleation	-38.80	-45.75	-51.51
	Warming	Ice melt	-2.43	6.31	13.23
LP2	Cooling	Ice nucleation	-40.83	-46.46	-54.36
	Warming	Ice melt	-14.06	-4.13	16.18
LP3	Cooling	Ice nucleation	-36.91	-40.12	-44.35
	Warming	Ice melt	-11.24	2.33	6.74
LP4	Cooling	Ice nucleation	-37.71	-43.95	-49.50
	Warming	Ice melt	-3.13	5.78	10.32
LP5	Cooling	Ice nucleation	-34.55	-39.63	-44.02
	Warming	Ice melt	-10.24	3.44	11.95
LP6	Cooling	Ice nucleation	-28.02	-32.61	-36.67
	Warming	Ice melt	-12.00	7.75	12.35
LP7	Cooling	Ice nucleation	-32.42	-36.64	-40.33
	Warming	Ice melt	-8.72	3.55	9.21
LP8	Cooling	Ice nucleation	-38.42	-44.44	-50.37
	Warming	Ice melt	-14.58	1.13	9.58
LP9	Cooling	Ice nucleation	-31.43	-36.83	-41.03
	Warming	Ice melt	-6.76	4.23	8.81

In this study the formation of ice nucleation was observed in the DSC thermogram at a temperature of -36.64ºC-Onset, -41.62ºC-Midpoint and -46.64ºC-End point for 9% moisture content control seeds (Table 2; Fig 2) and the temperature of (-34.76ºC-Onset, -40.81ºC -Midpoint and -45.43ºC-End point) for 10% moisture content control seeds (Table 3; Fig 3) Similarly the ice nucleation for the control seeds of 11% moisture content was observed at the temperatures of (-37.65ºC-Onset, -40.36-midpoint and -45.77ºC) (Table 2; Fig 4). Glass transition was not found in the control seeds of all the three-moisture content.

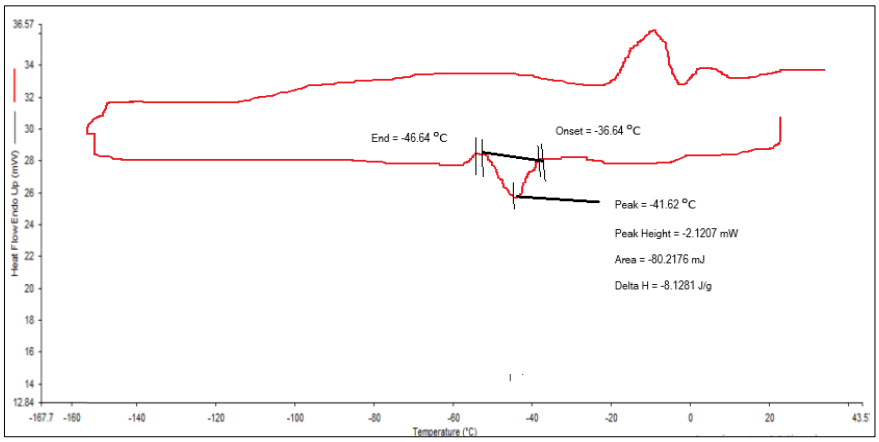

Fig 2: DSC thermogram of control (Pre-vitrified) seeds of Papaya CV. CO 8 at 9% moisture content

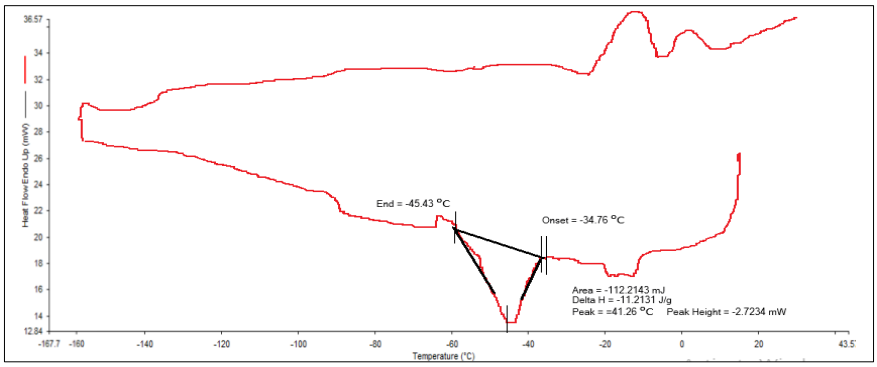

Fig 3: DSC thermogram of control (Pre-vitrified) seeds of Papaya CV. CO 8 at 10% moisture content

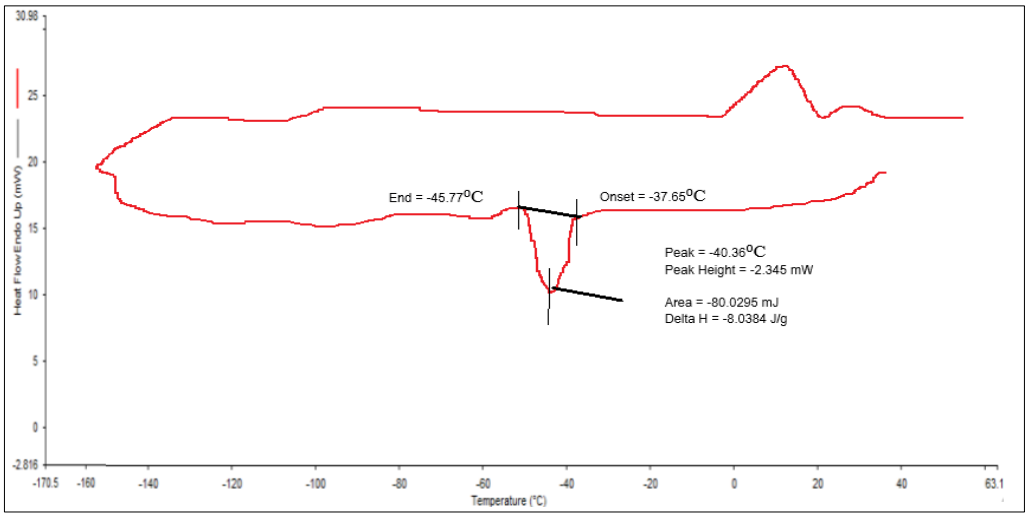

Fig 4: DSC thermogram for control (Pre-vitrified) seeds of Papaya CV. CO 8 at of 11% moisture content

This stated that the inter-cellular water was crystallized during cooling and ice nucleation event was noticed. These data suggested that the seeds of all the three moisture content require freeze induced dehydration treatments using vitrification solutions prior to storage of seeds in liquid nitrogen to avoid the cryo-injuries. The aforementioned thermal parameters were examined in the seeds of all three-moisture content subjected to different vitrification treatment combinations using DSC so as to develop optimum vitrification treatment. The DSC analysis revealed various ice nucleation and ice melting events. Among all the vitrification treatments the glass transition was observed in LP5 in 9% moisture content (-121.8ºC-Onset, -122.0ºC-midpoint and 122.21ºC-End point) (Table 2; Fig 5). Similarly, glass transition was also found in LP5 (-122.6ºC-Onset, -125.44ºC-midpoint and 122.21ºC-End point) (Table 3; Fig 6) and LP6 (-94.75ºC-Onset, 111.59ºC-midpoint and 119.76ºC-End point) (Table 3; Fig 7) of 10% moisture content. PVS2 exposure was very useful as it stabilizes the vitreous state of water thereby reducing the molecular stability and it also arrests the ice nucleation and thus improving the overall cell viscocity to a critical point leading to the glass formation.

According to the evaluation of the thermal properties obtained from the DSC study it could be stated that even though there was glass transition in LP5 of 9% and LP5 and LP6 of 10%, LP5 of 10% was identified as best treatment based on the results obtained from seed germination test. The thermal analysis was performed in the embryo of papaya (Nadarajan and Pritchard., 2014) [6]. Similarly, the DSC analysis was performed for optimization of cryopreservation of shoot tips of *Parkia speciosa* (Nadarajan, 2008) [5], Sugarcane (Shankar., 2018) [9], Banana (Sivakumar, 2020) [12] and Papaya (Wang *et al*., 2005). (Sharmela, 2020) performed thermal analysis in seeds and embryo of papaya.

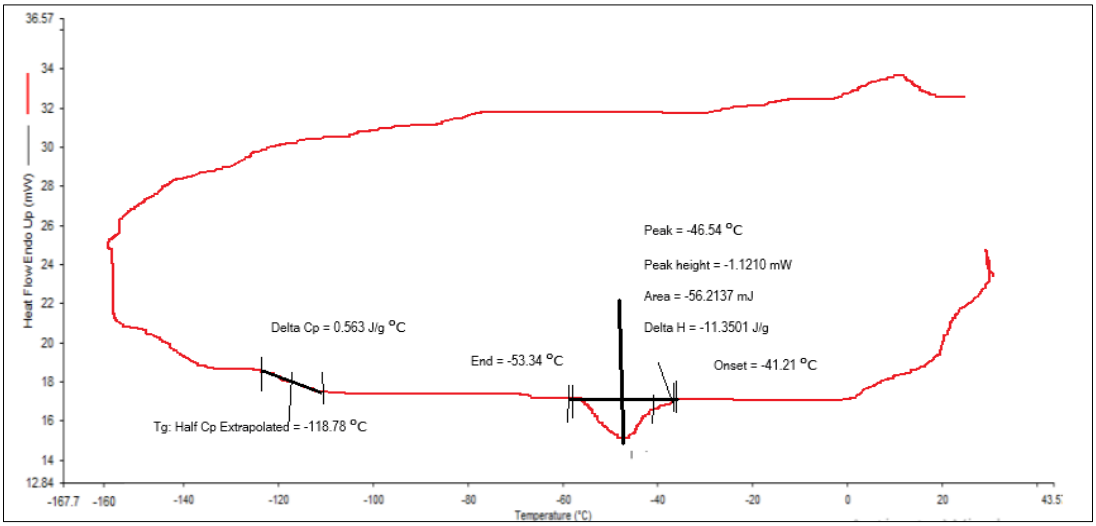

Fig 5: Thermogram curve showing glass transition in LP5 treatment in the seeds of 9% moisture content during cooling cycle

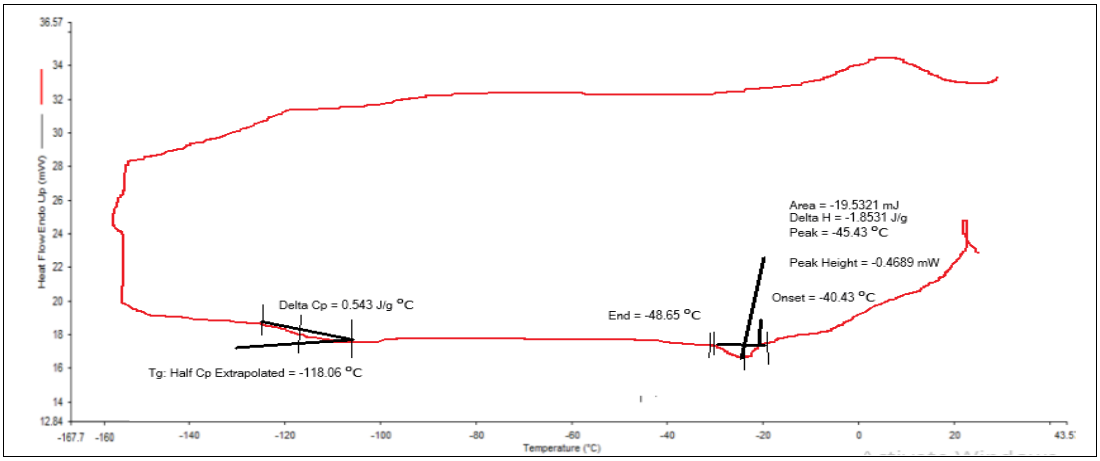

Fig 6: Thermogram curve showing glass transition in LP5 treatment in the seeds of 10% moisture content during cooling cycle

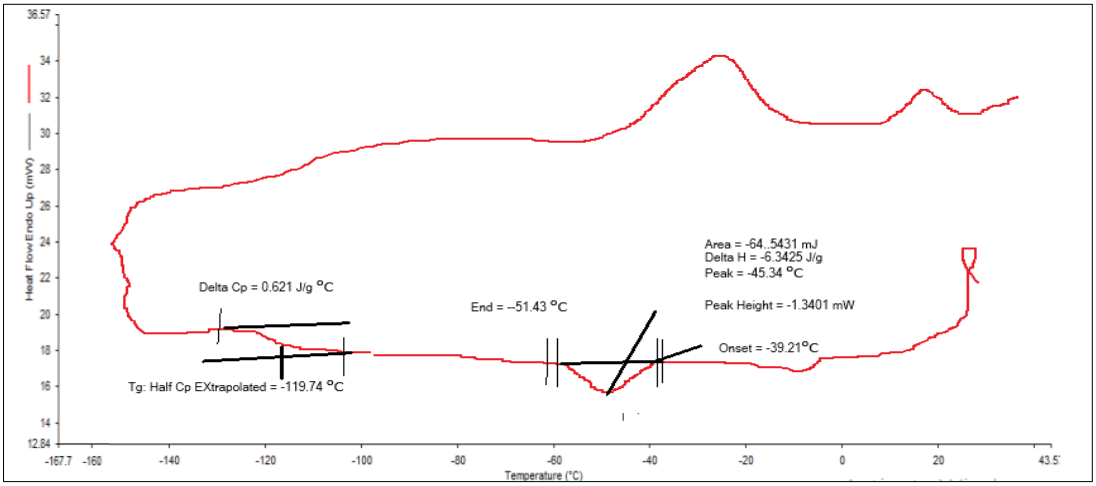

Fig 7: Thermogram curve showing glass transition in LP6 treatment in Papaya seeds at 10% moisture content during cooling cycle

4. Conclusion

The DSC analysis conducted for the papaya seeds stated that the seeds would be stored safely in liquid nitrogen if the moisture content is 10% and the seeds are treated with loading solution for 20 min and vitrified with PVS2 for 20 min. However further DSC studies have to be carried out for different crop species to get optimized vitrification treatment for safer cryo-conservation.

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