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Research Article

Standardization of vitrification treatments for safe cryo-conservation of banana (*Musa acuminata* Colla) cultivars using differential scanning calorimetry

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Abstract
Cryo-conservation is the most promising approach and reliable method for long term conservation of banana and plantain genetic resources. Prior to the development of a safe cryo-conservation procedure for highly hydrated tissues of any vegetatively propagated crop species such as banana, suitable vitrification treatments involving loading solution in combination with Plant Vitrification Solution 2 (PVS2) solution have to be standardized. In the present study, Differential Scanning Calorimetry (DSC) technique was attempted to identify the best vitrification treatment using shoot apices as storage explants in two banana cultivars namely Grand Naine and Red banana. The results showed that DSC thermogram curves detected the formation of ice nucleation at a temperature of -18.67°C for Grand Naine and -19.16°C for Red banana in the control shoot apices. Among the sixteen vitrification treatment combinations tested, the treatments, LP6, LP7, LP10, LP11, LP14 and LP15 were judged as best treatments for cryo-conservation of both banana cultivars as revealed by the formation of glass transition events in the vitrified shoot apices. This information obtained through DSC experiments would further provide scope for designing safe cryo-conservation system for banana. This is the first report to demonstrate the application of DSC to optimize the vitrification treatments that would be adopted for developing cryo-conservation protocol for *Musa* germplasm.

Keywords
Banana, shoot apices, Cryo-conservation, vitrification, Differential Scanning Calorimetry

INTRODUCTION

Banana is an important staple food crop for more than 400 million people in the developing countries. Cultivation of banana is being threatened by various pests and diseases, which significantly reduce its productivity. Therefore, banana breeding programmes should focus on the development of the high yielding, pest and disease resistant varieties to ensure the food and nutritional security. This could be achieved by continuous availability of the genetic resources of varied *Musa* species such as wild species and genetically diverse varieties. Banana being a vegetatively propagated crop species, genetic conservation is usually carried out by adopting two different methods namely field/clonal gene bank and *in vitro* gene bank. While the field gene bank conservation of banana is more difficult and laborious due to its occupation of space, repeated intercultural operations and damages caused by both natural calamities and biotic factors (Agrawal, *et al*., 2004), the utility of *in vitro* gene banks is also limited as it only enable short storage period of 3-5 years and provide the possibility of loss of materials through microbial contamination.

A promising approach for the safe and long term conservation of *Musa* genetic resources is through cryo-conservation, i.e. storage at ultra-low temperature, usually that of liquid nitrogen at -196°C. This safeguards the cultures without the risk of somaclonal variation (Reuveni *et al*., 1996) and human errors like contamination

and mislabeling during the labour intensive subculture events. Initially, banana cryo-conservation through *in vitro* grown shoot tip vitrification was attempted by Thinh (1999). Following this, a number of researchers have reported the cryopreservation in a wide range of banana genotypes by employing a variety of ultra-freezing procedures (Panis *et al*, 2005; Agrawal *et al*, 2008; Agrawal *et al*. 2014).

Cryo-conservation of biological tissues relies on the prevention of inter-cellular ice crystal formation to prevent the damage on cell membrane and its semi-permeability. The only way to prevent ice crystal formation at ultra-low temperature without an extreme reduction of cellular water content is possible only through vitrification (Sakai, 2000). During vitrification, the plant tissue obtains a glassy state to avoid the formation of lethal intra-cellular ice crystals in order to get optimal recovery and regeneration after cryopreservation. A successful vitrification requires a low water content that minimizes the ice crystallization in tissues **(**Sherlock *et al*, 2005). Hence, the important objective is to reduce water content in the target plant tissue before storage in liquid nitrogen under freezing temperature.

Standardization of cryopreservation protocol for any crop species is necessary as it is species and genotype-specific. To achieve this, it is essential to determine the optimal water content during dehydration, freezing in liquid nitrogen and thawing to avoid ice crystallization. Additionally, assessing the amount of freezable water and verifying the glass transition including the analysis of the thermal events during cooling and thawing cycles are the major key factors that would influence the development of an efficient and reliable protocol for cryo- conservation. This can be determined with the studies of thermal analysis of tissues with help of Differential Scanning Calorimetry (DSC) technique **(**Hammond *et al.,* 2019). DSC is a thermodynamical tool employed for direct assessment of the heat energy uptake, which occurs in a sample within a regulated increase or decrease in temperature. This technique is particularly applied to monitor the changes of phase transitions in tissues for cryo-conservation (Van Holde *et al*., 2006). The DSC instrument measures the temperature and heat flow associated with thermal transition in the tissue sample and provides the information about the endothermic or exothermic events. In addition, data on the most important thermal characteristics such as glass transition, ice nucleation, melting and re-crystallization are also generated which are highly useful for optimization of vitrification treatments (Zamecnik *et al*., 2019).

Recently, in our laboratory, Shankar (2018**)** successfully demonstrated the utility of DSC technique to optimize the vitrification treatments for the development of cryo-conservation protocol in crops such as sugarcane and cassava. As banana being a tropical plant having high water content in its tissue, there is a need to determine the safe water content and study the thermal behaviour of hydrated tissues before storing into liquid nitrogen **(**Benson, 2008). Hence, the present study was undertaken with a view to investigate the thermal properties of banana tissues, preferably the shoot apices, using DSC technique so that, the optimal vitrification treatments could be identified for the safe cryoconservation of banana cultivars.

MATERIALS AND METHODS

Two popular banana cultivars namely, Grand Naine and Red banana were chosen for the present study. Healthy suckers of both genotypes were collected at appropriate stage from the farmers' field in the Pagathur village, Sirumugai block, Coimbatore district.

After the collection of field grown suckers, they were brought to laboratory and trimmed to 2-5cm^3 volume to isolate the shoot apex under the laminar air flow chamber cabinet. Each excised shoot apex consisted of a thin layer of corm tissue along with 2-3 young leaf primordial. About 10mg of tissue were excised from each shoot apex, subjected for various vitrification treatment combinations at 25°C and subsequently analyzed for different thermal characteristics using Differential Scanning Calorimetry. Thermal behaviour studies in both control and vitrified shoot apices of both banana cultivars were performed using DSC 6000 (Perkin-Elmer, USA) with Pyris 13 software (**Fig.1**). Nitrogen was used as purge gas.

To confirm whether ice nucleation occurs due to phase transition, control shoot apices of banana cultivars were subjected for thermal analysis. Fresh shoot apices (2mm size) isolated from the field grown suckers weighing approximately 10mg were hermetically sealed in the aluminium pans of 50ìl size using Perkin-Elmer Crimper. Samples were then analyzed for their thermal characteristics using DSC 6000. During the DSC scan, the samples were first held at 30°C for one min, then cooled down from 30° C to -150°C at the rate of 10°C/min followed by holding the samples at -150°C for one min and re-warmed from -150°C to 30°C at the rate of 10°C/min. For each cultivar, two replications were maintained with 10 explants per replication.

For optimization of suitable vitrification treatments that could be applied for the development of cryo-conservation protocol, DSC was performed with shoot apices of both banana cultivars. The excised shoot apices were first treated with loading solution and then dehydrated with vitrification solutions in different combinations and time durations as detailed in **Table 1**. The Loading Solution (LS) comprised of 2M Glycerol and 0.4M sucrose and the Plant Vitrification Solution 2 (PVS) consisted of 30% Ethylene Glycol, 15% DMSO and 15% of glycerol and 0.4M sucrose (Sakai *et al*., 1990). The samples were analyzed for their thermal behavior for different vitrification treatments using DSC with the cooling and warming cycles as described previously for the control tissues.

Table1. Different loading solution and plant vitrification solution 2 treatment combinations for thermal analysis.

LP1	LS-0 min + PVS 2-20 min
LP2	LS-0 min + PVS 2-30 min
LP3	LS-0 min + PVS 2-60 min
LP4	LS-0 min + PVS 2-90 min
LP5	LS-10 min + PVS 2-20 min
LP6	LS-10 min + PVS 2-30 min
LP7	LS-10 min + PVS 2-60 min
LP8	LS-10 min + PVS 2-90 min
LP9	LS-20 min + PVS 2-20 min
LP10	LS-20 min + PVS 2-30 min
LP11	LS-20 min + PVS 2-60min
LP12	LS-20 min + PVS 2-90min
LP13	LS-30 min + PVS 2-20 min
LP14	LS-30 min + PVS 2-30 min
LP15	LS-30 min + PVS 2-60 min
LP16	LS-30 min + PVS 2-90min

Fig. 1. Differential scanning calorimetry with liquid nitrogen cooling system

RESULTS AND DISCUSSION

Cryo-conservation offers a viable alternative for the long-term maintenance of vegetatively propagated crops such as banana with minimum recurring costs. During cryo-conservation, the entire cellular metabolism is drastically arrested and biological deterioration is virtually hampered. In addition, cryo-conservation of vegetatively propagated species has a wide applicability both in terms of species coverage and the number of genotypes/varieties within a given species.

In spite of numerous advantages, cryopreservation techniques developed for many vegetatively propagated species including banana could not be reproducible across laboratories due to species- and genotype dependent nature of the protocols. This may be attributed to the fact that the effect of phase transition, as explained by the transition of liquid phase of cellular water into amorphous glassy state, has not been thoroughly investigated for many problem species. Because of these limitations, the cryo-techniques could not be extended to wide range of species/accessions maintained in various gene banks necessitating development of reproducible methods for potential use.

The success of the cryoconservation protocol mainly relies on the desiccation tolerance and ability of the tissue to

rejuvenate after the ice nucleation during cooling (Martinez *et al.,* 2000). Differential scanning calorimetry is a potential technique for non- invasive observation of the phase transitions (water) in the meristem tissue (Williams *et al.,* 1993). On the basis of the presence of freezable water content in biological tissues, thermal analysis is usually performed to predict the phase transition of water in the explants from liquid to amorphous glass state using the DSC technique. This is based on the fact that, whenever a material undergoes a physical change of state, heat is either liberated or absorbed. DSC principles precisely allow detecting the heat flow in a sample which is exposed to thermal gradients. The temperature of the sample holder and the reference holder will be kept the same, by the continuous adjustment of the instrument's thermal regulator. Concomitantly, a signal proportional to the difference between the heat input to the sample and that of the reference holder will be fed to the recorder which produces a thermogram. It is therefore possible to measure the heat changes associated with freezing and melting of water of biological tissues and, this information will be used to develop cryopreservation strategies (Benson *et al*., 2005). Therefore, the present study was attempted for the first time in banana cultivars namely, Grand Naine and Red banana to understand the thermal parameters associated with phase transition mechanism using shoot apices as explants using DSC and to identify the suitable vitrification treatment appropriate for the development of cryo-conservation protocol in the selected banana cultivars.

Table 2. Thermal characteristics as revealed by dsc in the shoot apices of banana cultivar grand naine during cooling and warming cycles

Thermal Events	Cooling (°C)		Warming (°C)	
Treatments	**Ice Nucleation**	**Glass Transition**	**Re- Crystallization**	**Melting**
Control	-18.67	-	-	8.46
LP1	-81.66	-	-82.21	-28.35
LP2	-85.18	-	-82.16	-28.15
LP3	-	-111.89	-73.98	-30.28
LP4	-	-	-70.32	-30.04
LP5	-72.74	-	-72.20	-24.16
LP6	-	-115.35	-57.35	-34.73
LP7	-	-118.22	-73.77	-31.13
LP8	-	-	-73.83	-29.57
LP9	-81.3	-	-82.02	-28.68
LP10	-	-119.86	-65.1	-32.41
LP11	-	-124.25	-76.94	-29.60
LP12	-	-	-68.87	-30.68
LP13	-80.1	-	-78.65	-27.75
LP14	-	-117.2	-77.13	-29.34
LP15	-	-119.84	-88.48	-25.92
LP16	-	-	-76.23	-28.12

Table 3. Thermal characteristics as revealed by dsc in the shoot apices of banana cultivar red banana during cooling and warming cycles

Thermal events	Cooling (°C)		Warming (°C)	
Treatments	**Ice Nucleation**	**Glass Transition**	**Re-Crystallization**	**Melting**
control	-18.41	-	-	-9.14
LP1	-82.09	-	-67.25	-31.81
LP2	-83.18	-	-77.63	-30.06
LP3	-	-118.76	-65.12	-32.85
LP4	-	-	-70.25	-30.44
LP5	-85.26	-	-64.83	-31.00
LP6	-	-116.35	-67.89	-28.33
LP7	-	-117.18	-66.16	-31.49
LP8	-	-	-68.6	-31.99
LP9	-81.3	-	-70.42	-31.17
LP10	-	-115.46	-69.51	-31.58
LP11	-	-118.65	-82.54	-28.31
LP12	-	-	-75.49	-30.37
LP13	80.1	-	-60.93	-32.8
LP14	-	-119.92	-78.73	-27.56
LP15	-	-121.98	-67.67	-31.02
LP16	-	-	-72.34	-29.94

In the present study, DSC thermogram curves detected the formation of ice nucleation at a temperature of -18.67°C for Grand Naine and -19.16°C for Red banana in the control shoot apex tissues (**Tables 2&3**). During melting, control shoot apices of Grand Naine and Red banana showed ice melting at a temperature of 8.97°C and 9.14°C in both the genotypes. Glass transition was not found in control shoot apices of both banana cultivars. This revealed that the inter-cellular water was crystallized during cooling and ice nucleation event was noticed. The re-crystallization of water in the shoot apices were not recorded during warming cycle in the control shoot apices of both cultivars (**Fig. 2**). These findings suggested that both species require freeze induced dehydration treatment using vitrification solutions prior to storage of the explants in liquid nitrogen to avoid the cryo-injuries.

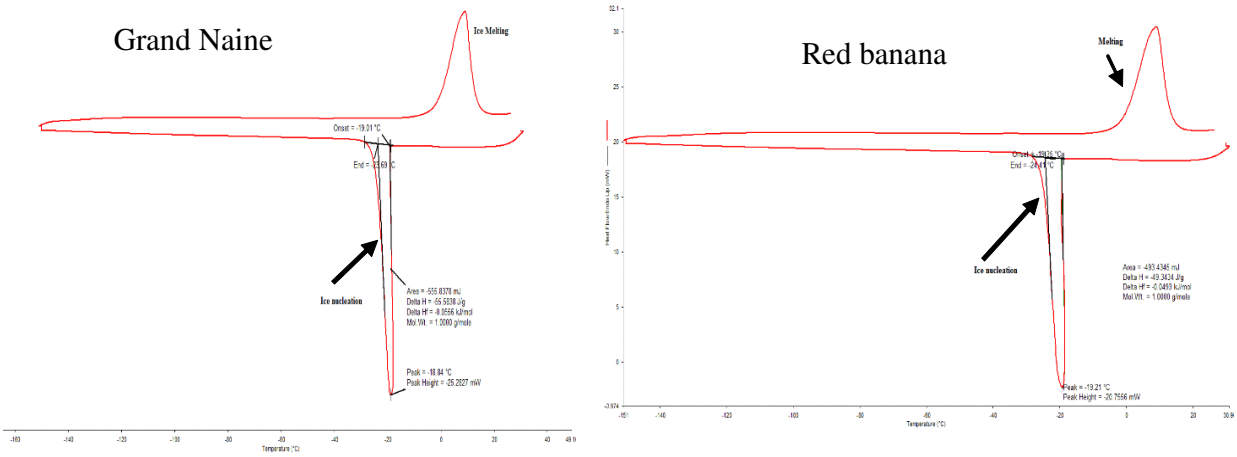

Fig. 2. DSC Thermogram curve for control shoot apices of banana cultivars.

In order to develop optimum vitrification treatments, the above thermal characteristics were studied in the shoot apices of banana cultivars subjected with different vitrification treatment combinations using DSC. The DSC analysis conducted in Grand Naine shoot apices revealed that during cooling cycle, varying degrees of ice nucleation temperature was observed in five treatments such as, LP1(-81.66°C), LP2(-85.18 °C), LP5(-72.74°C), LP9 (-81.30°C) and LP13(-80.10°C) (**Table 2**). In Red banana, same treatments LP1 (-82.09°C), LP2 (-83.17 °C), LP5 (-85.26°C), LP9 (-82.60°C) and LP13 (-83.47°C) also showed ice nucleation events during cooling process (**Table 3**). Among the different vitrification treatments tried, the glass transition (Tg) was observed in treatments namely, LP3 (-111.89°C), LP6 (-115.35°C), LP7 (-118.22°C), LP10 (-119.86°C), LP011 ((-124.25°C), LP14 (-119.06°C) and LP15 (-119.84°C) in Grand Naine (**Table 2; Fig 3-8**) and LP3(-118.76°C), LP6,(-116.35°C), LP7 (-119.02°C), LP10 (-115.46°C), LP011 (-117.76°C), LP14 (-119.06°C) and LP15 (-121.98°C) in Red banana (**Table 3; Fig 3-8**). Irrespective of loading solution treatments, 30min and 60min PVS2 exposure were able to stabilize the vitreous state of water thereby reducing the molecular mobility, arresting ice nucleation and improving the overall cell viscosity to a critical point leading to the stable glass formation. Since PVS2 is the most frequently used cryoprotectant for plant cryopreservation, it is not surprising that the glass transition temperature is observed in the successful vitrification treatments are correlated well with PVS2 Tg of -115°C to -125°C. As PVS2 changes freezing behavior of water remaining in the cells, water content of the shoot tip could be manipulated by exposure time of PVS2 (Volk *et al.* 2006). During warming cycle, recrystallization was noticed in all treatments at around - 32°C to -25° C in Grand Naine and -33°C to -26°C in Red banana. The ice melting invariably occurred at all treatments at around -38° to -17°C in both cultivars of banana.

On assessment of thermal characteristics data derived from DSC analysis, it could be concluded that, the vitrification treatments LP3, LP6, LP7, LP10, LP11, LP14 and LP15 were identified as the best treatments irrespective of the cultivars studied. Interestingly, the DSC results revealed that LP4, LP8, LP12 and LP16 treatments had no cooling events when shoot apices of Grand Naine and Red banana were vitrified in PVS2 for 90min (**Tables 2 & 3**). This may be due to the fact that increasing PVS2 treatment time to 90min would have completely eliminated the active water content in the tissues. Furthermore, the effect of cryoprotective chemicals became cytotoxic to tissues upon 90min exposure to PVS2 (Sherlock *et al.*, 2005). The application of thermal analysis in relation to study of phase transition effects for the development and validation of cryo-conservation protocol has been demonstrated in few vegetatively propagated crops such as Carrot (Dereuddre *et al.* 1992), *Ribes nigrim* (Benson *et al.*1996), *Parkia speciosa* (Jayanthi *et al.* 2008), *Allium*s (Zamecnik *et al.* 2011) and apple, potato, and garlic (Zamecnik *et al.* 2012).

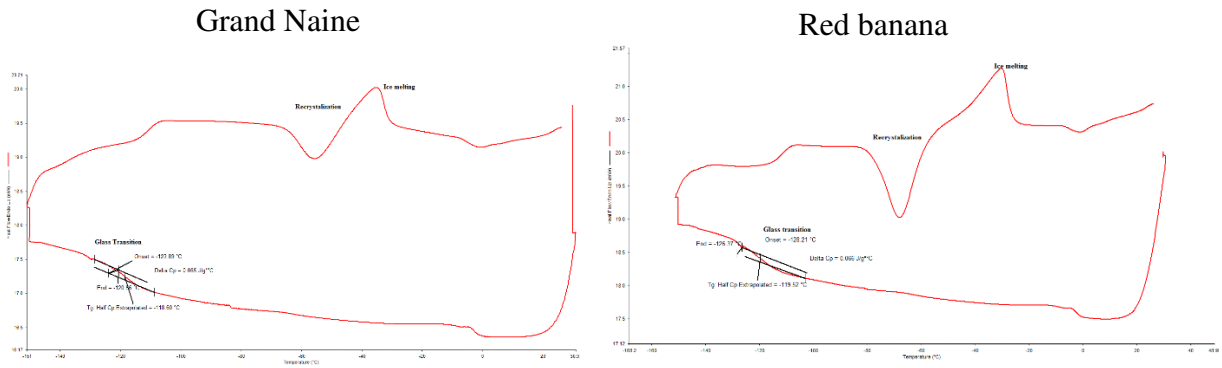

Fig. 3. DSC Thermogram curves showing glass transition events in LP6 treatment in the shoot apices of banana cultivars during cooling cycle

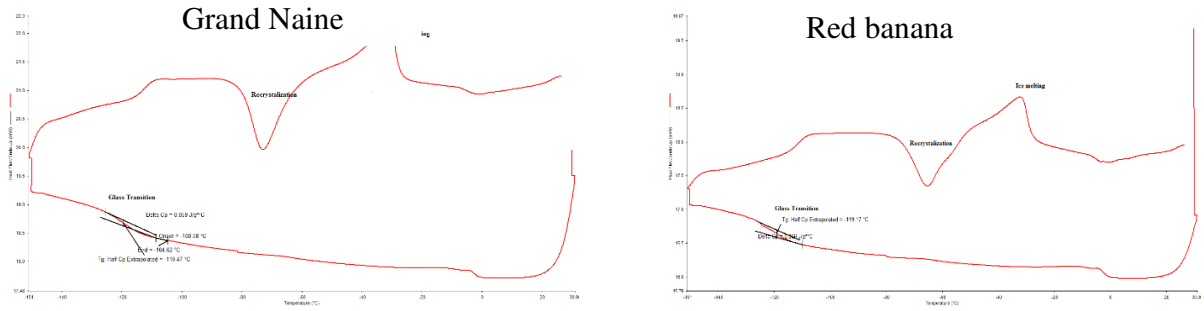

Fig. 4. DSC Thermogram curves showing glass transition events in LP7 treatment in the shoot apices of banana cultivars during cooling cycle.

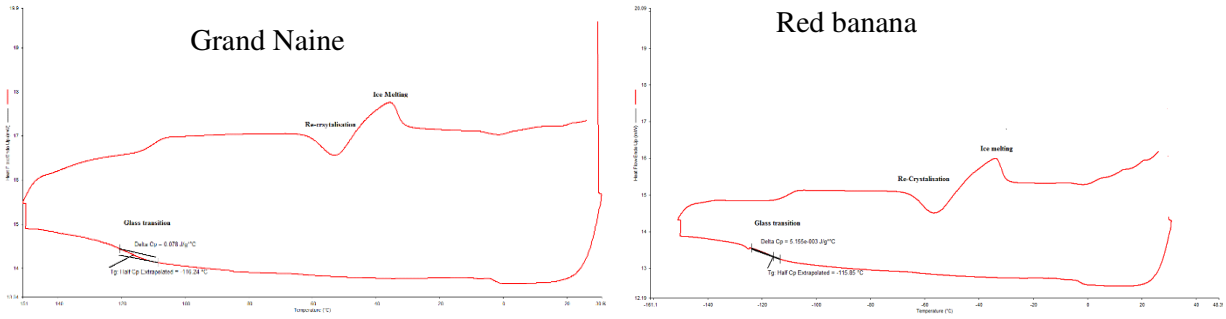

Fig. 5.DSC Thermogram curves showing glass transition events in LP10 treatment in the shoot apices of banana cultivars during cooling cycle.

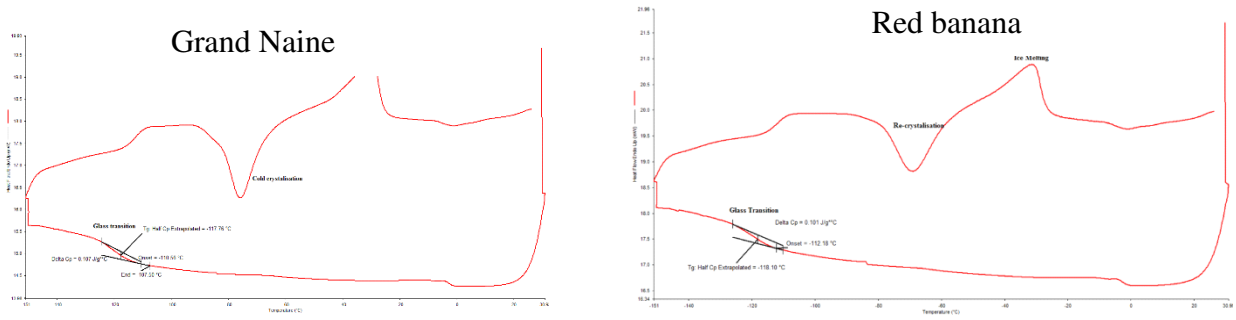

Fig. 6.DSC Thermogram curves showing glass transition events in LP11 treatment in the shoot apices of banana cultivars during cooling cycle.

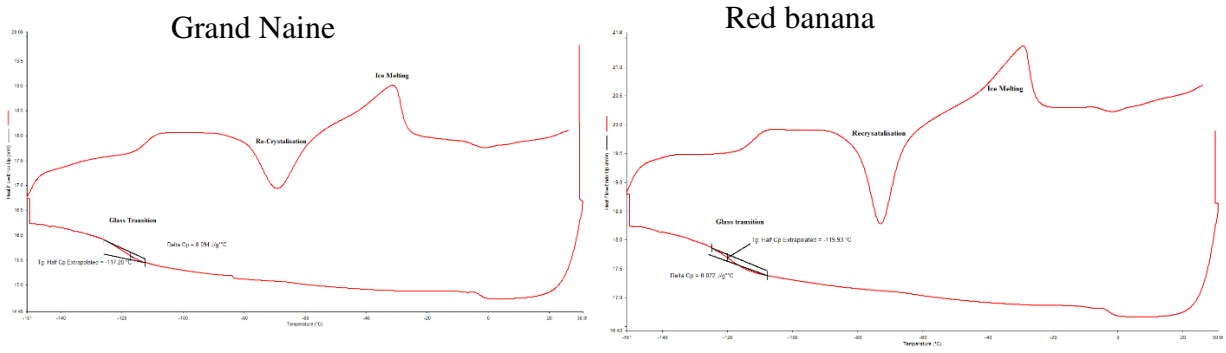

Fig. 7. DSC Thermogram curves showing glass transition events in LP14 treatment in shoot apices of banana cultivars during cooling cycle.

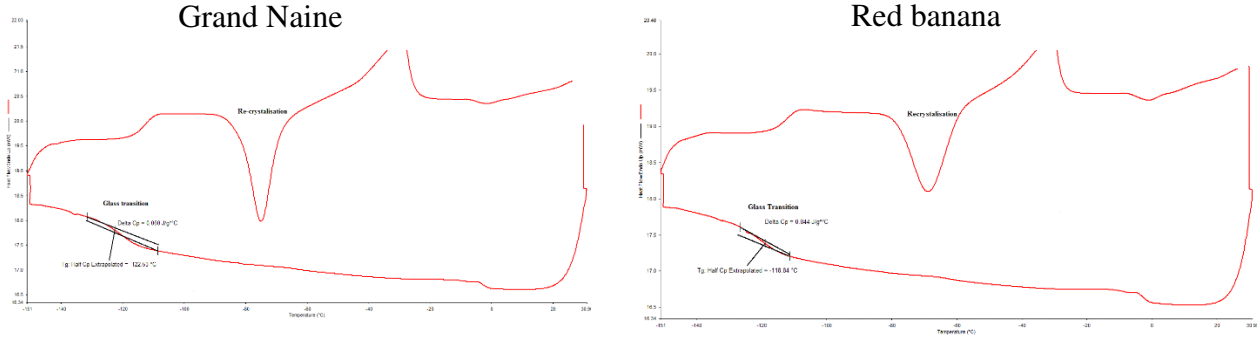

Fig .8.DSC Thermogram curves showing glass transition events in LP15 treatment in shoot apices of banana cultivars during cooling cycle.

In conclusion, DSC studies conducted for the first time in two banana cultivars have demonstrated that, the shoot apices as storage explants would be safely stored in liquid nitrogen if they are sufficiently vitrified in PVS2 solution for duration of either 30min or 60min. However, the results of the DSC should be further validated appropriately by conducting vitrification experiments with the optimized treatments for target crop species as regrowth of cryo-stored explants in the culture medium is the only parameter used to judge the success of vitrification based cryo-conservation system.

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