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Research Article

Studies on changes in the cellular pattern of cryopreserved explants of *Saccharum spp* and sugarcane cultivar

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Abstract
Cryopreserved samples in sugarcane generally require a long culturing period and can experience unexpected stress due to ultra-low freezing temperatures. This study aims to investigate the factors that hinder long-term storage by examining the anatomical changes that occur during the cryopreservation of meristem-derived axillary buds in sugarcane. Light microscopy was employed to analyze the microstructure of both cryopreserved and non-cryopreserved samples. The meristem-derived axillary buds from *Saccharum spontaneum* clones (IND 2019 2045 and IND 2019 2035) and the sugarcane cultivar Co 11015 were cross sectioned at various time intervals: 24 hours, 3 days, 7 days, 14 days, and 21 days after treatment with Liquid Nitrogen (LN) and subsequent rewarming procedures. This analysis aimed to infer the changes in cellular structures due to cryopreservation. The cross-sections of the meristem-derived axillary buds displayed damage to the epidermal and hypodermal regions at different treatment and rewarming durations. Additionally, vascular bundles were found to be highly disorganized and collapsed. The ultra-low temperature freezing likely contributed to ice nucleation within the cells, leading to the destruction of cellular integrity. Among the clones evaluated, Co 11015 demonstrated less damage compared to IND 2019 2045 and IND 2019 2035, suggesting that the commercial cultivar possesses a better capacity to withstand cryoinjury. Furthermore, utilizing analytical tools will enhance our understanding of the harm inflicted on biological tissues by cryopreservation. This knowledge will enable us to refine protocols for the successful regeneration of plantlets, ensuring the long-term conservation of germplasm.

Keywords: Cryopreservation, Light Microscopy, Meristem derived axillary buds, Sugarcane.

INTRODUCTION

Sugarcane is one of the most important cash crops globally, with over 80% of sugar production relying on this crop (Babu *et al.,* 2009). It is produced from *Saccharum* species, a perennial C4 plant that thrives in tropical and sub-tropical regions and is believed to have originated in New Guinea or Southeast Asia. Currently, more than 100 countries cultivate sugarcane, covering an area of approximately 26 million hectares. Each year, nearly 1.9 billion tons of fresh sugarcane are harvested, contributing to 40% of bioethanol production, and generating a total production value of USD 80 billion (Que *et al.,* 2023). India is the second-largest producer of sugarcane, responsible for 17.5% of the total area cultivated and 19.4% of global production, following Brazil.

The genetic makeup of sugarcane is crucial for enhancing its production. However, conventional breeding methods are limited due to its low flowering and seed production in certain climatic conditions, leading to a narrow genetic pool for selecting desirable traits (Zamir *et al.,* 2012). The primary challenge faced by sugarcane growers worldwide is vegetative propagation, which requires expensive, time-consuming, and labor-intensive replanting of field-grown plants every one to five years. Additionally, using lower-

quality planting material can reduce yields, necessitate more ploughing, and increase the risk of spreading systemic diseases. As the global population is projected to exceed 9 billion by 2050, there will be a heightened demand for energy and sugar, alongside greater risks such as rising temperatures, depletion of agricultural resources, and climate instability (Jaiswal *et al.,* 2023; Olsson *et al.,* 2023). Maintaining field collections of germplasm is impractical due to unpredictable weather, environmental hazards, pest and pathogen infestations, disease outbreaks, and the need for continuous monitoring. These factors can lead to the degradation of large germplasm collections (Jayabose *et al*., 2022a). Advanced conservation strategies, including tissue culture and micropropagation, have been developed to address these issues, allowing for rapid multiplication of germplasm and key varieties under limited growth conditions (Rai, 2022).

Cryopreservation—storing germplasm from non-orthodox seeds and vegetatively propagated species at ultra-low temperatures (-196 °C)—emerges as an alternative to *in-vitro* propagation (Martinez-Montero and Harding, 2015). Over the last 25 years, various cryopreservation techniques have been adopted for a wide range of commercially important plants, ornamentals, endangered species, forest trees, and plantation crops of both temperate and tropical origins (Engelmann, 2000). These procedures effectively eliminate viruses in economically significant species and preserve the viability of stored materials for extended periods (Bettoni *et al*., 2019). Recently, cryopreservation has gained attention for conserving and long-term storage of germplasm, overcoming challenges associated with field storage, space, maintenance, and risks related to stresses, diseases, slow growth, and contamination (Barraco *et al.,* 2011). An increasing number of laboratories are implementing these techniques to preserve secondary genetic resources (Reed, 2008). Plant specimens that have been tested and found healthy in *in-vitro* cultures can be kept in cryobanks in relatively small quantities, ensuring genetic stability with minimal maintenance. This involves storing plant specimens in liquid nitrogen and/or in the vapor phase of liquid nitrogen (at -135 °C) to ensure tissue preservation after thawing.

Cryobanking facilities must carefully manage the risks associated with cryopreservation to ensure sustainable backups for plant genetic resources. Accurate maintenance of liquid nitrogen levels is essential for effective cryogenic tissue storage. Cryopreservation procedures, including freezing, cooling, rewarming, and thawing, can disrupt cellular structures and membrane integrity. Therefore, it is crucial to optimize and standardize procedures for specific plant specimens, as there is no universal protocol (Benson, 2008). Various cryopreservation techniques have been developed for sugarcane explants; utilizing shoot tips may enhance the genetic stability of regenerated plants (Paulet *et al.,* 1993), while using meristem-derived axillary buds also improves regeneration in *Saccharum sp* (Jayabose *et al*., 2024).

However, achieving long-term storage and a successful standard technique for *Saccharum sp* is still in its early stages. Success in cryopreservation for crops like sugarcane remains challenging, often due to issues such as freezing stress and cell death. Under freezing stress conditions, ice nuclei typically form in the apoplast, the space within the cell walls of tissues. When cells are damaged by freezing, the ice can cause significant deformation of the cell wall (Panter *et al.,* 2020). The cell wall plays a crucial role in determining the shape and mechanical properties of plants as they grow in various environments (Cosgrove, 2022). Additionally, the cell wall serves as a dynamic primary barrier that contributes to the survival strategies of cells. The goal of cryopreserving plant explants is to halt cell metabolism, stabilize cellular structures, and limit molecular movement and chemical activity by solidifying the aqueous cytoplasm without forming ice crystals (Walters and Pence, 2021).

Each stage of cryopreservation—pre-cultivation, pre-treatment, freezing/rewarming, and recovery—can affect cell viability and the morpho-anatomical structure of the cells. Microscopic observation is one effective method for assessing the survival and integrity of cryopreserved explants (Kulus *et al.,* 2018). Halmagyi *et al.* (2017) used light microscopy to study the tolerance of tunica and corpus cells to ultra-low temperatures in tomatoes. They found that the cryopreserved samples required a prolonged period of culturing and experienced unexpected stress from freezing at these ultra-low temperatures.

This research aims to observe and analyze changes in cellular patterns during the cryo-storage of *Saccharum sp*. Furthermore, it seeks to understand the challenges associated with long-term storage.

MATERIALS AND METHODS

Plant Materials: The study focused on the cross-sections of axillary buds obtained from the meristem of *Saccharum spontaneum* L. clones *viz.,* IND-2019-2045 and IND-2019-2035, as well as the sugarcane cultivar Co 11015. These samples were maintained at the ICAR-Sugarcane Breeding Institute in Coimbatore, located at 77°E longitude and 11°N latitude. Fresh samples were regularly collected from the field and subjected to in-vitro micropropagation and subsequent cryopreservation techniques.

Methodology: A standardized micropropagation method for *Saccharum spontaneum*, including meristem-derived buds for each clone, was developed based on the protocol established by Jayabose *et al.* 2022b. The encapsulation dehydration technique was employed to cryopreserve the meristem-derived axillary buds from the clones IND-2019-2045, IND-2019-2035, and Co 11015.

Encapsulation: Sodium alginate is required for encapsulation techniques. To prepare the sodium alginate solution, 15 grams of sodium alginate were gradually added to 2.2 grams of MS media while stirring with a magnetic vortex to prevent clumping. The final volume of the solution was adjusted to 500 milliliters and sterilized using an autoclave. After cooling to room temperature, this solution was used for encapsulating the explants, along with 2% calcium chloride.

Preparation of Staining Solution: To investigate the changes in histology and cellular patterns of the axillary buds derived from meristems during cryopreservation, 0.02% toluidine blue stain was used (Pradhan Mitra and Loqué, 2014).

Preparation of samples for light microscopy: The histology of meristem-derived axillary buds (MDAB) was studied to analyze the effect of ultra-low temperature freezing on the cellular structure of samples stored in liquid nitrogen. The samples used for sectioning were obtained from different stages:

1. Untreated MDAB from subcultures kept as controls (without liquid nitrogen treatment).
2. Post-encapsulation dehydration of MDAB (MDAB encapsulated with sodium alginate).
3. Before pouring into recovery media, just after thawing.
4. Post-thaw culture (after thawing, the cryopreserved explants were cultured in recovery media in a petri dish, following the method of Jayabose *et al.* 2022b, and sections were taken at different time intervals: 3 days, 7 days, and 14 days).
5. Samples taken 24 hours, 3 days, 5 days, 7 days, and 14 days after treatment in liquid nitrogen and then transferred into recovery media.

Both the control and treated samples of MDAB were de-encapsulated in a laminar airflow hood to maintain aseptic conditions during the process. Details regarding the samples and treatment durations are provided in **Tables 1, 2, and 3**. The de-encapsulated MDAB were hand-sectioned using a sharp blade. Fine, thin sections were transferred onto clean slides with a dissection needle. The sections were stained evenly with toluidine blue stain using a dropper. After 1-2 minutes, any excess stain was removed by washing the sections with 70% ethanol. The adequately stained sections were then rinsed with distilled water to eliminate debris. The prepared sections were mounted on slides using glycerin and covered with a coverslip without causing damage. Finally, the mounted sections were observed under various objectives of a light microscope. This procedure was conducted for both the control and treated samples.

RESULTS AND DISCUSSION

Sugarcane has gained worldwide popularity due to its significant economic impact on sustainable energy production. Over the past 30 years, advancements in breeding and agronomic methods have led to notable increases in sugarcane yields (Cheavegatti-Gianotto *et al*., 2011). *In vitro* culture techniques have been widely utilized for thousands of plant species, including sugarcane. These methods have potential applications in germplasm multiplication, conservation, and transformation. They enable rapid plant multiplication in an aseptic environment, reduce space requirements, minimize genetic erosion, and lower labor costs (Martinez-Montero *et al*., 2012). However, cryopreservation is the only method for storing germplasm from vegetatively propagated species over extended periods. This technique requires temperature of about −196°C, typically achieved using liquid nitrogen. At this temperature, all cellular divisions, and metabolic processes cease, allowing plant material to be preserved without change. The cultures are preserved in small volumes, protected from contamination, and require minimal maintenance (Martinez-Montero *et al*., 2015).

Table 1. Observation of changes in pattern of cell structure in IND-2019-2045

Treatment	Nature of treatment of explants	Observation
Control	Without liquid nitrogen (LN)	Observed clear single layered epidermis, uninterrupted parenchymatous hypodermis and distinct vascular bundles
Treatment-1	After encapsulation dehydration of MDAB for 24 hours in Liquid Nitrogen treatment just after thawing	Epidermis is completely broken
Treatment-2	Exposure of explants in 24 hours of LN treatment- After 3 days in Culture media	Viable meristematic activity was observed
	After 7 days in Culture media	Loss of cells' meristematic activity and reveals discontinuous and disrupted cell layers.
Treatment-3	Exposure of explants in 14 days of LN treatment	the browning of cells of the outer epidermis
Treatment -4	Exposure of explants in 21 days of LN treatment	The vascular bundles are completely disorganized and collapsed

*Sample size-5 samples were taken for each treatment for histological studies

Table 2. Observation of changes in pattern of cell structure in IND-2019-2035

Treatment	Nature of treatment of explants	Observation
Control	Without liquid nitrogen (LN)	Observed clear single layered epidermis, uninterrupted parenchymatous hypodermis and distinct vascular bundles
Treatment -1	After encapsulation dehydration of MDAB for 24 hours in Liquid Nitrogen treatment just after thawing	Initiating shoot apical meristem region.
Treatment-2	Exposure of explants in 24 hours of LN treatment- After 3 days in Culture media	The vascular bundles are disorganized and collapsed
	After 7 days in Culture media	Cellular size has been reduced considerably near the hypodermis
	After 21 days in Culture media	The vascular bundles are completely disorganized and collapsed
Treatment-3	Exposure of explants in 3 days of LN treatment- After 21 days in Culture media	The regions of the epidermis and hypodermis are extremely disrupted
Treatment -4	Exposure of explants in 7 days of LN treatment- After 21 days in Culture media	The vascular bundles are completely disorganized and collapsed. The cortex also shows disorganization of cells.

*Sample size-5 samples were taken for each treatment for histological studies

Table 3. Observation of changes in pattern of cell structure in Co 11015

Treatment	Nature of treatment of explants	Observation
Control	Without liquid nitrogen (LN)	Observed clear single layered epidermis, uninterrupted parenchymatous hypodermis and distinct vascular bundles
Treatment-1	Exposure of explants in 3 days of LN treatment- After 3 days in Culture media	Epidermis, hypodermis, and Vascular bundles remain distinguished.
	Exposure of explants in 3 days of LN treatment- After 7 days in Culture media	It reveals slight tissue damages including ruptured epidermis, reduced cell size of Hypodermal cells and collapsed Vascular bundles
	Exposure of explants in 3 days of LN treatment- After 14 days in Culture media	Arrangement of cells of the epidermis and hypodermis is irregular, broken, and interrupted
Treatment- 2	Exposure of explants in 7 days of LN treatment- After 3 days in Culture media	The Vascular bundles weren't totally disorganized.
	After 7 days in Culture media	The vascular bundles are collapsed or distorted.
	After 14 days in Culture media	cellular disorganization in the cortex and towards the hypodermis the cells are nearing to necrosis
Treatment-3	Exposure of explants in 14 days of LN treatment- After 14 days in Culture media	Cortical region have reduced size and vascular bundles appear disorganized
Treatment -5	Exposure of explants in 21 days of LN treatment- After 14 days in Culture media	The vascular bundles are completely disorganized and collapsed.

*Sample size-5 samples were taken for each treatment for histological studies

Cryoprotocols are continually being refined to enhance cell viability, eliminate viral infections, and maintain genetic stability of the germplasm (Bajaj and Jian, 1995). Although cryopreservation has been effectively applied to conserve many plant species, the procedures and post-thaw culture can cause cryoinjuries to the samples. Evaluating the damage or changes in cryopreserved cells is essential for understanding how these procedures affect plant cell viability (Kushnarenko *et al*., 2010).

In the present study, cryopreserved samples of meristem-derived axillary buds began to show browning within 5-10 days after undergoing post-thaw culture on solid media for recovery. The untreated control samples demonstrated good plant regrowth, including multiple shoot formation (**Fig. 1**). The meristem-derived axillary buds (MDAB) from varieties IND 2019 2045, IND 2019 2035, and Co 11015 were sectioned using a sharp razor blade at various time intervals: 24 hours, 3 days, 7 days, 14 days, and

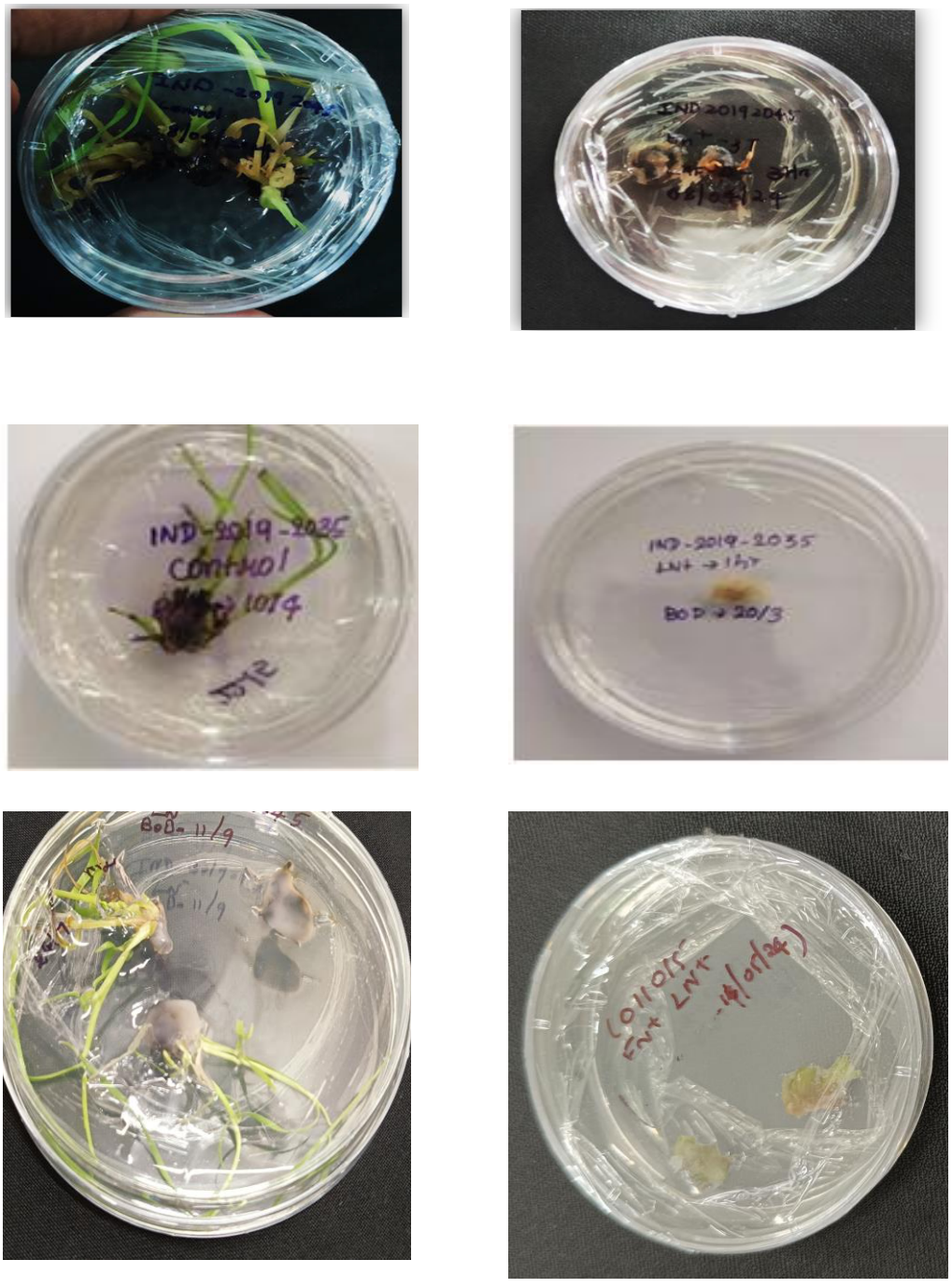


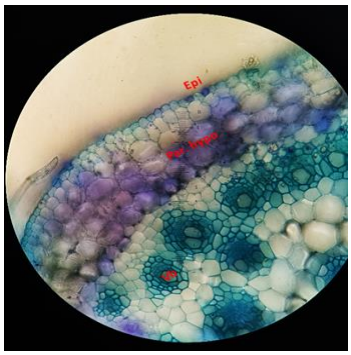
Fig. 1. MDAB of the clone IND 2019-2045, IND 2019-2035 and Co 11015 in solid recovery media (left- control, right- LN treated)

21 days after liquid nitrogen treatment and post-thaw procedures, to observe changes in the cellular structures of cryopreserved samples.

Response of IND-2019-2045 to different treatments: The cross-section of the control sample of MDAB from IND-2019-2045 remained unchanged in its cell structure (**Fig. 2a**). The cross-section showed an uninterrupted single-layered epidermis, a parenchymatous hypodermis with 2-3 layers of cells, and distinct vascular bundles containing metaxylem and protoxylem vessels surrounded by a protective sclerenchyma bundle sheath. All cryopreserved samples of IND-2019-2045 were sectioned after 24 hours of treatment in liquid nitrogen, revealing that the sample tissues had softened considerably, leading to cellular disorganization (**Fig. 2b**).

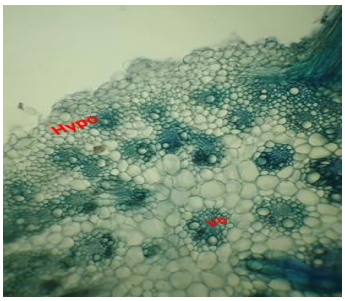
The dome of the shoot apical meristem was disturbed, and the cells in the apical dome began to brown, indicating a loss of their meristematic activity. Cells in the tunica and corpus regions exhibited abnormalities, particularly near the epidermis and leaf primordia showed signs of breakage. Cells at the base of the shoot apical meristem were less damaged. After three days of liquid nitrogen treatment, the cross-section showed that the epidermis had completely broken and the parenchyma cells of the hypodermis had shrunk. Some cells in the hypodermal and outer cortical areas were indistinct due to breakage and showing yellow-brown contents. Vascular bundles also exhibited significant size variation compared to the control and appeared collapsed (**Fig. 2c**). Following seven days of liquid nitrogen treated samples revealed that tissue damage and cellular breakdown. Most areas of the epidermis and hypodermis were ruptured, and cell separation may have contributed to impaired cell-to-cell communication. This disorganization could lead to alterations in metabolic pathways or the production of metabolites during the post-thaw culture period and its potentially contributing to the degeneration of cryopreserved MDABs (**Fig. 2d**). After 14 days of liquid nitrogen treatment, the arrangement of cells in the

2.a. Control



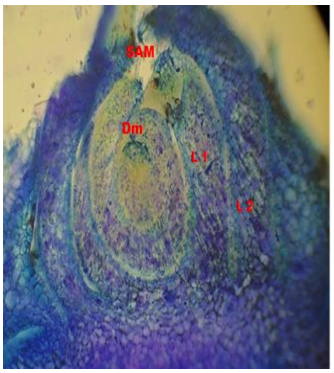
The figure shows (Epi)- epidermis which is single-layered and uninterrupted, (Par. Hypo)- Parenchymatous hypodermis with 2-3 layers of cells and distinct, (Vb)- Vascular bundles with distinct metaxylem and protoxylem vessels surrounded by protective sclerenchyma bundle sheath.

2.b. C.S. of MDAB after encapsulation dehydration and treatment in liquid nitrogen for 24 hrs and just after thawing. (Viewed under 10 X objective)



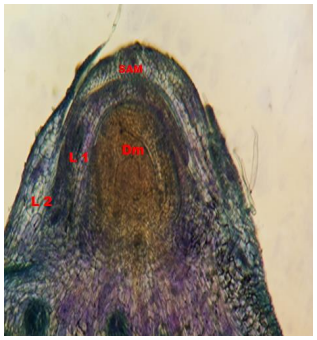
The figure shows the first type of damage of cryopreserved MDAB i.e., the epidermis is completely broken and parenchyma cells of hypodermis **(Hypo)** reveal shrinkage. vascular bundles **(Vb)** also show considerable variation in size compared to the control and appear more or less collapsed. Most of the cells are stained in blue than purple.

2.c. L.S. of MDAB showing the side-shoot apical meristem. (3 days after LN treatment)



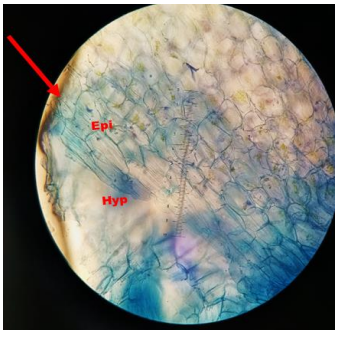
The figure shows the side-shoot apical meristem 3 days after LN treatment. (**Dm)-** the apical dome shows meristematic activity and (**L1)-** leaf primordia 1 and (**L2)-** leaf primordia 2 seem viable with meristematic activity.

2.d. L.S. of MDAB showing the side-shoot apical meristem (7 days after LN treatment)



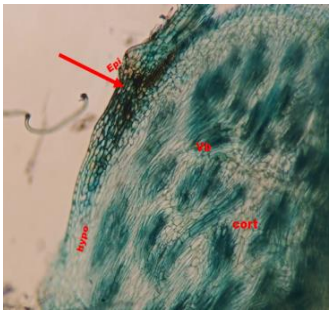
The figure shows the side-shoot apical meristem 7 days after LN treatment. (**Dm) -** the apical dome started browning, which indicates loss of cells' meristematic activity there. (**L1)-** leaf primordia 1 and (**L2)-** leaf primordia 2 reveals discontinuous and disrupted cell layers.

2.e. C.S. of MDAB after 14 days of LN treatment (viewed under 40 X objective)



The arrangement of cells of the epidermis **(Epi)** and hypodermis **(Hyp)** is irregular, broken, and interrupted. The red arrows show the browning of cells of the outer epidermis.

2.f. C.S. of MDAB after 21 days of LN treatment (viewed under 10 X objective)



The epidermis **(epi)** and hypodermis are extremely damaged and the red arrows show the browning of cells. There is a considerable reduction in cellular size near the hypodermis **(Hypo)** and cortex **(Cort).** The vascular bundles **(Vb)** are completely disorganized and collapsed.

Fig. 2. A Cross and Longitudinal section of Meristem derived axillary buds (MDAB) of Saccharum spontaneum clone, IND-2019-2045 under Light Microscope

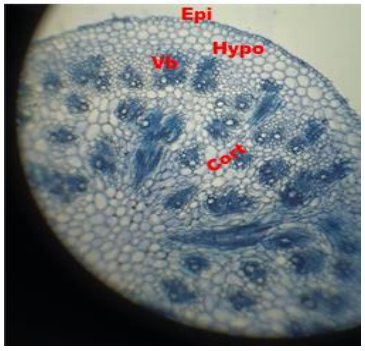
epidermis and hypodermis was irregular and disrupted. The outer epidermal cells began to turn brown (**Fig. 2e**). After 21 days of liquid nitrogen treatment and subsequent post-thaw procedures, the cross-sections revealed severe tissue damage (**Fig. 2f**). Our results suggest that the injuries observed in the recovery media are not solely due to the liquid nitrogen treatment but may also stem from the stress induced by the transfer into the recovery medium (Halmagyi *et al.,* 2017). The attempt to cryopreserve meristem-derived axillary buds has contributed to the onset of cryoinjuries in the samples after post-thaw culture.

Response of IND-2019-2035 to different treatments: The *S. spontaneum* germplasm accession IND-2019-2035 exhibited various changes in cell structure following treatment with liquid nitrogen. The cross-section of the MDAB control sample shows a distinct epidermis, a hypodermis consisting of 2-3 layers of parenchymatous cells, and well-organized vascular bundles (**Fig. 3a**). After 24 hours of LN treatment, immediately before transferring the MDAB to recovery media and following thawing, the cells retained their integrity and showed meristematic activity (**Fig. 3b**). The cellular structure damage varied after specific intervals at 3 days, 7 days, and 21 days post-thawing after the 24-hour of liquid nitrogen, as illustrated in **Fig. 3c-3e**. Samples treated with liquid nitrogen for 3 days and post-thawed after 21 days in recovery media, the cross-sectioned samples revealed that the epidermis and hypodermis regions were significantly disrupted, showing signs of browning. Notably, there was a considerable reduction in cell size, particularly in the hypodermis and cortex regions. Additionally, the vascular bundles were completely disorganized (**Fig. 3f**), while the samples that was in liquid nitrogen for 7 days and sectioned after 21 days of post-thaw culture revealed that the epidermal and hypodermal layers were disrupted. Besides, the vascular bundles were entirely disorganized and collapsed, with the cortex displaying disarray among its cells (**Fig.3g**). George *et al*. (2008) stated that the anatomy and morphology of tissue-cultured plants undergo significant changes, such as reduced diameter, limited development, and poorly developed vascular bundles, which may contribute to the disorganization of vascular bundles observed in both control and treated samples.

Response of Co 11015 to different treatments: The longitudinal section of control samples displayed active meristematic activity in the apical dome, while leaf primordia 1 and 2 appeared viable and also exhibited meristematic activity (**Fig. 4a**). A cross-section of 3 days of liquid nitrogen samples showed no significant damage during the subsequent 3 days post-thaw culture sample (**Fig. 4b**). However, 7 days post-thaw samples showed slight tissue damage, including a ruptured epidermis. The cell arrangement in the epidermis and hypodermis appeared irregular, broken, and disrupted (**Fig.4c**). Vascular bundles exhibited cellular damage at 14 days after post-thaw culture sample (**Fig. 4d**). The 7 days of liquid nitrogen-treated samples revealed that the epidermis was broken at several points, while the vascular bundles remained organized during 3 days post-thaw culture (**Fig. 4e**). Following 7-day post-thaw, culture samples showed that the cells near the hypodermal regions began to brown, and the vascular bundles were either collapsed or distorted (**Fig. 4f**). Fourteen days post-thaw culture samples revealed that significant cellular disorganization in the cortex, with hypodermal cells nearing necrosis (**Fig. 4g**). The samples obtained after 14 days of liquid nitrogen treatment indicated that the tissues were extremely soft with discontinuous epidermis and reduced cell size near the hypodermis. The vascular bundles further demonstrated disorganization. The 7 days of post-thaw culture samples revealed that both the epidermis and hypodermis continued to suffer damage, showing a significant reduction in cellular size near these regions. Vascular bundles appeared disorganized and collapsed. Cross-sections taken after 14 days in post thaw culture samples revealed considerable damage to most cellular membranes, particularly in the cortical region, where cell size had diminished and vascular bundles appeared disorganized (**Fig. 4h**). In samples obtained 21 days post LN treatment, the epidermis and hypodermis remained damaged. There was notable reduction in cellular size near the hypodermis and cortex, with vascular bundles disorganized and collapsed. Beyond 14 days, cross-sections illustrated severe damage to the epidermis and hypodermis, with cell browning observed. This was accompanied by a considerable reduction in cellular size near the hypodermis and cortex, and complete disorganization and collapse of vascular bundles (**Fig. 4i**). The findings indicate that following LN treatment and one week in recovery media, samples exhibited necrosis when observed under a light microscope. Treated samples also displayed differences in staining compared to control samples. These results align with the study on cryopreservation of apple meristems conducted by Kushnarenko *et al*. (2010). Cross-sections of meristem-derived axillary buds taken at various treatment durations and post-thaw timings showcased damage to the epidermal and hypodermal regions, with highly disorganized and collapsed vascular bundles. These findings indicate that ultra-low temperature freezing may contribute to ice nucleation within the cells, leading to the destruction of cellular integrity. Benson (2008) reported similar effects in studies on cryopreservation. Cellular heterogeneity, disruption of cellular membranes, disorganization of the apical dome, and abnormalities in leaf primordia are documented in lily shoot tips (Jung Yoon Yi *et al*., 2014), potato shoot tips (Kaczmarczyk *et al*., 2008), apple shoot tips (Kushnarenko *et al*., 2010), and chrysanthemum (Kulus *et al*., 2018).

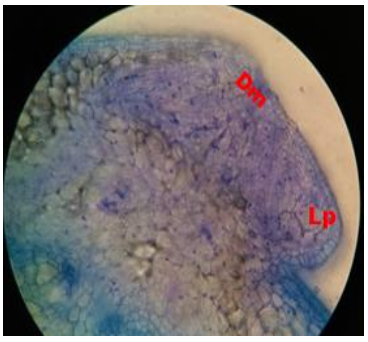
The findings in the present study indicate that the sub-apical tissues were not completely damaged. However, Kartha *et al.* (1982) reported that in *Manihot esculenta*, most of the sub-apical tissue was damaged, while the meristem and leaf primordia remained viable. As the

3.a. Control



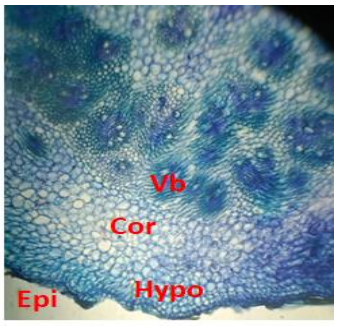
The C.S. of MDAB control exhibits a well-distinguished epidermis (Epi), 2-3 layered parenchymatous hypodermis (Hypo), and organized vascular bundles (Vb).

3.b. Cross section of MDAB- 24 hrs LN+, just after thawed



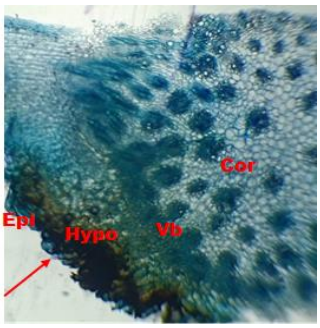
The figure shows an initiating shoot apical meristem region. Even after 24 hrs LN treatment followed by thawing, the cells are preserved and show meristematic activity.

3.c. Cross section of MDAB -24 hrs LN + and 3 days after thawed



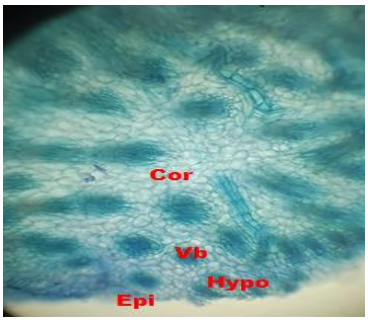
The epidermis **(epi)** and hypodermis **(Hypo)** are damaged. There is a considerable reduction in cellular size near the hypodermis **(Hypo)** and cortex **(Cort).** The vascular bundles **(Vb)** are disorganized and collapsed.

3.d. Cross section of MDAB -24 hrs LN + and 7 days after thawed



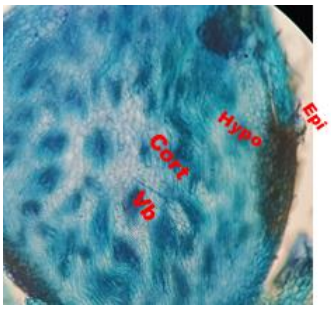
The epidermis (epi) and hypodermis are extremely disrupted and the red arrows show the browning. Cellular size has been reduced considerably near the hypodermis (Hypo) and cortex (Cort) here also. The vascular bundles (Vb) are completely disorganized.

3.e. Cross section of MDAB -24 hrs LN + and 21 days after thawed



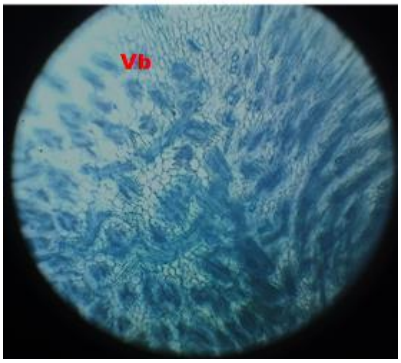
After 21 days of post-thaw culture, the MDAB's C.S. shows complete disruption of epidermal **(Epi)** and hypodermal **(Hypo)** regions. The vascular bundles **(Vb)** are completely disorganized and collapsed. The cortex also shows disorganization of cells.

3.f. C.S. of MDAB which is treated in LN for 3 days and 21 days after thawed



The regions of the epidermis **(Epi)** and hypodermis are extremely disrupted and the red arrows show the browning. Cellular size has been reduced considerably near the hypodermis **(Hypo)** and cortex **(Cort**) here also**.** The vascular bundles **(Vb)** are completely disorganized.

3.g C.S. of MDAB which is treated in LN for 7 days and 21 days after thawed



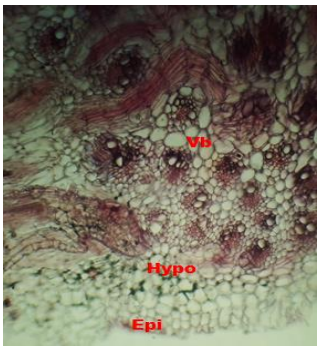
After 21 days of post-thaw culture, the MDAB's C.S. shows complete disruption of epidermal (Epi) and hypodermal (Hypo) regions. The vascular bundles (Vb) are completely disorganized and collapsed. The cortex also shows disorganization of cells.

Fig. 3. A Cross and Longitudinal section of Meristem derived axillary buds (MDAB) of Saccharum spontaneum clone, IND-2019-2035 under Light Microscope

4.a. Control

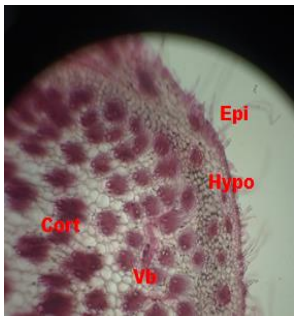
The figure on the left (L.S.) shows the side-shoot apical meristem of control. (**Dm)-** the apical dome shows meristematic activity and (**L1)-** leaf primordia 1 and (**L2)-** leaf primordia 2 seem viable with meristematic activity. The figure on the right shows (C.S.) (**Epi)-** epidermis which is single-layered and uninterrupted, (**Par. Hypo)**- Parenchymatous hypodermis with 2-3 layers of cells and distinct, (**Vb)-** Vascular bundles

4.b. C.S. of MDAB which is treated in LN for 3 days and 3 days after thawed



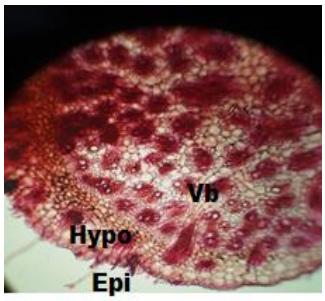
After 3 days post-thaw culture, the tissues doesn't show much differences. Epidermis**(epi),** hypodermis **(Hypo)** and Vascular bundles **(Vb)** remain distinguished.

4.c. C.S. of MDAB which is treated in LN for 3 days and 7 days after thawed



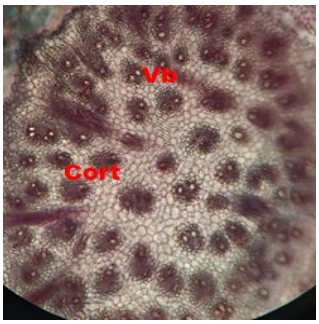
7 days after post-thaw culture, the C.S. reveals slight tissue damages including ruptured epidermis **(Epi),** reduced cell size of Hypodermal cells **(Hypo)** and collapsed Vascular bundles **(Vb).**

4.d. C.S. of MDAB which is treated in LN for 3 days and 14 days after thawed



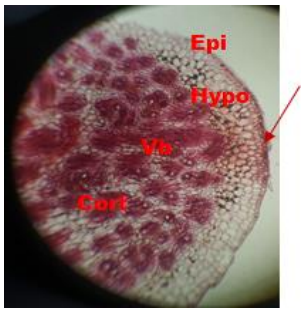
The arrangement of cells of the epidermis **(Epi)** and hypodermis **(Hyp)** is irregular, broken, and interrupted. Vascular bundles (**Vb**) also reveal cellular damage.

4.e. C.S. of MDAB which is treated in LN for 7 days and 3 days after thawed



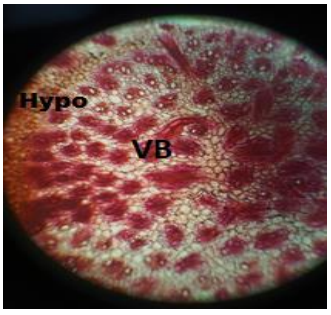
The epidermis **(epi)**was broken at some points of C.S. The Vascular bundles **(Vb)** weren't totally disorganized.

4.f. C.S. of MDAB which is treated in LN for 7 days and 7 days after thawed



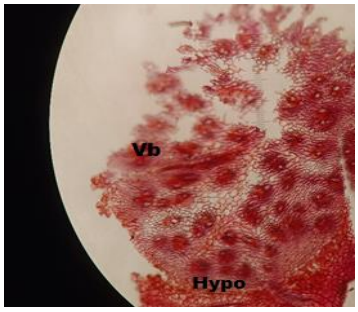
7 days past post-thaw culture the cells near the hypodermal regions **(Hypo)** are starting to brown (RED ARROW). The vascular bundles **(Vb)** are collapsed or distorted.

4.g. C.S. of MDAB which is treated in LN for 7 days and 14 days after thawed



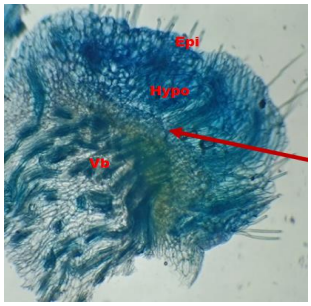
14 days after post-thaw culture, the MDAB C.S. shows cellular disorganization in the cortex and towards the hypodermis the cells are nearing to necrosis.

4.h. C.S. of MDAB which is treated in LN for 14 days and 14 days after thawed



After 14 days and beyond the C.S. reveals that most of the portion of cellular membranes are damaged. the cells in the cortical region have reduced size and vascular bundles (Vb) appear disorganized.

4.i. C.S. of MDAB which is treated in LN for 21 days and 14 days after thawed



The epidermis (epi) and hypodermis are extremely damaged and the red arrows show the browning of cells. There is a considerable reduction in cellular size near the hypodermis (Hypo) and cortex (Cort). The vascular bundles (Vb) are completely disorganized and collapsed.

Fig. 4. A Cross and Longitudinal section of Meristem derived axillary buds (MDAB) of Sugarcane variety, Co 11015

duration of post-cryopreservation culture extended, the sections displayed a bluer hue, despite toluidine blue being a double stain. This observation implied that ongoing stress from *in vitro* culture media transfer, cryopreservation techniques, and post-thaw cultivation may have induced biochemical changes within the cells. In plant specimens, cell walls with a high level of pectin typically exhibit a purple or pink stain; in contrast, those with lower pectin concentrations appear blue when processed with toluidine blue (Ribeiro and Leitão, 2020).

The primary components of the plant cell wall include pectin, hemicellulose, cellulose, and trace amounts of glycoproteins. While cellulose serves as the primary load-bearing element, various other components also influence the mechanical characteristics of the cell wall. In dicots, pectin is essential to the matrix and significantly affects both the mechanical properties and porosity of the cell wall (Cosgrove, 2022; Zhang *et al*., 2021). Pectin also plays a crucial role in cell adhesion. A comparison of control and treated MDAB samples reveals that cellular damage and membrane disruption were more pronounced in the treated samples. This may be due to a reduced concentration of pectin in the cell walls as a result of stress from post-cryopreservation procedures, which encourages cellular separation. Freezing injuries lead to the generation of free radicals, particularly reactive oxygen species (ROS). These free radicals attack the lipid components of cell membranes, leading to the formation of unstable lipid peroxides. Such lipid peroxides compromise the structural integrity of cellular membranes, affecting their fluidity and permeability (Martinez-Montero *et al.,* 2012). Martinez-Montero *et al.* (2012) indicating that damage to sugarcane callus cell membranes and the efflux of electrolytes may be attributed to reactive oxygen species. Damage caused by ROS weakens the lipid bilayer of cellular membranes, increasing their susceptibility to rupture, which jeopardizes cell viability and functionality. The disruption of membrane integrity has significant implications for transport processes, signaling, and overall cellular homeostasis (Martinez-Montero *et al*., 2012). Cells are injured by numerous and diverse causes from intrinsic and extrinsic sources; however, which activate one or more of four final common biochemical mechanisms leading to cell injury. Further in-depth studies in various biochemical and molecular mechanism could reveal the exact levels of cryo-protection measures that need to be taken to avoid such complication for long term conservation of sugarcane genetic resources.

Thus in the study, cryopreservation through encapsulation dehydration has been refined for axillary buds derived from the meristem of sugarcane. However, cryopreservation methods involve several complex steps, including the application of cryoprotectants, freezing at ultra-low temperatures, rapid thawing or rewarming, and subsequent transfer to recovery media. These processes can lead to cryoinjury in the treated samples, which may hinder or delay their regeneration. It is also clear that during the post-thaw culture phase, less cellular and membrane damage was observed in sugarcane cultivar Co 11015 compared to clones of *S. spontaneum*. Employing further analytical tools will enhance our understanding of the damage caused to biological tissues by cryopreservation. This knowledge could be instrumental in refining protocols for the successful regeneration of plantlets and ensuring the conservation of germplasm.

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