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# Alteration in Cell Ultrastructures During Cryopreservation: Transmission Electron Microscope Observation on Chinese Yam (*Dioscorea Polystachya* Turcz.)

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**ABSTRACT**

Changes in cell ultrastructures at different steps of cryopreservation protocol application under light microscope and transmission electron microscope (TEM) on Chinese yam (*Dioscorea polystachya* Turcz.) were studied. The specimen preparation for the examination was accomplished through steps of liquid fixation, dehydration, embedding and sectioning on an ultramicrotome followed by staining before observation. Following preculture treatment, an initial stage of vacuoles fragmentation, increasing numbers of amyloplasts containing starch granule and undulated nuclear envelopes were observed. Cells with small vacuoles containing high amount of dark-staining structures were observed after the dehydration treatment. Rapid rewarming of the explants was followed by some living cells with spherical shapes of nuclei and nucleoli, fragmented vacuoles containing electron dense globular and multi-membranous structures, osmiophilic bodies which were encountered along the plasmalemma, as well as some invaginations of the plasmalemma. Unloading treatment following rapid rewarming caused disappearance or inconspicuous appearances of cellular organelles in many cells. Several surviving cells showing disrupted nuclei; others have expanded nuclear envelopes at different sites. The protocol developed for cryopreservation should be simple and include only necessary steps that enable an optimum recovery. Addition of unnecessary steps can impose additional cellular injuries and reduce the quantity and quality (shoot or callus) of survival.

**Key words:** Cryopreservation, Cell ultrastructure, Chinese yam, TEM, Vacuole fragmentation, Osmiophilic bodies

## INTRODUCTION

Cell damages during cryopreservation of biological materials can be of physical and/or biochemical sources [1]. Physical damages, however, has been the attention of various studies. Intracellular ice causes physical damages to intracellular structures primarily through mechanical rapture of the membranes or by piercing the membranes and destroying their permeability [2-4]. Observations under transmission electron microscopy (TEM) showed that the lethal effect was caused by the large size rather the small size of ice crystals [5,4]. A few studies have been focusing on the plasma membrane as a place where the injuries take place during the cooling-rewarming cycle. Wiest and Steponkus [6] supported the theory of protoplast lyses caused by contraction and expansion of plasma membrane during cooling (dehydration) and rewarming. Stout *et al*. [2] indicated that membrane injury only takes place during cell contraction and does not depend on the membrane area expansion in the winter hardy Saskatoon serviceberry (*Amelachier alnifolia* Nutt.). Observation on the protoplast of *Secale cereale* L. under the TEM using freeze fracture technique indicated that when explants were cooled to the temperature for 50% survival, the injury in non-acclimated leaves was associated with several changes such as lateral phase separation in

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the plasma membrane, lamellae without particles lying next to the plasma membrane and regions of the plasma membrane and associated lamellae in the various stages of lamellar-hexagonal $_{II}$ transition. For acclimated leaves, the injury was associated with the occurrence of localized deviations in the fracture plane referred to as 'fracture-jump lesion' [7-9]. Observing the frozen-fixed rye cells under the electron microscope [10], described the mechanism involved in the cell ultra-structural damage as follows: at earliest state, there would be a role up and fusion of cellular membranes which is caused by dehydration and/or mechanical collapse of the cell in the frozen state. This fusion will subsequently form densely packed multibilayered vesicles (osmiophilic bodies). The membrane bilayers, eventually may lose their phospholipid lamellar lattice leading to cell damage (formation of amorphous lipids). Johnson-Flanangan and Singh [11] also observed irreversible formation of membrane multibilayer vesicles as well as endocytotic and exocytotic vesicle deletions on non-hardened cells of *Brassica napus* L., alfalfa and rye cells using conventional fixation technique. Furthermore, they indicated that for hardened cells, reversible exocytotic plasmalemma strand formation was observed. Grout and Henshaw [12] observed the shoot tips structure of potato after cryopreservation using conventional fixation method and showed that in cases of extreme damage, the structural organisation of the protoplast is totally destroyed, often accompanying cell wall rapture. In the severely damaged but unraptured cells, large numbers of small electron- transparent spaces appear in the cytoplasm, which might be due to large ice crystal formation. Surviving explants showed cells that have markedly electron-dense cytoplasm. In some areas, dark-stained cells were observed, which typically contained small vesicles scattered throughout the cytoplasm. Plasmalemma withdrawal from the cell wall and the outer nuclear membrane withdrawal from the surrounding cytoplasm also appeared. Such cells were never observed in the non-frozen control materials.

Studies comparing fixation techniques for the cells ultrastructural observation revealed that aqueous fixation caused some artefacts that could influence the interpretation of the results in comparison to freeze-substitution and high pressure freezing techniques [13,14]. The use of aqueous (chemical) fixation, however, has been continued and contributed to understanding of cellular and subcellular changes occurring during the cryopreservation processes [15,16,18]. This Microscopical examination on *Dioscorea* spp. after cryopreservation using the encapsulation/ dehydration method [19] indicated considerable numbers of damaged cells in the surviving apices after thawing. Electron-microscopical studies have also been done using this method [19]. Detail information, however, has not been reported. Understanding cell ultrastructural alteration (injuries) during cryopreservation could assist further improvement of a developed protocol. In the previous study [20] we developed a protocol combining vitrification and droplet methods, and obtained 30 to 50% shoot recovery for three different species of yams (*Dioscorea* spp.). This report examines the cell ultrastructural changes (injuries) at different steps of the developed protocol under TEM and discuss a possible improvement of the protocol based on the observation.

## MATERIALS AND METHODS

### Materials

The plant material used in this study was Chinese yam introduced from the Botanic Garden of University of Padua, Italy. The genotype (Yam 21) was previously identified as *D. oppositifolia* L. [21,22]. However, based on the recently published literatures, the name was changed into *Dioscorea polystahya* Turcz. [23,24]. The species (genotype) multiplied *in vitro* with a relatively constant rate (low standard error – [21] and therefore, the number of explants obtained for the experiments can be reasonably estimated. The explants produced from this genotype is also relatively simple to be dissected and prepared for microscopic observation. It is, therefore, practical to be used as experimental material in comparison to the other species used in cryopreservation experiments [20].

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**Experimental approach**

Samples for TEM were obtained from three experiments conducted in different time of the year, preparing three samples for each step of the protocol. Explants preparation was arranged so that all steps to be observed were fixed at the same date. Prior to observation under TEM the general performance of the cells in each sample was exposed under light microscope. Samples for the cellular observation under TEM were obtained from various steps of cryopreservation protocol as indicated in Table 1 (detailed description of the protocol is presented in [20]).

**Sample preparation for TEM**

Fixation, substitution, embedding, light and electron microscopic examination of the specimens were accomplished through the following procedure: for the primary fixation, pieces of specimen about two $mm^2$ in size were kept four hours at room temperature and 16 h at 4 °C in 50 mM cacodylate buffer (pH 7.2), containing 2.5% (v/v) glutaraldehyde and 2.0% (v/v) formaldehyde, after a vacuum-infiltration for a few seconds in the same medium. For the secondary fixation, specimens were transferred after two washes for 15 min with the used buffer to a solution of 1% (w/v) $OsO_4$ in 50 mM cacodylate buffer. The fixation of two hours is followed by two washes with buffer and two washes with distilled water. Dehydration of samples was done stepwise by exposing the explants to increasing concentration of ethanol. The steps were performed as follows: 30% (v/v) for three hours, 40% (v/v), 50% (v/v) and 60% (v/v) for 0.5 h each, 75% (v/v) for 15 h, 90% (v/v) and two times 100% (v/v) ethanol for one hour each. After two times dehydration with propylene oxide for one hour, the samples were infiltrated subsequently with Spurr (Plano GmbH, Marburg, Germany) as follows: 33% (v/v) Spurr resin in propylene oxide for four hours, 50% (v/v) for 15 h and 66% (v/v) for eight hours, followed by 100% (v/v) Spurr overnight. The samples were then transferred into BEEM capsules, kept there for six hours in fresh resin and polymerized at 70°C for 24 h.

Thin sections with a thickness of approximately 70 nm were cut with a diamond knife using a Leica UCT ultra microtome. Prior to examination in a Zeiss CEM 920A transmission electron microscope at 80 kV, the sections were contrasted with a saturated methanolic solution of uranyl acetate and lead citrate. For light microscopical examinations in a Zeiss Axiovert 135, semi-thin section with a thickness of 3 µm were cut and stained with a solution containing 1% methyleneblue and 1% borax.

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**Table 1: Steps of the cryopreservation protocol where the explants were obtained for TEM observation**

| Precondition ► | | Preculture ► | Cryoprotection ► | |
|---|---|---|---|---|
| Pregrowth | Cold acclimation | | Loading | Dehydration |
| 1-2 Months in M2 medium in 25°C room | 3 weeks in alternating 28°C day/5°C night | 3 days in M2 medium, 15% sucrose | 20 min. in loading solution | 20 min in PVS2 |
| **S1** | **S2** | **S3** | S4 | **S5** |
| **S12** | **S13** | | | |
| fresh cut explants cooled, rewarmed | fresh cut explants after acclimation, cooled, rewarmed | | | |

| Cooling ► | Rewarming ► | | Unloading ► | Survival ► | Regrowth |
|---|---|---|---|---|---|
| | Slow | Rapid | | | |
| in droplets of PVS2 on Al, into LN > 1h | 30 min in –90°C followed by 6 h in 2°C | 3 min in MS 3% sucrose solution, room temp. | 5 min in M2 40% sucrose solution | 1 week in M2 medium in the dark, 25°C | 1 month in MS medium in the light, 25°C |
| S6 | **S7** | **S8** | **S9** | **S10** | S11 |

**Notes**: Code numbers (steps/treatments) written in **bold**(graycolor) are those obtained for the TEM observation. Loading and PVS2 solutions [25]. MS [26]; M2, MS+0.1 mg/l naphtaleneaceticacid+ 2 mg/l bezylamynopurine [27] + 3% (w/v) sucrose + 0.2% (w/v) activated charcoal + 0.1% (w/v) agar; Al, aluminium foil; LN, liquid nitrogen.

## RESULTS

### General cellular performances on apical buds

Prior to observations under transmission electron microscope, semi-thin sections were made to observe the general condition of the meristemamtic tissues under the light microscope. This observation indicated that samples of cold acclimation treatment contained cells with larger size of vacuoles in comparison to that of control samples (Figure 1: S2 vs. S1). Number of cells with relatively larger vacuoles as well as the size of the vacuoles within the cells, however, seemed to decrease throughout the treatment from cold acclimation up to dehydration (Figure 1: S2, S3, S5). As well, number of cells with larger vacuoles increased from the apical dome to the lower part of the shoot (Figure 1: S1 –S13).

The ultrastructure of the control explants was represented by prominent nucleoli with spherical shapes situated in nuclei with slightly undulated envelopes. Various organelles such as proplastids, amyloplasts containing starch granule, mitochondria, and endoplasmic reticula were observed within the cytoplasm. Relatively small to moderately-sized vacuoles were observed in different cells (Figure 2a, b). Cold-acclimated explants showed cells with huge vacuoles covering the major part of the cytoplasm. Few cells with small vacuoles, however, were also observed (Figure 2c). The appearance of nucleoli, nuclei and other cellular organelles was somewhat similar to those of control cells except that cold acclimated cells exhibited fewer numbers of amyloplasts (Figure 2c, d) with less prominent appearance (Figure 2d vs. b).

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Following preculture treatment, an initial stage of vacuoles fragmentation, increasing numbers of amyloplasts containing starch granule and undulated nuclear envelope were observed (Fig. 3a). Mitochondria and endoplasmic reticula with slightly prominent appearances were also encountered (Fig. 3b). Cells with small vacuoles containing a high amount of dark-staining structures were observed after the dehydration treatment. Undulations of the nuclear envelopes were more pronounced than those exhibited after preculture treatment. Endoplasmic reticula were prominently appeared and amyloplasts were present in lower numbers as those shown after preculture treatment (Figure 3c, d). Slowly rewarmed explants contained cells with broken or swollen nuclei. No cell organelles were visible except starch granule, and in some cases with amyloplast. At some parts of the cellular membrane, empty spaces between cell wall and cytoplasm, and broken plasmalemma were observed, which can be interpreted as plasmolytic events (Fig. 4a, b). Rapid rewarming of the explants was followed by some living cells with spherical shapes of nuclei and nucleoli, fragmented vacuoles containing electron dense globular and multi-membranous structures, osmiophilic bodies which were encountered along the plasmalemma, as well as some invaginations of the plasmalemma. Other cell organelles such as amyloplasts containing starch granule and mitochondria were also encountered (Figure 4c, d, e).

Unloading treatment following rapid rewarming caused disappearance or inconspicuous appearances of cellular organelles in many cells. Several surviving cells showing disrupted nuclei; others have expanded nuclear envelopes at different sites (Figure 5a, b). After one week cultivation in the dark following rewarming and unloading, however, cells returned to their normal appearances similar to that of the control, with numerous cellular organelles including amyloplasts containing starch granule, mitochondria, endoplasmic retula and plasmodesmata (Fig. 5c, d). When cooled directly followed by rewarming, cells control and cold acclimation treatments showed severely disrupted cellular organelles. Although, the cell walls were still present separating one cell from the other, a closer look indicated no clear separation between cell wall and cytoplasm implicating a disruption of the plasmalemma (Fig. 6a - d).

**DISCUSSION**

Response of plant cells to cold acclimation has been indicated by various ultrastructural conversions including elimination and replacement of the large vacuoles by small protein storing vacuoles (protein bodies), which later decomposed and continuously diminishing number of amyloplasts and the amount of starch within them shown by the presence of translucent halo around the starch granule in poplar tree (*Populus* - [28] micro-vacuolation and increase in volume of cytoplasm accompanied by accumulation of cellular materials such as mitochondria and endoplasmic reticulum (ER), denser protoplasm and disappearance of starch in peach (*Prunus persica* L. - [29] irregular forms of the vacuoles reflecting a greater tonoplast surface in comparison to the spherical form of the control cells in alfalfa [30] as well as disappearance of starch granule in the meristematic dome and accumulation of sugar in sugar beet (*Beta vulgaris* L.) [31]. Three weeks cold acclimation treatment on *D. polystachya* in an alternating 5/28 °C temperature regime, implemented in the protocol, exhibited similar ultrastructural evidences as previously reported with respect to the presence of translucent halo around the starch grain and less prominent appearances of amyloplasts accompanied by increasing cellular solutes (protoplast possessing higher electron density) and the presence of numerous mitochondria. Opposite to the previous reports, however, larger vacuoles as compared to those found in control cells are mainly encountered.

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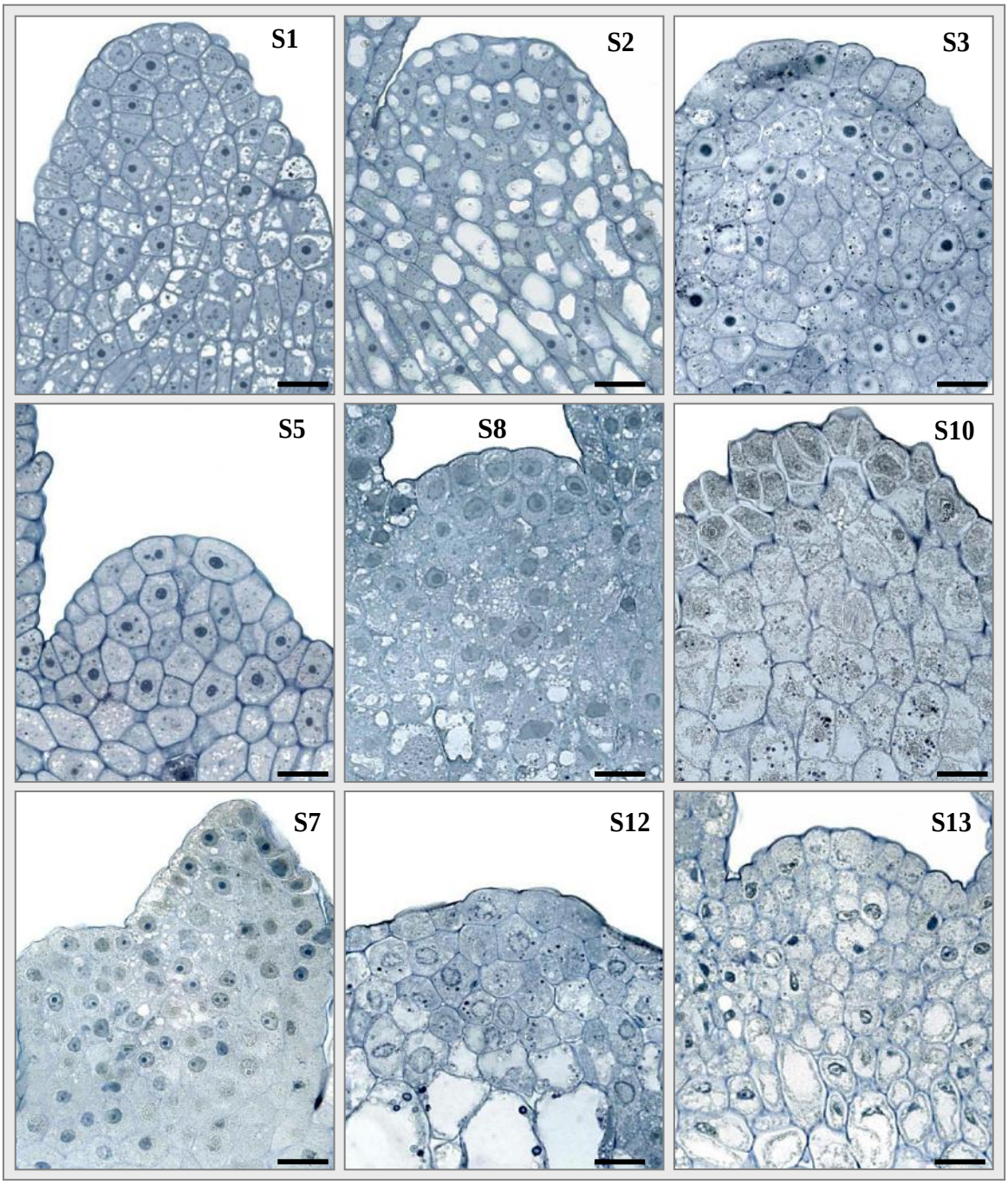


**Figure 1: Semi-thin sections of meristematic tissues after different steps of the cryopreservation protocol observed under the light microscope. Control (S1), cold acclimation (S2), preculture (S3), dehydration (S5), slow-rewarming (S7), rapid- rewarming (S8), one week after rewarming (S10), direct cooling from control (S12) and cooling after cold acclimation (S13). S2 shows highly vacuolated cells, which decrease in S3 and S5, moderate size of vacuole is indicated by S8, some cell divisions can be seen at S10 indicating an actively growing meristematic tissue. Bars indicate 20 µm.**

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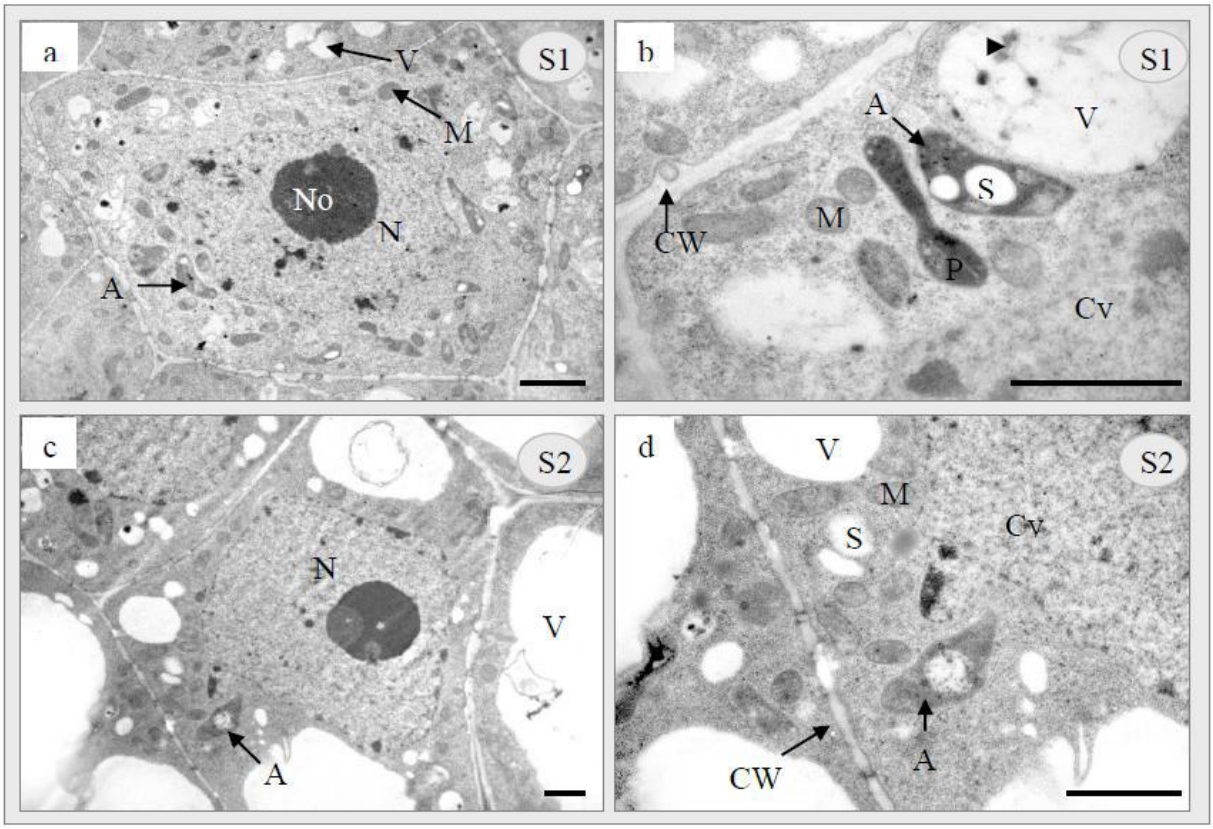


**Figure 2: Cells ultrastructural appearances in the control (S1) and after cold acclimation treatments (S2). (a) control cells showing a prominent nucleolus, nucleus and a number of organelles distributed within the cytoplasm. (b) closer look of several organelles i.e. amyloplast containing prominent starch granule. (c) cold acclimated cells with slightly more electron dense protoplasm, and moderate and small vacuoles surrounded by cells with large vacuoles. (d) numerous cellular organelles, i.e. starch granule with less prominent appearances. A amyloplast, Cw cell wall, ER endoplasmic reticulum, M mitochondrion, N nucleus, No nucleolus, P proplastid, S starch grain, V vacuole. Bars indicate 2 µm.**

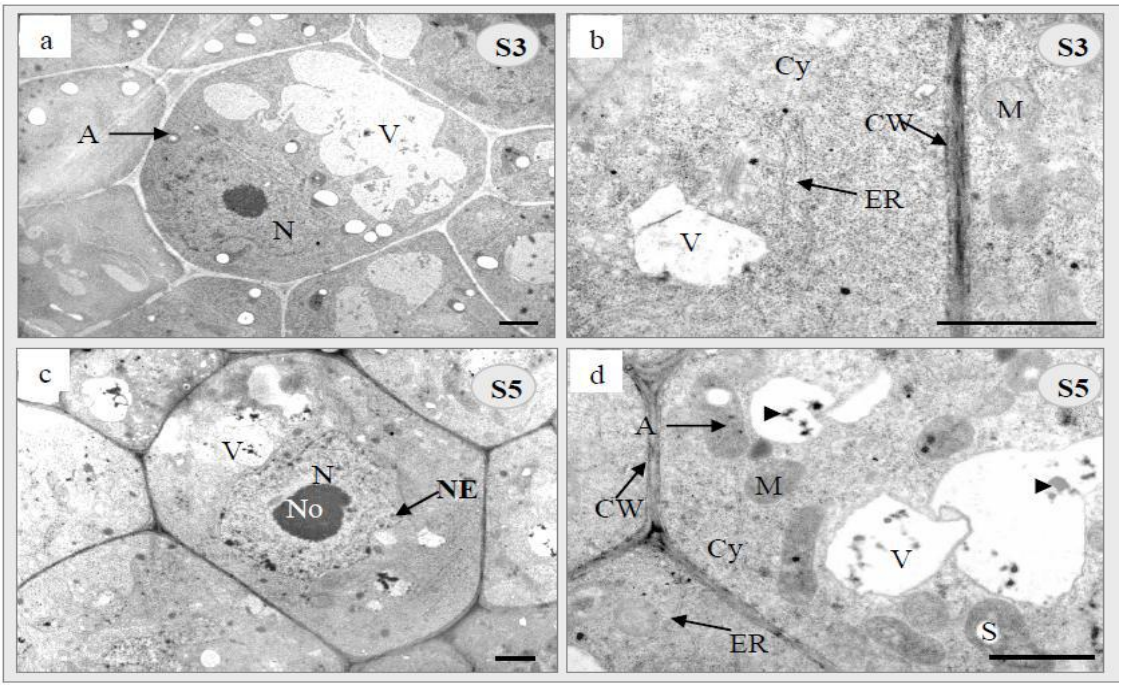


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**Figure 3: Cells ultrastructural appearances after preculture (S3) and dehydration (S5). (a) early stage of vacuole fragmentations, numerous starch granule with prominent appearances, slightly undulated nuclear envelope. (b) closer look of mitochondria and prominent ER. (c) fragmented vacuoles and undulated nuclear envelope. (d) closer look of vacuoles containing dark staining structures (arrow heads) and numerous cellular organelles. A amyloplast, CW cell wall, Cy cytoplasm, ER endoplasmic reticulum, M mitochondrion, N nucleus, NE nuclear envelope, No nucleolus, S starch grain, V vacuole. Bars indicate 2 µm.**

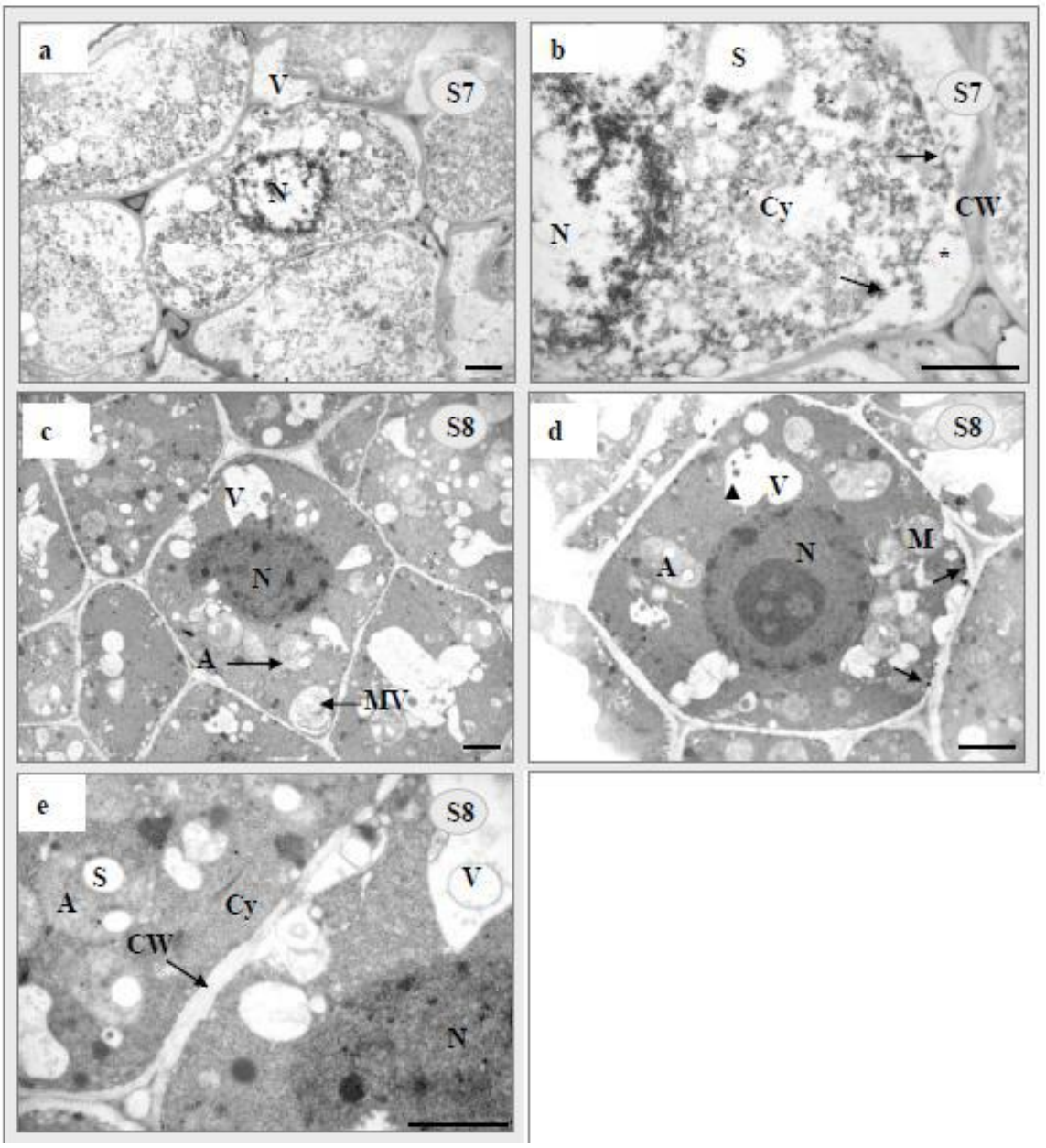


**Figure 4: Cells ultrastructural appearances after slow (S7) and rapid (S8) rewarming. (a) disruption of the nuclear envelope and disappearance of cellular organelles except inconspicuous amyloplasts containing starch granule, neighbouring cells indicating total disintegration of the cellular contents. (b) swollen nucleus, detachment of the cytoplasm from the cell wall (star), broken plasmalemma (arrow) at different sites. (c, d) living cells indicating intact cellular organelles including amyloplasts with starch granule, mitochondria, vacuoles containing electron dense globule (arrow head), nucleolus and nucleus with normal spherical shapes. As well, multi-membranous vesicle structures and osmiophilic bodies (arrows) were seen along the plasmalemma, which shows partially invaginations (star). (e)**

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**closer look of the cell wall and several cellular organelles. A amyloplast, CW cell wall, Cy cytoplasm, M mitochondrion, MV multi-membranous vesicle structure, S starch grain, V vacuole. Bars indicate 2 µm.**

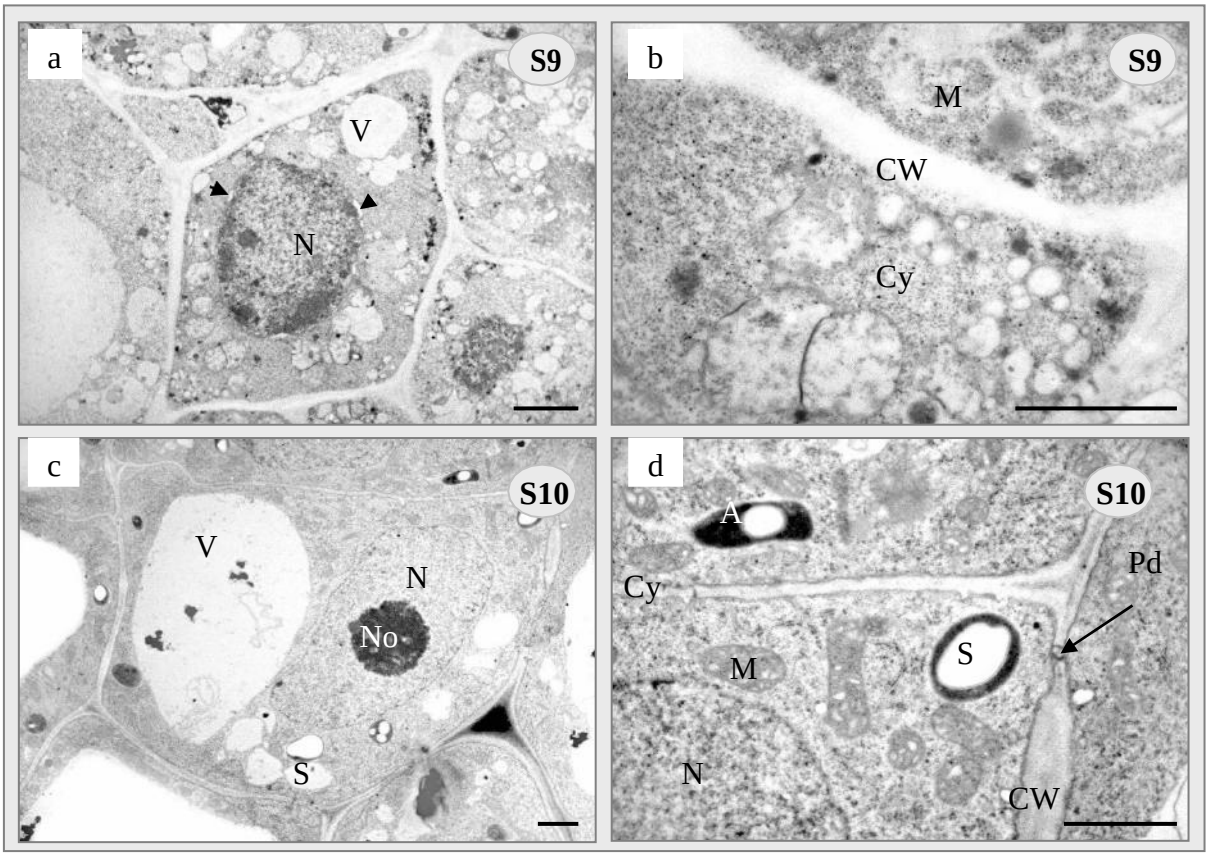


**Figure 5: Cell ultrastructural appearances after unloading (S9) and one week of culture in the dark (S10). (a, b) a nucleus with extended envelope at several sites (arrow heads), neighbouring cells with disrupted nuclei, cell organelles with inconspicuous appearance. (c, d) cells regain their normal condition with numerous cellular organelles indicating active metabolism processes. A amyloplast, CW cell wall, Cy cytoplasm, ER endoplasmic reticulum, M mitochondrion, N nucleus, No nucleolus, Pd plasmodesmata, V vacuole. Bars indicate 2 µm.**

Three days preculture in 15% sucrose, following the cold acclimation period, has initiated the fragmentation of the larger vacuoles and leave a very modest sign of plasmolysis, i.e. a slight undulation of the nucleus. In addition to this mild dehydration effect, such a long period of preculture has also increased starch accumulation within the cells as shown by the existence of numerous starch granule. These changes, together with the slightly more prominent appearance of ER, to some extent, confirmed the results of the previous studies on *Panicum maximum* Jacq. After three days preculture in 6% mannitol [32] on *Arabidopsis thaliana* (L.) Heynh. after one week preculture in medium with 1 M sucrose [33] and on banana (*Musa* spp.) after two weeks preculture in 0.4 M sucrose [18].

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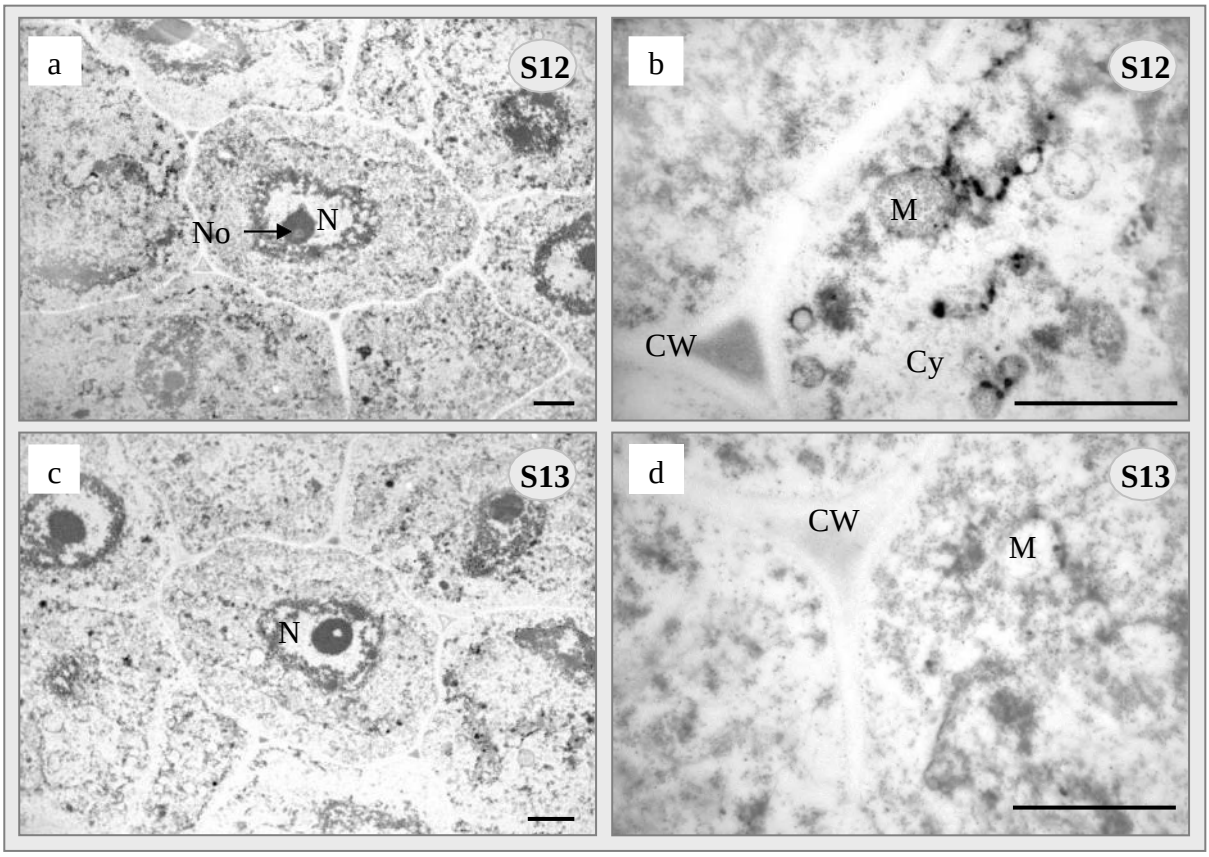


**Figure 6: Cell ultrastructural appearances after direct cooling of the control explants (S12) and direct cooling after cold acclimation (S13). (a) cells with intact cell wall, prominent nucleolus, nucleus with undulated envelope, no organelles appear within the cytoplasm, other cells show complete disintegration of cellular contents. (b) closer look of the cell wall and some inconspicuous cell organelles. (c, d) nucleolus and nucleus as well as plasmalemma with the similar appearances to those of a and b. CW cell wall, Cy cytoplasm, N nucleus. Bars indicate 2 µm.**

The purpose of dehydration treatment is to remove all freezable water from the cells in order to prevent or reduce intracellular ice crystal formation and to increase the cytosolic concentration. This cellular condition will enhance the formation of meta-stable glass upon immersion into liquid nitrogen. To avoid an osmotic shock after direct exposure to the highly concentrated dehydration solution, the explants are first treated with a mild concentration of a dehydration solution (loading). Ultrastructural evidence in this study indicated that at the end of the dehydration period, fragmentation of vacuoles has been completed leaving small vacuoles with irregular forms and causing a mild plasmolysis to the cells marked by undulation of the nuclear envelopes. The prominent appearance of ER accompanied by numerous mitochondria and fewer numbers of amyloplasts containing starch granule in comparison to the condition after preculture were also observed. Severely plasmolyzed cells or further signs of cellular injuries such as that reported previously [32,33,18] were not encountered at this stage of the protocol. The vitrification-based methods have been designed using ultra-rapid cooling and rewarming procedures. Such a cooling/rewarming rate will allow cells to rapidly pass the critical temperature in which ice crystals are formed and, therefore, prevent the cellular damages. Rapid cooling and rewarming, following the previous treatments in the present protocol seemed to have fulfilled its function. Many cells were still alive after rapid rewarming with the presence of amyloplasts and mitochondria in addition to nuclei and nucleoli in spherical forms. Several further ultrastructural changes, however, also took place at this stage. The presence of multivesicular structures as well as

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osmiophilic bodies observed along the plasmalemma next to its invagination represents the ultracellular events leading to disruptions of the tonoplast, the plasmalemma or other membranes (conversion to an amorphous stage - [10]. The electron-dense granules present in the vacuole in banana cells [18] after cryopreservation have been interpreted as an accumulation of toxic polyphenolic compounds which oxidise rapidly when the cells are exposed to stress conditions. The same feature was also encountered in the control cells and dehydrated cells in this study. Similar to banana, *Dioscorea* is also known to produce polyphenolic compounds. Opposite to rapid rewarming, slow or stepwise rewarming implemented in this study caused a total disruption of the cellular membranes. The main cause of this damage is the recrystallizations of vitrified solution (devitrification) resulting in mechanical rapture of the membranes or piercing the membranes and, thus, destroying their permeability [2,3,18].

Unloading in 40% sucrose after rewarming in 3% sucrose seemed to have imposed further damages into the cells as shown by the expansion of the nucleus cisternal spaces at different sites, which could lead to the rapture of nuclear envelope [18] Our preliminary test on *D. bulbifera* apical buds comparing the present protocol (5 min unloading in 40% sucrose after rewarming), direct rewarming in 40% sucrose (unloading solution) for 10 min [34]and rewarming in 3% sucrose for 3 min without unloading, found similar survival rates for the three different treatments. However, more shoots were developed on the treatment of rewarming with 3% sucrose without unloading than that with the two other treatment variants. An additional unloading step in this protocol is, therefore, not necessary and even detrimental since it imposes an additional osmotic excursion to the cells [35]. Despite further stress in the unloading step, a number of cells recovered and returned to the normal cellular metabolic activities. This phenomenon was indicated by the presence of prominent appearences of and numerous cellular organelles in addition to the normal forms of nuclei, nucleoli and plasmodesmata connecting one cell to the other, after one week cultured in semisolid medium. Previous studies have shown that cells resumed growth after a lag period of two days and grew at a rate comparable to the control [32]). Observation on banana, one-week culture after rewarming have also shown cryopreserved cells with similar appearances to that of control [18].

When the explants of control and after cold acclimation were cooled directly, none of the cells is alive. This fact was reflected in severe cellular damages indicated by disruption of most of the cellular membranes including plasmalemma, and disintegration of cellular contents, even though nuclei were still retained in some cells. It is demonstrated, therefore, that although cold acclimation might have improved cellular conditions causing hardiness to the cells, further steps in the protocol such as preculture, loading and dehydration combined with rapid cooling and rewarming are necessary for the survival and recovery of the explants after cryopreservation.

## CONCLUSIONS

Observation under light and transmission electron microscopes indicated that each step in the cryopreservation protocol caused certain ultrastructural alterations. These facts, indeed, should be taken into considerations in course of preparing the explants conditions before storage and in attempts to enhance cells recoveries after retrieval from liquid nitrogen. The protocol developed for cryopreservation, therefore, should be simple and include only necessary steps that enable an optimum recovery. Addition of unnecessary steps can impose additional cellular injuries and reduce the quantity and quality (shoot or callus) of survival. Omission of the unloading step in the protocol developed in this study might contribute to the improvement.

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