Effect of Encapsulation-Dehydration Cryopreservation on Histological Analysis of *Oncidium* Golden Anniversary orchid PLBs

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Effect of Encapsulation-Dehydration Cryopreservation on Histological Analysis of *Oncidium* Golden Anniversary orchid PLBs

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Abstract. *Oncidium* Golden Anniversary is a hybrid orchid that has been grown as cut flowers, potted plant and is valued for its attractive flower shapes and colours. New hybrid orchids are difficult to maintain and cultivate easily; therefore, cryopreservation technology has been applied to conserve the orchid plant genetic resources. This study aims to evaluate the impact of encapsulation-dehydration as one of the cryopreservation techniques on histological analysis of *Oncidium* Golden Anniversary orchid PLBs using stock, cryopreserved and non-cryopreserved PLBs. Results from the histological analysis indicated that the degree of plasmolysis due to dehydration and ruptured cells after freezing were the main factors affecting the viability of PLBs.

1. Introduction

One of the largest families in the flowering plants is Orchidaceae that have more than 800 genera and 25,000 to 35,000 species [1]. One of the species is *Oncidium* Golden Anniversary orchid which consists of hundred yellow flowers that have red-brown spots on the petals at each spray. The flowers are lesser in quantity but slightly bigger compared to common *Oncidium* flower. This hybrid flower of *Oncidium sarcatum* and *Oncidium sphacelatum* has been known to be grown as a cut flower, potted plant and is valued for its attractive features [1, 2]. The importance of orchid hybrids is due to its varieties in shapes and colours, a better quality of the plant, free-blooming, and longer shelf-life. Therefore, the development of the novel and hybrid orchid varieties has been rapidly growing. Generally, new orchid hybrids are difficult to maintain and cultivated conventionally [3]. This situation has raised the importance of conserving the hybrids that can preserve and safeguard the plants' genetic resources, especially for orchid hybrids that have the risk of a permanent lost [4,5]. Thus, cryopreservation

technology has been developed for long long-term conservation of orchid plants that are endangered and approaching extinction [4].

Cryopreservation refers to the storage of biological tissues in liquid nitrogen (-196°C) in which all the activities including biochemical, metabolic and cell division are terminated. This technique is suitable for a wide range of plant species because it uses limited space and maintenance and is also cost-effective for long-term conservation. At the same time, it causes no changes in genetic makeup, viability and vigour of the conserved materials [6, 7]. Encapsulation-dehydration is one of the methods that have been applied to orchids [8]. The key to successful cryopreservation is to control the level of injury in the cell structure [9, 10]. Membrane rupture caused by ice crystallisation, loss of enzyme activities, membrane denaturation and cell wall degradation will cause severe structural damage to the cells [11, 12]. Thus, it is important to identify the cell structure and control intracellular ice nucleation in cryopreservation protocol.

The main objective of this study is to investigate the impact of cryopreservation stages towards cell morphology of PLBs via histological analysis.

2. Material and methods

2.1. Plant Materials

Plant materials used for microscopic analyses were protocorm-like bodies (PLBs) of *Oncidium* Golden Anniversary orchid that is cultured in Plant Biotechnology Laboratory 310, School of Biological Sciences, USM.

2.2. Encapsulation-dehydration

All encapsulation dehydration treatments conducted were adapted from Engelmann et al. [13] with some modifications. PLBs were precultured on half-strength MS semi-solid media supplemented with 0.1 M sucrose prior to encapsulation with 3% (w/v) sodium alginate solution and polymerised in 0.1M calcium chloride solution. The beads were gradually transferred to osmoprotection media (0.15M and 0.25M) and dehydrated using silica gel for 4 hours. For cryopreserved PLBs, beads were exposed into liquid nitrogen for 1 hour and thawed rapidly at 40±2 ºC for 90 seconds before cultured on recovery media. Non-cryopreserved PLBs were directly cultured on recovery media which contained 20g/L sucrose.

2.3. Histological Analysis of PLBs Sample

PLBs were tested from different stages of cryopreservation such as stock, preculture, osmoprotection, dehydration, thawing, noncryopreserved (-LN) and cryopreserved (+LN) stages. Histological studies were adopted from Vyas et al. [14] method with some modification. Firstly, the PLBs were fixed with formaldehyde (FAA) consisting of 95% ethyl alcohol, glacial acetic acid, formaldehyde and water at ratio 10:1:2:7 for 7 days. Then, the PLBs were rinsed with distilled water for three times before dehydrated in graded series of ethanol (50%, 70%, 85%, 98%, 100%, absolute I and absolute II). Subsequently, the PLBs were exposed to xylene until the PLBs were transparent, followed by soaking them into the mixture of xylene and wax (Shandon Histoplast Pelletised Paraffin Wax) for 30 minutes in an oven at 60ºC. The PLBs were then treated with wax I, II and III for 1 hour respectively at 60ºC prior to block into a mould. The labelled blocks were sectioned at 11µm using manual rotary microtome to obtain a thin-sliced ribbon. The ribbons were mounted on a glass slide in a mounting bath before placed on a slide warmer for a minute. The slides were then dried overnight at 40°C in the oven. After that, the slides were stained with Periodic acid (0.5%), Shiff reagent and Naphthol Blue Black (1%). The slides were left for air-dried and mounted by a coverslip. The observation, imaging and photography of the slides were conducted using light microscope-compound (Olympus BX41, Japan) fitted with a camera (X-Cam61) using software analysis Image Processing version 5.1.

3. Results and discussion

Cross-section of PLB stock (Fig. 1A) indicates that some of the outer layers around cells were burst and damage while the inside cells remain intact and in isodiametric shape. The cells also resulted in voluminous dense stained nuclei. The apical meristem of PLBs was densely stained by Naphthol Blue Black (NBB), and starch accumulation was observed at cytoplasm, showing the presence of actively dividing cells and soluble protein accumulations. Similarly, PLB of the preculture stage remains intact with isodiametric cells (Fig. 1B). It has dense cytoplasm and stained nuclei that have confirmed the presence of soluble protein accumulations for cell division and exhibited meristematic cells which were actively dividing.

Addition of high concentration of sugar was always used as preculture treatment that caused a number of cellular alteration including expression of freezing-tolerant genes and increment of total soluble protein, proline and abscisic acid content [15]. Accumulation of sugar in the cells could help to keep plasma membrane integrity, thus stabilising proteins under stress condition [16]. One of the early physiological response of osmotically stressed cells is the increment of the protein level that is related to freezing tolerance [15]. During preculture treatment, sucrose penetrates cells, and the occurrence of starch has accumulated to supply energy for somatic embryo development, cell growth and proliferation [17]. Therefore preculture with high sucrose could accumulate starch and protect the cell [18]. Accumulation of starch grain in cells indicates the survival of post cryopreservation. These results were similar in *Brassidium* Shooting Star orchid study that has reported voluminous nucleus and denser cytoplasm in precultured PLBs [19].

Normally, cells show turgidity and the cytoplasm stays within the plasma membrane when an adequate amount of sucrose is used to desiccate. The PLBs is related to osmoprotection, and dehydration stages resulted in increased levels of plasmolysed cells within the cytoplasmic and meristematic regions (Fig. 1C-D). The cells were ruptured and undergone plasmolysis that has led them to collapse and shrink. Popov et al. [20] mentioned cells that dehydrated to a great extent due to extreme sucrose concentration will assist to plasmalemma rupture followed by cell death. The structural changes of the cells after excessively desiccated resulted in the protein to denature [21].

At thawing and cryopreserved stages, the cytoplasm and meristematic cells exhibited ruptured and had collapsed, composing of deformed and shrunken cells (Fig. 2A-B). The cytoplasm burst and released cellular contents into intercellular space. Fig. 2C-D indicated intact polyhedral isodiametric meristematic cells in non-cryopreserved PLBs. The nuclei and apical meristem of the PLBs were densely stained showed that it was actively dividing cells and accumulating soluble protein. Starch accumulations were also observed in the cytoplasm. This study has found that 4 hours of dehydration time resulted in plasmolysis of the cells, causing them to collapse and shrink. The cells were ruptured and had disorganised cellular. Poor dehydration during cryostorage will form crystal ice and caused cell injury. However, the damage PLBs still have the possibility to regenerate as long as there is a cell that can survive and develop a new cell [16]. A study by Ng and Saleh [22] reported that the area of dense cytoplasm on the anterior side of the PLB could form a sheath-leaf that would further enlarge and develop into true leaves.

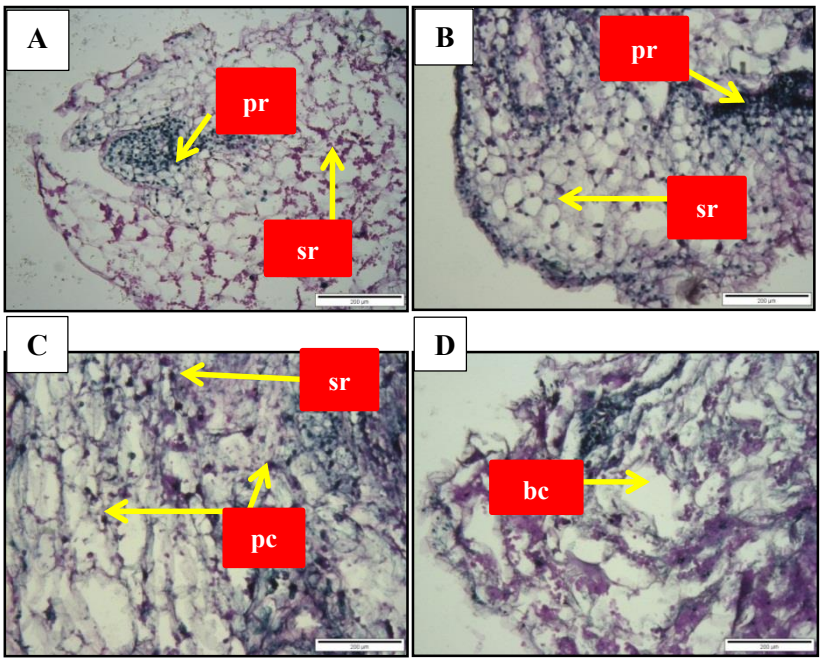

Figure 1. Histological section of PLBs of *Oncidium* Golden Anniversary orchid. (A) the stock of PLBs (B) PLB following preculture stage (C) PLB following osmoprotection stage and (D) PLB following dehydration stage. (pr: protein accumulation; sr: starch accumulation; pc: plasmolysed cell; bc: burst cell)

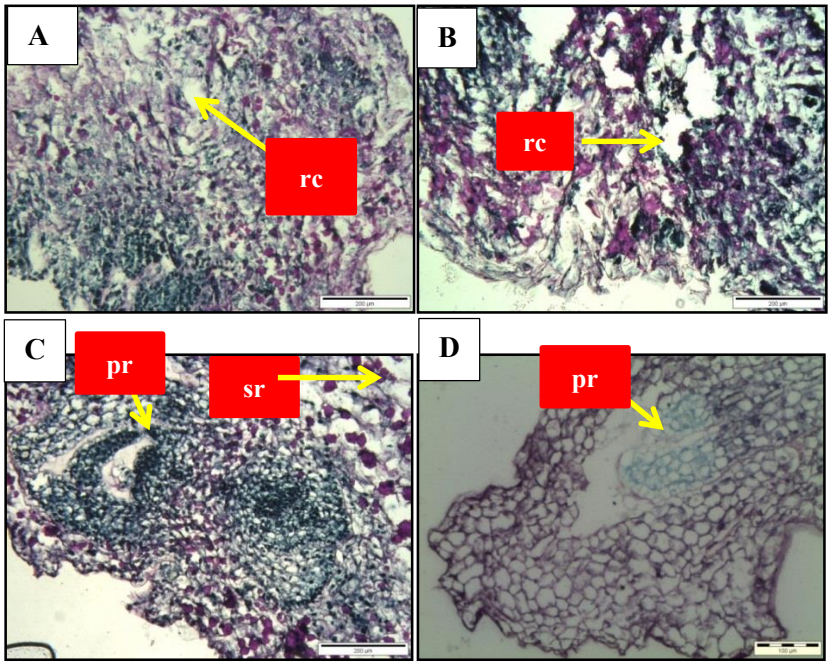

Figure 2. Histological section of PLBs of *Oncidium* Golden Anniversary orchid. (A) PLB following thawing stage (B) cryopreserved PLB and (C, D) non-cryopreserved PLB. (rc: ruptured cell; pr: protein accumulation; sr: starch accumulation).

Sucrose act as a cryoprotectant in stabilising membranes during cooling, followed by adding sucrose in preculture media is widely used in cryopreservation of plants [23,24,25]. Sucrose also decreases the water content of cell osmotically and help the formation of a glassy state [26]. In this study, precultured PLBs resulted in differentiation of proplastids into amyloplast containing starch. Helliot et al. [24] also previously reported that the alterations in the biomembranes, such as the undulations of the plasma membrane was caused by partial plasmolysis. Cell breakdown and cell death occurred when damages take place on the membrane during exposure of cells to the freezing condition [27]. The reason for cell death could also happen when ice crystals are formed. Thus, the free cellular water in cryopreserved cells should be cooled rapidly to prevent ice crystal formation.

4. Conclusion

Different cryopreservation stages of *Oncidium* Golden Anniversary PLBs showed different degree of plasmolysis and shrinkage of cells that resulted in cell damage. The morphology of the cells during cryopreservation affect the viability of the PLBs post-cryopreservation.

References

[1] Chugh S, Guha S and Rao I U 2009 Micropropagation of orchids: A review on the potential of different explants. *Scientia Horticulturae* **122** 507
[2] TH 2006 Orchids: *Oncidium* Golden Anniversary. Retrieved from http://orchidgdn.blogspot.com/2006/03/oncidium-golden-anniversary.html
[3] Kishor R, Sha Valli Khan PS and Sharma GJ 2006 Hybridisation and *in vitro* culture of an orchid hybrid *Ascocenda* _Kangla‘. *Scientia Horticulturae* **108** 66-73
[4] Kulus D and Zalewska M 2014 Cryopreservation as a tool used in long-term storage of ornamental species-a review. *Scientia Horticulturae* **168** 88–107
[5] Teixeira da Silva JA , Kim H and Engelmann F 2015 Chrysanthemum low-temperature storage and cryopreservation: A review. *Plant Cell, Tissue and Organ Culture* **120** 423-440
[6] Ogbu JU, Essien BA, Essien JB and Anaele MU 2010 Conservation and management of genetic resources of horticultural crops in Nigeria: Issues and biotechnological strategies. *Journal of Horticulture and Forestry* **2** 214-222
[7] Cruz-Cruz CA, Arnao GMT and Engelmann F 2013 Biotechnology and Conservation of Plant Biodiversity. *Resources* ***2*** 73-95
[8] Reed BM 2007 Cryopreservation—Practical Considerations. In B. M. Reed (Ed.), *Plant Cryopreservation: A Practical Guide*. USA: Springer.
[9] Uragami A, Sakai A and Nagai M 1990 Cryopreservation of dried axillary buds from plantlets of *Asparagus officinalis* L. grown *in vitro. Plant Cell Report* **9** 328-331
[10] Bekheet SA, Sota V, El-Shabrawi HM and El-Minisy AM 2020 Cryopreservation of shoot apices and callus cultures of globe artichoke using vitrification method. *Journal of Genetic Engineering and Biotechnology* **18** 1-8
[11] Aponte AAA and Tamayo MIS 2017 Changes in liquid phase and mechanical properties during osmodehydrofreezing of papaya (C*arica papaya* L.). *DYNA* **84** 208-213
[12] Nozawa M, Hatakeyama S, Sugimoto Y and Sasaki H 2017 Effect of surface condition to temperature distribution in living tissue during cryopreservation. *IOP Conf. Series: Materials Science and Engineering* **278** 1-7
[13] Engelmann F, Arnao MTG, Wu Y and Escobar R 2008 The development of encapsulation dehydration. In B. M. Reed (Ed.), *Plant Cryopreservation: A Practical Guide* USA: Springer. 59-75
[14] Vyas S, Guha S, Kapoor P and Rao IU 2010 Micropropagation of *Cymbidium* Sleeping Nymph through protocorm-like bodies production by thin cell layer culture. *Scientia Horticulturae* **123** 551-557
[15] Suzuki M, Ishikawa M, Okuda H, Noda K, Kishimoto T, Nakamura T, Isao O, Isao S and Tomoya A 2006 Physiological changes in gentian axillary buds during two-step preculturing with

sucrose that conferred high levels of tolerance to desiccation and cryopreservation. *Annals of Botany* **97** 1073-1081

[16] Feng CH, Cui ZH, Li BQ, Chen L, Ma YL, Zhao YH and Wang QC 2013 Duration of sucrose preculture is critical for shoot regrowth of *in vitro*-grown apple shoot-tips cryopreserved by encapsulation-dehydration. *Plant Cell, Tissue and Organ Culture* **112** 369–378

[17] Pinto G, Silva S, Neves L, Araújo C and Santos C 2010 Histocytological changes and reserve accumulation during somatic embryogenesis in *Eucalyptus globulus*. *Tree,* **24** 763-769

[18] Ganino T, Silvanini A, Beghé D, Benelli C, Lambardi M and Fabbri A 2012 Anatomy and osmotic potential of the Vitis rootstock shoot tips recalcitrant to cryopreservation. *Biologia Plantarum* **56** 78-82

[19] Mubbarakh SA, Rahmah S, Rahman ZA, Mohd Sah NN and Subramaniam S 2013 Cryopreservation of *Brassidium* Shooting Star orchid using the PVS3 method supported with preliminary histological analysis. *Applied Biochemistry and Biotechnology* **172** 1131-1145

[20] Popov AS, Popova EV, Nikishina TV and Vysotskaya ON 2006 Cryobank of plant genetic resources in Russian Academy of Sciences. *International Journal of Refrigeration* **29** 403-410

[21] Hoekstra FA, Golovina EA and Buitink J 2001 Mechanisms of plant desiccation tolerance. *TRENDS in Plant Science* **6** 431-438

[22] Ng CY and Saleh NM 2011 *In vitro* propagation of *Paphiopedilum* orchid through formation of protocorm-like bodies. *Plant Cell, Tissue and Organ Culture* **105** 193-202

[23] Kaczmarczyk A, Rutten T, Melzer M and Keller ERJ 2008 Ultrastructural changes associated with cryopreservation of potato (*Solanum Tuberosum* L.) shoot tips. *CryoLetters* **29** 145-156

[24] Helliot B, Swennen R, Poumay Y, Frison E, Lepoivre P and Panis B 2003 Ultrastructural changes associated with cryopreservation of banana (*Musa* spp.) highly proliferating meristems. *Plant Cell Report* **21** 690-698

[25] Miao NH, KanekoY and Sugawara Y 2005 Ultrastructural implications of pretreatment for successful cryopreservation of *Oncidium* protocorm-like body. *CryoLetters* **26** 333-340

[26] Hincha DK 1990 Differential effects of galactose containing saccharides on mechanical freeze-thaw damage to isolated thylakoid membranes. *CryoLetters* **11** 437-444

[27] Fuller BJ 2004 Cryoprotectants: The essential antifreezes to protect life in the frozen state. *CryoLetters* **25** 375-388