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Vitrification and histological analyses of protocorm-like bodies of V*anda* Kaseem's Delight orchid

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Abstract

This study was conducted to investigate the potential of cryopreserving protocorm-like bodies (PLBs) of *Vanda* Kaseem's Delight Orchid using plant vitrification solution 2 (PVS2). Measured parameters included the effect of PLB size (1-2 and 3-4mm), the effect of sucrose preculture concentrations and duration (0, 0.10, 0.25, 0.50 and 0.75 M, for 24 and 48 hours), the effect of dehydration duration (0, 10, 20, 30 and 40 minutes) and the effect of various unloading periods (0, 10, 20, 30 and 40 minutes) on survival of cryopreserved PLBs, as assessed using spectrophotometric 2,3,5-triphenyltetrazolium chloride (TTC) assay at 490nm. The PLBs were also subjected to a histological study to observe differences in both cryopreserved and non-cryopreserved PLBs of the orchid. The best results in the cryopreservation of PLBs of *Vanda* Kaseem's Delight was obtained when 3-4mm PLBs were precultured in VW medium supplemented with 0.1M sucrose for 24 hours, followed by a loading treatment, and 20 minutes of dehydration in PVS2 at 0°C, prior to cryostorage, and 30 minutes of unloading treatment after 90 seconds of thawing. Histological observations of cryopreserved PLBs indicated that most of the damages resulting from cryostorage occurred in the cell wall and nucleus of the cells.

Keywords: Histology; Loading; Preculture; Protocorm-like bodies; Unloading; TTC; *Vanda*; Vitrification.
Abbreviations: DMSO-dimethylsulfoxide; LN-liquid nitrogen; PLB-protocorm-like bodies; PVS2- plant vitrification solution 2; TTC-2,3,5-triphenytetrazolium choride; VW-Vacin and Went medium.

Introduction

The Orchidaceae is the largest family of flowering plants and estimates range from 17,000 to 35,000 species (Dressler, 1993), encompassing several times as many species as birds or mammals (Chase and Pillon, 2006). It consists of about 750 different genera with at least 25,000 native species and more than 30,000 cultivated hybrids (Hew and Yong, 2004). Many orchid species are in danger of extinction due to extensive disturbance of their natural habitat and indiscriminate harvesting of naturally growing plants. Hence, it is important to establish suitable and reliable techniques of conserving orchids as valuable germplasm (Hirano et al., 2005). Cryopreservation has long been considered an important tool for the long-term storage of plant germplasm (Hirano et al., 2005) as all metabolic processes and cell divisions are arrested at the low temperatures used (Matsumoto et al., 2001). Cryopreservation could play an important role in long-term storage of plant genetic resources because the method only requires minimal space and maintenance, and the desired plant material can be preserved for unlimited periods without alteration (Panis, 2008). Vitrification is a popular method of cryopreservation for orchid seeds as it is quick, simple, reliable, low-cost and practical for conserving many accessions of orchid seeds (Vendrame et al., 2007). The seeds of orchids such as *Dendrobium candidum* Wall. ex Lindl. (Wang et al., 1998), *Doritis pulcherrima* Lindl. (Thammasiri, 2000), and *Bletilla striata* Rchb. f. (Hirano et al., 2005a) have been preserved through direct immersion in liquid nitrogen. Vitrification avoids ice formation in cells through the use of highly concentrated cryoprotectants that increases viscosity in target cells, up to a point where glass formation is induced and water crystallization is bypassed (Pegg, 2010). A famous example of a cryoprotectant is plant vitrification solution 2 (PVS2), which combines dimethyl sulfoxide (DMSO), ethylene glycol and glycerol (Sakai et al., 1991a, b, 2008). The vitrification method was successfully applied to mature seeds of *Bletilla striata* and *Doritis pulcherrima* (Thammasiri, 2000), and immature seeds of *Bletilla striata* (Hirano et al., 2005a) and *Ponerorchis graminifolia* var. suzukiana (Hirano et al., 2005b). It is important to induce dehydration tolerance and reduce the resulting injuries in plant samples exposed to PVS2 to ensure successful cryopreservation of the explants (Hirano et al., 2009). The objectives of this study were to evaluate the effects of various sucrose concentrations (0.0, 0.10, 0.25, 0.50 and 0.75 M) on different sizes of PLBs (1-2 and 3-4mm) that were precultured for 24 and 48 hours; the effect of different dehydration periods (0, 10, 20, 30, and 40 minutes), conducted using PVS2 at 0^{o}C prior to storage in liquid nitrogen; and the effect of different unloading durations (0, 10, 20, 30, and 40 minutes). The PLBs were also subjected to a histological study to observe differences in both cryopreserved and non-cryopreserved PLBs of the orchid.

Results

Effect of Preculture on Cryopreserved PLBs of Vanda Kaseem's Delight

It was generally observed that the PLBs of *Vanda* Kaseem's Delight did not grow well in high concentrations of sucrose in this experiment. It was also observed that the absorbance

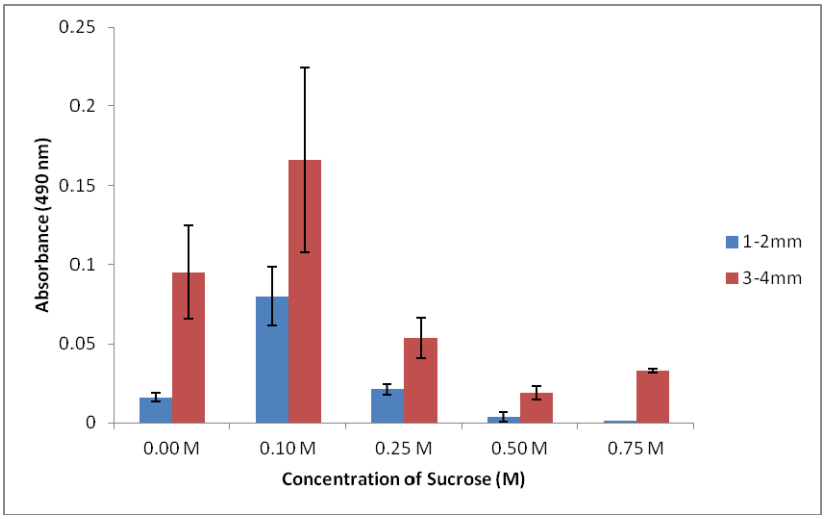

Fig 1. The effect of a 24-hour preculture on cryopreserved PLBs of *Vanda* Kaseem's Delight. Bars represent the standard deviation of absorbance at 490nm.

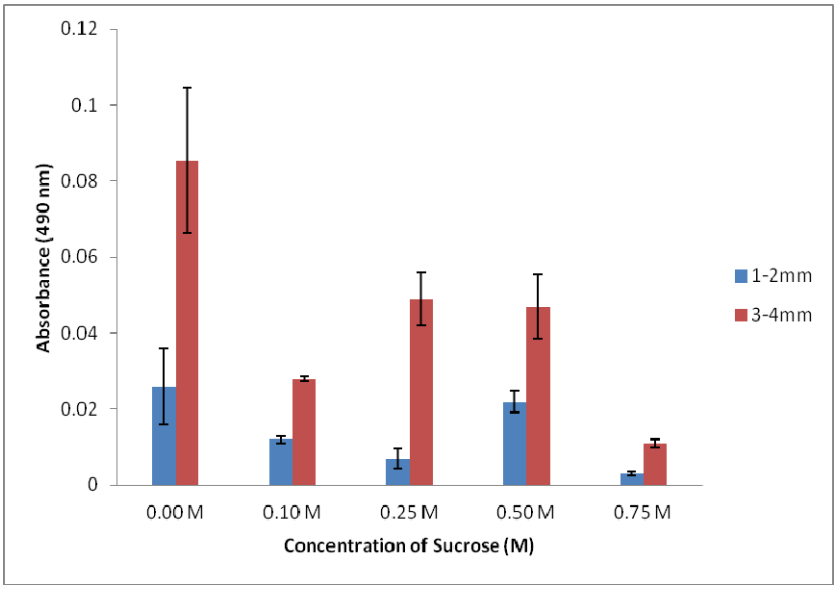

Fig 2. The effect of a 48-hour preculture on cryopreserved PLBs of *Vanda* Kaseem's Delight. Bars represent the standard deviation of absorbance at 490nm.

values obtained in this experiment fluctuated across the replicates tested, resulting in high variability of the results. The survival of cryopreserved PLBs increased for both size groups in the 24-hour preculture experiment when the preculture medium was supplemented with 0.1M sucrose, but decreased when concentrations above 0.1M were applied (Fig. 1). The highest absorbance value obtained in both the 24-hour and 48-hour preculture experiments was 0.166 when 3-4mm PLBs were precultured in 0.1M sucrose for 24 hours, while the lowest absorbance value (0.001) in the 24-hour experiment was recorded when 1-2mm PLBs were precultured in 0.75M sucrose. Hence, 3-4mm PLBs that were precultured in 0.1M sucrose were selected to proceed with the rest of the experiment.

Absorbance values obtained in the 48-hour preculture experiment fluctuated across the preculture concentrations. Here, the highest absorbance value (0.086) was obtained when 3-4mm PLBs were precultured in medium devoid of sucrose (0.0M, Fig. 2). The absorbance value then decreased at the 0.1M sucrose mark, and increased to 0.050 when 0.25M sucrose was used as the preculture agent. The lowest absorbance value (0.003) was obtained when 1-2mm PLBs were precultured in 0.75M sucrose.

Effect of Different Dehydration and Unloading Durations on Cryopreserved PLBs of Vanda Kaseem's Delight

In this experiment, the highest absorbance value (0.016) was obtained when the PLBs were dehydrated in PVS2 for 20 minutes prior to cryostorage, while the lowest absorbance (0.005) was obtained when the PLBs were subjected to 30 minutes of dehydration (Fig. 3). Hence, the subsequent

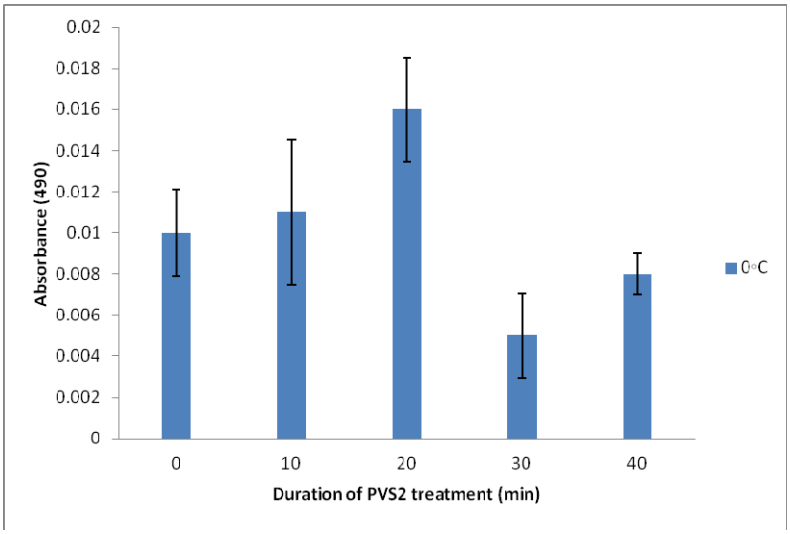

Fig 3. The effect of various dehydration periods on cryopreserved PLBs of *Vanda* Kaseem's Delight. Bars represent the standard deviation of absorbance at 490nm.

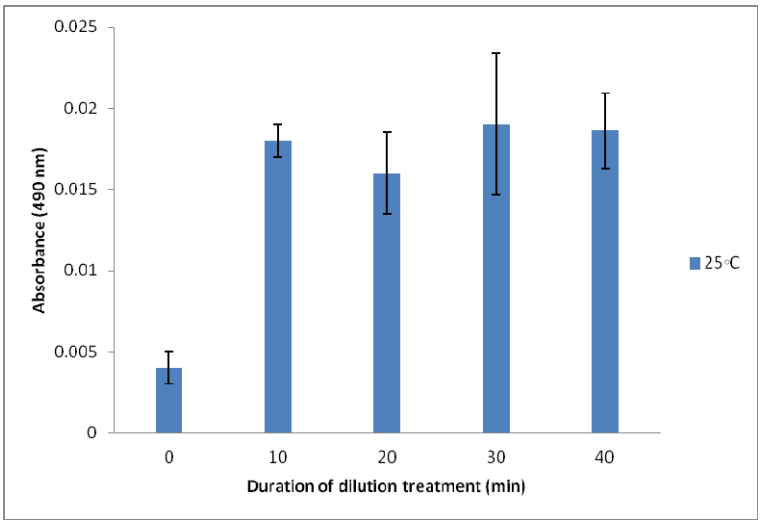

Fig 4. The effect of various unloading periods on cryopreserved PLBs of *Vanda* Kaseem's Delight. Bars represent the standard deviation of absorbance at 490nm.

experiments involved 20 minutes of dehydration in PVS2. Since no significant differences in survival were detected between PLBs subjected to unloading durations ranging from 10-40 minutes (Fig. 4), both non-cryopreserved and cryopreserved PLBs were unloaded for 10 minutes.

Histological Observations of Cryopreserved PLBs of Vanda Kaseem's Delight

Histological observations were conducted on cryopreserved PLBs that were subjected to recovery for three weeks, and untreated PLBs that were obtained from the stock culture. The structure and morphology of the nucleus, cell wall and cytoplasm could be clearly observed in the transverse sections of both groups of PLBs (Fig. 5 and Fig. 6). The cell wall structure and integrity were maintained in non-cryopreserved PLBs (Fig. 5). Sections of non-cryopreserved PLBs also displayed the presence of a single nucleus in each cell, surrounded by the cellular cytoplasm. However, ruptures and damages in the cell wall structure could be observed in cryopreserved PLBs, most likely as a result of ice crystallization occurring in the freeze-thaw cycle (Fig. 6). The cellular nuclei in cryopreserved PLBs appeared damaged and flocculated as well.

Discussion

Effect of Preculture on Cryopreserved PLBs of Vanda Kaseem's Delight

Highly concentrated sugar or sugar alcohols such as sucrose, sorbitol, mannitol, or trehalose are usually used as additives in preculture medium (Cho et al., 2007). Preculture was a necessary step in this experiment. However, low concentrations of sucrose (0.0M for 48 hours or 0.1M for 24 hours) was required as higher concentrations of sucrose or prolonged preculture of up to 48 hours produced low post-cryopreservation survival rates. Preculture was also deemed

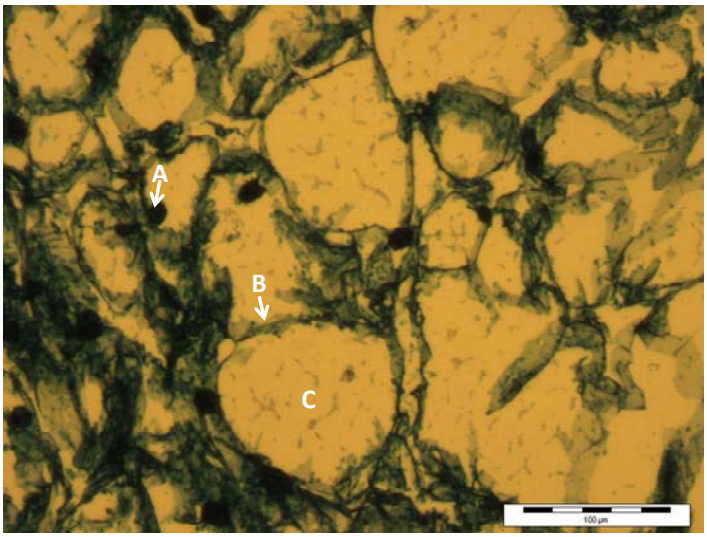

Fig 5. A histological section of a non-cryopreserved PLB of *Vanda* Kaseem's Delight, showing, (a) nucleus, (b) cell wall, and (c) cytoplasm. Non-cryopreserved cells were intact and polyhedral in shape.

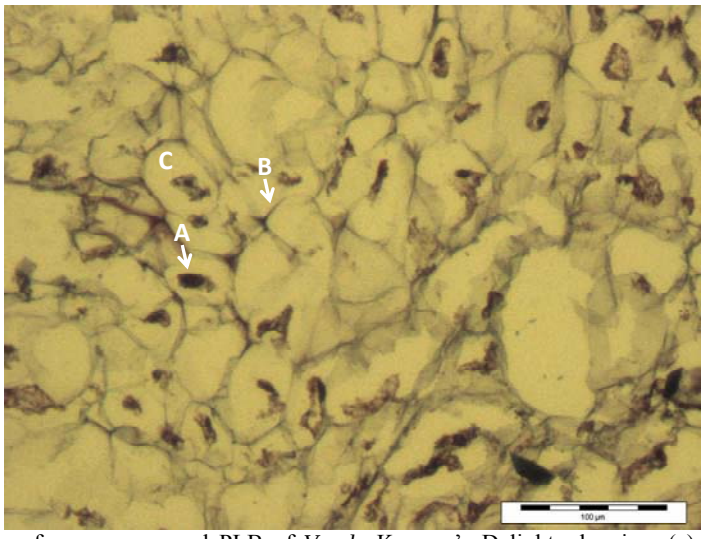

Fig 6. A histological section of a cryopreserved PLB of *Vanda* Kaseem's Delight, showing, (a) nucleus, (b) cell wall, and (c) cytoplasm. Cryopreserved cells displayed a loss of structural integrity as a result of the cooling and thawing process, as the cell wall was ruptured and many cells lost their cytoplasmic contents.

necessary in the vitrification of *Doritaenopsis* suspension culture, as cells which were not precultured showed very low TTC stainability (6.1%) and cells that were pretreated in New Dogashima medium supplemented with 0.1M sucrose for between three days to two weeks displayed significantly increased TTC stainability, with survival percentages reaching 51% after a preculture of seven days. The orchid cells were suggested to have acquired dehydration tolerance at that period and were in the log phase of their growth curve (Tsukazaki et al., 2000).

Effect of Different Dehydration and Unloading Durations on Cryopreserved PLBs of Vanda Kaseem's Delight

Although no significant differences were detected in the survival of PLBs of *Vanda* Kaseem's Delight when they were immersed for different periods of time in PVS2, 20 minutes of dehydration in PVS2 was deemed as necessary as it produced the highest survival in the experiment. An important factor in producing high post-cryopreservation survival rates in target explants is the optimization of exposure time to PVS2 prior to cryopreservation (Sakai et al., 2008). Tsukazaki et al.(2000) discovered that the use of both the loading solution and PVS2 was integral in the post-cryopreservation survival of *Doritaenopsis* suspension cells, as cells that were subjected to only a loading or dehydration treatment did not stain during the TTC assay. The use of both solutions was implicated in the successful cryopreservation of asparagus (Kohmura et al., 1992; Tsukazaki et al., 2000), Japanese horseradish (Matsumoto et al., 1994; Tsukazaki et al., 2000) and *Bletilla striata* (Ishikawa et al., 1997). Tsukazaki et al. (2000) discovered that the use of PVS2 was detrimental to the survival of *Doritaenopsis* suspension culture as the TTC stainability of the cells reduced to 80% when they were precultured in 0.056, 0.1, 0.2, 0.3 or 0.4M sucrose and immersed in PVS2, compared to cells that were simply precultured and not dehydrated (85%). However, cells that were not dehydrated did not survive the cryopreservation procedure at all. The team also did not observe any significant differences in the survival of the suspended cells

the dehydration in PVS2 was carried out at different time intervals of between 1-3 hours. The unloading solution is important in the removal of cryoprotectants and in the prevention of osmotic shock to target explants after the thawing step (Panis, 2008). The PVS2 is toxic to plants, and may cause ruptures and injuries to plant cells (Sakai et al., 2008). The efficiency of the unloading solution is influenced by its osmolarity (osmotic stress and efficiency of unloading) and by the duration of its application (accumulation of osmotic stress and amount of cryoprotectants effluxed) (Kim et al., 2006).

Histological Observations of Cryopreserved PLBs of Vanda Kaseem's Delight

The damages incurred by cryopreserved PLBs of *Vanda* Kaseem's Delight were also echoed in many other cryopreservation experiments involving various explants. Cells of *Gentiana spp.* that were cryopreserved displayed a range of ultrastructural damages. The nuclei appeared damaged, and the chromatin, detached from the inner nuclear envelope, displayed a flocculent structure. Additional observations also revealed that the mitochondria contained electron-lucent matrix and no cristae, with many of them degraded. The amyloplast, nuclear and mitochondrial envelopes displayed a loss of structure. Preculture in high concentrations of sucrose did not protect the cells against post-freezing damage, despite the reduction of cellular size and the degree of vacuolation (Mikuła et al., 2006). The cell wall is important in preventing ruptures in the plasma membrane when the protoplasts enlarge following the uptake of water (Evert and Esau, 2006). The resulting post-thaw loss in the cell wall function, which is to protect and shape the cells, may have caused the PLB cells to lose stability and function (Saini, 2010). The nucleus is a prominent spherical structure inside the cytoplasm of the cell and is the controlling centre of the cell (Saini, 2010), as it contains the hereditary material of a cell (Sastry et al. 2006). Hence, cells are only able to survive for a short time once their nuclei is removed (Enger and Ross, 2003) as the absence of a nucleus causes the loss of the genetic information needed to synthesize or repair the entire organelle (Clark, 2005).

Materials and Methods

Plant Materials

Protocorm-like bodies (PLBs) of *Vanda* Kaseem's Delight orchid were used to initiate multiplication of PLBs for this experiment. The PLBs were incubated under 16 hours photoperiod using 150 µmol m^{-2} s^{-1} cool white fluorescent lamps (Philips TLD, 36W) with the temperature set at 25 ± 2°C. The following experiments were conducted using the propagated PLBs.

Media Preparation

All media used in this experiment contained Vacin and Went (VW, 1949) components. The pH values of all media were adjusted to 5.7 prior to sterilization. The semi-solid culture medium was supplemented with fresh coconut water, tomato and Gelrite, while the semi-solid preculture media consisted of sucrose supplemented at the following concentrations (M): 0.0, 0.10, 0.25, 0.50, and 0.75. The loading solution (osmoprotectant) consisted of 0.4M sucrose and 2.0M glycerol. The PVS2 solution consisted of (w/v) 30% glycerol, 15% ethylene glycol, and 15% dimethylsulfoxide (DMSO), while the unloading solution consisted of 1.2M sucrose.

Preculture

Propagated PLBs were aseptically excised into two different sizes (1-2mm and 3-4mm), transferred to the preculture medium and incubated for 24 or 48 hours under 16 hours photoperiod using 150 µmol m^{-2} s^{-1} cool white fluorescent lamps (Philips TLD, 36W) with the temperature set at 25 ± 2°C.

Osmoprotection and Dehydration

Precultured PLBs were transferred into 2ml cryovials. The PLBs were osmoprotected with 1.5ml of the loading solution for 20 minutes, with fresh changes of the solution at 10 minutes intervals. The PLBs were then dehydrated in 1.5ml PVS2 for five different durations (0, 10, 20, 30 and 40 minutes) at 0°C. The cryovials were then placed onto cryocanes, plunged into liquid nitrogen (LN) and stored for 24 hours.

Unloading and Recovery

Cryostored PLBs were thawed for 90 seconds in a water bath set at 40±2°C. The PVS2 was discarded from the cryovials, and the PLBs were immediately immersed in 1.5ml of unloading solution for five different durations (0, 10, 20, 30 and 40 minutes) at room temperature, with fresh changes of the solution at half-time intervals. After the unloading treatment, the PLBs were placed on semi-solid culture medium affixed with a piece of sterile filter paper, followed by transfer to new medium without the filter paper after 24 hours. The PLBs were incubated in complete darkness for seven days under standard plant tissue culture conditions, followed by exposure to dim light for another week and exposure to standard tissue culture lighting and conditions for seven days. After three weeks of incubation, the PLBs were subjected to a survival test using TTC.

Survival Assessment via 2,3,5-triphenyltetrazolium (TTC) test

The TTC assay is based on the reduction of colourless TTC into insoluble red formazan by dehydrogenases located in the mitochondria of surviving cells. The TTC solution consisted of a 0.05M $Na_2HPO_4.2H_2O$ - KH_2PO_4 buffer set at pH 7.4, 0.6% (w/v) TTC and 0.05% (v/v) Tween 80 (Verleysen et al., 2004). After three weeks of recovery, cryostored PLBs were immersed in 2ml of the TTC buffer in complete darkness for between 18-24 hours at 30°C. The PLBs were then rinsed three times with sterile distilled water. The formazan was extracted from the PLBs with 7ml of 95% (v/v) ethanol in a water bath set at 80°C for 1 hour. The extracts were then cooled and made up to 7ml using 95 % ethanol. Absorbance values of the extracts were read at 490nm using a spectrophotometer (SHIMADZU spectrophotometer UV mini-1240), with 95% ethanol selected as the blank.

Histological Observations

Both cryopreserved and non-cryopreserved PLBs were fixed in FAA (formalin–acetic acid–alcohol) for 24 hours, dehydrated in a tertiary-butyl-alcohol series, embedded in paraffin wax, sectioned at 10µm thickness and stained with

0.5% safranin-O and 0.1% fast green (Jensen, 1962). The sections were viewed and photographed with a light microscope (Olympus, BX41-CCD).

Statistical Analyses and Experimental Design

The experiments were conducted in a completely randomized design and consisted of three replicates containing 10 explants for each parameter tested. Means were analysed through one-way analysis of variance and differentiated with Tukey's test, with the confidence intervals set at 95%.

Conclusion

The best result in the cryopreservation of PLBs of *Vanda* Kaseem's Delight was obtained when 3-4mm PLBs were precultured in VW medium supplemented with 0.1M sucrose for 24 hours, followed by a loading treatment, and 20 minutes of dehydration in PVS2 at 0°C prior to cryostorage, and 30 minutes of unloading treatment after 90 seconds of thawing. Histological observations of cryopreserved PLBs indicated that most of the cellular damages occurred in the cell wall.

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