

## Effect of preculture and PVS2 incubation conditions followed by histological analysis in the cryopreserved PLBs of *Dendrobium* Bobby Messina orchid

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### Abstract

In this study, we established a reliable method for cryopreservation of protocorm-like bodies (PLBs) of *Dendrobium* Bobby Messina by assessing factors involved such as preculture duration and plant vitrification solution 2 (PVS2). PLBs with the size range of 3-4 mm were selected from 4 weeks old cultures, precultured with half strength semi-solid MS media supplemented with 0.6 M sucrose at 25°C for 0 to 5 days. Precultured PLBs were then treated with a mixture of 2 M glycerol and 0.4 M sucrose supplemented with half strength liquid MS media at 25°C for 20 minutes. PLBs were then dehydrated with plant vitrification solution 2 (PVS2) at 0°C or room temperature for 0-140 minutes prior to storage in liquid nitrogen. PVS2 contains 30% (w/v) glycerol, 15% (w/v) ethylene glycol and 15% (w/v) DMSO supplemented with 0.4 M sucrose in half strength liquid MS medium. Following rapid warming in a water bath at 40°C for 90 to 120 seconds, the PLBs were washed with half strength liquid MS media supplemented with 1.2 M sucrose. Subsequently, the PLBs were cultured on half strength semi-solid MS media supplemented with 2% sucrose without the presence of any growth regulators. Triphenyl tetrazoliumchloride (TTC) spectrophotometrical analysis was used to assess the survival of the cryopreserved PLBs. The best preculture condition 0.6 M sucrose supplemented with half strength MS media for 1 day. The best PVS2 conditions using temperature and exposure duration was at 0°C for 60 minutes. Higher number of homogenous cell population was observed in cryopreserved PLBs comparative to non-cryopreserved PLBs.

**Keywords:** cryopreservation, orchid, *Dendrobium* Bobby Messina, PVS2.

**Abbreviations:** PLB, Protocorm-like bodies; LN, Liquid Nitrogen; MS, Murashige and Skoog Media; PVS2, Plant Vitrification Solution 2; DMSO, Dimethyl sulfoxide; TTC, 2,3,5-Triphenyltetrazolium chloride.

### Introduction

Cryopreservation is the storage of biological material at ultra low temperature (-196°C) in a cryogenic medium such as liquid nitrogen (Withers and Engelmann, 1997). At this ultra low temperature, all cellular divisions and metabolic processes are stopped which allows conservation for an unlimited period of time (Engelmann, 2004). Long term conservation of plant genetic resources using cryopreservation relies on freezing embryo or shoot tips. Embryo or shoot tips are complex structures with heterogeneous cellular composition. They will require cryogenic protective treatment to ensure conservation on their structural integrity and when dealing with species or hybrids with tropical origin, specific treatment is required to artificially induce cold tolerance because tropical plants do not develop cold tolerance mechanism and thus are highly sensitive towards low temperature (Engelmann, 2000). Water removal plays a central role in preventing freezing injury and in maintaining post thaw viability (Gonzalez-Arnao et al., 2008). Vitrification based cryopreservation method involves some degree of dehydration and desiccation before freezing by exposure of samples to highly concentrated cryoprotective solution (Engelmann, 2000). As results, most of the freezable water is removed during dehydration and subsequently cooling is performed rapidly by direct immersion into the liquid nitrogen, thereby inducing

vitrification of internal solutes (Engelmann, 2000). This simple and reliable method of cryopreservation has been developed by Sakai and his co-workers (Sakai et al., 1990). This method has been applied to different plant material such as grape (*Vitis*) and chestnut (*Castanea sativa*) (Pennycooke and Towill, 2000; Matsumoto and Sakai, 2003; Nieves et al., 2005; Ding et al., 2008; Tsai et al., 2009). Vitrification can be defined as the transition of water directly from liquid phase into an amorphous phase or glass while avoiding the formation of crystalline ice (Fahy et al., 1984). The Orchidaceae is a large flowering family that is recognized as an economically important commodity in international floriculture industry both as cut flower and potted plant (Kuehnle, 2007). Among various orchid categories in the family, *Dendrobium* have become increasingly popular due to its flower sprays, wide range of colors, sizes and shapes, year round availability, and long flowering life in several weeks to months (Kuehnle, 2007). In Malaysia orchid exports value about RM 40 Million annually, of which 11.7% comprise of *Dendrobium* production. Due to growing domestic and export markets, Malaysia has recorded increasing floricultural products from RM 0.73 million in 1992 to RM 2.77 million in 1995. At the continued growth rate of 6% per annum, productions are expected to reach RM 36 billion by the year 2010 (Jong et al., 1997). However,

increasing competition from established orchid producers such as the Philippines, Thailand, Taiwan and Singapore constantly overshadow that of Malaysia's (Kuehnle, 2007). Therefore, there is an urgent need of orchid conservation. Aseptic cultures of orchid supplemented with various nutrients can increase germination rate within 3 to 4 weeks after culture. Protocorm-like bodies (PLBs) can serve as plant material for cryopreservation (Yin and Hong, 2009). PLBs are well-differentiated tissues that are sometimes referred as orchid embryo that developed with two discrete bipolar structures, namely, the shoot and root meristems. Thus, these structures are able to convert to plantlets when grown on plant growth regulator (PGR)-free medium (Lee and Phillips, 1988). PLBs are preferred to be used as target tissues in cryopreservation due to large number of PLBs can be obtained within a relatively short period of time which provides plenty of materials to work with and have high capabilities to regenerate into plantlets (Sreeramanan et al., 2008). In cryopreservation of PLBs of *Dendrobium* Bobby Messina by vitrification method, explants of germplasm with 3-4 mm size range showed better viability comparative to size range 1-2 mm for both cryopreserved and non-cryopreserved PLBs. The best preculture concentration used in pretreatment media was 0.6 M sucrose (Antony et al., 2010). Therefore the aim of this study is to evaluate the effect of preculture duration and PVS2 temperature/duration on cryopreservation of *Dendrobium* Bobby Messina and to evaluate the histological observation in cryopreserved and non-cryopreserved PLBs.

## Results

### *Effect of various preculture durations on viability*

In cryopreservation of PLBs of *Dendrobium* Bobby Messina by vitrification method, PLBs with 3-4 mm size range showed better viability comparative to size range 1-2 mm for both cryopreserved and non-cryopreserved PLBs. From our previous study, the best preculture concentration used in pretreatment media was 0.6 M sucrose (Antony et al., 2010). Therefore, those conditions were further used in these subsequent experiments. All cryopreserved PLBs that were not precultured showed very low viability rate. Cryopreserved PLBs showed increasing viability rate when was precultured in half strength MS media supplemented with 0.6 M sucrose for 0-1 days. Contrarily, cryopreserved PLBs showed decreasing viability rate when were precultured in half strength MS media supplemented with 0.6 M sucrose for 2-5 days. Cryopreserved PLBs which were precultured in half strength MS media supplemented with 0.6 M sucrose showed highest viability rate when were precultured for 1 day comparative to other durations tested based on TTC Assay (Fig. 1). Therefore, 1 day preculture period combined with 0.6 M sucrose in preculture medium was subsequently used in the remaining experiment. All non-cryopreserved PLBs showed decreasing viability rate when was precultured in half strength MS media supplemented with 0.6 M sucrose for 0-5 days (Fig. 2).

### *Effect of PVS2 incubation temperatures and durations on viability*

To determine the optimal time and temperature of exposure to PVS2, cryopreserved PLBs were treated for 0-140 minutes with PVS2 solution at 0°C and room temperature prior to a plugged into the LN. Without PVS2 dehydration treatment at 0°C and room temperature, cryopreserved PLBs showed very

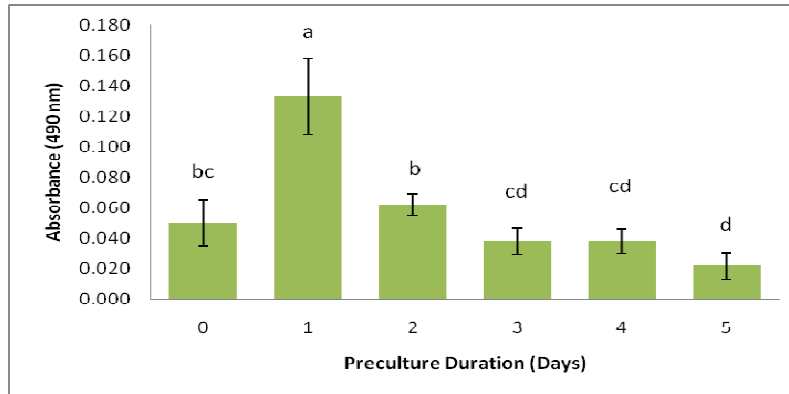
low viability rate. However, cryopreserved PLBs showed lower viability rate when were exposed to PVS2 solution at room temperature comparative to 0°C. In particular, PLBs showed highest viability rate when were exposed to PVS2 solution at 0°C for 60 minutes (Fig. 3). In addition, closer view of regenerated cryopreserved PLBs precultured on half strength semi-solid MS media supplemented with 0.6 M sucrose for 1 day and PVS2 treatment condition of 60 minutes at 0°C within 3 month in culture (Fig. 4). Therefore, dehydration with PVS2 solution at 0°C for 60 minutes was considered to be the best PVS2 treatment for PLBs cryopreservation of *Dendrobium* Bobby Messina. To determine the differences between cryopreserved PLBs and non-cryopreserved PLBs when were treated for 0-140 minutes with PVS2 solution at 0°C, results showed that there is no significant differences in viability between cryopreserved and non-cryopreserved PLBs (Fig. 5). To determine the differences between cryopreserved PLBs and non-cryopreserved PLBs when were treated for 0-140 minutes with PVS2 solution at room temperature, results showed that there is significant differences between cryopreserved and non-cryopreserved PLBs. The viability of non-cryopreserved PLBs was higher comparative to cryopreserved PLBs (Fig. 6).

### *Histology Analysis of cryopreserved and non-cryopreserved PLBs*

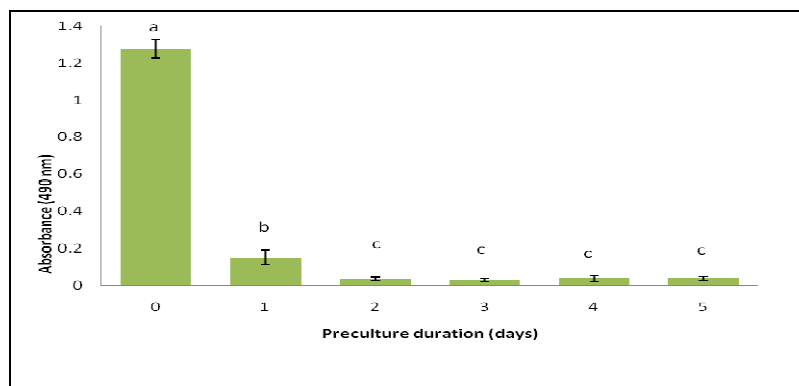
Histological observation of cryopreserved PLBs and non-cryopreserved PLBs (3 months after thawing) showed that the pack cell volume is higher in cryopreserved PLBs comparative to non-cryopreserved PLBs. Results also shows that the production of somatic embryos in cryopreserved PLBs is more rapid comparative to in non cryopreserved PLBs (Fig. 7; Fig. 8). At the 90<sup>th</sup> day after thawing, cross section of non-cryopreserved PLBs shows that the presence of complete outer layer of the cell wall (Fig. 7A). Cross section of cryopreserved PLBs shows the presence of cell wall with the breakage at the outer layer of the cell wall (Fig. 7B). Cross section of non-cryopreserved PLBs shows the presence of visible nucleus (Fig. 7C; Fig. 7E). Cross section of cryopreserved PLBs shows the presence of visible voluminous nucleus, denser cytoplasm and presence of more homogenous cell population comparative to non-cryopreserved PLBs (Fig. 7D; Fig. 7F). Cross section of non-cryopreserved PLBs shows that the cells were undamaged had a perfect polyhedral shape (Fig. 8A). Whereas, the cross section of cryopreserved PLBs shows that the cells were damaged and show symptoms of plasmolysis, nuclear shrinkage, rupture in cell wall and cell membrane (Fig. 8B). Cross section of cryopreserved PLBs also shows that the presence of raphid and storage materials around the nucleus (Fig. 8C; Fig. 8D). The storage material may contain protein and starch reserves.

## Discussion

In the present study, cryopreserved PLBs which were precultured in half strength MS media supplemented with 0.6 M sucrose showed highest viability rate when were precultured for 1 day comparative to other durations tested based on TTC Assay (Fig. 1). All non-cryopreserved PLBs showed decreasing viability rate when was precultured in half strength MS media supplemented with 0.6 M sucrose for 0-5 days (Fig. 2). Previous studies has shown that sucrose induce dehydration tolerance very effectively by means of osmotic



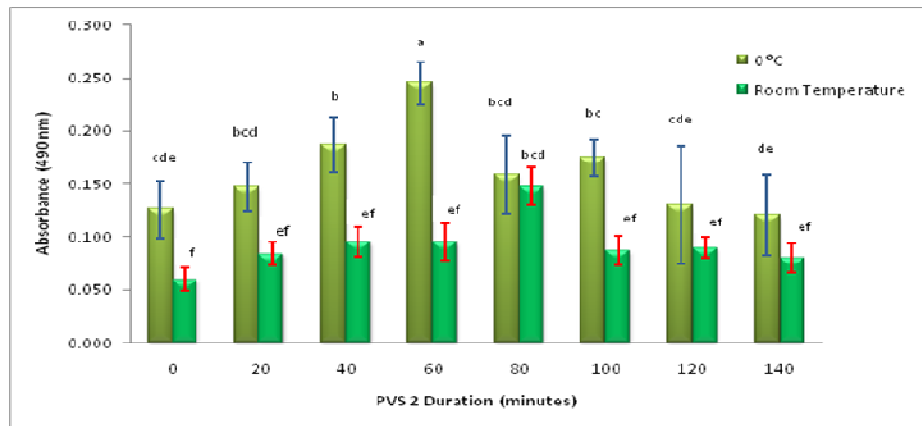
**Fig 1.** Effect of preculture duration on viability of cryopreserved PLBs precultures in 0.6M sucrose for 0-5 days. Results were analysed by one way ANOVA and means were compared by Tukey test. Vertical bars represent  $\pm$  SD of means of 6 replicates



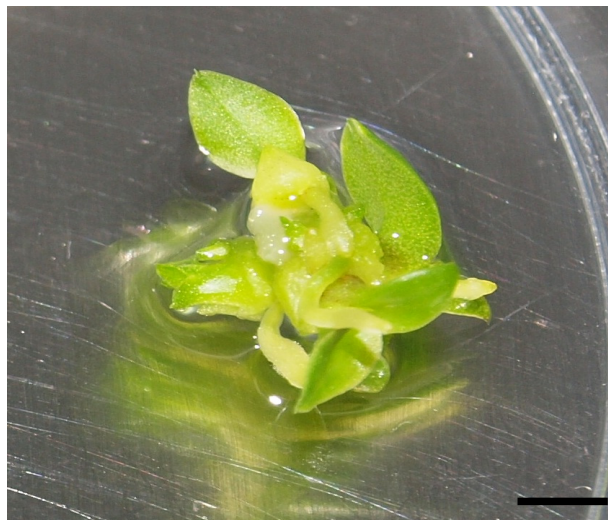
**Fig 2.** Effect of preculture duration on viability of non-cryopreserved PLBs precultured in 0.6M sucrose for 0-5 days. Results were analysed by one way ANOVA and means were compared by Tukey test. Vertical bars represent  $\pm$  SD of means of 6 replicates.

dehydration (Suzuki et al., 1994; Antony et al., 2010). It is long been reported that preculture duration also influence survival of cryopreserved plant tissues (Bouafia et al., 1996; Ishikawa et al., 1997). When potato shoot tips were precultured in 1 M sucrose over 1-7 days, preculture period of 2 days produced the highest survival of cryopreserved shoot tips (Bouafia et al., 1996). Previously, zygotic embryo of a Japaneses terrestrial orchid (*Bletilla striata*) were precultured with 0.3 M sucrose for 3 days and 50 to 60% of these embryos withstood preculture and developed into normal plantlets (Ishikawa et al., 1997). The rate of viability in precultured seed of *Lilium ledebourii* (Baker) was 50 % (Kaviani, 2010). However, when protocorms of *D. virgineum* were precultured in a modified Vacin and Went (1949) (VW) (Vacin and Went, 1949) liquid medium supplemented with 0.3 M sucrose for 3 days, the survival rate of cryopreserved protocorms was only about 15% (Pornchuti and Thammasiri, 2008). Similarly, low regrowth (13.33%) was observed from encapsulated shoot tips of *D. Walter Oumea* that were previously precultured on 0.3 M sucrose agar medium for 2 days (Lurswijidjarus and Thammasiri, 2004). All these finding suggested that different orchids exhibited varying levels of tolerance to high sucrose concentrations. In the present study, dehydration with PVS2 solution at 0°C for 60 minutes was considered to be the best PVS2 treatment for PLBs cryopreservation of *Dendrobium Bobby Messina* (Fig. 3, Fig. 4). Incubation period and temperature of the vitrification solution are two important factors affecting the survival of cryopreserved plant tissues (Hong et al., 2009).

Over exposure of plant tissues to the vitrification solution such as PVS2 may lead to chemical toxicity and excessive osmotic stress (Hong et al., 2009). The optimum exposure time for PVS2 varies with plant species and depends on the temperature during exposure (Hong et al., 2009). These factors determine the extent of cell dehydration and the amount of cryoprotectant permeated into the cells is determined by these factors (Wang et al., 2004). Many reports have shown that dehydration at 0°C can reduce toxicity of vitrification solution, compared with at room temperature, usually leading to higher survival (Yin and Hong, 2010). Also incubation time at 0°C was greatly extended compared with at room temperature, thus allowing much more flexibility in handling a large number of samples at the same time (Yin and Hong, 2010). The survival of *Dioscorea floribunda* was remarkably improved when was treated with PVS2 for 90 minutes at 0°C using vitrification method (Mandal and Sangeeta, 2007). The survival of *Dioscorea bulbifera* increased to 58% when was treated with PVS2 for 20 minutes using vitrification method (Leunufna and Keller, 2003). Therefore, it is clear that responses of different species to cryopreservation by vitrification method are different and this could be attributed to genotypic differences as well as differences in incubation period and temperature of vitrification solution. To determine the differences between cryopreserved PLBs and non-cryopreserved PLBs when were treated for 0-140 minutes with PVS2 solution at 0°C, results showed that there is no significant differences in viability between cryopreserved and



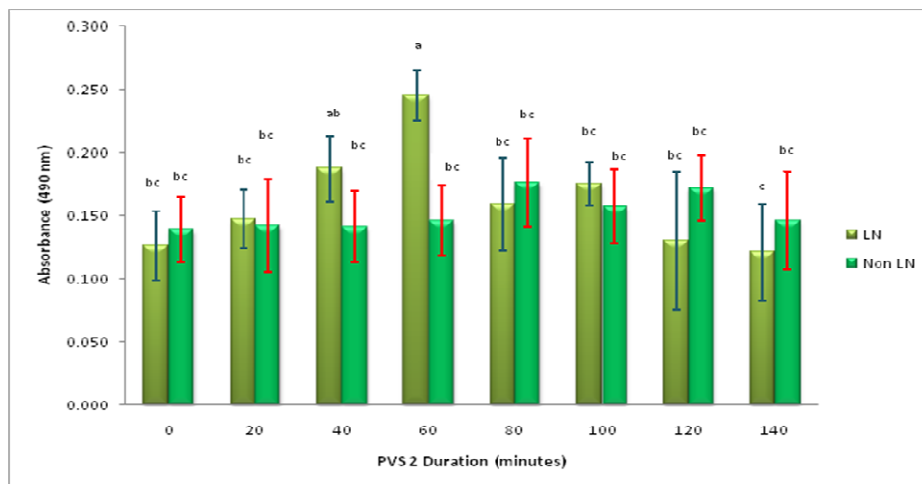
**Fig 3.** Effect of PVS2 in two temperatures and duration on viability of cryopreserved PLBs. Results were analysed by one way ANOVA and means were compared by Tukey test. Vertical bars represent  $\pm$  SD of means of 6 replicates



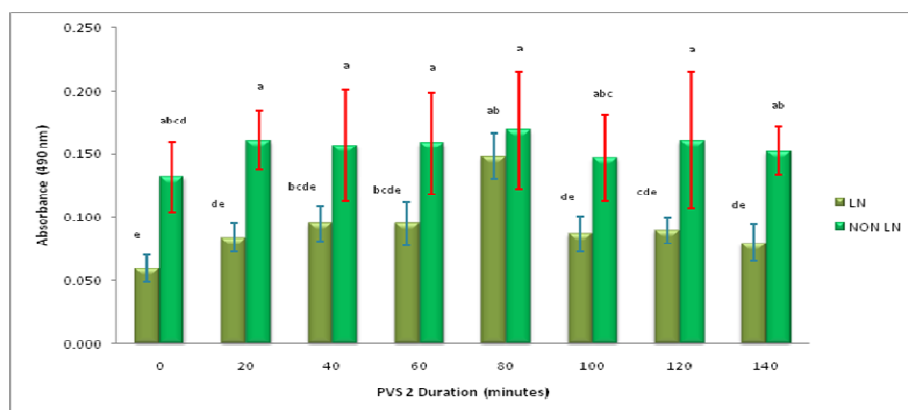
**Fig 4.** View of regenerated cryopreserved PLBs precultured on half strength semi-solid MS media supplemented with 0.6M sucrose for 1 day and PVS2 treatment condition of 60 minutes at 0°C. Growth recovery of cryopreserved PLBs after 2 month. Bar represent 1.25 mm.

non-cryopreserved PLBs (Fig. 5). This research results are in accordance with previous report on cryopreservation of embryonic calli of *Dioscorea bulbifera* that showed similar morphological indexes between cryopreserved and non cryopreserved embryonic calli of *Dioscorea bulbifera* (Yin and Hong, 2010). To determine the differences between cryopreserved PLBs and non-cryopreserved PLBs when were treated for 0-140 minutes with PVS2 solution at room temperature, results showed that there is significant differences between cryopreserved and non-cryopreserved PLBs. The viability of non-cryopreserved PLBs was higher comparative to cryopreserved PLBs (Fig. 6). In cryopreservation of *Dendrobium candidum* Wall ex Lindl, regrowth of control was initiated earlier and preceded faster than the cryopreserved PLBs (Yin and Hong, 2009). Therefore, this result obtained supports previous findings. In order to reveal the non-lethal and lethal damage produce by cryopreservation, histological observation was made on cryopreserved and non-cryopreserved PLBs of *Dendrobium* Bobby Messina. The cryopreserved PLBs actually grew from the initial explants which turned into brown initially due to cryopreservation. This is possible due to the fact that PLBs actually is composed of undifferentiated meristematic cells

that are able to regenerate into new meristems (Fig. 7; Fig. 8). The observations here that undifferentiated cells survive cryopreservation are in consistent with observation in pea embryonic exes (Wesley-Smith et al., 1995). Similar results were also obtained in cryopreservation of palm shoot tips (Bagniol et al., 1992). The physiological response of the cryopreserved explants is dependent upon the cryopreservation method (Volk and Caspersen, 2007). Microscopic observation provides valuable insight into the degree of plasmolysis that is occurring in the cells. This study demonstrates that the cellular nature of undifferentiated cells makes them less vulnerable to damages incurred during osmotic stress of cryoprotectant treatments (Volk and Caspersen, 2007). Cryoprotective treatment that favors survival of small meristematic cell in PLBs is most likely to produce high survival rate after LN exposure (Volk and Caspersen, 2007). Another important factor for the success of cryopreservation will be the water content in the cells. Meristematic cells usually contain few and small vacuoles with lower water content and therefore are more tolerant against water lost and freezing (Bagniol et al., 1992). In is vital to note that the tissue regrowth after cryopreservation cannot be the only criterion to determine the success of



**Fig 5.** Effect of PVS2 duration (0° C) on viability of cryopreserved and non cryopreserved PLBs. Results were analysed by one way ANOVA and means were compared by Tukey test. Vertical bars represent ± SD of means of 6 replicates.



**Fig 6.** Effect of PVS2 duration (room temperature) on viability of cryopreserved and non cryopreserved PLBs. Results were analysed by one way ANOVA and means were compared by Tukey test. Vertical bars represent ± SD of means of 6 replicates.

cryopreservation and for this reason the histological observation was done 3 month after thawing. In this study, histological observation revealed accumulation of mass homogenous cell population in cryopreserved PLBs comparative to non-cryopreserved PLBs (Fig. 7; Fig. 8). The mass cell population could be attributed due to selection process; elimination of non embryonic cells from cultures or it may be due to increase synchrony of development of embryonic cells (Salaj et al., 2011). Similar results were obtained in embryogenic tissue of *Pinus nigra* where larger number cell lines were generated in cryopreserved comparative in non-cryopreserved cell lines (Salaj et al., 2011).

## Materials and methods

### Plant Materials

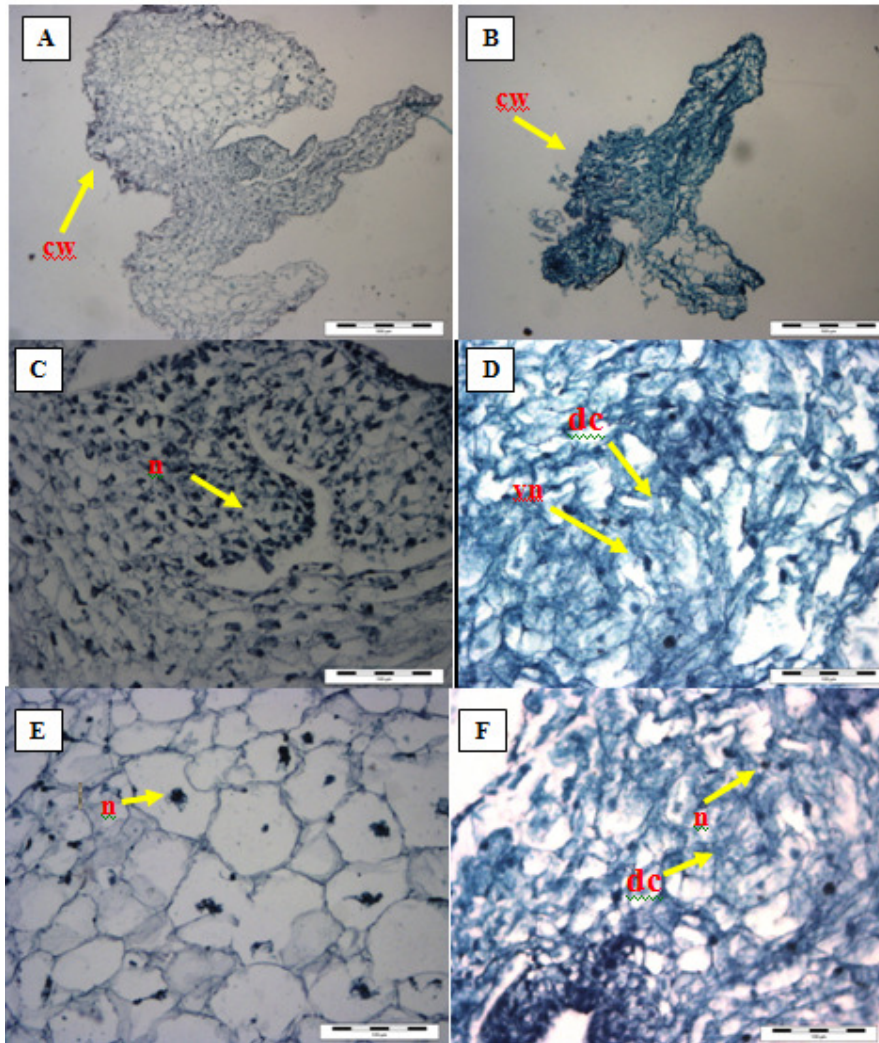
*In vitro* cultures of PLBs of *Dendrobium Bobby* Messina were selected for cryopreservation in this study (Antony et al., 2010).

### Preculture

For optimization preculture duration, 3-4 mm PLBs were selected from 4 weeks old culture and precultured in half strength semi-solid MS media (Murashige and Skoog, 1962) supplemented with 0.6 M sucrose at 25°C for 0 to 5 days under 16 hours photoperiod (Antony et al., 2010).

### Vitrification

Following preculture, the PLBs were dehydrated with 1.5 ml of loading solution (2 M glycerol supplemented with 0.4 M sucrose in half strength liquid MS media) in 2 ml cryotubes at 25°C for 20 minutes. Next, the PLBs were then dehydrated in 1.5 ml PVS2 solution at 0°C or 25°C for 0- 140 minutes. PVS2 solution contains 30% (w/v) glycerol, 15% (w/v) ethylene glycol and 15% (w/v) Dimethyl sulfoxide (DMSO) supplemented with 0.4 M sucrose in half strength liquid MS medium. After dehydration in PVS2 solution, the PLBs were resuspended in fresh 1.5 ml of PVS2 and were directly plugged into LN for minimum of 24 hours. Cryopreserved



**Fig 7.** Histological section of *Dendrobium* Bobby Messina PLB. A (500µm), C (100µm), E (100µm) shows the cross section of non-cryopreserved PLBs, whereas B (500µm), D (100µm), F (100µm) shows the cross section of cryopreserved PLBs. cw; cell wall, n;nucleus, vn; voluminous nucleus, dc; dense cytoplasm.

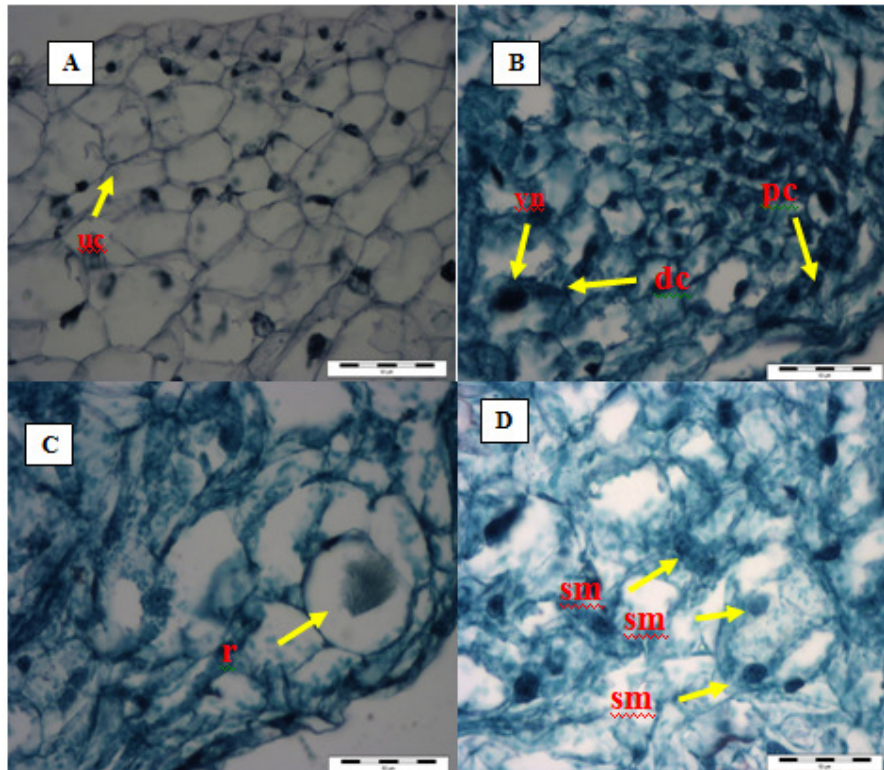
PLBs were thawed at 40°C for 90 to 120 seconds. After thawing, PVS2 solution was drained from the cryotubes and replaced with 1.5 ml of half strength liquid MS media supplemented with 1.2 M sucrose (Sakai et al., 1991) in which the PLBs were washed at 25°C for 20 minutes. In the non-cryopreserved experiment for preculture duration, the PLBs were subjected to all treatment except PVS2 treatment, cryostorage and thawing procedures. In the non-cryopreserved experiment for PVS2 duration, the PLBs were subjected to all treatment except cryostorage and thawing procedures. Both cryopreserved and non cryopreserved PLBs were then transferred onto a layer of sterilized filter paper disc over half strength semi-solid MS media supplemented with 2% sucrose and 2.75 g/L gelrite without the presence of any growth regulators at 25°C for 24 hours. After 1 day, the PLBs were transferred onto half strength semi-solid MS media supplemented with 2% sucrose and 2.75 g/L gelrite without the presence of any growth regulators. At the 1<sup>st</sup> week, the PLBs were kept in dark condition; 2<sup>nd</sup> week were kept in dim light condition and the 3<sup>rd</sup> week were exposed to 16 hours photoperiod (Antony et al., 2010).

#### **Viability Assessment and Statistical Analysis**

The survival of cryopreserved and non-cryopreserved PLBs were assessed based on growth observation after 3 weeks in culture and viability assay via triphenyl tetrazoliumchloride (TTC) spectrophotometrical analysis at 490 nm (Verleysen et al., 2004). Each consisted of 6 replicates per treatment with 10 samples each. All data were subjected to independent sample t-test, one way ANOVA and means were compared using tukey HSD test (Antony et al., 2010).

#### **Histology Analysis of cryopreserved and non-cryopreserved PLBs**

Histological analysis was performed for cryopreserved PLBs and non cryopreserved PLBs that were precultured in 0.6 M sucrose for 1 day and dehydrated in PVS2 at 0°C for 60 minutes. This was done after 3 months of growth recovery. First, series of transfer of PLBs into alcohol TBA was done with varying concentration (50-100%). After that, PLBs were treated with TBA I, TBA II and TBA III and left overnight.



**Fig 8.** Histological section of *Dendrobium* Bobby Messina PLB. A (50µm) shows the cross section of non-cryopreserved PLBs, whereas B, C, D (50µm) show the cross section of cryopreserved PLBs . uc; undamaged cells, vn; voluminous nucleus, dc; dense cytoplasm, r; raphid, sm; storage material, pc; plasmolysed cells.

Then, PLBs was exposed to xylene and Wax I,II, and III. Specimen was then blocked and sliced using 6 Micron Microtome (Leica RM 2135). Specimen was then stained with safranin and fast green. Slides were observed using light microscope (Olympus BX41).

### Conclusion

In vitrification method, PLBs of *Dendrobium* Bobby Messina precultured in half strength MS media supplemented with 0.6 M sucrose and exposed to PVS2 for 60 minutes at 0° C showed histological observation with higher number of homogenous cell population found in cryopreserved PLBs comparative to non-cryopreserved PLBs. In order to ensure greater success behind this method, molecular analysis of PLBs during the cryopreservation should be carried out in order to implement this protocol.

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