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Full Length Research Paper

Preliminary study on cryopreservation of *Dendrobium* Bobby Messina protocorm- like bodies by vitrification

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Protocorm-like bodies of *Dendrobium* Bobby Messina were cryopreserved by vitrification method. In this study, protocorm-like bodies (PLBs) with the size range of 1-2 and 3-4 mm were selected from 4 weeks old culture, pretreated with half strength semi-solid Murashige and Skoog (MS) media supplemented with 0.5 M sucrose at 25°C for 24 h. Pretreated 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 min. Osmoprotected PLBs were then dehydrated with plant vitrification solution 2 at 0°C for 20 min before storage in liquid nitrogen. After rapid warming in water bath at 40°C for 90 s, the PLBs were washed with half strength liquid MS media supplemented with 1.2 M sucrose and then cultured on half strength semi-solid MS media supplemented with 2% sucrose without the presence of any growth regulators. Survival of the cryopreserved PLBs was assessed based on triphenyl tetrazoliumchloride (TTC) spectrophotometrical analysis. The PLBs 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 pretreatment concentration used in pretreatment media was 0.6 M sucrose and 1.2 M sorbitol, respectively.

Key words: Cryopreservation, vitrification, *Dendrobium* Bobby Messina, Pretreatment.

INTRODUCTION

Germplasm conservation via traditional method is vital for maintenance of biodiversity and avoidance of genetic erosion, however, such method is time consuming and difficult to handle. Apart from that, important genotypes may be lost due to contamination, human errors during handling and mislabeling during culture (Engelmann, 1991). Thus, in order to overcome this problem, cryopreservation is an alternative germplasm preservation method.

Cryopreservation of biological material is based on the arrestment of cellular division and metabolic processes when biological materials are stored at ultra low temperature, usually at liquid nitrogen (-196°C). Storage at this temperature theoretically allows conservation for unlimited period of time (Gonzalez-Arno et al., 2008). Cryopreservation at -196°C in liquid nitrogen is an ideal tool which also offers phenotypic and genotypic stability, low cost, and minimum requirement of space and maintenance (Suzuki et al., 2008). The cryostorage may prevent any unfavorable changes that take place during successive passages *in vitro*. This method makes it possible to limit the number of passages, the risk of microbial contamination and somaclonal variation in stored cultures (Malabadi and Nataraja, 2006). However, it is vital to develop a cryogenic protocol where a large amount of recovery may be possible. Therefore, prevention of intracellular and extracellular ice formation during the cooling, storage and thawing is critical for successful cryopreservation. This is generally obtained by removing

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Abbreviations: PLB, Protocorm-like bodies; TTC, 2,3,5-triphenyltetrazolium chloride; PVS2, plant vitrification solution; MS, Murashige and Skoog media; DMSO, dimethyl sulfoxide; BAP, benzylaminopurine.

the water content from the cells before storage in liquid nitrogen (Fahy et al., 1987; Vertucci et al., 1991).

There are two types of cryopreservation protocols which differs based on their physical mechanism. In the classical or conventional cryopreservation method, freezing is performed in the presence of ice while in vitrification based protocol, freezing is performed without ice formation (Gonzalez-Arno et al., 2008). Vitrification based cryopreservation method offers a cost effective method as it does not require expensive equipments. The presence of highly concentrated solutions of cryo-protective agents prevents the formation of intra- and extracellular crystallization of ice in the cells and tissues as they pass rapidly through the temperatures where ice crystal growth occurs (Niino et al., 1992). In this method, when tissues were plugged into liquid nitrogen, the highly concentrated aqueous solution solidifies into a non-crystal, glassy state structure and therefore, ice crystal nucleation is impeded and the vitrified cells can escape the danger of intracellular freezing (Reed, 2008).

Vitrification techniques offer advantages such as greater recovery and it is a simple protocol (Lambardi et al., 2000; Turner et al., 2000, Reed, 2001). Positive results has been obtained for long term storage of embryonic tissues and somatic embryos with the use of vitrification for *Picea mariana* (Touchell et al., 2002) and, more recently, for *Picea sitchensis* (Gale et al., 2008). To date vitrification has been widely applied to wide range of orchids, such as zygotic embryo and immature seeds of *Bletilla striata*, (Hirano et al., 2005; Ishikawa et al., 1997), protocorm and protocorm-like bodies of *Dendrobium candidum* (Brian et al., 2002; Vacin and Went, 1949), shoot tips of *Dendrobium Walter Qumae* (Lurswijidjarus and Thammasiri, 2004), seeds of *Doritis pulcherrima* (Thammasiri, 2000), suspension culture cells of *Dorataenopsis* (Tsukazaki et al., 2000), *in vitro* grown shoot apices of *Arachnis* species (Gagliardi et al., 2003) and seed of *Bratonia* hybrid (Popov et al., 2004).

Hybridization in orchid is common in producing new and improved material, including new flower color, color pattern, flower size and a number of additional characteristics of commercial values. Over 100,000 commercial hybrids has been registered worldwide to date being grown as cut flowers and potted plant. The demand of orchid cut flowers increased with *Dendrobium* hybrid being commercially desirable due to the number of flower per inflorescence and recurrent flowering (Martin and Madassery, 2006).

Furthermore, the variety of flower color and color pattern and relatively short production cycle from seedling to full bloom plant for *Dendrobium* hybrids increase their commercial value (Vendrame et al., 2007). Protocorm-like bodies (PLBs) of orchids are small organs that readily develop into whole plants (Nikishina et al., 2007). PLBs actually have two ends directly opposite each other, the shoot region where the shoot appears and the root region where the root systems tend to be located.

The new cells tend to propagate on the surface of the original PLB to form a clump tissue. There are few reasons why the PLBs are used as the target tissues for cryopreservation since they are easily propagated *in vitro* which could provide plenty of materials to work with and secondly they are proved to be a reliable source of potentially regenerable tissues (Ishikawa et al., 1997).

Dendrobium Bobby Messina is one of a new orchid hybrid which has been reported to be grown widely as cut flower, potted plant and is valued for its attractive flower. There is no report so far on cryopreservation of *D. Bobby* Messina protocorm like bodies and therefore the aim of this study is to develop a cryopreservation protocol for *D. Bobby* Messina by vitrification method.

MATERIALS AND METHODS

Plant materials

In vitro cultures of PLB of *D. Bobby* Messina were selected for cryopreservation in this study. Stock cultures were cultured in half strength liquid (Murashige and Skoog, 1962) supplemented with 1 mg/L benzylaminopurine (BAP) and 2% sucrose. The pH was adjusted to 5.8 prior to autoclaving. The PLBs were grown at 25°C under 16 h photoperiod. The PLBs were then subcultured for every 4 weeks.

Pretreatment

For optimization of PLBs size, 1-2 and 3-4 mm were selected from 4 weeks old culture and pretreated respectively in half strength semi-solid MS media supplemented with 0.5 M sucrose at 25°C for 24 h under 16 h photoperiod. For optimization of pretreatment condition, PLBs selected from 4 weeks old culture were pretreated in half strength semi- solid MS media supplemented with different concentration of sucrose (0.0, 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 M) and sorbitol (0.0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 and 1.4 M) at 25°C for 24 h under 16 h photoperiod.

Loading, cryostorage, thawing, regeneration

After 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 min. Subsequently, the PLBs were then dehydrated in 1.5 ml plant vitrification solution 2 (PVS2) solution (Sakai et al., 1990) at 0°C for 20 min. 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 at 0°C for 20 min, the PLBs were resuspended in fresh 1.5 ml of ice cold PVS2 and were directly plugged into liquid nitrogen for minimum of 24 h. Cryopreserved PLBs were thawed at 40°C for 90 to 120 s. 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 min.

In the non-cryopreserved experiments, the PLBs were subjected to all treatment except PVS2 treatment, 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

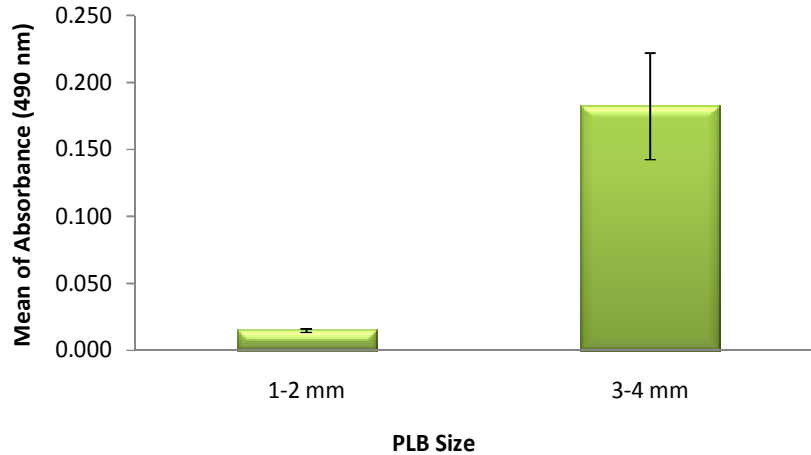


Figure 1. Effect of cryopreserved PLBs sizes on viability. Based on independent sample t test, P-value= 0.002. Vertical bars represent \pm SD of means of 3 replicates.

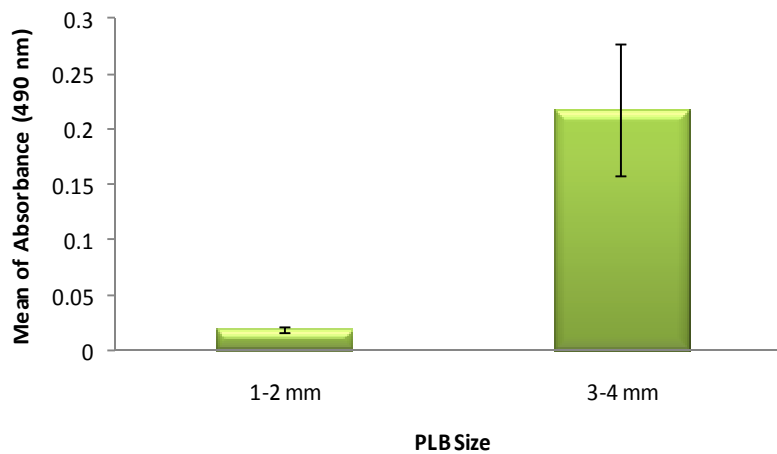


Figure 2. Effect of non-cryopreserved PLBs sizes on viability. Based on independent sample t test, P-value= 0.005. Vertical bars represent \pm SD of means of 3 replicates.

2.75 g/L gelrite without the presence of any growth regulators at 25°C for 24 h. After one 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 1st week, the PLBs were kept in dark condition; 2nd week they were kept in dim light condition and the 3rd week they were exposed to 16 h photoperiod.

Viability assessment and statistical analysis

After 3 weeks in culture, the survival of cryopreserved and non-cryopreserved PLBs were assessed based on growth observation and viability assay via triphenyl tetrazoliumchloride (TTC) spectrophotometrical analysis at 490 nm (Verleysen et al., 2004). Each experiment was repeated twice and consisted of 3 replicates per treatment with 10 samples each. All data were subjected to independent sample t-test, one way analysis of variance (ANOVA)

and means were compared using Tukey Honestly Significant Difference (HSD) test.

RESULTS

Effect of different PLB size range on viability

Effect of various PLBs size on viability of cryopreserved PLBs was investigated. The cryopreserved PLBs with size range of 3-4 mm showed higher viability comparative to PLBs with size range of 1-2 mm (Figure 1). Through independent sample t test, there was significant difference in PLBs with size range of 3-4 mm comparative to 1-2 mm. The non-cryopreserved PLBs with size range of 3-4 mm showed higher viability comparative to PLBs with

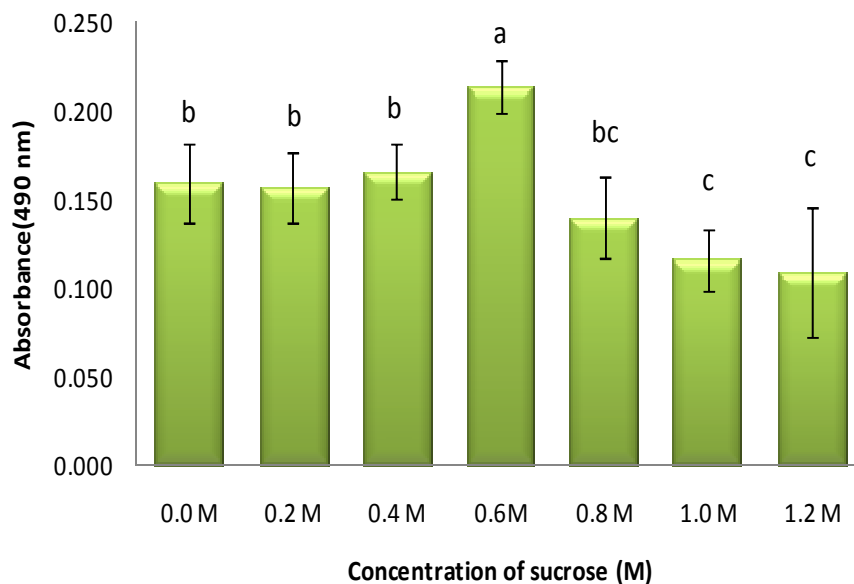


Figure 3. Effect of preculture sugar type (sucrose) on viability of cryopreserved PLBs. Results were analysed by one way ANOVA and means were compared by Tukey test. Vertical bars represent \pm SD of means of 3 replicates.

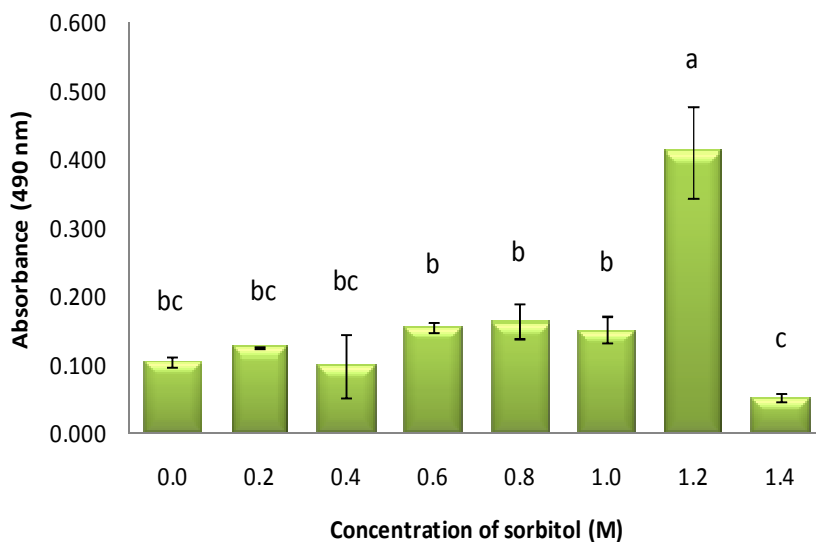


Figure 4. Effect of preculture sugar type (sorbitol) on viability of cryopreserved PLBs. Results were analysed by one way ANOVA and means were compared by Tukey test. Vertical bars represent \pm SD of means of 3 replicates.

size range of 1-2 mm (Figure 2). Through independent sample t test, there was significant difference in PLBs with size range of 3-4 mm comparative to 1-2 mm.

Effect of various pretreatment conditions on viability

Effect of various preculture conditions on viability of cryopreserved PLBs was investigated. Cryopreserved

PLBs that were precultured in sucrose (0.2 to 0.6 M) showed increase viability rate (Figure 3). In particular, cryopreserved PLBs which were pretreated in half strength semi-solid MS media supplemented with 0.6 M sucrose showed highest viability rate comparative to other sucrose concentration tested based on TTC Assay (Figure 3). Cryopreserved PLBs that were precultured in sorbitol (0.2 to 1.2 M) showed increase viability rate (Figure 4). In particular, cryopreserved PLBs which were



Figure 5. Regenerated cryopreserved PLBs precultured on half strength semi-solid MS media supplemented with 0.2 M sucrose. After 3 months of growth recovery stage. Bars represent 5 mm.

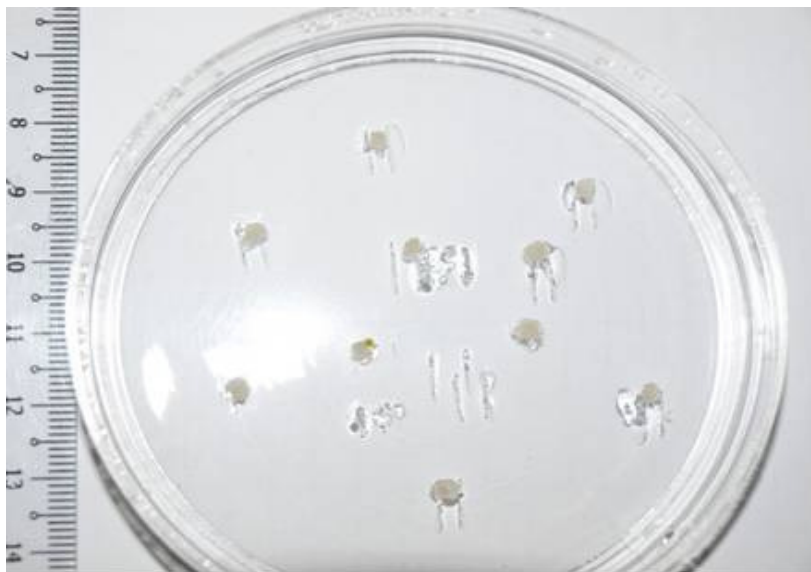


Figure 6. Regenerated cryopreserved PLBs precultured on half strength semi-solid MS media supplemented with 0.2 M sorbitol after 3 months of growth recovery stage.

pretreated in half strength semi-solid MS media supplemented with 1.2 M sorbitol showed highest viability rate comparative to other sorbitol concentration tested based on TTC Assay (Figure 4).

However, PLBs pretreated in half strength semi-solid MS media supplemented with 0.2 M sucrose showed

growth recovery within 3 months of culture (Figure 5). Contrarily, PLBs pretreated with half strength semi-solid MS media supplemented with 0.2 M sorbitol showed growth recovery within 3 weeks in culture (Figure 6). A closer view was taken from Figure 6 of regenerated cryopreserved PLBs precultured on half strength semi-solid

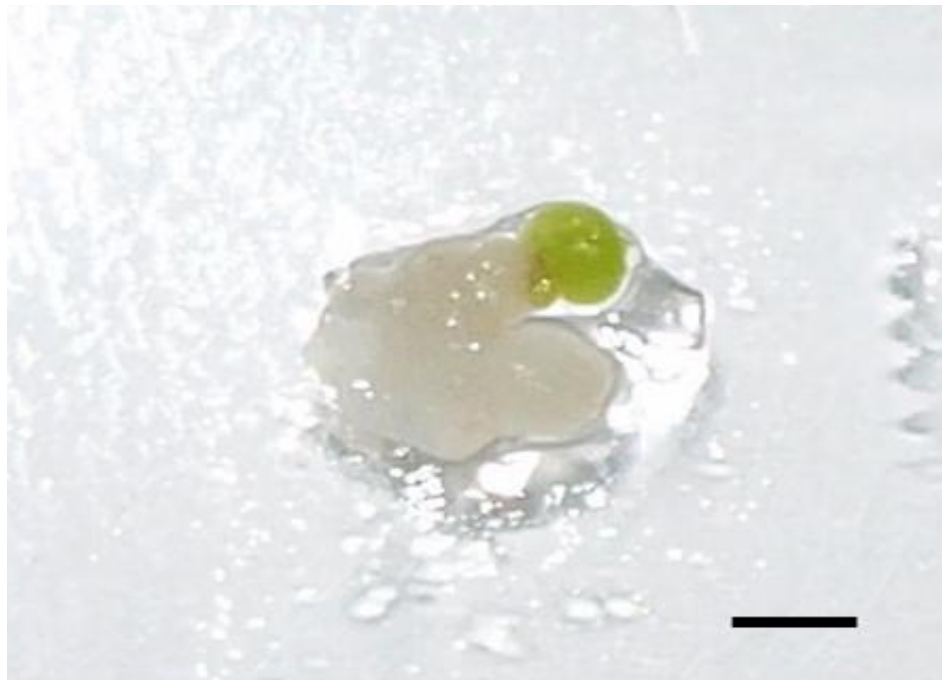


Figure 7. Closer view of regenerated cryopreserved PLBs precultured on half strength semi-solid MS media supplemented with 0.2 M sorbitol after 3 months of growth recovery stage. Bar represent 1.25 mm.

MS media supplemented with 0.2 M sorbitol within 3 weeks in culture (Figure 7).

DISCUSSION

This is the first preliminary report on successful cryopreservation of *D. Bobby Messina* protocorm-like bodies by vitrification method. There are several factors that can influence the post-thaw recovery of cryopreserved explants (Skerlep et al., 2008). The main ones are development phase of plant material, preculture condition and PVS2 exposure duration. In this report, the influence of PLBs size and preculture condition on post-thaw recovery was evaluated. The method reported in this protocol does not require sophisticated or costly equipments and leads to regeneration of protocorm like bodies.

The developmental phase of plant material has effect on cryopreservation (Li et al., 2009). Therefore, suitable physiological status of explants could be a key point to raise tolerance to liquid nitrogen treatment in cryopreservation (Takagi et al., 1997). In the present study, cryopreserved and non-cryopreserved PLBs with size range of 3-4 mm indicated better viability rate comparative to PLBs with size range of 1-2 mm based on TTC spectrophotometric assay. Explants which are too small often suffer with more mechanical injury (Boucaud et al., 2002). In cryopreservation of garlic shoot apices, explants with base diameter of 3 mm gave rise to more than 90% of post thaw regeneration (Baek et al., 2003).

Therefore, this supports our data that showed that bigger explant (3-4 mm PLB size) resulted in increase viability rate comparative to smaller explant (1-2 mm PLB size). This data obtained supports the view that smaller explants (1-2 mm) experience more mechanical injury during cryopreservation.

Preculturing in media containing sugar is a vital step in successful cryostorage of tissues using the vitrification procedure (Yin and Hong, 2009). High sugar content in the cytoplasm of the tissue aids in the establishment of vitrified state during cryopreservation and enables cells to tolerate dehydration that can cause freezing damage (Yin and Hong, 2009). Since, biological samples contain high amount of water which can cause mechanical injury due intracellular and extracellular ice formation during freezing and thawing, a reduction in water content is crucial prior to cryopreservation (Fabian et al., 2008; Yin and Hong, 2009). Preculturing with medium containing high sucrose (0.3 M) was reported to be useful in improving survival after cryopreservation of mature (Ishikawa et al., 1997) and immature (Hirano et al., 2005) orchid seeds. However, in cryopreservation of *D. candidum* Wall ex Lindl., preculture of PLBs in 0.75 M sucrose was found to be optimum (Yin and Hong, 2009).

In the present investigation, the PLBs pretreated in half strength semi-solid MS media supplemented with 0.6 M sucrose was significantly different comparative to media supplemented with other sucrose concentration tested (Figure 3). Therefore, this indicates that 0.6 M sucrose concentration was sufficient enough to dehydrate the

PLBs prior to cryopreservation. PLBs pretreated in half strength semi-solid MS media supplemented with 1.2 M sorbitol was significantly different comparative to media supplemented with other sorbitol concentration tested (Figure 4). Therefore, this indicates that 1.2 M sorbitol concentration was sufficient enough to dehydrate the PLBs prior to cryopreservation.

Therefore, data obtained from this study supports that a proper preculture step is crucial for the PLBs prior to cryopreservation. However, data obtained in this finding are different from optimum preculture concentration found in previous work. This may be best explained due to different orchid exhibiting varying level of tolerance towards sugar (Yin and Hong, 2009). In non-cryopreserved experiments of both sugar tested, the increasing concentration showed detrimental effect (data not shown). Similar situation was reported in cryopreservation of *Robinia pseudoacacia* when pretreated with medium supplemented with 0.7 M sucrose (Verleysen et al., 2005). Therefore, this result obtained supports previous findings.

However, growth observation indicated that PLBs pretreated in half strength semi-solid MS media supplemented with 0.2 M sucrose showed growth regeneration within 3 months in recovery period (Figure 5). However, PLBs pretreated in media supplemented with other sucrose concentration did not show any growth regeneration ability and PLBs turned brown after 3 weeks in recovery period. In contrast, PLBs pretreated in half strength semi-solid MS media supplemented with 0.2 M sorbitol showed growth regeneration after 3 weeks in recovery period (Figure 6).

A closer view was taken from Figure 6 of regenerated cryopreserved PLBs precultured on half strength semi-solid MS media supplemented with 0.2 M sorbitol within 3 weeks in culture (Figure 7). However, PLBs pretreated in media supplemented with other sorbitol concentration did not show any growth regeneration ability and PLBs turned brown after 3 weeks in recovery period. This is an added data to the previous findings.

Conclusion

The best PLBs size for cryopreservation of *D. Bobby Messina* was 3-4 mm range. The best pretreatment concentration when sucrose and sorbitol was applied in pretreatment media was 0.6 M sucrose and 1.2 M sorbitol, respectively.

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