Australian Journal of Crop Science AJCS
AJCS 5(13):1817-1822 (2011) ISSN:1835-2707

Selected potential encapsulation-dehydration parameters on *Dendrobium* Bobby Messina protocorm-like bodies using TTC analysis

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Abstract

Protocorm-like bodies (PLBs) of *Dendrobium* Bobby Messina were cryopreserved using an encapsulation-dehydration technique and analysed for survival using the 2,3,5-triphenyltetrazolium chloride (TTC) spectrophotometry analysis. PLBs with the size range of 1-2 and 3-4 mm were selected from four-week old cultures and pretreated with (0.0, 0.2, 0.4, 0.6, 0.8, 1.0, 1,2 M sucrose) and/or (0.0, 0.2, 0.4, 0.6, 0.8, 1.0, 1,2 M sorbitol) supplemented with half strength semi-solid MS media at 24°C for (0- 5 days) under 16 hours photoperiod. Precultured PLBs were encapsulated in 3% sodium alginate and 0.1M calcium chloride, both supplemented with half strength liquid MS media and 0.4M sucrose. The beads were osmoprotected in 0.75M sucrose supplemented with half strength liquid MS media on an orbital shaker (110 rpm) at 24°C for 24 hours under 16 hours photoperiod. Osmoprotected beads were dehydrated for (0-10 hours) hours in a parafilm-sealed culture jar containing 50g of oven-sterilized silica gel followed by rapid freezing in liquid nitrogen. After thawing in a 40 °C water bath for 90-120 seconds, the cryopreserved beads were cultured on growth recovery media containing half strength semi-solid MS media supplemented with 2% sucrose. Survival of the cryopreserved PLBs was assessed with the TTC spectrophotometric test. The highest survival of cryopreserved 3-4mm PLBs was obtained through the use of pretreatment medium supplemented with 0.4M sucrose, for three days and dehydrated for 9 hours with silica gel.

Keywords: Cryopreservation, Encapsulation-dehydration, *Dendrobium* Bobby Messina, Proocorm-like bodies, TTC analysis.
Abbreviations: PLB, Protocorm-like bodies; TTC, 2,3,5-Triphenytetrazolium choride; MS, Murashige and Skoog Media; BAP, Benzylaminopurine.

Introduction

Orchids exhibit an incredible range of diversity in size, shape and color of flowers. They are known for their long lasting and bewitchingly beautiful flowers which fetch a high price in both domestic and international markets (Saiprasad and Polisetty, 2003). Propagation of orchids using tissue culture techniques has been accomplished for more than a century and has resulted in the production of identical clones in many orchid genera (Arditti, 2008). The formation of protocorms from germinated seed and the subsequent induction of protocorm-like bodies (PLBs) or callus from the protocorm, stem-node, shoot-tip, leaf, root-tip, or root-tuber explants has turn into a reliable technique for propagating orchids (Park et al., 2003; Kosir et al., 2004; Anjum et al., 2006; Kalimuthu et al., 2007; Roy et al., 2007; Hong et al., 2008; Medina et al., 2009). Propagation by PLB formation is preferred by commercial growers of most orchid genera due to the large number of PLBs that can be obtained within a moderately short period of time. The large-scale propagation of PLBs can also be achieved via a bioreactor system (Park et al., 2000). PLBs are also the most widespread target tissue for genetic transformation studies in orchids because they can proliferate rapidly and have high capabilities to regenerate into complete plantlets (Liau et al., 2003; Sreeramanan et al., 2008). Additionally, PLBs can also serve as plant material for cryopreservation (Yin and Hong, 2009). PLBs are well-differentiated tissues that are frequently regarded as orchid embryos that develop with two discrete bipolar structures, namely, the shoot and root meristem. Therefore, these structures are able to convert to plantlets easily when grown on plant growth regulator (PGR)-free medium. Furthermore, the PLBs directly formed from meristem tissue will exhibit a higher genetic stability than those produced by callus (Lee and Phillips, 1988). Conservation of plant genetic resources ensures maintenance of agrobiodiversity (Gonzalez-Benito et al., 2004). Plant genetic resources conservation can be carried out either in the germplasm's natural habitat (*in situ*) or outside of the habitat (*ex situ*) (Frankel and Hawkes, 1975). *Ex situ* plant genetic resources conservation can be carried out in an efficient and economical way by cryopreservation.

Cryopreservation is the process of freezing living plant material (shoot tips, meristems, cells, somatic embryos, zygotic embryos etc.) at or near the temperature of liquid nitrogen (-196°C). At this temperature, physical and metabolic cellular processes are effectively stopped, hence trapping the living tissues in a state of suspended animation. The transfer of cells from room temperature to -196°C is done in such a way that the viability of the stored material is retained, so that their biological functions and growth can be reactivated after thawing and transfers to regrowth medium (Towill, 1991). Cryopreservation offers the only safe and cost effective option for long term conservation of plant genetic resources (Dixit et al., 2004; Engelmann, 2004). The

encapsulation-dehydration technique of cryopreservation developed by Fabre and Dereuddre (1990) is based on the technology for seed production. In this technique, the explants are encapsulated in beads, dehydrated and then cooled rapidly in liquid nitrogen (Sakai et al., 2000). This method is simple; inexpensive maintains the genetic stability of cryopreserved materials (Kartha and Engelmann, 1994). The beads are easy to handle and tissues are directly immersed into liquid nitrogen without being exposed to toxic cryoprotectants. Thus, this method solely depends on the presence of sugar in pretreatment as its cryoprotectant. The aim of this study is to develop a cryopreservation protocol for *Dendrobium* Bobby Messina through an encapsulation-dehydration technique.

Results

Diverse PLB size range on survival of cryopreserved PLBs via encapsulation dehydration method

Early investigations indicated that the 3-4mm PLBs showed higher survival when compared to 1-2mm PLBs in both cryopreservation and control experiments involving encapsulation dehydration method (Fig. 1; Fig. 2). Similar results were obtained in cryopreservation of *Dendrobium* Bobby Messina by vitrification method (Antony et al., 2010). Therefore, the optimisation of the other cryopreservation parameters was continued using 3-4mm PLBs.

Diverse pretreatment conditions on survival of cryopreserved PLBs via encapsulation dehydration method

All PLBs that were pretreated without the presence of sugar (0.0 M; sucrose or sorbitol) presented with low survival rates (Fig. 3; Fig. 4). Protocorm-like bodies which were pretreated in half-strength MS media with 0.4M sucrose prior to cryostorage presented with the highest survival rate when compared to media with other sucrose concentrations, as indicated by the TTC Assay (Fig. 3). Similarly, PLBs which were pretreated in half strength MS media with 1.0 M sorbitol prior to cryosyorage showed the highest survival rate when compared to other sorbitol concentrations (Fig. 4). However, morphological observations indicated that PLBs that were pretreated in sucrose showed better recovery capability compared to those pretreated in sorbitol. Hence, further experimentations involved only the use of sucrose, and no other pretreatment agents (Fig. 5). Similar results were obtained in cryopreservation of *Dendrobium* Bobby Messina when vitrification was applied whereby sucrose gave rise to better survival comparative to sorbitol (Antony et al., 2010) However, the best sucrose concentration applied in vitrification method was 0.6 M sucrose (Antony et al., 2010).

Diverse pretreatment duration on survival of cryopreserved PLBs via encapsulation dehydration method

Early investigations indicated that all PLBs that were not pretreated prior to cryostorage presented with very low survival rates. Cryopreserved PLBs showed an increase in survival when the pretreatment was conducted in half-strength MS medium with 0.4M sucrose for between one to three days, with the best survival rates obtained from three-day precultures (Fig. 6), based on the TTC assay. In contrast, PLBs presented with decreasing viability rates when the pretreatment was conducted in medium with 0.4M sucrose for five days.

Diverse dehydration periods on survival of cryopreserved PLBs via encapsulation dehydration method

Early study indicated that all PLBs that were not dehydrated prior to cryostorage presented with very low survival rates. Cryopreserved PLBs showed low survival rates when dehydrated (0-8 hours) based on the TTC assay (Fig. 7). However, in contrast, PLBs presented with high survival rate when were dehydrated for 9 hours. PLBs presented with decreasing survival rates when were dehydrated for 10 hours (Fig. 7).

Discussion

Different methods are tested in cryopreservation of most plant are due to the genotypic specificity of plants differs and different plants works well with only a specific method at times. Therefore, in cryopreservation of *Dendrobium* Bobby Messina, two cryopreservation methods namely, vitrification (Antony et al., 2010) and encapsulation dehydration are tested. However, only similar parameters were tested in both methods and these are generally those parameters tested in most cryopreservation procedures such as PLB size, preculture sugar type and concentrations, preculture duration and dehydration period. Many factors can influence the post thaw recovery of cryopreserved PLBs. The factors evaluated in the present study are the physiological state of explants and pretreatment conditions. Excised explants used for cryopreservation procedures must be at optimal physiological state in order to allow high level of dehydration tolerance and produce vigorous recovery after cryopreservation (Hirai and Sakai, 1999). Since the main objective of cryopreservation is to store material for the long term and subsequently produce true-to-type plants from the stored material, shoot-tips or meristems such as PLBs are the preferred experimental material. Meristematic cells contain comparatively small vacuoles with small amounts of water. Therefore, less water needs to be removed to prevent lethal ice crystallization compared to non meristematic cells (Panis et al. 2001). Meristematic tissues that survive cryopreservation includez shoot primordia of orchid (Na and Kondo 1996). In the present study, 3-4mm cryopreserved and non-cryopreserved PLBs presented with higher survival rates compared to 1-2 mm PLBs, based on the spectrophotometric-TTC assay (Fig. 1; Fig. 2). Therefore, the optimisation of the other cryopreservation parameters was continued using 3-4mm PLBs. Similar results were obtained in cryopreservation of *Dendrobium* Bobby Messina by vitrification method (Antony et al., 2010), *Dendrobium* Sonia 28 by PVS2 vitrification method (Hooi et al., 2010), *Phalaenopsis bellina* (Rchb.f.) christenson by encapsulation dehydration method (Khoddamzadeh et al., 2011), *Dendrobium* Sonia 17 by encapsulation dehydration method (Subramaniam et al., 2010) and *Brassidium* shooting star by encapsulation dehydration method (Yin at al., 2011). Therefore, these results support previous findings. Increased sugar in pretreatment medium leads to the accumulation of solute inside the cells, which maintains the integrity of the plasma membrane and inner membranes during dehydration and freezing (Plessis et al., 1993) and reduces the formation of ice crystals during cooling and thawing (Grospietsch et al., 1999). In this study, cryopreserved PLBs showed increasing survival rates when they were pretreated in medium with 0.2 to 0.4M sucrose. However, the survival rates decrease when the sucrose concentration increases from 0.6 to 1.2 M. Higher sucrose concentration in pretreatment enhances survival of

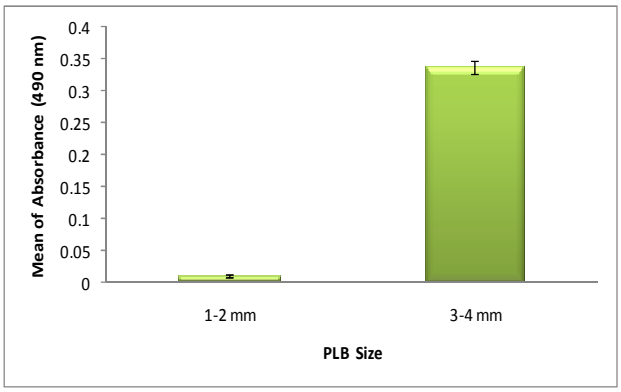

Fig 1. Diverse PLB size range on survival of cryopreserved PLBs via encapsulation dehydration method. Based on Independent sample t test, P-value= 0.002

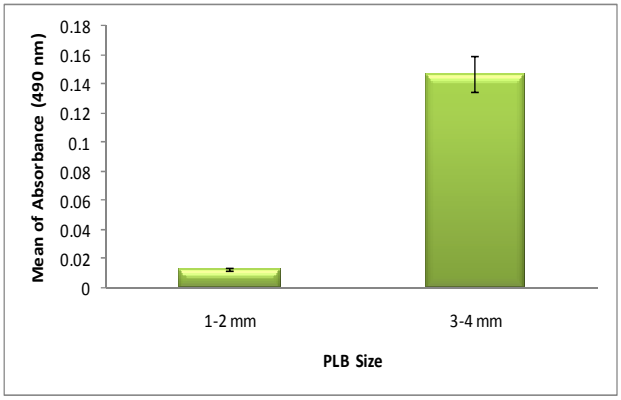

Fig 2. Diverse PLB size range on survival of non cryopreserved PLBs via encapsulation dehydration method. Based on Independent sample t test, P-value= 0.005.

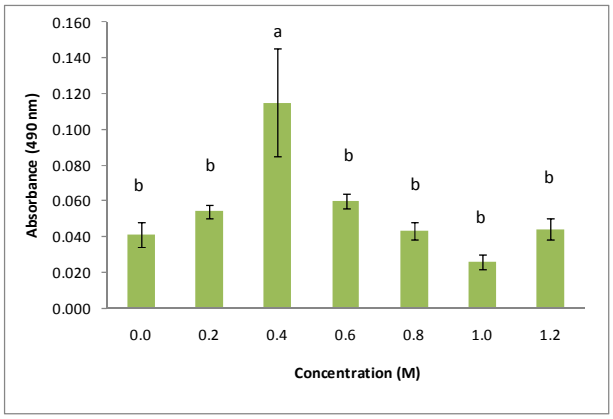

Fig 3. Diverse pretreatment conditions (sucrose) on survival of cryopreserved PLBs via encapsulation dehydration method**.** Results were analysed by one way ANOVA and means were compared by Tukey test.

cryopreserved explants but excessive concentration can be lethal. In the present study, the survival rate of PLBs that were pretreated in 0.4M sucrose was significantly different when compared to medium with other sucrose concentrations (Fig. 3; Fig. 4; Fig. 5). The PLBs also showed increasing survival rates when pretreated in 0.2 to 1.0M sorbitol. However, as the sorbitol concentration increased to 1.2 M the viability rates decreased. The survival rate of PLBs that were pretreated in 1.0M sorbitol was significantly different when compared to medium supplemented with other sorbitol concentrations (Fig. 4). Sucrose has been reported to be the most widely used sugar in cryopreservation techniques, hence its selection as the desired pretreatment agent in this study. Sucrose has been shown to maintain the plasma membrane integrity by substituting for water on the membrane surface, thus stabilizing protein under dry and freezing condition (Crowe et al., 1987). In cryopreservation of different orchids gave rise to different optimal concentrations such as; 0.6M sucrose preculture in *Dendrobium* Bobby Messina by vitrification method (Antony et al., 2010), 0.6M sucrose preculture in *Dendrobium* Sonia 28 by PVS2 vitrification method (Hooi et al., 2010), 0.75M sucrose preculture in *Phalaenopsis bellina* (Rchb.f.) christenson by encapsulation dehydration method (Khoddamzadeh et al., 2011), 0.5M sucrose preculture in *Dendrobium* Sonia 17 by encapsulation dehydration method (Subramaniam et al., 2010) and 0.8M sucrose preculture in *Brassidium* shooting star by encapsulation dehydration method (Yin at al., 2011). Therefore, these results suggest that different orchids may give rise to different optimal preculture conditions due to the genotypic differences of the plants involves. The viability of cryopreserved cells directly correlated with pretreatment duration (Bachiri et al., 1995). The viability of cryopreserved cells of *Catharanthus* and *Vaccinium pahalea* was found to be optimal if cells were pretreated for 3-7 days in 1M sucrose. The positive effect of extending durations of pretreatment with sucrose has been reported for *Dioscorea floribunda* (Mandal and Sangeetha, 2007). The present study presented with similar results in which PLBs pretreated in 0.4M sucrose for 3 days was significantly different comparative to other pretreatment durations tested (Fig. 6). 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 was pretreated with medium supplemented with 0.7M sucrose (Verleysen et al., 2005). The optimal water content of encapsulated tissues varies among plant species or even varieties mainly due to their difference in dehydration tolerance (Wang et al., 2000). Therefore, an optimization of the dehydration period was carried out to assure higher survival rates of cryopreserved plant tissues. The reduction of water content of synthetic seeds to minimal level is necessary step for cryopreservation of encapsulated explants (Bouafia et al., 1996). Cryopreservation by encapsulation dehydration techniques requires dehydration of encapsulated explants that have greatly reduce cell water but do not reach permanent wilting point and the cells vitrified at the temperature of liquid nitrogen will be able to regenerate (Benson, 1999). The low survival rate after dehydration with silica gel and the exposure to liquid nitrogen may be due to over-dehydration., which leads to inability of cryopreserved explants to rehydrate without cellular damage after thawing (Samia et al., 2002). In the present study, cryopreserved PLBs showed low survival rates when dehydrated (0-8 hours) based on the TTC assay (Fig. 7). However, in contrast, PLBs presented with high viability rate when were dehydrated for 9 hours. PLBs presented with decreasing viability rates when were dehydrated for 10 hours (Fig. 7). Similar results were obtained in cryopreservation of encapsulated shoot tips of *Dendrobium* Walter Qumae when dehydrated for 6-8 hours,

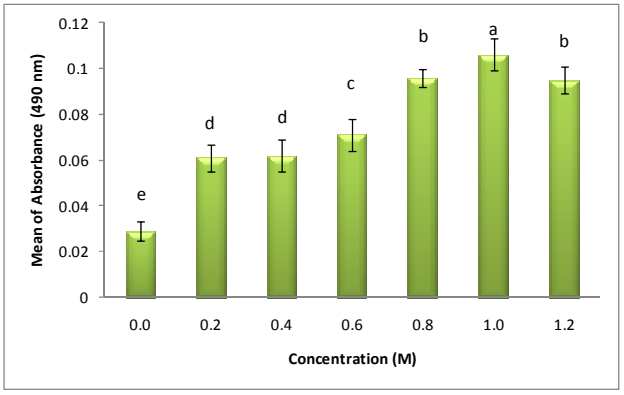

Fig 4. Diverse pretreatment conditions (sorbitol) on survival of cryopreserved PLBs via encapsulation dehydration method. Results were analysed by one way ANOVA and means were compared by Tukey test.

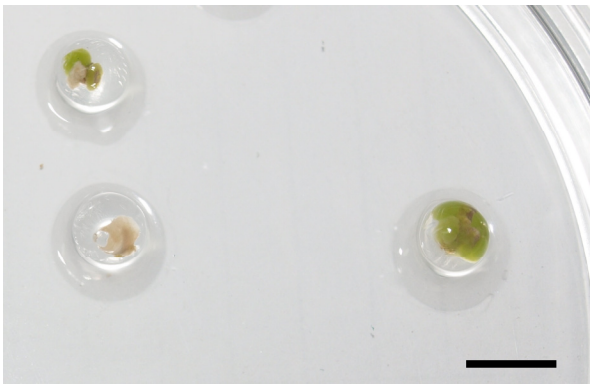

Fig 5. Closer view of regenerated cryopreserved PLBs pretreated on half strength semi-solid MS media with 0.4 M sucrose. After 3 months of growth recovery stage. Bars represent 0.3 mm.

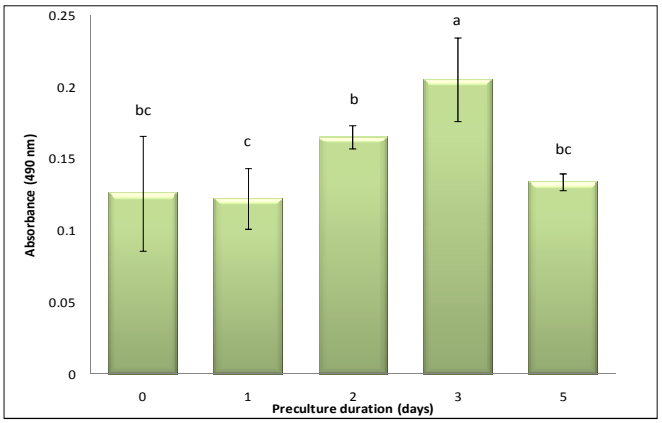

Fig 6. Diverse pretreatment duration on survival of cryopreserved PLBs via encapsulation dehydration method. Results were analysed by one way ANOVA and means were compared by Tukey test.

survival rate increased. However when dehydration period furthered till 10 hours, survival rate decreased drastically (Lurswijidjarus and Thammasiri, 2004).

Materials and methods

Plant Material

Protocorm-like bodies (PLBs) *in vitro* cultures of *Dendrobium* Bobby Messina were selected for cryopreservation in this study. Stock cultures were maintained in half strength Murashige and Skoog media (MS) (1962) with 1mg/L benzylaminopurine (BAP), 2% sucrose and 2.75 g/L Gelrite. The pH was adjusted to 5.8 prior to autoclaving. The PLBs were grown at 24°C under 16 hours photoperiod (Philips TLD, 36 W, 150µmol.m^{-2}.s^{-1}). The PLBs were then subcultured every 4 weeks (Antony et al., 2010).

Pre-treatment

For the optimisation of PLB size, 1-2 and 3-4 mm PLBs were selected from four-week old cultures and pretreated in half strength semi-solid MS media with 0.5M sucrose at 24°C for 24 hours under 16 hours photoperiod. For the optimisation of pretreatment sugar type and concentration, PLBs selected from four-week old culture were pretreated in half strength semi-solid MS media with different concentrations of sucrose or sorbitol (0.0, 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2M) at 24°C for 24 hours under 16 hours photoperiod. In the optimisation of pretreatment duration, PLBs selected from four-week old culture were pretreated in half strength semi-solid MS medium with 0.4M sucrose for 0, 1, 2, 3 and 5 days under 16 hours photoperiod (Antony et al.,2010).

Encapsulation, Osmoprotection, Dehydration

The PLBs were individually encapsulated in alginate beads (about 4-5 mm in diameter) by transferring them from liquid medium containing 3% sodium alginate into liquid 0.1M calcium chloride medium, both with 0.4M sucrose in half strength liquid MS media. After 30 minutes of gentle stirring, the beads were collected and osmoprotected in half-strength liquid MS medium with 0.75M sucrose on an orbital shaker (110 rpm) at 24°C for 24 hours under 16 hours photoperiod. Subsequently the beads were placed on a sterile filter paper in parafilm-sealed culture jar containing 50g of oven-sterilized (120°C) silica gel and were dehydrated in a laminar air flow cabinet for 4 hours.

Cryostorage, Thawing and Recovery

After dehydration, the beads were placed in 2ml cryovials and were directly plunged into liquid nitrogen for 24 hours. Cryopreserved beads were thawed in a water bath set at 40°C for 90 to 120 seconds. In the control experiments, the beads were subjected to all treatments except cryostrorage and thawing procedures. The beads were then cultured on half strength semi-solid MS media with 2% sucrose devoid of growth regulators for three weeks: the beads were placed in the dark for a week, followed by placement in dim light (95 lux) for another week, and 16 hours photoperiod (cool white fluorescent lamps [Philips TLD, 36 W] at 150µmol.m^{-2}.s^{-1}) in the third week.

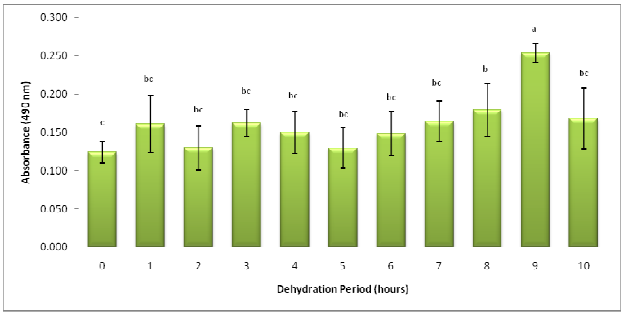

Fig 7. Diverse dehydration periods (0-10 hours) on survival of cryopreserved PLBs via encapsulation dehydration method. Results were analysed by one way ANOVA and means were compared by Tukey test.

Survival Assessment and Statistical Analysis

After 3 weeks of recovery, the survival of cryopreserved and non-cryopreserved PLBs were assessed based on growth observations and viability assay via the 2,3,5-triphenyltetrazolium chloride (TTC) spectrophotometrical analysis at 490nm (Verleysen et al., 2004). Each experiment consisted of six replicates per treatment, with each replicate containing 10 explants. All data were subjected to the Independent Sample t-test and One-Way ANOVA, and the differences in the means were compared using the Tukey HSD test (Antony et al., 2010).

Conclusion

The best PLB size for the cryopreservation of *Dendrobium* Bobby Messina by encapsulation dehydration method was 3-4 mm and the best pretreatment and dehydration condition for the PLBs involved the use of half-strength MS medium with 0.4M sucrose for three days and dehydrated for 9 hours.

Acknowledgements

This work was supported by Universiti Sains Malaysia Research University Research Grant Scheme (USM-RU) and National Science Fellowship (NSF).

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