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Cryopreservation technology for conservation of selected vegetative propagules

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ABSTRACT

Cryopreservation is the science of freezing biological materials and their subsequent storage at very low temperature in liquid nitrogen (-196°C). At such low temperature, metabolic rates are low or completely halted. Recently the need to conserve plant materials has increased tremendously due to extinction. Plants that produce orthodox seeds can be easily stored in the form of desiccated seeds at low temperature being the most convenient method to preserve plant germplasm. However, it is not applicable to crops that do not produce seed (e.g. bananas) or with recalcitrant or intermediate seed. In addition cryopreservation is now recognized as a powerful tool for the preservation of the unique genomic constitution of cultivars, tissue cultured lines and hybrids such as orchids. Various protocols, ranging from conventional slow freezing to the more modern vitrification based protocols have been established and utilized for various plant materials. To date there is still only limited number of plants whereby cryoconservation is readily used mainly because the techniques need to beadapted for each species. Therefore, continued efforts are needed in cryopreservation techniques to develop protocols for a wider range of plants. Conservation of plant germplasm has moved forward from the more costly slow cooling to vitrification based strategies. For effective adoption of the technique, simple reliable method with high regeneration ability has to be established. Our research in various plants has shown selection of plant material to be one of the most important steps in obtaining successful cryopreservation. In addition, different vitrification protocol can influence the success rate as well. This paper will highlight the importance of explant selection and the importance of method selection for successful cryopreservation using some selected crops.

***Keywords*:** Conservation, cryoconservation, propagule, vitrification

CASE STUDIES

Orchid protocorm-like bodies

Orchidaceae is a family of flowering plant with high ornamental value. Numerous orchid species exist to date, not to mention the number of hybrids that are being produced at high frequency. In order to produce true to type virus free plants, micropropagation is preferred for the commercial propagation of all types of orchids. Micropropagation through induction of protocorm-like bodies (PLBs) are considered as the efficient method of propagation because it can be rapidly proliferated where large number of PLBs can be produced in a short period of time (Sheelavanthmath et al. 2005) and each of them can be regenerated into a new plant (Park et al. 2000). PLB is actually the somatic embryo for orchid developed from nongametophytic somatic cells in culture medium (Sterk and de Vries 1993). Protocorm-like bodies of *Dendrobium* Shavin White were induced from *in vitro* shoot tip culture and the resulting PLBs were proliferated on ½ strength MS liquid medium supplemented with myo-inositol (50.0 $mg.l^{-1}$), glycine (1.0 $mg.l^{-1}$), nicotinic acid (0.25 $mg.l^{-1}$), pyridoxine HCL (0.25 $mg.l^{-1}$), thiamine HCL (0.05 $mg.l^{-1}$), ferrous sulfate (13.9 $mg.l^{-1}$), Na_2 EDTA (18.65 $mg.l^{-1}$), sucrose (15 $g\,L^{-1}$) and BAP (1.0 $mg.l^{-1}$).

During the above mentioned stage, PLB proliferation takes place but within a single flask PLBs of varying sizes can be observed. It is believed that for successful regeneration of PLBs, be it before or after cryopreservation, the best growth stage has to be selected and established. Based on the growth and developmental pattern of PLBs, they were separated into five categories based on size and morphology, namely d"2 mm (S1), >2 – 4 mm (S2), >4 – 6 mm (S3), >2 – 4 mm with shoot (S4) and >4 – 6 mm with shoot (S5) (Bustam et al., 2013). The PLBs were cultured for 8 weeks with frequent observation to determine the time taken for germination, germination percentage, time taken for conversion and conversion percentage. The size and morphology of PLBs resulted in variation in the ability to germinate. PLBs with shoot irrespective of the size (S4 and S5) germinated earlier, within 12 - 13 days as compared to PLBs without shoot (S1, S2 and S3) which required 27 - 29 days to germinate. It is the norm in literature to recommend the use of explant based on size, but this study has demonstrated that size alone is insufficient as conversion into plantlet was influenced by the morphology. Conversion into plantlet occurred

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between 31 - 44 days after culture in all treatments with S4 and S5 (with shoot) category having 85 – 90% conversion while PLBs without shoot (S1, S2 and S3) only had 30 – 50%. This study has shown that for successful conversion, PLBs with shoot irrespective of size has to be selected for rapid and uniform conversion which will than influence the survival after cryopreservation.

Oil palm polyembryoids

The importance of method selection for cryopreservation is illustrated using another monocot species *i.e.* oil palm polyembryoids. Palm oil is the prevalent source of edible oil in the world. It produces about seven times more oil than the best yields of other oil crops (Mielke, 1991) in terms of oil/hectare/year. It has been reported that tissue cultured oil palm resulted in 25–30% increase in oil production (Cochard et al., 2000), thus in recent years micropropagation of oil palm via somatic embryogenesis has become the key method for multiplication of oil palm elite genotypes. As the growth of somatic cell in culture cannot be arrested, cryopreservation offers a good means to do so. Oil palm polyembryoids were subjected to various methods of cryopreservation namely PVS2 vitrification, encapsulation vitrification and droplet vitrification. Based on our experience, selection of initial explant for cryopreservation was also prevalent in oil palm polyembryoids. Hence for this study, torpedo-shaped polyembryoids with haustorium as explained by Palanyandy (2013) (Figure 1a) was used in order to obtain rapid and uniform plantlets. Vegetative propagules are highly hydrated and therefore, the direct desiccation method is often unsuitable. The use of vitrification approach *i.e.* a form of chemical dehydration using a viscous solution to induce the glassy state during freezing instead of ice crystal formation is the preferred method. A concoction of chemicals termed vitrification solution is used to achieve this target. The most common vitrification solution reported in literature is PVS2 (Sakai et al., 1990). However, there are other recipes that have proven to be more suitable depending on the explant used. Whichever recipe used, they bear relatively the same consequence to the explant whereby the highly concentrated chemicals used causes a shock due to the high concentration. Hence often this effect is reduced by a preceding step termed 'Loading' which has a lower concentration of chemical in order to expose the explants first to milder conditions. The polyembryoids were exposed to six different Loading Solution (LS) for 1 h. MS basal medium was used as control, as all LS were prepared using the MS basal medium. The LS consisted of, L1 [2.0 M glycerol 0.4 M sucrose] (Nishizawa et al. 1992), L2 [1.5 M glycerol + 0.4 M sucrose + 5% (w/v) dimethyl sulphoxide (DMSO)] (Matsumoto et al., 1995), L3 [0.5 M glycerol + 0.3 M sucrose + 10% (w/v) DMSO] (Matsumoto et al., 1995), L4 [25% plant vitriûcation solution 2 (PVS2, Sakai et al. 1990) + (30% (w/v) glycerol 15% (w/v) ethylene glycol (EG) + 15% DMSO + 0.4 M sucrose] and L5 [10% (w/v) DMSO + 0.7 M sucrose] (Matsumoto et al., 1995). Amongst the different loading solutions tested, L5 which consist of 10% DMSO and 0.7 M sucrose gave the best response as compared to control, meaning to say damage due to exposure to LS was minimal. A further experiment to obtain the best time of exposure revealed that 30 min was sufficient to give the desired effect without damage to the cells (Suranthran et al.,

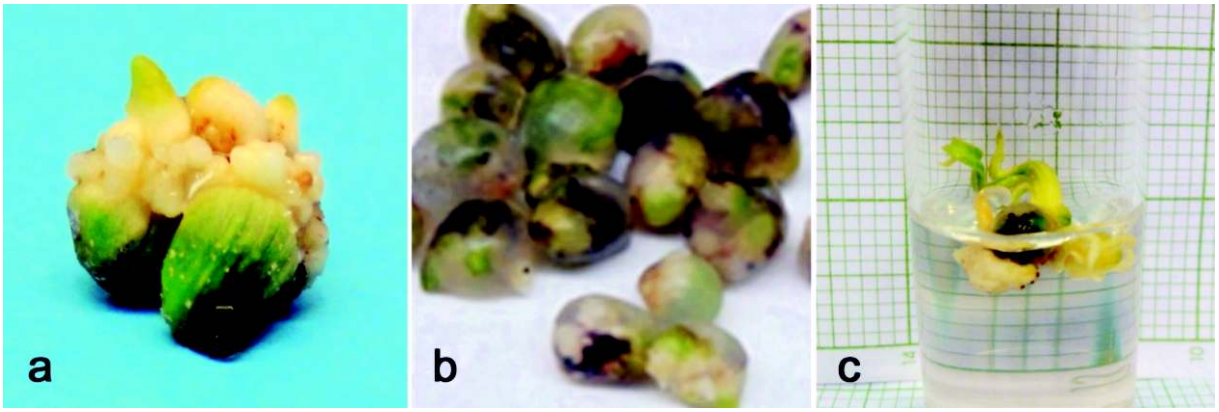

Fig. 1: a. The identified oil palm polyembryoid stage consisting distinct haustorium, torpedo shaped embryoids and secondary somatic embryo, optimal for cryoconservation; b. freshly encapsulated oil palm polyembryoid in calcium-alginate beads; c. post-cryo survival of polyembryoids with initiation of new shoot primordia.

2012). This was further tested for tolerance to Vitrification Solution (VS). The polyembryoid treated with L5 for 30 minutes were subjected to various VS namely, PVS (Uragami et al., 1989)], PVS2 (Sakai et al., 1990)], PVS3 (Nishizawa et al., 1993)], L-solution (Ishikawa et al., 1997)], Towill solution (Towill, 1990)] and Watanabe solution (Watanabe and Steponkus, 1995)] for 10 min. The final outcome of establishing the type of VS and exposure time gave 45% survival after exposure to liquid nitrogen when the polyembryoids were exposed to PVS2 for 5 min (Suranthran et al., 2012).

The polyembryoid is a structure that is highly meristematic and therefore the loss in meristematic region can be easily damaged due to exposure to highly concentrated toxic vitrification solution. In an attempt to try and minimize the effect, the encapsulation technique was used prior to cryopreservation. The isolated polyembryoid was placed in calcium-free liquid basal medium with 3% sodium alginate (Gonzalez-Arnao and Engelmann, 2006) and the mixture was dropped with a pipette into liquid culture medium containing 100 mM $CaCl_2$. The resulting beads (Figure 1b) were precultured on increasing concentration of sucrose 0.3M, 0.5M, 0.75M and finally 1.0M (transfer was done every alternate day) prior to subjecting them to liquid nitrogen exposure. At the end of 7 days in sucrose preculture liquid media, the beads were desiccated in the Laminar Air-Flow Cabinet for different periods. The results obtained were a vast improvement to that obtained for naked polyembryoid cryopreserved using the vitrification methods described earlier. The survival percentage after removal from liquid nitrogen was 73.3% (Fig 1c) if subjected to liquid nitrogen after ~9 hours of desiccation giving a moisture content of 23%. The protection provided by the alginate bead appeared to be necessary for avoiding the direct damage to the meristematic cells.

In recent times, the use of the droplet vitrification method has been mentioned to surpass the benefit of other techniques resulting in increased survival after cryopreservation. This approach was attempted on oil palm polyembryoids to see if it could improve the survival after cryopreservation to above 73%. Polyembryoids of oil palm were precultured on liquid MS medium supplemented with 0.5 M sucrose for 12 h. The polyembryoids were osmoprotected in loading solution [10% (w/v) DMSO plus 0.7 M sucrose] for 30 min at room temperature and then drenched in chilled droplets of PVS2 solution [30% (w/v) glycerol plus 15% (w/v) EG plus 15% (w/v) DMSO plus 0.4 M sucrose] for 10 min with individual polyembryoid placed on aluminium strips. Polyembryoids were then plunged quickly in liquid nitrogen and kept for 1 h. The polyembryoids were then thawed and unloaded (using 1.2 M sucrose solution) with subsequent transfer to regeneration medium which resulted in successful revival of ~80% (Gantait *et al*., 2014). Hence, by attempting different methods the survival of oil palm polyembryoids can be improved and increased from 45 to 80%.

This paper is presented in such a manner to show the importance of selection of explant in order to obtain good revival of the cryopreserved material. In addition the importance of the method selection for cryopreservation plays and important role as clearly shown in the case of oil palm polyembryoids.

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