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ORIGINAL ARTICLE

Cryopreservation of *Brassia rex* Orchid Hybrid Using DMSO Vitrification Technique

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ABSTRACT

The main objective of this study is to establish a preliminary cryopreservation technique for *Brassia rex* hybrid orchid. Cryopreservation was carried out using DMSO vitrification method using PLBs (protocorms like-bodies) derived from *in vitro* culture. There are two parts of experiment were conducted in this study. First part, *Brassia rex* PLBs was divided into two parameter sizes: size type 1 (0.00-0.30 mm) and size type 2 (0.31-0.61 mm) and were precultured on half strength Murashige and Skoog (1962) medium supplemented with various sucrose concentrations (control [0.06M], 0.10 M, 0.25 M, 0.50 M and 0.75 M) in two different time duration (24 and 48 hours). The viability of PLBs was determined by using 2,3,5-triphenyltetrazolium chloride (TTC test) and the best viability was obtained in size type 2 PLBs that was precultured in 0.5 M sucrose at 48 hours. Using these optimized parameters, the second part of the research was conducted. The PLBs were subjected to the 7.5% DMSO treatment for 0, 10, 20 and 30 min at two different temperatures (0ºC and 24ºC) prior to storage in liquid nitrogen (-196 ºC). The viability and the total chlorophyll content of PLBs were evaluated. Overall, the highest viability from cryopreserved PLBs was obtained with the following combinations: preculture in 0.5 M sucrose at 48 hours and 10 min dehydration with 7.5% DMSO treatment at 0 ºC. Thus, optimized cryopreservation technique for selected *Brassia rex* hybrid orchid can be carry out using PLBs precultured at 0.5 M sucrose for 48 hours and 10 min dehydration with 7.5% DMSO at 0 ºC.

Key words: *Brassia rex.* DMSO vitrification. PLBs

Introduction

The orchidaceae is one of the largest and most diverse families of flowering plants including up to one tenth of all flowering plant species in the world (Dressler, 1993). Although Orchidaceae is the largest family of plants (25000–35000 species), the International Union for Conservation of Nature and Natural Resources (IUCN) has put almost all its species on the list of endangered species known as the *Red Data Book* . In addition to the IUCN Red Data Book, the orchids are also under protection of the Convention on International Trade in Endangered Species of Wild Fauna and Flora.

The two main reasons underlie the situation are; the first is vulnerability of orchids because of their complex reproductive biology. The flowers of these highly evolved plants are often beautiful and sometimes bizarre with complex pollination mechanisms involving specific insect pollinator (Dressler, 1981). The second is powerful anthropogenic press (destruction of habitats). Many orchids are now considered to be risk of

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extinction as an indirect or direct result of human activities, which include habitat alteration or destruction and extraction of wild plants for trade. These processes and the phenomenal diversity of orchid have resulted in many threatened orchid species (Hagsater and Dumont, 1996). The impact of alternation or habitat destruction on orchid taxa will depend on its geographical distribution, habitat specificity, and population size (Rabinowitz *et al*., 1986). For example, *Epidendrum floridense*, a florida epiphytic orchid species appears to have nearly become extinct as a consequence of severe unseasonal frosts (Hagsater, 1993). Cryopreservation is one of the method of preserving important germplasm by storage at liquid nitrogen temperatures (-196°C) (Pritchard, 1984). Cryopreservation is found to be the most successful method for long-term storage of genetic resources especially in orchids.

The present study was carried out to establish a suitable method for the improvement of cryopreservation as a technique for long term conservation of endangered orchid species as well as commercially useful orchids. Therefore, the aim of this study was:

- to evaluate the effects of various sucrose concentrations (0, 0.1, 0.25, 0.5, and 0.75M) on explants that precultured at 24 and 48 hours,
- to study the effects of DMSO at different times (0, 10, 20, 30 min) and different temperatures (0 and 24 °C) prior to storage in liquid nitrogen,
- to establish efficient cryopreservation technique, the survival rate of explants after immersed in liquid nitrogen was determined. The viability of the explants was tested by staining with 2,3,5-triphenyltetrazolium chloride (TTC) and also with total chlorophyll determination.

Materials and Methods

Plant Materials:

The *in vitro*-grown protocorm-like bodies (PLBs) of *Brassia Rex* orchid hybrid were used in this study. This material was used as the starting material to initiate multiplication of shoots for the study. The cultures were incubated at 25±2ºC in a 16h photoperiod under cool white fluorescent lamps (Philips TLD, 36 W) at 150 μmol m^{-2} s^{-1}.

Excision and Pretreatment of PLBs:

The PLB clumps were aseptically dissected apart and measured into two parameters; size type 1 PLBs (0.0cm-0.3cm) and saiz type 2 PLBs (0.31cm-0.60cm). The dissected PLBs were transferred onto plastic Petri dish containing half-strength MS (Murashige and Skoog, 1962) solid media supplemented with sucrose according to their predetermined pretreatment concentrations (0.6M, 0.10M, 0.25M, 0.5M and 0.7M). Each sucrose concentration were placed six single PLBs and three replicates. Then the excised PLBs were then pretreated for 24 and 48hours.

Effect of Temperature (0ºc) During DMSO Dehydration Process in the PLBs Viability:

After PLBs were pretreatment with sucrose in part 1, the viability testing showed that size type 2 and 0.5 M sucrose concentration preculture for 48 hours had higher viability to survive. Therefore, this sucrose concentration and duration of pretreatment was used as pretreatment parameter to continue doing cryopreservation in part 2.

Therefore, size type 2 PLBs clumps were dissected apart aseptically in the laminar flow and the dissected PLBs were transferred onto plastic Petri dish containing half-strength MS semi-solid supplemented with 5.0 M sucrose concentration. Each Petri dish was placed eight single PLBs with eight replicates. The excised PLBs were then pretreated for 48hours and the samples of PLBs were transferred onto the cryovials. Each cryovials were placed 8 PLBs, therefore, overall had 8 cryovial with 8 PLBs each and then PLBs were immersed in a mixture of 2 M glycerol and 0.4 M sucrose dissolved in MS medium (pH 5.8) for 20min at room temperature. After removing the loading solution with a Pasteur pipette, 3 ml of DMSO solution 7.5% was added. The PLBs were subjected to the DMSO solution for 0, 10, 20 and 40 min at 0ºC. Each treatment contains positive control (+LN) and negative control (-LN). After the DMSO treatment, positive containing about eight single PLBs, were immediately plunged into liquid nitrogen (+LN) and held there for at least an hours. While, another negative control undergoes thawing process and directly cultured onto half-strength MS semi-solid medium (-LN).

After freezing in liquid nitrogen, the cryovials were then rapidly thawed in a water bath at 40ºC for 90

second. Then, the toxic DMSO solution was removed and replaced by an unloading solution for 30 min at room temperature. Next, PLBs were placed onto sterile filter paper on top of half-strength semi-solid MS medium containing 2.0g/L sucrose. After two days, PLBs that undergoes +LN and –LN were transferred onto regeneration medium without filter paper. The first two weeks of culture was always in the dark. After two weeks, survival rates were estimated.

Effect of Temperature (24ºC) During DMSO Dehydration Process in the PLBs Viability:

The experiment was exactly the same as experiment that carried out for 0ºC except that the PLBs were treated with DMSO 7.5% at 24ºC.

Thawing, Unloading and Regeneration:

After freezing in liquid nitrogen, the cryovials were then rapidly thawed in a water bath at 40ºC for 90 second. Then, the toxic DMSO solution was removed and replaced by an unloading solution for 30 min at room temperature. The unloading solution consisted of 1.2 M sucrose dissolved in MS medium (pH 5.8). Next, PLBs were placed onto sterile filter paper on top of half-strength semi-solid MS medium containing 2.0g/L sucrose. After two days, the PLBs were transferred onto regeneration medium without filter paper. The first two weeks of culture was always in the dark. After two weeks, survival rates were estimated.

Determination of Tissue Viability: 2,3,5-triphenyltetrazolium Chloride (TTC):

The 2,3,5-triphenyltetrazolium chloride (TTC) test was performed according to Rasmussan (1995). In this method, cell survival is estimated by the amount of formazan produced as a result of reduction of TTC. The amount of formazan produced by the frozen cells is expressed as a percentage (survival) of formazan produced by the control cell suspension. This reaction results in a pink colour. The procedure involves the following steps; buffer solution: 78% $Na_2HPO_4.2H_2O$ solution 0.05 M (8.9 g/L): 22% KH_2PO_4 solution 0.05 M (6.8 g/L).TTC solution: 0.18 M TTC dissolved in buffer solution. About three PLBs samples was put into 3 ml of TTC solution and incubated for 15-20 hours at 30ºC. The TTC solution was drained off and the PLBs washed with distilled water. Cells are centrifuged and extracted with 7 ml of ethanol (95%) in a water bath at 80ºC for 5 min. The extract is cooled and made to 10 ml volume with 95% ethanol. The absorbance (pink colour) is then recorded with a spectrophotometer (Spectro 22, Digital Spectrophotometer, Labomed. Inc.) at 530 nm.

Chlorophyll Determination (Harborne, 1973):

For the extraction of chlorophyll samples, 0.5g of fresh PLBs from each treatment samples were grounded with 1.0g of calcium carbonate ($CaCO_3$) power and 5 ml of 80% acetone solution at 4ºC using chilled mortar and pestle (in the ice box). Then, the ground extract was filtered with Bunchner funnel through the Whatmann No.1 filter paper, followed by washing the mortar and pestle with 80% acetone solution. Finally, the extraction volumes were added up to 25 ml using volumetric flask with 80% acetone solution. The absorbance readings were measured at 646nm and 663nm using spectrophotometer and the readings were taken 3 times for each sample. The chlorophyll contents (µg/ml) were calculated using formula as below:

Chlorophyll *a* = $12.21\ A_{663nm} - 2.81\ A_{646nm}$
Chlorophyll *b* = $20.13\ A_{646nm} - 5.03\ A_{663nm}$
Total chlorophyll content (µg/ml) = $17.30\ A_{646nm} + 7.18\ A_{663nm}$

$$\text{The chlorophyll concentration express on } \mu g/g = \mu g/ml \times \frac{\text{Extraction volume (ml)}}{\text{Fresh weigh (g)}}$$

Results and discussion

Effect of the PLBs Size on Viability Capacity after Sucrose Pretreatment:

The viability observations of the PLBs in Part I of the research was based on the TTC assay. Figure 1 and 2 showed a significant difference between two *Brassia rex* PLBs sizes, size type 1 (0.00-0.30 cm) and size type 2 (0.31-0.61 cm). The size type 2 PLBs (0.31-0.61 cm) shown to have higher absorbance when

compared to the size type 1 PLBs (0.00-0.30 cm).

In addition, there were no significant differences were obtained according to TTC assay between the sizes when they were compared according to their pretreatment concentrations. Based on these observations, the size type 2 PLBs (0.31-0.61 cm) were selected to continue Part II of the research. However, the first part of the research was plagued with high variances in the data obtained due to insufficient replicated that were employed for each treatment. Furthermore, probably that the absorbance obtained for the PLBs was a function of the size, the surface area and the amount of the tissues present in the samples, with size type 2 PLBs (0.31-0.61 cm) giving higher absorbance when compared to size type 1 PLBs (0.00-0.30 cm).

Effects of Preculture Duration and Sucrose Concentration on Brassia Rex PLBs Viability from Sucrose Pretreatment:

In Figure 1 and 2, the effect of sucrose preculture concentration on viability of *Brassia rex* PLBs is presented. The viability observation of PLBs in Part I of the research is based on the estimation of the amount formazan produced from the reduction of TTC due to the action of dehydrogenases in living cells or tissues. Comparing between the five sucrose concentrations (control [0.06M], 0.1 M, 0.25 M, 0.5 M, and 0.75 M) it was shown that sucrose concentration in the preculture medium should not exceed 0.5 M, in order to achieve high PLBs viability. A sucrose concentration of 0.75 M decreased significantly the viability of PLBs. This showed by the decrease of mean absorbance per milligram of PLBs at 0.75 M sucrose concentration (Fig. 1, 2). Among all concentrations of sucrose tested, the highest absorbance per milligram of PLBs was obtained using 0.50 M sucrose. Treatment with sucrose for 48 hours does not always significantly increase the viability of size type 2 PLBs (Fig. 4) whereas it significantly increases the viability of size type 1 PLBs (Fig. 3). Therefore, it can be recommended that 0.5 M sucrose preculture for 48 hours is the best sucrose preculture concentration and duration for viability of *Brassia rex* PLBs before DMSO vitrification treatment.

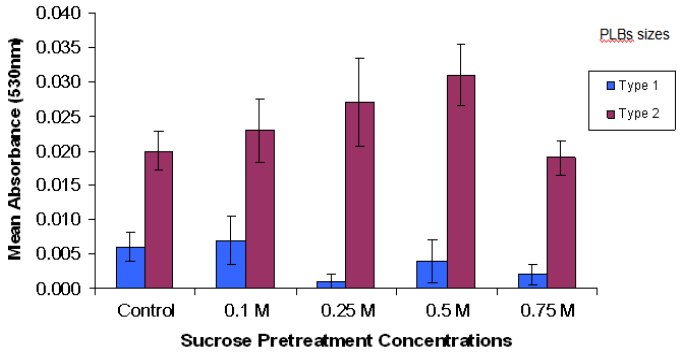

Fig. 1: Preculture of *Brassia rex* PLBs with different concentrations of sucrose for 24h. TTC stainability of the PLBs precultured in half strength MS semi-solid medium with control (0.06 M), 0.1, 0.25, 0.5, and 0.75M sucrose. Error bars represent the standard error of TTC stainability.

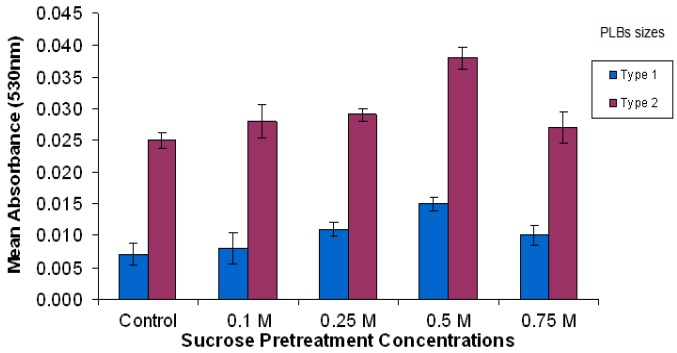

Fig. 2: Preculture of *Brassia rex* PLBs with different concentrations of sucrose for 48h. TTC stainability of the PLBs precultured in half strength MS semi solid medium with control (0.06 M), 0.1, 0.25, 0.5, and 0.75M sucrose. Error bars represent the standard error of TTC stainability.

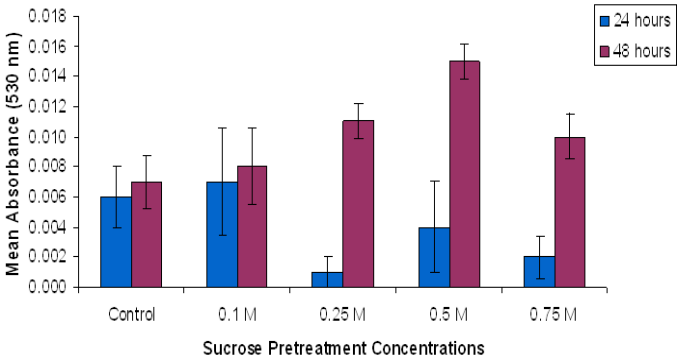

Fig. 3: Effect of sucrose concentration and duration of sucrose pretreatment in size type 1 *Brassia rex* PLBs. Error bars represent the standard error of TTC stainability.

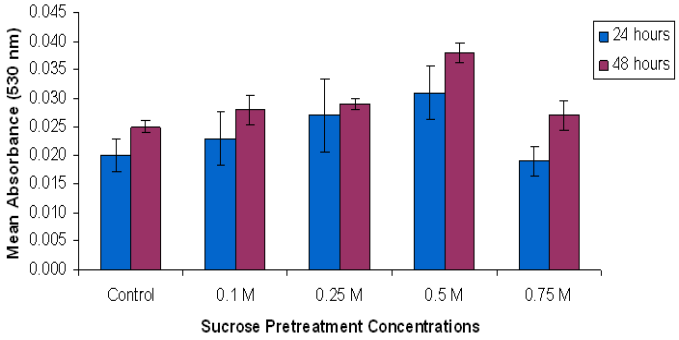

Fig. 4: Effect of sucrose concentration and duration of sucrose pretreatment in size type 2 *Brassia rex* PLBs. Error bars represent the standard error of TTC stainability.

Effects of Duration Exposure to DMSO Treatments at 24ºC and 0ºC on Brassia Rex Plbs Viability prior to cryopreserved (+LN) and non cryopreserved (-LN) PLBs:

For the TTC test, Figure 5 and 6 showed the viability of *Brassia rex* PLBs after exposure to variety length of DMSO treatment at two different temperature (24ºC and 0ºC), with (+LN) and without (-LN) their subsequent storage in LN. The inclusion of PLBs in DMSO vitrification maintained at 0ºC gave the best results in terms of viability of PLBs. It is interesting to note that the positive effect resulting from the treatment at 0ºC, with 10 minutes of exposure to DMSO treatment was more evident in PLBs which both subsequently underwent storage in liquid nitrogen (+LN) and does not undergoes storage in liquid nitrogen (-LN).

Figure 7 shows the effect of exposure of PLBs to DMSO treatment at 24ºC for times ranging from 0-30 min. The best protection of the PLBs from the damage caused by ultra-rapid freezing (+LN) was achieved at 30-min treatment in DMSO was applied. The highest PLBs viability tested by TTC assay after cryopreservation was obtained following a 30-min treatment with DMSO. However, the best viability of PLBs that does not underwent cryopreservation shown by 0-min of DMSO treatment. When comparing between cryopreserved (+LN) and non-cryopreserved (-LN) PLBs using various duration of DMSO treatments, it has been shown that non cryopreserved (-LN) gave the highest viability value. Whereas, for effect of exposure of PLBs to DMSO treatment at 0ºC for times ranging from 0-30 min, best protection of the PLBs from the damage caused by ultra-rapid freezing (+LN) was achieved when a 10-min treatment in DMSO was applied. The highest PLBs viability tested by TTC assay after cryopreservation was obtained following a 10-min treatment with DMSO. However, there are no significant different between cryopreserved (+LN) and non cryopreserved (-LN) PLBs viability at 10 min treatment. The mean absorbance per milligram for both cryopreserved (+LN) and non cryopreserved (-LN) PLBs at 10 min DMSO treatment gave the same values. The lowest viability of cryopreserved PLBs was recorded by 20 min exposure to DMSO but it shown an increasing pattern at 30 min DMSO treatment (Fig. 8). For the overall of Part II experiments, using TTC test, it can be concluded that size type 2 *Brassia rex* PLBs which was underwent 10 min DMSO treatment maintain at 0ºC have the best viability (Fig. 8).

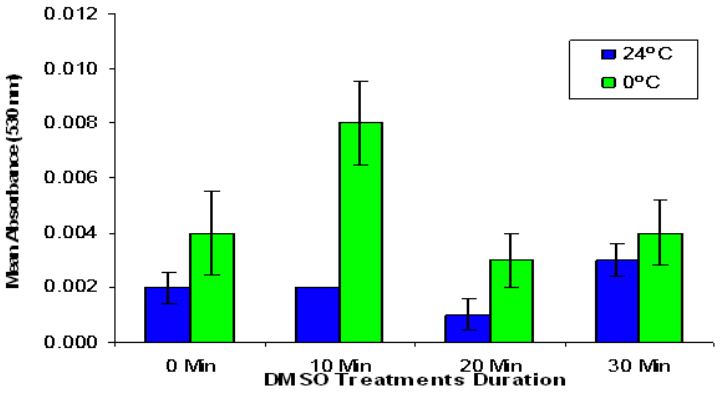

Fig. 5: Effect of exposure to DMSO treatment at 24ºC and 0ºC on PLBs viability from cryopreserved (+LN) *Brassia rex* PLBs. Viability of PLBs wasdetermined after 21 days recovery and the error bars represent the standard error of TTC stainability.

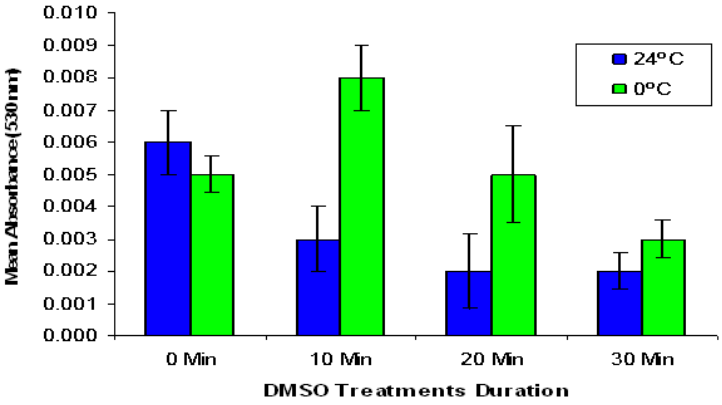

Fig. 6: Effect of exposure to DMSO treatment at 24ºC and 0°C on PLBs viability from non cryopreserved (-LN) *Brassia rex* PLBs. Viability of PLBs was determined after 21 days recovery and the error bars represent the standard error of TTC stainability.

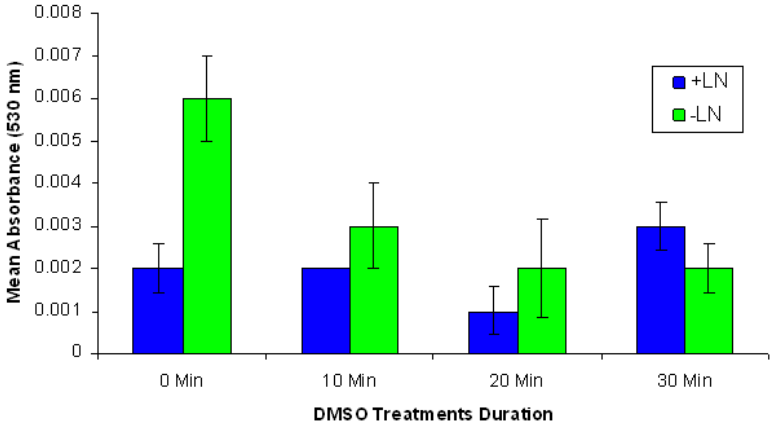

Fig. 7: Effect of exposure to DMSO treatment at 24ºC on PLBs viability from cryopreserved (+LN) and non cryopreserved (-LN) *Brassia rex* PLBs. Viability of PLBs was determined after 21 days recovery and the error bars represent the standard error of TTC stainability.

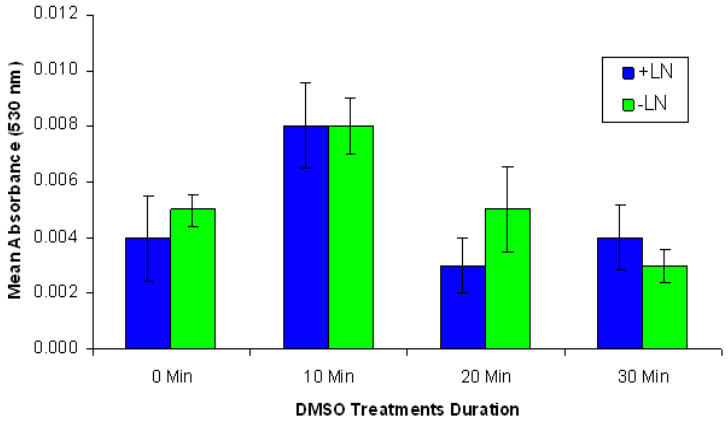

Fig. 8: Effect of exposure to DMSO treatment at 0ºC on PLBs viability from cryopreserved (+LN) and non cryopreserved (-LN) *Brassia rex* PLBs. Vibility of PLBs was determined after 21 days recovery and the error bars represent the standard error of TTC stainability.

Total Chlorophyll Determination:

Figure 9 and 10 show the total chlorophyll contents of PLBs that exposed to DMSO treatments at 0ºC and 24ºC for time ranging from 0-30 min with both cryopreserved (+LN) and without cryopreserved (-LN). The kinetic of the exposure was critical in term of chlorophyll contents after storage in LN. The highest chlorophyll content was achieved by non cryopreserved PLBs that exposed to 10 min DMSO treatment at 0ºC. The amount of chlorophyll *a* and chlorophyll *b* being detected in these PLBs has no significant different. Based on Figure11 and 12, non Cryopreserved PLBs have a significant different compared with cryopreserved PLBs. This figure clearly illustrates that total chlorophylls content for non cryopreserved PLBs (-LN) were higher than cryopreserved PLBs (+LN). If compared among cryopreserved PLBs, the highest chlorophyll content was achieved at 10 min treatment in DMSO treatment. Based on Figure 9 and 10, the amount of chlorophyll *b* was higher than the amount of chlorophyll *a* detected in the cryopreserved PLBs treated with 10 min DMSO treatment at 0ºC.

Besides, there was a significant different were obtained between the non cryopreserved PLBs when they are compared according to their treatment temperatures (0ºC and 24ºC) (Fig. 13). Treatment at 0°C gave the higher chlorophyll content compared to treatments at 24°C. Whereas, for cryopreserved PLBs (+LN), no significant differences were obtained between the temperatures (0ºC and 24ºC).

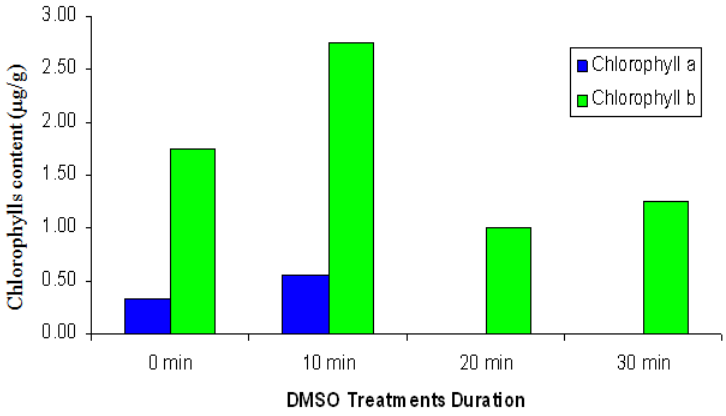

Fig. 9: Chlorophyll *a* and chlorophyll *b* content of cryopreserved *Brassia rex* (+LN) PLBs treated with DMSO treatment at 0ºC. Total chlorophyll content of PLBs was determined after 21 days of recovery using total chlorophyll determination (Harborne, 1973).

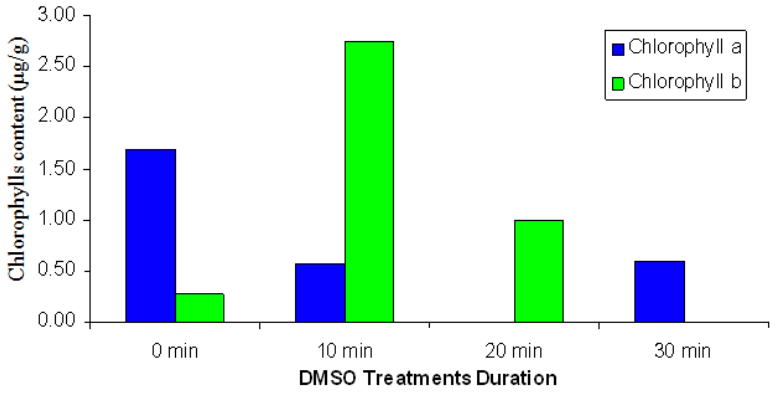

Fig. 10: Chlorophyll *a* and chlorophyll *b* content of cryopreserved *Brassia rex*(+LN) PLBs treated with DMSO treatment at 24ºC. Chlorophyll content of PLBs was determined after 21 days of recovery using total chlorophyll determination (Harborne, 1973).

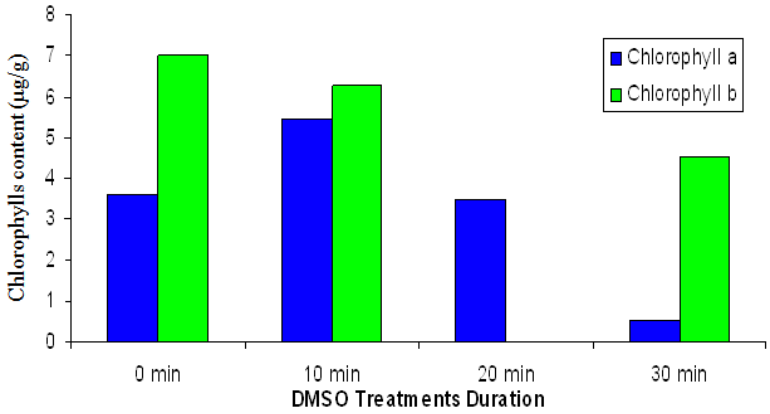

Fig. 11: Chlorophyll *a* and chlorophyll *b* content of non-cryopreserved *Brassia rex* (-LN) PLBs treated with DMSO treatment at 0ºC. Chlorophyll content of PLBs was determined after 21 days of recovery using total chlorophyll determination (Harborne, 1973).

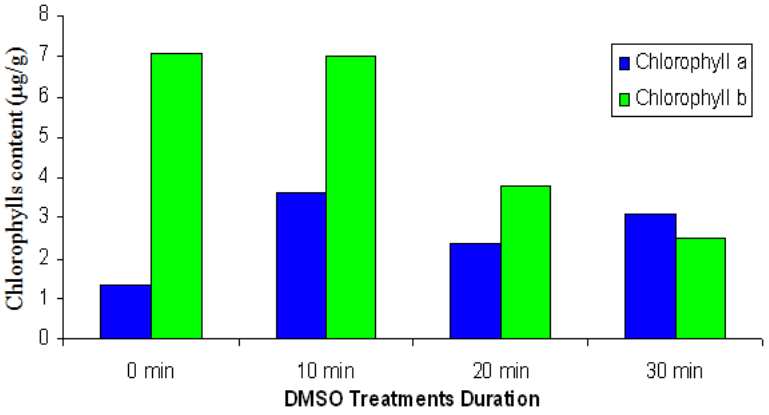

Fig. 12: Chlorophyll *a* and chlorophyll *b* content of non-cryopreserved *Brassia rex* (-LN) PLBs treated with DMSO treatment at 24ºC. Chlorophyll content of PLBs was determined after 21 days of recovery using total chlorophyll determination (Harborne, 1973).

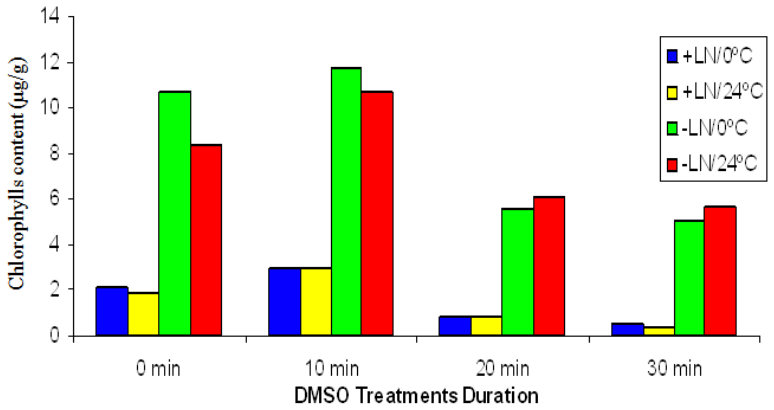

Fig. 13: Overall comparison of total chlorophyll content of non-cryopreserved and cryopreserved *Brassia rex* PLBs treated with DMSO treatment at 0°C and 24ºC. Chlorophyll content of PLBs was determined after 21 days of recovery using total chlorophyll determination (Harborne, 1973).

Discussion

Effect of the PLBs Sizes on Viability Capacity after Sucrose Pretreatment:

The control of water content of plant samples before freezing was the key factor in developing successful cryoprotection protocols as previously reported. When PLBs are not dehydrated sufficiently, freezing-injury can occur due to intracellular ice formation and when over-dehydrated, the osmotic stress can be damaging. The capacity to survive storage in LN is dependent upon many factors including genotype, physiological status and pre- and post-freezing manipulations (Lipavska and Vreugdenhil, 1996).

Therefore, the type and the sizes of explants choose also ones of the factors. Shoot explant, are widely used due to their high genetic stability, high survival and regrowth percentages. Shoot are characterized by their small dense and actively dividing cells and it ensures rapid multiplication rates after thawing. Besides, the low water content of shoot cells would propose a strong reason for choosing them as a basic plant material for cryopreservation (Ashmore, 1997). Manipulations of different sizes of the explants play an important role in the survivability of the tissues in the cryopreservation. Throughout the research, size type 2 PLBs (0.31-0.61 cm) had displayed better viability compared to the size type 1 PLBs (0.00-0.30 cm). This implies that the large PLBs size is able to withstand the entire DMSO vitrification protocol better that smaller PLBs. However, too large of explants also not good to used as starting material for cryopreservation since it have large vacuole volume and difficult to dehydrate it and the probability to form damage caused by crystallization of this water (Villalobos *et al*., 1993) and lower the post-thaw regeneration rates since the bigger explants might be attributed to less efficient heat exchange during cooling as well as thawing (Panis *et al*., 2005). Explants which are too small, often suffer from mechanical damage to the apical dome and also more difficult manipulation of small explants (Shibli *et al*., 2006) although , small cells aggregates have a higher freeze-tolerance than large cell aggregates (Lipavska and Vreugdenhil, 1996).

Effects of Preculture Duration and Sucrose Concentration on Brassia Rex PLBs Viability from Sucrose Pretreatment:

For successful cryopreservation, it is essential to avoid lethal intercellular freezing, which occur during rapid cooling in liquid nitrogen (Sakai and Yoshida, 1967). Extracellular freezing is considered as effective method of dehydrating living cells (Sakai *et al*., 1991). Freezing tolerance of explants can be increased by cold hardening and preculture in media with high level of osmotic agents before exposure to liquid nitrogen. Osmotic agents are materials that reduce the water potential of cells. The addition of osmotic agent such as mannitol, sucrose, sorbitol has been proved to be efficient in reducing growth and increasing the storage of many *in vitro* grown tissues of different plant species (Shibli and Al-Juboory, 2000; Wilson *et al*., 2000).

Sucrose is a major component of most tissues culture media. It functions as both a carbon or energy source and osmotic agent (Shibli, 2000). Pretreatment using sucrose plays a major role in improving the resistance of apices to both dehydration and freezing in LN (Swan *et al*., 1998; Shibli, 2006). However, the

growth of explants is dependent on the concentration of the sucrose. Usually, preculturing with high concentration of sucrose is very important in improving survival of cryopreserved cells and meristems (Uragami *et al*., 1990; Niino and Sakai, 1992; Matsumoto *et al*., 1994). The concentration and duration of exposure to the osmotic agent depend on the plant species and samples of the plant material (Lipavska, 1996; Ashmore, 1997). In these experiments that have been conducted, it showed that 0.50 M sucrose concentration gave the highest survival rate of *Brassia rex* PLBs. In addition, the increase of sucrose concentration to 0.75M reduced the viability of PLBs. Besides, the osmotic effect, sucrose acts by stabilizing membranes and protein during desiccation. High sugar concentrations in the cell cytoplasm help to establish a vitrified state during cooling and enable cells to tolerated dehydration that can cause freezing damage (Shibli *et al*., 2001). Mycock *et al*. (1995) reported that somatic embryos of date palm (*Phoenix dactylifera*) and (*Pisum sativum*) showed tolerance to dehydration and further freezing after treated with sucrose pretreatment.

Effects of Duration Exposure to Dmso Treatments at 24ºC and 0ºC on Brassia Rex PLBs Viability Prior to Cryopreserved (+ Ln) and Non-cryopreserved (- Ln) PLBs:

Vitrification techniques have been developed over the past ten years for different plant species. The vitrification (glass formation) procedure for cryopreservation eliminates the need for controlled slow freezing and permits cells and meristems to be cryopreserved by direct transfer into liquid nitrogen (Langis *et al*., 1989). At ultra low temperature, vitrification replaced freeze-induced cell dehydration (by the removal of all or a major part of the freezable cell water at room temperature or 0ºC) by a highly concentrated vitrification solution. The vitrification solution contains a high concentration of glycerol ethylene glycol or DMSO. In a few exceptional cases, a single cryoprotectant usually DMSO is effective, however, a cryoprotectant mixture consisting of DMSO, glycerol, and the third component such as sucrose, proline, mannitol or sorbitol is usually far more effective (Withers, 1991).

On the other hands, cryoprotectant must be non toxic to the plant cell at the proper concentration (Moges *et al*., 2003; Withers, 1991), have low molecular weight, readily miscible with water and able to penetrate cells rapidly (Ashmore, 1997). They also must be able to reduce the size of ice crystals and lower the freezing points of intercellular content which enable cells to withstand very low temperature without disruption of the plant cell membrane and cytoplasm. Whereas, in the case of duration exposure to DMSO treatment at 0ºC, it was determined that the optimal exposure time to DMSO cryoprotectant was 10 min because TTC test showed the highest viability on the PLBs are ones that expose to 10 min DMSO treatment compared to other parameters. It is appeared that 10 min of exposure to DMSO treatment was superior to 0, 20 and 30 min times of exposure since there is high viability of *Brassia rex* PLBs tested by TTC. However, for DMSO treatment at 24ºC, exposure time that recorded the highest viability of PLBs was determined at a 0 min treatment in DMSO for non cyopreserved PLBs (-LN). For the cryopreserved (+LN) PLBs, it was been observed that 30 min treatment with DMSO gave the highest PLBs viability. Reasons for this difference are not clear, could hypothesis that a long exposure of explants to highly concentrated vitrification solutions is potentially injurious because of the phytotoxic effects of individual components, or their combined osmotic effects on cell viability (Towill and Jarret, 1992), the duration of contact between explants and the vitrification solution is a critical parameter, in view with their high toxicity effect (Engelmann, 1997). The optimal dehydration treatment of different plant species with variable water content and membrane permeability can vary considerably. Takagi (2000) compared the behavior of several tropical species of banana and ranked banana among the sensitive plant speies since all meristems were killed by 10 min PVS2 treatment at room temperature and 70% of Taro meristems survived at 60 min treatment.

In the present study by using the TTC assay, the results show that at 0ºC exposure temperature to DMSO treatment given highest viability of *Brassia rex* PLBs compared to 24ºC of exposure for both cryopreserved (+LN) and non cryopreserved (-LN) PLBs. This have proved that performing the dehydration step at 0ºC instead of room temperature allows to reduced the toxicity of vitrification solution thus increase the surface exposure duration ensuring survival of size type 2 PLBs (Lipavska *et al*., 1996; Ashmore, 1997). At 0ºC, longer treatments are possible which especially benefits for tropical, dehydration sensitive plants like sweet potato (Plessis *et al*., 1996).

Total Chlorophylls Determination:

There are 2 type of chlorophyll pigments being study in this analysis; chlorophyll *a* and chlorophyll *b*. Chlorophyll *a* was the main pigment that assists the photosynthesis reaction while chlorophyll *b* was the accessory pigments that function to passes energy absorbed through the visible wavelength to chlorophyll *a* for the photosynthesis process. Chlorophyll *a* can be detected at the wavelength of around 662 nm while

chlorophyll *b* was around 644 nm (Lisiewska *et al.,* 2006).

From the experiments that have been conducted, the highest amount of chlorophyll content being detected derived from non cryopreserved *Brassia rex* PLBs which is underwent 10 min DMSO treatment at 0°C. Chlorophyll *b* concentration was observed to be higher that chlorophyll *a* concentration in all treatment parameters except for non cryopreserved PLBs which exposed to 30 min DMSO treatment at 24°C and 20 min DMSO treatment at 0°C. According to Lahai *et al*. (2003), can be hypothesis that chlorophyll *b* concentration may increase under the stressful conditions compared to chlorophyll *a*. The concentration of chlorophyll *b* was increase significantly under low light exposure. Smayda and Mitchell-Innes, (1974) reported that morphological, physiological and biochemical changes are expected in dark-stressed cells. The diatom *Fragilariopsis cylindrus* exposed to -1.8 °C and low light intensity showed decrease in photochemical efficiency, increase in cell concentration of chlorophyll *a* and *c*, and changes in expression of photosynthesis related genes (Mock and Valentin, 2004).

The light requirement during germination of orchid seed is species-specific, although this question is virtually not considered in the literature. Nikishina *et al.* (2001) published their finding that demonstrated a negative effect from light on initial stages of protocorm formation for nearly all species of rare tropical orchids. Protocorm of *C. reginae* showed better survival upon germination of seeds in darkness for 12 weeks (Harvais, 1973). According to Nikishina *et al*. (2001), the exposure of protocorms to light at early stages of development suppresses their growth in some species and become necessary only at later stages of development. During determination of the total chlorophyll contents of the samples, all the chlorophyll extraction works should preformed in the dim light environment and sometimes the test tubes that contain the chlorophyll extraction were covered with the aluminum foils in order to minimize the degradation and isomerization of chlorophylls. On the other hand, the addition of anhydrous calcium carbonate ($CaCO_3$) solution during grinding process was to adjust the pH of the sample extraction to pH 8-9 while preventing conversion of chlorophyll pigments to pheophytin pigments (Bellomo and Fallico, 2007).

Conclusion:

An optimal DMSO vitrification protocol for this hybrid orchid can be achieved using size type 2 PLBs (0.31-0.61 cm) pretreated with 0.5M sucrose for 48 hours, followed by loading treatment and dehydration with DMSO treatment prior to freezing. The optimal duration of exposure to DMSO treatment obtained from this research is a 10-min treatment with 7.5% DMSO at 0ºC condition. After one hour cryopreservation, a minimum requirement for recovery take placed about 21 days before survivability test. During recovery, samples are maintained in dark condition for 14 days and another 7 days in light condition. The size of PLBs selected, the duration and concentration of the sucrose pretreatment, the conditions of DMSO treatment (temperature and length of exposure) applied in this protocol, as well as the final viability and the total chlorophyll content in PLBs after entire treatment had proved to be greatly influential in determining the success of DMSO vitrification procedure for this hybrid orchid *Brassia rex.* Finally, even though cryopreservation is still routinely employed in a limited number of cases only, the development of new vitrification-based freezing techniques will make its application to a broad range of species possible. It can thus be expected that in the coming years, cryopreservation will be more frequently employed for long-term conservation of plant genetic resources.

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