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Cryopreservation of *Brassia rex* Orchid Shoots Using PVS2 Technique

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Abstract: *In vitro* grown shoots of *Brassia rex* orchid hybrid was cryopreserved by means of plant vitrification solution 2 (PVS2) technique. For the preculture treatment, the shoots were excised into two standard sizes of 0.5-1.0 and 1.0-1.5 cm and were precultured on half-strength Murashige and Skoog (MS) semi solid medium supplemented with different concentrations of sucrose (control (0.06 M), 0.1, 0.25, 0.5 and 0.75 M) for 24 and 48 h. For the PVS2 dehydration treatment, the 0.1 M precultured (48 h and 1.0-1.5 cm) shoots were chosen for further experiment where the shoots were dehydrated in PVS2 solution at various durations (5, 10, 15, 20, 25 and 30 min) at 0 and 24°C for positive and negative storage in Liquid Nitrogen (LN). The viability of the cryopreserved cells were determined by 2, 3, 5-triphenyltetrazolium chloride (TTC) assay and chlorophyll extraction techniques. The best condition of PVS2 treatment was at 20 min of PVS2 treatment at 0ºC prior to storage in liquid nitrogen. In chlorophyll determination based on chlorophyll assay, the highest concentration of total chlorophyll concentration (56.250 μg g^{-1}) was obtained from shoots that were dehydrated for 25 min in PVS2 solution at 0°C without storage in liquid nitrogen.

Key words: *Brassia rex,* PVS2, TTC assay

INTRODUCTION

Orchids have become is one of the most popular ornamental plants in the world. Orchid is one of the most unique, evolved and popular ornamental group of plants (Hieber *et al*., 2006). Unique flower shapes, fragrance, colours and keeping quality also render several orchid genera highly prized as commercial cut flowers and blooming potted plants (Hieber *et al*., 2006). The increasing demands for orchid's cut flowers act as a catalyst to the various breeding programmes of orchids. Orchids are threatened because of the unstoppable harvesting of wild type orchids which harm the existence of the wild species of orchids.

In the recent years, the valuable germplasm of tropical orchids which are in the virtue of extinction due to extensive disturbance of their natural habitat and unstoppable harvesting of naturally-growing plants need to be conserved (Thammasiri, 2005). Genetic resources of ornamental plants especially orchids are needed to be conserved due to their increasing threat of extinction. There are two major methods of preservation for endangered plant

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species, *in situ* conservation which maintains plants in their natural habitat and *ex situ* conservation which maintains plants under artificial conditions (Hirano *et al*., 2005). Cryopreservation is an alternative or a duplicate storage for the traditional *in situ* and *ex situ* germplasm conservation (Engelmann, 2000).

Cryopreservation is extensively used for the *ex situ* conservation of plant germplasm and is the method for preserving genetic resources that cannot be conserved by traditional seed banking (Ashmore, 1997). Cryopreservation in liquid nitrogen (-196°C) is a preservation method in which cell division and metabolic and biochemical processes are arrested (Nino and Sakai, 1992). Cryopreservation is one of the methods of preserving plants germplasm by storage in liquid nitrogen at very low temperature (-196°C). At this temperature, all cellular divisions and metabolic processes are stopped (Engelmann, 2000). Cryopreservation is the best option for the long-term storage of clonal germplasm and this technique developed over the last 25 years as well as the significant progress has been made (Engelmann, 2000). Recently, some simplified and reliable cryogenic procedures such as vitrification, encapsulation-dehydration and encapsulation-vitrification have been developed and the number of species or cultivars that have been cryopreserved has sharply increased (Sakai, 1997).

Brassia rex is one of the orchid hybrids in *Brassia* genus. *Brassia rex* is a hybrid from the hybridization between *Brassia verrucosa* and *Brassia gireoudiana* (Fig. 1). *Brassia rex* has unique flower structure, like spider and it is useful to cryopreserve *Brassia rex* for orchid floricultural industry. *Brassia rex* has high potential to become popular in international flower trade and can be marketed all over the world.

The objectives of this present study are:

- To evaluate the effects of various sucrose concentrations (control [0.06 M], 0.1, 0.25, 0.5 and 0.75 M) that precultured at two different durations (24 and 48 h)
- To study the effects of PVS2 at different durations (5, 10, 15, 20, 25 and 30 min) and temperature (0 and 24°C) prior to storage in liquid nitrogen
- To determine the viability and regrowth of explants after storage in Liquid Nitrogen (LN) by:
- 2, 3, 5-triphenyltetrazolium chloride (TTC) method

Fig. 1: *Brassia rex* orchid hybrid (Serdang Orchid Nursery, 2008). (Bar = 1.0 cm)

- Chlorophyll determination: Determination of chlorophyll a and chlorophyll b; Quantification of total chlorophyll content

MATERIALS AND METHODS

Plant Material

This study was conducted in the Plant Improvement laboratory in the School of Biological Sciences, Universiti Sains Malaysia. This research project was conducted from 7/7/08 to 23/10/08. 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 from the PLBs for the study. The cultures were incubated at 25±2°C in a 16 h photoperiod under cool white fluorescent lamps (Philips TLD, 36 W) at 150 μmol m^{-2} sec^{-1}.

Preculture of Shoots with Different Concentration of Sucrose (Control (0.6), 0.10, 0.25, 0.5 and 0.75 M)

The shoots of 2 months old were excised into 2 different lengths (0.5-1.0 and 1.0-1.5 cm) and preculture on half-strength MS (Murashige and Skoog, 1962) semi solid medium enriched with sucrose. Different concentrations of sucrose (control (0.06 M), 0.1, 0.25, 0.5 and 0.75 M) were added to the half-strength MS semi solid medium. The preculture process occurred for 24 and 48 h.

Treatment of Shoots with Presence of PVS2 and Absence of Liquid Nitrogen (LN) Storage

The dissected shoots (1.0-1.5 cm) were transferred to preculture media (0.1 M sucrose) and maintained in culture room (25±2°C) for 48 h. After that, the shoots were inserted into plastic cryovials (1.8 mL) and immersed with 1.4 mL of loading solution (2 M glycerol and 0.4 M sucrose in half-strength MS liquid medium) for 20 min at room temperature (22-25°C). Then, the shoots were dehydrated with 1.4 mL of vitrification solution, PVS2 (Sakai *et al*., 1990) which contain 30% (w/v) glycerol, 15% (w/v) ethylene glycol and 15% (w/v) dimethyl sulfoxide (DMSO) in half-strength MS liquid medium containing 0.4 M sucrose with pH 5.8. The precultured shoots were exposed to different incubation periods (5, 10, 15, 20, 25 and 30 min) at 0 and 24°C of PVS2. After treatment of PVS2, the shoots were immersed with 1.4 mL of unloading solution (1.2 M sucrose in half-strength MS liquid medium) for 30 min.

Treatment of Shoots with Presence of Both PVS2 and Liquid Nitrogen (LN) Storage

The dissected shoots (1.0-1.5 cm) were transferred to preculture media (0.1 M sucrose) and maintained in culture room (25±2°C) for 48 h. After that, the shoots were inserted into plastic cryovials (1.8 mL) and immersed with 1.4 mL of loading solution (2 M glycerol and 0.4 M sucrose in half-strength MS liquid medium for 20 min at room temperature (22-25°C). Then, the shoots were dehydrated with 1.4 mL of vitrification solution, PVS2 (Sakai *et al*., 1990) which contain 30% (w/v) glycerol, 15% (w/v) ethylene glycol and 15% (w/v) dimethyl sulfoxide in half-strength MS liquid medium containing 0.4 M sucrose with pH 5.8. The preculture shoots were exposed to different incubation periods (5, 10, 15, 20, 25 and 30 min) at 0 and 24°C of PVS2. Then, the plastic cryovials were immediately immersed into LN for 1 h. After treatment of PVS2, the shoots were immersed in 1.4 mL of unloading solution (1.2 M sucrose in MS liquid medium) for 30 min.

Thawing

After being stored for 1 h in LN, the shoots were thawed. The cryopreserved shoots were taken from LN tank and thawed into water bath at 40°C for 90 sec. Then, the shoots were removed from the plastic cryovials and placed on a sterile filter paper to drain out the PVS2 solution.

Growth Recovery

The cryopreserved shoots were transferred to Petri dish containing half-strength MS semi solid medium supplemented with sucrose and kept under dark condition. After 2 days, the shoots were removed to the new Petri dish and kept under dark condition for another 8 days. Then, the shoots were exposed to light for 4 days before viability test was done. All shoots were stored in a temperature controlled culture room maintained at 25±2°C with 16 h photoperiod under cool white fluorescent lamps (Philips TLD, 36W) at 150 µmol m^{-2} sec^{-1}.

2, 3, 5-Triphenyltetrazolium Chloride (TTC) Method

The viability of the cryopreserved tissues were measured by the 2, 3, 5-triphenyltetrazolium chloride (TTC) reduction with assay. The viability of shoots was determined by staining with TTC. The method is based on the reduction of colourless TTC into red formazan. The cryopreserved shoots were transferred to test tubes containing 2 mL of TTC solution and incubated for 15h at 30°C. The TTC solution was drained off and the cells were washed with distilled water. After that, cells were vortexed and extracted with 7 mL of 95% ethanol in water bath at 80°C for 5 min. The extract was cooled and made up to 10 mL with 95% ethanol. The absorbance (pink colour) was measured by using spectrophotometer (Spectro 22, Digital Spectrophotometer, Labomed. Inc.) at 530 nm.

Chlorophyll Determination (Harborne, 1973)

For the chlorophyll extraction, 0.1 g of shoots from each sample were grounded with 0.2 g of calcium carbonate ($CaCO_3$) powder and 1 mL of 80% acetone solution using chilled mortar and pestle. Then, the extract was filtered with Buchner funnel through the Whatmann No. 1 filter paper, followed by washing the mortar and pestle with 80% acetone solution. Finally, the volume was added up to 5 mL with 80% acetone solution. The absorbance readings were measured using spectrophotometer at 646 and 663 nm. The chlorophyll contents (µg mL^{-1}) were calculated using the following formula:

$$\text{Chlorophyll a } (\mu g\ mL^{-1}) = 12.21\ (A663\ nm) - 2.81\ (A646\ nm)$$

$$\text{Chlorophyll b } (\mu g\ mL^{-1}) = 20.13\ (A646\ nm) - 5.03\ (A663\ nm)$$

$$\text{Total chlorophyll content } (\mu g\ mL^{-1}) = 17.30\ (A646\ nm) + 7.18\ (A663\ nm)$$

The chlorophyll concentrations were express in µg g^{-1}:

$$\text{Conversion from } \mu g\ mL^{-1} \text{ to } \mu g\ g^{-1} = \mu g\ mL^{-1} \times \frac{\text{Extraction volume (mL)}}{\text{Fresh weight (g)}}$$

RESULTS AND DISCUSSION

Preculture of Shoots with Different Concentrations of Sucrose

Preculture is necessary for plant cryopreservation procedure. The viability of the shoots was measured by using TTC assay, based on absorbance reading at 530 nm using spectrophotometer.

In the 24 h preculture treatment, the viability increased in shoot sizes, 0.5-1.0 and 1.0-1.5 cm when the sucrose concentration increases from control (0.06 M) to 0.25 M (Fig. 2). However, the viability of shoots decreases at sucrose concentration of 0.5 and 0.75 M. In the 48 h preculture treatment, the viability of shoots for size 0.5-1.0 cm increases as sucrose concentration increases from control to 0.25 M (Fig. 3). However, the viability of shoots decreases at higher sucrose concentration of 0.5 and 0.75 M. For 1.0-1.5 cm shoots size, the viability of shoots increases at sucrose concentration of control to 0.1 M (Fig. 3). There was a slight decrease of the viability of shoots as sucrose concentration increases from 0.25 to 0.75 M.

Generally, it has been noticed that shoots of *Brassia rex* orchid hybrid that are preculture on control treatment of sucrose concentration showed very low TTC stainability for both preculture period of 24 and 48 h by using TTC assay. In this study, TTC stainability is higher when the shoots were precultured on control treatment of sucrose concentration at 48 h and size of 1.0-1.5 cm (Fig. 4). In contrast, TTC stainability was lower when the shoots were precultured on control treatment of sucrose concentration for 24 h for both sizes (0.5-1.0 and 1.0-1.5 cm). The TTC stainability of shoots that were precultured on control treatment of sucrose concentration at 48 h for size 1.0-1.5 cm also showing similar results (Fig. 4). Based on the result of TTC stainability at 530 nm, 0.1 M sucrose at 48 h (1.0-1.5 cm) appeared to be the most effective duration and concentration for pregrowth for PVS2 treatment with the highest viability at 0.071 absorbance value.

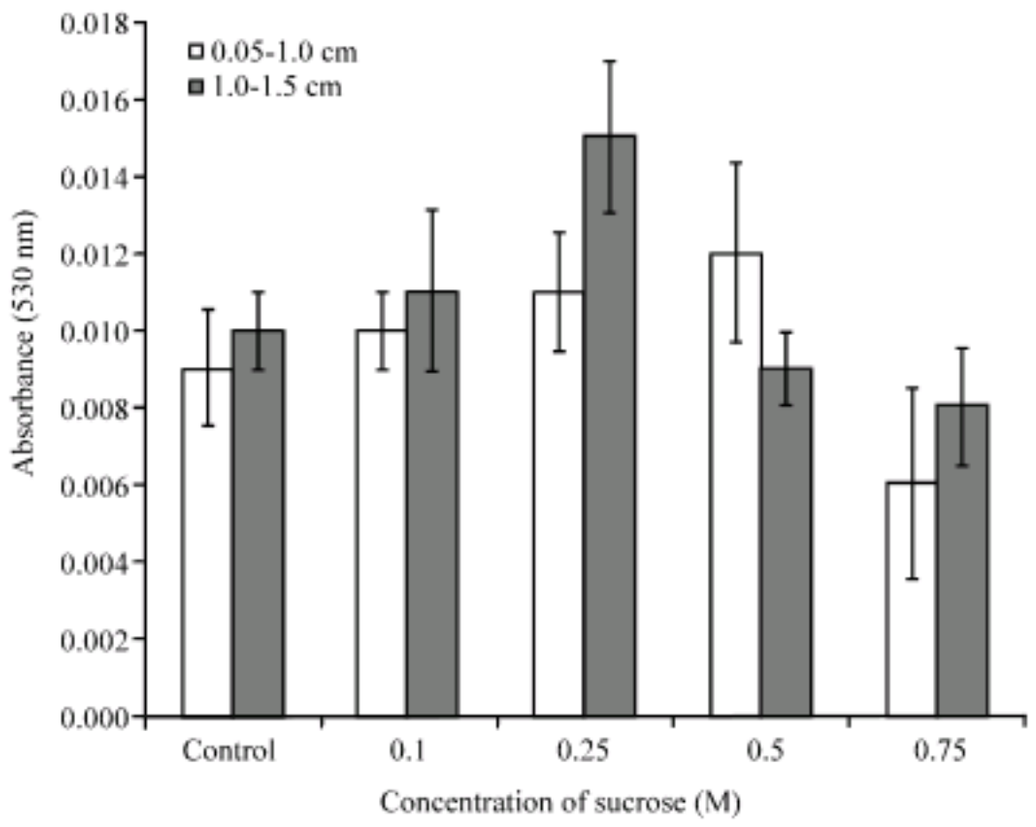

Fig. 2: Preculture of shoots with different concentrations of sucrose for 24 h. TTC stainability of the shoots precultured in MS semi-solid medium with control, 0.1, 0.25, 0.5 and 0.75 M sucrose. Error bars represent the standard error of TTC stainability

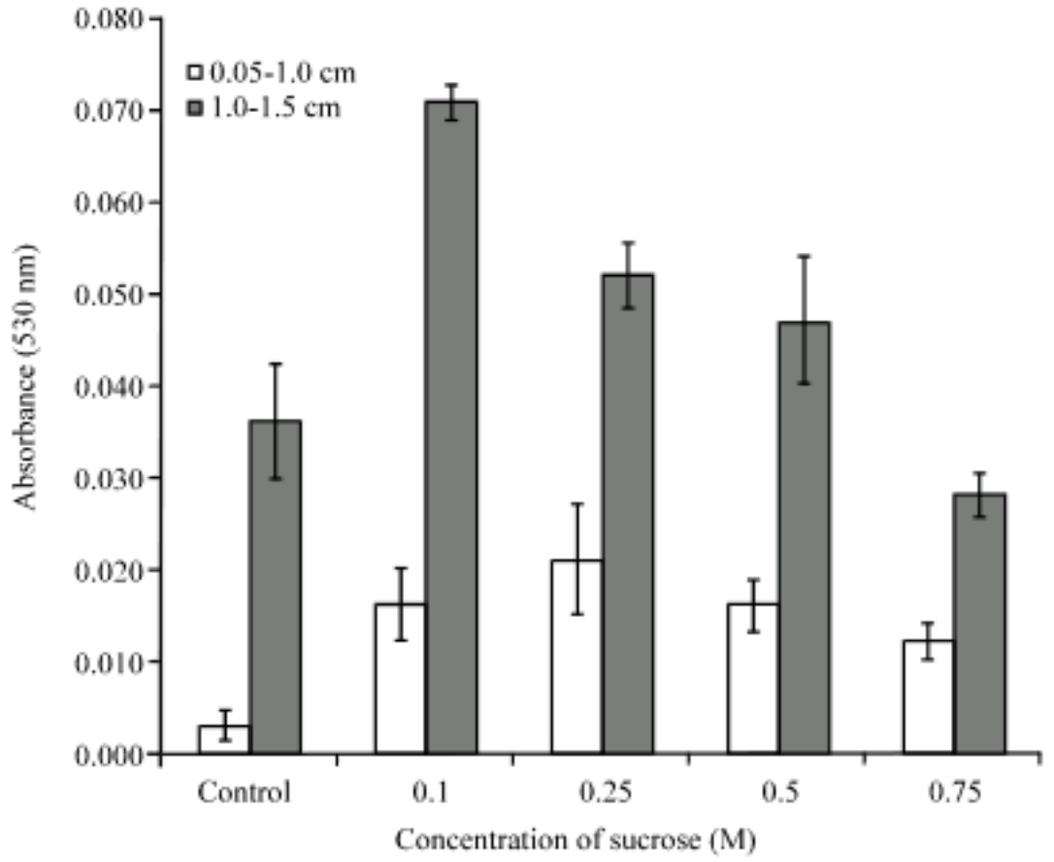

Fig. 3: Preculture of shoots with different concentrations of sucrose for 48 h. TTC stainability of the shoots precultured in MS semi-solid medium with control, 0.1, 0.25, 0.5 and 0.75 M sucrose. Error bars represent the standard error of TTC stainability

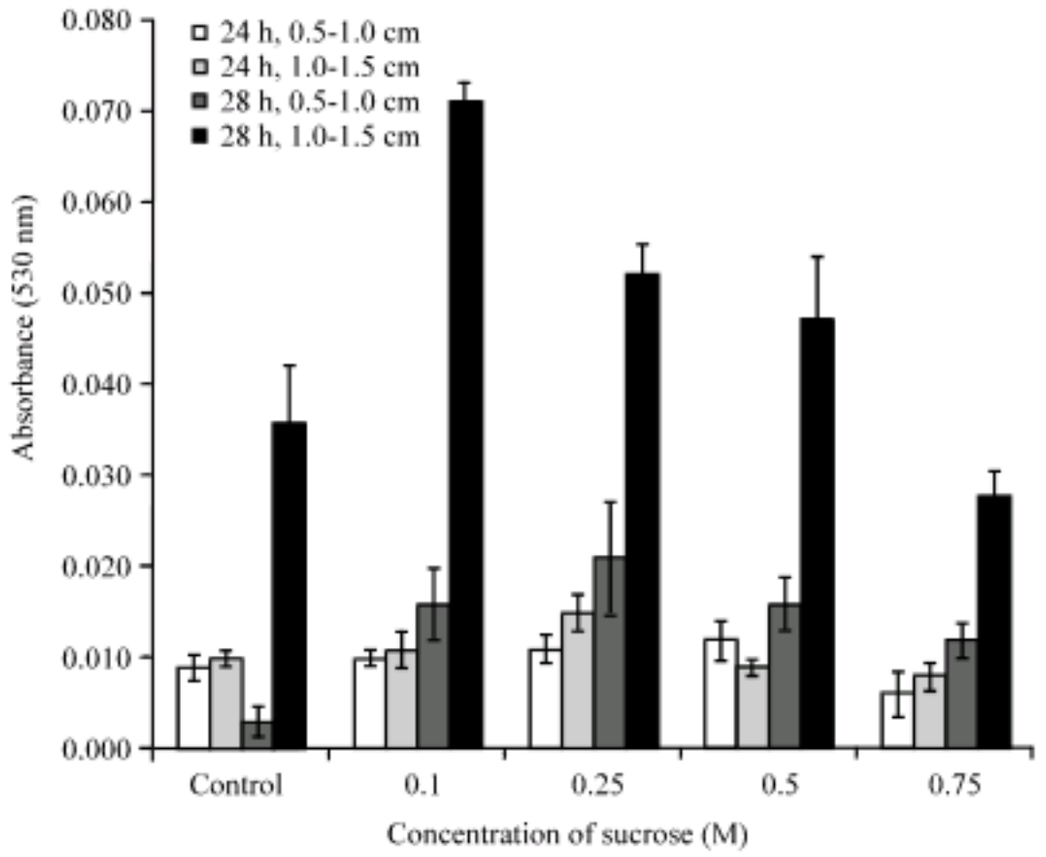

Fig. 4: Preculture of shoots with different concentrations of sucrose for 24 and 48 h. TTC stainability of the shoots precultured in MS semi-solid medium with control, 0.1, 0.25, 0.5 and 0.75 M sucrose. Error bars represent the standard error of TTC stainability

Treatment of Shoots with Presence of PVS2 and Absence of Liquid Nitrogen (LN) Storage

From the previous experiment, 0.1 M precultured shoots at 48 h (1.0-1.5 cm) were chosen and were dehydrated with PVS2 without storage in liquid nitrogen. In the 0°C dehydration

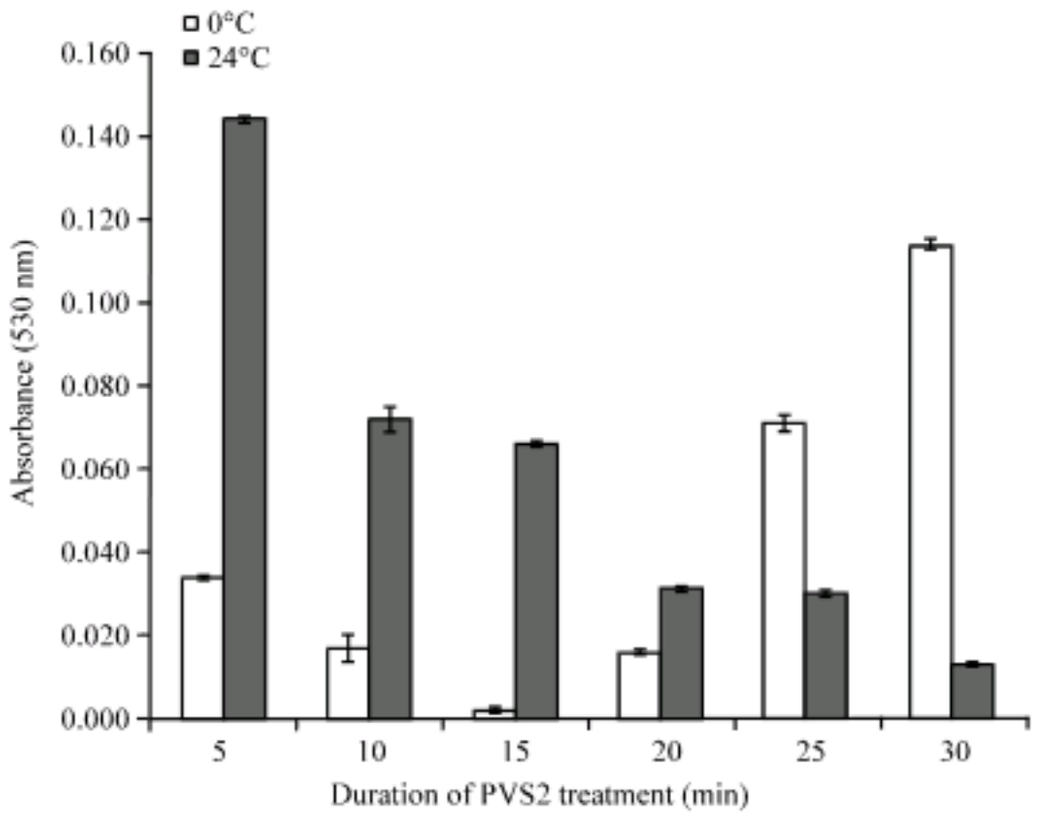

Fig. 5: Treatment of shoots with presence of PVS2 and absence of Liquid Nitrogen (LN) storage. TTC stainability of the shoots precultured in MS semi-solid medium with 0.1 M sucrose for 48 h. After preculture, shoots were dehydrated with PVS2 at 0 and 24°C with different durations without plunging into LN. Error bars represent the standard error of TTC stainability

treatment of PVS2, the viability of shoots decreases as the duration of PVS2 dehydration treatment increases from 5 to 15 min based on TTC assay (Fig. 5). However, the viability based on TTC assay increases from the range of 20 to 30 min.

Based on the result, for the dehydration treatment of PVS2 at 24°C, the viability of shoots based on TTC assay decreases as duration of PVS2 dehydration treatment increases from 5 to 30 min by (Fig. 9). The viability of shoots at 0.144 absorbance value which is the highest was obtained when the shoots were dehydrated in PVS2 at 24°C for 5 min, compare to other PVS2 treatment durations and temperature (Fig. 5).

Treatment of Shoots with Presence of Both PVS2 and Liquid Nitrogen (LN) Storage

From the previous experiment, 0.1 M precultured shoots at 48 h (1.0-1.5 cm) were chosen and were dehydrated with PVS2 solution prior to liquid nitrogen storage. The viability of cryopreserved shoots was determined by using TTC assay. In the 0ºC dehydration treatment of PVS2, there is no viability of shoots were obtained from the 5, 15 and 20 min of PVS2 treatments (Fig. 6). Based on TTC assay, the viability of cryopreserved shoots was low at 10 min and there was a rapid increase at 20 min of PVS2 dehydration treatment. The highest viability of shoots at 0.007 absorbance value was obtained from shoots that were dehydrated in PVS2 solution at 0ºC for 20 min (Fig. 10).

Based on TTC stainablity, for the 24°C dehydration treatment of PVS2, the viability of shoots increases at duration of 5 to 15 min prior to PVS2 dehydration treatments (Fig. 6). There is no viability of shoots at the 20 min of PVS2 treatment. The viability of shoots based on TTC stainability decreases as duration of PVS2 dehydration treatment increases at 25 and 30 min. Based on the result, the highest viability of shoots at 0.005 absorbance value was obtained from shoots that were dehydrated at 24°C for 25 min in PVS2 solution (Fig. 6). In comparison of treatment of shoots with presence of PVS2, for positive and negative Liquid Nitrogen (LN) storage, the highest

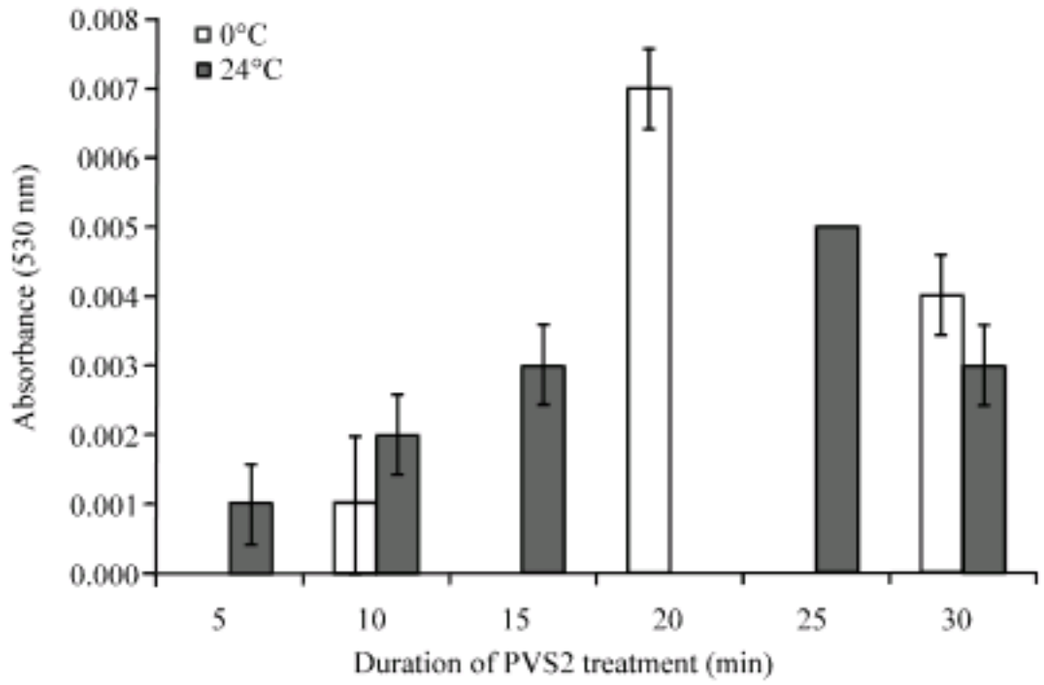

Fig. 6: Treatment of shoots with presence of both PVS2 and Liquid Nitrogen (LN) storage. TTC stainability of the shoots precultured in MS semi-solid medium with 0.1 M sucrose for 48 h. After preculture, shoots were dehydrated with PVS2 solution at 0 and 24°C with different durations and plunged into Liquid Nitrogen (LN). Error bars represent the standard error of TTC stainability

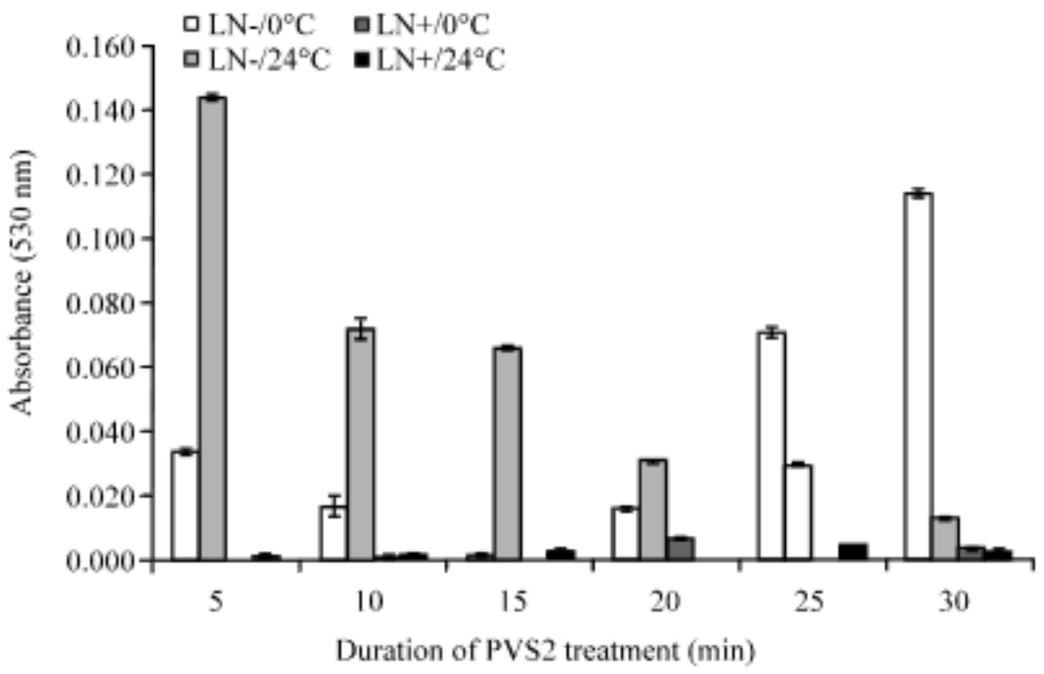

Fig. 7: Treatment of shoots with presence of PVS2 for positive and negative Liquid Nitrogen (LN) storage. TTC stainability of the shoots precultured in MS semi-solid medium with 0.1 M sucrose for 48 h. After preculture, shoots were treated with PVS2 at 0 and 24°C with different durations. Error bars represent the standard error of TTC stainability

viability of shoots at 0.144 absorbance value was obtained from shoots that were dehydrated in PVS2 solution at 24°C for 5 min without storage in Liquid Nitrogen (LN) by using TTC assay (Fig. 7).

Chlorophyll Determination of Shoots Dehydrated in PVS2 Solution with and Without Storage in Liquid Nitrogen (LN)

In dehydration treatment of PVS2 prior to liquid nitrogen storage at both 0 and 24°C, there were no chlorophyll a concentration could be obtained in the cryopreserved shoots

(Fig. 8). For dehydration treatment of PVS2 without liquid nitrogen storage at 0°C, the chlorophyll a concentration decreases from duration of 5 to 10 min and followed by rapid increasing at 15 min. There was a slight decrease at duration of 20 min. There was an increasing at duration of 25 min and followed by decreasing at 30 min (Fig. 8).

Based on chlorophyll determination analysis for dehydration treatment of PVS2 without liquid nitrogen storage at 24ºC, the chlorophyll *a* concentration of the shoots increases as the duration of dehydration treatment of PVS2 increases from 5 min to 25 min and there was a slight decrease when it reached 30 min (Fig. 8).

Based on chlorophyll determination analysis, there was no chlorophyll *b* concentration could be obtained in the shoots for dehydration treatment of PVS2 prior to storage in liquid nitrogen at both 0 and 24°C (Fig. 9). In dehydration treatment of PVS2 without liquid nitrogen

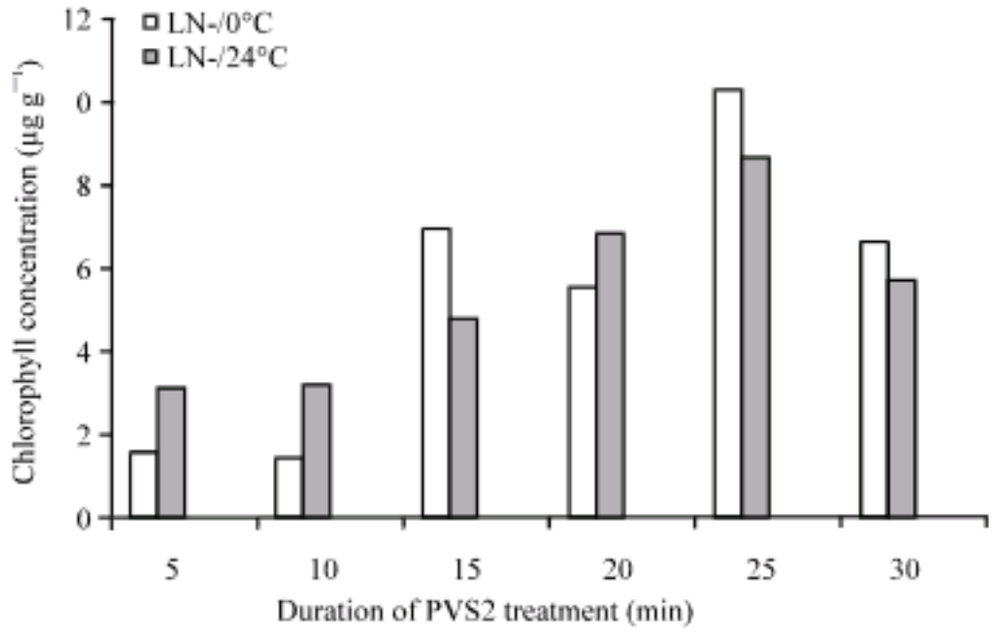

Fig. 8: Chlorophyll *a* concentration (µg g^{-1}) of the shoots. Chlorophyll *a* concentration of the shoots precultured in MS semi-solid medium with 0.1 M sucrose for 48 h. After preculture, shoots were dehydrated with PVS2 at 0 and 24°C with different durations

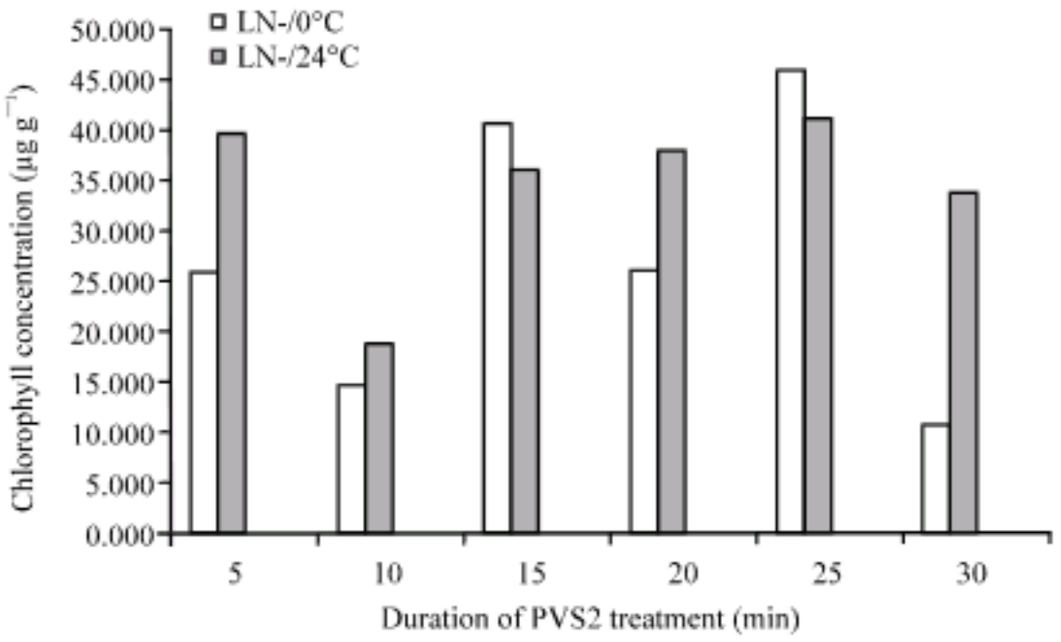

Fig. 9: Chlorophyll b concentration (µg g^{-1}) of the shoots. Chlorophyll b concentration of the shoots precultured in MS semi-solid medium with 0.1 M sucrose for 48 h. After preculture, shoots were dehydrated with PVS2 solution at 0 and 24°C with different durations

storage at 0°C, there was a slight decrease of chlorophyll b concentration from duration of 5 to 10 min. The concentration of chlorophyll b of the shoots increases at duration of 15 min of PVS2 dehydration treatment and there was a slight decrease when it reached 20 min. However, the concentration increases at duration of 25 min and followed by rapid decrease at 30 min (Fig. 9).

In dehydration treatment of PVS2 without storage in liquid nitrogen at 24°C, the chlorophyll b concentration of the shoots was high at duration of 5 min and decreases at duration of 10 min (Fig. 9). However, the concentration increases as the duration of PVS2 dehydration treatment increased from 15 to 25 min and there was a slight decrease at duration of 30 min of PVS2 dehydration treatment (Fig. 9).

In all treatments of PVS2 prior to Liquid Nitrogen (LN) storage, there was no chlorophyll concentration of the shoots that were obtained by using chlorophyll determination analysis (Fig. 10). For PVS2 dehydration treatment without liquid nitrogen storage at 0°C, the total chlorophyll concentration decreases as the duration of PVS2 dehydration treatment increases from 5 to 10 min. The total chlorophyll concentration of the shoots increases rapidly at duration of 15 min and slightly decreasing at 20 min of PVS2 dehydration treatment. There was an increasing at duration of 25 min and followed by rapid decrease at 30 min of dehydration treatment of PVS2 (Fig. 10).

For PVS2 treatment without liquid nitrogen storage at 24°C, the total chlorophyll concentration of the shoots decreases from 5 to 10 min of PVS2 treatments (Fig. 10). However, there was an increasing as the duration of PVS2 dehydration treatments increases from 15 to 25 min and followed by slight decrease at duration of 30 min. Based on the chlorophyll determination analysis, the total chlorophyll concentration of cryopreserved cells is the highest with 56.250 μg g^{-1} when the shoots were dehydrated in PVS2 at 0°C for 25 min compare to other PVS2 treatment durations and temperature.

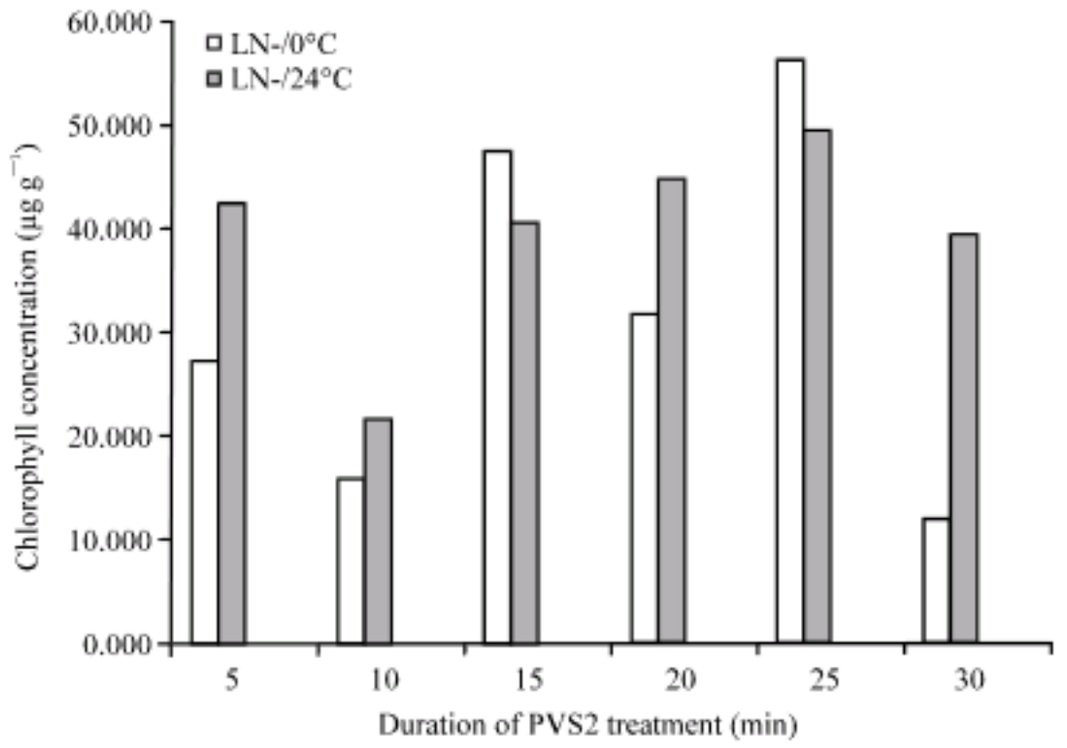

Fig. 10: Total chlorophyll concentration (μg g^{-1}) of the shoots. Total chlorophyll concentration of the shoots precultured in MS semi-solid medium with 0.1 M sucrose for 48 h. After preculture, shoots were treated with PVS2 at 0 and 24°C with different durations

Importance of Preculture Treatment of Plant Materials

Preculture of plants materials in media containing sucrose is a valuable method for improving survival of shoots in Liquid Nitrogen (LN) in the presence of cryoprotectants (Yamada *et al*., 1991; Nino and Sakai, 1992). The beneficial effect of sucrose in cryopreservation could be due to two effects (Steponkus *et al*., 1992). First, sucrose like other osmotic active substances such as mannitol has osmotic dehydration effect during treatment leading to reduced water content in the tissue (Tanaka *et al*., 2004). In addition, Dumet *et al*. (1993) reported that sucrose molecules can enter the plant cells. Preculture treatment with sucrose reduces water content in the cells and prevents ice crystallization. Preculture in sucrose enhanced the tolerance of the shoots against the treatment with PVS2. In this study, the highest viability of preculture of shoots of *Brassia rex* orchid hybrids with 0.1 M sucrose (Fig. 4) suggests the importance of sucrose as an additive of preculture medium.

A proof that sucrose is able to enter the cells has been provided by histological observations of intracellular accumulation of starch during preculture (Gonzalez-Arnao *et al*., 1993). The accumulation of sucrose within the tissue contributes to the viability when the freezable water is removed to the point of reaching the glassy state during vitrification in the presence of Liquid Nitrogen (LN) (Steponkus *et al*., 1992). When high concentration of sucrose was added to the preculture medium, it was assumed to act not only to induce intracellular dehydration via the osmotic effect but also acted to maintain the integrity of the plasma and the inner membrane during the freezing process (Blakesley *et al*., 1996).

Effects of Cryoprotectants

In this study, a loading solution consisting of 2 M glycerol and 0.4 M sucrose and PVS2 were used as the cryoprotectant. Sakai *et al*. (1991) and Nistrizawa *et al*. (1993) were reported that the loading solution was useful as a cryoprotectant in a simple (two-step) freezing method. Exposing cells to high concentrations of cryoprotective additives can be injurious and strategies have been devised to reduce their toxicity (Day *et al*., 2008). These include sequential of loading and unloading treatment to avoid osmotic shock and application at chilling temperatures (Day *et al*., 2008). The purpose of exposure of loading solution to the shoots is to prepare the shoots to undergoing PVS2 solution which has high concentration. The loading solution prevents the cells from osmotic shock and damage due to the exposure of high concentration of PVS2 solution. PVS2 alone was useful itself (Sakai *et al*., 1990). Cryoprotectant like PVS2 is used to protect and to recover the plant material after storage in LN (Sakai, 1995). PVS2 solution removes water from the cells and prevents formation of ice crystal in the cells upon storage in Liquid Nitrogen (LN).

The exposure of explants to the high concentrated vitrification solution is potentially injurious due to the phytotoxic effects of individual components or their combined osmotic effects on cell viability (Towill and Jarret, 1992). Steponkus *et al*. (1992) reported that exposure of protoplast to the vitrification solution for more than 5 min resulted in the complete disruption of the plasma membrane. However, in this study, the shoots that were dehydrated with PVS2 solution without storage in liquid nitrogen at 0 and 24°C for more than 5 min were healthy. The minimal period of exposure of cells in PVS2 solution has to be determined to protect the tissue from damage caused by high concentration of vitrification solution in the cells. As well as their physical protective properties, cryoprotectants also impart additional defences against cryoinjury as they can stabilize proteins and membranes and act as antioxidants (Fuller, 2004).

Effects of Temperature

Cryopreservation using ultra rapid rates of cooling usually involves a cryoprotective strategy, which allows cells and tissues to be directly plunged into liquid nitrogen (Day *et al*., 2008). First cooling rate and then warming rate, was found to be important determinants of survival shoots (Pegg, 2007). Mazur (2004) discovered that the rate of change of temperature was important because it controlled the transport of water across the cell membrane and hence, the probability of intracellular freezing. The rate of change of temperature in controls the osmolality of the surrounding fluid and also influences the rate at which water is transported out of the cells during cooling and into the cells during warming (Pegg, 2007).

If the cooling rate is too rapid for the membrane of the cell to transport sufficient water out of the cell, then the protoplasm will become supercooled and the greater the extent of supercooling, the cell is more likely to freeze internally. Slow warming allows the crystals to recrystallize and cause damage to the cells. During rapid warming, there is insufficient time for recrystallization happen and the ice simply melts. According to Pegg (2007), cells will differ in their cooling and warming requirements and cooling rate will interact with warming rate. If warming is carried out rapidly, there is insufficient time for devitrification to occur and post-thaw recovery is more likely to occur (Mazur, 2004).

Chlorophyll Determination of the Cryopreserved Shoots and Photoxidation

Concentrations of chlorophyll a and b can be determined by using chlorophyll assay. The most used assay to determine the concentrations of chlorophyll a and b in plant and algal materials was introduced by Arnon (1949) and Porra (2005). Previously, Comar and Zscheile (1942), assayed chlorophyll a and b in diethylether after extraction from leaves with acetone (Porra, 2005).

Acetone at 80% concentration is commonly used to extract chlorophylls and this method requires maceration of plant tissue and centrifugation prior to measurement (Tait and Hik, 2003). Wellburn (1994), has presented accurate extinction coefficients and relevant simultaneous equations for use with various solvents including DMSO, which is sometimes used as an alternative extractant (Porra, 2005). Acetone solvent gives very sharp chlorophylls absorption peaks and so is the solvent of choice for chlorophylls assays (Porra, 2005). There are several kinds of chlorophylls. Chlorophyll a is the main photosynthetic pigment of aerobic organisms (Devesa-Rey *et al*., 2009). All plants, algae and cyanobacteria which photosynthesize contain chlorophyll a (Devesa-Rey *et al*., 2009). Chlorophyll a is the molecule which makes photosynthesis possible. A second kind of chlorophyll is chlorophyll b, which occurs only in green algae and in the plants and its main function is to absorb light and transfer it to chlorophyll a (Devesa-Rey *et al*., 2009). This function is also carried out by the carotenoids, which are along with chlorophylls (Colyer *et al*., 2005).

Based on the photoxidation study, some of the cryopreserved shoots turn to complete or partially white in colour, indicating that there was enzymatic reaction following cryopreservation. It might due to production and oxidation of polyphenols commonly known as the effect of photoxidation. Bleaching of chlorophylls is an indication of photoxidation. The cryopreserved shoots could not regenerate and grown well due to photoxidation. Light constitutes the driving force for photosynthesis (Rajagopal and Carpentier, 2003). However, excess light causes the inactivation of the photosynthetic apparatus (Barber and Andersson, 1992) and contributes to photoxidation. Exposure of photosystem I (PS I) submembrane particles to strong light under low temperature altered the structure of chlorophyll-protein (CP) complexes and decreased photochemical activity and the efficiency of excitation energy migration (Rajagopal and Carpentier, 2003).

CONCLUSION

The cryopreserved shoots that were precultured on 0.1 M of sucrose concentration for 48h (1.0-1.5 cm) occurred as the most effective preculture treatment for the cryopreservation using PVS2-vitrification method with the highest viability at 0.071 absorbance value at 530 nm by TTC assay. The best condition of PVS2 treatment is 20 min of PVS2 dehydration treatment at 0°C prior to storage in Liquid Nitrogen (LN) at 0.007 absorbance value at 530 nm by TTC assay. In chlorophyll determination, the highest concentration of chlorophyll a (10.250 μg g^{-1}) was obtained from the shoots that were dehydrated in PVS2 for 25 min at 0°C without storage in liquid nitrogen by using chlorophyll determination analysis. For the concentration of chlorophyll b, 46.050 μg g^{-1} occurred as the highest concentration which was obtained from the shoots that were dehydrated for 25 min in PVS2 at 0°C without storage in liquid nitrogen. Based on the chlorophyll determination analysis, the total chlorophyll concentration of cryopreserved cells at 56.250 μg g^{-1} is the highest concentration when the shoots were dehydrated for 25 min in PVS2 at 0°C without storage in Liquid Nitrogen (LN) compare to other incubation periods and temperature.

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