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Optimisation of Cryopreservation Technique in *Mokara* Golden Nugget Orchid Using PVS2 Vitrification

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Abstract: The study was conducted to evaluate the effects of various sucrose concentrations on *Mokara* orchid shoots (0.5-1.0 and 1.0-1.5 cm) after precultured at 24 and 48 h. The 1.0-1.5 cm of *Mokara* shoots preculture at 0.25 M sucrose for 48 h was the best condition based on TTC assay and subsequently used for the following PVS2 vitrification treatment. The selected *Mokara* orchid shoots were subjected to PVS2 treatment at different time of exposure (min) and temperatures (0 and 24°C). The results showed that viability of shoots reached the highest absorbance value at 10 min and 24°C. However, for the overall treatment with the results shown that 0°C temperature treatment gave the higher absorbance value which could reduce the injurious effects of PVS2. For chlorophyll determination, cryopreservation of shoots at 0°C without LN (LN-) for 5 min exposure to PVS2 recorded as highest chlorophyll content. The result also shows that total chlorophyll a for shoot in all treatment were higher than chlorophyll b.

Key words: *Mokara*, PVS2, TTC, chlorophyll analysis

INTRODUCTION

Orchid is one of the most attractive groups of cut flower and ornamental plant. It is a beautiful and spectacular plant with very valuable esthetics of its colourful flowers. In the kingdom of flowering plants, orchid rule the emerald Earth, over 20,000 species found worldwide (Dressler, 1990). They are an endangered plant group since it is difficult to cultivate orchid using conventional method. Preservation of orchid germplasm is important to safeguard biodiversity and also to store elite plants, the latter being important for the development and maintenance of new cultivars (Bouman and De Klerk, 1990). Continuous replacement of various types of orchids with a limited number of selected cultivars is one of the main contributors to genetic erosion (Thammasiri, 2005; Vendrame *et al*., 2007). Therefore, the need for seed storage of high quality orchid's seed plays an important role for long-term conservation of orchid species, contributing to both preservation and distribution of germplasm at reduced cost (Thammasiri, 2005). Plant cryopreservation is an attractive alternative for the storing of orchid germplasm, consisting of conservation of the orchid's material at ultra-low temperature where the cell divisions and all other biological activities are completely put on a hold (Puchooa, 2004).

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Many commercial nurseries used *in vitro* systems for the rapid mass plant propagation of various orchids. A common practice among orchid growers is *in vitro* germination of hybrid seeds and most orchid seeds can either be readily germinated after harvest from the mother plant or stored for later germination (Pritchard and Seaton, 1993; Thammasiri, 2005; Hirano *et al*., 2005). Unfortunately, many *in vitro* problems occurred such as loosing of specific characters due to prolonged subculturing, contamination, somaclonal variation, intensive and high labour requirement and potential loss due to incubation failure. To overcome these problems, cryopreservation technique has attracted attention as a method to preserve orchid cultures and maintain their genetic integrity safely (Grout, 1995; Hirano *et al*., 2005; Vendrame *et al*., 2007).

Cryopreservation plays important roles for the long-term storage of plant genetic resources for future generations since requiring only a minimum of space and maintenance. With increasing interest in the genetic engineering of plants, the cryopreservation of cultured cells and somatic embryos with unique attributes is assuming greater importance. Cryopreservation was recently reported to offer real hope for enhancing the preservation of endangered, rare plants and potential hybrids (Touchell, 1995; Touchell and Dixon, 1996; Thammasiri, 2005). PVS2 is an appropriate and practical plant cryopreservation approach for conservation of many accessions of orchid tissues, being quick, simple, reliable and low cost. The exposure to PVS2 prior to cryopreservation contributed to minimize any possible damage by chemical toxicity, osmotic stress and ice crystallization (Vendrame *et al*., 2007).

One of most popular and famous orchid hybrid in Malaysia is tri-generic hybrid (crosses of three genera) which is known as *Mokara* Golden Nugget hybrid (Fig. 1). The aim of the present study are to establish an efficient technique for selected *Mokara* orchid hybrid conservation by using shoots culture, to evaluate the effect of various sucrose concentrations (0.6 (control), 0.10, 0.25, 0.5 and 0.75 M) on explants that precultured at 24 and 48 h, to study the effect of PVS2 at different time (5, 10, 15, 20, 25, 30 min) and temperature (0 and 24°C) prior to storage in liquid nitrogen and finally to determine the survival rate by using viability assessment based on TTC (Triphenyltetrazolium chloride) and chlorophyll determination methods.

Fig. 1: *Mokara* Golden Nugget hybrid (Bar = 1.0 cm)

MATERIALS AND METHODS

Plant Material

This study was conducted in the Plant Improvement Laboratory in the School of Biological Sciences, Universiti Sains Malaysia. The *Mokara* orchid shoots were used in this experiment (Fig. 2). This material was used as the starting material to initiated multiplication of shoots for the study. The cultured were incubated at 25±2°C in a 16 h photoperiod under cool white fluorescence lamps (Philips TLD, 36 W) at 150 µmol/m^2/sec.

Multiplication of PLBs

In order to have sufficient shoots culture for the whole study, MS (Murashige and Skoog, 1962) liquid media were used to multiply the explants. Fresh-growing PLBs were aseptically excised and transferred into the liquid media in conical flasks. The flasks were then tightly closed with aluminium foil, sealed with parafilm and put on rotary shaker for incubation of 2-3 weeks.

Experimental Designs

There were two main parts in this study. The first part was to determine the best sucrose concentration, time of culture and length of PLBs used in preculture that would produce best explants for next steps. Five different sucrose concentrations (0.06 M, 0.10 M, 0.25 M, 0.50 and 0.75 M) and two culture times (24 and 48 h) were tested. The PLBs were excised into two parameters of length, 0.01-0.20 cm and 0.21-0.40 cm. The second part was to determine the best time and temperature of PVS2 solution exposed to explants which would yield highest viability after LN storage. Six different exposure times were used, i.e., 5, 10, 15, 20, 25 and 30 min while two different temperatures applied were 0 and 24°C. Two viability tests were then employed in this part, namely TTC assay and chlorophyll analysis.

Triphenyltetrazolium Chloride Test (TTC Test)

In a TTC test, cell survival is estimated by the amount of formazan produced from the reduction of TTC due to the action of dehydrogenases in living cells or tissue. The 2, 3, 5-triphenyltetrazolium chloride (TTC) test was performed according to Rassmusen (1995).

Fig. 2: Shoots culture of the *in vitro Mokara* Golden Nugget hybrid. The bar in the bottom image represents 1.0 cm

Vitrification and Cryopreservation

There are seven steps in this experiment they are preculture, cryoprotectant (loading), dehydration (PVS2) and storage in liquid nitrogen, thawing, unloading and recovery. Following the best preculture treatment, *in vitro* shoots (1.0-1.5 cm) were implanted on Petri dish with 0.25 M sucrose MS medium. Six shoots were transferred into a 2 mL sterilized cryovial. About 1.5 mL of loading solutions was added using sterile micropipette and were incubate for 20-30 min. In this step, *Mokara* shoots were incubated in PVS2 solution for different duration (5, 10, 15, 20, 25 and 30 min) and temperature (0 and 24°C). After 20-30 min, half of loading solutions was removed. About 1 mL of PVS2 solutions were added into cryovials, solutions were mixed and were then removed. Approximately 1.5 mL PVS2 solutions were then added into cryovials and incubated for that time. For 0°C treatment cryovials were put in styrofoam which was filled with ice cube and incubated for that different time. After treatment with PVS2, the cryovials were put in the cryo holder and were then plugged into -196°C liquid nitrogen and held for at least 1 h. Non frozen shoots (-LN) were directly treated with unloading solution. The cryovials with frozen shoots were removed and warmed in 40°C water bath immediately for 90 sec till PVS2 become liquid again.

Growth Recovery after Cryopreservation Treatment

After thawing, half of PVS2 solutions were removed. About 1 mL of unloading solutions were added into cryovials, solutions were mixed and were then removed. Approximately 1.5 mL of loading solutions were then added into cryovials and incubated for 30 min. For regrowth, shoots were taken off the cryovials and bloated dry on the autoclaved filter paper. They were then implanted on to MS basal medium (20 g L^{-1} sucrose) in Petri dishes and transferred to standard culture room under dark condition for 2 days. After 2 days, shoots were transferred into new MS basal medium and stored again to standard culture room under dark condition for 8-12 days. Effect of cryoprotectan in different duration, temperature, without and post storage in liquid nitrogen were then measured using TTC test.

Chlorophyll Determination

The chlorophyll content determination was based on Harborne method (Harborne, 1973). In an ice box at 4°C using chilled mortar and pestle, 0.1g shoots was ground with 0.2 g of calcium carbonate ($CaCO_3$) powder and 1 mL of 80% acetone. The sample was then filtered through Buncher funnel by using Whatman no. 1 filter paper and washed with chilled 80% acetone. Finally, the extraction volumes were added up to 5 mL using volumetric flask with 80% acetone solution. Chlorophyll extracts was monitored with spectrophotometer (Spectro 22, Digital Spectrophotometer, Labomed. Inc.) at 646 and 663 nm to obtain the absorbance and readings were taken 3 times for each sample. The content of chlorophyll a and chlorophyll b were calculated using the following formula:

$$\text{Chlorophyll a } (\mu g\ g^{-1}) = 12.21(Abs_{663})-2.81(Abs_{646})$$

$$\text{Chlorophyll b } (\mu g\ g^{-1}) = 20.13(Abs_{646})-5.03(Abs_{663})$$

$$\text{Total chlorophyll } (\mu g\ g^{-1}) = 17.30(Abs_{646})-7.18(Abs_{663})$$

RESULTS AND DISCUSSION

Effect of Various Sucrose Concentrations

In order to increase the tolerance to LN, *Mokara* orchid shoot need to preculture in sucrose enriched MS medium. Sucrose participates in the improvement of dehydration and freezing tolerance in plant tissues (Grapin *et al*., 2003). The beneficial effect of sucrose in cryopreservation could be due to two factors (Steponkus *et al*., 1992). First, sucrose has like other osmotically active compounds, an osmotic dehydration effect during treatments leading to reduced water content in the tissue (Tanaka *et al*., 2004). Second, sucrose is able to enter the cell as shown also by historical observation of intercellular starch accumulation during precultured (Gonzalez-Arnao *et al*., 1993).

The following analysis was conducted using *Mokara* orchid shoots that were precultured in MS medium in the treatment of various sucrose concentrations (0.06, 0.1, 0.25, 0.5, 0.75 M) with different sizes (0.5-1.0 and 1.0-1.5 cm) and incubation durations (24 and 48 h). After excision into 2 different sizes, *Mokara* orchid shoots were incubated in semi-solid culture medium with different sucrose concentrations (0.06 (control), 0.1, 0.25, 0.5, 0.75 M) for 24 and 48 h and viability were then tested using TTC method. Figure 3 shows the absorbance value of shoots after pecultured for 24 h. The shoots with larger size that were precultured in 0.25 M sucrose had recorded the highest average absorbance (0.10). However, statistical analysis shows that there was no significant different between large shoot that were precultured in 0.25, 0.5 and 0.75 M sucrose. And the smaller shoot size (0.5-1.0 cm) which was precultured in 0.1 M sucrose had a lowest absorbance value (0.03).

For 48 h incubation, larger shoot size (1.0-1.5 cm) that were precultured in 0.25 M sucrose were also given the highest average absorbance value (0.13), the absorbance increases from control to 0.25 M and then decrease in 0.5 and 0.75 M (Fig. 4). The 0.75 M sucrose treatment of smaller shoot size was recorded as a lowest absorbance value (0.02). However, there was a difference in the absorbance obtained for both the sizes when each size was compared in general (Fig. 3).

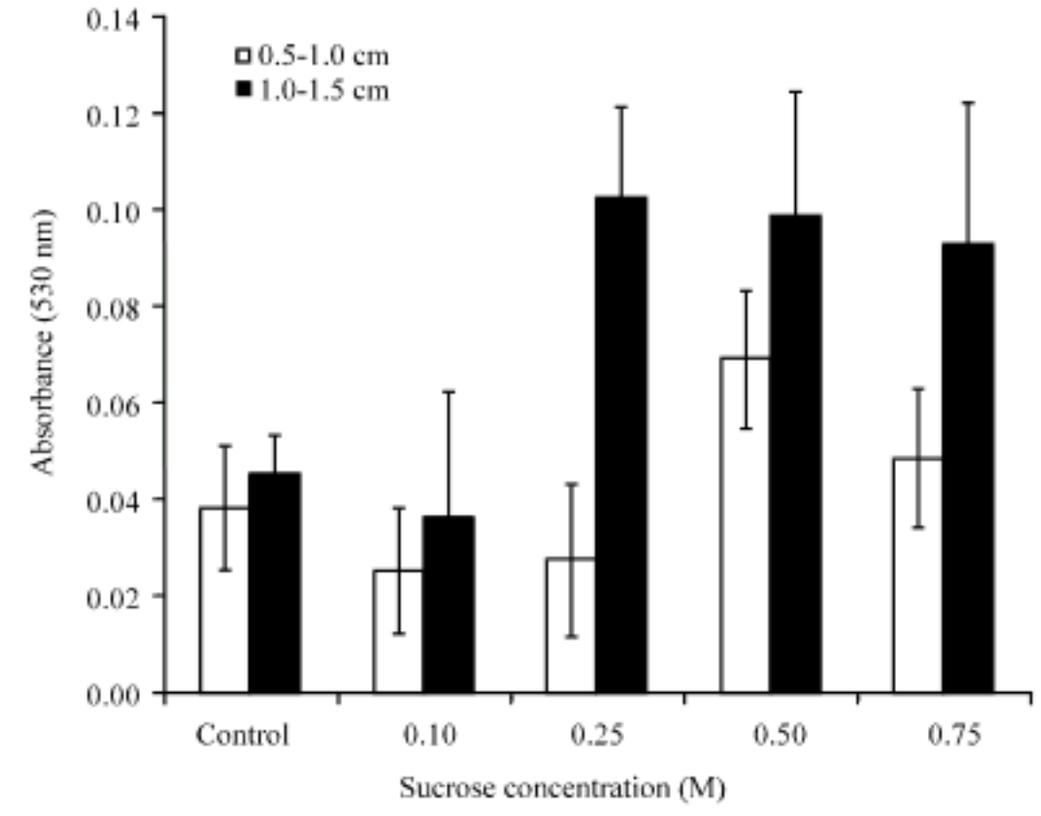

Fig. 3: Treatment of *Mokara* orchid shoots with various sucrose concentrations and different shoot size that was precultured for 24 h. Error bars show corresponding standard deviation

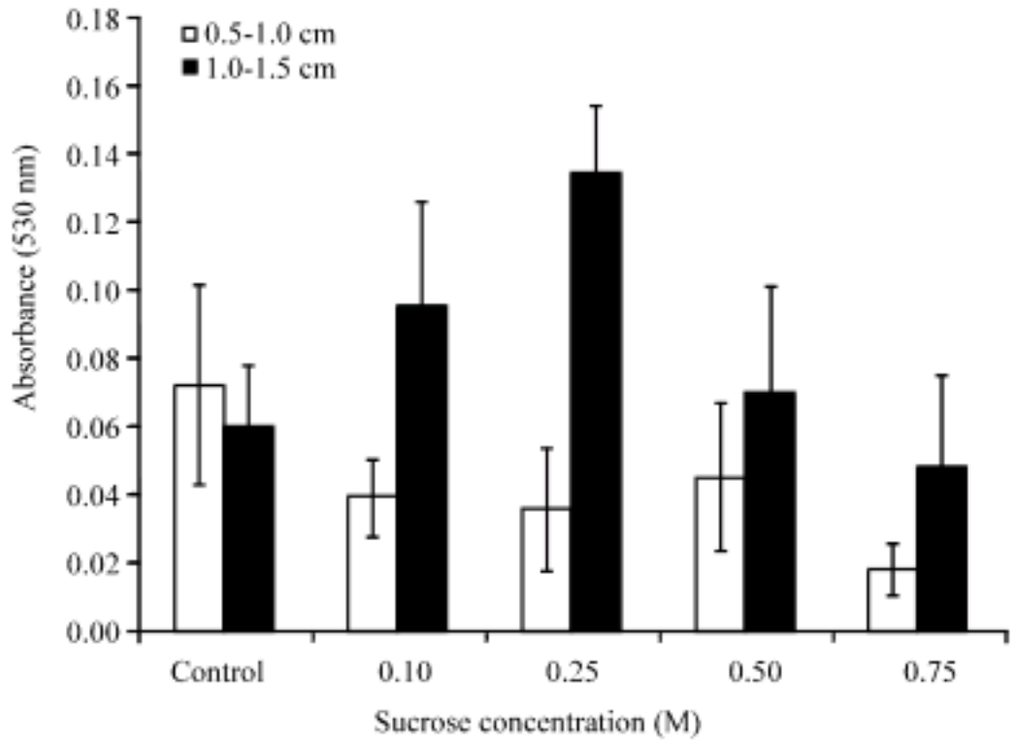

Fig. 4: Treatment of *Mokara* orchid shoots with various sucrose concentrations and different shoot size that was precultured for 48 h. Error bars show corresponding standard deviation

Figure 3 and 4 show the fluctuation or unstable data that results a high standard deviation. This fluctuation may caused by genetic factor or variation in explants since the experiment were not started from explants sterilization, the plant material had been cultured. Since the standard deviation was overlaps, there is no significant different of shoots that were precultured in 0.25 M compare to shoots was precultured in 0.5 and 0.75 M sucrose, suggesting that three concentrations yield similar viabilities in the pretreatment of the shoots.

It was evident that the absorbance obtained for the shoot was a function of the size, the surface area and the amount of the tissue present in the sample, with 1.0-1.5 cm giving higher absorbance when compared to the 0.5-0.1 cm orchid shoot. The preculture in media containing high sucrose concentration was important for the survival of *Mokara* orchid shoots after cryopreservation. At 48 h preculture period, shoots (1.0-1.5 cm) in media containing a sucrose concentration of 0.25 M was shown to produce the highest absorbance among all treatment (Fig. 5). The highest absorbance value was recorded by 0.25 M sucrose and followed closely by 0.01 M sucrose. The lowest absorbance value was recorded by 0.75 M sucrose but the result showed that there was no significant difference. Based on these observations, the large size (1.0-1.5 cm) of shoot tips after precultured for 48 h (Fig. 5) were selected for the subsequent experiment.

Effect of PVS2 at Different Time (5, 10, 15, 20, 25, 30 Min) and Temperature (0 and 24°C) Prior and Post Storage in Liquid Nitrogen

One of the keys to successful cryopreservation by vitrification is the careful control of dehydration and prevention of injury by chemical toxicity (Vendrame *et al*., 2007). Optimising the time of exposure or the temperature during exposure to PVS2 is important for producing a high level of shoot formation after vitrification. To determine the optimal time of exposure to PVS2, the best result from part I experiment (precultured *Mokara* orchid shoots in 0.25 M for 48 h) were treated with PVS2 at 24 and 0°C for different time (5, 10, 15, 20, 25, 30 min) before being plunged into LN and regrowth for two weeks under dark conditions. The treatment with PVS2 but without plunged into LN were used as a control. After two weeks recovery, viability of shoots was measured using TTC method and chlorophyll

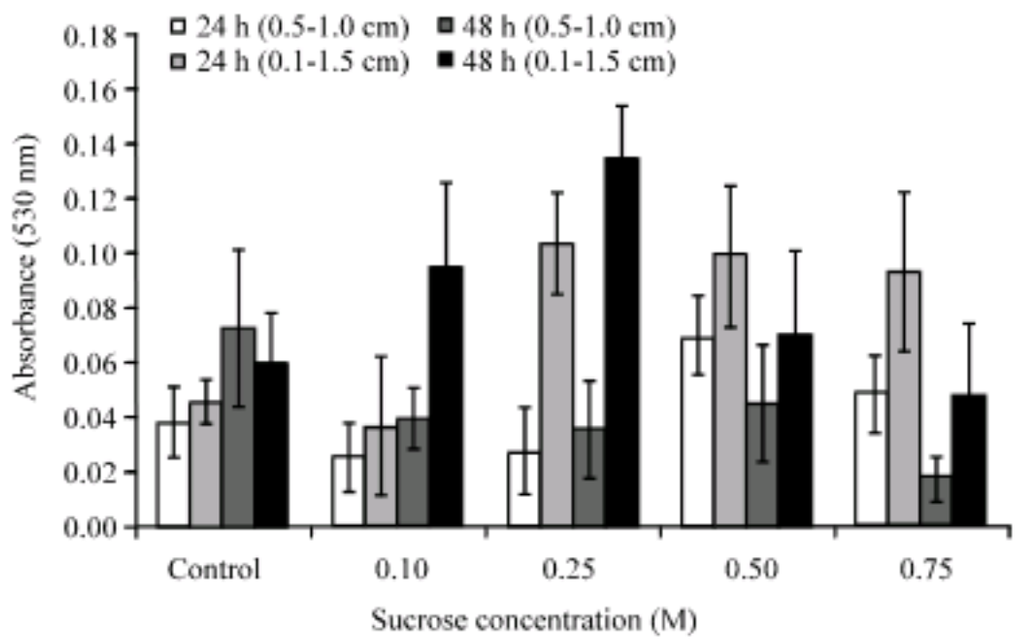

Fig. 5: Treatment of *Mokara* orchid shoots with various sucrose concentrations and different shoots size that was precultured for 24 and 48 h. Error bars show corresponding standard deviation

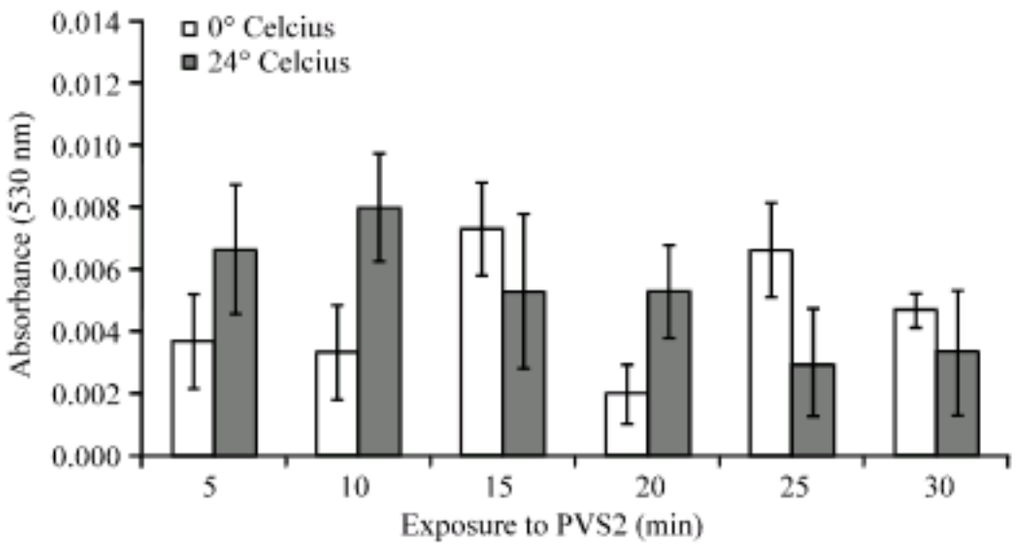

Fig. 6: Treatment of *Mokara* orchid shoots with the presence of both PVS2 and Liquid Nitrogen (LN). Error bars show corresponding standard deviation

determination. TTC staining is a reference method of the International Seed Testing Organization (ISTA) for testing seed viability and can be used to test biochemical activity of plant tissue after cold treatment (Van Waes and Debergh, 1986). The result will have the red colour, can either be visualized under a microscope (Pellet and Heleba, 1998) or analysed with a spectrophotometer (Harding and Benson, 1995) after extracted with 95% ethanol and boiled in 80°C water bath.

Figure 6 shows the effect of the exposure of shoots to PVS2 at two different temperatures (0 and 24°C) with (+LN) or without (-LN). Viability of shoot at 24°C reached the highest absorbance (0.008) at 10 min exposure, followed a slightly decreased at 15 to 30 min which could be due to the toxic effect (Fig. 6). Long exposure of explants to high concentrated vitrification solution is potentially injurious because of the phytotoxic effects of individual component or combined osmotic effect on cell viability (Towill and Jarret, 1992; Sakai, 2000). Steponkus *et al*. (1992) reported that exposure of protoplasts to the vitrification solution for more than 5 min resulted in the complete disruption of the plasma membrane. In the treatment at 0°C, control (shoots without plunged into LN) showed highest absorbance even though it was exposed to PVS2 treatment for longer time duration. These result show that vitrification does not cause additional loss

of shoot formation during dehydration by PVS2 (Fig. 6). For overall treatment, the result show that the almost 0°C treatment gave the better absorbance, which could reduced the injurious effects of PVS2.

Other observation that could be detected after cryopreservation treatment was the physical condition of shoots. After two weeks exposure to the light, some shoots was retained their colour and meanwhile some other shoots were bleached due the effect of photo oxidation.

Chlorophyll Determination

Chlorophyll is the pigment found in plants that give them their green colour and absorbs the light necessary for photosynthesis (Laasch and Weiss, 1989). There are actually 2 main types of chlorophyll, named a and b. They differ only slightly, in the composition of a side chain (in a it is -CH_3, in b it is CHO). Both of these two chlorophylls are very effective photoreceptors because they contain a network of alternating single and double bonds and the orbitals can delocalise stabilising the structure.

Cryopreservation treatment of *Mokara* orchid shoots at 0°C without LN (LN-) for 5 min exposure to PVS2 recorded as highest chlorophyll content. The shoot that did not subjected into LN almost gave highest chlorophyll content. Observation of photosynthetic activity in shoots of *Mokara* orchid hybrid subjected to liquid nitrogen allowed us to formulate that the deep-freezing method developed thus far inhibits activities of both photo systems to certain extent. This might be a potential cause for the lack of regeneration of plant material after its transfer to normal conditions. Despite strong inhibition of photosynthetic electron transport, observation indicates that the deep-freezing does not cause an immediate death of the shoots (Bukhov *et al*., 2006).

Figure 7a-d clearly show that total chlorophyll a for shoots in all treatment were higher than chlorophyll b, whereas for all treatments found that the chlorophyll b was zero.

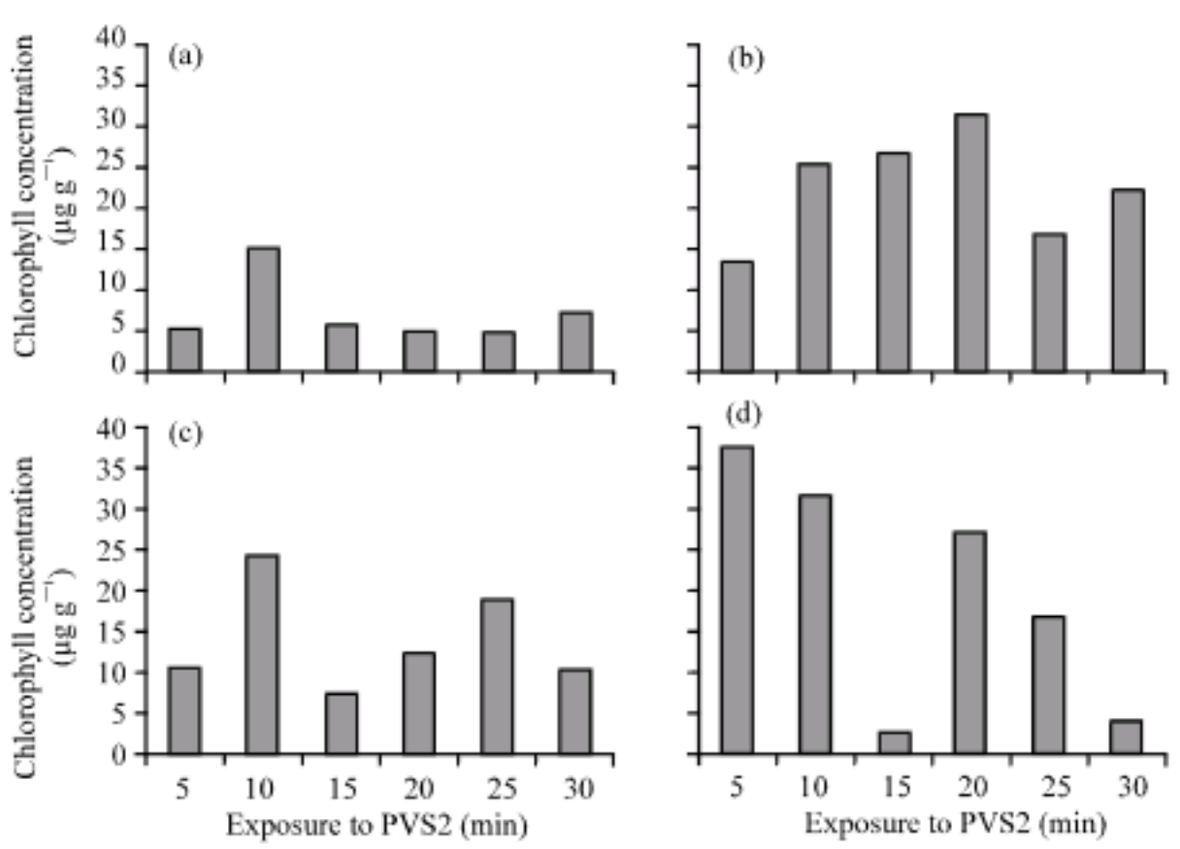

Fig. 7: Chlorophyll *a* and *b* of *Mokara* orchid shoots after treatment of PVS2 in different temperature and duration, without and post plunging into LN. (a) LN+ and (b) LN- are treated in 24°C; (c) LN+ and (d) LN- are treated in 0°C. The graph shows chlorophyll a give the higher value than chlorophyll b, the value of chlorophyll b was zero

The different side groups in the 2 chlorophylls tune the absorption spectrum to slightly different wavelengths, so that light that is not significantly absorbed by chlorophyll b, were captured by chlorophyll a, which absorbs strongly at that wavelength. Thus, these two kinds of chlorophyll complement each other in absorbing sunlight.

CONCLUSION

The procedure described for cryopreservation of *Mokara* Golden Nugget shoots is relatively simple and reliable whereby cryopreserved shoots could germinate and seedlings developed into normal healthy plants. *Mokara* orchid shoots precultured in 0.25 M sucrose for 48 h gave the best result for result in the first part. For the overall treatment, it was shown that 0°C treatment gave the highest absorbance value which could reduce the injurious effects of PVS2. For chlorophyll determination, cryopreservation of shoots at 0°C without LN (LN-) for 5 min exposure to PVS2 recorded as highest chlorophyll content. The result also shows that total chlorophyll a for shoot in all treatment were higher than chlorophyll b. Chlorophyll content for treatment at 0°C with LN (LN+) for 10 min exposure to PVS2 recorded as highest chlorophyll content. Cryopreservation method developed thus far inhibits activities of both photosystems to certain extent. Despite strong inhibition of photosynthetic electron transport, observation indicates that the freezing does not cause death of the shoots. The combination of optimized low temperature with dehydration treatments prior to cryopreservation showed to be necessary for the success of the orchid vitrification method in this study.

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