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Preliminary Study of DMSO Vitrification Technique of *Dendrobium* Sonia 28 Using Protocorm-Like Bodies (PLBs) Explant

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Abstract: *In vitro* grown shoot derived from protocorm-like bodies of *Dendrobium* sonia 28 hybrid were cryopreserved under liquid nitrogen condition, by means of DMSO vitrification method. Prior to the cryopreservation, the shoot were excised into two types of different length of 0.5-1.0cm and 1.0-1.5cm. Those entire excised shoot were grown on half-strength Murashige and Skoog (MS) semi solid medium. Upon DMSO vitrification method, the shoots were precultured (24 and 48 hours) at different sucrose concentrations (0.06M, 0.10M, 0.25M, 0.50M and 0.75M). Vitrification process proceeded with culturing of the shoots in a loading solution consist of mixture of 2M glycerol and 0.4M sucrose for 20 minutes at room temperature (28°C), followed by further dehydration process with DMSO for a different incubation duration (0 minute, 10 minutes, 20 minutes and 30 minutes) at temperature of 0°C and 24°C. The shoots were later plunged into liquid nitrogen. After recovering from the liquid nitrogen storage, the shoots underwent rapid thawing (40°C) and were grown in a regrowth semi solid medium for two days under dark condition. TTC analysis was carried out to determine the viability of the shoots after storage under liquid nitrogen. The highest absorbance value at 540nm was obtained using 2,3,5 triphenyl tetrazolium chloride (TTC) assay from the treatment of 1.0-1.5cm shoots precultured for 24 hours in 0.5M sucrose concentration MS semi-solid medium at 0°C for the 10 minutes incubation time in DMSO solution. The DMSO vitrification method was a crucial step in the orchid cryopreservation of *Dendrobium* sonia 28 hybrid shoot. Treatment of DMSO solution was proven to be capable of carrying out the dehydration process which was important for the survival rate of *Dendrobium* sonia 28 hybrid shoot undergoing cryopreservation at ultra low temperature (-196°C).

Key words: *Dendrobium* sonia 28 · DMSO Vitrification · TTC assay

INTRODUCTION

There are over 100,000 commercial hybrids registered worldwide, being grown as cut flowers and potted plants. In some tropical countries, orchids have become the major ornamental export crops and the demand for their cut flowers has been increasing by leaps and bounce over the pass few years. Recent data indicates that the demand for orchids alone, represent 8% of the global floriculture trade, with *Dendrobium* hybrids being most commercially desirable due to its number of flower per inflorescence and recurrent flowering [1].

Dendrobium hybrids have a wide range of colours: lavender, white, golden-yellow or even combinations of these colours and some rare species can be bluish, ivory, brilliant orange, scarlet or having exotic markings on the petals. *Dendrobium* orchids have relatively short production cycle from seedling to full bloom. *Dendrobium* orchids usually bloom several times a year regardless of seasons and due to that, they can easily be obtained all year round and is well suited to be used as excellent cut flowers for flower arrangement.

The loss of plant genetic resources is getting more and more serious and it attracts attentions worldwide due to the risk of losing important biodiversity. Continuous replacement of ancient varieties and wild type of orchids with a limited number of selected cultivars is one of the main contributors to genetic erosion [2]. Therefore

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the need for seed storage of high quality orchid's seed has surfaced. Seed storage plays an important role for long-term conservation of orchid species seed, contributing to both preservation and distribution of germplasm at reduced cost [2].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 [3]. The development and growth of the cryopreserved orchid is referred as '*frozen*'. Thus, the viability of the stored material is retained, so that their biological functions and growth can be reactivated after thawing and recovery stage which are among the protocols of cryopreservation technique. Besides from the benefits stated, applying cryopreservation as a mean for long-term germplasm storage also minimizes the risk of contamination and somaclonal variation of the plant [3]. Cryopreservation technique brings a thousand and one benefits in the field of long-term conservation of genetic materials as long as the protocols are followed strictly.

The Objectives of this Study Are:

- To establish an efficient techniques for *Dendrobium* sonia-28 using DMSO vitrification method using shoots culture,
- To evaluate the effects of various sucrose concentrations (0.058, 0.1, 0.25, 0.5, 0.75M) on explants which are precultured at 24 hours and 48 hours,
- To study the effects of DMSO at different time (0, 10, 20, 30 minutes) and temperature (0°C and 24°C) prior to the storage in liquid nitrogen,
- To determine the survival rate (viability) and the regrowth efficiency of the explants after undergoing cryopreservation procedure,
  - Viability assessment (TTC method),
  - Chlorophyll analysis: Determination of chlorophyll *a*, chlorophyll *b* and total chlorophyll content.

MATERIALS AND METHODS

Plant Material: The *in vitro*-grown protocorm-like bodies (PLBs) of *Dendrobium* sonia-28 orchid hybrid (Fig. 1) 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±2°C in a 16h photoperiod under cool white fluorescent lamps (Philips TLD, 36 W) at 150 µmol m^{-2} s^{-1}.

Fig. 1: Stock culture of the in vitro Dendrobium sonia-28 hybrid. The bar in the bottom image represents 1.0 cm.

Preparation of Shoots for Vitrification: Shoots from the conical flasks were taken out aseptically and excised precisely into 2 categories; 0.5-1.0cm and 1.0-1.5cm. The accurate excision was carried out with the using of ruler placed beneath the Petri dish where the shoot tips were under excision.

Determination of survival and viability: Viability of the plant materials were determined using 2,3,5-triphenyltetrazolium chloride (TTC) reduction with assay according to Ishikawa [4]. The estimation was done based on the turning of colourless TTC into pinkish red formazan produced in living cells due to reductase and dehydrogenase processes. The plant materials were inserted into test tubes and soaked with 2ml of TTC solution for 15 hours at room temperature. The TTC solution was later drained off and the shoots were washed with distilled water. The shoot tips were centrifuged and the cells were extracted with 7ml of 95% ethanol. The extract was cooled and top up to 10ml using 95% ethanol. The solutions were tested for absorbance using spectrophotometer (Spectro 22, Digital Spectrophotometer, Labomed. Inc.) at 540nm.

Preculture of Shoots with Sucrose: The excised shoot tips were then pre-cultured in sucrose enriched MS semi-solid [5] semi-solid medium. The effect of sucrose concentration on the growth of the shoots was tested by pre-culturing the excised shoot tips in MS-agar medium which consist of different level of sucrose concentration, 0.06M, 0.10M, 0.25M, 0.5M and 0.75M. The first set of Petri dishes were precultured for 24 hours with photoperiod of 16 hours under cool white fluorescent lamps (Philips TLD, 36W) at 150µmol $m^{-2}s^{-1}$ at temperature 25± 2°C while the second set of Petri dishes were precultured for 48 hours with photoperiod of

16 hours under the same condition as the first set. The optimized growth condition which produced explants with highest tolerance to DMSO vitrification was employed to proceed in next experiment.

DMSO Vitrification Technique: Cryopreservation of explants without liquid nitrogen storing: Four cryovials were loaded with 2-3 shoots each and filled up with loading solutions. The cryovials were then left for 20 minutes before the loading solutions were pipette out. After the withdrawal of loading solutions, the cryovials were then filled up with DMSO (Dimethyl Sulphur Oxide). The period of immersion of the shoot tips in the cryoprotectant solution varied from 0 minute, 10 minutes, 20 minutes and 30 minutes respectively. From then, the cryoprotectant solution was extracted and the cryovials were then filled up once again with unloading solutions for 20 minutes. Finally, the cryovials were dried out and the shoots were planted into 4 new half strength MS semi-solid agar medium according to their period of immersion in cryoprotectant solution and were kept incubating in dark condition at temperature 25°C. After two days, the shoots were transferred to another four fresh half strength MS semi solid agar medium. The Petri dishes were then kept back in dark condition for another two weeks. The procedure was repeated whereby the incubation of shoot tips in cryoprotectant solution was carried out in styrofoam where temperature was lowered to 0°C by filling in with ice cubes.

Cryopreservation of Shoots with Liquid Nitrogen Storing: Similar to the procedure done in the cryopreservation of explants in the absence of DMSO cryoprotectant treatment, four cryovials were loaded with 2-3 shoots each. Then loading solutions were filled into the cryovials for 20 minutes. After withdrawing the loading solutions, DMSO solution were pipette into the cryovials incubating the shoot tips in the cryovials for 0 minutes, 10 minutes, 20 minutes and 30 minutes respectively. After the duration of incubation, the cryovials were plunged immediately into liquid nitrogen tank for storage. The procedure was repeated whereby the incubation of shoots in cryoprotectant solution was carried out styrofoam where temperature was lowered to 0°C by adding in ice cubes.

Thawing and Unloading Treatment: After being stored under liquid nitrogen (-196°C) overnight, the shoots were defrost over the thawing process. The thawing took place in a water bath at 40°C for 90 seconds. The duration of the thawing process was critical as excessive warming may result in cell damage and lost of viability. The cryovials were then continuously swirled to cool down the wall of the cryovials. The solution in the cryotubes was pipette out and unloading solution was filled into the cryovials to wash off the cryoprotectants from the plant materials.

Regrowth and Recovery: After going through the thawing process, the fragile shoot tips were transferred carefully and aseptically in to four half strength MS semi solid agar medium according to their immersion period in DMSO solution. The Petri dishes were then kept in dark condition for 2 days before being transferred to another four new half strength MS semi solid agar medium for another 2 weeks, also in dark condition at temperature 25± 2°C.

Chlorophyll Determination Using Harborne Method [6]: To determine the chlorophyll content on the plant materials, the following procedures were taken. First, a few shoots were taken and grind using mortar and pestle. The grinding continued till sufficient amount of ground plant materials were obtained, 0.1g. The grinding continued with the addition of 1.0g calcium carbonate ($CaCO_3$) powder and 5ml of 80% acetone solution. All these procedures were carried out in an ice box where the temperature was lowered to 4°C. The ground extract was filtered with funnel through filter paper. The mortar and pestle were cleaned up after usage using 80% acetone solution. The absorbance of the ground plant materials were determined using spectrophotometer at 646nm and 663nm. The following calculation formula was applied to calculate the chlorophyll content of the ground plant materials.

Chlorophyll *a* = $12.21A_{663nm} - 2.81A_{646nm}$
Chlorophyll *b* = $20.13A_{646nm} - 5.03A_{663nm}$
Total chlorophyll content = $17.30A_{646nm} + 7.18A_{663nm}$

RESULTS AND DISCUSSION

The effects of preculture condition on the viability of plant shoots: In this study, plant shoots of *Dendrobium* sonia-28 were precultured in MS-semi solid medium which contains different level of sucrose concentration: 0.06M, 0.10M, 0.25M, 0.5M and 0.75M.

Length of the plant shoots to be cultured was set as another parameter. The plant shoots were categorized into two groups: 0.5-1.0cm and 1.0-1.5cm. The purpose of setting this parameter was to find out plant shoots at which length cultured in half strength MS semi solid medium with sucrose at which concentration, would give a higher viability to the plant shoots. The incubation

period for the pre-culture plant shoots were set to two different groups as well: 24 hours and 48 hours respectively. These three parameters set, correspond to each other with the purpose of finding out under which conditions, viability of plant shoots were at the highest. TTC (2,3,5-triphenyl tetrazolium) test method was applied and the viability of the plant shoots was compared according to their absorbance value at 530nm read using spectrophotometer. The comparison of the viability of plant shoots precultured under different condition was presented in bar graphs.

Figure 2 and 3 show the viability of plant shoots precultured at three parameters; length of plant shoots, period of culturing and sucrose concentration. All these three parameters bring effects to the survival rate of plant shoots in half strength MS semi-solid medium. As for the length parameter, both Figure 2 and 3 clearly indicated that the viability of the plant shoots with longer length (1.0-1.5cm) is much higher than the plant shoots with shorter length (0.5-1.0cm). Therefore, plant shoots with length of 1.0-1.5cm is adopted to be cultured for further studies application. For the incubation time period of 24 hours and 48 hours, both Figure 2 and 3 indicate that the survival rate of plant shoots precultured for 24 hours has higher viability, but the difference in the absorbance values are not significant. Hence, it is concluded that, incubation time period of 24 hours compared with 48 hours does not really bring much of an effect on the viability of the plant shoots growth.

The most important parameter in this preculture condition would be the sucrose concentration. Preculture of plant shoots in medium containing sucrose is a beneficial approach in the case of improving survival of plant shoots in LN with the presence of cryoprotectants [7,8]. Besides acting as the main carbon source in the half strength MS semi-solid medium, sucrose exhibits osmotic dehydration effect during treatment which resulted in the reduce of water content in the cells [9,10]. The accumulation of sucrose in the plant cells enhanced cell viability by removing the water content in the plant cells to the point of glassy state during vitrification in the presence of LN [11]. Last but not least, sucrose also plays a crucial role in the preservation of the membrane integrity [12] and protein structure [13] during dehydration. Hence, it is clearly stated that the presence of sucrose in the medium is important to the growth and survival of the plants. Too low of sucrose concentration might not carry out the dehydration process completely while too high of sucrose concentration would over dehydrate the plant cells render it completely dry, thus resulting in the dying of the plant cells. Therefore optimum concentration of sucrose is to be applied so that the dehydration process can be carried out without totally dry out the plant cells. Plant shoots with the length of 1.0-1.5cm that were cultured for 24 hours in the environment of 25± 2°C in a 16 hours photoperiod under cool white fluorescent lamps (Philips TLD, 36W) at 150μmol $m^{-2}s^{-1}$, in half strength MS semi-solid medium consisting of 0.5M sucrose concentration gives the highest absorbance value, thus the highest viability rate (Fig. 2, 3). Therefore, such condition is applied to grow plant shoots of *Dendrobium* sonia-28 for further studies.

The Effect of Incubation Period of Cryoprotectant to the Viability of the Plant Shoots: The viability of the plant shoots that are not subjected to storage in liquid nitrogen is much higher than those that undergo liquid nitrogen storing (Fig. 4, 5). This phenomenon is expected as the plant shoots without liquid nitrogen storage would not suffer any freezing damage as occurred in cryopreserved samples. Therefore, more of the plant shoots cells survived in the TTC viability test.

By considering the temperature of DMSO treatment, it could be observed that under room temperature (24°C), plant shoots that do not undergo cryopreservation storage display higher viability than plant shoots that were treated with DMSO under 0°C. As for those plant shoots that were subjected into cryopreservation in liquid nitrogen, the temperature of DMSO treatment prior to the cryopreservation, does not exhibit significant difference. In fact, for plant shoots that undergoes cryopreservation with DMSO treatment under 0°C for 10 minutes produces the highest absorbance value and thus highest viability rate. The period of incubation of the DMSO treatment brings tremendous effects on the viability of the plant shoots as well. Different incubation period of DMSO treatmenthave various effects on the viability of the plant shoots. Finding the optimal time period of treatment with a vitrification solution (DMSO) is equivalent to establishing a correct balance between toxicity and an adequate dehydration to reduce water content which reduces the chance of lethal ice formation in the regenerative tissue [14]. The exposure of explants to the high concentration of vitrification solution brings potentially harm as the accumulation of phytotoxic due to the effects of individual components or combined osmotic effects on cell viability [15].

Based on Figure 4 and 5 that prior to cryopreservation in liquid nitrogen storage, plant shoots subjected to 10 minutes of DMSO treatment under 0°C would give the highest viability value after two weeks of recovery.

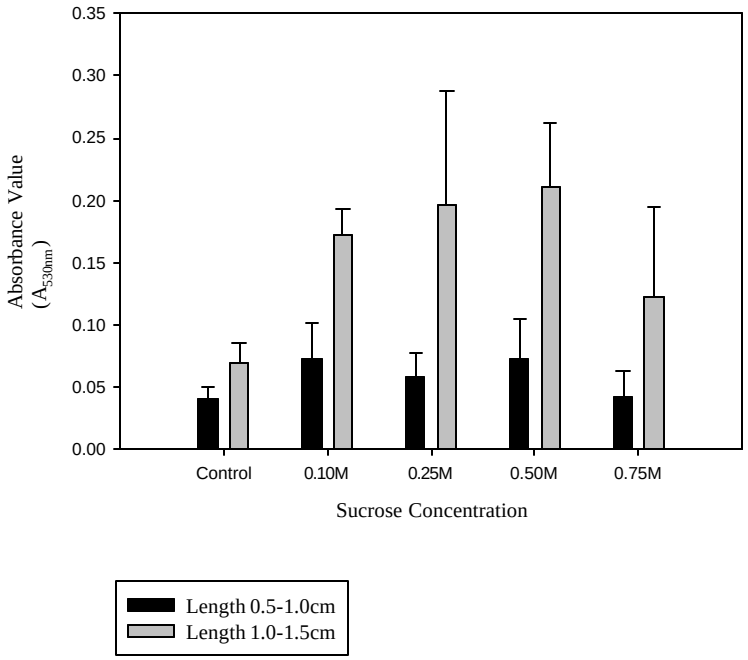

Fig. 2: TTC test method on the viability of plant shoots precultured for 24 hours with different sucrose concentration level of half strength MS semi-solid medium. The environment condition was 25± 2°C in a 16 hours photoperiod under cool white fluorescent lamps (Philips TLD, 36W) at 150µmol $m^{-2}s^{-1}$

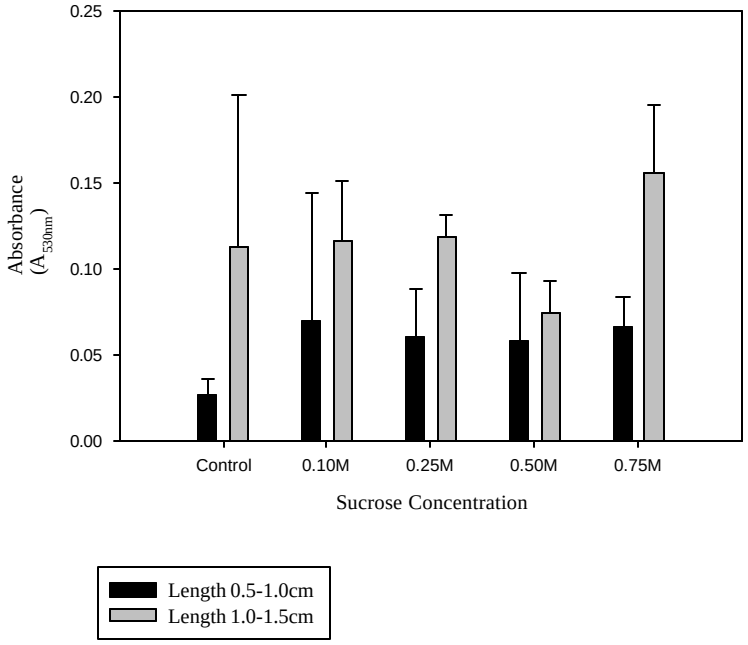

Fig. 3: TTC test method on the viability of plant shoots precultured for 48 hours with different sucrose concentration level of half strength MS semi-solid medium. The environment condition was 25± 2°C in a 16 hours photoperiod under cool white fluorescent lamps (Philips TLD, 36W) at 150µmol $m^{-2}s^{-1}$

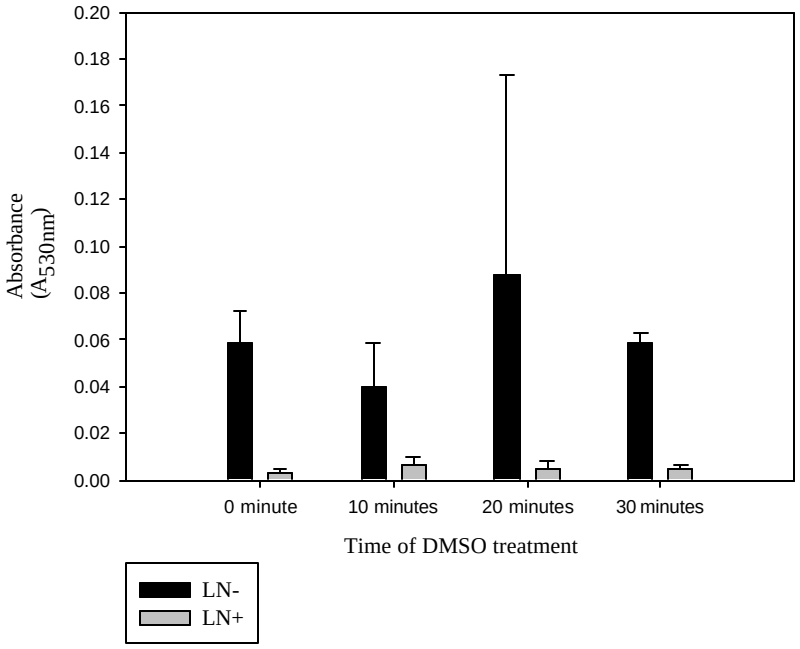

Fig. 4: Result of TTC assay on plant shoots after two weeks recovery from DMSO treatment at 0°C. The time of DMSO (cryoprotectant) treatment is crucial to the viability of the plant shoots

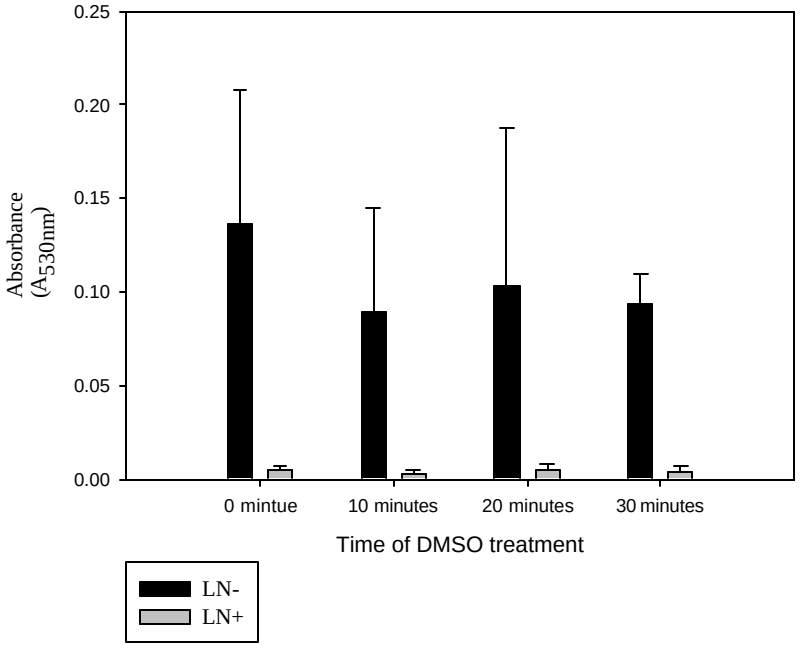

Fig. 5: Result of TTC assay on plant shoots after two weeks recovery from DMSO treatment at 24°C. The time of DMSO (cryoprotectant) treatment is crucial to the viability of the plant shoots

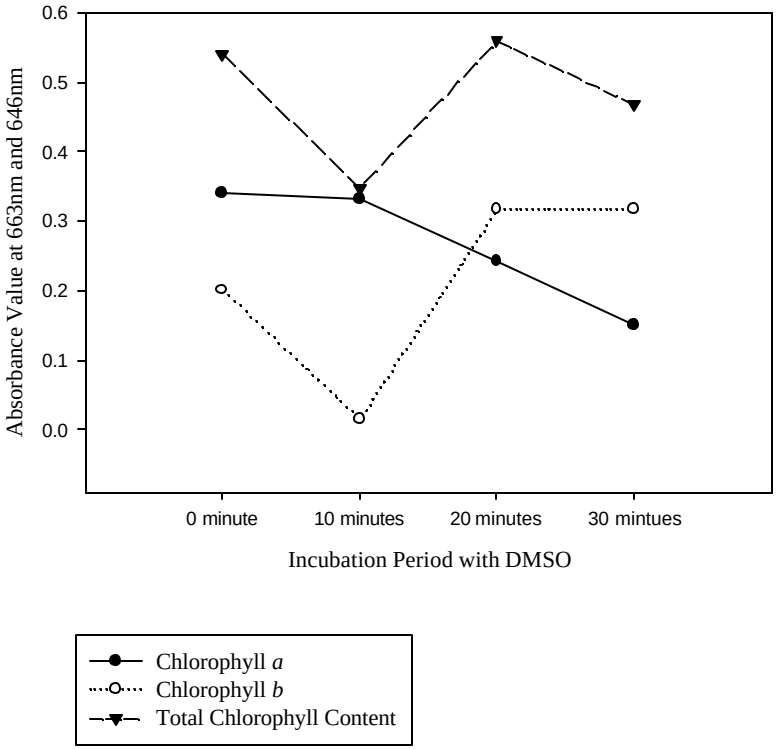

Fig. 6: Chlorophyll *a*, chlorophyll *b*, and total chlorophyll content of plant shoots after six-week recovery from DMSO treatment (without LN) at 0°C. The absorbance value was obtained using spectrophotometer at 530nm

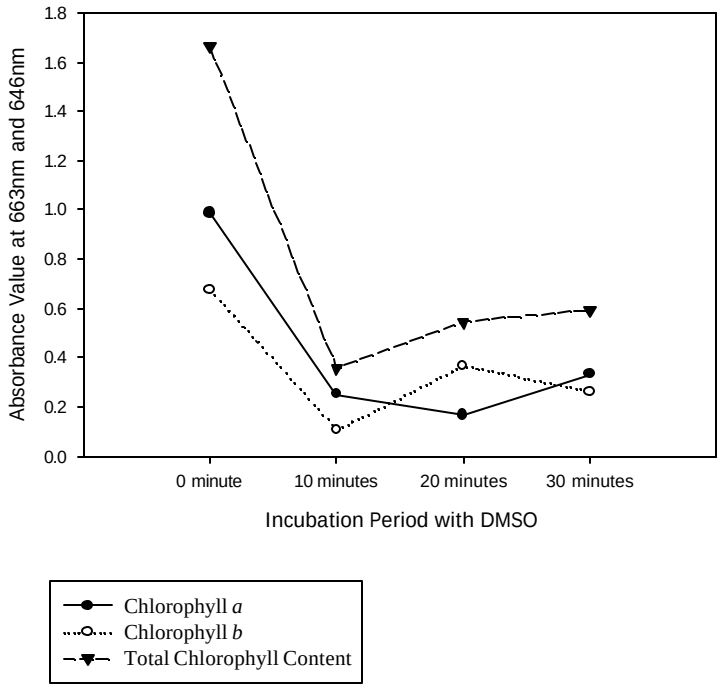

Fig. 7: Chlorophyll *a*, chlorophyll *b*, and total chlorophyll content of plant shoots after six-week recovery from DMSO treatment (without LN) at 24°C. The absorbance value was obtained using spectrophotometer at 530nm

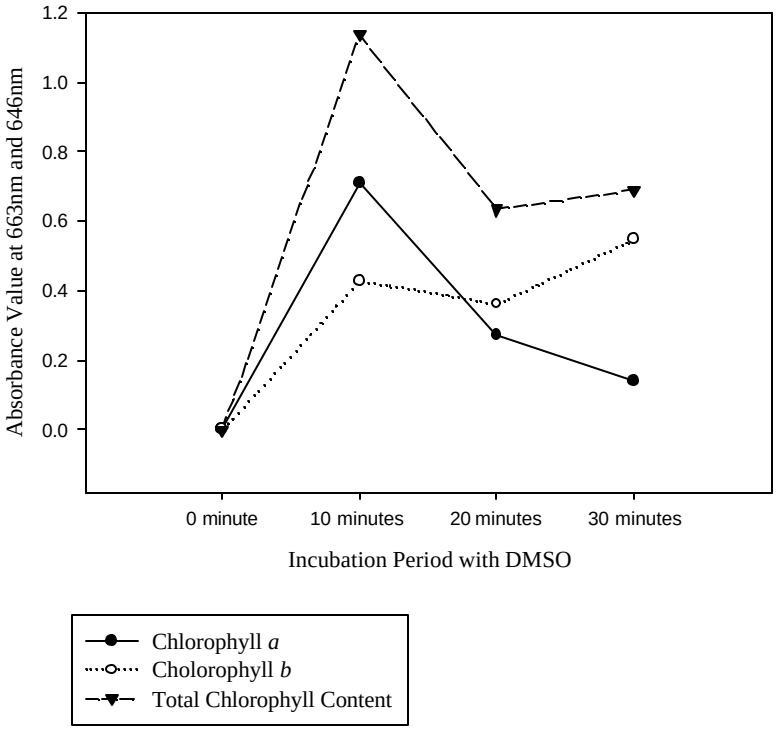

Fig. 8: Chlorophyll *a*, Chlorophyll *b*, and total chlorophyll content of plant shoots after six-week recovery from DMSO treatment (with LN) at 0°C. The absorbance value was obtained using spectrophotometer at 530nm

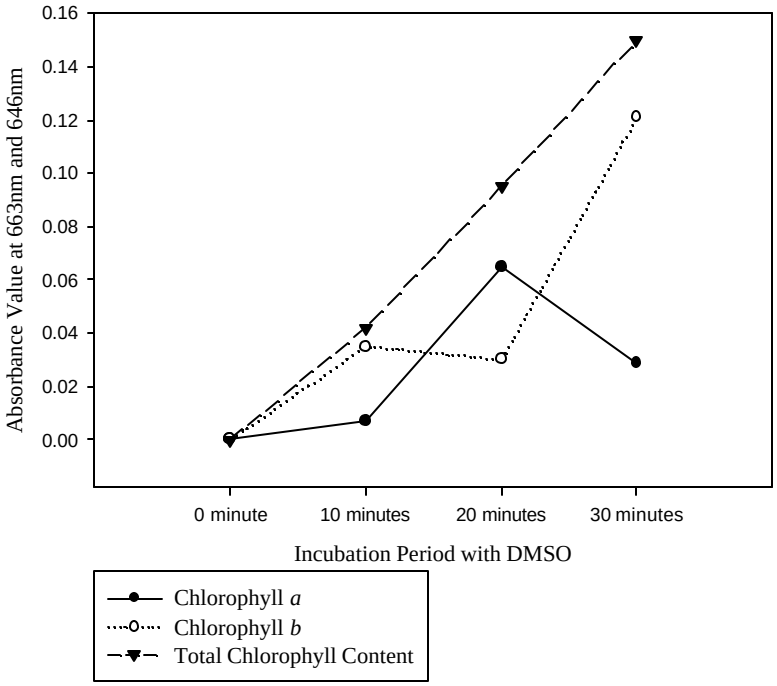

Fig. 9: Chlorophyll *a*, chlorophyll *b*, and total chlorophyll content of plant shoots after six-week recovery from DMSO treatment (with LN) at 24°C. The absorbance value was obtained using spectrophotometer at 530nm

Chlorophyll Analysis on Regrowth Shoots: Generally, all green plants need chlorophyll to carry out the process of photosynthesis in order to survive. Therefore, all living and growing plant cells should have chlorophyll pigments in them and the amount of chlorophyll which the plant possess should be proportional their health condition and growth rate. Healthy plants should have sufficient amount of chlorophyll which gives the colour of green on their leaves thus having a higher growth rate; plants which are not so healthy would present a much pale colour such as light green, yellowish or even whitish due to the insufficient amount of chlorophyll pigments in them, resulting in lower growth rate.

Chlorophyll analysis was carried out as an extra assessment to test the viability of the plant shoots. The analysis was applied on plant shoots after six weeks of recovery, so that to provide sufficient time for the plant shoots to regrowth and resume the production of chlorophyll pigments. Based on the graphs shown in Figure 6 to 9, overall it can be concluded that the plant shoots which did not undergo cryopreservation process (storage in liquid nitrogen tank) possess a much higher level of chlorophyll content, regardless of chlorophyll *a,* chlorophyll *b* or total chlorophyll content. This is expected due to the plant shoots that do not undergo cryopreservation process would not have to bear the stress caused by the ultra low temperature (-196°C) and therefore suffer no damages upon themselves. On the other hand, those plant shoots which undergo cryopreservation were put under harsh condition such as extreme coldness in liquid nitrogen tank, thawing process which might cause temperature shock and probably photo shock when they were being re-cultured under normal condition after being stored under dark condition in the liquid nitrogen tank.

From the Figure 8 and 9, it can be concluded that plant shoots shared a common starting point of 0 absorbance value. This can be explained by the fact that plant shoots that were expose to DMSO solution only for a short period (0 minute) and thus does not allow the cryoprotectant to incorporate into the cells. As a result the plant shoots were not protected when plunged into liquid nitrogen tank under extreme cold condition. Therefore, even after the recovery stage, too many damages had been done on to the plant shoot cells that were not protected by cryoprotectant. The survival rates of these plant shoots were tested to be 0 and thus no chlorophyll pigments could be found on them.

Regardless of the results on the survival rate of plant shoots which did not undergo cryopreservation, it is found that plant shoots that underwent incubation with DMSO solution for 10 minutes at the temperature of 0°C produced the highest viability rate among all the plant shoots that were plunged into liquid nitrogen tank for storage. The above finding is consistent to the chlorophyll analysis carried out where the analysis show that the recovered plant shoots which underwent incubation with DMSO solution for 10 minutes at the temperature of 0°C exhibits the highest content in chlorophyll which includes both chlorophyll *a* and chlorophyll *b*. It is utmost important that the cell viability of recovered plant shoots could be assess both quantitatively and qualitatively as it is a major protocol development and quality control for established routine procedures especially in the quality control within any gene bank for germplasm conservation.

The most common and widely used cell viability assessment is TTC test method, which is based on the concept of spectrophotometry [16]. This technique no doubt brings ambiguous result due to the fact that during the period shortly after thawing, the cells are rendered in the state of metabolic flux, which gives positive TTC assay characteristics regardless whether the cells are recovering or dying [16].

CONCLUSION

Based on this research study, it can be conclude that orchid shoots with the length of 1.0cm to 1.5cm exhibits the highest growth rate when pre-cultured for 24 hours at 25± 2°C in a 16 hours photoperiod under cool white fluorescent lamps (Philips TLD, 36W) at 150µmol $m^{-2}s^{-1}$ in half strength MS semi-solid medium which consist of 0.5M of sucrose concentration. Sucrose besides acting as the main carbon source, also contributes in the process of dehydration of the plant cells, which is a critical protocol in cryopreservation.

After two weeks of recovery, another TTC test was brought upon the plant shoots and it was found that plant shoots that undergo cryostorage in liquid nitrogen display a lower viability rate compared to those which does not undergo cryostorage in liquid nitrogen. Besides, the research project also shows that when the plant shoots are subjected to cryoprotectant solution (DMSO vitrification treatment) in optimum duration prior to cryostorage, the survival rate of the cryopreserved plant shoots increased. It is observed that plant shoots which are subjected to 10 minutes of DMSO treatment at 0°C displayed the highest viability rate among all the plant shoots that undergo cryostorage. Four weeks after the TTC testing, another test is carried out to support the result obtained, chlorophyll analysis. Every living plant

needs chlorophyll to perform photosynthesis which is critical for the survival of the plant, hence the survival rate of the plant is considered to be proportional to the amount of chlorophyll it possesses. After six weeks recovery from cryostorage, the plant shoots should be able to resume its production of chlorophyll pigment depending on its viability. The chlorophyll analysis test gives consistence result with the TTC test earlier that recovered plant shoots which underwent incubation with DMSO solution for 10 minutes at the temperature of 0°C exhibits the highest viability rate. In conclusion, conservation of *Dendrobium* sonia 28 by cryopreservation using shoots has been established. The effects of sucrose concentration on the orchid shoots and their preculture duration have been evaluated. The effects of DMSO treatment at various time length have been determined and finally the viability of the regrowth orchid shoots has been assessed via viability assessment (TTC method), chlorophyll analysis (chlorophyll *a,* chlorophyll *b* and total chlorophyll content.

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