# SURVIVABILITY AND PLANT REGENERATION STUDIES OF A *Vanda* ORCHID'S NON- ENCAPSULATED AND ENCAPSULATED PROTOCORM- LIKE BODIES (PLBS)

By

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A project report submitted to the Department of Biological Science

Faculty of Science

Universiti Tunku Abdul Rahman

In partial fulfillment of the requirement for the degree of

Bachelor of Science (Hons) Biotechnology

May 2014

## ABSTRACT

**SURVIVABILITY AND PLANT REGENERATION STUDIES OF A *Vanda* ORCHID'S NON- ENCAPSULATED AND ENCAPSULATED PROTOCORM- LIKE BODIES (PLBS)**

**Goh Yun Qiang**

*Vandas* are a group of well- known orchids which have a high market value. In this study, encapsulated and non- encapsulated PLBs of *Vanda* orchids were treated with different parameters and in different conditions (room temperature and liquid nitrogen). In the study of encapsulated PLBs, the PLBs were encapsulated by 3 % (w/v) of sodium alginate. The encapsulated PLBs were tested with different osmotica (sucrose, sorbitol and mannitol) and thawing temperatures (30 $^{o}$C, 40 $^{o}$C, 50 $^{o}$C and 60 $^{o}$C). In this study all cryopreserved PLBs did not survive, only the non- cryopreserved encapsulated PLBs survived. Sorbitol was the most suitable osmoticum, in which the survivability and germination percentage of non- cryopreserved encapsulated PLBs was 68.3 ± 2.3 % and 21.7 ± 2.4 %, respectively after four weeks. Besides, 40 $^{o}$C was the most suitable temperature, in which the survivability and germination percentage of non- cryopreserved encapsulated PLBs was 50.0 ± 4.7 % and 8.3 ± 2.3 %,

respectively after four weeks. On the other hand, the non- encapsulated PLBs were tested on the pre- culture duration (0 day, 1 day and 3 days) as well as different osmotica (sucrose, sorbitol and mannitol) and thawing temperatures (30 $^{o}$C, 40 $^{o}$C, 50 $^{o}$C and 60 $^{o}$C). The non- encapsulated PLBs were pre- cultured in sucrose solution. Then, the non- encapsulated PLBs were first treated with loading solution (LS) and further treated with plant vitrification solution 2 (PVS2). In this study, all the non- cryopreserved or cryopreserved non- encapsulated PLBs were dead within three days of culture. In conclusion, the encapsulated PLBs might be more suitable for long- term storage than non- encapsulated PLBs under room temperature condition. At this stage, cryopreservation was not suitable for the storage of *Vanda* PLBs. Further research is required to look for a suitable cryopreservation protocol.

## ACKNOWLEDGEMENT

I would like to express my greatest gratitude towards my supervisor, Dr. Tee Chong Siang for his encouragement, support and patience, and spending a lot of his time on guiding me throughout this project. He has been inspiring a figure who has shaped my study interest in plant tissue culture. Besides that, I would like to thank the laboratory assistant managers, Ms. Luke, Ms. Nurul and Ms. Choo for their technical supports. Furthermore, I would like to extend my appreciation to Mr. Tan Si Nan, a master student who willing to share his experience and being there to lend a helping hand when things were difficult. Special thanks to my lab mates for their cooperation and supports when problems occur. Most important of all, a big thanks to my beloved family, for their unconditional love, both emotionally and financially. Last but not least, I would also like to acknowledge those unmentioned ones who have help and supported me in any way during this project.

## DECLARATION

I hereby declare that the project report is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

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(GOH YUN QIANG)

## APPROVAL SHEET

This project entitled "**SURVIVABILITY AND PLANT REGENERATION STUDIES OF A *Vanda* ORCHID'S NON- ENCAPSULATED AND ENCAPSULATED PROTOCORM- LIKE BODIES (PLBS)**" was prepared by GOH YUN QIANG and submitted as partial fulfillment of requirements for the degree of Bachelor of Science (Hons) Biotechnology at Universiti Tunku Abdul Rahman.

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**PERMISSION SHEET**

It is hereby certified that **GOH YUN QIANG** (ID No: **10ADB0667**) has completed this final year project entitled "**SURVIVABILITY AND PLANT REGENERATION STUDIES OF A *Vanda* ORCHID'S NON-ENCAPSULATED AND ENCAPSULATED PROTOCORM- LIKE BODIES (PLBS)**" under the supervision of Dr. Tee Chong Siang (Supervisor) from the Department of Biological Science, Faculty of Science.

I hereby give permission to my supervisor to write and prepare manuscripts of these research findings for publishing in any form, if I do not prepare it within six (6) months from this date, provided that my name is included as one of the authors for this article. The arrangement of the name depends on my supervisors.

Your truly,

_____

(GOH YUN QIANG)

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| | |
|---|---|
| mm | Millimetre |
| M | Molar |
| min | Minute |
| mM | Millimolar |
| psi | Pounds per square inch |
| w/v | Weight per volume |
| % | Percentage |
| $^{o}$C | Degree Celcius |
| DMSO | Dimethyl sulfoxide |
| HCl | Hydrochloric Acid |
| MS | Murashige and Skoog |
| NaOH | Sodium Hydroxide |
| PLBs | Protocorm- like bodies |
| PVS2 | Plant vitrification solution 2 |

# CHAPTER 1

## INTRODUCTION

Orchids exhibit a massive range of diversity in size, shape and flower color. They can be found in tropical jungle, on the exposed slopes of rock cliffs, on the shoreline above high tide, at the edge of the desert, on the foothills of Himalayas, and even in the Arctic (Teoh, 2005). Orchids are famous with their long-lasting and bewitchingly beautiful flowers which fetch a high price in international floriculture market as compared to other flowering plant families (Saiprasad and Polisetty, 2003). It is known as one of the most diverse flowering plant families, with over 800 genera and 25,000 species (Chungh, Guha and Rao, 2009). In Penisular Malaysia alone, there are approximately 120 genera and also 800 species (Teo, 2009).

The fascinating charisma of orchids has attracted many people to make growing orchids as their hobby. However, today growing orchids is no longer just a hobby, but it is an international business. In the world floriculture trade, orchids covered around 8% of sales so it has great potential to change the economic landscape of a country (Chungh, Guha and Rao, 2009). According to Hossain (2008) orchids are the second most well known cut flower and potted flower plants in the ornamental industry.

Other than using orchids as the decorative materials, some orchids also have been used in food industry. For example, European consumes orchid tubers to acquire the amylopectins, which gives high concentration of glucose (Gott, 2008). The most well- known orchids use for food industry is *Vanilla* orchids. *Vanilla* orchids are grown for culinary use. Dried pods of several species of *Vanilla* can be used in food flavoring. In addition, patients on dialysis can use the unique flavor of these orchids to improve appetite (Bulpitt, Yan, Bulpitt and Wang, 2007). Other than *Vanilla* orchids, *Gastrodia elata, Dendrobium* species and *Bletilla striata* orchids are commercially grown in China to supply for the large Chinese herbal medicine industry (Bulpitt, Yan, Bulpitt and Wang, 2007).

Since orchids exhibit high popularity in global floriculture industry, cultivation of orchids contributing to many countries' economy. Malaysia is one of the main orchid exporters in the world (Kumaria and Tandon, 2007). Many Asia countries such as Singapore, Japan, Taiwan and Thailand are great competitors to Malaysia as orchid exporter. Thus, it is important for Malaysia to create new orchid hybrids and improve the production of orchids in order to survive in this competitive floriculture industry.

In order to meet the demand of the world orchid floriculture industry, mass production of orchids is essential. There are many ways of propagating orchids. The conventional vegetative propagation of orchids is not suitable for mass production because it is time consuming and expensive (Saiprasad and Polisetty, 2003). Orchid propagation by using seeds will lead to production of heterozygous plants (Chungh, Guha and Rao, 2009). In addition, orchid seeds are minute in size, with reduced endosperm and require of an association with mycorrhizal fungi for germination which cause difficulty in germinating orchid efficiently (Saiprasad and Polisetty, 2003). Thus, a more promising orchid propagation technique will be through meristem culture of orchids to produce disease- free, high quantity and quality plant materials with desired traits. Furthermore, by using plant tissue culture, orchids can be cultured irrespective of season and weather (Singh, 2009).

Normally, micropropagation of orchid is conducted through protocorm- like bodies which are obtained from germinating embryos and somatic tissues. Micropropagation of orchids through protocorm- like bodies (PLBs) is an important strategy in obtaining massive genetically similar orchid plants and for the improvement of plant quality (Paek, Hahn and Park, 2011). Despite micropropagation is effective in producing large number of orchid clones in relatively short duration, yet it is subjected to unpredictable mutations or somaclonal variation which can occur during the process of multiplication

(Khoddamzadeh et al., 2010). Somaclonal variation can be caused by many reasons such as the type of media, types of plant growth regulator, type of explants used and frequency of subculturing performed (Reuveni, Israeli, Degani. and Eshdat, 1986). Somaclonal variation leads to morphological or physiological changes in the tissue cultured plants which are mostly an unfavorable phenomenon in the ornamental industry (Chen and Chen, 2007). Since accumulation of somaclonal variation in micropropagtion is high, an efficient technique to maintain the genetic stability should be established to reduce it.

Besides, the population of orchids in natural habitats is reducing. Thus, an effective orchids' propagation and germplasm conservation techniques should be established to protect the wild orchids. Orchids are threatened for various reasons such as the increase adverse biotic influences, socio- economic development and uncontrolled commercial exploitation of forest wealth. Other factors affecting orchid population loss include improper use of land, deforestation and general exploitation natural resources (Kumaria, and Tandon, 2007). According to *International Union for Conservation of Nature* (IUCN) Red List of Threatened Plants, there are more than 34,000 plant species at risk of extinction, among these, 1,779 species belong to the Orchidaceae family such as *Vanda scandens*, *Vanilla phalaenopsis, Oncidium lancifolium*, etc. (Clark, Elliott, Tingley and Biro, 2002). Thus, immediate action on conservation and preservation action should be taken to protect these unique species.

Production of synthetic seed via encapsulation or plant organs can be served as an efficient tool for storage, plant conservation, and transportation of planting materials (Germana et al, 2011). It has been decades that the synthetic seed technology has been used to produce several commercial crops, for instance seedless watermelon, seedless grape, seedless cucumber, seedless jack, corn, soybean, genetic modified tomato and banana (Redenbaugh, Viss, Slade and Fuji, 1987). For orchids, Sarmah, Borthakur and Borua (2010) reported that *Vanda coerulea* Griff. Ex. Lindl plant propagation could be produced from encapsulated PLBs derived from six- month- old axenic leaves. Besides, based on their findings, the encapsulated PLBs could be an alternative tool for orchid germplasm conservation.

In order to store plant tissues or planting materials for a long period, cryopreservation can be a useful alternative technique. The use of liquid nitrogen with ultra- low temperature (-196$^{o}$C) is able to stop almost all biological activity and deterioration of the preserved plant tissues (Ching, Antony, Poobathy and Subramaniam, 2012). However, the successful use of cryopreservation involves many factors, such as the starting materials, pretreatement conditions, cryoprocedures, the use of cryoprotectants and post- thaw treatments. These factors are dependence on plant species. Therefore, a detailed study on individual plant species approach should be carried out in order to accomplish successful cryopreservation for each species.

Thus, the study was conducted with the objectives:

- to study the effects of the preculture time, the osmotica and thawing temperatures on the survival and plant regeneration of *Vanda* orchid's non-encapsulated PLBs, and
- to study the effects of the osmotica and thawing temperatures on the survival and plant regeneration of *Vanda* orchid's encapsulated PLBs.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 An Introduction to Orchidaceae

In Plant Kingdom, seed plants generally separated into two groups which are gymnosperms, the plants that lack of flowers and angiosperms, the flowering plants (Audesirk, Audesirk, and Byers, 2002). Orchidaceae, the orchid family, forms one of the largest families of angiosperms (Martin and Madassery, 2006). Orchidaceae can be further divided into five monophyletic subfamilies: Apostasioideae, Cypripedioideae, Epidendroideae, Orchidoideae, and Vanilloideae (The Plant List, 2010). The diverse Orchidaceae consists of more than 800 genera and 25,000 species (Florida Museum of Natural History, 2013).

Orchidaceae are monocots perennial herbs with simple leaves and parallel veins. They are well known for the rich diversity of their flower structures (Encyclopedia of Life, 2013). Their beauty, strange shapes and long life attract an abundance of attention. The orchid plant size varies greatly; a whole plant in bloom may only be the size of a nickel, others may have huge flowers with wispy petals (30 inches long) or sprays of smaller flowers (12-14 feet long) (Encyclopedia of Plant, 2010). The unique orchid flower structure allows orchids to be differentiated from the other flowering plants. As shown in Figure 2.0, the

floral parts of orchid plants are arranged in two whorls: the outer whorl is known as the sepals and the inner whorl the petals. One of these petals is modified into a structure, lip or labellum, which allows the pollinators to land. In the centre of the flower, the female and male sexual parts are combined into a fingerlike- structure called the column (Hugh and Hew, 1995). Column gives orchid flowers bisexual characteristic, each flower havs both male and female organs (Yam, 1996). The male reproductive organs include anther and filament while the female reproductive organs include stigma and style.

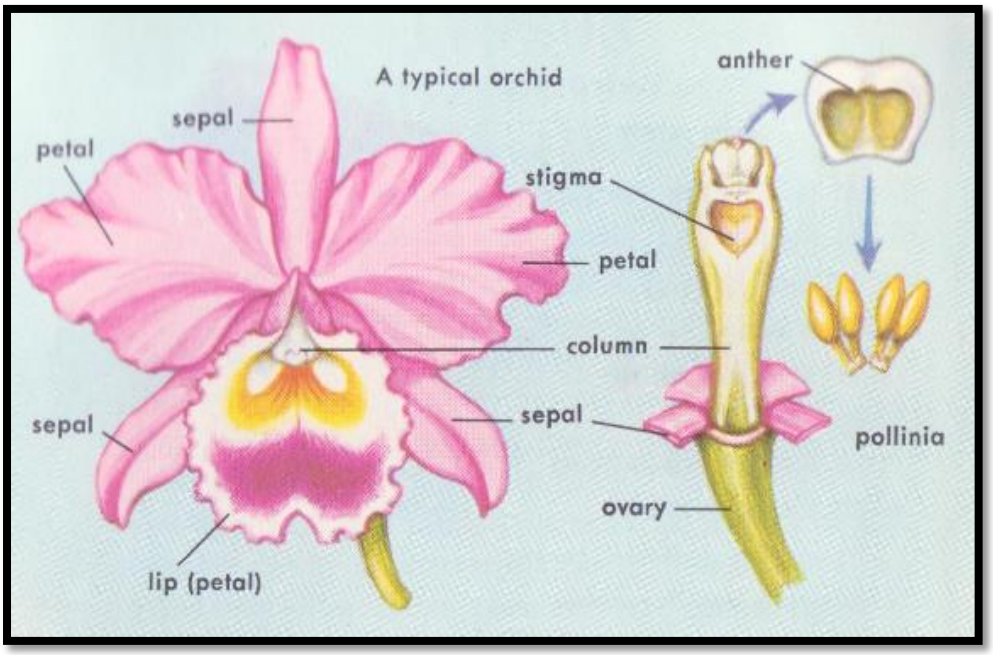


**Figure 2.0:** Typical orchid flora structure (Hugh and Hew, 1995).

The unique lip structure of orchid flower allows the landing of pollinating insects such as flies, wasps and bees for pollination (Yam, 1996). After pollination, fertilization occurs and fruits are formed. When the fruits ripened, it released masses of dust- like seeds. Orchid seeds are the smallest and most numerous in flowering plants (Swamy, Kumar, Ramakrishna, and Ramaswamy, 2004). Although orchids produce numerous seeds but orchid seeds are too minute to store nutrients for germination. Generally, orchid seeds do not contain endosperm and have no organized embryo (Venamy Orchids, 2002). So most orchid seeds can only germinate with a fungal symbiont (Encyclopedia of Life, 2013).

## 2.2 Sympodial and Monopodial Orchids

Orchids have two distinct growth patterns, sympodial and monopodial (Figure 2.1). Sympodial orchids grow by producing new growth via pseudobulbs, a succession of roots or bulb like stems (Allikas, 2009). Each new pseudobulb formed from the base of the pseudobulbs before it that connected by the rhizome (Proverbs, 2003). Pseudobulbs store water which as able to prolong sustainability of orchids in a dry environment (Allikas, 2009). The pseudobulbs can swell or shrink as moisture is stored or withdrawn. Most orchid genera are sympodial such as the *Catellya*, *Cymbidium*, *Dendrobium* and *Oncidium* (Hannah, 2012).

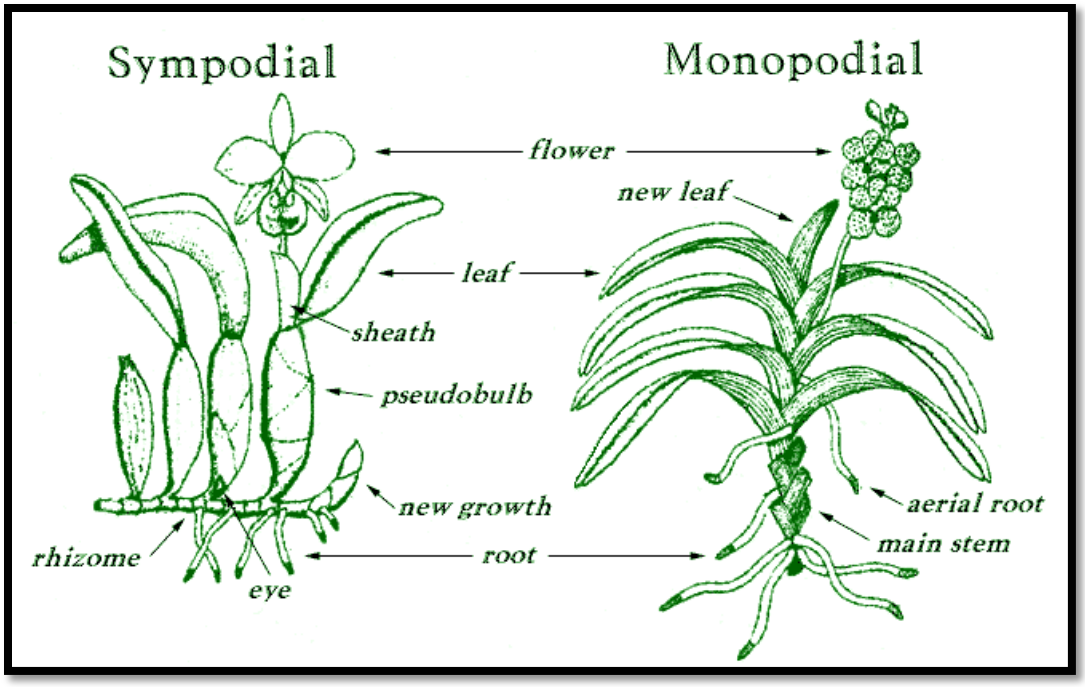


**Figure 2.1**: Sympodial and monopodial orchids (Allikas, 2009).

Monopodial orchids grow as a single upright stem with one leaf following another on the opposite sides of the center (Hannah, 2012). Monopodial orchids continuously grow upwards. It is significantly grow taller than the sympodial orchids, some may reach many feet in height under the ideal condition (Allikas, 2009). As monopodial orchids do not have pseudobulbs apart from their thick leaves and roots, so frequent watering is essential to maintain the survivability of these orchids. The examples of monopodial orchids are *Phalaenopsis*, *Vanda*, *Ascocenda* and *Angreacum* (Allikas, 2009).

## 2.3 Habitats of Orchids

Orchids are extremely adaptable and able to survive in all climates except for frigid and arid extremes. Orchids can be found in semi-desert regions, near the seashore and in the tundra (Rain Forest, 2013). However, orchids are more common in mature forest, open scrublands and swamps (George, 2012). Since orchids can grow in vast habitats, the way orchids grow in nature also can be used to differentiate these flowering plants.

Most of the orchids do not grow in the ground but instead grow naturally on trees. This kind of orchids is known as epiphytic orchids (Allikas, 2009). Epiphytic orchids are not parasites, thus, they do not absorb nutrients from the host plant. They only depend on the host plants for support and exposure to sunlight (Proverbs, 2003). They obtain their moisture and nutrients from air, rain and debris (Alikas, 2009). Epiphytic orchids favor situations where there is warmth, humidity and air movement to create suitable condition for their growth and reproduction. Thus, epiphytic orchids are mainly found in rainforest which generally has high rainfall and reliable seasonal changes (Introduction to Austrialian Orchidaceae, n.d.). *Vanda, Cattleya, Phalaenopsis and Brassia* are common examples of epiphytes (Dharmani, 2013).

Lithophytic orchids are another type of orchids that do not grow on the ground. They can be found growing with its roots attached to rocks or growing in extremely rocky terrain where there is scant humus matter (McDonald, 1999). Lithophytic orchids are rare and not commonly cultivated (Alikas, 2009). They are commonly grow in south Florida and along the Gulf Coast where high humidity and warm temperature that sustain their growth almost all year (McDonald, 1999). An example of a lithophyte is *Sophronitis* (Dharmani, 2013).

The last major group of orchids is the terrestrial orchids. Terrestrial orchids grow in the ground, soil (Allikas, 2009). There are some terrestrial orchids which are semi-terrestrial. Semi- terrestrial orchids grow on the ground with the exception that their root system does not penetrate into the soil beneath the ground surface but rather make their way through the layer of fluffy humus and leaves that lie on the surface of the ground (Orchid Basics, 2013). Terrestrial orchids does not rely on other trees to obtain sunlight, thus they need an opened space such as wet sclerophyll, open forest, shrubby forest, woodland and coastal shrubs to obtain sufficient sunlight for photosynthesis (Introduction to Austrialian Orchidaceae, n.d.). *Cymbidiums* and most of the slipper orchids are the examples of terrestrial orchids (Dharmani, 2013).

## 2.4 *Vanda* Genus Orchids

*Vandas* are well-known genus of orchids which are fast growing and frequent bloomers (Canadian Orchid Congress, 2003). Nevertheless, they have magnificent flowers with broad colors ranging from white, green, orange, red and burgundy (Cheam, Antony, Abdullah. and Perumal, 2009). *Vanda*s, monopodial orchids, usually bloom every few months and the flowers will last for two to three weeks (Canadian Orchid Congress, 2003 & Allikas, 2009).This member of genus are mostly epiphytic but some are lithophytic or terrestrial and they survive well with warm and full- sun habitats (Dharmani, 2013). However, some *Vanda*, can grow in the cool highlands. Hence, they can be found in India, Himalaya, Southeast Asia, New Guinea, south China and northern Australia (Cheam, Antony, Abdullah. and Perumal, 2009).

*Vandas* have thick stem and roots. The leaves may different in sizes and morphologies according to their habitats. Some *Vanda*s leaves are flat, strap-shaped and close together. Others may be terete and fleshy (Cheam, Antony, Abdullah. and Perumal, 2009). Generally, *Vanda* can be classified into three groups based on their leaves structure, strap- leaved, semi- terete and terete. Strap- leaved *Vandas* have broader, flat leaves, while terete *Vandas* have round, pencil-shaped leaves. Then, for semi-teretes *Vandas* are hybrids between the two, with an intermediate leaf shape (American Orchid Society, 2013).

Sufficient sunlight allows *Vandas* to grow healthily and produce large leaves and flowers (Canadian Orchid Congress, 2003). The growth of *Vandas* also will be affected by other conditions such as temperatures, water and humidity. *Vandas* can tolerate a wide range of temperature, from dry to cool weather, but they grow optimally at temperature 18$^{o}$C at night, and maximum of 35 $^{o}$C during day (Canadian Orchid Congress, 2003). In terms of water, *Vandas* can be watered daily in warm and sunny situation. However, their roots must be dried quickly and 80 % humidity is ideal condition for *Vandas'* growth (American Orchid Society, 2013).

## 2.5 Plant Tissue Culture

In plant science, "tissue culture" is commonly used to describe all types of *in vitro* plant cultures, namely callus, cell, protoplast, anther, meristem, embryo and organ culture that are able to regenerate and propagate into entire plant (Loyola-Vargas and Vazquez-Flota, 2005). Plant tissue culture generally refers to the technique of growing and multiplication of cells, tissues and organs on defined solid or liquid media under an aseptic and controlled environment (Singh, 2009). The success of plant tissue culture is greatly depending on the totipotency of plant cultures. Totipotency of plant is the ability of a single cell to divide, to differentiate and regenerate into a whole plant (Davey and Anthony, 2010).

In nature, plants are propagated either sexually (through seeds) or asexually (through vegetative propagation) (Jha and Ghosh, 2005). Conventional seed propagation is less desirable due to its long juvenile period before flowering (Decruse, Gangaprasad, Seeni and Menon, 2003). Moreover, vegetative propagation is time consuming and might be expensive (Saiprasad, and Polisetty, 2003). Thus, the rapid production of planting materials via plant tissue culture technique certainly offers advantages over the traditional propagation method. Micropropagation is one of the important applications of plant tissue culture techniques in plant propagation.

Micropropagation is the rapid production of high quality, disease-free and uniform planting materials. By using this technology, plants can be grown anywhere, irrespective of a season and weather, on a year-round basis (Singh, 2009). Through micropropagation which high quality and healthy planting from plant tissue culture has created new opportunities in global trading for producers, farmers and nursery owners (Singh, 2009).

Orchids, as a popular ornamental plant, exhibit high demand in global floriculture industry. Plant tissue culture can be a promising propagation techniques for orchids (Preil, 2003). Due to that the propagation of orchids from seeds is undesirable as the heterozygosity of seeds, minute seed size, lack of endosperm and the requirement of an association with mycorrhizal fungi for germination

(Saiprasad and Polisetty, 2003), an effective and reliable techniques for orchids propagation should be established.

## 2.6 Synthetic Seeds Technology

The use of synthetic seeds or artificial seeds or encapsulated seeds in plant propagation has evolved as an alternate and potentially more efficient method to conventional micropropagation (Dixon and Gonzales, 1994). Synthetic seeds can be defined as artificial encapsulated somatic embryos, shoot buds, cell aggregates, or any other tissues that are able to regenerate into a whole plant. These encapsulated explant tissues also can be used for sowing as a seed which are able to regenerate into a plant under *in vitro* or *ex vitro* conditions (Ara, Jaiswal. and Jaiswal, 2000).

Encapsulation is a process that uses the chelating potential of alginates in the presence of bivalent ions (mainly $Ca^{+}$) to produce gel capsules or synthetic seeds, in which the explants are embedded (Davey and Anthony, 2010). For synthetic seed production, explants are placed in liquid medium of alginate solution that devoid of calcium ions. The explant is transferred drop wise by a pipette into the calcium chloride solution. Once the alginate drops come into contact with calcium chloride solution, synthetic seeds are formed (Davey and Anthony, 2010).

In current synthetic seed technology, two types of synthetic seeds can be produced which are desiccated and hydrated synthetic seeds (Ara, Jaiswal and Jaiswal, 2000). The desiccated synthetic seed which is desiccation tolerant can be produced via somatic embryos either naked or encapsulated in polyoxyethylene glycol (Polyox$^{Y}$) then proceed with desiccation (Ara, Jaiswal and Jaiswal, 2000). On the other hand, hydrated synthetic seeds are produced by encapsulating the somatic embryos of desiccation-sensitive plant species in hydrogel capsules.

Synthetic seeds technique serves as low cost and high- volume propagation. Besides, the small sized synthetic seeds are convenient in storage and transportation (Saiprasad and Polisetty, 2003). Moreover, *in vitro* storage of synthetic seeds able to reduce the frequency of sub-culturing and able to preserve the unique germplasm (Mohanty et al, 2012). Thus, the use of synthetic seeds will be an ideal technique in maintaining the cloned plant materials (Saiprasad and Polisetty, 2003). Synthetic seeds of orchids are commonly produced by encapsulation of protocorm- like bodies (Saiprasad and Polisetty, 2003).

### 2.6.1 Applications of Micropropagation

Micropropagation is an area of plant tissue culture which has gained maximum attention of its potential commercial application, even though it still has limitations for wider commercial uses. In fact, micropropagated plantlet production rates are not always satisfactory and not warrant high investment (Sicurani, Piccioni, and Standardi, 2001). In addition, the final product of this technology only represented by small amount of plantlets which require specific and expensive management until commercialization and final delivery (Sicurani, Piccioni, and Standardi, 2001). In order to achieve time and cost saving procedure, a possible way to reduce these problem is by using synthetic seeds.

Many economically profitable crops were propagated via synthetic seeds. For an example, banana which is conventionally propagated by suckers is now replaced with synthetic seed produced by the excised shoot tips (Bapat, n.d.). High percent germination of these synthetic seeds was achieved. Other than banana, other important plants such as mulberry, rice cardamom, sandalwood, grape and apple were also successfully propagated via this synthetic seed technology (Ara, Jaiswal and Jaiswal, 2000). Nevertheless, ornamental plants such as orchids also applied synthetic seed technology for propagation.

### 2.6.2 Conservation of Germplasm

Deliberate destruction for urban development in many countries caused fast dwindling in natural repositories of germplasm. In order to compensate this problem, many researches have been done on finding appropriate techniques for conserving plant germplasms. Conventionally, seed storage has been widely used as method for plant germplasm conservation (Evans, Coleman and Kearns, 2003). However, many species cannot be conserved by this method due to their seeds are not able to tolerate dehydration and/or cold condtion (e.g. coffee), or they are only able to propagate vegetatively (e.g. banana.) (Evans, Coleman and Kearns, 2003). Thus, plant tissue culture has provided an alternative system for *in vitro* conservation of germplasms.

Synthetic seed technology is not mainly applied on the plant propagation but it is also used in the conservation of germplasm of plants. Plants that normally use this technology face the self- incompatibility and long breeding cycles problems (Ravi and Anand, 2012). Besides, conservation of these types of plants by vegetative method are very difficult. For conservation of plants, conventionally the seed banks conserve the plants by growing them in the fields which is space and cost consuming. Thus, the synthetic seed technology that retains the plants germplasm in small space, under the controlled conditions and without the danger of natural disasters would be preferable (Ravi and Anand, 2012).

In *in vitro* conservation of germplasm, cultures can be maintained either in slow-growth storage or by cryopreservation. Slow- growth storage reduces the frequency of subcultures and saves on labor and media costs. Slow- growth can be achieved via various methods such as low temperature (2- 8$^{o}$C), low light, low oxygen, dessication or culture on minimal medium with growth retardants (Evans, Coleman and Kearns, 2003). On the other hand, cryopreservation is achieved by storing cultures in extremely low temperature of liquid nitrogen (-196$^{o}$C). At this temperature, all cellular process is effectively stopped and germplasm can be stored for very long duration. However, many different procedures must be applied that either use cryoprotectants and controlled cooling or apply dehydration followed by rapid freezing to ensure the survivability of cultures (Evans, Coleman and Kearns, 2003).

*In vitro* conservation of plant germplasms are commonly conjugated with synthetic seed technology. This is because the extra coated layer provides protection on plantlet tissues from physical damages just like the naturally formed seed coat that function as endosperm protection in embryo development and seed germination. Furthermore, the coating layer which acts as reservoir of nutrients would aid the survival and rapid growth of the conserved plant (Sign et al., 2006).

If synthetic seed is used in slow- growth storage, it can be directly stored in low temperature (2- 8$^{o}$C). However, if synthetic seed is used in cryopreservation, extra treatment must be applied. This is because cryopreservation will cause the intracellular ice- crystal formation which damages the plant cells and it is irreversible (Sharma, Shahzad and Jamie, 2013). Thus, synthetic seed will normally complement with some other strategy to reduce the intracellular ice-crystal formation such as two- step freezing, simple dessication, encapsulation-dehydration, vitrification and encapsulation- vitrification (Sharma, Shahzad and Jamie, 2013).

# CHAPTER 3

# MATERIALS AND METHODS

## 3.1 Plant Materials

A *Vanda* hybrid orchid, *Vanda* Kasem's Delight x *Vanda* Gordon Dillon, protocorm- like bodies (PLBs) were cultured in the modified half- strength of Murashige and Skoog (MS) medium. The PLBs were transferred into fresh half- strength MS medium for multiplication monthly. Then, PLBs in 3- 4 mm length would be selected from the 1-2 months old *in vitro* culture, where the PLBs were loosely connected with each other. The PLBs were placed in a covered petri dish before subjecting them to the following studies.

## 3.2 Preparation of Culture Medium

Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) was modified and prepared as the culture basal medium in all the studies (Appendix A). The modified half- strength MS medium consisted of half- strength of macronutrients and micronutrients and full- strength of vitamins and iron disodium ethylenediamine tetraacetate ($FeNa_2EDTA$). Then, approximately 3 % (w/v) of sucrose was dissolved to the medium. The pH of the modified half- strength MS medium was adjusted to pH $5.7 \pm 0.1$ using either sodium hydroxide (NaOH) or

hydrochloric acid (HCl). Approximately 0.8 % (w/v) agarose was then added into the half- strength MS medium. Lastly, the half- MS medium was autoclaved at 121$^{o}$C and 15 psi for 15 min.

### 3.3 Preparation of Encapsulated Protocorm- Like Bodies (Synthetic Seeds)

The excised PLBs (3- 4 mm) were dipped into 3 % (w/v) sodium alginate solution (dissolved in half- strength MS medium). Then, suitable sized nozzle pipette was used to withdraw the PLBs from the sodium-alginate solution and were transferred into 75 mM calcium chloride solution (dissolved in half- strength MS medium). Only one PLB was transferred each time and it was left in the 75 mM calcium chloride solution for 30 min with occasional shaking. After that the alginate encapsulated PLBs or synthetic seed was formed. The calcium chloride solution was decanted. The collected synthetic seeds were rinsed with distilled-water for three times. Then, the synthetic seeds were blot dried on the sterile filter papers.

Before transferring them into cryotubes, synthetic seeds were separated into two groups; one group was kept at 25 $^{o}$C in the dark condition while another group was plunged into liquid nitrogen (-196 $^{o}$C). After one day of storage, the synthetic seeds were thawed at 40 $^{o}$C for 2 min. The synthetic seeds were then removed

from the cryotubes and blot dried with the sterile filter paper before culturing on the medium. All cultures were kept under a culture room with photoperiod of 16 hours and 8 hours of dark condition, at $25 \pm 2\,^{o}C$.

### 3.3.1 Parameters Investigated

Two parameters were investigated on encapsulated PLBs which were the different osmotica and thawing temperatures.

#### 3.3.1.1 Osmotica

Three different types of osmotica, sucrose, mannitol and sorbitol, were studied for the survivability of the synthetic seeds. In this study the osmotica used to prepare the sodium alginate solution and calcium chloride soultion were modified according to the experiment. Sucrose, mannitol or sorbitol was used individually in each solution in the study. The survivability and plant regeneration of encapsulated PLBs were observed and recorded weekly. The morphological changes of the encapsulated PLBs were also recorded. The experiment was repeated once with triplicate. Each replicate consist of 10 encapsulated PLBs.

#### 3.3.1.2 Effect of Thawing Temperature

Four thawing temperatures, 30 $^{o}$C, 40 $^{o}$C, 50 $^{o}$C and 60 $^{o}$C, were studied for the survivability and plant regeneration of the synthetic seeds after storing in liquid nitrogen for one day. The survivability and plant regeneration of encapsulated PLBs were observed and recorded weekly. The morphological changes of the encapsulated PLBs were also recorded. The experiment was repeated once with triplicate. Each replicate consist of 10 encapsulated PLBs.

### 3.4 Preparation of Non-Encapsulated Protocorm- Like Bodies (PLBs)

The excised PLBs (3- 4 mm) were immersed in half- strength MS liquid medium containing 0.5 M sucrose for 3 days. After 3 days, the PLBs were transferred into LS solution (half- strength MS liquid medium containing 2 M glycerol plus 0.4 M sucrose) at 25 $^{o}$C for 40 min. Then, the PLBs were further treated with plant vitrification solution 2, PVS2 [30 % (w/v) glycerol, 15 % (w/v) ethylene glycol, 15 % (w/v) dimethyl sulfoxide (DMSO) and 0.4 M sucrose dissolved in half-strength MS medium]. The PLBs were first treated with 60 % PVS2 (diluted from prepared PVS2 solution using half- strength MS medium) at 0 $^{o}$C for 30 min and followed by 100 % PVS2 at 0 $^{o}$C for 40 min.

Finally, the PLBs were transferred into cryotubes with fresh 100 % PVS2. The cryotubes with PLBs were separated into two sets; one set was kept in 25 $^{o}$C in dark condition while another set was plunged into liquid nitrogen (-196 $^{o}$C). After one day of storage, the PLBs were thawed at 40 $^{o}$C for 2 min. The PLBs were removed from the cryotubes and blot dried with sterile filter paper before culturing onto the prepared half- strength MS media. All cultures were kept under a culture room with photoperiod of 16 hours and 8 hours of dark condition, at 25 $\pm 2$ $^{o}$C.

### 3.4.1 Parameters Investigated

Three parameters were investigated on non- encapsulated PLBs which were the preculture duration, osmotica and thawing temperature.

### 3.4.1 .1 Preculture Duration

The preparation of PLBs was similar as described in section **3.3**. However, there was slight alteration on the preculture duration. Three preculture durations, 0 day, 1 day and 3 days, were studied for the survivability and plant regeneration of the PLBs. The survivability and plant regeneration of PLBs were observed and recorded weekly. The morphological changes of the PLBs were also recorded.

The experiment was repeated once with triplicate. Each replicate consist of 10 non- encapsulated PLBs.

### 3.4.1.2 Osmotica

The preparation of PLBs was similar as described in section **3.3**. Three different types of osmotica, sucrose, mannitol and sorbitol, were studied for the survivability and plant regeneration of the PLBs. In this study the osmotic used to prepare preculture solution, LS solution and PVS2 solution were modified according to the experiment. Sucrose, mannitol or sorbitol was used individually in each solution in the study. The survivability and plant regeneration of PLBs were observed and recorded weekly. The morphological changes of the PLBs were also recorded. The experiment was repeated once with triplicate. Each replicate consist of 10 non- encapsulated PLBs.

### 3.4.1.3 Thawing Temperature

The preparation of PLBs was similar as described in section **3.3.** Four thawing temperatures, 30 $^{o}$C, 40 $^{o}$C, 50 $^{o}$C and 60 $^{o}$C, were studied for the survivability and plant regeneration of the PLBs after they were stored in liquid nitrogen for one day. The survivability and plant regeneration of PLBs were observed and

recorded weekly. The morphological changes of the PLBs were also recorded. The experiment was repeated once with triplicate. Each replicate consist of 10 non- encapsulated PLBs.

### 3.5 Data Collection and Statistical Analysis

The recorded survivability and plant regeneration of non- encapsulated and encapsulated PLBs were calculated as shown below:

Percentage of non- encapsulated / encapsulated protocorm- like bodies (PLBs) that survived (%) =

$$\frac{Number\ of\ survival\ non-encpsulated\ or\ encapsulated\ PLBs}{Total\ number\ of\ cultured\ non-encpsulated\ or\ encapsulated\ PLBs}\ x\ 100\%$$

Percentage of germinated non- encapsulated / encapsulated protocorm- like bodies (PLBs) that survived (%) =

Note: For encapsulated PLBs, PLBs that managed to break the encapsulation considered as germinated PLBs.

$$\frac{Number\ of\ germinated\ non-encpsulated\ or\ encapsulated\ PLBs}{Total\ number\ of\ cultured\ non-encpsulated\ or\ encapsulated\ PLBs}\ x\ 100\%$$

The collected data were subjected to analysis of variance (one-way ANOVA) and Tukey's multiple range tests using IBM SPSS Statistic 22.

# CHAPTER 4

# RESULTS

## 4.1 Investigating the Parameters Affecting the Survivability and Plant Regeneration of Encapsulated Protocorm- Like Bodies (PLBs)

Generally, encapsulated PLBs were prepared to study the effects of different types of osmotica and thawing temperatures after cryopreserved in the liquid nitrogen for one day. The non- cryopreserved encapsulated PLBs were prepared as the control of this study. The morphology changes of encapsulated PLBs particularly the color was recorded; PLBs in fresh greenish color indicated as viable and healthy PLBs and PLBs in white color were assumed as dead PLBs.

### 4.1.1 Osmotica

In this study, encapsulated PLBs were prepared in the half- strength MS medium containing either 3 % (w/v) sucrose or sorbitol or mannitol. Firstly, all three types of osmotica were able to form oval shaped, clear and transparent encapsulated PLB seeds (Figure 4.1 a, b and c). However, after one day cryopreserved in liquid nitrogen, the alginate of encapsulated PLBs turned into semi-transparent (Figure 4.2 a, b and c). The non-cryopreserved encapsulated PLBs remained in clear and transparent condition.

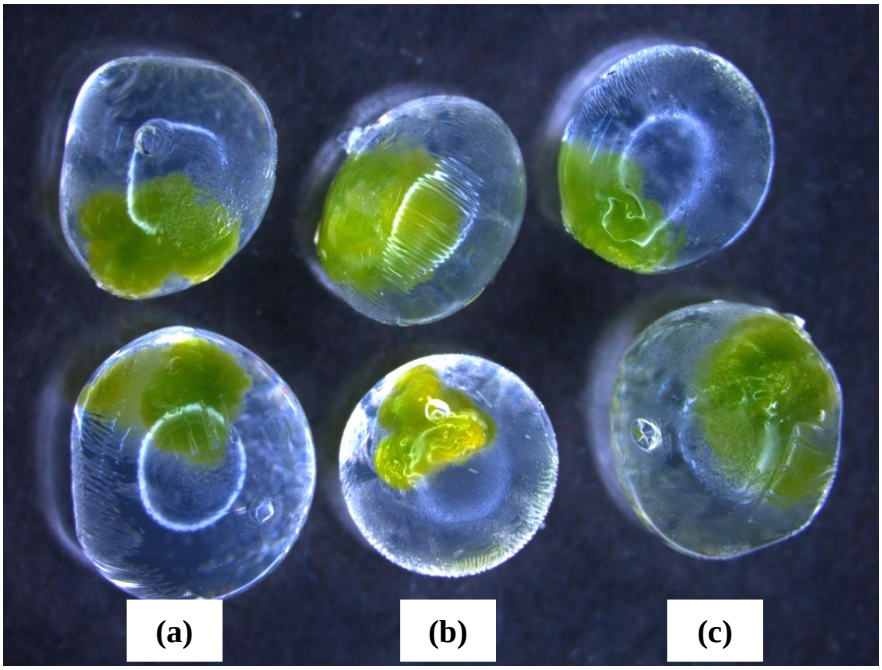


**Figure 4.1** Encapsulated PLBs prepared from three different types of osmotica, (a) sorbitol, (b) mannitol and (c) sucrose.

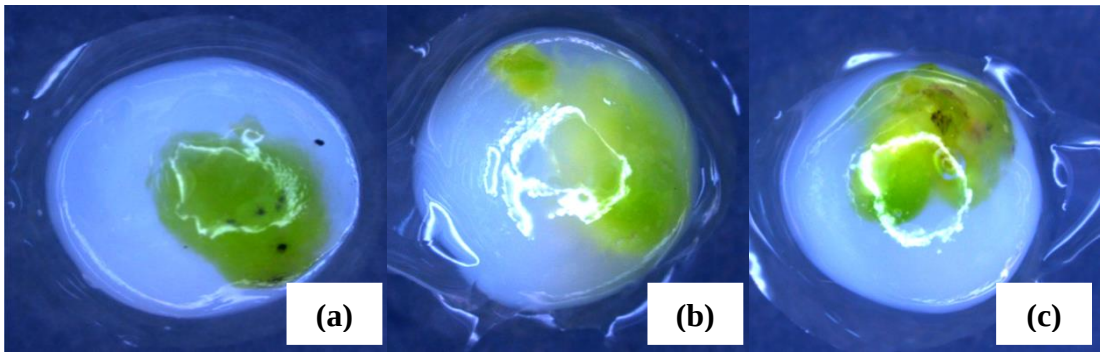


**Figure 4.2** Encapsulated PLBs produced from three different types of osmotica after one day cryopreservation in liquid nitrogen, (a) sorbitol, (b) mannitol and (c) sucrose.

The survivability and plant regeneration of cryopreserved and non- cryopreserved encapsulated PLBs were investigated in this study. For the encapsulated PLBs that were not cryopreserved, most of the encapsulated PLBs survived and showed fresh green color (Figure 4.3 a, b and c). Based on Table 4.1, the survival percentage was highest in the medium containing 3 % (w/v) sucrose, 96.7 ±4.7 %,

and followed by sorbitol, 90.0 ± 14.1 %, and mannitol, 86.7± 18.9 %. The survived encapsulated PLBs were continuously observed for four weeks. A significant decrease on the survivability of encapsulated PLBs was observed. The highest survival percentage of encapsulated PLBs was found when sorbitol was used, 68.3 ± 2.3 %, and followed by sucrose, 65.0 ± 2.4 %, and mannitol, 55.0 ± 21.2 %. The PLBs multiplied inside the alginate gel and some of the encapsulated PLBs were gradually germinated.

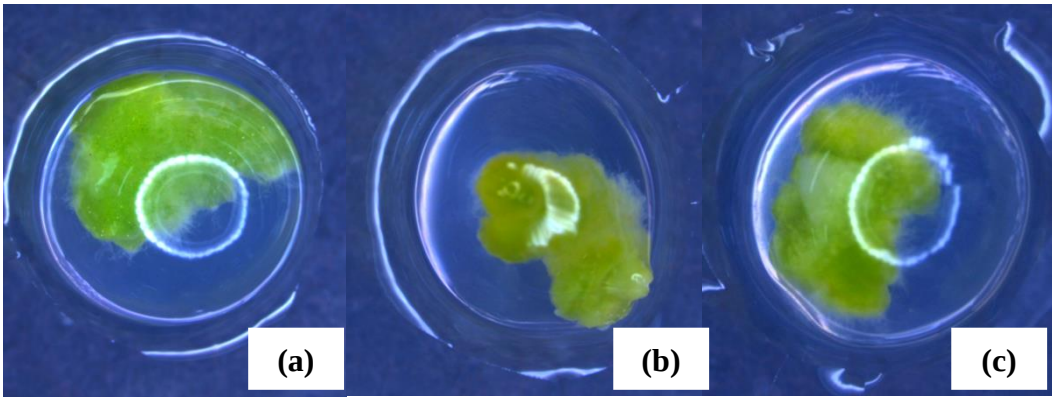


**Figure 4.3** The effects of different osmotica on the non- cryopreserved encapsulated PLBs (a) sucrose, (b) sorbitol, and (c) mannitol, after one day of culture.

**Table 4.1** The effects of different osmotica on the survival of non- cryopreserved encapsulated PLBs of *Vanda* hybrid after four weeks.

| Osmotica | Survival of PLBs (%) | |
|---|---|---|
| | After 1 day | After 4 weeks |
| Sorbitol | 90.0 ± 14.1[a] | 68.3 ± 2.3[a] |
| Mannitol | 86.7 ± 18.9[a] | 55.0 ± 21.2[a] |
| Sucrose | 96.7 ± 4.7[a] | 65.0 ± 2.4[a] |

Note: Mean ± standard deviation (SD) of triplicate per treatment for one repeats, followed by the same letter are not significantly different at ($p \leq 0.05$) according to Tukey's HSD test.

After four weeks, some of the non- cryopreserved encapsulated PLBs managed to break the alginate gel and germination occurred (Figure 4.4 a, b and c). Based on Table 4.2, the percentage of germination for the encapsulated PLBs in all three different osmotica, sorbitol, mannitol and sucrose, were low. The highest germination percentage of encapsulated PLBs was 21.7 ± 2.4 % (sorbitol), followed by 16.7 ± 0.0 % (mannitol) and 10.0 ± 0.0 (sucrose).

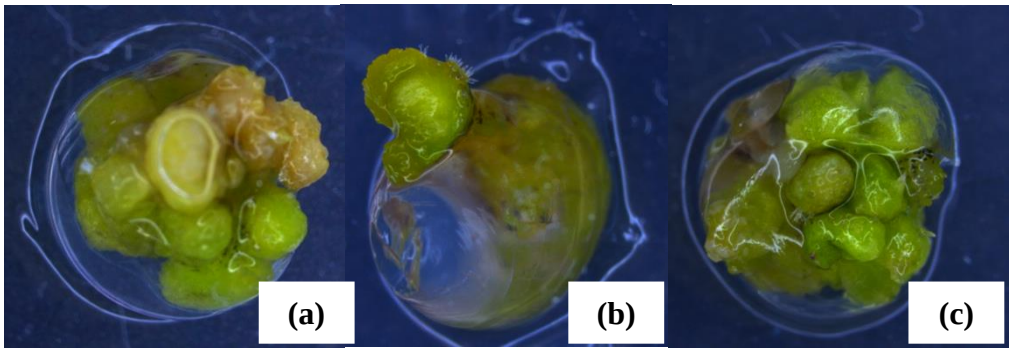


**Figure 4.4** The germination of non- cryopreserved encapsulated PLBs. (a) sucrose, (b) sorbitol and (c) mannitol, after four weeks of culture.

**Table 4.2** The germination of non-cryopreserved encapsulated PLBs in different osmotica of *Vanda* hybrid after four weeks.

| Osmotica | Germinated encapsulated PLBs (%) |
|---|---|
| Sorbitol | $21.7 \pm 2.4^{b}$ |
| Mannitol | $16.7 \pm 0.0^{b}$ |
| Sucrose | $10.0 \pm 0.0^{a}$ |

Note: Mean ± standard deviation (SD) of triplicate per treatment for one repeats, followed by the two different letters are significantly different at ($p \leq 0.05$) according to Tukey's HSD test.

Based on Table 4.3, all cryopreserved encapsulated PLBs prepared and cultured in the half- strength MS medium containing either sucrose or sorbitol or mannitol showed white color indicating the death of PLBs (Figure 4.5 a, b and c).

**Table 4.3** The effects of different osmotica on the survival of cryopreserved encapsulated PLBs of *Vanda* hybrid after one day.

| Osmotica | Survival of PLBs (%) |
|---|---|
| | After 1 day |
| Sorbitol | $0.0 \pm 0.0^{a}$ |
| Mannitol | $0.0 \pm 0.0^{a}$ |
| Sucrose | $0.0 \pm 0.0^{a}$ |

Note: Mean ± standard deviation (SD) of triplicate per treatment for one repeats, followed by the same letter are not significantly different at ($p \leq 0.05$) according to Tukey's HSD test.

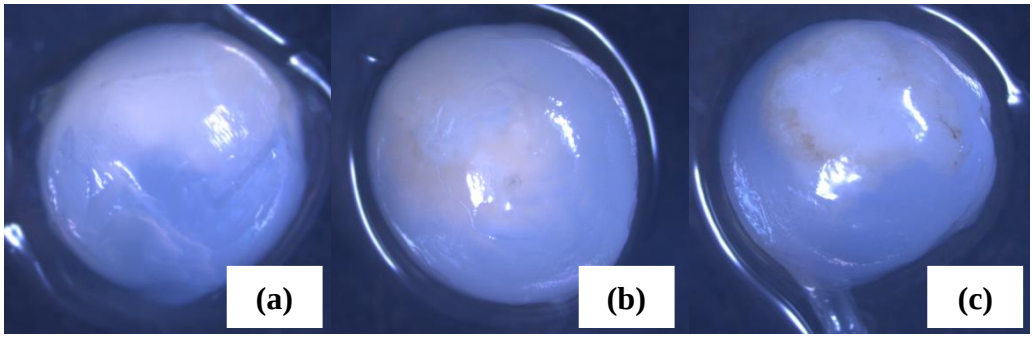


**Figure 4.5** Effects of different osmotica on the cryopreserved encapsulated PLBs. (a) sucrose, (b) sorbitol and (c) mannitol, after one day of culture.

Based on the results, the storage of encapsulated PLBs in liquid nitrogen did affect the survivability and plant regeneration of PLBs. Only the non-cryopreserved encapsulated PLBs were able to survive. Besides, the three osmotica tested, sorbitol, mannitol and sucrose, also affected the survivability and germination of the non- cryopreserved encapsulated PLBs.

### 4.1.2 Thawing Temperatures

In this study, the survivability and germination of non- cryopreserved and cryopreserved encapsulated PLBs were studied. For the encapsulated PLBs that were not cryopreserved, some of the encapsulated PLBs survived and showed fresh green in color (Figure 4.6 a, b and c). Based on Table 4.4, after one day, the highest survival percentage was at 60 $^{o}$C, 93.3 ± 1.4 %, and followed by 30 $^{o}$C (86.7 ± 1.4 %), 50 $^{o}$C (83.3 ± 1.4 %) and 40 $^{o}$C (71.7 ± 2.1 %). The survived encapsulated PLBs were observed for four weeks and a decrease on the survivability of encapsulated PLBs was observed. The highest survival percentage was found when the encapsulated PLBs were incubated at 40 $^{o}$C, 50.0 ± 1.4 %, and followed by 50 $^{o}$C (40.0 ± 1.4 %), 60 $^{o}$C (38.3 ± 2.1 %) and 30 $^{o}$C (33.3 ± 1.4 %).

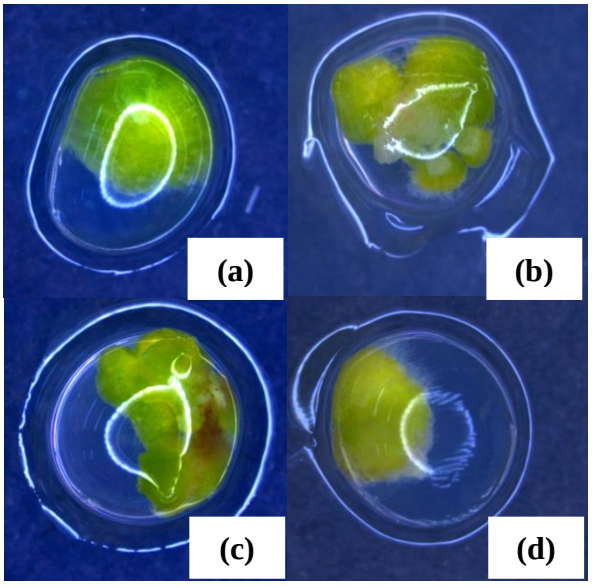


**Figure 4.6** Effects of different temperatures on the non- cryopreserved encapsulated PLBs (a) 30 $^{o}$C, (b) 40 $^{o}$C, (c) 50 $^{o}$C and (d) 60 $^{o}$C, after one day of culture.

**Table 4.4** The effects of different temperatures on the survival of non-cryopreserved encapsulated PLBs of *Vanda* hybrid after four weeks.

| Incubation temperatures ($^{o}$C) | Survival of PLBs (%) | |
|---|---|---|
| | After 1 day | After 4 weeks |
| 30 | 86.7 ± 4.7[a] | 33.4 ± 4.7[a] |
| 40 | 71.7 ± 7.1[a] | 50.0 ± 4.7[a] |
| 50 | 83.4 ± 4.7[a] | 40.0 ± 4.7[a] |
| 60 | 93.4 ± 4.7[a] | 38.3 ± 7.1[a] |

Note: Mean ± standard deviation (SD) of triplicate per treatment for one repeats, followed by the same letter are not significantly different at ($p \leq 0.05$) according to Tukey's HSD test.

Based on the result obtained (Table 4.5), only very low percentage of the non-cryopreserved encapsulated PLBs (8.3 ± 2.3 %) treated with 40 $^{o}$C managed to break the alginate gel after four weeks of culture. The PLBs treated with the other temperatures remained viable but did not germinate (Figure 4.7 a, b and c).

**Table 4.5** The germination of non-cryopreserved encapsulated PLBs treated with different temperatures of *Vanda* hybrid after four weeks.

| Thawing temperature ($^{o}$C) | Germinated encapsulated PLBs (%) |
|---|---|
| 30 | 0.0 ± 0.0[a] |
| 40 | 8.3 ± 2.3[b] |
| 50 | 0.0 ± 0.0[a] |
| 60 | 0.0 ± 0.0[a] |

Note: Mean ± standard deviation (SD) of triplicate per treatment for one repeats, followed by the two different letters are significantly different at ($p \leq 0.05$) according to Tukey's HSD test.

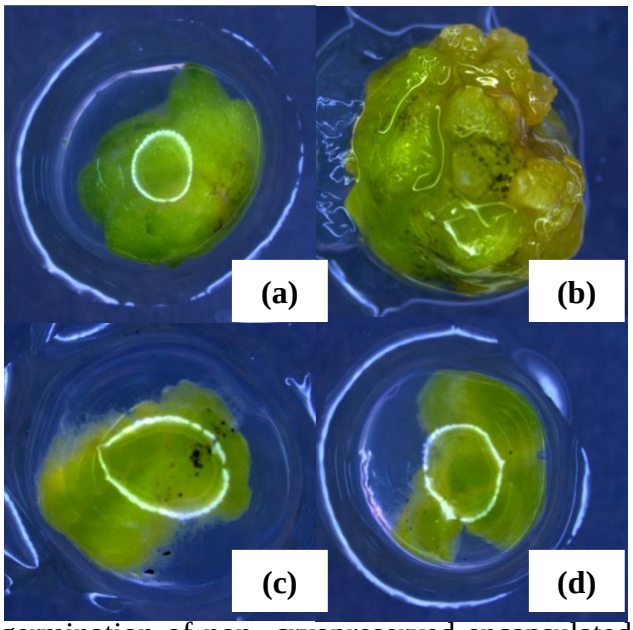


**Figure 4.7** The germination of non- cryopreserved encapsulated PLBs at (a) 30 $^{o}$C, (b) 40 $^{o}$C, (c) 50 $^{o}$C and (d) 60 $^{o}$C, after four weeks of culture.

For cryopreserved encapsulated PLBs, encapsulated PLBs were stored in liquid nitrogen for one day. Based on the Table 4.6, all PLBs thawed at 30 $^{o}$C, 40 $^{o}$C, 50 $^{o}$C and 60 $^{o}$C turned into white color after one day of culture indicating the death of PLBs (Figure 4.8 a, b and c).

**Table 4.6** The effects of different thawing temperatures on the survival of cryopreserved encapsulated PLBs of *Vanda* hybrid after one day.

| Thawing temperatures ($^{o}$C) | Survival of PLBs (%) |
|---|---|
| | After 1 day |
| 30 | 0.0 ± 0.0[a] |
| 40 | 0.0 ± 0.0[a] |
| 50 | 0.0 ± 0.0[a] |
| 60 | 0.0 ± 0.0[a] |

Note: Mean ± standard deviation (SD) of triplicate per treatment for one repeats, followed by the same letter are not significantly different at ($p \leq 0.05$) according to Tukey's HSD test.

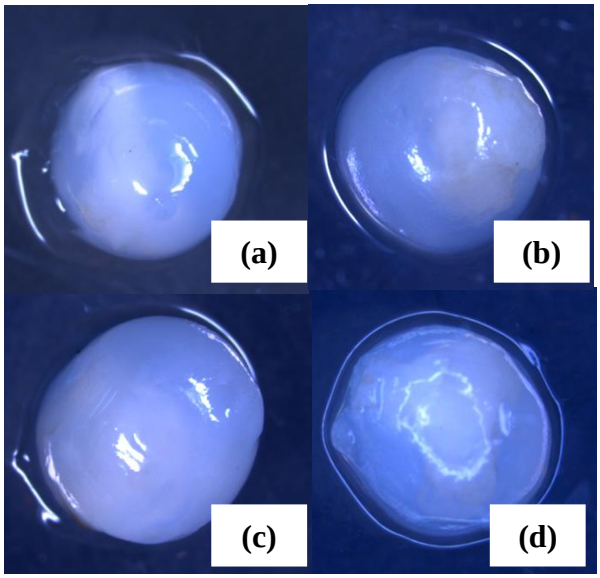


**Figure 4.8** Effects of different thawing temperatures on the cryopreserved encapsulated PLBs (a) 30 $^{o}$C, (b) 40 $^{o}$C, (c) 50 $^{o}$C, and (d) 60 $^{o}$C, after one day of culture.

Based on the results, the storage of encapsulated PLBs in liquid nitrogen did affect the survivability of PLBs. All cryopreserved PLBs died but the non-cryopreserved encapsulated PLBs were able to survive. Besides, for non-cryopreserved PLBs treated with different temperatures (30 $^{o}$C, 40 $^{o}$C, 50 $^{o}$C and 60 $^{o}$C), they showed different percentage of survivability and germination ability.

## 4.2 Investigating the Parameters Affecting the Survivability and Plant Regeneration of Non- Encapsulated Protocorm- Like Bodies (PLBs)

Generally, the non- encapsulated PLBs (PLBs without encapsulation with sodium alginate) were tested on the pre- culture duration, types of osmotica and also

thawing temperature after cryopreserved in the liquid nitrogen. The non-cryopreserved non- encapsulated PLBs were prepared as the control of the study. The morphology changes of non- encapsulated PLBs particulary the color was recorded; PLBs in fresh greenish color indicated as viable and healthy and PLBs in white color were assumed as dead PLBs.

### 4.2.1 Pre- culture Duration

In this study, non- encapsulated PLBs were pre- cultured at different durations, 0 day, 1 day and 3 days. For non- cryopreserved non- encapsulated PLBs, the results obtained are shown in Table 4.7. All the non- cryopreserved non-encapsulated PLBs in three pre- culture durations (0 day, 1 day and 3 days) showed fresh greenish color after one day of culture indicating the viability of PLBs (Figure 4.9 a, b and c). However, the fresh greenish color of the non-cryopreserved non- encapsulated PLBs did not retain for too long as the greenish color of PLBs gradually faded. After three days, all the non- cryopreserved non-encapsulated PLBs (pre- cultured for 0 day, 1 day and 3 days) turned into white color which indicated the death of PLBs (Figure 4.9 d, e and f).

**Table 4.7** The effects of different pre- culture duration on the survival of non-cryopreserved non- encapsulated PLBs of *Vanda* hybrid after three days.

| Pre- culture Duration (Days) | Survival of PLBs (%) | |
|---|---|---|
| | After 1 day | After 3 days |
| 0 | 100.0 ± 0.0[a] | 0.0 ± 0.0[a] |
| 1 | 100.0 ± 0.0[a] | 0.0 ± 0.0[a] |
| 3 | 100.0 ± 0.0[a] | 0.0 ± 0.0[a] |

Note: Mean ± standard deviation (SD) of triplicate per treatment for one repeats, followed by the same letter are not significantly different at ($p \leq 0.05$) according to Tukey's HSD test.

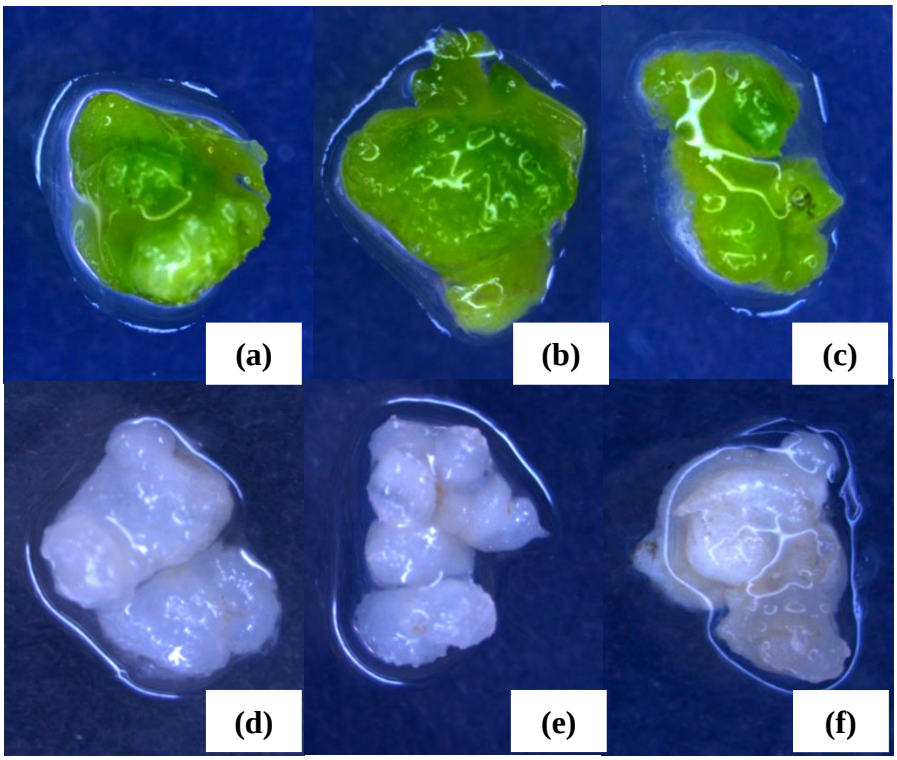


**Figure 4.9** Effects of different pre- culture duration on the non- cryopreserved non- encapsulated PLBs (a) 0 day, (b) 1 day and (c) 3 days pre- cultured PLBs after one day of culture; (d) 0 day (e) 1 day and (f) 3 days pre- cultured PLBs after three days of culture.

Based on the results obtained, Table 4.8, all the cryopreserved non- encapsulated PLBs in three pre- culture durations (0 day, 1 day and 3 days) showed fresh greenish in color after one day of culture indicating the PLBs were still viable (Figure 4.10 a, b and c). However, the fresh greenish color of the cryopreserved non- encapsulated PLBs did not retain for too long. The fresh greenish color of PLBs gradually faded. After three days of culture, all the cryopreserved non-encapsulated PLBs pre- cultured for 0 day, 1 day and 3 days turned into white color which indicated the death of PLBs (Figure 4.10 d, e and f).

**Table 4.8** The effects of different pre- culture duration on the survival of cryopreserved non- encapsulated PLBs of *Vanda* hybrid after three days.

| Pre- culture Duration (Days) | Survival of PLBs (%) | |
|---|---|---|
| | After 1 day | After 3 days |
| 0 | $100.0 \pm 0.0^{a}$ | $0.0 \pm 0.0^{a}$ |
| 1 | $100.0 \pm 0.0^{a}$ | $0.0 \pm 0.0^{a}$ |
| 3 | $100.0 \pm 0.0^{a}$ | $0.0 \pm 0.0^{a}$ |

Note: Mean ± standard deviation (SD) of triplicate per treatment for one repeats, followed by the same letter are not significantly different at ($p \leq 0.05$) according to Tukey's HSD test.

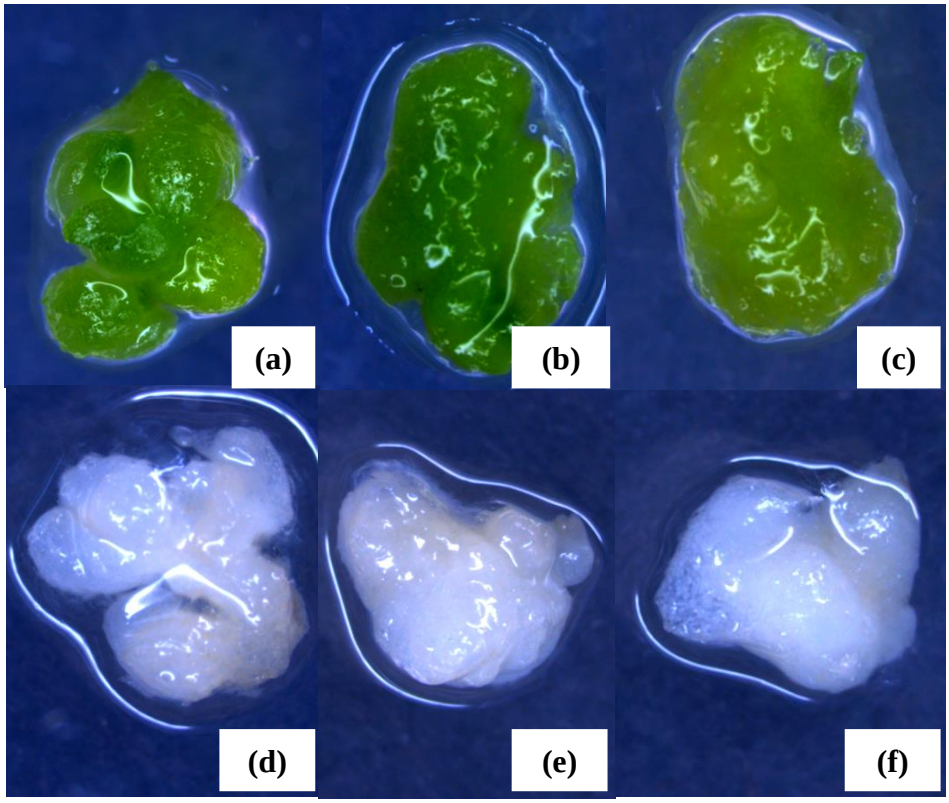


**Figure 4.10** Effects of different pre- culture duration on the cryopreserved non-encapsulated PLBs (a) 0 day, (b) 1 day and (c) 3 days pre- cultured PLBs after one day of culture; (d) 0 day (e) 1 day and (f) 3 days pre- cultured PLBs after three days of culture.

Based on the results, the effect storage of the non- encapsulated PLBs in liquid nitrogen on the survivability and plant regeneration could not be determined as similar results were obtained from the PLBs without cryopreserved. Similarly, the effect of three different pre- culture durations (0 day, 1 day and 3 days) on the survivability and plant regeneration could not be determined as both the non-cryopreserved and cryopreserved non- encapsulated PLBs were dead after three days of culture.

### 4.2.2 Osmotica

In this study, non- encapsulated PLBs were treated with different osmotica, sucrose, sorbitol and mannitol, in preparing pre- culture solution, LS solution and PVS2 solution (as described in Chapter 3, section 3.3.1.1). For non-cryopreserved non- encapsulated PLBs, the result obtained are shown in Table 4.9. All the non- cryopreserved non- encapsulated PLBs in three osmotica (sucrose, sorbitol and mannitol) showed fresh greenish color after one day of culture indicating the PLBs were still viable (Figure 4.11 a, b and c). However, the fresh greenish color of the non- cryopreserved non- encapsulated PLBs did not retain for too long as the greenish color of PLBs gradually faded. After three days, all the non- cryopreserved non- encapsulated PLBs treated with sucrose, sorbitol and mannitol turned into white color which indicated the death of PLBs (Figure 4.11 d, e and f).

**Table 4.9** The effects of different osmotica on the survival of non- cryopreserved non- encapsulated PLBs of *Vanda* hybrid after three days.

| Osmotica | Survival of PLBs (%) | |
|---|---|---|
| | After 1 day | After 3 days |
| Sucrose | $100.0 \pm 0.0^{a}$ | $0.0 \pm 0.0^{a}$ |
| Sorbitol | $100.0 \pm 0.0^{a}$ | $0.0 \pm 0.0^{a}$ |
| Mannitol | $100.0 \pm 0.0^{a}$ | $0.0 \pm 0.0^{a}$ |

Note: Mean ± standard deviation (SD) of triplicate per treatment for one repeats, followed by the same letter are not significantly different at ($p \leq 0.05$) according to Tukey's HSD test.

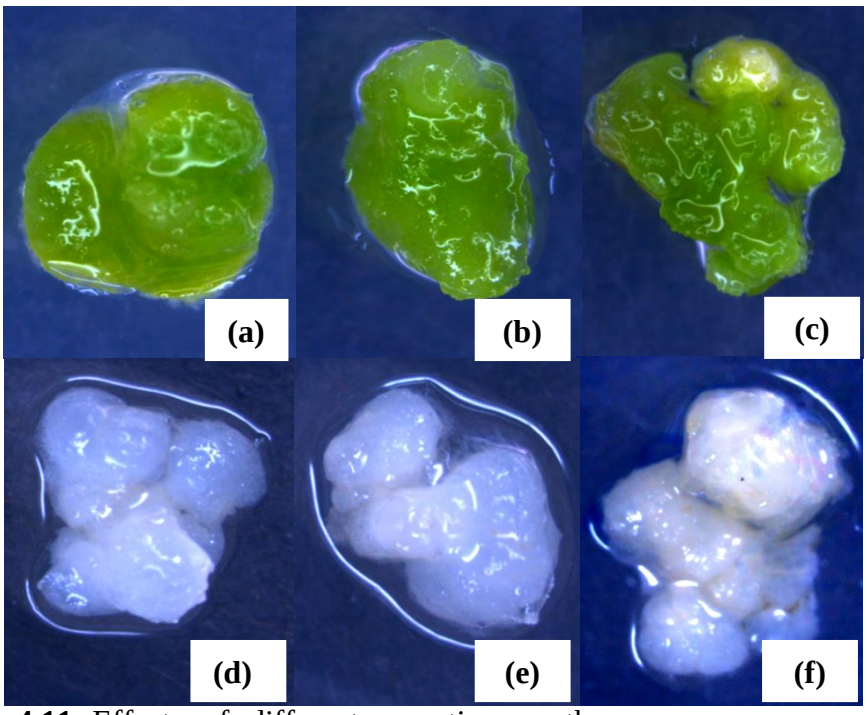


**Figure 4.11** Effects of different osmotica on the non- cryopreserved non-encapsulated PLBs (a) sucrose, (b) sorbitol and (c) mannitol after one day of culture; (d) sucrose (e) sorbitol and (f) mannitol after three days of culture.

Based on the result obtained, Table 4.10, all the cryopreserved non- encapsulated PLBs in three osmotica (sucrose, sorbitol and mannitol) showed fresh greenish color after one day of culture indicating the viable of PLBs (Figure 4.12 a, b and c). However, the fresh greenish color of the cryopreserved non- encapsulated gradually faded within three days. After three days of culture, all the cryopreserved non- encapsulated PLBs treated with either sucrose or sorbitol or mannitol turned into white color, which indicated the death of PLBs (Figure 4.12 d, e and f).

**Table 4.10** The effects of different osmotica on the survival of cryopreserved non- encapsulated PLBs of *Vanda* hybrid after three days.

| Osmotica | Survival of PLBs (%) | |
|---|---|---|
| | After 1 day | After 3 days |
| Sucrose | 100.0 ± 0.0[a] | 0.0 ± 0.0[a] |
| Sorbitol | 100.0 ± 0.0[a] | 0.0 ± 0.0[a] |
| Mannitol | 100.0 ± 0.0[a] | 0.0 ± 0.0[a] |

Note: Mean ± standard deviation (SD) of triplicate per treatment for one repeats, followed by the same letter are not significantly different at ($p \leq 0.05$) according to Tukey's HSD test.

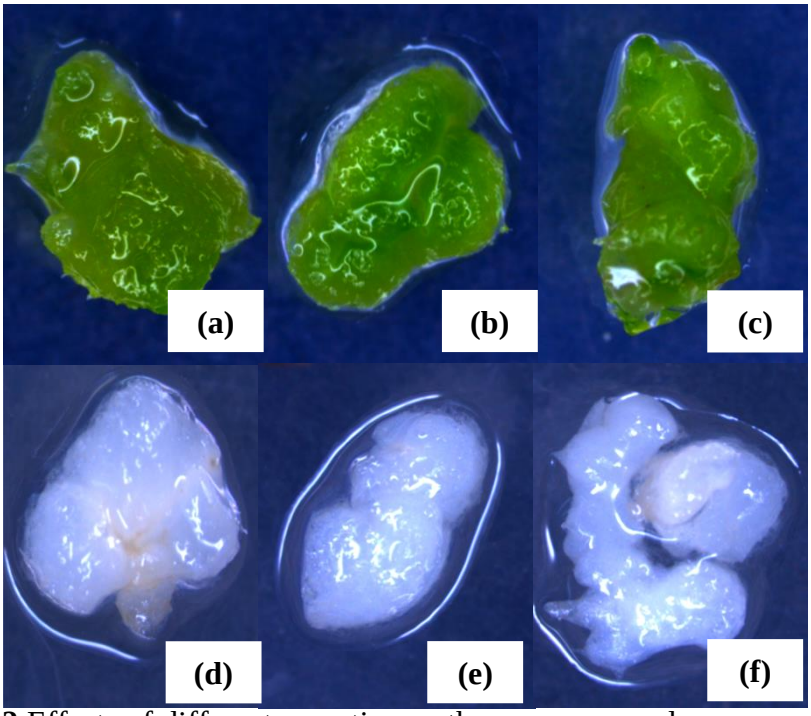


**Figure 4.12** Effects of different osmotica on the cryopreserved non- encapsulated PLBs (a) sucrose, (b) sorbitol and (c) mannitol after one day of culture; (d) sucrose (e) sorbitol and (f) mannitol after three days of culture.

Based on the results, the effect of storage for the non- encapsulated PLBs in liquid nitrogen on the survivability and plant regeneration could not be determined as the similar results were obtained from the non- cryopreserved PLBs. Similarly, the effect of different osmotica (sucrose, sorbitol and mannitol) on the survivability and plant regeneration could not be determined as both the non-cryopreserved and cryopreserved non- encapsulated PLBs were dead after three days of culture.

### 4.2.3 Thawing Temperatures

In this study, non- encapsulated PLBs were treated with different thawing temperatures, 30 $^{o}$C, 40 $^{o}$C, 50 $^{o}$C and 60 $^{o}$C. For non- cryopreserved non-encapsulated PLBs, the results are showed in Table 4.11. Similarly, all the non-cryopreserved non- encapsulated PLBs treated with 30 $^{o}$C, 40 $^{o}$C, 50 $^{o}$C and 60 $^{o}$C showed fresh greenish color after one day of culture (Figure 4.13 a, b, c and d). Again, the fresh greenish color of the non- cryopreserved non- encapsulated PLBs gradually faded and all PLBs died after three days of culture (Figure 4.13 e, f, g and h).

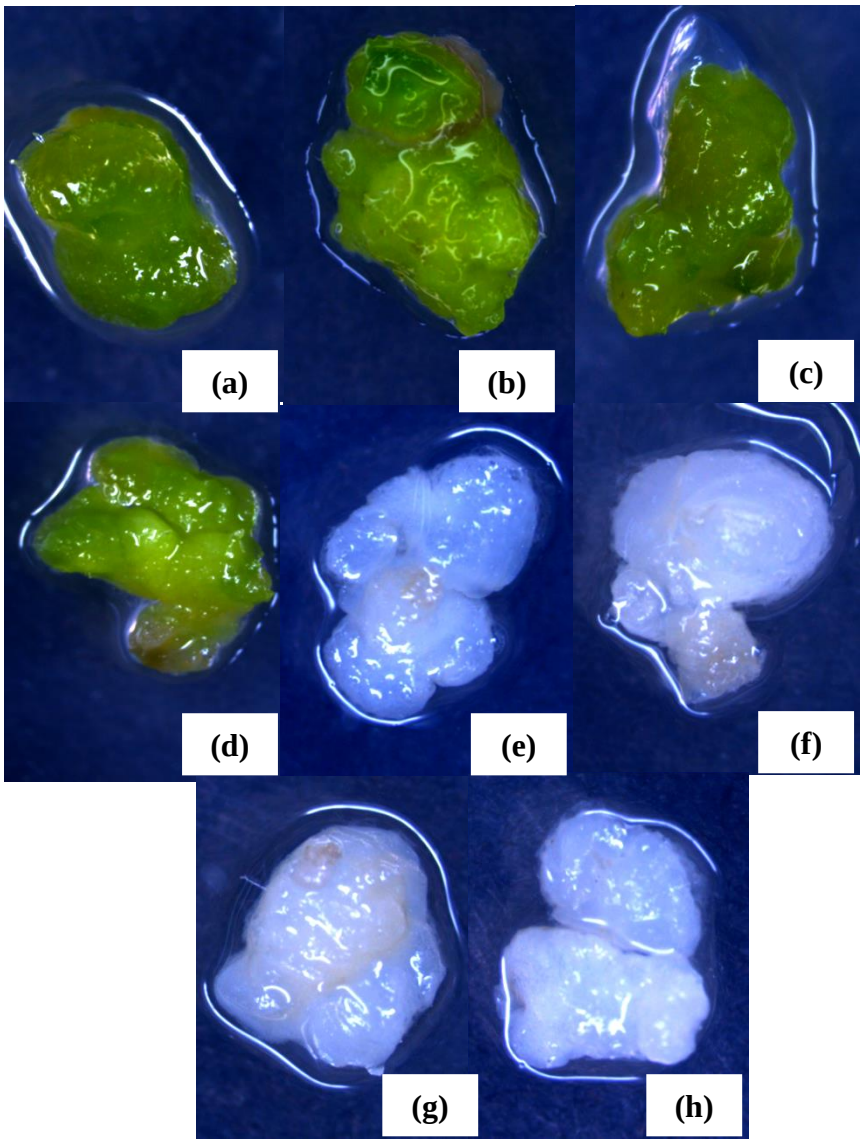


**Figure 4.13** Effects of different temperature on the non- cryopreserved non-encapsulated PLBs (a) 30 $^{o}$C, (b) 40 $^{o}$C, (c) 50 $^{o}$C, (d) 60 $^{o}$C, after one day of culture; after three days all PLBs turned white (e) 30 $^{o}$C, (f) 40 $^{o}$C, (g) 50 $^{o}$C, and (h) 60 $^{o}$C.

**Table 4.11** The effects of different temperatures on the survival of non-cryopreserved non- encapsulated PLBs of *Vanda* hybrid after three days.

| Thawing Temperatures ($^oC$) | Survival of PLBs (%) | |
|---|---|---|
| | After 1 day | After 3 days |
| 30 | $100.0 \pm 0.0^a$ | $0.0 \pm 0.0^a$ |
| 40 | $100.0 \pm 0.0^a$ | $0.0 \pm 0.0^a$ |
| 50 | $100.0 \pm 0.0^a$ | $0.0 \pm 0.0^a$ |
| 60 | $100.0 \pm 0.0^a$ | $0.0 \pm 0.0^a$ |

Note: Mean ± standard deviation (SD) of triplicate per treatment for one repeats, followed by the same letter are not significantly different at ($p \leq 0.05$) according to Tukey's HSD test.

Based on the result obtained, Table 4.12, all the cryopreserved non- encapsulated PLBs thawed using different temperatures (30 $^oC$, 40 $^oC$, 50 $^oC$ and 60 $^oC$) remained fresh greenish and viable (Figure 4.14 a, b, c and d). However, the fresh greenish color of the cryopreserved non- encapsulated PLBs gradually faded. After three days, all the cryopreserved non- encapsulated PLBs thawed at 30 $^oC$, 40 $^oC$, 50 $^oC$ and 60 $^oC$ turned into white color indicating the PLBs were dead (Figure 4.14 e, f, g and h).

**Table 4.12** The effects of different thawing temperatures on the survival of cryopreserved non- encapsulated PLBs of *Vanda* hybrid after three days.

| Thawing Temperatures ($^oC$) | Survival of PLBs (%) | |
|---|---|---|
| | After 1 day | After 3 days |
| 30 | $100.0 \pm 0.0^a$ | $0.0 \pm 0.0^a$ |
| 40 | $100.0 \pm 0.0^a$ | $0.0 \pm 0.0^a$ |
| 50 | $100.0 \pm 0.0^a$ | $0.0 \pm 0.0^a$ |
| 60 | $100.0 \pm 0.0^a$ | $0.0 \pm 0.0^a$ |

Note: Mean ± standard deviation (SD) of triplicate per treatment for one repeats, followed by the same letter are not significantly different at ($p \leq 0.05$) according to Tukey's HSD test.

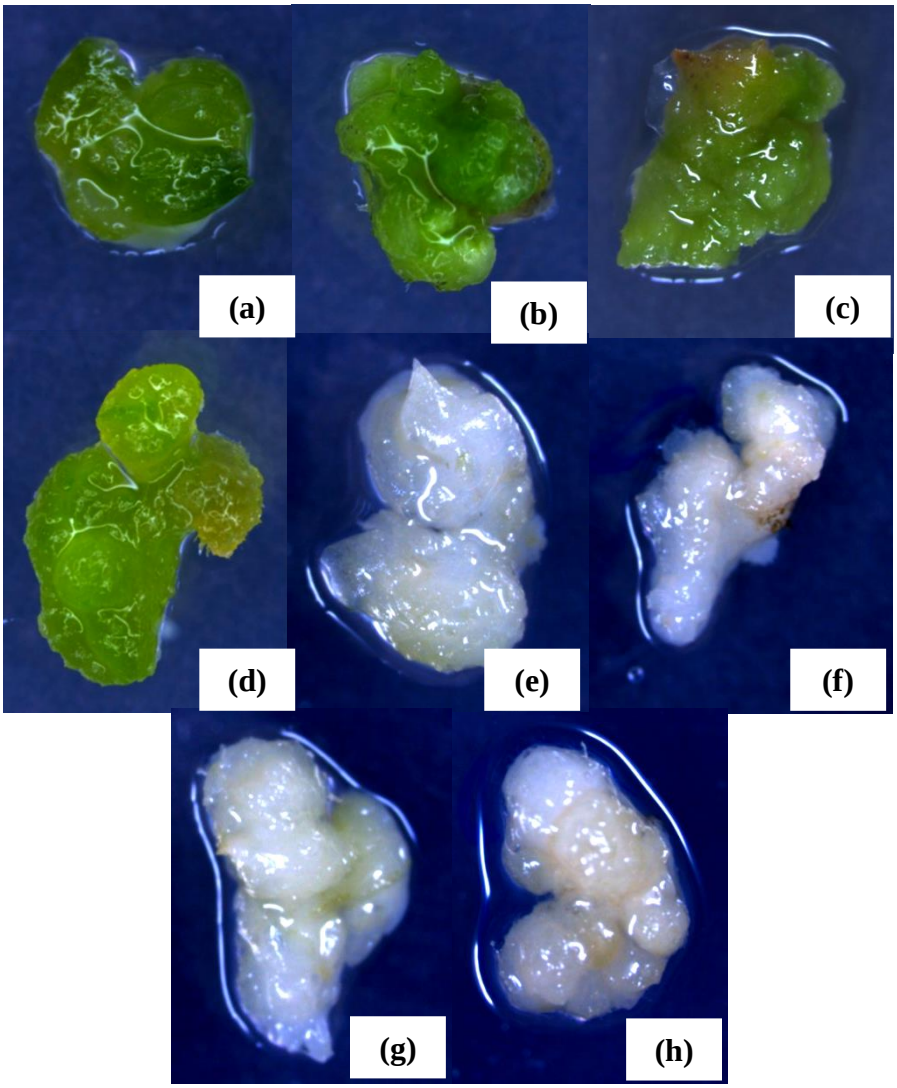


**Figure 4.14** Effects of different thawing temperature on the cryopreserved non-encapsulated PLBs (a) 30 $^{o}$C, (b) 40 $^{o}$C, (c) 50 $^{o}$C, (d) 60 $^{o}$C, after one day of culture; after three days all PLBs turned white (e) 30 $^{o}$C, (f) 40 $^{o}$C, (g) 50 $^{o}$C, and (h) 60 $^{o}$C.

Based on the results, the effect of storage of the non- encapsulated PLBs in liquid nitrogen did not the survivability and plant regeneration could not be determined as the similar results were obtained from the PLBs without cryopreserved. Similarly, the effect of different temperatures (30 $^{o}$C, 40 $^{o}$C, 50 $^{o}$C and 60 $^{o}$C) on the survivability and plant regeneration could not be determined as both the non-cryopreserved and cryopreserved non- encapsulated PLBs were dead after three days of culture.

# CHAPTER 5

# DISCUSSION

## 5.1 Viability Study of Encapsulated Protocorm- Like Bodies (PLBs) under Different Conditions

### 5.1.1 Osmotica

The formation of encapsulated PLB seeds may appear in different textures, shapes and transparency. According to Sign et al. (2006), different morphologies of encapsulated shoot tips will form depending on the concentration of sodium alginate and calcium chloride used. In their study, 3 % of sodium alginate polymerized in 75 mM calcium chloride produced clear, firm, round, uniform sized and easy handling encapsulated shoot tips of *Phyllanthus amarus.* Generally, sucrose, mannitol and sorbitol are carbohydrates which play a role in *in vitro* cultures as energy and carbon source (George et al, 2008). These osmotica would not affect the shape of encapsulated PLBs seeds. All the encapsulated PLBs seeds shared the same shape and texture as shown in this study.

Mannitol and sorbitol are sugar alcohols that usually are not used as the carbon source for plant growth. However, they are normally employed as an osmoticum to modify the water potential of the culture medium (George et al, 2008). On the other hand, sucrose is commonly used as carbon source and rarely used as an

osmoticum. However, like other osmotica, sucrose can also exhibit osmotic dehydration effect which can lead to the reduced water content in the cell (Tanaka et al., 2004). In this study, mannitol, sorbitol and sucrose, as osmotic agent, might increase the osmotic stress of the PLBs which could lead to cell plasmolysis and slower cell division (Mohanty et al., 2012). In this study, the non- cryopreserved encapsulated PLBs did not show significant difference among all the osmotica tested in terms of survivability according to the one- way ANOVA statistical analysis. Indirectly, it showed that the type of osmotica did not affect the survivability of the encapsulated PLBs.

However, the osmotica used might slow down the germination of the encapsulated PLBs due to the withdrawal of water from the plant tissues. In this study, the non- cryopreserved encapsulated PLBs only showed slight difference among all the osmotica tested in terms of germination. Similar result was obtained in the study of Mohanty et al. (2012) where the encapsulation of *Dendrobium nobile* LindI PLBs in 0.1 M sucrose and mannitol showed similar germination efficiency. Huda, Rahman and Bari (2007) also reported that the germination of encapsulated nodal segments of *Solanum melongena* with 0.1 M of sorbitol, mannitol and sucrose did not significantly affect the germination from encapsulated plant tissues.

In this study, all the cryopreserved encapsulated PLBs were dead after one day of culture. The death of the encapsulated PLBs was most probably due to the intracellular ice formation during cryopreservation (Vasanth and Vivier, 2011). According to Wen et al (2010), the main reason of cryopreservation failure was normally linked to the irreversible fatal caused by intracellular ice formation. Intracellular ice formation causes cell wall rupture, rupture of epidermis, protoplast outflow and irregular nucleus shape and lead to viability lost of plant cells (Kavani, 2011).

Furthermore, the inadequate concentration of osmoticum will also lead to the death of plant cells (Vasanth and Vivier, 2011). Excessive dehydration will cause the cells facing "solute effect" (such as pH changes, increasing electrolyte concentrations, protein denaturation, membrane phase transition and macromolecular interaction) whereas insufficient dehydration will cause the intracellular ice crystal formation (Lurswijidjarus and Thammasiri, 2004). When a suitable concentration of osmoitcum is used, the water from the plant cells would be removed via osmosis (George et al, 2008). Thus, when the plant cells are cryopreserved the formation of intracellular ice crystals would be reduced and the survivability of plant cells would increase. However, suitable concentration of osmoticum must be chosen in order to obtain optimal dehydration of plant cells (Vasanth and Vivier, 2011).

### 5.1.2 Thawing Temperature

The thawing process after cryopreservation is very important in determining the survivability of plant cells. Rapid thawing should be carried out after cryopreserved plant cells are taken out from liquid nitrogen (Simione, 2009; Baginiol and Engelmann, 1992). However, thawing plant tissues at high temperature will provoke rapid cellular rehydration which creates osmotic shock to the plant tissues and cause cell death (Baginiol and Engelmann, 1992). The rapid thawing causes stress crack and fractures in the preserved plant cells (Poobathy, Sinniah, Rathinam, and Subramaniam, 2013). On the other hand, low thawing temperature will extend the thawing period that allow recrystallization phenomena to occur and also will lead to cell death (Baginiol and Engelmann, 1992). Thus, a suitable thawing temperature must be chosen appropriately.

In this study, the non- cryopreserved encapsulated PLBs were treated at different temperatures as a control to observe the effect of the temperature on the PLBs. The highest survivability was obtained at 40 $^{o}$C among all temperatures investigated. Besides, only the PLBs treated with 40 $^{o}$C showed germination after 4 weeks of culture. At this stage, 40 $^{o}$C could be more suitable for PLBs germination from the encapsulation and the different temperatures used did not adversely affect the survivability of PLBs.

For the cryopreserved encapsulated PLBs tested with different thawing temperatures, all the PLBs were not able to survive. Cryopreserved plant tissues are extremely fragile and require gentle handling and immediate placement into pre-warmed water bath. When thawing the cryopreserved plant tissues, temperature and duration of thawing are the important factors that will determine the viability of plant tissues. In this study, no suitable thawing temperature could be identified as the cryopreservation process might have killed the PLBs due to the reason as describe at section 5.1.1.

However, based on the study of Baginiol and Engelmann (1992), the best thawing temperature for date palm was 40 $^{o}$C as it might allow the rapid rewarming and avoid recrystalization phenomena. The recrystalization phenomena occurred in thawing process will induce lethal damages on the plant tissues (Baginiol and Engelmann, 1992). As 40 $^{o}$C was found to be suitable for non- cryopreserved PLBs germination in this study, after optimizing the cryopreservation process, this might be a suitable thawing temperature for *Vanda* PLBs.

In addition, the death of cryopreserved encapsulated PLBs may due to the lack of post- cryopreservation treatment. The cryopreserved encapsulated PLBs can undergo suitable unloading process (or post- thaw treatment) to increase survivability. This unloading process is essential in removal of cryoprotectants (in this study, dimethyl suiphate (DMSO) was used) and prevent osmotic shock of

PLBs after thawing step (Panis, 2008). If PVS2 is not removed after thawing, toxic PVS2 may cause rupture and injuries on plant cells (Sakai, Hirai and Niino, 2008). Ching, Antony, Poobathy and Subramaniam (2012) reported that thawed encapsulated *Dendrobium* Sonia-*28* PLBs was treated with unloading solution, 1.2 M sucrose solution for 20 min before culturing on the half- strength MS medium which showed a high survivability. On the other hand, the survivability of the cryopreserved encapsulated PLBs can be post- culture in nutritive culture medium. Vasanth and Vivier (2011) reported that the increase in the duration of the post- culture in nutritive Nitsch and Nitsch medium (NN) increased the survivability of cryopreserved encapsulated grapevine somatic embryogenic callus compared to the non- treated cryopreserved encapsulated grapevine somatic embryogenic callus. They also showed that liquid medium was more suitable in post- culturing treatment.

## 5.2 Viability Study of Non- Encapsulated Protocorm- Like Bodies (PLBs) under Different Conditions

### 5.2.1 Pre- culture Duration

Pre- culture is a process used to enhance the cells tolerance to dehydration and subsequent freezing in ultra low temperature (Sharaf, Shibli, Kasrawi and

Baghdadi, 2012). In the cytoplasm of cells, high concentration of sugar and protein solutes enable a glassy- solid state to occur during cryopreservation and the cells are able survive when exposed to liquid nitrogen (Yin and Hong, 2009). Thus, pre- culture can reduce the water content in the cells and prevent ice crystallization (Zainuddin et al, 2011). In many cryopreservation research studies, not only pre- culturing the PLBs before cryopreservation would enhance the survivability but suitable pre- culture solution concentration and duration also will improve the survivability (Mohanty et al, 2012).

For orchids, different species of orchids have different level of tolerance towards sugar (Yin and Hong, 2009). Poobathy, Hesam, Julkifle and Subramaniam (2012) reported that *Vanda* Kseem's Delight was best pre- cultured in 0.1 M of sucrose solution for 24 hours before storage in liquid nitrogen. In a study carried out by Ching, Antony, Poobathy and Subramaniam (2012), they obtained positive effect of prolonging the pre- culture duration up to six days in 0.5M of sucrose solution. The non- cryopreserved and cryopreserved encapsulated *Dendrobium* Sonia- *28* PLBs survivability increased three- fold after six days of pre- culture as compared to a one day pre- culture. However, in this study, the survivability of the cryopreserved or non- cryopreserved non- encapsulated PLBs were not within the expectation, where all the non- encapsulated PLBs were dead.

The death of PLBs may due to the pre- culture solution containing high concentration of sucrose, 0.5 M. According to Halmagyi and Pinker (2006), high concentration of sucrose causes cells to dehydrate excessively and reduces the survivability of explants. This statement is supported by the study of Tianm, Rathinam, Chan and Subramaniam (2009), where they reported cryopreserved *Dendrobium* Sonia- *28* PLBs showed highest survivability when pre- culture in 0.25 M sucrose solution and beyond 0.25 M of sucrose solution, the survivability of PLBs decreased. In addition, Poobathy, Sinniah, Mahmood and Subramaniam (2013) also reported that sucrose concentration higher than 0.4 M caused the death of the *Dendrobium* Sonia- 28 PLBs. Besides, high sucrose concentration would cause tissues blackening and retard the proliferation of PLBs (Panis et al, 1996).

### 5.2.2 Osmotica

Sucrose is most often used as the carbon source in media. Other sugars such as mannitol and sorbitol also have been used in cryotechnology research (Sharaf, Shibli, Kasrawi and Baghdadi, 2012). In general, these sugars function as an osmotic regulator which stabilizes cellular membranes and maintain the turgor in cells (Sharaf, Shibli, Kasrawi and Baghdadi, 2012; George et al, 2008). Cells cultured in media containing these osmotic regulators will have mild to moderate dehydration stress which stimulate metabolic change of the plant cells and

improve dehydration and freezing tolerance. However, in this study, no conclusion could be made based on the effect of osmotica used as all PLBs died after one day of culture.

In this study, different osmotica were manipulated in pre- culture solution, loading solution and PVS2 solution (as described in Chapter 3, 3.3.1.2). Among these solutions, loading solution and PVS2 solution were involved in the cryoprotective treatment. PLBs were treated with loading solution for 40 min, 60 % PVS2 for 30 min and 100 % of PVS2 for 30 min. In which glycerol (in loading solution) and DMSO (in PVS2) were added as act as cryoprotectant that protect cells from freezing damage (Subramaniam, 2010). Suitable cryoprotective treatments should be conducted to increase the survivability and regeneration of PLBs.

Many researches have reported that the exposure duration to the cryoprotective solution and temperature of cryoprotective solution are the main factors that cause the death of plant cells. The optimization of exposure time of PVS2 is important to achieve the high survival of PLBs after cryopreservation (Sakai, Hirai and Niino, 2008). Overexposure of plant cells to highly concentrated PVS2 is potentially creating phytotoxic effects in the cells which will affect the cell survivability (Sakai, 1997). Poobathy, Izwa, Julkifle and Subramaniam (2013) reported that *Dendrobium* Sonia- *28* PLBs treated with PVS2 solution for 20 min

had highest survivability after cryopreservation. Beisdes, Poobathy, Hesam, Julkifle and Subramaniam (2012) also reported that the exposure duration of PVS2 on the *Vanda* Kaseem's Delight PLBs in 20 min had the highest survival percentage. Thus, the death of PLBs resulted from this study could be due to the overexposure PVS2 solution to the PLBs.

PVS2 contains DMSO which is toxic to plant cells. However, the toxicity can be reduced by reducing the temperature during exposure (Subramaniam, 2010)**.** Thus, the temperature of PVS2 during exposure will affect the survivability of PLBs. Many reports have shown that PVS2 treatment at 0 $^{o}$C can reduce the toxic formation and have higher survivability percentage of plant cells. Yin and Hong (2009) research reported that *Dendrobium candidum* PLBs treated with PVS2 at 0 $^{o}$C had almost 20 % higher survivability as compared to *Dendrobium candidum* PLBs that were treated with PVS2 at room temperature. In this study, non-cryopreseved PLBs were exposed to PVS2 at room temperature for one day while cryopreseved PLBs were plunged into liquid nitrogen for one day. Thus, the death of non- cryopreseved PLBs may due to toxic effect of DMSO in the PLBs.

### 5.2.3 Thawing Temperature

There are not many researches studying the effect of different thawing temperatures for plant after cryopreservation. Generally the range of thawing temperature used is 30- 40 $^{o}$C. Many researchers conducted their researches based on this thawing temperature range. Poobathy, Sinniah, Mahmood and Subramaniam (2013) in their study of cryopreserving *Dendrobium* Sonia- *28* PLBs used 40 $^{o}$C to thaw the PLBs and the cryopreserved PLBs were successfully recovered. Besides, Subramaniam et al (2011) also reported that the cryopreserved *Dendrobium* Sonia- *17* PLBs were thawed at temperature ranged at 38- 40 $^{o}$C. However, in this study, non- cryopreserved and cryopreserved non-encapsulated PLBs failed to survive in all four thawing temperatures.

The death of non- cryopreserved and cryopresered PLBs may due to the exposure of light to the PLBs after thawing. In this study, non- cryopreserved PLBs were kept in dark for one day when the cryopreserved PLBs were kept inside liquid nitrogen for one day. Based on the chemiluminescence analysis, expose light on cryopreserved cells right after thawing will increase oxygen singlets in the cells (Baginiol and Engelmann, 1992). The light causes an oxidative effect which will promote browning and necroses of the cells (Baginiol and Engelmann, 1992). The high concentration of oxygen will combine with highly reactive intermediates will cause the overproduction of reactive oxygen species (ROS) which caused the

death of plant tissues (Ledford and Niyogi, 2005). For example, the cryopreserved *Dendrobium* Sonia- *28* PLBs were bleached and died within 2- 3 days after exposure of light (Poobathy, Sinniah, Mahmood and Subramaniam, 2013). Thus, the death of non- cryopreserved PLBs that kept in dark also may due to the oxidative effect. However, the oxidative effect can be reduced by supplying activated charcoal in the medium (Baginiol and Engelmann, 1992).

## 5.3 Further Studies

The encapsulated seed technology is a very effective way to carry out micropropagation or conservation study. Studies or researches can be done to improve or increase the survivability of the encapsulated PLBs. Furthermore, studies on the use of suitable phytohormones can also be conducted in order to obtain higher germination yield the of encapsulated PLBs.

The study to cryopreserve the encapsulated seeds can be further optimized and developed. There are many cryopreservation techniques that can be studied such as freezing, ultra rapid freezing, vitrification, encapsulation/ dehydration and encapsulation/ vitrification. Additional research can be done on finding suitable cryopreservation techniques to conserve orchids.

Besides, the survivability of post- thawed cryopreseved plant tissues can be improved by culturing them in a dark condition. Temporarily reducing the cryo-samples from exposure to light immediately after cryopreservation has been shown to increase post-cryogenic survival due to the removal of photo oxidative stress in the plant (Kaczmarczyk et al., 2012). Besides, optimizing the duration of cryopreserved plant tissues in dark condition can be investigated too.

# CHAPTER 6

# CONCLUSION

Generally, there was no significant effect shown on the survivability of non-cryopreserved encapsulated PLBs when different types of osmotica were applied. However, the PLBs treated with sorbitol showed a higher survivability, 68.3 ± 2.3 %, and germination, 21.7 ± 2.4 %, on the encapsulated PLBs. For the study of effect of different temperatures, only the non- cryopreserved encapsulated PLBs survived. The highest survivability, 50.0 ± 4.7 %, and germination, 8.3 ± 2.3 %, were observed for the encapsulated PLBs treated with 40 $^{o}$C. However, for the cryopreserved encapsulated PLBs, all PLBs were dead. Cryopreservation performed in the study might kill the PLBs.

On the other hand, the non- encapsulated PLBs were studied on the effect pre-culture duration, osmotica and also thawing temperatures. In all three tested parameters, the non- cryopreserved and cryopreserved non- encapsulated PLBs showed the same results. All PLBs in both situations only managed to survive on the first two days of culture. The death of non- encapsulated PLBs might cause by the high concentration of pre- culture solution, overexposure of plant vitrificaiton solution 2 (PVS2), formation of reactive oxygen species in the PLBs and the ultra- low temperature during cryopreservation in liquid nitrogen (-196 $^{o}$C).

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## Appendix A

Nutrient composition for MS basal medium

| Nutrients | Concentration of stock (mg/L) |
|---|---|
| **Macroelements** | |
| $NH_4NO_3$ | 1650.00 |
| $KNO_3$ | 1900.00 |
| $CaCl_2.2H_2O$ | 440.00 |
| $MgSO_4.7H_2O$ | 370.00 |
| $KH_2PO_4$ | 170.00 |
| **Microelements** | |
| KI | 0.83 |
| $H_3BO_3$ | 6.20 |
| $MnSO_4.4H_2O$ | 22.30 |
| $ZnSO_4.7H_2O$ | 8.60 |
| $Na_2MoO_4.2H_2O$ | 0.25 |
| $CuSO_4.5H_2O$ | 0.025 |
| $CoCl_2.6H_2O$ | 0.025 |
| **Iron salts** | |
| $FeNa_2EDTA$ | 37.08 |
| **Vitamin B5** | |
| Myo- inositol | 100.00 |
| Nicotinic acid | 1.0 |
| Pyridoxine HCl | 1.0 |
| Thiamine HCl | 1.0 |
| **Sucrose** | 30000.00 |
| **pH medium** | 5.7 |

## Appendix B

The statistical analysis by One- Way ANOVA using Tukey's HSD test ($p < 0.05$) for comparison of the percentage of survival and germination of non-cryopreserved encapsulated PLBs using different osmotica.

**Osmotica**

**Homegenous Subsets**

**a) Percentage of Survival (After One Day)**

Tukey HSD

| Osmotica | N | Subset for alpha = 0.05 |
|---|---|---|
| | | 1 |
| Mannitol | 2 | 86.665 |
| Sorbitol | 2 | 90.000 |
| Sucrose | 2 | 96.665 |
| Sig. | | .770 |

Means for groups in homogeneous subsets are displayed.
Harmonic Mean Sample Size = 2.000

**b) Percentage of Survival (After Four Weeks)**

Tukey HSD

| Osmotica | N | Subset for alpha = 0.05 |
|---|---|---|
| | | 1 |
| Mannitol | 2 | 55.000 |
| Sucrose | 2 | 65.000 |
| Sorbitol | 2 | 68.334 |
| Sig. | | .770 |

Means for groups in homogeneous subsets are displayed.
Harmonic Mean Sample Size = 2.000

**c) Percentage of Germination (After Four Weeks)**

Tukey HSD

| Osmotica | N | Subset for alpha = 0.05 | |
|---|---|---|---|
| | | 1 | 2 |
| Sucrose | 2 | 10.000 | |
| Mannitol | 2 | | 16.683 |
| Sorbitol | 2 | | 21.667 |
| Sig. | | 1.000 | 0.70 |

Means for groups in homogeneous subsets are displayed.
Harmonic Mean Sample Size = 2.000

## Appendix C

The statistical analysis by One- Way ANOVA using Tukey's HSD test ($p < 0.05$) for comparison of the percentage of survival and germination of non-cryopreserved encapsulated PLBs using different thawing temperature.

**Thawing Temperature**

**Homegenous Subsets**

**a) Percentage of Survival (After One Day)**

Tukey HSD

| Osmotica | N | Subset for alpha = 0.05 |
|---|---|---|
| | | 1 |
| 40 | 2 | 71.700 |
| 50 | 2 | 83.350 |
| 30 | 2 | 86.650 |
| 60 | 2 | 93.350 |
| Sig. | | 0.53 |

Means for groups in homogeneous subsets are displayed.
Harmonic Mean Sample Size = 2.000

**b) Percentage of Survival (After Four Weeks)**

Tukey HSD

| Thawing temperature | N | Subset for alpha = 0.05 |
|---|---|---|
| | | 1 |
| 30 | 2 | 33.350 |
| 60 | 2 | 38.300 |
| 50 | 2 | 40.000 |
| 40 | 2 | 50.000 |
| Sig. | | .114 |

Means for groups in homogeneous subsets are displayed.
Harmonic Mean Sample Size = 2.000

**c) Percentage of Germination**

Tukey HSD

| Thawing temperature | N | Subset for alpha = 0.05 | |
|---|---|---|---|
| | | 1 | 2 |
| 30 | 2 | 0.000 | |
| 50 | 2 | 0.000 | |
| 60 | 2 | 0.000 | |
| 40 | 2 | | 8.350 |
| Sig. | | 1.000 | 1.000 |

Means for groups in homogeneous subsets are displayed.
Harmonic Mean Sample Size = 2.000