CRYOPRESERVATION OF *ASCOCENDA* WANGSA GOLD ORCHID USING PVS2 VITRIFICATION METHOD

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by

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LIST OF ACRONYMS AND ABBREVIATIONS

%	Percent/percentage
µL	Microlitre
µM	Micromolar
$\mu mol.m^{-2}\cdot s^{-1}$	Micromole per meter square per second
2,4-D	2,4-dichlorophenoxyacetic acid
A	Absorbance
AFLP	Amplified Fragment Length Polymorphism
ANOVA	Analysis of variance
BAP	6-benzylaminopurine
Bp	Base pairs
cm	Centimetre
Cw	Cell wall
dc	Damage cell
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DP	Direct plunging
DV	Droplet-vitrification
ED	Encapsulation dehydration
EDTA	Ethylenediaminetetra acetid acid
EV	Encapsulation vitrification
FAA	Formaline-acetic acid-alcohol
g	Gram
g g	force/ gram for gravitational

g/L	Gram per litre
gc	Guard cell
h	Hour
H_2O	Water
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
ic	Intact cell
IFEs	*In vitro* fragmented explants
is	isodiametric
KH_2PO_4	Monopotassium phosphate
LN	Liquid nitrogen
M	Molar
$M^{-1}.cm^{-1}$	Molar per centimetre
mg/L	Milligram per litre
min	Minute
min^{-1}	per minute
mm	Millimetre
mM	Millimolar
MS	Murashige and Skoog
n	Nucleus
Na_2HPO_4	Disodium hydrogen posphate
NaOH	Sodium hydroxide
nm	Nanometre
nu	Nucleolus
O_2	Oxygen

°C	Degrees celcius
OD	Optical density
PGR	Plant growth regulator
PLB	Protocorm-like body
PLBs	Protocorm-like bodies
Pps	Periplasmic space
PVS2	Plant vitrification solution 2
PVS3	Plant vitrification solution 3
RM	Ringgit Malaysia
rm	rupture membrane
s^{-1}	per second
SCoT	Start codon targeted
SEM	Scanning electron microscope
st	Starch
TBA	tertiary-butyl alcohol
TDZ	Thidiazorun
TTC	2,3,5-triphenyltetrazolium chloride
U/g	Unit per gram larutan vitrifikasi tumbuhan 2 (PVS2),
$U.mg^{-1}$	Unit per milligram
$U.mL^{-1}$	Unit per millilitre
uc	Undamaged cell
$	US dollar
V	Volt
w/v	Weight over volume

PENGKRIOAWETAN ORKID *Ascocenda* Wangsa Gold MENGGUNAKAN KAEDAH VITRIFIKASI PVS2

ABSTRAK

Satu kaedah pengkrioawetan larutan vitrifikasi tumbuhan 2 (PVS2) yang effisien telah dibangunkan untuk jasad seperti protokom (JSP) *Ascocenda* Wangsa Gold orkid. Kesan bahan tambahan organik seperti air kelapa, homogenat ubi kentang dan pisang ke atas pertumbuhan JSP *Ascocenda* Wangsa Gold telah dikaji. Bilangan JSP diaruh dari setiap eksplan telah dikira selepas empat minggu rawatan. Penambahan 2% homogenat ubi kentang, 2% pisang dan 15% air kelapa ke dalam media Vacin dan Went didapati adalah kultur medium terbaik untuk menginduksi JSP yang menghasilkan sebanyak 94.7%. Parameter yang dinilai termasuk size protokom, kepekatan sukrosa, kesan tempoh pendedahan kepada PVS2, tempoh pencairan, suhu, dan keadaan kultur berdasarkan bacaan penyerapan TTC dan melalui pemerhatian penjanaan semula protokom. Kadar pemulihan sebanyak 33.3% telah diperolehi selepas 2 bulan apabila JSP telah didedahkan pada PVS2 selama 30 min. Kadar pemulihan telah meningkat kepada 47% apabila dicairkan pada 45°C selama 85s. Kadar pertumbuhan semula yang paling tinggi (53.3%) telah diperolehi apabila JSP didedahkan kepada rawatan gelap 7 hari sebelum dipindahkan ke fotokala 16/8 jam cahaya/gelap. Analisis histologi dan imbasan elektron mikroskop telah dijalankan untuk mengkaji kerosakan yang boleh mengubahkan struktur JSP *Ascocend*a Wangsa Gold yang telah dikrioawet dan tidak dikrioawet. Kajian menunjukkan bahawa hanya sel-sel embriogenik dapat diselamatkan pada JSP yang dikrioawet selepas rawatan pembekuan dan pencairan. Kaedah Start codon targeted (ScoT) DNA telah digunakan untuk mengkaji kestabilan genetik *Ascocenda* Wangsa Gold PLB yang dirawat, tidak dirawat dan tumbuhan kawalan. Empat primers

bermaklumat telah dipilih daripada 16 primers ScoT-DNA berdasarkan keupayaan mereka untuk menghasilkan jalur monomorphic jelas daripada kandungan genetik protokom asli. Analisis SCoT-DNA menunjukkan bahawa JSP dikrioawet dan tidak dikrioawet adalah genetik yang sama seperti protokom kawalan. kaedah pengekacaan yang dibangunkan dalam kajian ini adalah kaedah yang boleh dilaksanakan dan selamat untuk mengukuhkan pemuliharaan germplasma orkid ini untuk tujuan komersial.

CRYOPRESERVATION OF *Ascocenda* Wangsa Gold ORCHID USING PVS2 VITRIFICATION METHOD

A**BSTRACT**

An efficient plant vitrification solution 2 (PVS2) cryopreservation method was developed for protocorm-like bodies (PLBs) of *Ascocenda* Wangsa Gold orchid. The effects of organic additives such as coconut water, potato and banana homogenates on PLBs proliferation of *Ascocenda* Wangsa Gold in different media culture were studied. The numbers of PLBs induced from each explant were counted after four weeks of treatment. Addition of 15% of coconut water, 2% of potato and banana homogenate into Vacin and Went medium was found to be the best media culture for PLBs induction at 94.7%. Parameters assessed included the preculture sucrose concentration effect of PVS2 exposure periods, thawing duration, temperature, and culture conditions based on 2, 3, 5-triphenyltetrazolium chloride absorbance readings and regrowth rates. A regrowth rate of 33.3% was obtained after 2 months when the PLBs were dehydrated in PVS2 for 30 min. The growth rate was improved to 47% when thawed at 45°C for 85 s. The highest growth rate (53.3%) was obtained when the PLBs were subjected to a 7-day dark treatment before being transferred to a 16-h/8-h light/dark photoperiod. Histological and scanning electron microscopy analyses were conducted to study the lethal and non-lethal damages of cryopreserved and non-cryopreserved PLBs of *Ascocenda* Wangsa Gold. The studies showed that embryogenic cells only survived in cryopreserved PLBs after freezing and thawing treatments. Start codon targeted (SCoT) markers were used to study the genetic stability of cryopreserved, non-cryopreserved and control plantlets of *Ascocenda* Wangsa Gold. Four informative primers were selected from 16 SCoT primers based on their capability to produce clear and reproducible

monomorphic bands. The SCoT analyses showed that the cryopreserved and non-cryopreserved PLBs were genetically similar as control plant. The vitrification method developed in this study is a feasible and safe method for strengthening the germplasm conservation of this orchid for commercial purposes.

CHAPTER ONE

INTRODUCTION

Orchidaceae is a highly diverse angiosperm family in plant kingdom and consists of 35,000 orchid species, distributed more than 150,000 orchid hybrids and over 1,000 distinct genera (Dressler, 1993; Yue et al., 2006). Generally, orchids are famous for their natural beauty appearance and also attracting the interest of numerous producers and information collection of their cultivation. The orchid plants can grow abundantly in tropical countries especially in the Southeast Asia due to the high humidity. Recently, ornamentals and cut flower (e.g., orchids, pitcher plants) have been successfully exported with estimated value of RM 150 million every year in Malaysia (Ibrahim and Normah 2013).

Orchids are economically important in horticulture, floristry and they command the top most value as cut flower and potted plants. In 2012, the Department of Foreign Trade Thailand reported the total global trade value of wholesale orchid has increased up to US$504 million (Cheamuangphan et al., 2013). The demand of hybrid orchids has been increasing due to their flower colour and shape, fragrance, size, vase life and texture. Therefore, the valuable germplasm and resources of ornamental plants like orchid species and the hybrids to be stored and maintained to avoid their extinction.

The multiplication of orchids through *in vitro* propagation is more efficient than vegetative methods and sexual propagation (Arditti and Ernst, 1993; Talukder et al., 2003). Tissue culture technique is widely applied for multiplication, plant germplasm storage and maintaining plant biodiversity. Recently, many techniques are available for the conservation of plant genetic resources of endangered species.

Nutrition applied in the media and the growth regulator highly influence the success rate of plant tissue culture especially for orchids. Generally, the media consists of carbon source, minerals, salts, vitamins, water and growth regulators (Murdad et al., 2010). The addition of organic additives also significantly improvises the current tissue culture media into commercial production (Ichihashi and Islam, 1999). The plant material like somatic embryo or protocorm-like bodies (PLBs) are able to yield mass production due to the meristematic tissue region which has the capability to regenerate into whole plantlets (Park et al., 2002).

Cryopreservation is the best method to conserve orchid germplasm in liquid nitrogen under ultralow temperature (-196°C) for indefinite time. At such temperature, the plant material will not undergo any biochemical process, metabolic activity or cell division process. Thus, the plant material can be maintained without genetic alteration or deterioration for unlimited time (Shibli et al., 2004; Zhu et al., 2006). Cryopreservation is the only method that ensures the long- term conservation of non-orthodox seed species, potential hybrids, endangered, unique, vegetatively propagated plants and rare plants (Touchell, 1995; Touchell and Dixon, 1996; Thammasiri, 2005). In cryopreservation, the plant material requires less maintenance (refilling LN), low cost and small volume storage.

The water removal in all cryopreservation method is required to avoid the freezing injury and preserve the viability of post-thawed cryopreserved materials. The water content in plant cells is higher due to their large vacuole structure. Thus, the elimination of water can be done by dehydration process (Panis et al., 2005; Benson, 2008; Engelmann, 2011). The addition of cryoprotectant at high concentration and removal of water from the explant through osmotic dehydration will enhance the cell viscosity of the plant material (Benson, 2008). The

cryopreserved plants involved in several stages, like the establishment of *in vitro* cultures, conditioning of these tissues, the addition of cryoprotectant, exposure of cultures to ultra-low temperature, re-warming and regeneration of plant cells and tissues (Bajaj, 1995).

Vitrification refers to the glass- forming process in which liquid turns to solid without undergoing crystallization (Fahy et al., 1984; Engelmann et al., 2008). Vitrification treatments with PVS2 or PVS3 method were mostly carried out in plant cells through the elimination of intra-and extracellular freezable water. This occurs when explants are exposed to the highly concentrated cryoprotective solution prior to rapid cooling by direct immersion in liquid nitrogen. This method can be applied to complex structures such as embryo and shoot apices (Withers and Engelmann, 1997). In order to undergo cooling rate and colligative cryoprotection, an explant must be able to withstand extracellular freezing, chilling and osmotic stress. Generally, vitrification protocols require an organism to be tolerated desiccation injury and high osmotic stress (Harding, 2004). The vitrification-based methods like PVS2 and droplet-vitrification has been efficiently and successfully applied on a large number of tropical and temperate species (Engelmann et al., 2008). The dehydration period relies on explants initial water content and its tolerance towards the dehydrating process by exposed in the silica gel or dried in a laminar hood (Fabre and Dereuddre, 1990; Heine-Dobbernack et al., 2008).

Various cryogenic treatments resulted in physical, chemical or physiological damages and stress to plant tissues, which could change the genomic contents of the explants. This variation could take place during micropropagation, preculture and up to regeneration step in cryopreservation procedure (Harding, 2004; Martín and González-Benito, 2005). One of the simplest and fastest molecular maker detection

of variation is the start codon targeted (SCoT-DNA) polymorphism DNA technique (Collard and Mackill, 2009). The SCoT-DNA DNA markers are formed by polymerase chain reaction (PCR) amplification using single primer that are developed from short conserved region found in plant genes surrounding ATG translation site (Collard and Mackill, 2009; Luo et al., 2010; Guo et al., 2012).

The long-term conservation method is essential for the maintenance of desired genotype and phenotypes of *Ascocenda* orchid industry. *Ascocenda* Wangsa Gold orchid is a combination of *Ascocentrum* parents with vast size flower of *Vanda* parents. The cultivation of *Ascocenda* species is common in Thailand, India, Myanmar and Philippines. *Ascocenda* Wangsa Gold is a hybrid orchid where no study has been conducted on development of PVS2 vitrification method.

1.1 Objectives

The objectives of this study are:

a. To identify suitable medium to enhance the protocorm-like bodies (PLBs) proliferation of *Ascocenda* Wangsa Gold's orchid,

b. To optimise various parameters for plant vitrification solution 2 (PVS2) method using PLBs,

c. To perform post-cryopreservation analyses which include histological and scanning electron microscopy (SEM) techniques of the cryopreserved plantlets,

d. To confirm genetic stability of the cryopreserved plantlets using the SCoT DNA marker analysis.

CHAPTER TWO

LITERATURE REVIEW

2.1 Orchid morphology and distribution

Orchidaceae is a spectacularly diverse and largest group of angiosperm family and covers up more than 1/3 of monocot species (Arditti, 1992; Gustafsson et al., 2010). The orchid name is originally derived from ‘orchis’ the Greek word refers to the male testicle because of the root tubers shape. It includes about 800 genera covering 35,000 of orchid species. Orchids are very compatible across two different species. In 1856, the first artificial hybrid was made in England and now over 150,000 hybrids have been created (Yue et al., 2006). Orchid represents the symbol of luxury, beauty and love for centuries. Initially for Greeks, the orchid symbolized virility, "the plant of the king's fragrance" called by Chinese.

The Orchidaceae is widely distributed all over the world. The overview of distribution of orchid population at different region: Tropical America (250 to 270 genera), tropical Asia (260 to 300 genera), tropical Africa (230 to 270 genera), Oceania (50 to 70 genera), Europe and temperate Asia (40 to 60 genera) and North America (20 to 26 genera) (Wu et al., 2009). In Asia, about 10,000-15,000 orchid species were discovered to be 120 exotic species (Jezek, 2003). So far, 2000 orchid species belonging to 120 genera have been identified in Malaysia (Hamdan, 2008).

Nearly 70% of orchids are epiphytic (grow non-parasitically on other plants or objects for support), 25% are geophytic and about 5% are litophytic (Gravendeel et al. 2004; Teoh, 2005; Hsu et al., 2011). Most of the lithophytes grow on rock or mossy forest areas and very small amount of saprophytes are living with support of

decaying matter (Banks, 1999; Teoh, 2005). The terrestrial orchids grow on ground level and can be found mostly around temperate region.

The orchid plant can be easily distinguished from other flowering plants by the arrangement of three elements called pollen, column and their small seeds. Both hybrids and wild orchids have similar characteristics. For example, orchids are bilaterally symmetrical flower, column is placed where the reproductive organ are contained, placed of sepals and petals and the lip parts in orchids provides a stand for the orchids pollinator (Jezek, 2003).

Orchids are grouped into two different growth patterns such as monopodial or sympodial and that distinguish their growth habits (Morel, 1974). The growth habits of monopodial and sympodial are depends on their apical meristem progression or its growing points. Monopodial orchid has a single stem and the apical meristem region will grow annually without the pseudobulbs existence. The shoots will be growing indefinitely and the flower stem arise from the base of axil leaf in monopodial orchid (Dressler, 1993). Examples of monopodial orchids with the same type of growth patterns are *Phalaenopsis* and *Vandas*. For sympodials, the orchids grow horizontally and producing new shoots from main growing point (old rhizome). It is known as pseudobulbs which store the nutrients and water for support the plant to survive long droughts (Morel, 1974). Sympodial orchids are including *Dendrobium, Cattleya, Oncidium* and *Cymbidium*.

2.2 Orchid trade market and significance

The demand of orchids in both national and international markets is high due to their longevity, size, and bloom colour (Saiprasad et al., 2004). In the past 60 years, the orchids have been used as cut flowers and now orchids are characterized as ornamental plants which almost comprised 8% of global floriculture trade (as been reviewed by Arditti, 1992). In 2012, orchid's wholesale importation and exportation value has dramatically increased up to 40-60% around the world (Cheamuangphan et al., 2013). Asian countries like Taiwan, Malaysia and Singapore are involved and contributing for orchid global trade market while Thailand is the leading exporter of orchids as cut flower (Ooi, 2005; Cheamuangphan et al., 2013).

There are several orchids being used in food and cosmetic industry. For example, *Vanilla pompano, Vanilla planifolia* and *Vanilla tahitensis* cultivated commercially to be used as spices and flavoring agents. Besides, some orchid species like *Anacamptis, Tetramicra bicolour* and *Orchis* are used as flavour in ice creams and milk in South America and Turkey (Arditti, 1992). On the other hand, dried tubers are consumed as Salep by Europeans (Arditti, 1992) while, fresh tubers are made into juice by Africans (Linder and Kurzweil, 1999). Moreover, leaves of *Lipais japonica* is consumed as vegetable by Koreans (Arditti, 1992).

Most orchid species act as herbal medicine with approximately 20% species are native to China (Go and Hamzah, 2011). Since orchids like *Gastrodia* elata contains '*Tian ma,* it can be widely used against diarrhea, fever and influenza, crooked eyes and gastrointestinal disease (Arditt, 1992). In Malaysia, the orchid *corymborkis veratrifolia* (Reinw) Blume which is well known as 'Hanching Ali'

used to treat cure bladder illness and the pigeon orchid or *Dendrobium crumenatum* helped to treat earaches (Arditti, 1992; Go and Hamzah, 2011).

2.3 *Ascocenda* genus

Ascocenda is a man-made and attractive orchid hybrid, a cross between *Vanda* and *Ascocentrum* (V x Asctm). *Ascocenda*, an orchid species plays important role as cut-flower in the ornamental industry for countries like Thailand due to their colour, size and shapes, able to travel long distance, bloom persistence, hence maintained the top ten positions as cut flowers in the global trade market (Martin and Madassery, 2006). This genus is evergreen compact epiphytes and grows upright with narrow oviform leaves. *Ascocenda* genus is axillary clusters contains 6 to 7 open flowers. They bloom twice a year with showy and long lasting flowers. The flowers are intensely coloured, often covered with contrasting colours.

Ascocenda orchid species are very common in cultivation with combination of contrast colour of *Ascocentrum* parents and larger flower of *Vanda* parents (Ranjetta et al. 2009). Many are found their origin in India, Thailand, Malaysia, Philippines and the Myanmar. *Vanda* orchid genus is florally and considered the most evolved orchid. The *Vanda* species are highly prized in the horticulture trade due to their fragrant, intensely colored flowers, long-lasting and showy (Ranjeeta et al. 2009).

Ascocentrum known as small genus and belongs to orchid family. Mostly, this genus can be found in humid, warmer to intermediate climates countries like Myanmar, Malaysia, Java, Philippines, Bornea and Thailand. They are common among orchid cultivators due to similarity with the compact *Vanda*-like orchid species (Ranjetta et al., 2009).

2.3.1 *Ascocenda* Wangsa Gold

Ascocenda Wangsa Gold is one of the artificial epiphyte *Ascocenda* orchid genus resulting from the hybridization between *Vanda* Rasri Gold and *Ascocentrum* Ram Indra (Plate 2.1). This orchid considered to be highly desirable because of the traits combination expressed by both the parents: the large flower size of *Vanda* genus resembles promising progenitors for making a variety of cut flower orchid hybrids and bright colour combination of *Ascocentrum*, which is considered an important traits for producing miniature *Vanda* hybrids. *Ascocenda* Wangsa Gold is yellow flower orchid that commercially crucial in floricultural market and possesses upright, oviform leaves, mild fragrance with 4 to 6 petals.

Plate 2.1: *Ascocenda* Wangsa Gold orchid flower. Bar represents 2cm**.**

2.4 Modes of Conservation

Conservation of plant biodiversity is crucial to maintain the ecological environment stability and preserve the genetic resources of new cultivars (Kaviani, 2011). The plant biodiversity conservation can be achieved by *ex situ* or *in situ* techniques. The *in situ* conservation preserves the cultivated and domesticated species in their natural habitations. This type of habitats provides the species to develop their distinctive structures and characteristics with minimum management and optimum ecosystem (Tay, 2007; Lauterbach et al., 2012). Nevertheless, the human development activities and natural disaster lead to the heavy loss or deterioration in species number and ecosystem. Hence, the *in situ* conservation methods alone are not sufficient for saving threatened species.

Additional approaches, such as storage in gene or seed banks, botanical gardens and *in vitro* collection, field gene collection, complement the conservation program for the plant biodiversity (Rao, 2004). The *ex situ* conservation method is equally important as *in situ* conservation for preserving plant biodiversity (Ramsay et al., 2000). Both *in situ* and *ex situ* are not exclusive but complementary. They offer various alternatives for conservation, however, the selection of the suitable strategy is based on the number of criteria; also include the biological nature and characteristics of the species and the feasibility of selected methods (Engelmann, 2012).

Currently, the biotechnological methods have been applied to conserve endangered, crop ornamental, rare, forest and medicinal species, allowing the conservation of pathogen-free material, short and long-term of genetic diversity. The *in vitro* conservation method is significant for non-orthodox and vegetative

propagated plant species (Engelmann, 2011). *In vitro* method also offers minimum space, low cost maintenance, safe, easy storage system. The valuable genetic material of the wild species preserved through *in vitro* technique and the molecular genetic stability studies is investigated (Tandon and Kumaria 2005). The *in vitro* culture is can be considered as more stable conservation method, where plant materials are cultivated under sterile and constant environmental factors. It also plays important part within *ex situ* strategies to maintain the desire genotype and normal propagules like recalcitrant seeds which may not suitable and effective for long-term storage. Hence, these involve the practice of conventional micropropagation methods, cryopreservation and slow growth techniques.

2.5 Plant tissue culture

Tissue culture technique is a process of multiplication, field collection and preserves the plant germplasms. This technique is very beneficial for preserving plant biodiversity, including endangered and rare species, genetic resource of vegetatively propagated species and recalcitrant seeds and biotechnology products (Bunn et al. 2007; Engelmann, 2012). The propagation of explants with high multiplication rates can be obtained through tissue culture technique under aseptic environment. Following both alternative morphogenic pathways; somatic embryogenesis and shoot organogenesis tissue culture well developed, propagated and able to obtained regeneration of more than 1000 variety plant species (Villalobos and Engelmann, 1995).

The nutrition such as mineral, vitamins, salt, water and growth regulators applied in orchid tissue culture media is significantly changes the success rate of orchid culture (Murdad et al., 2010). Apart from micronutrient and macronutrient,

the addition of carbon source in to the medium offers the energy to the plant materials, particularly when they are not able to photosynthesize own food during the primary stage of plant tissue culture (Al-Khateeb, 2008). The presence of carbon source in the tissue culture media also influenced the growth of plant. The carbon source can be in the form of complex or simple sugar (Akter et al., 2007).

Sucrose is commonly used in the tissue culture media as a carbon source. The use of organic additives is advisable for orchid culture medium to improvise the recent orchid tissue culture media to commercial productions (Ichihashi and Islam, 1999). The organic additives not only supply a natural carbon source to the tissue culture media, but it also contains natural hormones, vitamins, phenol, protein and fibers (Gnasekaran et al. 2010). Akter et al. (2007) reported that organic additives promote more shoots, leaves, PLBs and increases the mass of somatic embryos. The organic additive also promotes the growth of asymbiotic seeds and the regeneration of plantlets (Tawaro et al., 2008).

Plant tissue culture is an efficient technology for the crop improvement and development of the production of gametoclonal and somaclonal variants. The micropropagation method is a process of isolation of beneficial variants in well-adapted quality yielding genotypes with improved disease resistance and high stress tolerance capabilities (Brown and Thorpe, 1995). Several callus cultures promote clones that have an inheritable feature which is different from those biological parent plants. This is lead to somaclonal variability occurrence with better-quality varieties (George, 1993).

2.6 Plant Cryopreservation

The ideal method and valuable tool to date for the long-term conservation of plant is cryopreservation, meaning immersion of biological materials in liquid nitrogen (LN) without losing viability. During cryopreservation, cell division, biochemical and metabolic processes are halted and thus the explant can be preserved for a long period of time without any deterioration or modification (Sakai, 2004; Zhu et al., 2006). Cryopreservation method is based on slow metabolism, non-injurious reduction and biological deterioration or alteration of plant materials by the temperature reduction up to -196°C (Sakai, 2004). After liquid nitrogen storage, the plant materials able to be recovered and regenerated into a whole plant (Engelmann, 2011).

Cryopreservation method also offers the explants can be stored for an indefinite time with minimum storage requirements and low cost (Engelmann, 2012). It only needs work inputs at early stage during sample preparation and cooling (Engelmann, 1997; Sakai, 2000). During storage, it only requires refilling of LN with less maintenance. Cryopreservation process needs only less number of replicates for efficient plant conservation system and prevent the plant material from bacterial and fungal infections (Sakai et al., 2000; Gonzalez-Arnoa et al., 2008).

The ultimate reason of cryopreservation method is to preserve the plant genetic resources (Benson, 1999). Various plant materials were successfully developed in plant cryopreservation such as seeds, shoots, meristem, zygotic and somatic embryo, protocorm-like bodies (PLBs), callus, *in vitro* fragmented explants (IFEs) and protocorm (Panis and Lambardi 2005; Benson, 2008; Gonzalez-Arnao et al., 2009; Salaj et al., 2011). Plant cells is difficult to freeze due to their complex cellular structure and the large vacuole with high amount of water content (Heine-

Dobbernack et al., 2008). Prevention of intracellular ice formation highly required with manipulation of water state such as liquid, glass and ice conditions.

Recently, several cryopreservation techniques have been developed to plant species such as desiccation, encapsulation and vitrification-based method [PVS2, PVS3 or droplet vitrifications] (Tsai et al., 2009). In plant cryopreservation study, the success rate of desiccation (air-drying) method is very low compared to other methods (Wusteman et al., 2002). This is due to direct exposure of explant to dehydration process. In several research, vitrification and desiccation methods produced low growth rate in orchid culture (Bian et al., 2002). However, encapsulation- vitrification and encapsulation- dehydration methods were comparatively regenerate higher of plantlets after cryopreservation treatments (Yin and Hong, 2009). Hence, a appropriate protocol is required to achieve high amount of recovery of cryopreserved plants especially in testing new plant.(Reed et al., 2006).

2.7 Orchid cryopreservation

Orchids are one of the utmost diverse angiosperm family and involves at risk of extinction and destruction. According to Sanford (1974), the orchid seeds can be stored at freezing point 22 to 27°C or desiccator at 46°F and preserve the viability of seeds for more than two years. The efficient manner of orchid conservation is through *in vitro* germplasm storage banks of complete plants which are derived from isolated cells and organs. Thus, such types of storage displayed high priority part in the preservation of plant species threatened due to extinction. Advances in biotechnology could conserve the orchid germplasm at cryogenic temperatures for long-term storage (Kong and Von Aderkas, 2011).

Orchid germplasm is cryopreserved and stored by using three different types of methods: vitrification, encapsulation–dehydration and air desiccation (Hirano et al., 2009). The studies showed that several seeds of orchids like *Dendrobium candidum* Wall. ex Lindl. (Wang et al., 1998); *Doritis pulcherima* Lindl. (Thammasiri, 2008) and *Bletilla striata* Rchb.f (Hirano et al. ,2005) can stored directly in liquid nitrogen without any pretreatment (Vendrame et al., 2007). In contrast, some orchid seeds undergo some water elimination to obtain optimal moisture content prior to LN storage (Hirano et al., 2009). The most effective method for orchid seed cryopreservation is vitrification which is simple, reproducible, low-cost, quick and reliable (Vendrame et al., 2007).

In vitrification process, only small amount of water need to dispose and this can be done by optimal exposure time in PVS2 solution (Hirano et al., 2009). Water removal is essential and it can be done with either through direct dehydration for zygotic embryos or chemical dehydration for organized meristematic regions (Le Bras et al., 2014).

Protocorm of orchids has unique developmental cycle and develops directly from the seed (Bukhov et al., 2006). The major problem in PLBs is formation of high amount of unbound water around their cells (Wang et al., 1998). This unbound water can formed ice crystals during LN storage and post-thawing which can cause lethal damages to cryopreserved cells (Bukhov et al., 2006). However, main issue that still controversial here is whether the cell death is take place instantly during the freeze-thaw cycle or promoted later step by the injuries found from the earlier part (Bukhov et al., 2006).

In vitrification technique, adequate exposure time to cryoprotective solution (loading, PVS2 and unloading) is relying on the plant sample. In research conducted

by Ishikawa et al. (1997), about 60% germination rate of zygotic embryo of *Bletilla striata* was obtained when after precultured on ND medium added with 0.3M sucrose (3 days) and dehydrated for 3h with PVS2 solution. In other study conducted by Hirano et al. (2009) stated that PVS2 exposure for 30 min or more exposure duration can increase the regeneration rate of *Phaius tankervillae* seeds. *Oncidium flexuosum* Sims seeds germination rate was improved 41.2 to 78.4% when exposed for 60 min directly to PVS2 solution with 1% phloroglucinol antioxidant (Galdiano et al., 2012).

2.8 The role of water and ice nucleation

The osmotic equilibrium and the state of water associated to the movements of water in and out of the plant cells are the main parameters for a successful cryopreservation study (Mazur, 2004). Water elimination plays an essential role in avoiding freezing injury and also in preserving post-thaw viability. Ice nucleation disturbs the osmotic, plant structural and also the colligative integrity of plant cells causing mechanical damage and physical ruptures (Benson, 2008). Thus, the formation of intracellular ice nucleation can be prevented through the freeze induced dehydration, evaporative desiccation and osmotic dehydration. Whereas, extracellular formation, such as freeze induced dehydration, removes the molecules of free liquid water by an osmotic gradient from the plant cytoplasm into intracellular spaces, where crystallization takes place.

The dehydration process causes the cellular concentration of solutes in plant cell rises and during cooling it converts to ice crystal. This situation is named as 'glassy state' means it solidifies without crystallization. At this stage, Taylor et al. (2004) reported that the molecules of water are amorphous and not well organized structure, but hold the physical and mechanical properties of a solid. The possibility

of water molecules to revert from glassy state to liquid and/or devitrify to ice are high (Benson, 2008).

Generally, cryopreservation protocols use several chemicals like plant vitrification solutions 2 (Sakai et al., 1990), sucrose and dimethyl sulfoxide (DMSO) to reduce the free water content in plant cells. In PVS2, both components like DMSO and glycerol are membrane and cell wall penetrable and it maintain the cellular osmolality to prevent the ice formation (Benson, 2008). Sucrose is a disaccharide and it's a bigger molecule that able to penetrate the outer layer of cell wall (Tao and Li, 1986). Meanwhile, sucrose is not able to pass through the plasma membrane due to the bigger size of molecule. When the cells are at frozen stage, sucrose is concentrated at the cell wall space. Thus, the plant cell structure is maintained from from freeze-induced desiccation. Here, the outer surface of plasmalemma was protected by buffer layer formation between the protoplast and cell wall (Tao and Li, 1986; Anthony et al., 2013). The dehydration process leads to the cell water content reduction and increase the cell viability without ice crystallization process (Bhat et al., 2005). .

2.9 General cryopreservation techniques

Orthodox seeds and dormant buds have natural dehydration process and do not require any pretreatment in cryopreservation protocol. However, most of the plant material employed in cryopreservation such as calluses, cell suspension, shoot tips and embryos contain high amount of free water in cell and extremely sensitive to cryo-injury. Thus plant cells have to be dehydrated artificially to safe from harmful damages caused by crystallization process.

The physical mechanisms applied in the techniques are vary in classical and new cryopreservation protocol. In classical cryopreservation technique cells are dehydrated based on freeze-induced method. While new cryopreservation techniques use vitrification method in which internal solutes form a glass thus ice crystal formation during storage in liquid nitrogen is avoid. Freezing can be done with presence of ice, whereas in vitrification-based method; freezing usually performed without ice formation.

2.9.1 Controlled rate cooling

The controlled rate cooling objectives are to optimize two injurious components like the ice formation and colligative solution effects damage. This step involves pre-freezing temperature and the temperature changes using programmable freezer that can reduce from 4- 40 °C prior to liquid nitrogen immersion (Reinhoud et al., 2000). The rate of cooling is important in determining the cell survival (Mazur, 2004).

The cells are unstable and do not survive if the cooling rate is too slow or too fast. Thus, optimizing these two types of cryo-injuries are the crucial to success. During LN storage, the optimal rate of cooling is employed to the cells, formed extracellular ice, a differential water gradient is developed across the plant cell membrane and the water moves outside from intracellular.

This is an important factor to reduce the amount of free water available in the cells. In contrast, extracellular ice has an important cryoprotective part as its formation will indirectly decreases the possibilities of ice nucleating in the plant cell. Hence, the plant cells can survive, although the extracellular ice formed and harmful, especially in the complex multicellular tissues (Benson, 2008). However, the

intracellular ice formation is causing lethal. The cell's solutes became too concentrated at slow cooling rate and it causing colligative damage (Benson, 2008).

The sufficient amount of water in the plant cell is highly required to eliminate the ice formation and colligative injury. Thus, the cooling rates need to be optimized for every plant species. The central key is to stabilize the two types of injuries by optimizing the cooling rates so that sufficient amount of water is eliminated to avoid the colligative injury. Hence, the amount of water does remain is so minimal that prevent ice formation and the cytoplasm become vitrified state (Benson, 2008 ; Gonzalez-Arnao et al., 2008).

2.10 PVS2 Vitrification method

The study of plant tissue cryopreservation is more challenging than animal cell cryopreservation due to the existence of cell wall in plant and requires combination of several cryoprotectants (Kulus and Zalewska, 2014). In vitrification method, sample is exposed in a loading cyoprotectants after preculture treatment in order to increase the sample resistance level. This step is important for the sample to overcome highly concentrated compounds such as DMSO and glycerol in vitrification protocol. According to Jitsopakul et al. (2012), pretreatment with loading solution is crucial and effective for *Vanda tricolor* cryopreservation. Sekizawa et al. (2011) highlights the significance of suitable loading solution (LS) selection because cryoprotectant highly influence on the water content in tissues and dehydration tolerance of the samples.

The main problem encountered in plant cryopreservation is a high amount of free water content in dedifferentiated cell cultures (Kaczmarcyk et al., 2011). In contrast, the plant cells should content low amount of free water molecules in order

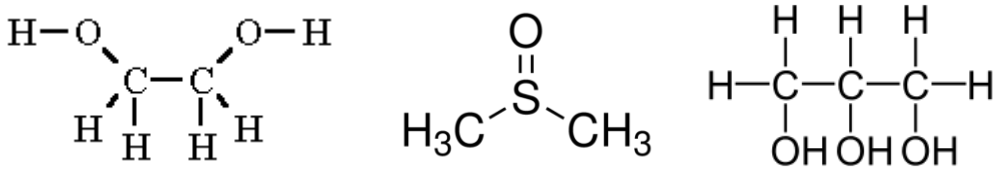
to survive and regenerated into whole plant after cryopreservation treatment (Reed, 2008). There are two different types of phase transition transformed liquid to solid phase in aqueous solution which known as vitrification and ice nucleation (Sakai et al., 2008). The ice nucleation or ice formation is a process where changes of liquid phase into ice crystal that promotes osmotic and physical damages and injured the cellular function in plant after cryopreservation treatment (Fleck et al., 2006).

Vitrification can be defined as process that resulting in 'glass formation' meaning the conversion of liquid to solid phase without the presence of crystallization (Sakai et al., 2008; Hong et al., 2009). Accordingly, cells or tissues that are sufficiently slow frozen turn into "vitrified". This process involves the use of a cryoprotectant or medium which has contain high amount of solute concentration to start with. Hence, the ice formation does not take place in any portion of the sample. The glassy state in cryopreserved cell is extremely viscous and it stops the chemical reaction in the cell as well (Day et al., 2008).

The successful cryopreservation of biological material can be performed with absence of ice crystal formation which could lead damages to plant cell membrane and lost their semi-permeability in liquid nitrogen storage. The free water in the cell is a challenging process since the rate of ice crystal formation is relatively high even at the below their freezing point (Sakai et al., 2008). Thus, dehydration process is highly required to eliminate the excess water retained in plant cell which causing the crystal to formed. Generally, the dehydration process can be done in vitrification-based cryopreservation through concentrated cryoprotective solution application during cryopreservation process. The main purpose of apply concentrated cryoprotective solution is to prevent damages and cell injuries during liquid nitrogen immersion. Two type of cryoprotectant is applicable based on the capability to

diffuse across plant cell membranes: colligative or penetrating and osmotic or non-penetrating. The penetrating/colligative cryoprotectant penetrates across cell membranes and builds a situation for a reduction of the cell water content usually at temperature adequately low to minimize the detrimental effect of the concentrated solutes on the plant cells. There are many colligative/ penetrating cryoprotectant solution available such as dimethyl sulfoxide (DMSO), glycerol, propylene glycol and ethylene glycol. Whereas, non-penetrating cryoprotectant not able to across the cell membranes. It will degrade and lower the freezing point between -10 to -20 and reduces the rate of freezing water osmotically.

In vitrification method, some concentrated cryoprotectant commonly used such as PVS2 (15% (w/v) ethylene glycol, 15% (w/v) DMSO, 30% (w/v) glycerol and 0.4M sucrose) liquid medium (Sakai et al., 1990) and PVS3 (50% (w/v) sucrose and 50% (w/v) glycerol) liquid basal medium (Nishiziwa et al. 1993). Both cryoprotectants are glycerol based solution and the advantage of these agents are easily supercool below temperature -100°C and form vitrified or metastable glass stage at temperature -115°C (Sakai and Engelmann, 2007). The permeability of DMSO and ethylene glycol is fast across cell membrane, good glassy state formation with low ice-blocking tendency (Figure 2.1) .



Ethylene glycol DMSO Glycerol

Figure 2.1: The cryoprotectant chemical structures of DMSO, ethylene glycol and glycerol (Falbe and Regitz, 1998)