DEVELOPMENT OF CRYOPRESERVATION IN *Dendrobium* Bobby Messina USING PVS2 VITRIFICATION AND ENCAPSULATION-DEHYDRATION METHODS

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by

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Specially Dedicated to:

My Parents

Mr and Mrs James Antony Ratnam

My Late Grandparents

Mr and Mrs Ratnam; Mr and Mrs Maria Soosay

My Supervisor

Dr. Sreeramanan Subramaniam

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LIST OF ACRONYMS AND ABBREVIATIONS

%	Percentage
ºC	Temperature
µL	micro liter
µM	micromolar
AFLP	amplified fragment length polymorphism
BAP	Benzylaminopurine
bp	base pairs
cm	centimeter
DMSO	Dimethyl suphoxide
DNA	Deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DSC	differential scanning calorimeter
EDTA	Ethylenediaminetetraacetic acid
g	Gram
g/L	gram per Liter
ISTA	International Seed Testing Organisation
KCI	potassium chloride
LN	Liquid nitrogen
+LN	Cryopreserved
-LN	Non-cryopreserved
m	meter

M	Molar
mg/L	Milligram per Liter
$MgCl_2$	magnesium chloride
mL	milliliter
mm	millimeter
mM	mili molar
MS	Murashige and Skoog
NADPH	nicotinamide adenine dinucleotide phosphate
nm	nanometer
PCR	Polymerase chain reaction
PGR	Plant growth regulator
PLB	Protocorm-like body
PVS2	Plant Vitrification Solution 2
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic acid
ROS	reactive oxygen species
rpm	Rotation per minute
SAM	Shoot apical meristem
SD	Standard deviation
SEM	scanning electron microscope
SI	Similarity Index
TBE	Tris/Borate/EDTA
Tg	transition temperature

*T*m	Melting temperatures
Tris-HCl	Tris- Hydrochloride
TTC	triphenyltetrazoliumchloride
UV	ultraviolet
v/v	volume/ volume
VNTR	Variable Number Tendem Repeats
W	Watt
w/v	weight / volume

PEMBANGUNAN KRIOAWETAN UNTUK *Dendrobium* Bobby Messina MENGGUNAKAN KAEDAH VITRIFIKASI PVS2 DAN PENGHIDRATAN PENGKAPSULAN

ABSTRAK

Dua kaedah baru krioawetan iaitu pengkrioawetan vitrifikasi dan pengkapsulan-pengeringan digunakan ke atas jasad seperti protokom (PLB) *Dendrobium* Bobby Messina, orkid yang bernilai tinggi di Malaysia di mana kemandiriannya diawasi melalui ujian 2,3,5-trifeniltetrazolium klorida (TTC) dan melalui pemerhatian pertumbuhan. Dalam kaedah vitrifikasi, parameter-parameter yang telah dioptimumkan adalah saiz protokom, kepekatan prakultur, jangka masa prakultur, suhu larutan vitrifikasi tumbuhan 2 (PVS2) dan jangka masa inkubasi PVS2. Dalam kaedah pengkapsulan-pengeringan, parameter-parameter yang telah dioptimumkan adalah saiz protokom, kepekatan prakultur, jangka masa prakultur, dan jangka masa pengeringan dengan menggunakan gel silika. Untuk krioawetan kaedah vitrifikasi, protocol optimum berdasarkan analisis TTC dan pemerhatian pertumbuhan adalah protokom bersaiz 3-4 mm yang telah diprakultur dengan 0.2M sukrosa untuk satu hari dan dirawat dengan campuran 2M gliserol dan 0.4M sukrosa dibekalkan dengan media separa pepejal MS pada suhu 25°C selama 20 minit dan berikutnya dikeringkan dengan larutan vitrifikasi tumbuhan 2 (PVS2) pada suhu 0°C selama 20 minit sebelum dikrioawet dalam cecair nitrogen (LN). Menyusuli pemanasan dalam air bersuhu 40°C selama 90 saat, protokom-protokom dicuci dengan media cecair MS dibekalkan dengan 1.2M sukrosa. Berikutnya, protokom-protokom dikultur dalam media separa pepejal MS dibekalkan dengan 2% sukrosa tanpa sebarang regulator pertumbuhan. Kaedah vitrifikasi optimum telah berjaya untuk memelihara

orkid ini dan telah menghasilkan pertumbuhan pada protokom yang tidak melalui pengkrioawetan sebanyak 100% dan yang telah dikrioawet sebanyak 40% untuk *Dendrobium* Bobby Messina. Untuk krioawetan kaedah pengkapsulan-pengeringan, protokol optimum berdasarkan analisis TTC adalah protokom bersaiz 3-4 mm yang telah diprakultur dengan 0.4M sukrosa untuk 3 hari. Kemudian, protokom-protokom dikapsulkan dalam 3% alginat natrium dan 0.1M kalsium klorida, di mana kedua-duanya dibekal dengan media cecair MS dan 0.4M sukrosa. Kemudian, kapsul telah diosmolindungi dalam 0.75M sukrosa dibekalkan dengan media cecair MS di orbital shaker (110 rpm) pada suhu 25 °C selama 24 jam di bawah 16 jam fotoperiod. Kapsul yang telah diosmolindungan dikeringkan selama 9 jam dalam jar kultur yang mengandungi 50g silika gel dan mengikuti krioawetan dalam cecair nitrogen (LN). Mengikuti pemanasan cepat dalam air pada suhu 40°C untuk 90 saat, kapsul yang telah dikrioawet di kultur pada media pertumbuhan yang mengandungi media separa pepejal MS yang telah dibekal dengan 2% sukrosa. Bagaimanapun, protokom-protokom kaedah pengkapsulan-pengeringan yang telah dioptimumkan menunjukkan tiada pertumbuhan pada protokom yang telah dikrioawet dan yang tidak dikrioawet. Berdasarkan observasi histologi, dalam kaedah vitrifikasi dan pengkapsulan-pengeringan, sel-sel homogenus kelihatan banyak bilangannya dan sitoplasma pula kelihatan lebih padat pada protokom yang telah dikrioawet berbanding dengan yang tidak dikrioawet. Pengimbasan mikroskop electron (SEM) menunjukkan kerosakkan yang kurang pada protokom-protokom yang telah melaui kaedah vitrifikasi berbanding protokom-protokom stok kultur dan ini memberi kelebihan kepada kaedah vitrifikasi untuk digunakan sebagai kaedah pemeliharaan germplasme. Sebaliknya, SEM menunjukkan kerosakkan teruk pada protokom-protokom yang telah dikrioawet dan yang tidak dikrioawet melalui kaedah pengkapsulan

pengeringan berbanding dengan protokom stok kultur dan merupakan sebab tiada pertumbuhan dengan kaedah ini. Bagaimanapun, analisis genom yang menggunakan 6 primer menunjukkan bahawa protokom-protokom yang telah dikrioawet dan tidak dikrioawet yang melaui kaedah vitrifikasi adalah setia secara genetic kepada pokok induk. Bagaimanapun, 3 primer menunjukkan polimorfisme dan 1 primer menunjukkan separa polimorfisme di antara protokom-protokom yang telah dikrioawet dan tidak dikrioawet berbanding protokom-protokom stok kultur.

DEVELOPMENT OF CRYOPRESERVATION IN *Dendrobium* Bobby Messina USING PVS2 VITRIFICATION AND ENCAPSULATION-DEHYDRATION METHODS

ABSTRACT

Two new cryopreservation techniques, PVS2 vitrification and encapsulation-dehydration, were applied on PLBs of *Dendrobium* Bobby Messina, highly-prized orchid in Malaysia, with survival monitored through observations of growth and the 2,3,5-triphenyltetrazolium chloride (TTC) assay. In the vitrification method, the parameters optimized were PLBs size, preculture concentration, preculture durations and PVS2 incubation temperature and duration. In the encapsulation-dehydration method, the parameters optimized were PLBs size, preculture concentration, preculture duration and dehydration period with silica gel. In cryopreservation of *Dendrobium* Bobby Messina by vitrification technique, the optimized protocol based on TTC spectrophotometrical analysis and growth recovery were 3-4mm PLBs precultured in 0.2M sucrose for 1 day, treated with a mixture of 2M glycerol and 0.4M sucrose supplemented with half strength liquid MS media at 25°C for 20 minutes and subsequently dehydrated with plant vitrification solution 2 (PVS2) at 0°C for 20 minutes prior to storage in liquid nitrogen. Following rapid warming in a water bath at 40°C for 90 seconds, the PLBs were washed with half strength liquid MS media supplemented with 1.2M sucrose. Subsequently, the PLBs were cultured on half strength semi-solid MS media supplemented with 2% (w/v) sucrose without any growth regulators. The optimized vitrification method was successful in preserving this orchid as it produced growth recovery in non-cryopreservaed and cryopreserved PLBs up to 100% and 40% for *Dendrobium* Bobby Messina. In

cryopreservation of *Dendrobium* Bobby Messina by encapsulation-dehydration technique, the optimized protocol based on TTC spectrophotometrical analysis were 3-4mm PLBs precultured in 0.4M sucrose for 3 days. Then, precultured PLBs were encapsulated in 3% (w/v) sodium alginate and 0.1M calcium chloride, both supplemented with half strength liquid MS media and 0.4M sucrose. The beads were osmoprotected in 0.75M sucrose supplemented with half strength liquid MS media on an orbital shaker (110 rpm) at 25°C for 24 hours under 16 hours photoperiod. Osmoprotected beads were dehydrated for 9 hours in a parafilm-sealed culture jar containing 50g of oven-sterilized silica gel followed by rapid freezing in liquid nitrogen. Following rapid warming in a water bath at 40°C for 90 seconds, the cryopreserved beads were cultured on growth recovery media containing half strength semi-solid MS media supplemented with 2% (w/v) sucrose. However, optimized encapsulation-dehydration method showed no growth in non-cryopreserved and cryopreserved PLBs. Based on histological observation, in vitrification and encapsulation-dehydration methods, there are higher amout of homogenous cell population and denser cytoplasm in cryopreserved PLBs comparative to non-cryopreserved PLBs. Scanning electron micrograph in vitrification method, showed lesser damage in cryopreserved and non-cryopreserved PLBs by vitrification method comparative to PLBs stock culture which makes it a possible technique for germplasm conservation. In contrast, scanning electron micrograph showed severe damages in cryopreserved PLBs and non-cryopreserved PLBs comparative to the PLBs stock culture which in return could be the possible reason of no regrowth in encapsulation-dehydration method. However, genomic analyses using 6 primers indicated that cryopreserved and non-cryopreserved PLBs from vitrification method were genetically faithful to the mother plant. However, 3

primers showed polymorphism and 1 primers indicated partial polymorphism between the cryopreserved and non-cryopreserved PLBs comparative to the stock culture PLBs.

CHAPTER ONE

INTRODUCTION

Malaysia with the most biologically diverse country in the world, has about 12 500 species which comprise of the flowering plants. These include species of valuable and profitable timber, fruits and other plants that are able to produce natural products (Noor et al. 2011).

An overall of 8321 plants have been added to the Red List of Threatened Species during the period of 1996 to 2004 (IUCN 2004). In addition, plants that are recorded as critically damaged have increase to about 60%. In the wild species, *in situ* conservation is considered to be a traditional method. However, the *ex situ* technique can be used to complement the *in situ* method. In addition, this method can be the only method to conserve certain extremely endangered and rare species (Ramsay et al. 2000). In *ex situ* technique, botanical garden plays an important role in conserving plants. UNEP (1995) made an estimated that botanic gardens generally makes about more than one third conservation of world's flowering plants, among which Botanic Gardens Conservation International recognized more than 15000 threatened plants. *Ex situ* conservation of plants is normally is in the form of field collection. However, there is a major drawback in the field collection, such as their safety is being threatened. Most plant diversity is concerned on plant hybrids. This is due to the world biodiversity has declined to an extraordinary rate (Engelmann 2011).

Biotechnology leads to the production of new category of germplasm which comprise of hybrids from special genotype with beneficial attributes (Engelmann 1992; Engelmann 2011). In general, these plant hybrids are valuable and

are not easy to be produce. Therefore, a competent method in conserving them becomes our central aim today. In terms of preserving the important plants today, there is effort has been made to advance the quality and security of conservation offered by field genebanks and botanic gardens. However, it is apparent that alternative approach to genetic conservation is needed and therefore, since 1970s interest has turned to the possibilities offered by biotechnology, in particular *in vitro* method comprising of cryopreservation (Engelmann 2011).

Orchidaceae is a family with 20,000 to 30,000 species and is the largest family in the plant kingdom (Godo et al. 2010). Orchids are one of the most captivating clusters of ornamental plants and several novel cultivars have been produced by interspecific and intergeneric hybridization in order to generate plants with exotic and elegant flowers. Due environmental disruption, succession of natural habitats and over exploitation of horticultural, numerous orchid species are endangered today (Godo et al. 2010). Therefore, the importance of orchid hybridization becomes a concern today. The most universal use of orchids is as ornamentals and cut flowers. However, only few orchid plants are attractive enough for direct utilization as ornamentals. Orchids hybrids are used as ornamental due to they have beautiful foliage, shiny, multicoloured and decorated with prominent veins in elaborate patters. Orchids from several genera such as *Dendrobium, Anoectochilus*, *Goodyera*, *Ludisia* (Haemaria) and *Macodes* are used widely. Orchids used indoor and outdoor landscapes are often employed to create a special effect such as exotic surroundings, feeling of luxury and unique environment (Arditti 1992). Mostly, the flowers were made into corsages. In the orchid industry, trade in plants has always been and remains an important aspect. Nevertheless, in the past, this trade was limited to species, seedlings and matured plants. The development of

clonal propagation procedures and hybridization of orchid such as *Dendrobium* hybrid has increased this market (Arditti 1992). In return, it had brought a large numbers of outstanding cultivars which are mass-propagated by growers. Another form of orchid exploitation is the selection of some species or hybrids as national flowers such as *Peristeria elata* in Panama (Arditti 1992).

The world export and import trade of orchid cut flowers and plants exceeds US $150 million dollars. Among this, about 80% are cut orchids and the remaining 20% is composed of pot plants. Orchids alone account for 3% of total fresh cut flower imports to the European Union, with a wholesale value of 21 million Euro. Since, Japan is the world's largest importer of cut orchids, Asia dominates the world trade. The main producers of orchids are Thailand, Singapore and Malaysia. In 2001, the main exporters were Thailand with exports valued at US $50 million, Singapore US $7.7 million, Malaysia US $2.8 million, New Zealand US $830,000 and Italy US $652 000 (Pizano et al 2005).

There are many different *in vitro* conservation methods that are in use which is dependent on storage duration requested. In short term storage method, the growth is been reduce and the number of intervals between subcultures is been increased. In long term storage, the only feasible method is cryopreservation whereby plants are stored at ultra low temperature, using liquid nitrogen (-196°C). At this ultra low temperature, all cellular divisions and metabolic processes are halted. Therefore, theoretically the plant material can be stored without alteration or modification for an unlimited period of time. Apart from that, cultures are stored in small volume, confined from contamination and require incredibly limited maintenance. Therefore, cryopreservation is the only technique currently presented to guarantee the safe and cost efficient long term conservation of plant germplasm (Engelmann 2011).

Orthodox seeds or dormant buds exhibits natural dehydration processes and can be cryopreserved without any pretreatment. Nevertheless, cell suspensions, calluses, shoot tips and embryos that are cryopreserved contains high amount of cellular water and are susceptible to freezing injury in view of the fact that most of them are not inherently freezing tolerant (Mazur 1984). In order to keep them away from damages caused by crystallization of intracellular water into ice, cells have to be dehydrated (Mazur 1984; Benson et al. 2008). There are two methods in cryopreservation which are classical and new cryopreservation techniques (Wither and Engelmann 1998). A classical technique involves freeze induced dehydration while new techniques are based on vitrification. Vitrification is defined as the transition of water directly from liquid phase into amorphous phase or glass while avoiding crystallization of ice (Fahy et al. 1984). Cryopreservation has been employed to a wide range species through the implementation of wide range of methods (Engelmann 2011). The advantages of this technique are simplicity in procedure and wide applicability. In order to achieve high growth in cryopreservation protocol, the optimization of several key factors plays a large role. The basic factors for the success in cryopreservation are physiological status of the plant material, preculture and cryoprotective treatments, cooling and rewarming rates and growth recovery conditions (Engelmann 2011).

In order to get a better understanding on cryopreservation, this study was conducted to develop a cryopreservation protocol by vitrification and encapsulation-dehydration techniques of *Dendrobium* Bobby Messina for germplasm conservation. This study covers a several factors to be optimized prior to the vitrification and encapsulation-dehydration protocol development.

1.1 Objectives

Therefore, the objectives of this study are:

a. To optimize cryopreservation protocol by PVS2 vitrification and encapsulation-dehydration method of *Dendrobium* Bobby Messina for long term germplasm conservation,

b. To investigate histological and scanning electron microscope analysis of cryopreserved and non-cryopreserved PLBs comparing to PLBs stock culture for both PVS2 vitrification and encapsulation-dehydration methods in order to observe the morphological changes following cryopreservation,

c. To carry out RAPD analysis of cryopreserved and non-cryopreserved PLBs comparing to PLBs stock culture for PVS2 vitrification method to conform the genetic fidelity.

CHAPTER TWO

LITERATURE REVIEW

2.1 Orchid Morphology

Orchidaceae is the largest family of flowering plants, with an estimated 800 genera and at least 24000 species (World Checklist of the Monocotyledons 2006). Distributed throughout all continents except Artantica and particularly numerous and diverse as epiphytes in the wet tropics, orchidaceae have long fascinated biologists by their remarkable range of life history strategies, floral and vegetative morphology and pollination syndromes (Fay and Chase 2009).

Orchidaceae is a very polymorphic family whose great diversity is associated to the many different circumstances under which it evolved and the numerous niches it occupies at present. There are morphological similarities which exist, and some of the differences are variations on a common theme. There are two general forms or habits that can be distinguished among the orchids which are monopodial and sympodial. Some of examples of monopodial orchids are *Neofinetia*, *Aerides* and *Vanda* and their characteristics are as follow (Arditti 1992):

- A sole shoot apex, for an example, one growth axis,
- limitless growth and continuous elongation,
- Absence of rhizomes,
- No new growths from stem (cane) bases,
- Adventitious root production on stems between nodes,
- Lateral (meaning axillary) inflorescences,

- Branching between nodes.

The stems of several monopodial orchids can reach a height of 3m or more. Apart from that, monopodial species may be only 30 to 40cm. A number of monopodial orchids, such as *Vanilla* and *Gastrodia* which grows as vines and can be 20m long. Some of examples of sympodial orchids are *Brassavola glauca*, and their characteristics are as follow (Arditti 1992):

- Rhizomes top row, second and third from left which grow forward, may branch and produce leaf bearing stems, roots, normal and scale-like leaves, as well as new growth from axillary buds, as in *Paphiopedilum* and *Cypripedium*,

- Upright stems or pseudobulbs which may complete their growth within a certain period and cease to elongate after that. The pseudobulbs are food and water-storage organs. Both stems and pseudobulbs are variable in terms of size and form,

- Terminal or lateral inflorescences (acranthous and pleuranthous growth types spectively).

- Short, underground rhizomes that produce very reduced leaf-bearing stems, roots, normal and scale-like leaves, and new growth from axillary buds, as in *Paphiopedilum* and *Cypripedium*.

2.2 *Dendrobium* Orchids

The genus *Dendrobium* (*Dendrobium* : Orchidaceae) is one of the most vital genera in the orchid family with 1190 species as listed by the Royal Botanic Gardens, Kew, UK (Wang et al. 2009). *Dendrobiums* are well distributed in tropical and subtropical Asia and North-Australia and exhibits unique ecological diversification. They occur in terrestrial, epiphytic and lithophytic life forms. Besides, for many centuries in Asian countries *Dendrobiums* substances have been used in traditional medication (Bulpitt et al. 2007). *Dendrobiums* are also among the most popular orchids for commercial production as cut flower and pot plants (Wang et al. 2009).

There are more than 8000 novel *Dendrobium* hybrids and cultivars have been produced in horticulture through interspecific hybridization since the 18th century. This is generally done to obtain novel flower and better morphological characteristics. Hybridization in orchid is a universal mean in producing novel and better material, including new flower colour, colour pattern, flower size and a number of additional characteristics of commercial values. There are over 100 000 commercial hybrids has been registered worldwide to date being grown as cut flowers and potted plant. The requirement of orchid as cut flowers increased with *Dendrobium* hybrid being commercially desirable due to the number of flower per inflorescence and recurrent flowering (Martin and Madassery 2006; Wang et al. 2009).

Furthermore, the diversity of flower colour and colour pattern and relatively short production cycle from seedling to full bloom plant for *Dendrobium* hybrids enhanced their commercial value (Vendrame et al. 2007; Wang et al. 2009).

2.3 *Dendrobium* Bobby Messina

Dendrobium Bobby Messina (Plate 2.1) which is a sympodial orchid is one of a new *Dendrobium* orchid hybrid from Indonesia with the parentage of (Imelda Romualdez × Jaquelyn Thomas). The characteristic of a sympodial orchid is mentioned in Section 2.1. This orchid has been reported to be grown widely as cut flower, potted plant and is valued for its attractive flower. The height of the plant is generally about 30cm during the first flowering. The spikes carry about 8 beautiful blooms. The plant is generally easy to grow. The flowers measures about 8cm in diameter. The petal and sepal is in deep red in colour. Flowers are generally arranged in compact manner on the stalk. *Dendrobium* Bobby Messina is also closely related to *Dendrobium* Sharifah Fatimah and *Dendrobium* Tun Ku Imran (Khosravi et al. 2009).

Plate 2.1: *Dendrobium* Bobby Messina. Scale bar represents 10 cm.

2.4 Micropropogation of Orchids and Protocorm-Like Bodies (PLBs)

Naturally, germination of orchid seeds is dependent upon the formation of a symbiotic relationship with fungi which usually supplies nutrients and carbohydrates to the orchid seeds (McKendrick et al. 2000; Yoder et al. 2000; Godo et al. 2010). On the other hand, this relationship can be simulated *in vitro* by co-culturing orchid seeds with compatible fungi (symbiotic seed germination) and has been established with diverse orchid species (Shimura and Koda 2005; Zettler et al. 2005; Batty et al. 2006; Johnson et al. 2007; Stewart and Kane 2007, Yagame et al. 2007; Stewart and Kane 2010).

Asymbiotic seed germination or orchid micropropogation can be done, in which seeds are cultured without fungi but instead with nutrient rich media enriched with mineral nutrients, carbohydrates such as sucrose and organic compounds. Symbiotic seed culture is valuable for analyzing fungal specificity and nutrient flow between symbionts. On the other hand, asymbiotic culture is useful for scientist to manipulate and analyze the effects of specific compounds and environmental conditions on seed germination (Johnson et al. 2011).

Micropropagation is the production of microplants through tissue culture by which is generated through initiation of meristematic material such as seedlings, shoot apices, shoot buds and PLBs from mature plants into the culture system. Micropropagation generally has five distinct stages which are; Stage 1, preparation of *in situ* donor material; Stage 2, initiation including surface sterilization of explants; Stage 3, shoot multiplication such as optimisation of proliferation media; Stage 4, root induction on micro-cuttings which can be done *in vitro* or *ex vitro*;

Stage 5, acclimatization of rooted shoots or unrooted micro-cuttings to *ex vitro* conditions.

Propagation of orchids by means of tissue culture techniques has been used for more than a century and has resulted in the production of uniform clones in many orchid genera (Ng and Saleh 2011). Carbohydrate used by seeds serves several functions throughout germination. Mainly, stored carbohydrates serve as an energy resource that aids in germination and seedling growth (Go´recki et al. 1996; Young et al. 1997; Bonfil 1998; Kitajima 2003; Obendorf et al. 2009). In addition, carbohydrates also works as a signaling molecules relating in the regulation and integration of numerous main biochemical pathways that influence germination, seed dormancy and seed reserve mobilization (Karrer and Rodriguez 1992; Perata et al. 1997; Finkelstein and Lynch 2000). Exogenous carbohydrates are required for *in vitro* orchid seed propagation is almost generally accepted by scientist in this field. In addition, plants that that is able to germinate without carbohydrates shows limited post germination development (Johnson et al. 2011).

On the other hand, apart from using seeds as an explants in orchid micropropogation, the formation of protocorms from germinated seed and the following induction of protocorm-like bodies (PLBs) or callus from the protocorm, stem-node, shoot-tip, leaf, root-tip or root-tuber explants has become a dependable method for propagating orchids (Park et al. 2003; Kosir et al. 2004; Anjum et al. 2006; Kalimuthu et al. 2007; Roy et al. 2007; Hong et al. 2008; Medina et al. 2009). Micropropagation through PLB formation is preferential by commercial growers of most orchid genera due to the numerous PLBs can be obtained within a moderately short period of time (Park et al. 2000; Young et al. 2000; Yam and Arditti 2009). The

large-scale propagation of PLBs can also be achieved by using bioreactor systems (Park et al. 2000). PLBs are also the most common target tissue for genetic transformation studies in orchids since they can proliferate rapidly and have high potential to regenerate into complete plantlets (Liau et al. 2003; Sreeramanan et al. 2008). Additionally, PLBs can also serve as plant material for cryopreservation (Nikishina et al. 2007; Yin and Hong 2009; Khoddamzadeh et al. 2011). PLBs are well-differentiated tissues that are at times regarded as orchid embryos that develop with two discrete bipolar structures, the shoot and root meristem. Therefore, these structures are able to convert to plantlets easily when grown on plant growth regulator (PGR)-free medium. Furthermore, the PLBs directly formed from meristem tissue will exhibit a higher genetic stability than those produced by callus culture (Lee and Phillips 1988).

2.5 Germplasm Conservation

2.5.1 *In situ* conservation method

In situ conservation method is conservation of ecosystem and natural habitats and maintenance and recovery of viable populations of plants in natural surrounding and in the case of domesticated or cultivated species, in the surrounding where they have developed their distinctive properties (UNEP 1992). Even though, this is a primary method of protecting germplasm, however this method is not always sufficient to guarantee survival of certain species (Pence 2010). The *in situ* conservation method includes genetic reserve area and on-farm conservation (Mandal et al. 2000).

2.5.2 *Ex situ* conservation method

Although plant conservation is achieved most effectively through the management of wild population and natural habitats (*in situ* conservation), *ex situ* method can be used to complement *in situ* method and in some instances may be the only choice for some plants (Maunder et al. 1998; Ramsay et al. 2000; Pence 2010). The world's 1600 botanic gardens and arboreta cultivate an estimated 80 000 or more than one third of world's flowering plants (UNEP 1995). Many of these plants were not collected specifically for conservation purpose and do not necessary contain a large number of genetic diversity (Maunder et al. 2001a; Ramanathan Rao and Hodgkin 2002; Pritchard et al. 2004).

Ex situ conservation offers advantages such as germplasm will be easily assessable and there can be constant evaluation of plant grown. The disadvantages is the high cost of establishment and maintenance, plants are exposed to risk of diseases, pests, natural catastrophes and vandalism (Noor et al. 2011). *Ex situ* conservation involves several types inclusive of seed genebank, DNA storage or conservation genetics, field genebank such as botanical garden and in *vitro* conservation involving cryopreservation (Mandal et al. 2000).

Difficult to store germplasm comprising of intermediate and recalcitrant species, orthodox seeds and vegetatively propogated plant species are easily conserved using seed genebank method. This method stored seeds at low moisture content (3 to 7%) and at sub-zero temperatures (-18°C/ -20°C) the most convenient and widely used method of genetic conservation (Mandal et al. 2000; Pritchard 2007).

The conservation genetics defined as an applied science which involves the application of evolutionary and molecular genetics to biodiversity conservation. Generally, IUCN recommends conservation of biological diversity at three levels which are genes, species and ecosystem diversity. Genetics conservation contributes to all three levels. However, the storing of DNA for plant genetic resources is one of the future technologies and requires more research study. The regeneration of an entire plant from DNA cannot be done for now but however DNA could be stored and single or small number of genes can be utilized using biotechnological techniques. The maintenance of an entire genome through DNA storage is still a challenge for scientist (Mandal et al. 2000; Frankham 2010).

The conservation of germplasm in the field genebank involves collecting of plant material and planting in the orchad or field such as botanical gardens situated in a different location. Living collections implemented in botanical gardens has got a long historical record. Majority horticulturally amenable taxa were collected in botanical garden (Cohen et al. 1991; Linington and Pritchard 2001; Maunder et al. 2004). There is a rapid increase in the last two decades in a number of *ex situ* facilities such as botanical gardens contributing to the conservation of endangered plant species or in particular managed for conservation purposes (Gomez-Campo 1985; Maunder et al. 2001b; Wyse Jackson 2001; Smith and Hawtin 2003; Cohrane 2004; Havens et al. 2006). The suggestion of the dynamic role of botanical gardens in conservation of endangered plants has been proposed at the first and second International Congresses for Nature Protection held in 1923 and 1931 has been reviewed by Heywood (1991).

However, reported that due to global and climate change, botanical gardens faces both challenge and prospect (Heywood 2011). Apart from climate change there

are other drawbacks in this method, such as poor genetic management. This is due to the representation of species by only a few individuals, lack of information on accession sampling locality and mislabeling (Hurka 1994). When the numbers of stored accessions for each plant are low and are insufficiently documented, this collection method is extremely at risk of random genetic drift, artificial selection and mutation accumulation. Apart from the, the plant species are also vulnerable to infestation by pathogens (Volis and Blecher 2010).

2.6 Principle of Cryopreservation in Classical Cryopreservation and New Cryopreservation Method

Cryopreservation is known as storing of biological material at ultra-low temperatures normally at the temperature of the liquid nitrogen, LN, (-196°C). Currently this is the only feasible technique for long-term germplasm conservation of vegetatively propagated plant species. At low temperature, all cellular divisions and the metabolic processes of cryopreserved cells are halted. Consequently, the plant materials can be stored as theoretically for unlimited period of time (Bajaj 1995; Towill 1996; Engelmann 2000; Burritt 2008). Cryopreservation technique offers various advantages compared to other conservation methods such as stability of plants phenotypic and genotypic characteristics, minimal storage space and maintenance requirements (Engelmann 1997a; Martinez et al. 1999; Sakai et al. 2000; Gonzalez-Arnoa et al. 2008).

The development of cryopreservation protocol in both classical and new cryopreservation methods for wide range of plants is a difficult challenge in applying the theory to a practical outline by taking into consideration the physiological

complexicty of the species concern and the cryoprotective parameters to ensure high survival. Generally in development of a cryopreservation protocol, several important factors are in concern such as water behaviour, cryoinjury and cryoprotection (Benson et al. 2008; Day et al. 2008).

Water exists in four states such as liquid, glass, solid and vapour. The formation of this state is greatly influenced by temperature. The manipulation of the liquid, glassy and solid (ice) states of water is the central goal of cryopreservation in order to avoid intracellular ice formation (Benson et al. 2008; Day et al. 2008). There are two main approaches in cryopreservation.

The first is generally known as traditional or controlled rate freezing. This requires the control of extracellular ice crystallization (Day et al. 2008). Extracellular ice crystallization is a process described as nucleation by which it is the point at which ice crystals are initiated (Day et al. 2008). In the absence of circumstances that permit water molecules to aggregate, water can supercool to temperatures below zero (Day et al. 2008).

The second approach is generally known as vitrification. The second approach in cryopreservation requires the cell to reach a critically high viscosity such that on contact to freezing temperatures, water forms a vitrified state (Benson et al. 2008; Day et al. 2008). Glasses are highly viscous solidified liquids, amorphous, metastable and non-crystalline, lack of organized structure, thus glasses are less damaging to cells compared to ice (Day et al. 2008). Glass formation also involves thermal changes of which the most significant is the glass transition temperature, (Tg), which is the temperature at which a glass is initiated. Glasses are metastable and their actions in biological tissues is highly complex as water can devitrify and convert back to ice (Wolfe at al. 2002; Block 2003; Benson et al. 2008; Day et al.

2008). Knowledge of the thermal events associated with ice nucleation, melting and the Tg is very useful in developing cryopreservation protocols. Thermal analysis using a differential scanning calorimeter (DSC) may be employed to study and optimize vitrification procedures (Benson et al. 2008; Day et al. 2008).

Tolerance to cryopreservation in plants is dependent upon the ability to overcome cryoinjury. There are two factors were involved which are ice formation and colligative damage in overcoming cryoinjury (Mazur 1965). Ice promotes structural and osmotic damages in cells and in return causes mechanical injury (Fleck et al. 2006). Colligative injury is the extreme concentration of solutes which in return is damaging to cellular function.

Successful cryopreservation protocols are dependent upon the use of cryoprotective strategies and cooling rates applied in the cryopreservation protocols (Fahy et al. 2004; Fuller 2004; Volk and Walter 2006; Benson 2008). There are two main types of chemical cryoprotectants which are penetrating and non-penetrating by which they are frequently used in combination.

Traditional controlled rate cooling protocols uses penetrating cryoprotectants such as dimethyl sulphoxide (DMSO) or glycerol, even though their permeabilities differ among cell types. As cells are exposed to controlled cooling, for an example, −1°C/min ice forms extracellularly causing the removal of intracellular water (Meryman et al. 1977; Mazur 1984; Fahy et al. 2004; Fuller 2004; Volk and Walter 2006; Benson 2008). This is advantageous as it reduces the amount of water available for initiating lethal ice crystals. It is essential to induce extracellular ice nucleation as from this point onwards there is control over the excursion of water. Furthermore, when the water content is removed, the freezing point becomes increasingly depressed allowing more time for water to exit the cell (Meryman et al.

1977; Mazur 1984; Fahy et al. 2004; Fuller 2004; Volk and Walter 2006; Benson 2008). However, balance should be achieved by which the dehydration does not result in excessive, deleterious concentration of solutes and this is where colligative cryoprotectants plays it role. These chemicals penetrate cells and act as cellular solvents as they protect against the detrimental concentration of solutes that occurs as water is lost from the cell and obviates the deleterious and potentially lethal reduction in cell volume. Following these treatments, when cells are finally exposed to terminal freezing temperatures and liquid nitrogen, the intracellular solution becomes too concentrated that it becomes vitrified. On the other hand, if some water remains available for nucleation, the ice crystals formed are so small that they are harmless (Meryman et al. 1977; Mazur 1984; Fahy et al. 2004; Fuller 2004; Volk and Walter 2006; Benson 2008).

When slow to moderate cooling rates are applied, ice usually nucleates extracellularly causing a vapor pressure deficit between the outside and inside of the cell and retains osmotic equilibrium as water passes out of the cells. Rates of water loss are determined by cooling rate, the dynamics of extracellular ice nucleation and cryoprotection. Mathematical equations and theoretical models of thermodynamic behavior and membrane permeability (Rubinsky and Pegg 1988; Chuenkhum and Cui 2006) have very important applications in optimizing controlled rate cooling and cryoprotective protocols for animal and human cells (Gilmore et al. 1995). However, their application in plant, algal and microbial cells are difficult due to the presence of cell walls, extracellular mucilage and other components.

Cryopreservation using ultra rapid rates of cooling usually involves a cryoprotective approach which allows cells and tissues to be directly plunged into the liquid nitrogen. This approach normally requires the formation and stabilization of a

vitrified state by which is achieved by attaining a critically high cell viscosity. This state is achieved by osmotic removal of water or the addition of highly concentrated penetrating and non-penetrating cryoprotectants (Benson and Bremner 2004; Fuller 2004). Non-penetrating cryoprotectants have multiple protective roles whereby they depress the freezing point, cause osmotic dehydration and reduce the amount of water available for freezing. Non-penetrating cryoprotectants may also impair ice nucleation by restricting the molecular mobility of water molecules extracellularly (Fahy et al. 2004; Fuller 2004; Volk and Walter 2006; Benson 2008).

Cryopreservation by this method is quite different to controlled rate cooling by which it uses the protective strategy for the formation of extracellular ice to initiate the process of freeze-dehydration. Vitrified systems comprise of two types, which are partially vitrified by which the solution external to the cell forms ice while the intracellular component remains ice free or totally vitrified by which both the external and intracellular compartments become vitrified and in return this cryopreservation procedure is in the absence of ice (Benson and Bremner 2004; Fuller 2004). Cryoprotectants also have got additional physical protective properties such as stabilizes proteins and membranes and act as antioxidants in order to protects against cryoinjury (Benson and Bremner 2004; Fuller 2004).

Rewarming of cryopreserved samples in both cryopreservation methods also requires stringent optimization as the formation of small innocous ice crystals are capable of growing to a size that may cause injury. Likewise, relaxation of glasses during rewarming can rupture fragile and rigid cell structures or they can devitrify and form ice if they are rewarmed too slowly (Pegg 2007; Day et al. 2008).

By applying these general principles of cryobiological theory, it is now possible to consider the development of different types of cryopreservation protocols in order to conserve a particular biological resource (Day et al. 2008).

2.7. Classical Cryopreservation

Classical Cryopreservation protocols is based on regulating cooling and manipulating the colligative cryoprotectants followed by cooling steps intercepted by the manual, automatic or passive induction of ice nucleation. Survival can be improved by preculture and cold hardening treatments applied before cryopreservation by which it usually simulates natural acclimation responses.

For example, culturing algal cells at lower than normal growth temperatures (Morris 1976) or applying biochemical additives to enhance stress tolerance in plants (Luo and Reed 1997). Post-storage survival in mammalian and human cells can be improved by adding antioxidant supplements to the freezing media (Fuller 2004; Marco-Jiménez et al. 2006). Modifications to post-storage culture can also significantly enhance the level and rate of recovery (Day and Brand 2005). Morris et al. (1999) demonstrated that human spermatozoa can be successfully cryopreserved by manipulating the cooling regime to provide non-linear changes in temperature with time over critical temperatures. Classical cryopreservation techniques have been successfully applied to undifferentiated culture systems such as cell suspensions and calluses (Kartha and Engelmann 1994; Withers and Engelmann 1998) and apices of cold-tolerant species (Reed and Uchendu 2008).

Factors critical to controlled cooling methods are cryoprotectant composition in which a penetrating colligative additive must be included as well as cooling

regime. Different cell types have specific optimal cooling rates that are largely determined by balancing the two components. Computerized, controlled rate programmable freezers provides the most reliable means of controlling cooling parameters which are the loading temperature, the first cooling ramp, temperature of initiating ice nucleation with an optional hold, a second or third optional cooling ramp, an intermediate terminal transfer temperature at or near the point of homogeneous ice nucleation, a hold, transfer to liquid nitrogen, storage in liquid or vapor phase liquid nitrogen and rapid rewarming. Computerized programmable freezers have the advantage of providing data outputs allowing each run to be documented for protocol development and quality control purposes (Mazur 1965).

2.8 New Method based on Vitrification

2.8.1 Vitrification

At the glass transition temperature (Tg) molecular motion ceases and a liquid becomes a glassy solid. In controlled rate cooling it is possible that on exposure to freezing temperatures the intracellular viscosity of the cell is sufficiently high that cells become vitrified inside, while remaining frozen extracellularly. This is a partially vitrified system and although the glassy state may account in part for survival, ice nucleation is still required to evoke osmotic, freeze-induced dehydration. Ice-free cryopreservation, initially pioneered for animal cells (Fahy et al. 1984) and applied to plants (Sakai 2004) and algae (Harding et al. 2004) involves a very different cryoprotective strategy such that ice formation is inhibited both inside and outside the cell, resulting in total vitrification. The glassy state offers great

benefits for larger and structurally complex heterogeneous tissues for which it is difficult to optimize colligative cryoprotection and cooling rates.

Vitrification does not require controlled cooling and the need for programmable freezing apparatus is circumvented making cryopreservation more amenable to researchers from non-specialist laboratories. However, as glass formation usually requires the excessive concentration of solutes and cells must be able to tolerate dehydration. Pathways to achieve vitrified and frozen states are frequently interdependent and increasingly protocols are being developed that incorporates component of colligative cryoprotection and vitrification procedures (Benson 2008)**.** The conventional approach to vitrification developed for human and animal systems uses mixtures of traditional liquid cryoprotectants such as DMSO, glycerol, ethylene glycol, polyols and sugars at high concentrations. Those that penetrate the cell cumulatively increase solute concentration while non-penetrating additives act synergistically by withdrawing water osmotically. Their combined effect enhances the overall viscosity of the cell.

Exposing cells to high concentrations of cryoprotective additives can be injurious and strategies have been devised to reduce their toxicity. These include their sequential treatment such as preculture, loading and unloading treatments to avoid osmotic shock during the application at chilling temperatures. The steps during cryopreservation by vitrification method includes preculture, loading, PVS2 treatment, liquid nitrogen, thawing and unloading treatments (Plate 2.2).

A preculture treatment with sugar at a lower concentration has proved to considerably to increase dehydration tolerance and to lessen the mechanical stress caused by the subsequent treatment with highly concentrated plant vitrification solutions (Gonzalez Arnao et al. 2008). The loading treatment with mixture of 2M

glycerol supplemented with 0.4M sucrose in liquid media (Nishizawa et al. 1993) applied for 20 min at room temperature is very effective to enhance osmotolerance (Sakai 2004). Among all the vitrification solutions, of which plant vitrification solution number 2 (PVS2) is the most widely used, have been applied to many different types of plant genetic resources (Sakai 2004; Gonzalez Arnao et al. 2008). PVS2 was developed by Sakai et al. (1990) which contains (30% glycerol [w/v], 15% ethylene glycol [w/v], 15% DMSO [w/v]) in liquid media supplemented with 0.4M sucrose. Direct contact of samples to any vitrification solution frequently leads to harmful effects due to the toxicity caused by their high concentration, approximately more than 7M for PVS2 (Gonzalez Arnao et al. 2008). The PVS2 treatment will be followed by immersion into the liquid nitrogen followed by rapid rewarming and unloading steps to remove cryoprotectants prior to growth recovery of the plant involves (Sakai 2004).

Vitrification has been developed for cryopreservation of cell suspension, somatic embryo and apices of several species (Sakai 1993). This procedure has been developed for apices, cell suspensions and somatic of numerous different species (Sakai and Engelmann 2007; Sakai et al. 2008). In addition, cryopreservation by vitrification was successful in a number of plants such as somatic embryos and shoot tips of asparagus (Uragami et al. 1989), shoot tips of cassava (Charoensub et al. 1999), *Dendrobium* sonia-28 (Hooi et al. 2010) and white mulberry (Padro et al. 2011).

The advantage in this method is no controlled freezing apparatus required. However, due the concentrated cryoprotective solution are highly toxic, therefore the duration of the treatment and the dilution of the cryoprotectant should be optimized prior to protocol development. This techniques is however far more easy to use in

handling large number of materials (Villalobos and Engelmann 1995). However, this technique has several disadvantages such as vitrification solutions are toxic to many plants and consequently cellular damages are possible to occur. Thus, it requires careful timing in solution changes (Dixit et al. 2004).