CRYOPRESERVATION OF *Oncidium* Golden Anniversary's PROTOCORM-LIKE BODIES USING ENCAPSULATION-DEHYDRATION METHOD

SUHANA ZAKARIA

UNIVERSITI SAINS MALAYSIA

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by

SUHANA ZAKARIA

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LIST OF ACRONYMS AND ABBREVIATIONS

%	Percentage
(w/v)	Weight per volume
+LN	Cryopreserved
°C	Degree Celcius
µg/g	Micro gram per gram
µM	Micromolar
ABA	Abscisic acid
APX	Ascorbate peroxidase
ASH	Ascorbic acid
BAP	6-Benzylaminopurine
bp	Base pair
$CaCl_2$	Calcium chloride
CAT	Catalase
DAMD	Directed Amplification of Minisatellite Region DNA
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
EG	Ethylene glycol
g/L	Gram per liter
GB	Glycine betaine
GSH	Glutathione
GSH	Glutathione
H_2O_2	Hydrogen peroxide
ISSR	Inter Simple Sequence Repeat
ISTA	International Seed Testing Organisation
LA	Lipoic acid
-LN	Non-cryopreserved
lTCL	Longitudal Thin Cell Layers
mg/L	Miligram per liter

mM	Milimolar
mm	Milimeter
NAA	1-Naphthaleneacetic acid
NADP+	Nicotinamide adenine dinucleotide phosphate
nm	Nanometer
O_2●	Superoxide radicals
^oC/min	Degree celcius per minute
OD	Optical Density
OH●	Hydroxyl radicals
PCR	Polymerase chain reaction
PLBs	Protocorm-like Bodies
POX	Peroxidase
PVP	Polyvinylpyrrolidone
PVS	Plant Vitrification Solution
RAPD	Random Amplification of Polymorphic DNA
ROS	Reactive oxygen species
rpm	Rotation per minute
SE	Standard Error
SEM	Scanning Electron Microscope
SOD	Superoxide dismutase
TCLs	Thin cell layers
TEM	Transmission Electron Microscope
Tm	Temperature
TTC	2,3,5-triphenyl tetrazolium chloride
tTCL	Transverse thin cell layers
USD	United States Dollar
µmol/g	Micro mol per gram
$\mu mol.m^{-2}.s^{-1}$	Micromole per metre square per second
$mM^{-1}cm^{-1}$	Milimolar per centimeter

m^2s^{-1}	Metre square per second
UmL^{-1}	Unit per mililiter
1^o	Primary
$^1O_2$	Singlet oxygen
2^o	Secondary

KRIOAWETAN JASAD SEPERTI PROTOKOM *Oncidium* Golden Anniversary MENGGUNAKAN KAEDAH PENGKAPSULAN-PENDEHIDRATAN

ABSTRAK

Oncidium Golden Anniversary ialah hibrid orkid yang mempunyai nilai ekonomi yang tinggi dalam pasaran bunga di seluruh dunia. Walau bagaimanapun, orkid hibrid ini sukar untuk dijaga dan ditanam secara konvensional, menyebabkan meningkatnya risiko kehilangan kekal. Oleh itu, teknik krioawetan telah dibangunkan untuk pemuliharaan orkid secara jangka masa panjang. Kajian ini bertujuan untuk menghasilkan percambahan jasad seperti protokom (PLB) dan membangunkan kaedah pengkapsulan-pendehidratan untuk krioawetan hibrid orkid *Oncidium* Golden Anniversary. Media yang terbaik untuk percambahan pesat PLB adalah di dalam media MS dengan setengah kekuatan separa pepejal yang ditambah dengan 20 g/L sukrosa dan 1 mg/L BAP dengan menggunakan sama ada keseluruhan PLB, separuh bulatan PLB dan lapisan sel nipis melintang (tTCL) PLB sebagai eksplan. PLB yang diperolehi digunakan sebagai bahan permulaan dalam kaedah pengkapsulan-pendehidratan dan dianalisa dengan menggunakan ujian spektrofotometri 2,3,5-triphenyltetrazolium klorida (TTC) dan peratusan kemandirian. Beberapa pemerhatian penting yang telah dinilai adalah saiz PLB, keadaan pra-kultur, media natrium alginat, larutan kalsium klorida, tempoh penghidratan dan penambahan antioksidan. Kemandirian yang maksimum pada kadar 93.3% diperoleh apabila saiz PLB 3-4 mm diprakulturkan di atas media MS setengah kekuatan ditambah dengan 0.3 M sukrosa dan 0.1 µM melatonin selama 1 hari, dikapsulkan dengan 3% natrium alginat yang mengandungi 0.4M sukrosa,

dipolimerkan dalam larutan 100mM kalsium klorida yang mengandungi 0.056M sukrosa, didehidratkan menggunakan silika gel selama 4 jam sebelum disimpan di dalam cecair nitrogen selama 1 jam. PLB yang telah dikrioawetkan dinyahbekukan dengan cepat pada suhu 40±2°C selama 90 saat dan dikulturkan di atas media penjanaan semula MS setengah kekuatan tanpa antioksidan. Dalam usaha untuk mendedahkan kerosakan yang terhasil daripada rawatan krioawetan, analisa biokimia, mikroskopik dan molekul ditentukan terhadap PLB yang dikrioawetkan dan tidak dikrioawetkan serta dibandingkan dengan PLB yang tidak dirawat. Analisa biokimia menunjukkan ketidakstabilan aktiviti enzim antioksidan (CAT, APX, POX) di dalam PLB semasa pelbagai tahap krioawetan yang menunjukkan keputusan yang tidak konsisten dan kemandirian yang rendah selepas krioawetan. Sementara itu, penurunan kandungan klorofil, karotenoid dan porphyrin menunjukkan penurunan proses fotosintesis di dalam sel. Analisa histologi menandakan faktor utama yang mempengaruhi kebolehhidupan PLB adalah tahap plasmolisis di dalam PLB yang disebabkan oleh penghidratan dan kerosakan sel selepas pembekuan. Analisa daripada pengimbasan mikroskop elektron mendedahkan saiz PLB yang dikrioawetkan dan tidak dikrioawetkan mengecut sementara kajian ultrastruktur daripada kaedah pengkapsulan-pendehidratan yang optima menunjukkan PLB yang dikrioawetkan mengalami kerosakan yang menyebabkan tiada pertumbuhan sel selepas pembekuan. Akhir sekali, analisa DAMD dan ISSR membuktikan kewujudan polimorfisma sebanyak 7.4% dan 7.32%, masing-masing di dalam PLB yang dikrioawetkan. Pemerhatian ini menunjukkan bahawa beberapa kajian lanjut perlu dilakukan untuk meningkatkan lagi pertumbuhan semula PLB yang telah dikrioawetkan.

CRYOPRESERVATION OF *Oncidium* Golden Anniversary's PROTOCORM-LIKE BODIES USING ENCAPSULATION-DEHYDRATION METHOD

ABSTRACT

Oncidium Golden Anniversary is an orchid hybrid that has high economic value in flower market worldwide. However, this new orchid hybrid is difficult to maintain and cultivate conventionally thus increase the risk of permanent loss. Therefore, cryopreservation technique has been developed for long-term conservation of the orchids. This study was aimed to establish the proliferation of protocorm-like bodies (PLBs) and to develop encapsulation-dehydration method for cryopreservation of *Oncidium* Golden Anniversary hybrid orchid. The best media for rapid proliferation of PLBs was in half-strength semi-solid MS media supplemented with 20 g/L sucrose and 1 mg/L BAP using either whole PLBs, half-moon PLBs and transverse thin cell layer (tTCL) PLB as an explant. The PLBs produced were used as starting material in encapsulation-dehydration method and analysed by 2,3,5-triphenyltetrazolium chloride (TTC) spectrophotometry test and survivability percentage. Some important parameters have been assessed were PLB size, preculture condition, sodium alginate media, calcium chloride solution, dehydration duration, and the addition of antioxidants. The maximum survivability with 93.3% was obtained when 3-4mm PLB size was precultured on half-strength MS media supplemented with 0.3M sucrose and 0.1µM melatonin for 1 day, encapsulated with 3% sodium alginate contained 0.4M sucrose, polymerized in 100mM calcium chloride solution consisting of 0.056M sucrose, dehydrated using silica gel for 4 hours before stored for 1 hour in liquid nitrogen. The cryopreserved PLBs were

thawing rapidly at 40±2°C for 90 seconds and cultured on half-strength MS regrowth media without antioxidants. In order to reveal the damages produced by cryopreservation treatments, biochemical, microscopic and molecular analyses were determined in both cryopreserved and non-cryopreserved PLBs comparing with untreated PLBs. Biochemical analyses showed the fluctuation of antioxidant enzyme activities (CAT, APX, POX) in PLBs at various cryopreservation stages explained the inconsistent outcomes and low survivability post-cryopreservation. Meanwhile, reduction of chlorophyll, carotenoid and porphyrin contents resulted in a reduction of photosynthesis process in the cells. Histological analysis indicated that the main factors affecting the viability of PLBs were a degree of plasmolysis in PLBs due to dehydration and rupture of cells after freezing. Scanning electron microscopy analysis revealed that cryopreserved and non-cryopreserved PLBs shrinking in size while ultrastructural studies of optimized encapsulation-dehydration method show cryopreserved PLBs cells were suffered from damages that resulted in no growth of cells after freezing. Finally, DAMD and ISSR analyses confirmed the occurrence of 7.4% and 7.32% polymorphism, respectively in the cryopreserved PLBs. This research indicates that some further study need to be done to improve regrowth of cryopreserved PLBs.

CHAPTER 1

INTRODUCTION

The Orchidaceae is one of the largest and most diverse families of the plants are well known for their attractive colours and unique flower shapes among angiosperms. They are exclusive because of exotic floral patterning with distinct organ identity and structures. Orchids are an economically important horticultural commodity in countries such as Malaysia, Thailand, Singapore, and Australia (Chugh et al., 2009). Orchids have high demand either by potted floriculture or cut flowers because of their exotic beauty and long time span of usability.

One of the famous orchids is from *Oncidium* genus that have over 400 species belongs to the subfamily Epidendroideae of the orchid family (Orchidaceae) (Chase et al., 2009). Common name for *Oncidium* orchid is "Dancing Ladies" for their distinctive floral design as it looks like ladies are dancing by wearing a dress. It has bright yellow flowers with red brown markings on the petals borne in shower of hundred on each spray (Mahmood et al., 2011). Malaysia and Taiwan become the major *Oncidium* producers in the world including hybrid orchids (Shen & Chen, 2012). *Oncidium* Golden Anniversary orchid is a hybrid between *Oncidium sphacelatum* and *Oncidium sarcatum.* Each spray consists of hundred yellow flowers that have red-brown spots on the petals. The flowers are slightly bigger but lesser in quantity compared to common *Oncidium* flower such as "Golden Shower". This orchid hybrid has been known to be grown as cut flower, potted plant and is valued for its attractive flower (TH, 2006). In addition, the development of the novel and hybrid orchid varieties has been rapidly growing (Sim et al., 2007). The importance of orchid hybrids is due to reasons involving better quality of plant, free-blooming,

varieties in shapes and colours and longer shelf-life (Kishor et al., 2006). Therefore, tissue culture method has been commercially exploited by growers to produce rare and hybrid orchid in mass production and at the same time to fulfil the orchid demand.

Generally, new orchid hybrids are difficult to maintain and cultivated conventionally (Kishor et al., 2006). This situation has raise the importance of conserving the hybrids by developing an efficient method that can preserve and safeguard the plants' genetic resources to ensure a continuous supply of the hybrid to meet consumer demand and at the same time conserve orchid plants that currently endangered and nearing extinction (Kulus & Zalewska, 2014). Orchid hybrids extinction will increase the risk of a permanent lost (Teixeira da Silva et al., 2015). Therefore, various cryopreservation technology has been developed for long-term conservation of orchids either hybrids or nearing extinction orchid species.

Cryopreservation refers to the storage of biological tissues in liquid nitrogen (-196°C) in which all the activities including biochemical, metabolic and cell division are terminated. Therefore plant material can be stored for unlimited periods of time. This technique only need limited space and very limited maintenance but cause no changes in viability, vigour and genetic makeup of the conserved materials (Ogbu et al., 2010). Cryopreservation is suitable for wide range of plant species and the only technique ensuring the safe and cost-effective for long-term conservation (Cruz-Cruz et al., 2013). Few types of cryopreservation methods have been applied to orchids such as encapsulation-dehydration, encapsulation-vitrification, vitrification, and droplet vitrification (Reed, 2007).

Compared to other methods, encapsulation-dehydration method is simple and easier to manipulate the samples by alginate embedding storage of large number of delicate explants. Moreover, this method provides a direct protection of explant during dehydration, produce higher regrowth of cryopreserved explants, and need a very simple freeze-thawing process (Paul et al., 2000). This method does not utilize the use of toxic cryoprotectants such as dimethylsulphoxide (DMSO) and ethylene glycol which are detrimental to explant and can affect their survivability post thawing (Hirata et al., 1998). The presence of nutritive matrix surrounding the explants can provide a nutrient to the explants and promote regrowth after thawing (Panis & Lambardi, 2005).

Successful of cryopreservation technique depends on few factors such as physiological status of plant materials, preculture step, dehydration step, thawing process and growth recovery condition (Engelmann, 2004). All these factors have to be optimized in order to minimize cellular damage as well as intracellular and extracellular ice crystal formation that caused by sequential treatments in cryopreservation technique. Higher amount of osmotic and chemical stresses resulting in changes of cells ultrastructural, morphological, biochemical and genetic variations in cryopreserved material (Larkin & Scowcroft, 1981; Harding, 2004; Benson, 2008). The changes occurred resulting in stresses from excision, osmotic injury, desiccation, freezing and thawing stages lead to the production of reactive oxygen species (ROS). ROS-induces stress is harmful to cells and can cause in oxidative damage in plant systems and cell death in cryopreservation samples (Uchendu et al., 2010a).

Plant possess complex biochemical activities to counteract external stress include antioxidants enzyme such as catalase (CAT), peroxidase (POX), and

superoxide dismutase (SOD), and non-enzymatic antioxidants like ascorbic acid (ASH) and reduced glutathione (GSH), carotenoids and flavonoids (Chen et al., 2014). By understanding the plant defence mechanism in response to various stress emerged from cryopreservation treatments, this will help to improve growth recovery of plant cell that may enhance success of cryopreservation (Ren et al., 2013). Besides, freezing at very low temperature will cause severe structural damage to cells, as well as membrane rupture that caused by ice crystallization, loss of enzyme activities and membrane denaturation and cell wall degradation (Aponte & Tamayo, 2017). One way to screen the impact of each step of the cryopreservation method is by ultrastructural study of cells. The understanding of cell ultrastructure during cryopreservation could help to improve regrowth and reduce degree of cellular injuries in the cells (Miao et al., 2005).

In different circumstances, cryopreservation could also induce somaclonal variations in cryopreserved samples (Harding, 2004). Variation in plant morphology, number of chromosomes, accumulation of gene mutations, level of gene expression in ribonucleic acid, protein profiles and molecular changes in DNA sequences should be avoided in cryopreservation in order to conserve true-to-type plant (Larkin and Scowcroft, 1981). Thus, genetic stability evaluation using molecular markers such as Directed Amplification of Minisatelite DNA (DAMD) and Inter Simple Sequence Repeats (ISSR) could be used as successful genetic studies methods in cryopreservation (Mehrotra et al., 2012).

The current study involved development of cryopreservation technique by using encapsulation-dehydration method for conservation of phenotypic and genotypic features of *Oncidium* Golden Anniversary orchid, supported with biochemical analyses, ultrastructural studies and genetic stability evaluations. Till

date, no research has been conducted on the development of cryopreservation method for this orchid hybrid.

1.1 Research objectives

The objectives of the research were:

i. To improve the proliferation of protocorm-like bodies (PLBs) of *Oncidium* Golden Anniversary by optimizing MS medium and using thin cell layers (TCLs) technique.

ii. To optimize cryopreservation condition in *Oncidium* Golden Anniversary orchid using encapsulation-dehydration method.

iii. To analyse biochemical changes in *Oncidium* Golden Anniversary PLBs at different cryopreservation stages by using different types of biochemical analyses.

iv. To investigate the cell morphology of PLBs at different cryopreservation stages through histological, scanning electron microscope (SEM) and transmission electron microscope (TEM) analyses.

v. To assess the genetic stability of cryopreserved and non-cryopreserved PLBs compared to the stock culture PLBs through Direct Amplification of Minisatellite DNA regions (DAMD) and Inter Simple Sequence Repeat (ISSR) DNA molecular analyses.

CHAPTER 2

LITERATURE REVIEW

2.1 Orchid

The Orchidaceae is one of the largest families of flowering plants that have more than 800 genera and 25,000 to 35,000 species. The ease of hybridization means more than 100,000 hybrids has been created which more than any other floricultural crop (Khoddamzadeh et al., 2011a). It being the most economically significant world cut flower and pot plants with their beauty designated has enchanted and fascinated people. Several countries like Thailand, Australia, Singapore and Malaysia involve in this million dollar horticulture industry (Chugh et al., 2009). In 2005, Malaysia was ranked as the third world orchid-producing countries behind Thailand and Singapore with an export earnings amounting to USD 13 million annually (Shen & Chen, 2012). Regarding to Hank (2015), Malaysia was a fourth top countries that exporting cut orchids to European Union countries including United Kingdom.

Orchids are grown as ornamentals and appreciated for their beautiful long shelf life and long lasting flowers. Orchids also display an incredible range of diversity in shape, size and colour that produce exotic, attractive and beautiful flowers (Saiprasad & Polisetty, 2003; Chugh et al., 2009). Normally, orchids often used as indoor and outdoor landscape to generate a special effect of exotic surroundings, feeling of luxury and unique environment (Dalayap et al., 2011).

Orchids have the typical flowers of monocotyledonous plants, which consist of four whorls of floral organs such as petaloid sepals, petals, anthers and pistils (Goh & Arditti, 1985). They are exclusive because have exotic floral patterning with distinct organ identity and structures to distinguish from other flowering plants.

Orchids have only one functional stamen, fused with styles and stigmas into a structure called the column or gynostemium. They also have pollen grains that bound together by viscin threads in masses and packaged as a pollinium for effective pollination. The median petal located opposite to a column act as a landing platform for insect pollinators is often modified to form a gaudy and variable labellum or lip. These special features assist pollinators to move pollen grains from one flower to another, contributing to the success of the Orchidiaceae during the evolutionary development of flowering plants (Yu & Xu, 2007).

Besides famous in ornamental industries, orchids also used as food (tubers of *Cynorchis* and *Eulophia*), spice (vanilla) and herbal medicines by many different cultures and tribes (Arditti, 1992). Several species that termed as jewel orchids and belong to genera *Anoectochilus, Goodyera, Ludisia* and *Macodes* are grown for their beautiful foliage (Chugh et al., 2009).

2.2 *Oncidium* orchid

2.2.1 Botany

Oncidium genus with over 400 species belongs to the subtribe Oncidiinae and subfamily Epidendroideae of the orchid family (Orchidaceae). *Oncidium* are sympodial and have little in common with each other within genera about their characteristics flower shape. *Oncidium* is one of the four best known orchids of the worlds besides *Dendrobium, Cattleya and Phalaenopsis* because of high horticultural value flowers (Wang et al., 2007a). Shen and Chen (2012) reported that Malaysia and Taiwan were the major *Oncidium* producers in the world. This orchid become economically important crop and is cultivated for cut flowers and potted plant (Mengxi et al., 2011). A common name for *Oncidium* is “Dancing Ladies” for their

distinctive floral design as it looks like ladies are dancing by wearing a dress. It has bright yellow flowers with red brown markings on the petals borne in shower of hundred on each spray (Mahmood et al., 2011).

Oncidium is a sympodial orchid in which the apical meristem is terminated and growth is continued by one or more lateral meristems. This orchid has pseudobulbs that store water thus can survive for prolonged periods without water until the medium dries out. New pseudobulbs will grow directly from previous pseudobulbs and multiple growth lead a single horizontal stem called rhizome. Roots will grow from the rhizome. This orchid grows in bright lights but not under direct sun to avoid scorching of the leaves. Under natural conditions, it needs about 3 years to reach sexual maturity and its flowering being precisely regulated by temperature (Chase et al., 2009). This orchid need to be maintained under light intensity below 300 mol $m^{-2}s^{-1}$ and the adequate temperature ranges from 22 to 32^{o}C for their optimal growth (Shen & Chen, 2012).

2.2.2 *Oncidium* Golden Anniversary orchid

Oncidium Golden Anniversary (Plate 2.1) is a hybrid between *Oncidium sphacelatum* and *Oncidium sarcatum* was registered in January 1979 by Corbet. It has bright yellow flowers with red-brown markings on the petals borne in shower of hundred on each spray and can last for about a month. The flowers are slightly bigger but lesser in quantity compared to common *Oncidium* flower such as "Golden Shower". This orchid hybrid have been known to be grown as cut flower, potted plant and is valued for its attractive flower (TH, 2006).

Numerous novel orchid cultivars have been formed by interspecific and intergeneric hybridization to produce elegant and exotic flowers. Orchids hybrids

Plate 2.1: *Oncidium* Golden Anniversary orchid. Bar represent 1cm.

have a beautiful foliage, shiny, multicoloured and decorated with prominent veins in pattern thus making it as a consumer preference (Antony et al., 2014a). Therefore, it is important and practical value for the orchid industry to generate hybrids with improved floral characters (Yu & Xu, 2007). Since this hybrid only common with growers, it needs to be conserving to maintain the orchid hybrid. Otherwise, any form of threats such as habitat destruction, climate change, or by unsustainable harvest for horticulture, food or medicine (Fay, 2018) in future could cause a total loss of this hybrid. Thus this explains the importance to conserve aesthetic hybrid such as *Oncidium* Golden Anniversary orchid through effective cryopreservation method.

2.3 Plant Tissue Culture Technology

2.3.1 Micropropagation of orchids

Plant tissue culture technology is used to regenerate and propagate complete plants from growing isolated plant cells, tissues and organs under control conditions. It depends on the situation of cell totipotency, in which single cell have ability to divide, to produce all the differentiated cells characteristic and to regenerate into a whole plant (Iliev et al., 2010). Growing plant in *in vitro* condition, with knowledge of plant material and culture condition will confirm clonal propagation of genetically superior genotypes of important plant. The development of efficient micropropagation protocol is beneficial in accelerating plant production, producing more uniform plants and reducing labour costs (Jheng et al., 2006). Furthermore, tissue culture techniques offer a safe mean to internationally exchange plant material, allow supply of valuable material for wild population recovery and enable establishment of extensive collections using minimum space (Cruz-Cruz et al., 2013).

Micropropagation has become an important part of many plants because its advantages in multiplication system. This technique is found to be more effective than sexual propagation and other vegetative method (Talukder et al., 2003). Conventional vegetative propagation techniques are time consuming and expensive. On the other hands, propagation of orchids by seed is undesirable due to the heterozygosity of seed, presence of reduced endosperm, minute seed size, and requirement of an association with mycorrhizal fungi (Saiprasad & Polisetty, 2003; Sungkumlong & Deb, 2008). Therefore, tissue culture techniques provide a solution for producing large number of propagules within a limited period of time especially for slow growing plant species like orchid (Puchooa, 2004). Jheng et al. (2006) successfully enhance growth and regeneration of *Oncidium* Grawer Ramsey by establishment of embryogenic callus cultures. Approximately, 6,000 PLBs could be generated from initial culture of 1g callus within 2 months, subsequently developed into plants in 4 month after two subcultures.

Tissue culture also used in studying the regulation of growth and organized development through investigation of structural, physiological, biochemical and molecular bases underlying developmental processes (Cangahuala-Inocente et al., 2004; Barraco et al., 2014; Li et al., 2014). Histological observation of *Oncidium* Grower Ramsey provide a platform to researchers to study the effect of media composition in enhancing PLBs growth and at the same time showed the suitable callus line used for transformation to produce genetically uniform and stable transgenic plants (Jheng et al., 2006). Besides, ultrastructural analysis provides useful information of cellular response towards cryopreservation in order to investigate cellular process in D*endrobium* PLBs (Antony et al., 2014b).

Moreover, tissue culture technique has been applied in the conservation and development of rare orchids, cut flowers, medicinal plants and forest species (Arditti & Ernsnt, 1993). *In vitro* conservation is essential for vegetatively propagated and for non-orthodox seed plant species either for short-, medium- and long-term conservation that allow the conservation of pathogen-free material, elite plants and genetic diversity (Cruz-Cruz et al., 2013). Conservation and availability of genetic resources provide basic support for breeding novel cultivars either by conventional or genetic engineering programs. Thus, long-term conservation of plant genetic resources such as cryopreservation has long been considered since this technique have minimal requirement for storage space, and maintenance of genetic integrity of stored materials (Benson, 2008). For example, Bustam et al. (2015) conserved endangered terrestrial orchid, *Caladenia latifolia,* by development of cryopreservation for primary (seed generated) and secondary (adventitious) protocorms.

The success of tissue culture technology depends on the culture media and plant materials used. The culture media must supply all the essential nutrients for plant growth. It contains macronutrients, micronutrients, vitamins, iron, carbon sources, plant growth regulators (PGRs) and gelling agents (if solid). The composition of the culture media depends upon plant species, the explants and the aim of the experiments (Iliev et al., 2010). In general, some modification may require achieving genotype-specific by manipulating the concentration of growth regulators or by addition of specific components to the culture medium. Hong et al. (2008) studied on the promotion of direct somatic embryogenesis of *Oncidium* by adjusting five types of carbon sources, and found that 30 g/L of sucrose and 20g/L was the optimum carbon requirement for *Oncidium* Gower Ramsey and *Oncidium* Sweet

Sugar, respectively. Plant growth processes and plants adaptation to the environment are influence by plant growth regulators. The most important PGRs in plant growth are auxin and cytokinin but it depends on plant species. For example, asymbiotic germination of *Chloraea crispa orchid responded well in* Van Waes medium supplemented with 0.1 mg/L of 6-benzylaminopurine (BAP) (Quiroz et al., 2017). Direct regeneration of PLBs from leaf apices of *Oncidium flexuosum Sims* (Orchidaceae) obtained when cultured in a medium containing 1.5µM TDZ under dark condition (Mayer et al., 2010) while the suitable media for rapid micropropagation of Vu Nu orchid (*Oncidium* sp.) was on MS medium supplemented with 0.75 mg/L NAA (Minh et al., 2017).

Selection of suitable starting materials also contributes to the success of micropropagation. Chen and Chang (2000) reviewed that different parts of plant such as flower stalk internodes, leaves, roots, stems and protocorm-like bodies (PLBs) have been commonly used as an explant in orchid micropropagation. However, micropropagation through induction of PLBs is considered as the efficient method of propagation because it can result in rapid proliferation in which large number of PLBs can be produced in a short period of time (Mengxi et al., 2010; Sopalun et al., 2010; Ferreira et al., 2011; Bustam et al., 2015; Romero et al., 2018). Yang et al. (2010) demonstrated on the production of PLBs of *Oncidium* 'Sugar Sweet' with bioreactor and regeneration through micropropagation to produce mass propagules of the orchid.

2.3.2 Protocorm-like bodies (PLBs)

Protocorm-like bodies (PLBs) are well-differentiated tissues that can develop to two distinct bipolar structures known as shoot and root meristem. Consequently, these structures are able to convert to plantlets when grown on suitable nutrient medium (Piria et al., 2008; Ng & Saleh, 2011). Arditti and Ernst (1993) described that protocorm-like bodies are explant such as the tissues or callus of orchids form structures similar to protocorms *in vitro*. PLBs on the other hand are structures similar to protocorm but originating from vegetative tissues (Gantait et al., 2012a). The process of direct and indirect embryogenesis in orchids occurs throughout the formation of PLBs.

PLBs are well-differentiated tissues that sometimes regarded as transitional structures connecting embryo and plant of orchid (Ng & Saleh, 2011). Propagation of orchid by PLBs formation is a preferred option because a large number of PLBs can be obtained in large scale production at a short period of time and sustainable manner. Interestingly, single PLB can proliferate and regenerate rapidly into large number of complete plantlets (Liau et al., 2003). This is due to PLB having single or multiple meristematic zone with the ability to regenerate into new plant (Gantait et al., 2012a; Mohanty et al., 2012).

PLBs are somatic embryo for orchid in which initiated as a small globule like structure which eventually will increase in size and appear like a protocorm. Lee et al. (2013) reported that PLBs should be regarded as somatic embryos because PLBs show characteristic similar to the zygotic counterparts in terms of early division pattern of embryogenic cells, globular shape and wall protein. Thus, the somatic embryogenesis of orchid was termed as protocorm-like bodies (PLBs) based on its appearance (Bustam et al., 2015). PLBs can be induced using various parts of plant

such as shoot tips (Gnasekaran et al., 2010), leaves (Mayer et al., 2010), seeds (Mohanty et al., 2012), stem nodes (Tan et al., 2013), and protocorms (Romero et al., 2018) which are able to regenerate directly into plantlets. PLBs can be subcultured on proliferation media in order to maintain the PLBs or be harvested to regenerate into plantlet through the plantlet regeneration medium. However, the suitable stage for regeneration depends on PLBs size (Bustam et al, 2015).

Protocorm-like bodies are the best clonal propagules to use for orchid micropropagation. The use of PLBs enables the selection of uniform for morphological, physiological and biochemical studies (Mahmood et al., 2011). Besides using PLBs in micropropagation (Martin & Madassery, 2006; Zhao et al., 2008; Romero et al., 2018), PLBs also used as starting material for synthetic seed (Flachsland et al., 2006; Ching et al., 2012; Antony et al., 2014b) and in cryopreservation (Bukhov et al., 2006; Galdiano et al., 2013; Bustam et al., 2015).

2.3.3 Thin cell layers (TCLs)

Orchids were once considered as difficult to propagate plants *in vitro*. However, thin cell layers (TCLs) technology has been introduced making mass clonal propagation easier and more reproducible. In TCLs culturing, very small longitudinal (lTCL) or transverse (tTCL) sections are excised from different plant organs (Texeira da Silva, 2003). Normally, longitudinal TCL (lTCL) comprise a single tissue type, but sometimes may also be found two types of tissues, while transverse (tTCL) section contain a few cells from different tissue types. The cut surface of the PLBs would attributed to wounded surface which claimed to produce callus as a repair mechanism system, while quiescent unwounded cells near the cut surface become active and initiate cell proliferation (Imaseki, 1985). Generally, small

size of explant that less than 1-2mm in thickness was prepared from differentiated plant organ to be cultured (Van, 1980). This technique is suitable to produce mass of clonal from a limited source.

Previously, thin layer were used for studying the morphogenetic events during organ formation (Van, 1980). However this concept has now being exploited as a method for rapid plant multiplication. TCLs is a simple system in which organ interaction is suppressed and a minimum tissue interaction is maintained because the explant has a small size and being composed to few cell layers (Nhut et al., 2001). The advantages of TCLs technology are to produce high frequency of organ regeneration and to decrease time interval required to produce a desired organ. Teixeira da Silva (2003) estimated that a single transverse TCL could produce more than 80,000 plantlets a year compared to 11,000 plantlets produced by a conventional shoot tip method. Only a small amount of plant material and medium volume were required for this system. Therefore TCLs technology has been one system that has advanced in tissue culture, making mass clonal propagation easier and more reproducible.

Numbers of plants have resulted in successful of propagation using TCLs technology. A review by Teixeira da Silva (2013a) mentioned about comparison between three protocol of tissue culture used to subculture *Cymbidium* hybrids PLBs. Protocol 1 considered as a conventional method by using the whole 1^{o} PLB to induce 2^{o} PLB. Whereas in protocol 2, the 1^{o} PLB was cut symmetrically into half and called as half-moon PLB before place the cut-surface down on the medium. Meanwhile, protocol 3 was thin cell layer technique in which the PLB was cut into longitudinal (lTCL) and transverse (tTCL) prior culture on the medium. From the observation, PLBs from TCL technique showed the highest 2^{o} PLBs production after

9 month period compared to other protocol. As a result, it was proven that TCLs technology contributes major advances in clonal micropropagation of orchids. Monja-Mio and Robert (2013) investigated application of TCLs technology on *Agave fourcroydes* and found that thin tissue segment of transversely TCL (tTCL) from *in vitro* plant stem gave the best embryogenic response. Each plantlet can generate a minimum of 100 embryos in 60 days of culture, thus making this technology useful for large-scale micropropagation. This tTCL system also increases multiplication rate of threatened *Ceropegia bulbosa* shoot (Dhir & Shekhwat, 2014) and *Cymbidium* orchid (Teixeira da Silva, 2013b).

2.4 Germplasm conservation

Global orchid conservation has imbalance number of rare and endangered orchids species. Same with other specified plant species, orchid are facing a rapid decline due to loss of natural habitats as results of land clearing for agriculture, over-harvesting from natural populations for medicinal and horticultural used, and in recent decades, because of climate change (Bustam et al., 2015). Thus, conservation of plant germplasm is essential to sustain the exploitation of biological resources, stability of ecological environment and the breeding of new cultivars (Lambardi et al., 2000). Preservation of endangered and rare plant species involve two major methods known as *in situ* and *ex situ* conservation.

In situ conservation involves conservation in the natural habitats such as wilderness areas, reserves, and protected areas and within traditional farming systems. These approaches allow plant species to grow in their natural habitats where evolutionary processes continue to operate; thus making it a dynamic system (Ogbu et al., 2010). Advantages of this approach includes conserving a large range of

potentially interesting alleles, enhance and ensure sustainable use of genetic variation for present and future human needs, assures protection of associated species and facilitates research on species in their natural habitats. Besides grow in their natural habitat in protected areas and national reserve, *in situ* conservation can be carried out on-farm in the areas where landraces and local varieties are cultivated. Participation of active farmers are required to conserve landraces and traditional farmers varieties. This method of approach is significance to the wild relatives of crop plants, tree crops and forest species. However, *in situ* approach also have disadvantages that involved cost, size and maintenance aspect such as over-exploitation, competition from invasive alien species, genetic drift and inbreeding as well as human disturbances. In addition, a danger of genetic wipe out as a result of natural disasters also is in consideration (Radhika et al., 2014). In such situations, the alternative way to conserve diversity is to maintain it via *ex situ*.

Ex situ conservation involves removal of the plant genetic resources from their natural habitat and placing them under artificial storage conditions (Withers & Engelmann, 1998). This approach includes classical methods such as seed storage in seed banks, filed gene banks and botanical gardens. *Ex situ* conservation involve species recovery programs of restoration and reintroduction by providing the opportunity to study the biology and to understand the threats to endangered species. It also has the advantages of preserving plant material and making it available for research purposes, without damaging the natural populations (Temitope, 2013). Besides, biotechnological methods like *in vitro* storage and cryopreservation can be an alternative approaches for *ex situ* conservation of certain plant materials that not suitable using classical method (Radhika et al., 2014).

In vitro conservation involve slow growth technique offer a medium-term storage option, avoiding risk of losses of germplasm on field gene bank that cause by disease attacks and natural disasters. This method is used in vegetatively propagated species, non-orthodox seeded species and wild species which produce little or no seeds (Ogbu et al., 2010). Basically, plant tissue culture is focused on formulating media for each genotype that would show optimal response in term of growth rate of the explants. However, when tissue culture is employed for conservation, the focus is to devise a medium that would decrease the growth rate of explants. Number of methods used includes use of retardant agents, osmotic regulators, minimal growth media, reduction in oxygen concentration, and maintenance under reduced temperature and light intensity (Radhika et al., 2014). Example of research that use slow growth conservation includes *Heliconia champneiana* cv. Splash under different light spectra (Rodrigues et al., 2018).

Cryopreservation is used for long-term conservation of germplasm of vegetatively propagated and recalcitrant seed species. This technique refers to the storage of biological tissues at ultra-low temperature, usually liquid nitrogen (-196°C) in which cell division, metabolic and biochemical activities are arrested, thus, plant material can be stored without changes for unlimited periods of time. This technique only need limited space and very limited maintenance but cause no changes in viability, vigor and genetic makeup of the conserved materials. Interestingly, this technique eliminates the need to test stored materials frequently, thus making cost effective storage (Ogbu et al., 2010).

2.5 Plant cryopreservation methods

There are two types of plant cryopreservation methods include classical and new cryopreservation method. Classical cryopreservation method also termed as controlled freezing, slow freezing or two step freezing method are based on slow cooling method. New cryopreservation method based on vitrification technique in which the latest approach for plant conservation purpose (Engelmann, 2004). Generally, different cryopreservation methods have varying technical details.

2.5.1 Classical cryopreservation method - Slow freezing method

Slow freezing or simple freezing method is the classical cryopreservation technique that was developed in 1970s to 1980s (Kaviani, 2011). In this method, samples (shoot tips, embryo and cells) are packed in cryotube or straw, and treated with cryoprotectans. This sample are then inoculated on ice at -7^{o}C and frozen at -0.3-0.5 ^{o}C/min to -40^{o}C using a programmable freezer for dehydration before immersed into liquid nitrogen (Kumu et al., 1983). During slow freezing method, ice crystals are formed thus have harmful to the cells. Formation of intracellular ice crystals can damage the cell wall and structure, while extracellular precipitation of water as ice crystal increases the salt concentration to the level that can cause damage to the cells. Therefore these potential harmful factors must be avoided throughout the process to enhance survival of the cells (El-Danasouri & Selman, 2005).

Slow freezing method combine the application of penetrating cryoprotectant and controlled freeze dehydration by cold, sugar hardening or osmotic dehydration. Cold acclimation has changes membrane composition subsequently increasing dehydration tolerance of the cell. A usual characteristic in cold-acclimated plants is the increased concentration of soluble sugar which is known to have important

function in osmoprotection, cryoprotectant, and metabolization of other protective substances during cryopreservation (Kaviani, 2011). During freeze-induced dehydration, extracellular ice formation withdraws free liquid water molecules through an osmotic gradient from cytoplasm to intercellular spaces (Benson, 2008). Consequently, dehydration process cause the cellular concentration of solutes rises and becomes too high to nucleate to ice crystal during cooling, thus produce glassy state condition in the cell.

Other way to protect the cells is by introducing cryoprotectants such as dimethylsulfoxide (DMSO), glycerol, ethylene glycol or sucrose. This chemical solute can decrease free water content in cells (Sakai, 2000). DMSO and glycerol can enter into cells and protect cellular integrity to avoid ice formation. Whereas sucrose can penetrate and concentrated in the cell wall space during cells are frozen, thus protect protoplasts from freeze-induced dehydration. This is because it form buffer layer between cell wall and the protoplast to protect the outer surface of the plasma membrane (Tao & Li, 1986). Another main factor in slow freezing method is cooling speed. The slower cooling rates producing the higher survival rates (Benson, 2008). Combination of slow freezing with pretreatment such as cold acclimation and addition of cryoprotectants has proven to be effective for *Pyrus* germplasm conservation (Bell & Reed, 2002).

Slow freezing method mainly used for freezing undifferentiated cultures such as cell suspensions and calluses (Kartha & Englemann, 1994). However, this method is less efficient in larger explant sizes such as zygotic embryos, somatic embryos and shoots. These classical techniques are operationally complex since they require the use of sophisticated and expensive programmable freezers although sometimes,

slow-freezing step can be performed with a domestic or laboratory freezer (Ramya et al., 2014).

2.5.2 New cryopreservation method – Encapsulation dehydration

From the early 1990s, several fast-cooling approaches which were based on vitrification were developed**.** This technique is able to cryopreserve tropical species and temperate germplasm which are intolerant to desiccation and extremely low temperatures (Sakai, 2000). There are different vitrification-based methods available and these include encapsulation-dehydration, vitrification, encapsulation–vitrification, dehydration, pregrowth, pregrowth–dehydration, droplet freezing, and cryo-plate (Cruz-Cruz et al. 2013).

Encapsulation-dehydration method is based on the technology developed for producing synthetic seed which is encapsulation of explant in calcium alginate beads (Reed, 2007). Usually, culture of explants on sucrose enriched medium (0.3-0.7%) before encapsulation will improves survival of cryopreserved plants. Then, it involves the incubation of explants in Na-alginate solution. A drop of alginate solution with explants subsequently release into a complexity agent such as calcium chloride ($CaCl_2$) where bead hardening occur in 20-30 minutes. During this period, an ion-exchange process takes place by replacement of sodium ions with calcium ions forming calcium alginate. Concentration of calcium alginate and calcium chloride contribute to the hardening of the beads and it may vary with different explants as well as with different plant species (Rai et al., 2009). Normally, 3% of Na-alginate and 50-100mM $CaCl_2$ solution being the most suitable concentration used. Addition of mineral salt, organic substances, plant growth regulators or antioxidants is acceptable in alginate beads. Afterwards, encapsulated explants are

precultured in high sucrose concentration and partially desiccated to 20-30% of moisture content before immersed to liquid nitrogen. Physical desiccation is carried out either with silica gel or in the air flow of the laminar flow cabinet (Paulet et al., 1993). Plate 2.2 represented a general diagram of encapsulation-dehydration stages in cryopreservation treatment.

Encapsulated explants allow exposure to extreme treatments and stress such as preculture with high sucrose concentrations and desiccation to low moisture contents that would be highly damaging to non-encapsulated explants. After removal of freezable water from the cells, vitrification of internal solutes takes place during rapid exposure to liquid nitrogen, thus avoiding lethal intracellular ice crystallization (Engelmann et al., 2008). Liquid nitrogen is a cryogenic fluid that can cause complete rapid freezing on contact with living tissue as minimum as an hour of exposure especially for small sample (Reed et al., 2005). Vitrification can be defined as the transition of water directly from the liquid phase into an amorphous phase or glass, though avoiding the formation of crystalline ice (Fahy, 1987). This system is amorphous, which lacks of organized structure but possesses the mechanical and physical properties of a solid (Taylor et al., 2004). Vitrification of water in biological systems is dependent on increased cell viscosity in which increased viscosity inhibits the coming together of water molecules to form ice. As a result, vitrification prevents the injurious problems associated with intracellular ice formation. However, vitrified systems are particularly unstable during thawing as changes in molecular mobility and energy may be sufficient to permit water molecules to rearrange and form ice (Benson, 2008).

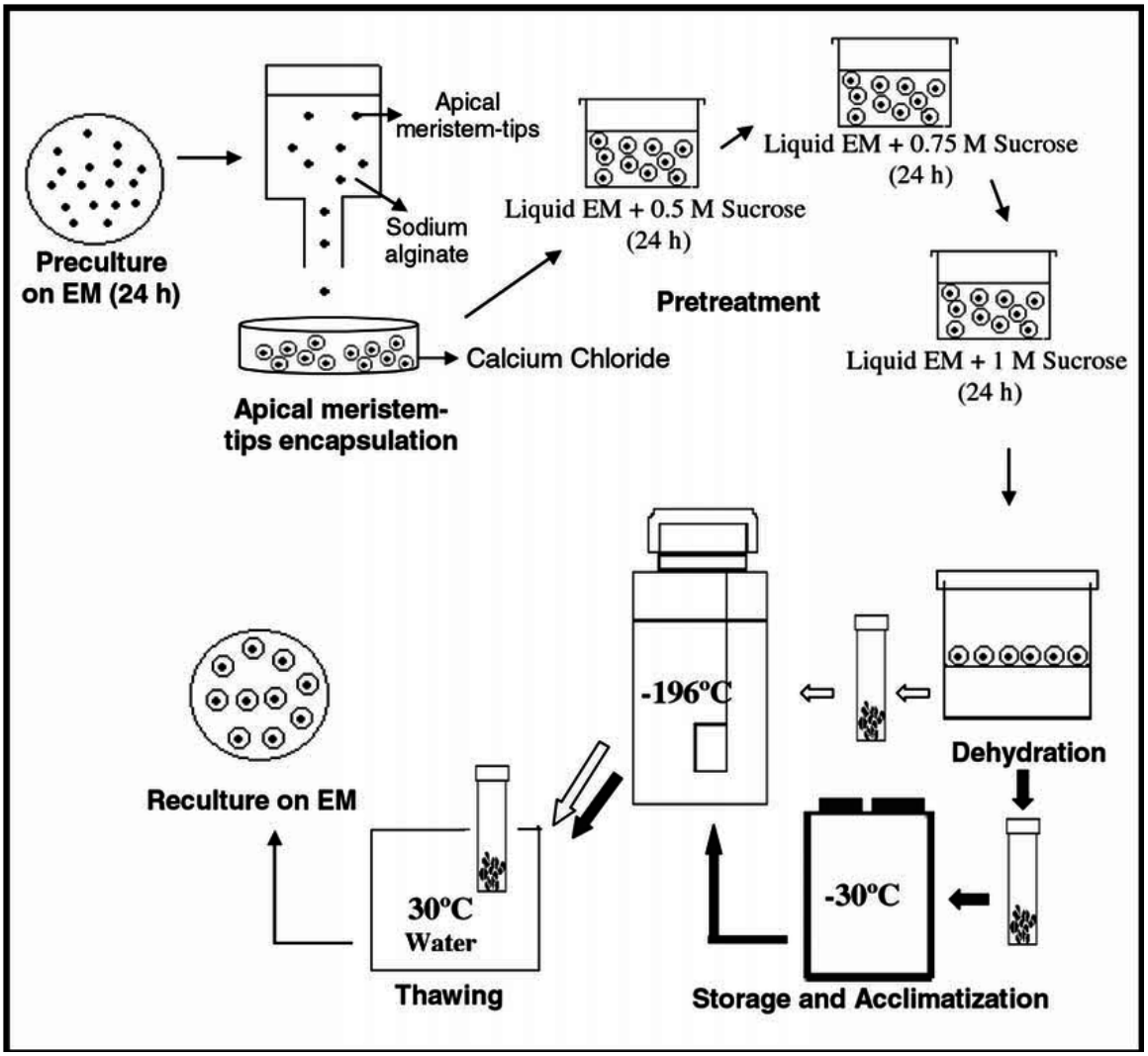

Plate 2.2: Representative diagram of encapsulation-dehydration treatment stages.
(Scocchi et al., 2004)