PVS2 DROPLET-VITRIFICATION CRYOPRESERVATION TECHNIQUE FOR *Aranda* Broga Blue ORCHID PROTOCORM-LIKE BODIES

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PVS2 DROPLET-VITRIFICATION CRYOPRESERVATION TECHNIQUE FOR *Aranda* Broga Blue ORCHID PROTOCORM-LIKE BODIES

by

KHOR SOO PING

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LIST OF ABBREVIATIONS

ABA	Abscisic acid
AFLP	Amplified fragment length polymorphism
APX	Ascorbate peroxidase
ATG	Adenine, thymine and guanine
BIP1	Luminal-binding protein 1 precursor
bp	Base pairs
BSA	Bovine serum albumin
CAT	Catalase
CO	Carbonyl
CPAs	Cryoprotective agents
cv.	Cultivar
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DSC	Differential scanning calorimetry
EDTA	Ethylenediaminetetraacetic acid
FDA	fluorescein diacetate
GB	Glycine betaine
GPX	Glutathione peroxidase
GSH	Glutathione
HPLC	High performance liquid chromatography
ISSR	Inter simple sequence repeat
JA	Jasmonic acid

LA	Lipoic acid
LEA	Late-embryogenesis abundant
Lindl.	Lindley
LN	Liquid nitrogen
MDA	Malondialdehyde
MDHA	Monodehydroascorbate
MS	Murashige and Skoog
OEE1	Oxygen-evolving enhancer protein 1
PASW	Predictive Analytics Software
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PGR	Plant growth regulators
PLB	Protocorm-like body
PLBs	Protocorm-like bodies
PUFA	Polyunsaturated fatty acids
PVP	Polyvinylpyrrolidone
PVS2	Plant vitrification solution 2
PVS3	Plant vitrification solution 3
RAPD	Random Amplified Polymorphic DNA
Rchb.f.	Reichenbach
ROS	Reactive oxygen species
SA	Salicylic acid
SCoT	Start Codon Targeted
SOD	Superoxide dismutase
SSR	Single sequence repeats

Ta	Annealing temperature
Taq	*Thermus aquaticus*
Tm	Melting temperature
TBE	Tris-Borate-EDTA
TPF	Triphenylformazan
TTC	2,3,5-triphenlytetrazolium chloride
UV-B	Ultraviolet-B
UV-Vis	Ultraviolet-visible
vtc-1	*vitamin c-1*
VS	Vitrification solution
VW	Vacin and Went

LIST OF SYMBOLS

a	Extinction coefficient of H_2O_2 at 240nm ($39.48M^{-1}cm^{-1}$)
ED	Encapsulation-dehydration
EV	Encapsulation-vitrification
$^1O_2$	Singlet oxygen
$O_2^{\bullet-}$	Superoxide
OH•	Hydroxyl radical
OOH•	Hydroperoxyl
Tg	Glass transition temperature

TEKNIK KRIOAWETAN TITISAN VITRIFIKASI PVS2 UNTUK JASAD SEPERTI PROTOKOM ORKID *Aranda* Broga Blue

ABSTRAK

Teknik krioawetan titisan vitrifikasi Plant Vitrification Solution 2 (PVS2) telah dibangunkan untuk pemeliharaan jasad seperti protokom (JSP) orkid *Aranda* Broga Blue berdasarkan tindak balas eksplan kepada faktor kritikal yang berbeza dalam krioawetan. Kesan saiz JSP, kepekatan sukrosa dan tempoh pre-kultur, vitrifikasi dua-langkah atau tiga-langkah, media pemulihan pertumbuhan dan tempoh pendedahan PVS2 telah dinilaikan. Peratusan pemulihan pertumbuhan yang terbaik (5%) telah diperolehi apabila JSP bersaiz 3-4mm dikultur dalam media separa pepejal Vacin and Went (VW) (1949) yang mengandungi 0.2M sukrosa selama 3 hari, diikuti oleh osmo-perlindungan dalam larutan pemuat selama 20 minit, dehidrasi dalam PVS2 selama 20 minit pada 0°C, pemindahan JSP kepada titisan PVS2 atas jalur kertas aluminium, penyimpanan dalam cecair nitrogen (LN) untuk minimum 30 minit, pencairan dalam larutan penyahmuat dan pengeraman selama 20 minit, dan akhirnya pertumbuhan semula dalam media VW10 (media separa pepejal VW ditambah dengan 1.5% air kelapa, 0.2% pisang dan 0.2% homogenat kentang). Asid askorbik luaran yang diuji pada julat kepekatan dari 0.284-0.852mM (50-150mg/L) ditambah di peringkat prakultur, pemuatan, penyahmuatan dan pemulihan pertumbuhan adalah tidak berkesan untuk meningkatkan pertumbuhan JSP yang dikrioawetkan. Induksi jumlah kandungan protein larut yang singnifikan diperhatikan dalam eksperimen krioawetan tanpa asid askorbik luaran sepanjang peringkat dehidrisi (prakultur, pemuatan dan PVS2) berbanding dengan kultur *in vitro*. Jumlah kandungan protein larut JSP tanpa asid askorbik luaran didapati lebih tinggi dengan

signifikan berbanding dengan peringkat krioawetan ditambah dengan asid askorbik terutamanya di prakultur, pemuatan, PVS2, dan penyahmuatan untuk kedua-dua eksperimen krioawetan dan bukan-krioawetan. Aplikasi asid askorbik luaran dalan peringkat pemuatan, +penyahmuatan dan +pertumbuhan semula memberi kesan yang signifikan kepada aktiviti-aktiviti superoxide dismutase (SOD) JSP. Tidak ada perubahan yang signifikan dalam aktiviti-aktiviti catalase (CAT) dan ascorbate peroxidase (APX) dikesan pada perlbagai peringkat krioawetan diuji dengan kepekatan asid askorbik luaran yang berbeza. Penilaian ultrasturktur JSP pulih daripada rawatan krioawetan yang berbeza diperhatikan menggunakan mikroskop transmisi elektron (TEM) mencadangkan bahawa JSP menggunakan mekanisme yang berbeza untuk bertahan terhadap rawatan krioawetan yang tertekan. Rawatan pre-kultur dengan 0.2M sukrosa selama 3 hari semasa krioawetan menghasilkan JSP yang ditumbuh dengan strukur sel menyerupai JSP kawalan yang tidak dirawat. Penilaian kestabilan genetik menggunakan penanda DNA RAPD dan SCoT membuktikan bahawa eksplan dijana dari JSP yang dikrioawet adalah serupa dengan genetik JSP kawalan yang tidak dirawat.

PVS2 DROPLET-VITRIFICATION CRYOPRESERVATION TECHNIQUE FOR *Aranda* Broga Blue ORCHID PROTOCORM-LIKE BODIES

ABSTRACT

Plant Vitrification Solution 2 (PVS2) droplet-vitrification cryopreservation technique has been developed for the preservation of of *Aranda* Broga Blue orchid PLBs based on the response of explants to different critical factors in cryopreservation. Effects of PLB size, preculture sucrose concentrations and durations, choice of vitrification solutions, two-step or three-step vitrification, growth recovery medium and PVS2 exposure durations were assessed. The best growth regeneration percentage (5%) was obtained when 3-4mm PLBs were precultured in semi-solid Vacin and Went (VW) (1949) medium containing 0.2M sucrose for 3 days, followed by osmoprotection in loading solution for 20 minutes, dehydration in PVS2 for 20 minutes at 0°C, transfer of PLBs to PVS2 droplets on aluminum foil strips, LN storage for minimum of 30 minutes, thawed in unloading solution and incubate for 20 minutes, and finally growth regeneration in VW10 medium (semi-solid Vacin & Went medium supplemented with 1.5% coconut water, 0.2% banana, and 0.2% potato homogenate). Exogenous ascorbic acid tested at concentration range from 0.284-0.852mM (50-150mg/L) added in preculture, loading, unloading and growth recovery stages was not effective to improved growth recovery of cryopreserved PLBs. Significant induction of total soluble protein was observed in cryopreservation experiment without exogenous ascorbic acid throughout dehydration (preculture, loading and PVS2) compared to *in vitro* culture. Total soluble protein of PLBs without exogenous ascorbic acid was found to be significantly higher compared to cryopreservation stages supplemented with ascorbic

acid particularly preculture, loading, PVS2, and unloading stages for both cryopreservation and non-cryopreservation experiment. Application of exogenous ascorbic acid in loading, +unloading and +GR stages significantly affect superoxide dismutase (SOD) activities of PLBs. No significant changes of catalase (CAT) and ascorbate peroxidase (APX) activities were detected at various cryopreservation stages tested with different exogenous ascorbic acid concentrations. Ultrastructural assessment of PLBs recovered from different cryopreservation treatment by transmission electron microscopy (TEM) suggested that PLBs utilized different mechanism to survive stressful cryopreservation treatment. Preculture treatment with 0.2M sucrose for 3 days during cryopreservation produced regenerated PLBs with similar cellular structure which resembles the untreated control PLBs. Genetic stability assessment using RAPD and SCoT DNA marker proved that explants regenerated from cryopreserved PLBs was genetically identical to the untreated control PLBs.

CHAPTER 1

INTRODUCTION

Orchidaceae which comprised the most abundant of all angiosperm families, are widely used as ornamental plants and some species are famous for its medicinal value. *Aranda* genus orchids are intergeneric hybrids bred between *Vanda* and *Arachnis* genera (Gantait and Sinniah, 2012; Suis *et al.*, 2015), consisted of the major part of ornamental orchids industry in Malaysia back in the 1970s until mid 1990s (Zainol, 2003). *Aranda* Broga Blue is one of the latest *Aranda* hybrid produced between *Mokara* Wangsa Gold and *Vanda coerulea* to freshen the orchid cut flower industry in Malaysia (Suis *et al.*, 2015).

Different conservation strategies has been employed to preserved threaten plant species facing extinction, as well as to safeguard species richness biodiversity that provide the sources of foods, pharmaceutical constituents and crop breeding industry as well (Panis and Lambardi, 2006). Cryopreservation refers to the storage and maintenance of biological materials at ultralow temperature of liquid nitrogen (LN), which is at -196°C or its vapour phase at -150°C (Kartha, 1985; Kaczmarczyk *et al.*, 2011; Popova *et al.*, 2016), has been escalating to become a commonly used method for *ex situ* conservation (Niino and Arizaga, 2015). This method offers a long term conservation strategies, as ultralow temperature halted all metabolic activities and cell division under cryogenic storage (Cruz-Cruz *et al.*, 2013; Popova *et al.*, 2016). Thus, somaclonal variation or genetic changes of plant materials which may occur during serial subculturing can be prevented (Cruz-Cruz *et al.*, 2013). Besides endangered and threatened plant species, cryopreservation method is also applied in conserving recalcitrant or dehydration sensitive species, where seeds are not storable

or extremely scarce (Kaczmarczyk *et al.*, 2012). Moreover, the utilization of cryopreservation method has been extended in many specific cultivars of commercially important crops such as potato and banana or ornamental plants with unique phenotypes such as chrysanthemum and orchids (Panis *et al.*, 2005; Kaczmarczyk *et al.*, 2011; Kaczmarczyk *et al.*, 2012).

To achieve successful cryopreservation, it depends on the capability of the plant germplasm to withstand different stresses incurred and accumulated at every stage of cryopreservation treatment (Poobathy *et al.*, 2009). The main concern is to minimized chances of ice crystal formation caused by cellular water during storage in liquid nitrogen, thus water removal in plant cells is the primary role of avoiding freezing injury and improve post-thaw viability (Gonzalez-Arnao *et al.*, 2008; Cruz-Cruz *et al.*, 2013). Water removal of plant cellular water can be performed by various dehydration treatments via air drying, freeze drying, cold hardening and incubation in highly concentrated vitrification solution (Panis and Lambardi, 2006).

Vitrification based-cryopreservation involves dehydration of plant materials before plunging into liquid nitrogen at a fast and ultra rapid cooling rate. Vitrification refers to the physical process by which a highly concentrated cryoprotectants supercools to very low temperatures and solidifies into a metastable glassy state without undergoing ice crystallization at a practical cooling rate (Fahy *et al.*, 1984; Sakai and Engelmann, 2007; Sakai *et al.*, 2008). Manipulation of vitrification cryopreservation protocol has been done to suit the choice of plant materials, and at present there are several widely used vitrification-based cryopreservation protocol such as encapsulation-dehydration, encapsulation-vitrification, vitrification, dehydration, pregrowth, pregrowth-dehydration, droplet-vitrification and cryo-plate method (Cruz-Cruz *et al.*, 2013). Each protocol has its pros and cons depending on

availability and simplicity, suitability of the plant materials as well as the capability of producing post-cryopreservation viability (Kim *et al.*, 2012).

The latest vitrification protocol such as droplet-vitrification and cryo-plate method which utilized the concept of ultra rapid cooling and warming rates, improve the efficiency in achieving vitrified state of cryopreserved materials, thus minimizing detrimental ice crystallization formation (Panis *et al.*, 2005; Kaczmarczyk *et al.*, 2012). The probability of obtaining glassy vitrified state is higher during cooling and de-vitrification can be avoided upon thawing (Panis *et al.*, 2005), which contributed to high viability of cryopreserved materials.

Besides freezing injuries, there are many factors that affect growth recovery of cryopreserved explants (Kaczmarczyk *et al.*, 2012). Although prevention of ice crystals is important, however explants excision, manipulation and over dehydration could be detrimental to explants which affect post-thaw survival. Vitrification based-protocol often required the application of vitrification solution containing mixture of cryoprotective agents (CPAs) such as glycerol, dimethyl sulfoxide (DMSO), and ethylene glycol (Kulus and Zalewska, 2014). Some of the CPAs are thought to possessed toxicity to plant cell at high concentration. All these factors led to the production of reactive oxygen species (ROS) in plant cells, causing oxidative stress.

ROS-mediated oxidative stress arised from sequential dehydration during cryopreservation treatment was known as the main factor that caused cell death in cryopreserved materials (Georgieva *et al.*, 2014). High level of toxic ROS such as singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet-}$) and hydroxyl (OH•) cause cellular functions and properties modification through oxidation and inhibition of proteins, DNA destruction and induction of lipid peroxidation (Foyer and Noctor, 2005; Gill and Tuteja, 2010; Sharma *et al.*, 2012). Meanwhile, under

optimal plant growth conditions, ROS performed effectively as important signalling molecules that regulates cellular metabolism and response in plants (Sharma *et al.*, 2012) such as photosynthesis and photorespiration, plant growth, cell cycle, programmed cell death and hormonal signalling (Mittler *et al.*, 2004; Gill and Tuteja, 2010). Moreover, ROS has been shown to induce plant defence mechanism during various abiotic and biotic stresses, by influencing expression of defence gene, modifying transcription factors and cellular reprogramming (Apel and Hirt, 2004; Foyer and Noctor, 2005; Boguszewska and Zagdańska, 2012). To sustain ROS at non-toxic level to act as signalling messenger, plant possessed complex scavenging system that detoxify excess ROS production consisted of both enzymatic and non-enzymatic antioxidants (Gill and Tuteja, 2010). The enzymatic antioxidants included superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione peroxidase (GPX) (Apel and Hirt, 2004; Sewelam *et al.*, 2016), while non-enzymatic antioxidants composed of ascorbic acids, glutathione, tocopherol, carotenoids and phenolic compounds (Apel and Hirt, 2004; Gill and Tuteja, 2010; Sharma *et al.*, 2012). During cryopreservation studies, exogenous antioxidants such as ascorbic acid and glutathione, has been proven to be beneficial in improving survival of cryopreserved explants (Uchendu *et al.*, 2009; Uchendu *et al.*, 2010). By revealing the plant defence mechanism in response to various abiotic stresses emerged from cryoprotectants and different cryopreservation treatments, this will also help to improve growth recovery of plant cell that may enhance success of cryopreservation (Ren *et al.*, 2013; Zhang *et al.*, 2015a).

Cryopreservation, which include a series of stressful treatments to plants, were found to influence ultrastructure of plant cellular components at different cryopreservation stages as revealed in the studies of asparagus embryogenic cell

suspensions (Jitsuyama *et al.*, 2002), *Cosmos atrosanguineus* shoot tips (Wilkinson *et al.*, 2003), and banana meristem (Helliot *et al.*, 2003). It was found that plant tolerance during cryopreservation treatment was accompanied by plasmolysis (Jitsuyama *et al.*, 2002), fragmentation of endoplasmic reticulum (Mikuła *et al.*, 2005), reduced and contracted vacuoles (Zhang *et al.*, 2014), and accumulation of starch grains (Miao *et al.*, 2005; Zhang *et al.*, 2014). All these events that occurred are due to the loss of water content in cells during various dehydration treatments, which allow sufficient vitrification and minimize intracellular ice crystallization during storage in LN. Without proper cryoprotection, freezing and thawing treatment lethally influenced the plant cellular structure characterized by nuclear envelope rupture, chromatin condensation, swelling of mitochondria, reduction of mitochondrial matrix density, disintegration and ruptured of cell wall (Mikuła *et al.*, 2005; Wen *et al.*, 2012; Heringer *et al.*, 2013). As reported by Sershen *et al.* (2012a), cellular injuries were identified at preculture stage as the damaging factor, proven that cryo-injury may not be attributed to the effects of freeze-thaw process alone. By understanding the changes of cell ultrastructure of plant cells during cryopreservation is one of the crucial factor to improve the technique of plant cryopreservation with reduced degree of cellular injuries (Xu *et al.*, 2006).

Besides focus on developing optimal cryopreservation technique that confirmed successful regeneration of cryopreserved materials, it is also important to assess whether genetic stability of regenerants were maintained and identical to the original untreated control prior cryo-storage (Harding, 2004). Risk of genetic variation might occur due to series of potentially stressful treatment imposed to plant materials during cryopreservation such as severe dehydration treatment, exposure to toxic cryoprotectants and drastic temperature changes (Zhai *et al.*, 2003; Harding,

2004). Genetic stability of regenerants has been confirmed using various molecular markers such as random amplified polymorphic DNA (RAPD), inter simple sequence repeat (ISSR), and amplified fragment length polymorphism (AFLP). A newly developed gene-targeted molecular marker named Start Codon Targeted (SCoT) marker described by Collard and Mackill (2008) was designed based on the short conserved region of plant genes surrounding the ATG start codon, has gained popularity quickly for genetic diversity assessment.

The current study involved development of cryopreservation technique for the preservation of genotypic and phenotypic features of *Aranda* Broga Blue orchid hydrid, supported with biochemical analyses, ultrastructural studies and genetic stability assessments.

1.1 Research objectives

The objectives of this research were:

I. To develop droplet-vitrification cryopreservation technique that produce efficient plant recovery and regeneration system for PLBs of *Aranda* Broga Blue orchid,

II. To determine effects of exogenous ascorbic acid in cryopreservation stages on growth recovery, total soluble protein and antioxidant enzymes activities of PLBs of *Aranda* Broga Blue orchid,

III. To compare ultrastructural differences of regenerated cryopreserved *Aranda* Broga Blue orchid PLBs treated with different cryopreservation treatment using Transmission Electron Microscopy (TEM),

IV. To determine genetic stability of explants regenerated from cryopreserved *Aranda* Broga Blue orchid PLBs using RAPD and SCoT molecular markers.

CHAPTER 2

LITERATURE REVIEW

2.1 Orchids

With more than 25,000 species in 880 genera, Orchidaceae denotes to the most species-rich family of all angiosperm families (Swarts and Dixon, 2009a; Popova *et al.*, 2016). Orchids are widely distributed worldwide in the tropical and subtropical region and many of them are inherently rare or locally restricted (Merritt *et al.*, 2014). Moreover, there are more native orchids waiting to be discovered. Orchids are famous as ornamental plants due to its enormous flower size, shape and colours. Today, growing orchids is more than just a hobby, it is an international business covering around 8% of the global floriculture trade and this figure worth approximately US$ 40 billion (Martin and Madassery, 2006; Popova *et al.*, 2016).

In Malaysia, orchid planting industry has export values of up to RM700 million per year with *Dendrobium* orchids cultivated as the major orchid cut flowers for export (Cheah, 2014; Ahmad *et al.*, 2015). Meanwhile, oriental *Cymbidiums* are popular in China, Taiwan, Korea and Japan as horticultural plant with high economic value (Popova *et al.*, 2016). Wide varieties of orchid hybrids were also cultivated between different species to produce hybrids that inherited unique characteristics of parents and they are economically important to the floriculture industry.

Besides using as ornamental plants, orchids were also widely propagated for its precious medicinal value. The Chinese were the first to describe and use orchid as medicinal plants dated back in 3000 to 4000 years ago (Bulpitt, 2005; Pant, 2013). It has been reported that orchid contains biological active compounds that has

therapeutic effect, which is important in pain treatment, curing wounds, anti-inflammatory activity, and anticancer or antitumor as well (Gutierrez, 2010). All parts of the orchid has been used as medicine, including their leaves, roots, flowers, pseudobulbs, tuber, rhizomes and even the whole plants (Gutierrez, 2010; Pant, 2013; Popova *et al.*, 2016). In China, *Dendrobium nobile, Bletilla striata* and *Gastrodia elata* were widely used as traditional Chinese medicine, and they have been commercially propagated which comprised of the major part of herbal industry (Bulpitt, 2005; Pant, 2013).

Besides that, *Anocetochilus formosanus* Hayata, a jewel orchid which is native from Taiwan, is known as the 'King medicine' because it contains diverse pharmacological effect (Hossain, 2011). It has been reported that this jewel orchid can be used to lower high blood pressure, to treat diabetes and possessed antitumor and antivirus properties (Chang *et al.*, 2007).

2.1.1 *Aranda* orchids

Aranda orchids, which referred to intergeneric hybrids between two orchid species genera *Vanda* and *Arachnis*, appeared as the foundation of orchid industry in the tropics (Gantait and Sinniah, 2012; Suis *et al.*, 2015). *Vandas* produce short inflorescences bearing flowers with broad petals and sepals, meanwhile *Arachnis* possessed relatively longer inflorescences with spider-like flowers of narrow petals and sepals (Lee, 1991).

Aranda orchid hybrid comprised of the most important commercially grown orchid in Malaysia orchid industry back in the 1970s till mid 1990s (Zainol, 2003; Suis *et al.*, 2015) and it has been reported that more than 170 first generation hybrids

have been bred artificially (Lee, 1991). Some of the clones of these hybrids have been selected for commercial production purpose (Goh and Kavaljian, 1989).

2.1.2 *Aranda* Broga Blue orchid hybrid

Zainol (2003) reported that in Malaysia, there are declining of *Aranda* hybrid cultivation for the production of cut flowers due to the lacking of suitable new hybrids. A new orchid hybrid namely *Aranda* Broga Blue, is produced from the cross between *Mokara* Wangsa Gold and *Vanda coerulea* to refresh the cut flower industry (Suis *et al.*, 2015) (Plate 2.1). One of the parent *Vanda coerulea*, being one of the famous *Vanda*s has been parent to so many internationally renowned orchid hybrids (Kishor and Sharma, 2008) due to its incredibly characteristics of fairly deep blue colour, flower shape and size (Thammasiri and Soamkul, 2007). According to Simmler *et al.* (2009), *Vanda coerulea* carries antioxidant and anti-inflammatory properties. In addition, extracts of *Vanda coerulea* has been patented in recent years and used as cosmetic active agent with skin hydrating and anti-aging properties (Andre *et al.*, 2011; Cauchard *et al.*, 2011).

Aranda Broga Blue orchid hybrid has the potential in becoming one of the famous cut flowers in the floriculture industry and the potential to be used for beneficial secondary metabolites production. Thus, a suitable preservation method that conserved all the important features of this orchid hybrid is considerable important and *in vitro* cryopreservation strategy will be a promising approach. With increasing interest in the potential of *Aranda* orchids, the preservation of cultured cells and somatic embryos with unique attributes is assuming greater importance.

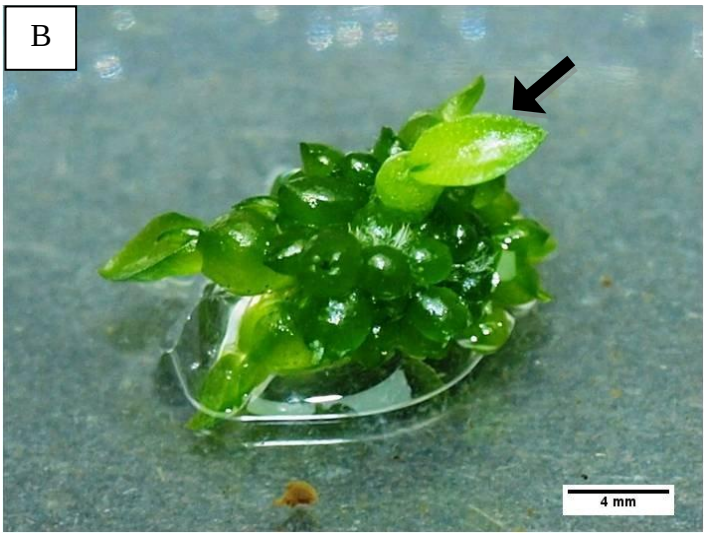

Plate 2.1. The orchid hybrid *Aranda* Broga Blue.
A. Inflorescences.
B. Clumps of PLBs with regenerated young leaves (arrow).

2.2 Orchid conservation

Orchidaceae, being the most abundant of all angiosperm family, contained a larger portion of threatened genera with many of the species facing extinction (Swarts and Dixon, 2009b). The high level of extinction threat to many orchid species is due to the wide and dynamic range of intrinsic and extrinsic factors (Swarts and Dixon, 2009a,b). Extrinsic factors include loss and degradation of natural ecosystem, deforestation, environment and habitat change, weed invasion and illegal collection (Swarts and Dixon, 2009a,b; Merritt *et al.*, 2014). On the other hand, intrinsic factors refers to disruption of biotic interdependency between the orchid species and other organism and this factor is now seen as increasing the risk to extinction in rare species (Swarts and Dixon, 2009b).

Orchids are highly specific to their insect pollinators, and they possessed a unique life cycle requiring an association with specific mycorrhizal fungi during the early stages of development (Popova *et al.*, 2016). Global warming and climate change is predicted as the factor that cause the major threat to pollination service and networks, leading to disruption of the orchid communities and this is particularly serious in mountain and tropical regions, orchid biodiversity hotspots in Asia and Latin America (Seaton *et al.*, 2010; Popova *et al.*, 2016). The conservation strategies must as well involve conserving the orchid communities, therefore it is important for the conservationist to understand and aware of the orchid life history traits, ecology and evolution that may benefits the long term sustainability (Swarts and Dixon, 2009b; Seaton *et al.*, 2010; Popova *et al.*, 2016).

Besides *in situ* conservation of the orchid natural habitat, a range of *ex situ* conservation strategies has been established for the conservation purpose.

Traditionally, botanical gardens lead the primary conservation project by maintaining and documenting living whole plant collections. However, the problems associated with living collections is that the restricted size of gene pool of most species in cultivation, and many of the species are not 'long-lived' (Nash *et al.*, 2003; Seaton *et al.*, 2010; Popova *et al.*, 2016). Seed banking has been increasingly being adopted in *ex situ* conservation of orchids (Merritt *et al.*, 2014). The orchid seeds are regarded as possessing orthodox behaviour which can withstand dry storage for long term (Seaton and Pritchard, 2003; Seaton *et al.*, 2010; Merritt *et al.*, 2014) and it allows the storage of large number of seeds in a small volume, making it a sufficient storage capacity without needing large facilities (Seaton *et al.*, 2010). Nevertheless, there has been report demonstrated that low temperature storage of seed capsules is ineffective and produce poor germination rate (Batty *et al.*, 2001; Merritt *et al.*, 2014; Popova *et al.*, 2016). Seed banking is not suitable for all orchid species, as some of the short lived orchid seeds might lost viability along the storage period, which in turn affecting the germination potential.

In vitro micropropagation of germinated orchid seeds served as an alternative conservation strategies driven by the development and advanced of plant biotechnology (Popova *et al.*, 2016) and this method indeed frequently produced more plants than remain in wild populations (Swarts and Dixon, 2009a,b; Seaton *et al.*, 2010). *In vitro* tissue culture allowed the germination of orchid seeds in an aseptic environment (Cruz-Cruz *et al.*, 2013) and germinated orchid explants can be subsequently subcultured for mass propagation. This technique has been widely used for large scale multiplication of threaten orchid species for conservation purposes as well as in commercially important orchids (Popova *et al.*, 2016). Several disadvantages were associated with the use of *in vitro* conservation technique. Firstly,

it is labour-intensive and require high cost maintenance; moreover, chances of phenotypic and genotypic variation might occur during repeatedly subculture (Khoddamzadeh *et al.*, 2011; Popova *et al.*, 2016). Consequently, cryopreservation method which involves the storage of explants in liquid nitrogen has been developed to prolong the storage longevity of orchid germplasm, which is more cost effective, safe and reliable.

2.3 Plant Cryopreservation

In recent years, the awareness of conserving valuable plant biodiversity especially to those extinct plant species has been raised and many conservation programs were stimulated for the concern about losing precious genetic resources (Paunescu, 2009). Besides conserving plants facing extinction, the purpose of plant genetic resources conservation is to safeguard species richness biodiversity that provide the sources of foods, pharmaceutical constituents and crop breeding industry as well (Panis and Lambardi, 2006). Development and advancement of plant biotechnology have provided powerful tools to support and enhance the efficiency of conservation and management of plant biodiversity (Cruz-Cruz *et al.*, 2013).

Cryopreservation, requiring little space and maintenance, is touted as an important tool for long-term storage of plant genetic resources especially for future generations (Sakai *et al.*, 2008), and it has progressively becoming a widely used method for *ex situ* conservation (Niino and Arizaga, 2015). Cryopreservation involves the storage of biological materials in liquid nitrogen (LN), which is at -196°C or its vapour phase at -150°C (Kartha, 1985; Kaczmarczyk *et al.*, 2011; Popova *et al.*, 2016). It offers safe and cost-effective option for long-term

conservation of genetic resources in many plant species (Engelmann, 2004). At this ultra-low temperature of liquid nitrogen (-196°C), all the cell division, metabolic activities and biochemical process of cells will be arrested and can be stored for unlimited periods without any deterioration or modification (Kaczmarczyk *et al.*, 2011; Popova *et al.*, 2016). As a result, plant germplasm can be preserved in such a state for a long period with its genetic stability and regeneration potential maintained. Moreover, cryopreservation has been applied for cryotherapy of shoot tips to produce pathogen free explants, by killing the virus infected tissue located distance away from the less infectious cells located at the meristematic zone of apical dome (Wang and Valkonen, 2009).

The success of the cryopreservation technique depends on the capability of germplasm to tolerate the stresses incurred and accumulated at each stage of the cryopreservation procedure (Poobathy *et al.*, 2009). General cryopreservation protocol involves the application of cryoprotectants and treatments prior and subsequent to freezing, to protect and recover the germplasm material during and after storage in liquid nitrogen (Verleysen *et al.*, 2004). The main concern in any cryopreservation technique is to prevent crystal formation caused by cellular water in explants during rapid cooling in liquid nitrogen. Ice crystal formation in plant cells cause irreversible damage to cell membranes and thus destroy their semi permeability. This destructive phenomenon can be avoided through dehydration, where the cell cytosol of explants will be concentrated and cellular water will be reduced to protect the cells from intercellular ice crystallization. The methods of achieving dehydration includes air drying, freeze drying, cold hardening and suspension in high concentrated vitrification solution (Panis and Lambardi, 2006). Physical mechanisms of cell dehydration are different upon the cryopreservation

technique employed and they can be classified into classical and new cryopreservation technique (Engelmann, 2004; Gonzalez-Arnao *et al.*, 2008; Engelmann, 2011).

2.3.1 Classical or conventional cryopreservation method

The classical cryopreservation technique also termed as controlled freezing, slow freezing or the two-step freezing methods, which involves cooling down samples in slow rate until a defined pre-freezing temperature, subsequently immersed into liquid nitrogen for storage (Engelmann, 2004, 2011). As temperature decreasing nearing to 0°C, the cells and its external medium supercool, followed by extracellular medium ice formation. Meanwhile, inner content of cells remained unfrozen, probably due to the cell membrane and cell wall that act as barrier to prevent ice crystals formed in the intercellular space and freezing in the cytoplasm (Engelmann, 2011; Vendrame *et al.*, 2014).

Extracellular freezing and ice formation around the cells facilitate intracellular water removal due to the difference in water vapour pressure, led to a mechanism known as freeze induced desiccation (Vendrame *et al.*, 2014). Application of cryoprotectants are not necessary in this technique, however, some experiments include low concentration cryoprotectants to further improve dehydration and growth recovery (Kaczmarczyk *et al.*, 2012). Slow-cooling method was known to be a suitable cryopreservation method for unorganized tissue. Complex plant tissues such as shoot tips composed of different cell types, which possessed different water and solute movement within and between plant cells, thus sufficient dehydration is particularly difficult to achieve (Gonzalez-Arnao *et al.*,

2008; Kaczmarczyk *et al.*, 2012). Moreover, to control the cooling rate (0.5-2.0°C/ minute), this technique generally requires the use of expensive programmable freezers, which is very high cost to maintain in the long run (Engelmann, 2011).

2.3.2 New cryopreservation method- Vitrification

In contrast, the new generation of cryopreservation technique applied freeze-avoidance mechanism known as vitrification. Vitrification is defined as the physical process by which highly concentrated cryoprotectants supercools to very low temperatures and solidifies into a metastable glassy state without undergoing ice crystallization at a practical cooling rate (Fahy *et al.*, 1984; Sakai and Engelmann, 2007; Sakai *et al.*, 2008).

Vitrification was proposed as a method to replace freeze-induced cell dehydration for the cryopreservation of biological materials because it would avoid the potentially detrimental effects of extracellular and intracellular freezing that will damage the cell (Sakai and Engelmann, 2007; Sakai *et al.*, 2008). Due to high viscosity of glass, all chemical reactions and pH alteration that require diffusion of water are arrested at ultra low temperature, thus in this conditions the biological material can be remained stable for a long time without additional tissue collapse (Gonzalez-Arnao *et al.*, 2008; Sakai *et al.*, 2008). Dehydration of plant cells can be performed in two ways under pre-freezing temperature, by exposing explants into highly concentrated vitrification solutions or physical air desiccation method (Engelmann, 2011). Subsequently, vitrification can be achieved by immersing dehydrated explants into liquid nitrogen, and the resultant solid retains the random

molecular arrangement of a liquid in the plant cell, but has the mechanical properties of a solid (MacFarlane, 1987; Gonzalez-Arnao *et al.*, 2008).

Vitrification-based cryopreservation technique offer several advantages over classical freezing technique. As opposed to classical freezing, vitrification technique allows the application of complex organ such as shoot tips and embryos which contain a variety of cell types (Engelmann, 2011). Furthermore, less complex vitrification technique excludes the need of control freezing programmable freezer, thus reduce the cost of operation. Besides that, this technique required only minor modification for different cell types and hence broadens the potential of applicability (Engelmann, 2011). However, the main challenge of vitrification technique occurred during its application to desiccation sensitive explants as the critical step to achieve survival is the dehydration step but not during the freezing step (Engelmann, 2011). As a result, the key to successful survival is to acquire dehydration tolerance in plant cells especially in recalcitrant plant species or cell types to withstand further freezing stress imposed during cryo-storage.

There has been reported that cells and explants that were conditioned to withstand severe dehydration especially during exposure to highly concentrated vitrification, were able to withstand following freezing and rewarming procedure with little or no additional loss of survival (Yamada *et al.*, 1991; Niino *et al.*, 1992; Matsumoto *et al.*, 1994). The cells that need to be cryopreserved must be in a physiologically optimal status for the acquisition of dehydration tolerance to produce recovery after thawing from liquid nitrogen (Withers, 1979; Dereuddre *et al.*, 1988; Sakai *et al.*, 2008). Desiccation tolerance of the chosen explants can be acquired by optimizing several parameters in vitrification cryopreservation procedure, which include preconditioning (cold hardening), preculture, osmoprotection with loading

treatment, vitrification solution exposure conditions (duration and temperature), and even post warming handling are crucial for subsequent plant cell recovery (Sakai *et al.*, 2008).

The concept of vitrification has allowed further modification and evolution of different vitrification-based cryopreservation method. Several simple, reliable and efficient protocols have been developed and they include: encapsulation-dehydration, encapsulation-vitrification, vitrification, dehydration, pregrowth, pregrowth-dehydration, droplet-vitrification and cryo-plate method. Choice of suitable protocol is relatively plant species specific and it also depends on the expertise, equipments and facilities available. The number of species that were successfully cryopreserved has rapidly increased over the years due to the progressive research, development and improvement of new cryopreservation techniques (Radha *et al.*, 2012). Currently, cryopreservation has been successfully applied for over 200 species including endangered plants, commercially important crops and plant with high ornamental value (Benson, 2008a; Popova *et al.*, 2016).

Over the years, the trend of cryopreservation method has moved from slow freezing towards ultra-rapid cooling cryopreservation technique. The latest droplet-vitrification and cryo-plate technique which utilizing ultra rapid cooling and warming rate, were gaining considerably high interest among cryobiologist due to its high and efficient explants recovery.

2.3.2(a) Droplet-freezing and droplet-vitrification

One of the main factors that contribute to post thaw viability of cryopreserved explants is through the removal of water from explants to avoid ice

crystallization formation and freezing injury (Gonzalez-Arnao *et al.*, 2008). Ice crystallization is often a major problem in cryopreservation because ice crystals possessed branch like needle or sharp edges, which will lead to mechanical damage of the plant cell structure and compromise cell integrity upon freezing (Benson *et al.*, 2006).

Cryopreservation technique applied back in the 1970s utilizing slow freezing method, initiate extracellular ice nucleation. As the freezing progresses, ice nucleated on the surface of cells will form water vapour deficit and force intracellular water to move out from the cells, and this phenomenon was termed as cryo-dehydration (Benson *et al.*, 2006). Vitrification, which refer to the solidification of liquid phase into metastable glass solid at glass transition temperature (Tg) without ice crystallization (Fahy *et al.*, 1984), was later applied in plant cryopreservation using asparagus culture cells in the 1989 by Uragami *et al.* (1989).

Vitrification cryopreservation can prevent water crystallization formation by using high viscosity cryoprotectant to remove intracellular water through osmotic pressure from cells. Plant cells when composed of highly concentrated solutes, prevent remaining water molecules in the cell to rearrange and form ice crystals (Benson *et al.*, 2006). However, formation of intracellular ice crystals is unavoidable if the freezing rate is slow where the mobility and diffusion of water molecules is still available before the plant cells achieve freezing state. In addition, de-vitrification might occur upon slow rewarming when metastable glass returning to either liquid or crystalline structure, which in turn reduce the viability of thawed explants (Benson *et al.*, 2006; Gonzalez-Arnao *et al.*, 2008). This can be controlled by manipulating the tissue water content as well as freezing and warming rates.