THE DEVELOPMENT OF ENCAPSULATION-DEHYDRATION AND VITRIFICATION PROTOCOLS FOR PROTOCORM-LIKE BODIES (PLBs) OF *Dendrobium* sonia-28

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by

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LIST OF ABBREVIATIONS

A	Adenine
AFLP	Amplified Fragment Length Polymorphism
AFPs	Antifreeze proteins
ANOVA	Analysis of variance
APX	Ascorbate peroxidase
ATP	Adenosine triphosphate
BAP	6-Benzyladenopurine
bp	Base pairs
BSA	Bovine serum albumin
BSV	Banana streak virus
C	Cytosine
C_3	C_3 carbon fixation pathway
CAT	Catalase
CIAT	International Center for Tropical Agriculture
CIP	International Potato Centre
CITES	Convention on International Trade in Endangered Species of Wild Fauna and Flora
CMV	Cucumber mosaic virus
Co.	Company
cv.	Cultivar
DHAR	Dehydroascorbate reductase
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate

DSC	Differential scanning calorimetry
EC	The Enzyme Commission number
EDTA	Ethylenediaminetetraacetic acid
EHT	Extra high tension
ESEM	Environmental scanning electron microscope/microscopy
et al.	*et alia*
EtBr	Ethidium bromide
FAO	Food and Agriculture Organization
FDA	Fluorescein diacetate/Food and Drug Administration
Fe	Ferum/iron
Fig.	Figure
G	Guanine
GPOD	Guaiacol peroxidase
GR	Glutathione reductase
HSPs	Heat shock proteins
Inc.	Incorporation
INIBAP	International Network for the Improvement of Banana and Plantain
IPGRI	The International Plant Genetic Resources Institute/Bioversity International
IVF	*In vitro* fertilisation
Lindl.	Lindley
L	Laevorotatory/litre
L.	Linnaeus
LEA	Late-embryogenesis-abundant proteins
LHCP	Light harvesting complexes

LN	Liquid nitrogen
LP	Leaf primordium/primordia
Ltd.	Limited
M	Mean
MDHAR	Monodehydroascorbate reductase
$MgCl_2$	Magnesium chloride
MS	Murashige and Skoog
NAA	1-naphthaleneacetic acid
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NBT-2HCl	Nitroblue tetrazolium 2-hydrochloride
nptII	Neomycin phosphotransferase II gene
OD	Optical density
P700	Pigment 700
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PEG1000	Polyethylene glycol 1000
PEG6000	Polyethylene glycol 6000
PGD	Polyethylene glycol-glucose-DMSO
PLB	Protocorm-like body
PLBs	Protocorm-like bodies
POD	Peroxidase
PSI	Photosystem I
PSII	Photosystem II
PVP	Polyvinylpyrrolidone
PVS2	Plant vitrification solution 2

RAPD	Rapid amplified polymorphic DNA
Rchb. f.	Reichenbach
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
Rubisco	Ribulose-1,5-bisphosphate carboxylase oxygenase
SAM	Shoot apical meristem
sam1	S-adenosylmethionine synthetase 1 gene
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Scanning electron microscope/microscopy
SI	Similarity index/indices
SOD	Superoxide dismutase
sp.	Species (singular)
spp.	Species (plural)
Taq	*Thermus aquaticus*
T	Thymine
TBA	Tertiary butyl alcohol
TBE	Trishydroxymethylaminomethane-borate-ethylenediaminetetraacetic acid
T-DNA	Transfer DNA
TE	Trishydroxymethylaminomethane-ethylenediaminetetraacetic acid
TM	Trademark
Tris-HCl	Trishydroxymethylaminomethane-hydrochloride

TTC	2,3,5-triphenyltetrazolium chloride
tTCL	Transverse thin cell layers
UK	United Kingdom
UNCED	United Nations Conference on Environment and Development
USA	United States of America
UV	Ultraviolet
Var.	Variant
VP SEM	Vapour pressure scanning electron microscopy

LIST OF SYMBOLS

Δ	Difference/increment
%	Percent/percentage
±	Plus or minus
+	Plus/with
−	Minus/without
/	Division/or
×	Hybridised with/times
16/8	16 hours light/8 hours darkness photoperiod
ºC	Degrees Celsius
ºC.min^{-1}	Degrees Celsius per minute
a	Extinction coefficient of the oxidation of hydrogen peroxide at 240nm (39.4M^{-1}.cm^{-1})
A_{240}	Absorbance at 240nm
A_{260}	Absorbance at 260nm
A_{280}	Absorbance at 280nm
A_{490}	Absorbance at 490nm
A_{560}	Absorbance at 560nm
cm	Centimetre
Cu	Copper
df	Degrees of freedom
ε	Average molar absorption co-efficient
ºF	Degrees Fahrenheit
FW_e	Fresh weight of plant tissues in the catalase assay
g	Gram

$gH_2O.g^{-1}$ DW	Gram water per gram dry weight
$g.l^{-1}$	Gram per litre
H^+	Hydrogen ion
H_2O	Water
H_2O_2	Hydrogen peroxide
KCl	Potassium chloride
KH_2PO_4	Potassium dihydrogen phosphate
kDa	Kilodalton
kV	Kilovolt
L	litre
$lm.W^{-1}$	Luminous efficacy, the ratio of luminous flux to power
λ_{max}	Maximum wavelength
M	Molar
$M^{-1}.cm^{-1}$	Molar per centimetre
mg	Milligram
$mg.L^{-1}$	Milligram per litre
$mg.mL^{-1}$	Milligram per millilitre
min^{-1}	Per minute
mL	Millilitre
mM	Millimolar
Mn	Manganese
Mn^{3+}	Manganese ion
mm	Millimetre
$[mol.L^{-1}]^{-1}.cm^{-1}$	litre per mole per centimetre
$\mu E.m^{-2}.s^{-1}$	Microeinstein per metre square per second

µg	Microgram
µg.mL^{-1}	Microgram per millilitre
µL	Microlitre
µm	Micrometre
µM	Micromolar
µmol.m^{-2}.s^{-1}	Micromole per metre square per second
N	Normality
$Na_2HPO_4.2H_2O$	Disodium hydrogen phosphate dihydrate
N_{xy}	Number of monomorphic RAPD bands between the control and treatment groups
N_x	Total number of RAPD bands in the control group
N_y	Total number of RAPD bands in the treatment group
nm	Nanometre
O_2	Molecular oxygen
O_2•	Superoxide radical
•OH	Hydroxyl radical
O_2^1	Singlet oxygen
p	p-value
®	Registered trademark
SD	Standard deviation
t	t-test value
T_d	Exothermic devitrification temperature
T*g*	Glass transition temperature
T_m	Melting temperature
U.g^{-1}	Enzyme units per gram

$U.\mu L^{-1}$	Enzyme units per microlitre
$U.mg^{-1}$	Enzyme units per milligram
$U.mL^{-1}$	Enzyme units per millilitre
V	Volt
V_e	Volume of the buffer in the catalase assay
V_f	Final volume in the catalase assay reaction mixture
V_0	Volume of catalase extract used in the catalase assay reaction mixture
v/v	Volume over volume
W	Watt
w/v	Weight over volume
Zn	Zinc

PEMBANGUNAN PROTOKOL-PROTOKOL PENGKAPSULAN-PENDEHIDRATAN DAN VITRIFIKASI UNTUK JASAD SEPERTI PROTOKOM (PLBs) *Dendrobium* sonia-28

ABSTRAK

Kajian ini menilai kesan penggunaan kaedah pengkrioawetan vitrifikasi dan pengkapsulan-pengeringan ke atas kemandirian jasad seperti protokom (PLB) hibrid orkid *Dendrobium* sonia-28. Kadar kemandirian protokom ditentukan melalui pemerhatian penjanaan semula protokom dan ujian 2,3,5-trifeniltetrazolium klorida (TTC). Kadar kemandirian yang tertinggi (16.0% penjanaan semula) bagi kaedah vitrifikasi diperolehi apabila protokom bersaiz 3-4mm dirawat seperti berikut: dikultur dalam media separa pepejal Murashige dan Skoog (1962) yang ditambah dengan sukrosa berkepekatan 0.4M, dimuat selama 20 minit dalam larutan pemuat, dinyahhidrat untuk 50 minit pada 0°C dalam larutan plant vitrification solution 2 (PVS2), dikrioawet dalam cecair nitrogen (LN) untuk 24 jam, dipanaskan dalam air bersuhu 40±2°C selama 90 saat, dinyahmuat dalam larutan sukrosa 1.2M selama 20 minit, dan dipulihkan dalam media separa pepejal MS yang ditambah dengan 2g.L^{-1} serbuk arang. Semua media ditambah dengan asid askorbik berkepekatan 0.6mM. Protokom yang dikrioawet didedahkan secara beransur-ansur kepada cahaya (kegelapan dalam minggu pertama, keamatan cahaya sebanyak 3.4μmol.m^{-2}.s^{-1} untuk tiga minggu seterusnya dan 150μmol.m^{-2}.s^{-1} pada minggu-minggu berikutnya). Kadar daya kemandirian terbaik (85.5% apabila diperhatikan untuk tanda-tanda kemerahan) dalam pengkapsulan-pengeringan dan pengkrioawetan diperolehi apabila protokom bersaiz 3-4mm dirawat seperti berikut: dikultur dalam media yang mengandungi 0.5M sukrosa selama 24 jam, dikapsulkan selama 30 minit menggunakan cecair

natrium alginat 3% (berat/isipadu) yang ditambah dengan sukrosa 0.4M dan kalsium klorida 0.1M, diosmolindung dalam larutan sukrosa 0.75M selama 24 jam pada suhu 25°C, dinyahhidrat dalam 50g gel silika selama empat jam, dikrioawet selama 24 jam, dipanaskan dalam air bersuhu 40±2°C untuk 90 saat, dan dipulihkan dalam media separa pepejal MS yang ditambah dengan 1mg.L^{-1} 6-benziladenopurin (BAP). Protokom tersebut didedahkan secara beransur-ansur kepada cahaya (kegelapan dalam minggu pertama, keamatan cahaya sebanyak 3.4µmol.m^{-2}.s^{-1} dalam minggu kedua dan 150µmol.m^{-2}.s^{-1} seterusnya). Analisis biokimia protokom yang dilakukan pada setiap peringkat kaedah vitrifikasi menunjukkan penurunan dalam kandungan protein protokom yang dikrioawet dengan perkembangan protokol, manakala peningkatan dicatatkan dalam aktiviti enzim catalase dan superoxide dismutase. Aktiviti catalase dan superoxide dimutase masing-masing memuncak pada peringkat pemulihan pertama (130.28U.g^{-1} protein) dan kedua (148.03U.mg^{-1} tisu protokom). Analisis RAPD protokom yang diperolehi dari kultur pembiakan, eksperimen kawalan pengkapsulan-pengeringan dan vitrifikasi, dan sampel protokom yang dikrioawet menunjukkan bahawa protokom yang dikrioawet melalui kaedah vitrifikasi tidak mengalami peyimpangan daripada kandungan genetik protokom asli, manakala protokom yang dikrioawet melalui kaedah pengkapsulan-pengeringan mempunyai kandungan genetik yang tidak stabil. Analisis histologi dan pengimbasan mikroskop elektron (SEM) protokom yang dikrioawet menunjukkan bahawa kerosakan akibat kitar pembekuan dan pemanasan berlaku pada sel parenkima protokom, dan hanya sel embryo yang mampu diselamatkan daripada rawatan pengkrioawetan.

THE DEVELOPMENT OF ENCAPSULATION-DEHYDRATION AND VITRIFICATION PROTOCOLS FOR PROTOCORM-LIKE BODIES (PLBs) OF *Dendrobium* sonia-28

ABSTRACT

The vitrification and encapsulation-dehydration methods of cryopreservation were applied on protocorm-like bodies (PLBs) of the orchid hybrid *Dendrobium* sonia-28, with survival assessments conducted through growth observations, visual- and spectrophotometric 2,3,5-triphenyltetrazolium chloride (TTC) assays. The best survival (16.0% regeneration) for vitrification was obtained when 3-4mm PLBs were precultured in semi-solid half-strength MS medium supplemented with 0.4M sucrose, loaded in a loading solution for 20 minutes, dehydrated for 50 minutes at 0°C in plant vitrification solution 2 (PVS2), cryopreserved in liquid nitrogen (LN) for 24 hours, thawed in a 40±2°C water bath for 90 seconds, unloaded in 1.2M sucrose for 20 minutes, and regenerated in semi-solid half-strength MS medium containing $2g.L^{-1}$ charcoal, with all media supplemented with 0.6mM ascorbic acid. The PLBs were gradually exposed to light (darkness in the first week of recovery, exposure to $3.4\mu mol.m^{-2}.s^{-1}$ for the subsequent three weeks and $150\mu mol.m^{-2}.s^{-1}$ thereafter). The best viability rate (85.5% when observed for any signs of redness) for encapsulation-dehydration was obtained when 3-4mm PLBs were pretreated in semi-solid 0.5M sucrose medium, encapsulated for 30 minutes using 3% (w/v) liquid sodium alginate medium supplemented with 0.4M sucrose and 0.1M calcium chloride, osmoprotected in 0.75M sucrose solution for 24 hours at 25°C, dehydrated using 50g heat-sterilised silica gel for four hours, cryopreserved for 24 hours, thawed in a 40±2°C water bath for 90 seconds, and regenerated in semi-solid half-strength

MS medium followed by a transfer to medium supplemented with 1mg.L^{-1} 6-benzyladenopurine (BAP), with gradual exposure to light (darkness in the first week of recovery, exposure to 3.4µmol.m^{-2}.s^{-1} in the second week and 150µmol.m^{-2}.s^{-1}thereafter). Biochemical analyses of PLBs subjected to the vitrification-cryopreservation experiment indicated a general decrease in the total protein content of cryopreserved PLBs with progression of the protocol. A general increase was recorded in the catalase and superoxide dismutase activities, with both peaking at the first (130.28U.g^{-1} plant tissue) and second (148.03U.mg^{-1} protein) recovery stages respectively. The RAPD analyses of PLBs obtained from the stock culture, control encapsulation-dehydration and vitrification experiments, and frozen samples from encapsulation-dehydration and vitrification experiments indicate that PLBs that were vitrified and cryopreserved were genetically similar to the stock culture, while those that were encapsulated, dehydrated and cryopreserved were not genetically stable. Histological and scanning electron microscopy (SEM) analyses of vitrified and cryopreserved PLBs indicated that the freezing and thawing cycles inflicted damages to the parenchymal cellular regions of the PLBs. Only embryogenic cells survived the treatment.

CHAPTER 1

INTRODUCTION

The Orchidaceae, a large flowering family, is economically important as cut flowers and potted plants in the international floriculture industry (Arditti, 1992; Kuehnle, 2007; Khosravi *et al.*, 2009). The orchid genus *Dendrobium* is increasingly popular due to its floriferous flower sprays, wide spectrum of colours, sizes and shapes, year-round availability, and long flowering life (Kuehnle, 2007; Khosravi *et al.*, 2009). *Dendrobium* sonia-28, a hybrid resulting from the cross between two different hybrids, *Dendrobium* Caesar and *Dendrobium* Tomie Drake, is prized for its pink-coloured and good cut flowers (Van Rooyen Orchids Catalogue, 2007). Categorised as a *Dendrobium Phalaenopsis* orchid, the hybrid is popular as potted plants due to their long-lasting flower sprays. Various explants of the orchids are able to form structures similar to protocorms or zygotic embryos *in vitro* (Arditti and Ernst, 1993; Mayer *et al.*, 2010). Known as protocorm-like bodies or PLBs, they are produced through the process of direct or indirect embryogenesis in orchids (Arditti and Ernst, 1993; Begum *et al.*, 1994; Zhao *et al.*, 2008; Mayer *et al.*, 2010), and are able to be mass-produced in short periods of time for the propagation of orchid plants (Morel, 1963, 1974).

Somaclonal variation in *in vitro* plants may occur due to genetic or epigenetic reasons and is imminent with prolonged and numerous subcultures for plant multiplication or regeneration (Häggman *et al.*, 2008). In order to preserve the unique genomic constitution of various cultivars of plant species such as yam, taro and garlic and ornamental plants such as lily and orchids, many of them are propagated vegetatively in field collections and *in vitro*. Cryopreservation, however,

is the 'ultimate' preservation method as plant tissues placed under such conditions may be preserved for unlimited durations of time without undergoing alterations (Panis, 2008).

Ice-free cryopreservation is one of the main approaches in preserving plant germplasm (Benson, 2004), and various cryoprotectants have been considered in the application of the vitrification technique on plant and algal germplasm (Harding *et al.*, 2004; Benson *et al.*, 2008; Benson, 2008). Cell viscosity is enhanced through the addition of cryoprotective substances at high concentrations, and by removing water from the target explants through evaporative desiccation and/or osmotic dehydration (Benson, 2008). Vitrification can be defined as "the solidification of a liquid brought about not by crystallization but by an extreme elevation in viscosity during cooling" (Fahy *et al.*, 1984; González-Benito *et al.*, 2004). A solution turns into an amorphous glassy solid, or glass, as a result of vitrification. Vitrification can be achieved in plant cells through the reduction in intra- and extracellular freezable water, and this occurs when plant tissues are exposed to highly concentrated cryoprotective mixtures or to physical dehydration prior to rapid cooling by direct immersion in liquid nitrogen. Vitrification techniques can be applied to complex structures such as embryos and shoot apices (Withers and Engelmann, 1997; González-Benito *et al.*, 2004). Two types of vitrification techniques exist: vitrification (in literal terms) and encapsulation-dehydration. The techniques may also be combined to conserve explants (González-Benito *et al.*, 2004).

More than one method of dehydration exists for encapsulation-dehydration treatments. Encapsulated explants can be exposed to a defined amount of silica gel in hermetically-sealed glass jars for a specific period of time (Swan *et al.*, 1998), or dried in a laminar air flow bench (Fabre and Dereuddre, 1990; Bachiri *et al.*, 1995;

Heine-Dobbernack *et al.*, 2008), prior to storage in liquid nitrogen. The dehydration duration relies on an explant's initial water content and its tolerance towards the drying process. The best final moisture content usually ranges from 20% to 30% of the initial wet weight of the beads (Heine-Dobbernack *et al.*, 2008).

The main objective of any cryoprotective vitrification strategy is to heighten cell viscosity until ice formation can be inhibited and water is vitrified on exposure to cryogenic temperatures. Highly concentrated cryoprotective solutions such as glycerol are very viscous and are easily supercooled to temperatures below –70°C. The formation of glass in the system is also said to prevent further dehydration in cooled samples as glass is presumed to possess lower water vapour pressure than the corresponding crystalline solid (Burke, 1986; Sakai *et al.*, 2008). Various combinations of cryoprotectants had proven to be successful in the early stages of slow cooling experiments (Reed and Uchendu, 2008). A cryoprotective mixture known as PGD, comprising of 10% polyethylene glycol (PEG), 8% glucose and 10% dimethylsulfoxide (DMSO, w/v), reduced the toxicity of the solution and increased post-cryopreservation survival rates of callus cultures of *Saccharum* sp. (Ulrich *et al.*, 1979), while the combination of DMSO and sorbitol improved recovery rates of cryopreserved cells of *Catharanthus roseus* (L.) Don. (Reed and Uchendu, 2008). The action of combining various cryoprotectants reduces the toxicity of individual components (Chen *et al.*, 1984), as attested by Smith (1983) and Tao and Li (1986), with both groups reporting encouraging results when using combinations of DMSO, glucose and PEG (Reed and Uchendu, 2008). A modification of the plant vitrification solution 2, or PVS2 (Sakai *et al.*, 1990; Sakai *et al.*, 1991; Reed and Uchendu, 2008), was successfully applied in the controlled rate cooling procedure for *Prunus* rootstocks (Brison *et al.*, 1995; Reed and Uchendu, 2008).

Differences can be observed in the types of cryoprotection technique conferred on the target cells and the resulting injury to the sample from the technique. In the case of controlled rate cooling and colligative cryoprotection, an organism must be able to withstand chilling, extracellular freezing and osmotic stress. However, vitrification requires the organism to tolerate high osmotic stress and desiccation injury (Harding *et al.*, 2004; Benson *et al.*, 2007). Many simplified methods of cryopreservation have been developed for the long-term storage of embryogenic callus clumps, allowing direct immersion in LN. For instance, vitrification-based and encapsulation-dehydration procedures were developed for embryogenic cultures of important crop and plant species such as *Citrus* spp., *Olea europaea*, *Fraxinus* spp., *Quercus* spp., *Oryza sativa* (Lambardi *et al.*, 2008).

Plants are able to sense and respond to the abiotic or biotic stress that is subjected on them by communicating the stress signal to downstream components that leads to target genes being switched on and/or off (Reddy *et al.*, 2008). Osmotic injuries may occur during a cryopreservation procedure from the use of highly-concentrated additives, which cause toxicity, while evaporative dehydration could result in desiccation sensitivity. Cellular injuries may also occur on thawing as a result of devitrification and ice formation. The application of various cryoprotective strategies for cryobanking therefore relies on the resistance of the target organism or cell type to cryoinjuries, and the potentially deleterious effects of cryoprotection (Harding *et al.*, 2004; Benson *et al.*, 2007). Reactive oxygen species (ROS) are generated when plants possess impaired stromal metabolism, but experience highly energized primary photochemistry. The ROS cause membrane damage through the formation of radicals, which often occurs during dehydration and cryopreservation (Margesin *et al.*, 2007). Earlier studies have indicated that plants are able to limit

damages incurred from dehydration by maintaining the physiological integrity in the desiccated state and possessing repair mechanisms to recover from the damage upon rehydration (Bewley, 1995; Reddy *et al.*, 2008). Microorganisms, plants, and animals synthesize various intracellular compatible solutes such as polyols and sugars for both external and internal protection against intracellular freezing (Gounot and Russell, 1999; Margesin *et al.*, 2007). Plants also possess efficient antioxidant systems to scavenge ROS during low and/or high temperature stress (Liu and Huang, 2000; Djanaguiraman *et al.*, 2010). Examples of such enzymes include superoxide dismutase (SOD), catalase (CAT), peroxidases (POD), and gluthatione reductase (GR) (Yordanova *et al.*, 2004).

Cryopreservation may cause physical, physiological or chemical damages and stress to plant tissues, which may potentially contribute to changes in the genomic contents of the explants. However, the variations may also be caused by the entire cryopreservation procedure, beginning from the explant propagation and preculture, right up to the regeneration step, instead of originating from the cryostorage step itself (Harding *et al.*, 2004; Martín and González-Benito, 2005). Examples of techniques used in the assessment of genetic stability in cryopreserved plants include Random Amplified Polymorphic DNA (RAPD) analysis (Jokipii *et al.*, 2004; Benson *et al.*, 2007).

The cryopreservation of PLBs of *Dendrobium* sonia-28 presents a unique opportunity of preserving the desired genotypes and phenotypes of the orchid that promotes its desirability and marketability. No research has been conducted on the development of an encapsulation-dehydration or vitrification protocol for this orchid hybrid.

1.1 Objectives of research

The objectives of this research project were:

- To optimise the encapsulation-dehydration and vitrification methods for the orchid hybrid *Dendrobium* sonia-28.
- To develop an efficient plant recovery and regeneration system for the orchid hybrid *Dendrobium* sonia-28.
- To observe the effects of the vitrification treatment upon the PLBs of *Dendrobium* sonia-28 through histological and scanning electron microscopy (SEM) preparations.
- To detect and quantify the total protein, catalase and superoxide dismutase contents in PLBs of the orchid hybrid *Dendrobium* sonia-28 subjected to each stage of the vitrification experiment.
- To assess the genetic stability of encapsulation-dehydration and vitrification-treated *Dendrobium* sonia-28 orchid plantlets using the RAPD technique.

CHAPTER 2

LITERATURE REVIEW

2.1. Orchids: geography, morphology and importance

"The growing of orchid is not only an economically important industry but also an important factor in keeping many individuals sane and happy in this disturbing world" (Sanford, 1974).

Orchidaceace is one of the largest families of flowering plants in the world with an estimated 800 genera (Arditti, 1992), 25,000 to 30,000 species and more than 150,000 artificial hybrids (Yue *et al.*, 2006). Orchids can be found in almost all the regions of the world, except in the deserts and perpetually icy regions (Jezek, 2003). The word 'orchid' was derived from the Latin word *orchis*, literally translated as testicle, due to the similarity in shape of the tubers of some European terrestrial species to the male genitalia. As many as 90% of the world's orchid populations are found in the tropical climatic regions: Asia (10,000 to 15,000 species), Central America (1,000 species), South America (6,000 to 8,000 species) and Africa (2,000 species). About 700 species can be found in Australia, 200 species in North America and another 200 species in Europe. Not all orchids are thermophilic, as orchids can be found at the lowland, montane or submontane levels. For instance, some species of the *Coelogyne* can be found as high as 3,000m above the sea level in the Himalayas, while orchids from the genera *Lemboglossom* and *Odontoglossum* can be found at 4,000m above the sea level in the South American Andes (Jezek, 2003).

Theories on the high number of members in the Orchidaceae family largely centre on the relatively young age of the family, as it has been speculated that the

first orchid species arrived 50 to 60 million years ago, compared to the rise of the Angiosperms about 130 million years ago (Jezek, 2003). Hence, there is still 'time' for the family to branch out, evolve and expand. This theory is also supported by the fact that many orchids of the same or different genera are able to interbreed without much difficulty, giving rise to many viable variants that are completely different from the parents, and yet are still able to reproduce, despite the genetic irregularities found in some species or hybrids. Many hybrids are the results of human interference, rather than random interbreeding (Jezek, 2003). Both wild orchids and hybrids have the following four characteristics: bilaterally symmetrical flowers, sticky masses of pollen grains called pollinia, minute seeds containing undeveloped embryos with no nutritive materials and the ability of seeds to only germinate with the presence of a symbiotic fungus under natural conditions (Jezek, 2003).

Orchids are also categorised into two different groups: monopodial or sympodial (Morel, 1974). The classification of orchids as monopodial or sympodial depends on the development of their apical meristems or growing points. The meristem is self-perpetuating and ensures both the growth of stems and the differentiation of new leaves. As the cells divide, the offspring remains in the meristem and when new differentiated organs are formed, the meristem is perpetuated to these other parts. When the growth of the apical meristem is continuous, the orchid plant structure is known as monopodial. In other orchids, a lateral meristem starts to bud when the main growing point stops dividing, hence creating a structure known as sympodial (Morel, 1974). Examples of monopodial orchids include *Phalaenopsis* and *Vanda*, while sympodial orchid include *Dendrobium* and *Cattleya* (Morel, 1974).

Almost all orchids are listed under the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) as threatened and endangered (Nikishina *et al.*, 2007). The Russian Red Book listed 44 out of 123 wild orchid species in 1990, and the list is currently expanded to include another 22 species, with some enlisted as extinct ("Spisok zhivotnykh i rastenii, podpadayushchikh pod deistvie SITES [List of Animals and Plants Which Fall under SITES Jurisdiction]", 1998; Nikishina *et al.*, 2007). The orchids are susceptible to extinction as they possess highly vulnerable complex reproductive biology that involve specialized cross-pollination, long developmental cycles of up to 17 years, and obligate symbioses with specialized fungi necessary for the germination of microscopic embryos lacking in reserve compounds. Furthermore, orchids are also vulnerable to anthropogenic effects, and are frequently harvested for their decorative flowers and curative properties of the plant parts (Perebora, 1998; Golovkin *et al.*, 2001; Nikishina *et al.*, 2007).

Orchids represent 8% of the global floriculture trade, and over 100,000 registered commercial hybrids are grown as cut flowers and potted plants (Martin and Madassery, 2006; Vendrame *et al.*, 2007). Many orchids, although expensive, are highly in demand in the national and international markets due to their diversity in terms of size, shape, flower colour and longevity (Saiprasad *et al.*, 2004). Clonal propagation of orchid plantlets had been previously conducted through the conventional backbulb culture, which could take about 10 years in order to raise a good-sized propagation (Blowers, 1964; Morel, 1974). The cut flower industry is programmed in such a way that the required flower is ready on the exact day it is needed. Therefore, there is a need for pure lines or clones of the orchid hybrids as these are able to give exact responses to the cultural treatments. The task of raising a

clone of orchids with uniform characteristics for industrial cultivation is difficult as many cultivated orchids, being complex hybrids, are highly heterozygous in nature, hence the difficulty in breeding pure lines out of them (Morel, 1974).

In vitro propagation of orchids has emerged as an option for rapid propagation of commercially valuable cultivars as the conventional *in vivo* vegetative propagation presents with problems such as slow multiplication rate, high financial demand and insufficient production of clones within a short timeframe (Saiprasad and Polisetty, 2003; Martin and Madassery, 2006). *In vitro* culture has also made it possible to preserve orchids, since the advent of asymbiotic seed germination (Kulikov and Filippov, 1998; Andronova *et al.*, 2000; Nikishina *et al.*, 2001; Nikishina *et al.*, 2007). However, the maintenance of *in vitro* collections requires manual labour and causes the accumulation of somaclonal variations and phenotype-based involuntary selections (Butenko, 1999), which result in the homogeneity of the orchid population (Ivannikov, 2003) and depletion of the gene pool (Nikishina *et al.*, 2007).

2.2. Orchids of the genus *Dendrobium*

The genus *Dendrobium*, established by Olaf Swartz in 1799, consists of the largest diversity of interesting specimens in terms of horticultural importance, and is made up of more than 1100 species distributed throughout the world, ranging from Southeast Asia to New Guinea and Australia, making it the second-largest orchid genus in the orchid family after *Bulbophyllum* (Puchooa, 2004). *Dendrobium* orchids are classified as crassulacean acid metabolism (CAM) plants (He *et al.*, 1998), as the light-dependent decarboxylation of citrate or malate is a crucial source of carbon

dioxide for alleviating photoinhibition in those plants (Haag-Kerwer *et al.*, 1992; He *et al.*, 1998). Orchids of the genus *Dendrobium* are epiphytes possessing connected stems known as pseudobulbs, each with the capacity of producing one or few inflorescence (Yue *et al.*, 2006).

Dendrobium, possessing traits such as high number of flowers per inflorescence and flowering recurrence, is usually propagated sexually by seeds and asexually by division of offshoots, and occupy one of the top positions in the cut flower industry (Martin and Madassery, 2006). The genus *Dendrobium* accounts for about 80% of the total micropropagated tropical orchids (Debergh and Zimmerman, 1991; Saiprasad *et al.*, 2004). The genus' commercial value lies in the variety of flower colours and patterns, and the relatively short production cycle from seedling to a full bloom plant. Most *Dendrobium* hybrids produce flowers that are lavender, white or golden-yellow in colour, with some having combinations of these colours (Puchooa, 2004). Rare specimens may consist of bluish, ivory, brilliant orange or scarlet flowers, with exotic markings. Most evergreen species of *Dendrobium* do not produce fragrance; while some deciduous species such as *D. superbum*, *D. pierardii* and *D. parishii* may produce fresh citrus-like scents, or smell of raspberries (Puchooa, 2004). Although popular as ornamentals, many *Dendrobium* species such as *Dendrobium loddigesii* also possess medicinal value but are limited in their distribution as a result of their minimal ecology study (Yue *et al.*, 2006).

International production of potted *Dendrobium* plants spiked up in the last few years, with large-scale production occurring in the Netherlands, Germany, China, Taiwan, Thailand, Philippines, Unites States, and Japan (Puchooa, 2004). Mass production of hybrids of *Dendrobium* is conducted via *in vitro* germination of hybrid seeds as most orchid seeds readily germinate after direct harvest from the

mother plant or can be stored for later germination (Vendrame *et al.*, 2007), and by protocorms (Hawkes, 1970; Saiprasad *et al.*, 2004). Problems associated with the genus include low or no seed setting and germination, and heterozygous seedling progenies that are not true-to-type plants of hybrid cultivars (Martin and Madassery, 2006). Other problems include minute seed size, presence of reduced endosperm, and the requirement of an association with mycorrhizal fungi (Saiprasad and Polisetty, 2003).

2.3. *Dendrobium* sonia-28

Dendrobium sonia-28, a hybrid resulting from the cross between two different hybrids, *Dendrobium* Caesar and *Dendrobium* Tomie Drake, is prized for its pink-coloured and good cut flowers (Plates 2.1A and B). Categorised as a *Dendrobium Phalaenopsis* orchid, the hybrid are popular as pot plants due to their long-lasting flower sprays. Growth conditions for the evergreen and warm-growing hybrid include good air movement and strong light (Van Rooyen Orchids Catalogue, 2007).

Martin and Madassery (2006) succeeded in introducing a micropropagation technique that promoted commercial level production of *Dendrobium* hybrids sonia-17 and 28, two highly priced cut flower hybrids through direct shoot induction from foliar explants and subsequent PLBs induction and plant regeneration. The PLBs of *Dendrobium* sonia-28 follows the following developmental phases: the pro-meristematic stage from between eight to 10 days old, the leaf primordia stage from between 13 to 15 days old and the formation of the first embryonic leaves at between 18 to 20 days old (Saiprasad and Polisetty, 2003). Puchooa (2004) has shown that

Dendrobium sonia is amenable to large-scale propagation by using liquid medium for PLB initiation and applying supplements such as banana, wood chips, sand, rock sand, coconut coir and vermiculite for plantlet regeneration.

2.4. Orchid protocorm and protocorm-like bodies (PLBs)

The current orchid clonal propagation scene is based on the regeneration of newly-formed protocorms through protocorm sections (Morel, 1974; Saiprasad and Polisetty, 2003). The growth of a new bud upon a protocorm is absolutely identical to the growth of a seedling (Plate 2.2), with the leaves produced at the tip of the bud and the appearance of a root at the base of the bud when the plantlet is about 1cm long (Morel, 1963, 1974). It has been shown that protocorms that were sectioned into a few parts and subcultured into new medium regenerated into a new protocorm clump instead of differentiating into a bud. This enables the growth of protocorm cultures to be maintained for an unlimited period of time, and at a fantastic rate. For instance, each protocorm of the orchid *Cymbidium*, when sliced into four, will be able to regenerate at a factor of eight to more than a billion plantlets in nine months, if each sliced piece regenerates into two new protocorms (Morel, 1974).

In a study conducted by Morel (1974) to determine the nature of protocorm proliferation, adult protocorms 2.5-3.0mm in diameter were sliced into four sections in a plane at a right angle with the main axis. The sections were then peeled by removing a sheet of cells comprising the epidermis and three to four layers of the subepidermal cells. When all the sections were subcultured into new media, only the epidermal fragments proliferated and formed new protocorms. The central parenchyma remained alive for a few months, with no cell divisions observed.

An anatomical study conducted on *Cymbidium* protocorms indicated that cell divisions occurred periclinally in the outer cell layers, beginning within or below the subepidermal layer, but rarely in the epidermis itself (Morel, 1974). The divisions induce the random proliferation of between two to six active meristematic cells on the protocorm surface, giving rise to small protocorms within a few days. The leaf primordium forms first as a result of the differentiation of the protocorm cells, followed by the formation of a procambial strand below the leaf primordium, with the apex still undifferentiated. Although a few leaf primordial and procambial strands are formed in the initial stage, none of the pairs will grow and proliferate further until one of them forms a growing point with leaves in a distichous phyllotaxy. The growth of the other pairs is then inhibited, and resumes only when excised. However, a few pairs may develop together, forming a large protocorm with several buds (Morel, 1974).

Various orchid explants that were subjected to *in vitro* micropropagation have produced bodies which appeared similar to seedling protocorms in terms of their structure and growth (George and Debergh, 2008). Termed as 'protocorm-like bodies' (PLBs) by many orchid enthusiasts and workers, these somatic protocorms may not be visibly similar to seedling protocorms, for instance, in terms of their colour on synthetic growth media, but they are considered as a manifestation of embryogenesis as they can be derived directly from zygotic embryos and on various orchid explants or other PLBs, comparable to somatic embryogenesis (Champagnat and Morel, 1972; Norstog, 1979; George and Debergh, 2008). Protocorm-like bodies are versatile orchid organs that can be induced from various orchid explants, for instance from axillary buds, flower stalks, cell suspension and callus cultures in the case of *Doritaenopsis* (Tsukazaki *et al.*, 2000; Islam *et al.*, 2003).

In a study conducted by Vyas *et al.* (2010) to perform micropropagation of PLBs of *Cymbidium* Sleeping Nymph through transverse thin cell layers (tTCL), SEM studies also showed that new PLBs were formed from the peripheral region of the tTCL. Confocal laser scanning micrograph showed deeply stained fluorescing prominent nuclei in the subepidermal parenchymatous tissue of the tTCL after 10 days of culture, indicating meristematic activity of the cells. Newly formed PLBs showed tightly packed smaller cells with large fluorescing nuclei, while cells at the central region of the tTCL failed to fluoresce, indicating senescence, possibly due to degeneration of the nuclei. The cells in the subepidermal region of a 30-day old PLB were of two sizes: small polyhedral towards the centre and periphery and large ones between the two layers of polyhedral cells. The cells were compactly packed with no intercellular spaces. The formation of the PLBs was traced to the small polyhedral cells that were found to be meristematically active as observed through confocal microscopic study. Histological analysis of the tTCL indicated that the PLBs formed after 30 days of culture from the subepidermal parenchymatous region of the tTCL.

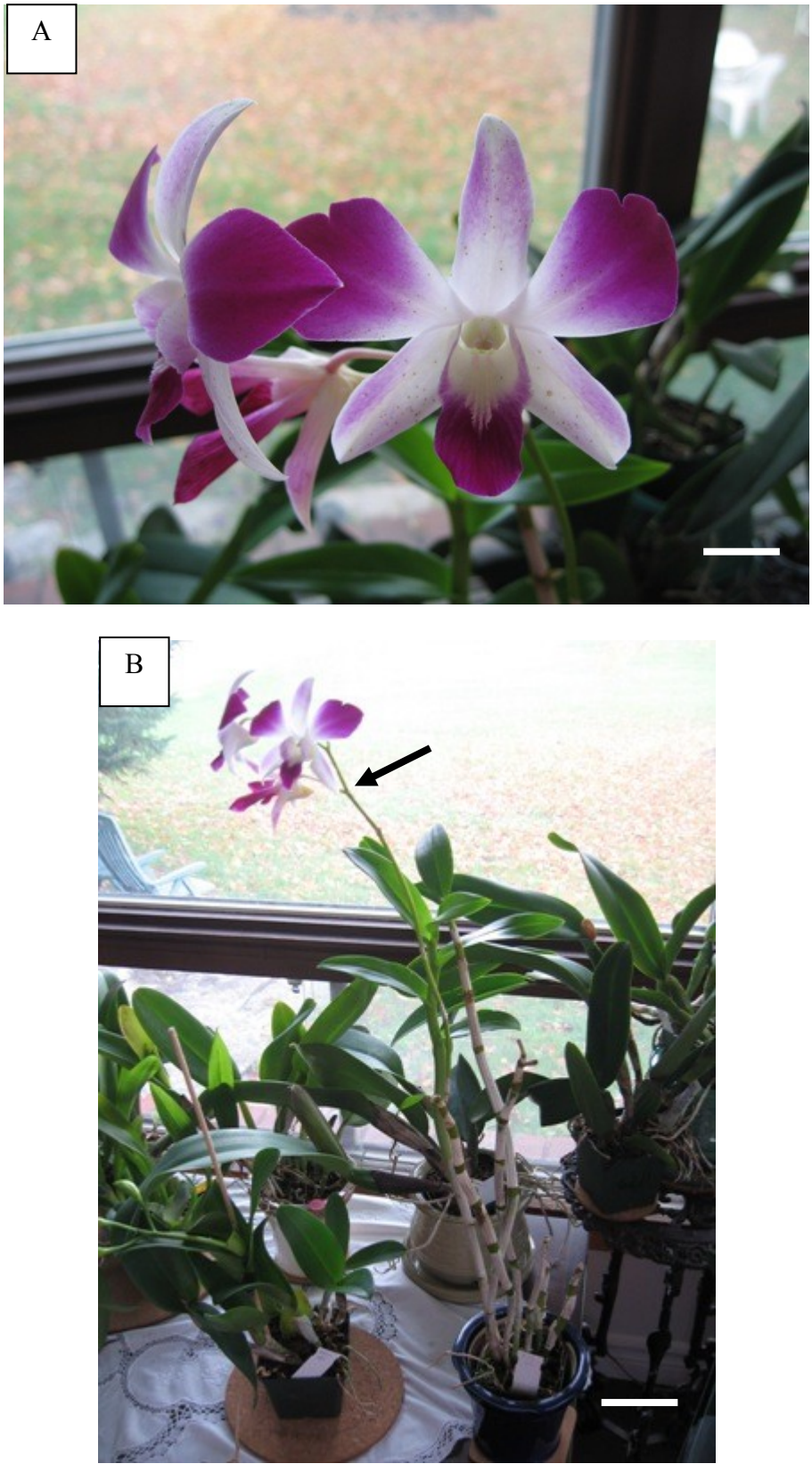

Plate 2.1. The orchid hybrid *Dendrobium* sonia-28 (OrchidBoard.com, 2007).
A. Inflorescences. Bar = 1cm.
B. A potted plant (arrow). Bar = 10cm.

Plate 2.2. The *in vitro* proliferation of PLBs and plantlets from a single PLB (arrow) of *Dendrobium* sonia-28 within three months of culture on semi-solid half-strength MS medium supplemented with 2% (w/v) sucrose and 0.2% (w/v) charcoal. Bar = 1cm.

2.5. Types of conservation

The preservation of plant genetic resources can be conducted through *ex situ* and/or *in situ* conservation. According to Article 2 of the Convention on Biological Diversity (UNCED, 1992; Engelmann, 2000), *in situ* conservation involves the preservation and recovery of viable populations of species in their natural ecosystems and habitats, including the maintenance and recovery of their natural surroundings, for instance through genetic reserves, on-farm and home garden conservation. For domesticated or cultivated species, *in situ* conservation takes place in the surroundings where their characteristic properties were developed (Maxted *et al.*, 1997; Engelmann, 2000). Plant samples maintained under *in situ* conditions will continually experience change by environmental factors, ageing and evolutionary progression (Benson *et al.*, 2007).

Ex situ conservation is defined as the conservation of components of biological diversity outside of their natural habitats (Maxted *et al.*, 1997; Engelmann, 2000). *Ex situ* cryobanks complements the *in situ* conservation of biodiversity by capturing the genetic and physiological state of the organism at the point of introduction into the cryobank (Benson *et al.*, 2007). Examples of *ex situ* conservation include seed storage, *in vitro* storage, DNA storage, pollen storage, field genebanks and botanical gardens (Maxted *et al.*, 1997; Engelmann, 2000). The preservation of strains from endangered and at risk provenances have emerged as an integral application of *in vitro*-cryopreserved culture collections (Benson *et al.*, 2007).

2.6. The cryopreservation theory and history

In vitro cryopreservation, a component of the *ex situ* conservation, involves the storage of viable cells at ultra-low temperatures (-196ºC), usually in gas-phase or liquid nitrogen (Benson *et al.*, 2007). The metabolic activities of the cells are assumed to be arrested at such temperatures, hence stabilising the cells for indefinite periods as long as the liquid nitrogen supply is maintained. Survival of cells after the cryogenic treatment is common to an amazingly diverse range of species, and the *in vitro* cryobank is one of the most extreme low-temperature environments that an organism or its component will ever encounter on earth (Benson *et al.*, 2007).

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). Great importance is currently placed on preserving cultured cells and somatic embryos that express unique characteristics due to the increasing interest in plant genetic engineering (Sakai *et al.*, 2008). Cryopreservation may also assist in the preservation of endangered and rare plants (Touchell, 1995; Touchell and Dixon, 1996; Sakai *et al.*, 2008). A simple and yet reliable cryopreservation method could facilitate widespread storage of cultured cells, meristems, and somatic embryos (Sakai *et al.*, 2008).

Cryopreservation is the only technique that provides a safe, efficient and cost-effective long-term storage option that facilitates the conservation of plant genetic resources (Engelmann *et al.*, 2008). Cryopreservation can be thought of as either a primary or secondary mode of storage of plant samples, and not as the sole source of clonal plant preservation (Reed, 2008). Primary storage is conducted for embryogenic cultures that may lose their capacity for embryo formation with time, as

the cultures can be revived and used to produce more embryos in the future. Secondary storage through cryopreservation acts as a secure backup for living plant collections in the conservation of plant genetic resources (Reed, 2008).

Cryopreservation research began through efforts of freezing and preserving animal cells. Freezing is defined as the conversion of liquid water to crystalline ice, resulting in the concentration of dissolved solutes in the remaining liquid phase and the precipitation of solutes exceeding their solubility limit. Animal cells were only successfully frozen, with their structure and function intact, after 1948, with the discovery of a general method that allows the freezing of many types of animal cells. Freezing injury theories then capitalised on ice crystals piercing or teasing apart the cells and intracellular structures, destroying them through direct mechanical action. Polge *et al.* (1949) (as reviewed by Pegg, 2007), serendipitously discovered that the inclusion of between 10–20% of glycerol enabled the spermatozoa of roosters to survive prolonged freezing at -80°C. Glycerol was said to reduce the amount of ice formed in tissues through an increase in the total solute concentration, in the same way that antifreeze (ethanediol) reduces the amount of ice forming in the cooling system of an automobile engine (Pegg, 2007).

The freezing of an aqueous solution was also recognized to increase solute concentration in the reducing volume of the remaining solution, hence causing injury to the tissues as well (Pegg, 2007; Benson *et al.*, 2008). As reviewed by Pegg (2007), this idea, termed as the solution effect, was propagated by James Lovelock, who in a series of publications in the 1950's (Lovelock, 1953a, b) provided strong evidence that salt concentration is the cause of freezing injury to cells, instead of ice, and that glycerol protects tissues from this damage through the modulation of the rise in salt concentration during freezing. Hence, the effectiveness of glycerol, or of any similar

cryoprotectant, is dependent on factors such as the high penetrability of the cryoprotectant in the target cells; the solubility of the compound in water, even at low temperatures in order to effectively depress the freezing temperature; and low toxicity of the compound to allow its use at high concentrations required to produce these effects. Compounds sharing these cryoprotective traits include glycerol, DMSO, ethanediol, and propanediol (Pegg, 2007). These discoveries kick-started modern cryobanking techniques (Fuller, 2004; Leibo, 2004) and its use for assisted reproduction via *in vitro* fertilization (IVF) and cell, tissue and organ storage (Benson *et al.*, 2007).

Plant cryopreservation is a relatively new field as the first reports on successful explant cryostorage was published by Sakai (1960) involving silver birch twigs, and by Quatrano (1968) using *in vitro* cultured flax cells (Benson *et al.*, 2007). Maximov pioneered studies of plant cryopreservation by highlighting the importance of sugars in natural freeze tolerance (Diller, 1997; Benson *et al.*, 2007). The 1980s witnessed the advent of controlled rate cooling cryopreservation, which is based on freeze-induced dehydration (Sakai, 1985; Kartha and Engelmann, 1994; Engelmann, 1997b; Engelmann *et al.*, 2008) and involved pretreatment with cryoprotectants. The procedure was especially successful on many temperate plants, with the same level of success not seen when many tropical plants were tested (Haskins and Kartha, 1980; Bagniol *et al.*, 1992; Engelmann *et al.*, 2008). This led to the development of vitrification-based protocols in the 1990s (Engelmann, 2000, 2003; Engelmann *et al.*, 2008). The implementation of improved cryoprotection strategies has also assisted phytodiversity cryopreservation efforts considerably (Day *et al.*, 2005; Benson *et al.*, 2007; Benson, 2008).

2.7. The role of water and ice in cryopreservation

The issue on how liquid water (H_2O) associate to form ice is still a point of contention (Franks, 1972; Mazur, 2004; Benson *et al.*, 2008; Benson, 2008). It is important to control or avoid intracellular ice nucleation, also known as seeding, in any cryopreservation protocol. Ice nucleation can cause mechanical injuries and physical ruptures as a result of the damage to the structural, osmotic and colligative integrity of cells, while colligative damage results from the increase in solute concentrations that disrupt cellular function (Benson, 2008).

2.7.1. Extra- and intracellular ice formation

The initiation of ice crystals in cryopreserved explants relies on the moderation of various factors between the extracellular and intracellular components (Benson *et al*., 2008; Benson, 2008). Water rarely freezes at 0°C, contrary to the popular belief, and supercooling of the water molecules occurs at freezing points below zero in the absence of templates that allow H_2O molecules to aggregate. Homogeneous ice nucleation occurs at around –40°C, which is the lowest possible supercooling temperature in most biological systems. At this stage, water molecules aggregate and form an "ice embryo" of a critical size that is thermodynamically capable of growing a crystal, creating an ordered matrix that releases energy as the latent heat of fusion and forming ice as heat is produced. Following the ice nucleation event, ice crystals grow into complex clusters that form networks with other crystals, causing exponential ice growth as more water molecules participate in complex alignments with each other (Benson *et al*., 2008; Benson, 2008).

Freezing usually occurs when the energy levels are favourable towards ice formation and when ice nucleation templates are available (Benson *et al*., 2008; Benson, 2008). Intracellular ice nucleation occurs as a result of the increase in local thermal properties of a system that energetically induces water molecules to approach each other and form sufficient hydrogen bonds to enable initiation of an embryonic ice crystal. With the exception of a rapid decrease in temperatures, ice normally forms in extracellular regions, as water molecules move to the extracellular area in order to establish osmotic equilibrium when a deficit is created between the partially frozen components outside of the cell and the unfrozen components inside the cell (Benson *et al*., 2008; Benson, 2008). Cells undergoing extracellular ice formation shrink, and the rate of water loss can hence be expressed as a function of cell size (Benson, 2008).

The cellular water movement, which occur through diffusion and aquaporin pores, is affected by the plasma membrane's permeability and osmotic gradient (Tyerman *et al.*, 2002; Benson, 2008). This phenomenon is more apparent in animal cells compared to plant cells which are rigid with the cell wall defining their sizes and shapes. Solutes become more concentrated as liquid water is removed from the system to form ice, hence lowering the temperature at which further ice is formed. This could be attributed to the impediment in the ability of the remaining water molecules to interact and form crystals, as solute molecules become increasingly concentrated. When the water freezing temperature remains above the temperature of homogeneous ice formation, but is still reducing, the freezing temperature of water becomes depressed to a point known as the eutectic, when the whole system solidifies and no further change can occur as all available water has frozen (Benson *et al*., 2008; Benson, 2008). The eutectic point has a few characteristics: the point

presents with coordinates for temperature and concentration and applies to two or more substances that are able to form solids with one another and lower each other's freezing point to a minimum temperature. Hence, the balance between the exit of intracellular water, ice formation and cell solute concentration is important for successful cryopreservation (Benson, 2008).

2.7.2. Impact of extracellular water movements on colligative cryoprotection

It is important to take into account the effect of solute concentration on freezing point in order to understand colligative cryoprotection (Benson *et al.*, 2007, 2008). When water is withdrawn from the cell due to the formation of more extracellular ice, the intracellular freezing point is depressed further, eventually compromising the integrity of the cell when the cellular water content approaches a potentially injurious minimum cell volume. Penetrating cryoprotectants become effective at this point as they will be equally distributed at the same concentration across the cell when added at relatively high concentrations, hence contributing to the overall osmolality of the cell (Benson *et al.*, 2007, 2008). The level of water to be frozen in order to achieve osmotic equilibrium and the extent to which dehydration occurs in the cell are therefore less, with the former enumerated through counting the total number of osmotically active components of a solution on the basis of osmoles of solute per kilogram of solvent (Benson *et al.*, 2007).

Colligative additives also depress freezing point, reducing the rate of ice nucleation while enhancing cellular viscosity (Benson *et al.*, 2007, 2008). The cellular viscosity inhibits ice crystal growth, and any crystals produced at the freezing point may be so few and small they produce insignificant effect on the cell

(Meryman and Williams, 1985; Benson *et al.*, 2007). The increased viscosity also raises the glass transition temperature (T*g*), causing possible vitrification of intracellular water and preserving the cells in a partial "glassy state." A glass is a metastable, amorphous solid liquid which lacks crystalline structure and is less damaging to cells (Benson *et al.*, 2007, 2008).

Taylor *et al.* (2004) (as reviewed by Benson, 2008) evaluated the behaviour of water in various transitional states, and its influence on explants' survival with respect to the relative stabilities of the amorphous, liquid and solid phases of water and their potential for causing thermo-mechanical stresses in biological tissues. Glasses are normally unstable in nature and their labile properties include cracking or fractures which can damage larger organs and tissues such as seeds. Ice crystal formation and devitrification, often erroneously observed as harmless, may occur as glasses undergo relaxation during thawing, and this is easily observed in cryovials when transparent glasses become opaque when ice is formed (Taylor *et al.*, 2004; Benson, 2008).

Rapid thawing rates are usually advocated in vitrification experiments to avoid ice nucleation risks when the samples pass through their T*g* (Benson, 2008). This step, however, should be preceded with caution as rapid rewarming rates can lead to stress cracks and fractures in the preserved explants. Instead, two rewarming phases could be applied, for instance a short (1–2 seconds) slow thawing phase at room temperatures allowing glasses to relax without forming stress fractures, followed by rapid thawing in a water bath at 45°C to avoid passage through an ice phase while ensuring speedy transition from glass to liquid phase. Empirical optimisation of these phases could be necessary for each biological system and its cryoprotective regime (Benson, 2008).