EFFECTS OF DROPLET-VITRIFICATION CRYOPRESERVATION METHOD ON *Ludisia discolor* FOR LONG-TERM GERMPLASM CONSERVATION

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EFFECTS OF DROPLET-VITRIFICATION CRYOPRESERVATION METHOD ON *Ludisia discolor* FOR LONG-TERM GERMPLASM CONSERVATION

by

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LIST OF SYMBOLS

%	Percentage
+LN	With Liquid Nitrogen (cryopreserved)
–LN	Without Liquid Nitrogen (non-cryopreserved)
µ mol/s	Micromole per Second
℃	Degree Celsius
A	Absorbance
A_{490}	Absorbance at 490 nm
cm	Centimeter
g	Gram
g/L	Gram per Liter
H_2O	Water
H_2O_2	Hydrogen Peroxide
KH_2PO_4	Potassium Dihydrogen Phosphate
M	Molar
mg/L	Milligram per Liter
mL	Milliliter
mm	Millimeter
µg/mL	Microgram per Milliliter
µmol/s	Micromole per Second
µM	Micromolar
µ mol $m^{-2}s^{-1}$	Micromole per Square Meter per Second
$Na_2HPO_4.2H_2O$	Disodium Hydrogen Phosphate Dihydrate
nm	Nanometer
O_2	Oxygen

$^1O_2$	Singlet Oxygen
$O_2^{\bullet -}$	Superoxide
OH•	Hydroxyl Radical
pH	Potential Hydrogen
T_a	Annealing Temperature
T_m	Melting Temperature
™	Trademark
US$	US Dollar
v/v	Volume per Volume
w/v	Weight per Volume

LIST OF ABBREVIATIONS

AFLP	Amplified Fragment Length Polymorphism
ANOVA	Analysis of Variance
BC	Burst Cell
BIP1	Luminol-Binding Protein 1 Precursor
bp	Base pair
BSA	Bovine Serum Albumin
C	Control Stock Culture
CITES	Convention on International Trade in Endangered Species of Wild Fauna and Flora
CPAs	Cryoprotective Agents
CW	Cell Wall
DAMD	Directed Amplification of Minisatellite DNA
DMRT	Duncan's Multiple Range Tests
DNA	Deoxyribonucleic Acid
et al.	Et Alia
FAA	Formalin: Acetic acid: 95% alcohol
FeCH	Ferrochelatase
FW	Fresh Weight
GluTR	Glutamyl-tRNA Reductase
IC	Intact Cell
ISSR	Inter Simple Sequence Repeats
IUCN	International Union of Conservation of Nature and Natural Resources
LED	Light-Emitting Diode
LN	Liquid Nitrogen
MgCH	Magnesium Chelatase

MS	Murashige and Skoog
N	Nucleus
NAA	Naphthalene Acetic Acid
Nu	Nucleolus
OEEI	Oxygen-Evolving Enhancer Protein 1 Chloroplast Precursor
P	Plastid
PCR	Polymerase Chain Reaction
PLBs	Protocorm-Like Bodies
Ply	Plasmolysed Cell
PM	Plasma Membrane
RAPD	Random Amplified Polymorphic DNA
ROS	Reactive Oxygen Species
RM	Ruptured Membrane
SC	Shrink Cell
SE	Standard Error
SI	Similarity Index
SSR	Simple Sequence Repeats
TBA	Tertiary-Butyl Alcohol
TDZ	Thidiazuron
TEM	Transmission Electron Microscope
TTC	Triphenyl Tetrazolium Chloride
UV	Ultraviolet
V	Vacuole

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KESAN KAEDAH KRIOAWETAN TITISAN-VITRIFIKASI PADA *Ludisia discolor* UNTUK PEMULIHARAAN GERMPLASMA JANGKA PANJANG

ABSTRAK

Ludisia tergolong dalam kumpulan orkid terestrial yang tumbuh perlahan dan ditanam kerana mempunyai daun hiasan yang menarik, biasanya dirujuk sebagai 'Orkid Permata'. Orkid ini semakin berkurangan dalam populasi semula jadi melalui kehilangan habitat dan pengumpulan orkid liar secara sembarangan. Oleh itu, krioawetan sebagai pendekatan penyimpanan jangka panjang menawarkan strategi pemuliharaan pelengkap yang melindungi daripada kehilangan secara tidak sengaja. Walaupun beberapa kaedah krioawetan telah dibangunkan untuk beberapa genera keluarga Orchidaceae, kaedah krioawetan untuk spesies *Ludisia* belum didokumenkan sebelum ini. Oleh itu, kajian ini melaporkan percubaan pertama penyimpanan jangka panjang *L. discolor*, menggunakan kaedah titisan-vitrifikasi. Kajian ini bertujuan untuk mengkaji kesan pengkrioawetan orkid *L. discolor* disokong dengan analisis kualitatif menggunakan mikroskopi, biokimia, dan molekul. Parameter kritikal seperti kepekatan sukrosa dan tempoh prakultur, tempoh dan suhu larutan vitrifikasi tumbuhan 2 (PVS2), medium pemulihan pertumbuhan, dan spektrum diod pemancar cahaya (LED) berbeza telah dinilai. Kemandirian tunas aksil ditentukan menggunakan analisis spektrofotometri 2,3,5-triphenyltetrazolium klorida (TTC) dan pemeriksaan secara visual. Kemandirian optimum diperolehi apabila 4 – 5 mm tunas aksil diprakultur dalam medium Murashige dan Skoog (MS) separuh kekuatan yang mengandungi 0.2 M sukrosa selama 24 jam, diikuti oleh osmo-perlindungan dalam larutan pemuatan selama 20 minit, dehidrasi dalam larutan PVS2 selama 10 minit pada

0 °C, dan diinkubasi dalam nitrogen cecair selama 1 jam. Selepas itu, tunas aksil telah dicairkan dengan cepat dalam larutan penyahmuat dan dipindahkan ke medium pemulihan pertumbuhan yang ditambah dengan 0.05 µM melatonin, yang membawa kepada peluang kemandirian yang lebih baik (16.67 %) untuk *L. discolor* yang telah dikrioawet. Cahaya putih sejuk (400 – 700 nm) dan kombinasi biru dan merah pada 1:1 (puncak panjang gelombang masing-masing pada 440 dan 660 nm) adalah spektrum yang paling sesuai untuk kemandirian tunas aksil *L. discolor* yang dikrioawet. Tekanan osmotik dan pengeluaran berlebihan spesies oksigen reaktif (ROS) semasa peringkat krioawet boleh mengakibatkan kecederaan krio dan kemandirian yang rendah apabila peningkatan paras prolin (5.51 µmol/g), katalase (85.64 U/g protein), peroksidase (565.37 U/g protein), dan aktiviti peroksidase askorbat (12.19 U/g protein) dikesan selepas peringkat dehidrasi, prakultur, pencairan, dan, pemuatan. Penilaian histologi ke atas tunas aksil mendapati pecah dan kerosakan pada struktur dinding sel mengurangkan peluang kemandirian. Penilaian kestabilan genetik menggunakan penanda DNA DAMD dan ISSR masing-masing mempunyai sampel yang stabil sebanyak 83.78 dan 95.35 % apabila dibandingkan dengan tumbuhan kawalan. Kedua-dua penanda DNA mengesan polimorfisme dalam tunas aksil yang dikrioawet dan yang tidak dikrioawet dengan lebih kepekaan dalam DAMD (mengesan 16.22 % jalur polimorfik dalam tunas aksil yang dikrioawet). Keputusan ini mengesahkan bahawa, dengan perancangan dan ujian yang teliti, *L. discolor* sesuai untuk penyimpanan jangka panjang.

EFFECTS OF DROPLET-VITRIFICATION CRYOPRESERVATION METHOD ON *Ludisia discolor* FOR LONG-TERM GERMPLASM CONSERVATION

ABSTRACT

Ludisia belongs to a group of slow-growth terrestrial orchids cultivated for their attractive ornamental leaves, commonly referred to as 'Jewel Orchids'. They are steadily dwindling in their natural population through habitat loss and indiscriminate collection of wild orchids. Therefore, cryopreservation as a long-term storage approach offers a complementary conservation strategy that safeguards against accidental loss. Although some cryopreservation methods have been developed for several genera of the Orchidaceae family, the cryopreservation method for *Ludisia* species has not been documented before. Therefore, this study reported the first attempt of long-term storage of *L. discolor*, using a droplet-vitrification method. The present study sought to examine the effects of cryopreserving *L. discolor* orchid supported with qualitative analyses using microscopy, biochemical, and molecular. Critical parameters such as preculture sucrose concentrations and durations, plant vitrification solution 2 (PVS2) durations and temperatures, growth recovery medium, and different light-emitting diode (LED) spectra were assessed. The survivability of axillary bud was determined using 2,3,5-triphenyltetrazolium chloride (TTC) spectrophotometric analysis and visual inspection. The optimal survival was obtained when 4 – 5 mm axillary buds were precultured in half-strength Murashige and Skoog (MS) medium containing 0.2 M sucrose for 24 hours, followed by osmoprotection in loading solution for 20 minutes, dehydration in PVS2 solution for 10 minutes at 0 °C, and incubated in liquid nitrogen for 1 hour. Subsequently, axillary buds were

rewarmed rapidly in dilution solution and transferred to a growth recovery medium supplemented with 0.05 µM melatonin, which led to an improved survival chance (16.67 %) for cryopreserved *L. discolor*. Cool white light (400 – 700 nm) and the combination of blue and red at 1:1 (peak at 440 and 660 nm, respectively) were the most suitable spectra for surviving cryopreserved *L. discolor* axillary buds. The osmotic stress and the overproduction of reactive oxygen species (ROS) during cryopreservation stages may result in cryoinjuries and poor survival, as increased levels of proline (5.51 µmol/g), catalase (85.64 U/g protein), peroxidase (565.37 U/g protein), and ascorbate peroxidase activities (12.19 U/g protein) were detected after dehydration, preculture, rewarming, and loading stage. Histological assessment of axillary buds found ruptures and damages in the cell wall structure hindered the chances of survival. Genetic stability assessment using DAMD and ISSR DNA markers attained stable samples of 83.78 and 95.35 % when compared to control plantlets, respectively. Both DNA markers detected polymorphism in both cryopreserved and non-cryopreserved axillary buds with more sensitivity in DAMD (detected 16.22 % of polymorphic bands from the cryopreserved axillary buds). These results confirmed that, with careful planning and testing, *L. discolor* is suitable for long-term storage.

CHAPTER 1

INTRODUCTION

One of the most diverse and globally distributed flowering plant families is the Orchidaceae (Christenhusz & Byng, 2016) with up to 972 species in 159 genera recorded in Peninsular Malaysia alone (Ong et al., 2017; Besi et al., 2023). *Ludisia* belongs to a group of terrestrial orchids cultivated for their attractive ornamental leaves, commonly referred to as 'Jewel Orchids'. These ground-dwellers are native to China and Southeast Asia, including Malaysia (Cheah, 2020).

The global orchid market stands firm with over US$ 4 billion of commercial orchid trading (Zhang et al., 2018) for numerous purposes, including medicine and food, as well as ornamental plants (Hinsley et al., 2018; Teoh, 2019). Given their unique symbiotic relationship with mycorrhizal fungi, specific pollinators, and their poor germination rates, most species are found in explicit habitats (Zhang et al., 2018; Gale et al., 2019). As a result, the population of certain species are steadily dwindling across the ecosystem due to over-exploitation, loss of habitat, indiscriminate collection, and degradation.

Substantial development and extensive progress of *in vitro* germination and propagation technology were achieved to support *in situ* conservation efforts, which could not solitarily preserve all the orchids (Shiau et al., 2005; Chugh et al., 2009; Poobathy et al., 2019). However, the maintenance of *in vitro* orchids collections through repeated subcultures requires intensive labour and is costly, apart from the growing risks of somaclonal variations and contamination in the materials over time (Kulus & Zalewska, 2014; Coelho et al., 2020). As a result, these limitations have led to the advanced development of safer and more stable long-term conservation

strategies via the cryopreservation of plant organs and tissues (Reed, 2017; Popova et al., 2023).

Cryopreservation refers to the ultra-low temperature storage of biological materials, usually in liquid nitrogen (LN) at –196 °C (Sakai & Engelmann, 2007). Theoretically, all cellular division (including cell suspensions, seeds, lateral and axillary buds, and stems apices) and metabolic activities of biological materials (such as respiration) cease to function at such temperature conditions, reducing the risk of somaclonal variations or genetic alterations, thus, providing indefinite conservation (Benson, 2008). Practically, biological materials of commercially valuable crops, plants with horticultural values, and endangered plants have survived various durations of storage in LN, ranging from 1 – 48 hrs (Mikuła et al., 2011; Edesi et al., 2020) to 1.5 – 28 years (Caswell & Kartha, 2009; Mikuła et al., 2011; Volkova et al., 2015; Beulé et al., 2018).

One of the recently adopted and continuously developed orchid cryopreservation methods is the droplet-vitrification (González-Arnao et al., 2020), which combines the rapid cooling and warming approach in the presence of cryoprotectant mixtures containing dimethyl sulfoxide (DMSO), ethylene glycol, and sugar, such as glucose and sucrose, at high concentration to prevent ice nucleation and growth (Panis et al., 2005).

Despite its effective use, the vitrification-based methods lead to various stressful conditions, including treatment with highly concentrated cryoprotectants, dehydration, excision, and osmotic and freezing injuries from rapid temperature changes that disrupt the growth after rewarming. This leads to the accumulation of Reactive Oxygen Species (ROS) and consequently generates oxidative stress

(Uchendu et al., 2010; Antony et al., 2019). The ROS, which includes singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), superoxide radical ($O_2^{\bullet-}$), and hydroxy radical (OH•), are generated in the chloroplasts, peroxisomes, and mitochondria (Suzuki et al., 2012) at an equilibrium state under typical growth environments. However, the overwhelming generation of ROS would exceed the capacity of the antioxidants to restore cellular impairment, which could stimulate biological degradation (Suzuki & Mittler, 2006). Thus, precautionary steps are applied to promote cell tolerance and growth recovery, for example, through sugar addition in the preculture media (Bissati et al., 2020), combining different cryoprotectants, altering the duration of exposure, the surrounding temperature (Elliott et al., 2017), light source parameters (Edesi et al., 2017), and adjusting the growth recovery media either through substance addition (Uchendu et al., 2014; Diengdoh et al., 2019) or the removal of growth hormones (Gupta & Reed, 2006).

Nevertheless, particular uncertainties related to the harmful effects on the cryopreserved tissues or cells remain a focal point of discussion. Cell damage could take place during the cooling-rewarming stages or from the accumulation over several hours to days. In view of this, various analytical tools, such as histological observations and biochemical analyses, can be employed to reveal further understandings of the influence of cryopreservation at the cellular level. In addition, the superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) are antioxidants that protect the plant cells by scavenging excess ROS and can be used as biomarkers to determine the defence mechanism as a result of the various imposed oxidative stress throughout the sequential cryopreservation stages (Poobathy et al., 2013; Antony et al., 2019).

Plant tolerance was indicated by the accumulation of starch grains, abnormal membrane structures, cell plasmolysis, and cell walls after being exposed to ultra-low temperatures (Mubbarakh et al., 2014a; Wesley-Smith et al., 2014). Apart from that, it is essential to modify the cryopreservation protocols through the incorporation of various stressing conditions, including clonally stable plants, to enhance the survival and regeneration process. Previously, genetically-stable regenerants have been verified through numerous molecular markers, for example, the Directed Amplification of Minisatellites DNA (DAMD), Inter Simple Sequence Repeats (ISSR), Random Amplified Polymorphic DNA (RAPD), and Start Codon Targeted Polymorphism (SCoT). Other reports had demonstrated the use of cryopreservation without or with minimal genetic changes in the regenerated *Dendrobium* orchids (Antony et al., 2015; Bhattacharyya et al., 2015), date palm (Purayil et al., 2018), kiwi fruit (Zhang et al., 2020), and violet (Żabicki et al., 2021).

1.1 Rationale and significance of study

A long-term storage approach via cryopreservation is highly suggested to preserve the slow-growing jewel orchids, which are facing extinction as a result of overharvesting and rapid loss of habitat. While most attention has been given to developing the micropropagation method using seeds and nodal segments, maintaining the delicate tissue cultures is laborious, unaffordable, and vulnerable to *in vitro* contaminations.

Despite the implementation of various cryopreservation methods on various genera of the Orchidaceae family, which include desiccation, preculture desiccation, encapsulation-dehydration, vitrification, and droplet-vitrification, none of the reports has focused on the development of cryopreservation techniques for *Ludisia* species.

Hence, there is an urgency to explore an alternative conservation strategy to protect the endangered jewel orchid species from the imminent threat of extinction.

1.2 Research objectives

The main aim of this study is to investigate the feasibility of an *ex situ* conservation strategy of *L. discolor* via the droplet-vitrification cryopreservation method. The following objectives were outlined in order to accomplish the aim of this study:

I. To assess the feasibility of *L. discolor* axillary buds to the proposed droplet-vitrification cryopreservation technique,

II. To identify changes in the biochemical properties of *L. discolor* at different cryopreservation stages through different biochemical assessments,

III. To examine changes in the cellular morphology of *L. discolor* using histological and transmission electron microscope (TEM) analyses,

IV. To evaluate and compare the genetic stability of cryopreserved and non-cryopreserved *L. discolor* with that of the stock culture via DAMD and ISSR molecular markers.

CHAPTER 2

LITERATURE REVIEW

2.1 Biodiversity and popularity of orchids

One of the most plenteous and dispersed flowering plant families in the world is the Orchidaceae (Christenhusz & Byng, 2016) with up to 972 species in 159 genera recorded in Peninsular Malaysia alone (Go et al., 2015; Ong et al., 2017; Besi et al., 2019; Besi et al., 2023). In fact, Penang Hill boasts at least 136 documented species from the Orchidaceae family (Go et al., 2011). The high variability among orchid species and habitats contributes an essential role in plant biodiversity worldwide with the most diverse orchid species was reported in the Andes of Colombia and Ecuador as well as in the tropical rainforests of Borneo, Madagascar, New Guinea, and Sumatra (Cribb et al., 2003; Swarts & Dixon, 2009).

Although orchids are flowering plants with diversely and highly evolved taxon, the distribution of most of the species is limited in certain habitats given their symbiotic association with specialised pollinators, mycorrhizal fungi, and poor germination rates (Zhang et al., 2018). Recently, Go et al. (2020) produced a case study related to the orchid extinction in Malaysia, which highlighted the gradually dwindling population of orchids due to several factors, especially loss of habitat, over-collection of wild orchids, and the lack of suitable methods for domestication. Furthermore, the growing global market for orchid trading has multiplied the demand for orchid-cut flowers and potted plants (Pal et al., 2016), which has transformed into industrial-scale commerce in a number of countries, including Malaysia, Singapore, Australia, and Thailand (Chugh et al., 2009).

The ancient Chinese civilisation was the first to recognise the medicinal value of orchids (Pant, 2013) from the native *Cymbidium* and *Dendrobium* species to treat various illnesses, including allergies, diabetes, and fatigue (Ng et al., 2012; Liu et al., 2014). All parts of the plant are utilised for medicinal purposes including flowers, bulbs, leaves, and roots (Pant, 2013). In addition, the components of orchids are frequently utilised in the food and beverages industry, such as vanillin extracts of *Vanilla planifolia* seed pods. Realising the beneficial use of orchids, numerous orchid species are commercially traded as food ingredients, medicinal value, and ornamental plants (Popova et al., 2016; Hinsley et al., 2018; Teoh et al., 2019).

Small and large industries have relied on conventional and advanced breeding programmes that exploit the available genetic resources for orchid propagation (Paek & Murthy, 2002; Liu et al., 2014). At the same time, there is a continuous interest among the public and scientific community to acquire wild species for novel gene combinations in which over a hundred new variations are registered annually. While orchids are considered the most evolved vascular plants in comparison to other vascular plants, the once abundantly-available flowering plant is declining in numbers and is on the brink of extinction (Hinsley et al., 2018). Moreover, orchids are susceptible to biotic and abiotic fluctuations due to their life cycle, which is associated with specific mycorrhizal fungi during the preliminary growth stages (Krumov et al., 2022), selective insect pollinators (such as the *Pieris rapae* butterfly and *Bombus pseudobaicalensis* bumblebee) (Zhang et al., 2010; Sugiura & Takahashi, 2015), and tiny seeds with limited food reserve (Arditti & Ghani, 2000; Arditti & Ghani, 2013; Popova et al., 2016).

2.2 Jewel orchid

Jewel orchids are grouped under the subfamily Orchidoideae and are closely related to the tribe Cranichidea and subtribe Goodyerinae. The orchids are terrestrial and naturally rooted in soil with some being preferable to tropical climates while others favour temperate conditions (Hayden, 2016; Sudin & Isa, 2024). Jewel orchids are known for their impressive and splendid ornamental leaves and not for their small discreet white flowers (Gangaprasad et al., 2000).

The tribe Cranichideae consists of approximately 1600 species encompassing 90 different genera. The tribe possesses unique storage organs, including fleshy roots at the nodes on creeping or underground rhizomes that clump together in their natural habitat (Figueroa et al., 2008). The majority of the tribe members are terrestrial and commonly thrive on shaded forest floors.

Jewel orchids make up only five genera, namely, *Anoectochilus*, *Dosinia*, *Goodyera*, *Ludisia*, and *Macodes* (IUCN/SSC Orchid Specialist Group, 1996), which all belong to the same subtribe Goodyerinae (Figure 2.1). Besides, members of the subtribe Goodyerinae are characterised by their horizontal stems with velvety leaves and creeping rhizomes (Figueroa et al., 2008). The subtribe is native to the tropical regions of Southeast Asia although certain species, such as *Goodyera pubescens*, are found in Eurasia and North America (Hayden, 2016). The morphological similarities among the subtribe species make it difficult to identify beyond the flowering period (Chen & Shiau, 2015). In addition, jewel orchids are able to cross-pollinate, such as those reported between *A. formosanus* and *A. koshunensis* (Cheng et al., 1998; Chen & Shiau, 2015) and between *A. formosanus* and *L. discolor* (Chou & Chang, 2004;

Chen & Shiau, 2015), which is an ongoing effort to restore the population in the ecosystem (Chen & Shiau, 2015).

Figure 2.1 A collection of jewel orchids such as *Anoectochilus* sp., *Goodyera* sp. and *Ludisia* sp. (Scale bar = 5 cm)

Figure 2.2 *L. discolor* orchid. (Scale bar = 5 cm)

2.2.1 *Ludisia discolor* (Ker Gawl.) A. Rich

Previously known as *Haemaria discolor*, *L. discolor* orchids is a member of the subtribe Goodyerinae and widely renowned as Jewel orchids (Figure 2.2) due to its unique colouration and distinctive leaves pattern (Shiau et al., 2005). However, there are insufficient records on the availability of *L. discolor* in Malaysia as a result of their drastically shrinking population (Go et al., 2011) mainly due to land conversion and habitat fragmentation (Go et al., 2011; Masum et al., 2017). *Ludisia* orchids are harvested as a substitute component (adulterant) to the *Anoectochilus* that are well-known for their medicinal properties given the similar morphologies between both species (Hu et al., 2019). This was verified through a phylogenetic study via the nuclear Internal Transcribed Spacer (ITS) regions and a chloroplast matK sequence, which indicate the close association between the genus *Anoectochilus* and *L. discolor* (Chen & Shiau, 2015).

The recently documented *L. ravanii* (Cootes & Tiong, 2013) and *L. discolor*, are the only two species that have been identified in the genus (Govaerts et al., 2021). The perennial herb, which grows up to 10 – 25 cm tall, is well-dispersed across South China and throughout Southeast Asia, including Laos, Myanmar, the Philippines, Sumatra, and Thailand, with only one species available in Peninsular Malaysia (Cheah, 2020). The wild *L. discolor* prefers to thrive on shaded forest floors but can also grow on leaf litter and rocks (Botanic Gardens of South Australia, 2014; Poobathy et al., 2019). Moreover, the seeds of the slow-growing plant mature after 2 – 3 years (Shiau et al., 2005) and begin to flower between late December and March (Chou & Chang, 2004).

Zheng and colleagues (2013) reported that the Hmong ethnic community harvests the whole *Ludisia* plant to treat infections, while the Li community administers the *Ludisia* plant to heal external injuries (Zheng & Xing, 2009). The leaf extracts are rich in amino acids, such as glutamine, asparagine, and threonine (Shiau et al., 2005). Statistically, the trade data indicates that wild orchid plants were exported to China and Korea at US$ 1 – 9/kg and US$ 40/kg, respectively (The Agrobiodiversity Initiative in the Lao PDR, 2010). The export of *L. discolor* from the Amazonian regions between the years 2005 and 2014 is worth approximately US$ 0.9 million/year at US$ 17.33/single living orchid plant (Sinovas, 2017).

2.3 Orchid conservation strategies

The population of certain orchid species are declining and is immensely difficult to retrieve. The greatest risk to most of the native angiosperms in Malaysia is the destruction and loss of habitat due to land conversion (Masum et al., 2017). Realising the potential harm of these species, they are included in the Red Data Book of the International Union of Conservation of Nature and Natural Resources (IUCN) and listed in Appendix I or II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) as part of the conservation initiatives to preserve the biodiversity of wild orchids (Popova et al., 2016). Govaerts et al. (2021) construed that almost all wild orchids are included in Appendix I of CITES and are highly emphasised for conservation efforts given the massive habitat disruption. In addition, it was predicted that two crucial factors could threaten the biodiversity of orchids in Latin America and Asia, which include severe climate change and global warming (Seaton et al., 2010; Popova et al., 2016). Therefore, preserving the species richness requires a comprehensive understanding of the orchid's life-history traits,

ecology, and evolution (Swarts & Dixon, 2009; Seaton et al., 2010; Popova et al., 2016).

Popova et al. (2016) reported that the conventional *ex situ* preservation approaches, for example, the establishment of the Royal Botanic Gardens, Kew, United Kingdom, conserve the delicate orchid species by maintaining a well-documented living orchid collection with up to 2700 species. Seed banking is also another viable *ex situ* conservation option for plants, including orchids (Dolce & González-Arnao, 2020). However, the storage of certain orchid species under low-temperature conditions was relatively ineffective (Pritchard & Seaton, 1993; Merritt et al., 2014). For instance, the germination rate of *Phaius tankervilleae* seeds was decreased following the storage of the seeds at 4 °C for 6 months (Hirano et al., 2009).

In comparison to seed banking, field gene banks provide beneficial and easy access to plants, which are required to undergo germination and grow before ready for utilisation. Nevertheless, the larger space requirement and greater labour cost limit the full potential of field gene banks as a preferable conservation strategy (Rao, 2004). Apart from that, numerous environmental stresses and plant diseases could also jeopardise the integrity and purity of the collections in botanical gardens (Seaton et al., 2010). Furthermore, seed-feeding flies, *Japanagromyza tokunagai* had severely reduced over 95 % seeds production of the five endangered Japanese orchids, including *Cymbidium macrorhizon* and *Epipactis helleborine* var. *sayekiana* (Suetsugu et al., 2016), which are the only plant documented to host these flies. Consequently, it is paramount to synchronise *in situ* and *ex situ* techniques into a coherent conservation approach in order to conserve the best plant genetic resources for future global breeding programs.

Several *in vitro* orchids conservation strategies have been applied via micropropagation and cryopreservation. The vegetative propagation of the medicinal orchid, *Dendrobium nobile* using tissue cultures and reintroducing them to nature is one of the examples of *in vitro* conservation approach (Bhattacharyya et al., 2016). Despite the *in vitro* methods are broadly applied for large-scale conservation purposes and commercialisation of endangered orchid species (Popova et al., 2016), the available techniques require high-cost maintenance and are labour-intensive, the possibility of phenotypic and genotypic variations increases with each repeated subcultures (Khoddamzadeh et al., 2011; Popova et al., 2016). These concerns induce the urge to develop safer, cheaper, and more reliable conservation techniques, such as cryopreservation, involving the use of LN to boost the storage life of orchid germplasm.

2.4 Cryopreservation of plants

Cryopreservation refers to the ultra-low temperature storage of biological materials, usually in LN at −196 °C (Sakai & Engelmann, 2007) in which all metabolic activities, including cellular division (seeds, stems apices, lateral or axillary buds, and cell suspensions) and respiration, cease to operate, thus, minimising the exposure to somaclonal variations or genetic alterations and ultimately, in theory, providing long-term conservation (Benson, 2008). Although Walters and colleagues (2004) argued that the possible deterioration of biological materials at ultra-low temperatures remains high, another study by them (Walters et al., 2004) estimated that the storage of fresh lettuce seeds in vapour and liquid phase LN recorded half-lives of approximately 500 and 3400 years, respectively, which were longer compared to the anticipated storage duration of any method available.

To date, the use of cryobanks is the only *ex situ* method for long-term conservation of vegetatively propagated (clonally reproducing) species as well as for desiccation-sensitive (recalcitrant) species or short-lived seeds (Engelmann, 2004; Pritchard, 2007; Ballesteros et al., 2023). Cryopreservation is currently employed as a large-scale alternative strategy to support the field collections of numerous crops, such as the accessions of 507 apples at Julius Kühn-Institute (JKI), Institute for Breeding Research on Fruit Crops, Dresden, Germany (Höfer, 2015; Wang et al., 2018a), 1100 accessions of banana at Bioversity International, Leuven, Belgium (Panis et al., 2020), 1158 accessions of garlic at the National Agrobiodiversity Centre (NAAS), Suwon, South Korea (Kim et al., 2012), 1470 accessions of mulberry at National Institute of Agrobiological Sciences (NIAS), Tsukuba, Japan (Fukui et al., 2011) and 1533 accessions of potato at the International Potato Centre (CIP) in Lima, Peru (Vollmer et al., 2017).

The rapid advance in plant cryobiology throughout the 1990s has seen the development of shoot tip cryopreservation methods based on vitrification and dehydration processes, namely vitrification, encapsulation-vitrification, and encapsulation-dehydration (Sakai & Engelmann, 2007; Lambardi & Shaarawi, 2017). Vitrification is defined as the synthesis of metastable glasses under rapid LN cooling without the occurrence of ice crystallisation within the plant cells, which normally occurs when treated with viscous and highly concentrated cryoprotectant mixtures (Fahy & Wowk, 2015; Wang et al., 2020). The vitrified metastable characteristic enables the plant to easily convert and return to its original state. Additionally, the pre-conditioning of the explants through a succession of specific growth conditions or solutions prior to storage in LN would inhibit the formation of ice crystals, stimulating

a greater tolerance and adaptation of the explants to freezing conditions and minimising the risk of cell damage due to ice crystallisation (Wang et al., 2020).

2.4.1 Droplet-vitrification method

The droplet-vitrification method was first introduced by Kartha et al. (1982) based on the cryopreservation of cassava shoot tips (*Manihot esculenta*) in DMSO droplets. The method takes advantage of the rapid cooling rates through the use of droplet freezing and the properties of the vitrification solution, which successfully cryopreserved the shoot tips (Panis, 2019). Additionally, the first effective cryopreservation application using the Plant Vitrification Solution 2 (PVS2) in the droplet-vitrification technique was reported by Pennycooke and Towill (2000) on sweet potato shoot tip, while Panis et al. (2005) further optimised the method for the cryopreservation of banana. Wang et al. (2021) illustrated an overview of the major steps proposed by Panis et al. (2005) for the droplet vitrification of shoot tip cryopreservation. As shown in Figure 2.3, the proposed method includes cryo-plate and cryo-mesh vitrification-based techniques. Moreover, several studies have revealed the successful application of the proposed method for various species and plant materials, such as grapevine axillary buds (Pathirana et al., 2016), bulbil primordia of garlic (Kim et al., 2006), adventitious roots from ginseng (Le et al., 2019), and shoot tips of blackcurrant (Rantala et al., 2020) and kiwifruit (Pathirana et al., 2020).

One of the fundamental principles of plant cryopreservation is vitrification, which involves the formation of glass without crystallisation (Benson, 2008). A glassy condition minimises physicochemical alterations and completely prevents the formation of ice crystals, which pose lethal freeze injuries to the cells, hence, ensuring

the conservation and survival of living tissues (Zamecnik et al., 2021). Numerous compositions of the specialised cryoprotecting solution have been established to effectively treat the plant cells and protect them from the harsh cryogenic temperatures (Benson, 2008). A cryoprotectant should exhibit at least three main characteristics before it can be considered to be used for vitrification, which include an exceptional glass-forming ability, a strong dehydration strength on a colligative basis to dehydrate the plant cells and stimulate the vitrification state, and non-toxic to the plants. Although the toxicity of PVS2 is a contentious point, it remains a significantly effective vitrification solution for plant shoot tip systems (Volk & Walters, 2006).

Comparatively, the droplet-vitrification protocols, including the preculture and dehydration stages, are more or less similar to the conventional vitrification method (Roque-Borda et al., 2021). The ultra-rapid freezing protocol was adopted from the droplet-freezing method and integrated with the vitrification method. Prior to the direct immersion in LN, the explants are placed on a piece of aluminium foil before a micro-drop of approximately 3 – 6 µL of vitrification solution is applied to encircle the sample. The higher thermal conductivity of the aluminium foil than polypropylene plastic cryovials provides a rapid heat transfer during the process. The latest study by Roque-Borda and colleagues (2021) revealed that the application of the droplet-vitrification method in several up-to-date established cryoprotocols for various plants signifies the status of the droplet-vitrification as the most popular approach for agronomic plants in the *Solanum* genus and is comprehensively researched in terms of long-term storage option.

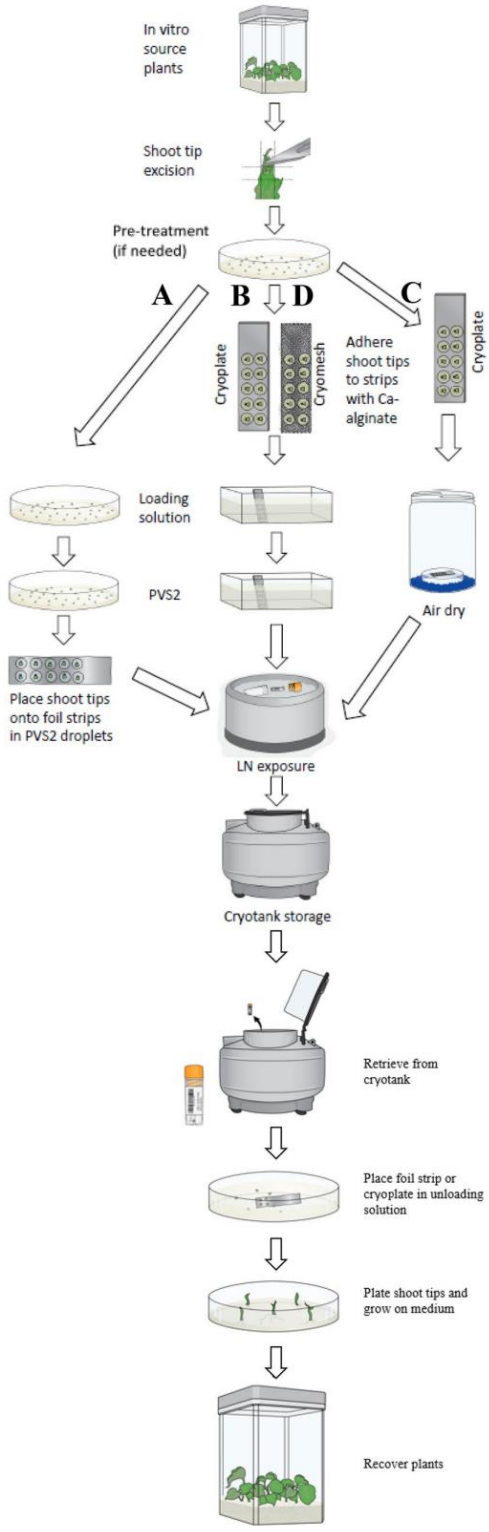

Figure 2.3 Major steps of shoot tip cryopreservation. (A) Droplet-vitrification, (B) cryo-plate (vitrification), (C) cryo-mesh, and (D) cryo-plate (desiccation). (Wang et al., 2021)

2.5 Parameters involved in cryopreservation

A complete cryopreservation approach includes a series of steps comprising the preculture, osmoprotectant, dehydration, ultra-cooling, rewarming, and recovery. Note that each plant species produces varying responses to a specific treatment. Hence, optimising the respective step is vital to achieving an optimum recovery rate.

2.5.1 Selection of plant materials for cryopreservation

One of the crucial parameters that affect cryopreservation is the selection of suitable plant material in terms of size, physiological condition, cell composition, and growth response. Typically, homogeneous samples are applied to enhance the possibility of achieving a uniform and positive outcome, while freshly-dissected tissues are frequently vulnerable to mechanical damage (frequently detected through browning or blackening) and more exposed to further damage via cryogenic treatments (Benson & Harding, 2012).

The physiological conditions of the plants are also inclined to influence the success rate of cryopreservation. In view of this, actively growing mother plant samples are normally selected to ensure the presence of actively dividing meristematic cells in the sample (Engelmann, 2004) and increase the possibility to avoid unfavourable genetic variations (Kulus & Zalewska, 2014). Previously, Gogoi et al. (2013) recorded up to 70 and 72 % regeneration of cryopreserved protocorms using two *Cymbidium* species (*C. eburneum* and *C. hookerianum*, respectively) with sufficient acclimatisation. Moreover, Kulus (2018) successfully achieved a 100 % survival rate using a developed cryopreservation protocol for shoot tips of Lady Orange chrysanthemum. Conversely, Carmona-Martín et al. (2018) recorded a

genetically stable range of 42 – 84 % recovery of *Asparagus officinalis* L. rhizome buds. In the current study, the axillary buds, which comprise small groups of meristematic cells accumulated at the leaf axils (Hopkins & Hüner, 2010), were selected as the primary material for the cryopreservation process.

2.5.2 Successive cryopreservation steps

Each cryopreservation step contributes to the survival rate of the cryopreserved explants. Additionally, the cryo-capability of the respective species in terms of their tolerance to dehydration and toxicity of the cryoprotectant influences the effectiveness of the method (Sakai et al., 1990; Tirado-Pérez & Sandoval-Cancino, 2022).

2.5.2(a) Preculture

The cold hardening treatment is applied to enhance the tolerance of temperate plants to cryopreservation. Generally, the mother plants are incubated at low-temperature conditions for around 2 – 3 weeks to stimulate the accumulation of intracellular solute, leading to a robust growth recovery post-cryopreservation (Benson, 2008). Due to the limitations of cold hardening treatment, including time-consuming and the use of highly sophisticated instruments, preculture of plant materials on media supplemented with high sugar concentration is the preferred method over the cold hardening treatment (Bachiri et al., 2000; Dumet & Benson, 2000). The preculture is also beneficial to treat tropical plants that are difficult to survive (Normah et al., 2019).

The addition of high sugar or sugar alcohol concentration in precultures substantially elevated the cell tolerance during the dehydration and cooling steps (Kim

et al., 2006; Feng et al., 2013). The cell membrane integrity is preserved as water molecules on the membrane surface are replaced with hydroxyl groups from the sugar accumulation, which also maintain the stability of proteins during dehydration (Crowe et al., 1987; Bachiri et al., 2000). Sucrose is a commonly used in the vitrification-based cryopreservation protocols (Popova et al., 2020; Wang et al., 2020; Ozkaya et al., 2022) since sucrose pre-culture permits the adjustment of the sugars and the total soluble protein in the cells that are essential for the regeneration of cells following cryopreservation (Bachiri et al., 2000; Jitsuyama et al., 2002; Wang et al., 2003). Nevertheless, overexposure to sucrose at a high concentration could cause extreme dehydration, leading to cell plasmolysis and rapture of plasmalemma (Popov et al., 2006; Popova et al., 2016). Therefore, it is essential to optimise the sucrose concentration and the preculture duration to achieve optimum survival of the cryopreserved plants.

2.5.2(b) Dehydration prior to LN storage

The duration of incubation and temperature setting are two vital parameters that contribute to the survival rate of the cryopreserved plant tissues after being subjected to the vitrification solutions (Mohanty et al., 2012a). It is crucial to determine the appropriate exposure duration during the vitrification procedures to ensure sufficiently balanced dehydration and chemical toxicity level (Sakai & Engelmann, 2007). As mentioned earlier, PVS2 is a frequently used cryoprotectant in plant cryopreservation research (Sakai et al., 1990), which consists of 30 % weight over volume (w/v) glycerol, 15 % (w/v) ethylene glycol, and 15 % (w/v) DMSO in 0.4 M basal medium.

Most cryopreservation techniques require the use of chemical substances that serve as protective agents, which are referred to as cryoprotectants (Kulus & Zalewska, 2014). Cryoprotectants that are utilised in vitrification cryopreservation techniques are also known as vitrification solutions (VS), which are composed of cryoprotective agents (CPAs), for example, DMSO, ethylene glycol, glycerol, and sucrose (Elliott et al., 2017). Considering that the intracellular formation of ice crystals is widely acknowledged as detrimental cellular stress that results in irreversible injury, it has been long presumed that CPAs are able to alter the transition of liquid water into ice (Elliott et al., 2017). However, the formation of ice crystals is harmless under normal conditions, such as during the growth of cells in standard growth media.

The utilisation of additives in the vitrification solutions could lead to osmotic injuries (for non-penetrating components), cryoprotectant toxicity, and devitrification, while osmotic or evaporative dehydration could induce desiccation sensitivity in the explants. Thus, the use of a mixture of various additives (Kulus & Zalewska, 2014) would minimise the toxicity effects of any single additive during cryoprotection, reducing the potential damages during the evaporative drying, and facilitate the stabilisation of the formed glasses. According to Benson (2008), the applied vitrification solutions on plants are mostly a combination of penetrating and non-penetrating cryoprotectants. In several plant vitrification protocols, cryoprotectants are added and excess water is removed via the evaporative desiccation and osmotic dehydration in order to optimise the survival rate of the explants post-cryopreservation (Popova et al., 2016).

One of the profound challenges that cryobanks face is that cryopreservation protocols are species - and even genotype - specific (Popova et al., 2016; Vujović et

al., 2024). In practice, this means that a cryopreservation protocol developed and well-adapted to one crop often cannot be used for cryopreserving another crop without preliminary optimization. As cryopreservation is a labour-intensive, multistep process for introducing plant material into the cryobank, there is no generic time for LN storage. Depending on the size and design of the storage vessels, the holding time of LN tanks ranges from a few hours to a few weeks (Acker et al., 2017). Additionally, published literatures (Rajasegar et al., 2015; Popova et al., 2016; Roostika et al., 2024; Vujović et al., 2024) recommended a storage duration of at least 1 hr in order to prioritized on optimizing the cryopreservation protocols. Once the protocols were established with regenerative abilities of cryopreserved explants, manipulating LN to observe the correlation between storage duration and genetic stability can then be conducted (Caswell & Kartha, 2009; Mikuła et al., 2011; Volkova et al., 2015; Beulé et al., 2018).

2.5.2(c) Growth recovery

Explants are susceptible to experiencing abnormal growth in the preliminary post-thaw recovery stage, causing an increased genetic instability and damage (Towill & Bajaj, 2002). As a result, it is imperative to use the growth recovery media to restore impaired tissues and shoot formation directly from the cryopreserved explants shoot apex (Popova et al., 2023). Therefore, selecting a proper growth recovery medium plays a crucial role in the growth recovery of the plant since the recovery media regulates the growth, totipotency, and development of tissues and cells, and at the same time, the use of a balanced recovery media ensures a continued post-thaw survival (Towill & Bajaj, 2002; Popova et al., 2016).

The effects of physical parameters, including exposure to light, should also be considered. Since most damage repair takes place under dark conditions, the exposure to light influences the post-thaw recovery of plant tissues. The quantity and quality of the light source (photoperiod, intensity, and spectral composition) influence the morphogenetic responses of the *in vitro* plants (Yoon et al., 2007; Edesi et al., 2017; Mølmann et al., 2019). Previously, short periods of dark incubation post-thawing recorded positive effects in terms of plantlet regeneration (Mohanty et al., 2012a; Mohanty et al., 2012b). In another study, altering the light spectra prior to LN storage and during the recovery post-cryopreservation enhanced the overall survival rate and recovery process (Yoon et al., 2007; Edesi et al., 2016; Mølmann et al., 2019).

Furthermore, shoot tips of potato cultivars were subcultured under an intense light source (130 µmol/m^2s above the culture vessel) before the cryopreservation was carried out and achieved a substantially greater recovery post-cryopreservation (Yoon et al., 2007). Oppositely, Edesi et al. (2017) showed that blue Light-Emitting Diodes (LEDs) promoted the growth potential, photomorphogenesis, and successive survival post-cryopreservation of potato clones. Meanwhile, Mølmann et al. (2019) proposed that the specific red and far-red LEDs were effective during green-sprouting for maximum inhibition of sprout growth in the same species. However, the impact of modification of light source on the efficiency of the cryopreservation method is less studied.

It was observed that successful recovery depends on the extent of antioxidant protection from harmful ROS, which regularly occurs post-LN incubation (Chen et al., 2015). The incorporation of vitamins as additive components in the culture media, for instance, tocopherol and ascorbic acid, was shown to minimise oxidative damage.