DEVELOPMENT OF V CRYO-PLATE METHOD OF *LUDISIA DISCOLOR* (KER GAWL.) A. RICH FOR LONG-TERM GERMPLASM CONSERVATION

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UNIVERSITI SAINS MALAYSIA

2022

DEVELOPMENT OF V CRYO-PLATE METHOD OF *LUDISIA DISCOLOR (*KER GAWL.) A. RICH FOR LONG-TERM GERMPLASM CONSERVATION

by

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Thesis submitted in fulfilment of the requirements
for the degree of
Master of Science

March 2022

ACKNOWLEDGEMENT

First of all, I would like to express my utmost gratitude to my main supervisor Prof. Dr. Sreeramanan Subramaniam. He continuously encouraged and supported me both academically and emotionally whenever I'm demotivated, and it's been the biggest key to complete my studies. My next gratitude to my co-supervisors, Dr. Chew Bee Lynn and Dr. Ranjetta Poobathy (QIUP) for their advice and guidance in upgrading my project. I am blessed to be a member of Lab 101, D32, USM. I thank all my great lab mates, colleagues, and lab assistants who helped me throughout this beautiful journey. The biggest thanksgiving goes to my family for their immense love and support. Thanks to my parents, Mr. Selva Rajan and Mrs. Boomathi. Special love to my sisters Ms. Rehasri and Ms. Krubasri, always been on my side and supported me emotionally during ups and downs. My sincere gratitude to the School of Biological Sciences, Universiti Sains Malaysia, allows me to continue my studies here by providing the laboratories facilities as required for my research. I extend my gratitude to the Ministry of Higher Education for the FRGS grant. My sincere appreciation for the grants from MMICARE Association (Miss Malaysia India Care Association) and Agricultural Crop Trust without which this research wouldn't be materialised. Out of all, I finally thank god for the good health, mind, knowledge, and strength to endure all the difficulties I faced throughout this research.

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LIST OF ACRONYMS AND ABBREVIATION

%	Percent/ percentage
°C	Degree celcius
°C/min	Degree celcius per minute
µL	Microlitre
$\mu mol.m^{-2}.s^{-1}$	Micromole per metre square per second
AFLP	Amplified fragment length polymorphism
ANOVA	Analysis of variance
BC	Burst cell
bp	Base pair
$CaCl_2$	Calcium chloride
cm	Centimetre
CW	Cell wall
DAMD	Directed amplification of minisatellite DNA
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
FAA	Formalin-acetic-acid-alcohol
g	Gram
g/L	Gram per litre
h	Hours
IC	Isodiametric cells
IN	Intact cells
ISSR	Inter simple sequence repeat
kb	Kilobyte
KH_2PO_4	Monopotassium phosphate
LN	Liquid nitrogen
LS	Loading solution
m	Metre
min	Minute
ml	Millilitre
mM	Millimole
MS	Murashige-Skoog
N	Nucleus
Na_2HPO_4	Disodium phosphate
NAA	Naphthalene acetic acid
ng/µL	Nanogram per microlitre
nm	Nanometre
Nu	Nucleolus
OD	Optical density
P	Plasmolysed
PCR	Polymerase chain reaction

PVS2	Plant vitrification solution 2
PVS3	Plant vitrification solution 3
QTL	Quantitative trait locus
RAPD	Random amplified polymorphic DNA
ROS	Reactive oxidative species
RS	Unloading solution
RT	Room temperature
SC	Shrink cell
SCoT	Start codon targeted
Sec	Second
SG	Starch granule
STR	Short tandem repeats
TBA	Tert-butanol
TBE	Tris/ Borate/ EDTA
TDZ	Tidiazuron
TTC	Tetrazolium triphenyl tetrazolium chloride
US	US dollar
V	Volt
v/v	Volume per volume
VNTR	Variable number of tandem repeats
VS	Vitrification solution
W	Watt
w/v	Weight per volume

PEMBANGUNAN KAEDAH V KRIO-PLAT UNTUK *LUDISIA DISCOLOR* (KER GAWL.) A. RICH BAGI PEMULIHARAAN GERMPLAMA JANGKA PANJANG

ABSTRAK

Ludisia discolor adalah sejenis orkid permata yang terkenal kerana daun yang menarik daripada bunganya. Orkid ini bukan sahaja digunakan sebagai tumbuhan hiasan, ia juga dieksploit untuk nilai khasiatnya. Populasi *L. discolor* sedang menyusut disebabkan oleh kutipan hasil yang sangat tinggi dari habitatnya. Kaedah V krio-plat diperlukan untuk pemeliharaan jangka panjang orkid ini. Tujuan kajian ini adalah untuk membangunkan protokol kaedah V krio-plat pada tunas aksil, menyiasat analisis mikroskopi eksplan yang dikrioawet, menganalisis perbezaan kandungan air dan perbezaan genetik di antara sampel kawalan dan eksplan yang diawet. Sejumlah enam (6) parameter telah dioptimum dalam kajian V krio-plat ini iaitu kepekatan prakultur, tempoh prakultur, jenis larutan muatan, tempoh dalan larutan PVS2, suhu pencairan dan tempoh pencairan. Pentaksiran kemandirian eksplan *L. discolor* yang diawet telah diambil menggunakan spektroskopi TTC dengan nilai kuantiti 490nm. Kemandirian terbaik telah didapati dengan tunas aksil di kultur dalam 0.25M prakultur untuk tempoh 48 jam, 0.3M sukrosa + 0.5M gliserol + 10% DMSO larutan muatan, dehidratan dalam laruran PVS2 untuk 20 minit dan pencairan dengan suhu 40°C untuk 90 saat. Analisis histologi dan mikroskop elektron penghantaran telah digunakan untuk meninjau kerosakkan intrasel dalam eksplant diawet berbanding dengan kultur kawalan, Analisis histologi dari sampel dikrioawet dan tidak krioawet menunjukkan bahawa terdapat perbezaan struktur daripada sampel kawalan. Mikroskop elekton penghantaran juga menunjukkan perbezaan struktur di antara sampel kawalan dan diawet. Tunas aksil yang di-krioawet telah mengalami kerosakkan yang banyak menurut kedua-dua analisis histologi dan mikroskop elektron penghantaran. Seterusnya, analisis kandungan air telah diambil pada setiap langkah yang terlibat dalam kaedah V krio-plat. Langkah dihidrasi dengan PVS2 mempunyai kandungan air yang paling rendah. Akhirnya, analisis molekular dilakukan dengan penanda DAMD, ISSR dan SCoT. Daripada analisa tersebut jumlah peratus polimorfisme DAMD, ISSR dan SCoT adalah 12.5%, 4.17%, and 10.53% pada tunas aksil yang dikrioawet. Manakala, jumlah peratus polimorfisme DAMD, ISSR dan SCoT yang diperolehi adalah 6.15, 5.77, and 10. SCoT telah dipilih sebagai penanda yang terbaik dalam menentukan polimorfisme untuk *L. discolor* berbanding dengan DAMD and ISSR.

DEVELOPMENT OF V CRYO-PLATE METHOD OF *LUDISIA DISCOLOR* (KER GAWL.) A. RICH FOR LONG-TERM GERMPLASM CONSERVATION

ABSTRACT

Ludisia discolor is a jewel orchid that is well known for the striking leaves than its flowers. Other than being used as an ornamental plant, this orchid is also being exploited for its medicinal properties. Hence, *L. discolor* facing possible depletion in its natural habitat lately due to over-harvesting. Recently, the latest cryopreservation methods such as V cryo-plate is needed for long-term germplasm conservation. This study aims to develop a V cryo-plate cryopreservation method for *L. discolor* axillary buds to perform microscopy analysis of cryopreserved explants, determine the water content changes, and genetic stability between control and treated explants. Six (6) parameters have been optimized in this V cryo-plate method which are preculture concentration and duration, types of loading solution, duration on PVS2, thawing temperature, and thawing duration. Viability assessment of treated explants was evaluated by using TTC spectrophotometric at 490nm. The best viability for *L. discolor* was obtained when the axillary bud was subjected to 0.25M preculture media for 48 hours, 0.3M sucrose + 0.5M glycerol + 10% DMSO loading solution, dehydration with PVS2 for 20 minutes, thawed in 40°C for 90 seconds. In contemplating the intercellular damages in treated *L. discolor* explants compared to control, histological, and transmission electron microscopy (TEM) was carried out. Histology analysis showed that cryopreserved and non-cryopreserved were structurally different from the control samples. Similarly, transmission electron microscopy displayed structural differences between treated (cryopreserved & non-cryopreserved) and control axillary buds. The cryopreserved axillary buds from histology and TEM analysis appeared to be severely damaged due to freezing. Next, water content analysis was done in each step involved in V cryo-plate method in both cryopreserved and non-cryopreserved samples. PVS2 dehydration step exhibited the lowest water content for both treated samples. Finally, molecular analysis using DAMD, ISSR, and SCoT DNA markers was done to confirm the genetic stability in the treated explants compared to the control. It was found that the polymorphism occurrence in DAMD, ISSR, and SCoT was detected 12.5%, 4.17%, and 10.53% in cryopreserved samples. However, the polymorphism in non-cryopreserved axillary buds in DAMD, ISSR, and SCoT was obtained at 6.15%, 5.77%, and 10%, respectively. SCoT was chosen as the best molecular marker in detecting polymorphism in *L. discolor* compared to DAMD and ISSR.

CHAPTER ONE

INTRODUCTION

The Orchidaceae is the most prominent family in flowering plants, and it consists of approximately 20,000 orchids (Moreira and Isaias, 2008). Not only being cultivated for ornamental purposes, but orchids are also widely believed to have medicinal values. According to Ibrahim et al. (2012), orchids have been exported as one of the major cut flowers from Malaysia with an estimated value of RM 150 million per year.

Ludisia discolor (Ker Gawl.) A. Rich orchid is known as "Jewel orchid', are an endangered native terrestrial orchid species of Southeast Asia, and can be found in Malaysia (Poobathy et al., 2019). *L. discolor* is renowned for its captivating leaves with shiny, intrinsic reticulations. The orchid is a slow-growing perennial herb that flowers annually in the wintery months of October, November, and December (Tseng et al., 2006). The plantlets mature and reproduce through seed production after two to three years of growth. Commonly propagated through seeds, wild *L. discolor* plants usually undergo low germination rates, and currently face the danger of extinction caused by overharvesting (Ket et al., 2004; Lim et al., 2013). *L. discolor* is classified as a medicinal herb that is being used to cure several diseases such as tuberculosis, hemoptysis, neurashania and loss of appetite. Due to its high medicinal values this plant is widely exploited and now it is in the verge of extinction (Liu et al., 2021).

Plant cryopreservation technology is known as a reliable *ex-situ* conservation method in conserving this orchid. It has been marked as an important technique for genetic conservation of endangered, hybrids, and unique and rare orchid species for future selection and breeding purposes (Thammasiri, 2005). Cryopreservation is an ideal long-term germplasm conservation technique because it is not time-consuming, requires minimum space, cost, and labour saving as compared to other germplasm conservation strategies (Suzuki et al., 2008; Engelmann, 2011). Cryopreservation, conducted at low temperatures of liquid nitrogen (-196°C) has the potential for preservation by reducing the metabolism of the target genetic resource to such levels that all biochemical processes and biological deterioration are significantly reduced (Engelmann, 2011).

Cryopreservation method based on vitrification phenomenon, the cells and meristems must be sufficiently dehydrated with concentrated chemicals such as loading solution, (LS), plant vitrification solution (PVS2), and unloading solution (RS) to avoid lethal injury from immersion in liquid nitrogen (LN) (Thammasiri, 2020). Plants contain a high amount of free water content which tends to turn into intercellular ice crystals when directly plunge into LN. Intercellular ice causes lethal injuries to the cellular membranes and reduces the survivability of the explants. Thus, the dehydration step is crucial for a plant sample before subjecting them to ultra-low temperature (Benson, 2008; Engelmann, 2011). Other than dehydration, rewarming or thawing process also should be handled carefully. Rapid rewarming is needed to attain a vitrified state and avoid devitrification (ice melts and reforms crystals) (Mazur, 1984; Fahy et al., 1984). Although there are few other cryopreservation techniques, the vitrification method is very effective because it can warm and cool rapidly and maintain the explants in the glass state (Thammasiri, 2020).

V cryo-plate method is the latest cryopreservation method, created by Yamamoto et al. (2011), by combining encapsulation-dehydration and droplet-vitrification methods. The advantage of using the V cryo-plate method is that it is simple-to-use, has rapid cooling and warming rate, and causes lower explant injury, resulting in a high recovery percentage (Niiino, 2013; Salma, 2014; Matsumo, 2015). The full set of V cryo-plate method involves preculture of excised explants on solidified medium with optimised sucrose concentration for a specified duration at room temperature (RT), LS, PVS2, rapid cooling and rapid warming of samples in a water bath, rewarming in RS, and finally regeneration under *in vitro* condition. Preliminary viability assessments were conducted using the 2,3,5-triphenyltetrazolium chloride (TTC) assay (Whiters 1985). The benefits of this technique include ease of performance, repeatability, and the lack of requirement for sophisticated equipment (Mikula et al., 2006).

Successful cryopreservation depends on the optimization of few factors to minimise the intercellular damage caused by a series of steps involved in it (Engelmann, 2004). More osmotic and chemical stress causes an alteration in a cryopreserved cell in terms of ultrastructure, morphological, and molecular compared to the control cells (Harding, 2004; Benson, 2008). Thus, water content analysis was done in this experiment because water content plays a crucial factor in determining post-cryopreservation recovery rate (Sakai and Engelmann, 2007).

Other than that, ultrastructural analysis is also vital in cryopreservation studies. This analysis helps to understand the changes that happened in the ultrastructural level of a cell during cryopreservation, which could enhance the survivability rate and avoid lethal injuries (Miao et al., 2005). Explant being frozen at ultra-low temperature cause extreme stress and damage to the cells such as membrane rupture and denaturation, deterioration of enzyme activity, and cell wall degeneration.

Nonetheless, cryopreservation can also cause genetic variation in cryopreserved samples (Harding, 2004). Genetic variation happens when the alleles of genes in DNA vary in a gene pool of a species. Variation at the molecular level should be avoided to obtain a true-to-type plant. Genetic variation can be determined by using molecular markers. Directed amplification of minisatellite DNA (DAMD), inter simple sequence repeats (ISSR), and start codon targeted (SCoT) are the markers that can be used for molecular studies in cryopreservation. DAMD and ISSR markers are very useful tools for genetic diversity studies in plants. They exhibit a comprehensive depiction of the degree of diversity (Purayil et al., 2018). However, SCoT is widely used to evaluate phylogenetically and produce better resolvability than other markers (Etminan et al., 2016).

1.1 Problem statements

Ludisia discolor is a slow-growing plant, commonly propagate through seeds. This plant is overharvested lately because of its medicinal properties and is used for traditional medicine. This forces wild orchids to face extinction. Hence, there is a need to address this problem through cryopreservation as it acts as one of the promising tool for long-term germplasm conservation.

1.2 Research objectives

The objectives of this research are:

1. To develop an efficient PVS2 V cryo-plate cryopreservation technique for *Ludisia discolor* axillary buds,

2. To determine histology and transmission electron microscope (TEM) analyses in the cryopreserved explants,

3. To carry out water content analysis and genetic stability for control and treated explants using DAMD, ISSR, and SCoT-DNA markers.

CHAPTER TWO

LITERATURE REVIEW

2.1 Orchids

Orchids belong to the family Orchidaceae, and it is the largest group in angiosperm. Recent estimation shows a range of 25,000-35,000 species in 5 subfamilies and 150,000 artificial hybrids are available (Cribb, 2003; Yue, 2006; Sarmah, 2017). The word orchid emerged from a Greek term, *orchis*, meaning testis, an association made by Theophrasus (370-285 a.c.) due to the similarity of the Mediterranean's underground bulbs orchid and mammalian organ. Orchid (*Ian bua*) is a symbol of intellect, humble, sustain, austere and straightforward according to the Chinese (Teo, 2005).

Orchid classified under the class Liliopsida has multiple structural organizations, flower arrangements, forms, and colours, divided into the subfamilies Apostasioideae, Cranichideae, Cypripedioideae, Epidendroideae, and Orchidoideae. Cranichideae and Orchidoideae contain many terrestrial orchids, while the other three subfamilies host the majority of epiphytic species (Dressler, 1993; Moreira and Isaias, 2008). Subfamily Orchidoideae distinguished their multiplicity in terms of the exclusive adaptations found in their anatomy and morphology (IUCN/SSC Orchid Specialist Group, 1996). They own complex life cycle, which consists specific pollination syndromes, and interdependent mycorrhizal requirement in seed germinations (Mahendran and Narmatha Bai, 2015). Orchids vastly have occupied the earth in areas ranging from the rainforests, cloud forests, tundra, semi-desert scrubs, and the desert (Wraith et al., 2020).

An orchid plant consists of inflorescence, leaves, stems, rhizomes, and roots (Kramer, 1975; Moreira and Isaias, 2008). Among the total number of orchids worldwide, epiphyte makes up to 75% (Moreira and Isaias, 2008). Orchids are found as epiphytes, lithophytes, terrestrial, or saprophytes (Moreira and Isaias, 2008) which have a structural difference in vegetative organs (Pabst and Dungs, 1975; Weston et al., 2005). Generally, epiphytes own aerial root that helps to anchor the plant on substrates and take up nutrients (Moreira and Isaias, 2008). However, terrestrial orchids obtain required nutrients from the soil and have any of the following three root adaptation: roots involved in the absorption, fixation, and storage of nutrients; roots involved in both absorption and fixation of nutrients; or roots that function only as nutrient storage and reservoir (Moreira and Isaias, 2008). Tubers of terrestrial plants can withstand conditions without water (Dressler, 1993; Moreira and Isaias, 2008).

Orchids are often mistaken as parasites that depend on trees for nutrients. However, the reality is that orchids are epiphytes that rely on trees only for support. Orchids are available in various shapes that determine their fancy names. The variations observed in orchids are very diverse that at first glance, one could not comprehend that all the individual orchids belong to the same family. Cross-pollination by man is way effective than natural pollination, and almost 100000 hybrids have been produced from this last century (Teoh, 2005).

Plenty of orchids from other parts of the world were introduced in south-east Asia 80 years ago and now part of our garden flora. Some countries like Thailand and Singapore have pioneered in hybridisation and growing orchids. In Malaysia, the family orchidaceae comprises more than 10% (972 species 143 genera) of the entire Flora of Peninsula Malaysia (8300) (Ong, 2017). Orchidaceae represent 15% of all the flowering plants, making them the largest plant family in Peninsular Malaysia (Kiew et al., 2010; Ong et al., 2011).

The largest threat to most of the native angiosperms in Malaysia is the declination or loss of habitat due to changes in land use (Lim et al., 2013). Orchids are exploited for their economic value, especially in horticulture and floristry (Mahendran and Narmatha Bai, 2015). Orchids were once extensively used as traditional medicine, as many presumed their efficacy as a treatment for various ailments (Barragán-Zarate et al., 2020; De, 2020). An example of a rare orchid used for its medicinal purpose is *Aphyllorchis montana* (Sinu et al., 2012; Mahendran and Narmatha Bai, 2015). Native to India and popular in the Ayurvedic treatment system (Thalla et al., 2013), the orchid's extract is used to treat cough, colds, diabetes, and anaemia, and for general body strength (Prajapati et al., 2003; Pentela et al., 2012; Thalla et al., 2013; Mahendran and Narmatha Bai, 2015). Hence, lots of orchids worldwide are categorised as threatened due to overcollection for the reasons above (Mahendran and Narmatha Bai, 2015).

2.2 Jewel orchid

Tribe Cranichideae, composed of approximately 1500 species which are classified into 90 generas. Around 95% of these orchids are grown in tropical regions globally (Dressler, 1993; Chase et al., 2003; Pridgeon et al., 2003; Figueroa et al., 2008). Orchids from cranichideae have storage organs such as plump roots, which could be found at the nodes on creeping or underground rhizomes, clumping in their natural habitat (Figueroa et al., 2008). Most members of this tribe are terrestrial. The preferred condition for this tribe is under a deep shady area. Hence, they are widely tracked in leaf litter on the forest floor. There are saprophytes present in this subtribe such as *Cystorchis aphylla*, but others have similar morphology to tiny bamboo plants. Orchids of this tribe, namely Goodyerinae and Tropidiinae, possess sectile pollinia (Freudenstein and Rasmussen, 1997). Orchids from subtribe Goodyerinae, members of Cranichideae, have horizontal stems which are surrounded by a rosette of leaves (Figueroa et al., 2008).

Jewel orchids are the most prominent members of the group. An orchid popularly grown for its leaves than its flower is referred to as a jewel orchid (Gangaprasad et al., 2000). *Anoectochilus, Dosinia, Goodyera, Ludisia,* and *Macodes* are the genera classified as jewel orchid (IUCN/SSC Orchid Specialist Group, 1996). All the jewel orchid member comes under the tribe Cranichideae and subtribe Goodyerinae, which makes them immensely related to one another (Hayden, 2016). Although some of them are temperate, jewel orchids are terrestrial plants, and some are a tropical plant (Hayden, 2016). These varieties' foliage is unique and attractive than their relatively small flowers (Thanh et al, 2012).

Members of jewel orchids can cross-pollinate. Some examples are between *A. formosanus* and *A. koshunensis* (Cheng et al., 1998; Chen and Shiau, 2015), and between A. *formosanus* and *L. discolor* (Chou and Chang, 2004; Chen and Shiau, 2015). *Ludochilus* Jin-Chai, a hybrid registered under the International Orchid Registrar of the Royal Horticultural Society in 2005, was virtually indistinguishable from *A. formosanus*. The *Anoectochilus* genus is closely linked to *L. discolor*, as elucidated through the ITS sequences and matK pseudogene. Both molecular methods were suitable in differentiating orchids that belong to the subtribe Goodyerinae (Chen and Shiau, 2015).

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

The genus *Ludisia*, previously known as haemaria belongs to the tribe Erythrodeae. These orchids are found in many places in Asia. Due to the blood-red colour sheathing bracts (Schultes and Pease, 1963), it was named as αίμα or "haima" in Greek by Lindley (1826) which translates as blood. *Ludisia discolor* (Mandarin: Xue-ye-lan or Cai-ye-lan) is known as a jewel orchid due to its striking leaf pattern with intricate reticulations than its tiny white flowers (Shiau, 2005; De, 2014). It is native to southern China and Burma's regions through the Malay Archipelago, Sumatra, Java, and other islands of Indonesia (Teo 1978).

L. discolor thrives under low light conditions, grow on mossy rocks and leaf litters near streams, and sloped of loamy soil or red clay (The Agrobiodiversity Initiative in the Lao PDR, 2010). It is a slow-growing perennial plant, and seedlings mature and reproduce through seeds after 2 - 3 years of growth (Hawkes 1970 cited in Shiau, 2005). This orchid's blooming period is from late December to March (Chou and Chang, 2004). *L. discolor* is the only species that belongs to its monotypic genus, Ludisia (Hayden, 2016). This species is widely distributed and has a long history in horticulture, a wide variety of veins, and interveins are expected to be found in cultivated plants. *L. discolor* is easily grown in greenhouse and indoor and greatly valued in floriculture for its attractive foliages (Watson, 2011).

Fully matured *L. discolor* could reach heights up to 10-25 cm. This orchid has an upright stem with 2-5 leaves which measure 1.5-2.2 cm from the base, growing at the petiole. Five silvery-red veins are found on fleshy, apex acute, or mucronate leaves abaxially pale red and adaxially blackish green. The shape of the leaves are oblong-lanceolate to elliptical and are between 3-7 cm and 1.7-3 cm in length and width respectively. The inflorescences of the plant are pubescent and possess two to three sterile bracts. The rachis, measuring 3-8 cm, may possess laxly few to more than 10 flowers. The flower produced is white with red-tinged. The plant flowers from February to April each year (Chen et al., 2009). Green foliage with red shades is famous among the other variety, dark purple with unclear veins. There is also an anthocyanin-missing cultivar of *L. discolor,* which has light green leaves with white veins similar to Goodyera (Hayden, 2016).

L. discolor (Plate 2.1) is known as guāng slā by the Li ethnic group of Mount Yinggeling, Hainan Island (Wang, 2004; Zheng & Xing, 2009), as 'ya bai lai' or 'yna bai lai' in Laos, and as 'phak bia chang', 'wan nam thong' or 'wan ron thong' in Thailand (The Agrobiodiversity Initiative in the Lao PDR, 2010). *L. discolor*, together with *Pholidota chinensis*, was traditionally used to treat coughs and strengthen weak lungs (Tsui, 1992; Chou and Chang, 2004). The extract by toasting the whole plant is used by Li ethnic community to apply onto external injuries (Zheng and Xing, 2009). However, the Lao community believes that fresh or boiled *L. discolor* helps to reduce aging. Some useful components that have been found in the leaf extract of this orchid are amino acids such as asparagine, glutamine, histidine,

Plate 2.1: ***L. discolor*** **from greenhouse.** Scale bar represents 5cm

serine, and threonine (Arditti, 1992; Shiau et al., 2005). *L. discolor* is resistant to heat, insects, and diseases, contrary to *Anecthochilus formosanus*.

In Laos, the entire plant is dug out from the ground to harvest the rhizomes or roots, and collections may range between 200-300 g per person per day, depending on the orchid population density and specific habitats. According to Lim (2013), *L. discolor* faces depletion in numbers due to over-harvesting and not sufficiently strict management rules. The orchid can be propagated by seeds and rhizomes, with the latter simply propagated by division. Trade data of this orchid indicate that the wild plants are exported to China for between US$1-9/kg in and US$40/kg to Korea (The Agrobiodiversity Initiative in the Lao PDR, 2010). The estimated price proxy of *L. discolor* orchid species exported from the Amazon region in 2005-2014 was 9,900,000 USD, where the pricing for one live plant was 17.33USD (Sinovas, 2017).

2.4 Germplasm conservation

Conservation of plant genetic resources is essential for food security and agro-biodiversity (Kaviani, 2011). According to Rao (2004), new and high-yielding varieties of plants that can withstand environmental pressure could be produced by selecting and breeding from genetic diversity. It is important to use a larger scale of genetic diversity worldwide as the demand for plant-related industries increases year by year.

However, landrace sustainability in developed and developing countries is at risk causing many plant species to face extinction lately (Heraty & Ellstrand, 2016). Hence, their preservation is crucial for plant breeding programs which will eventually become a source for medical, food, and crop protection industries (Panis and Lambardi, 2006). Although orchid utilization has a significant impact on the local and state economy, uncontrolled destruction of natural populations of terrestrial orchids for commercial purposes significantly endangers the existence of some species (Kreziou et al., 2016).

Germplasm conservation can be divided into two: *In situ* conservation and *ex situ* conservation. *Ex situ* and *in situ* act as the conservation initiative to reduce the loss in diversity centres (Heraty & Ellstrand, 2016).

2.4.1 In situ conservation

In situ conservation means protecting and rehabilitating a species' viable population in its original habitat without relocating them. It maintains the content at its original site, where it was discovered, and continues the evolutionary cycle (Malhotra et al., 2019). However, due to high risks from forest destruction *in situ* conservation method could not guarantee the conservation of a species (Lauterbach et al., 2012).

In situ activities permits the conservation of geographical partitioned genetic diversity which maintains the potential for local environmental-evolutionary adaptation (Vincent et al., 2019).

2.4.2 *Ex situ* conservation

Ex situ method perhaps protect the species diversity that could be used for reviving programme in case of extinction (Lauterbach et al., 2012). *Ex situ* conservation involves preserving and reproducing threatened organisms under limited or fully designed environments in places such as zoos, gardens, and nurseries (Jaisankar et al., 2018). *Ex situ* conservation could be done in a few ways like gene banks, botanical gardens, and reserves provide a controlled environment mainly to conserve the germplasm of the organism (Heraty & Ellstrand, 2016).

Growing plants in gardens and nurseries help the researchers to physically evaluate them in such aspects as total yield, length, and potential disease (O'Brien et al., 2011). However, there are some drawbacks to these repositories. Repositories are expensive and demand constant supplies in space, human resources, administration, and materials to maintain diversity. Moreover, plants that have been grown in repositories are more prone to diseases and pests (O'Brien et al., 2011).

2.5 Plant Cryopreservation

As for long-term germplasm conservation of biotechnology plant products, non-orthodox seeds, and vegetatively propagated species, cryopreservation is considered a safe and cost-effective option (Engelmann, 2004). The plant cryopreservation technique using various parts of a plant took up in the 1960s and continues to date (Reed, 2017). During the cryopreservation process, cells or tissues are preserved in ultra-low sub-zero temperature, -196°C (boiling point of liquid nitrogen) in such a way that the viability is retained following re-warming (Kaczmarczyk et al., 2012). Cryopreservation, conducted at low temperatures, has the potential for 'undefined' preservation by reducing the metabolism of the target genetic resource to such levels that all biochemical processes and biological deterioration are significantly reduced (Panis, 2018).

Adding only a small volume of LN weekly in cryo-dewars is the only maintenance required in cryostorage. Low labour maintenance, usage of minimum space, and costs are some other advantages of the cryopreservation technique. The labour maintenance is lower compared to the living collection and even compared to looking after the tissue culture samples at room temperature. The risk of contamination is also greatly reduced once the sample is cryogenically stored, and its genetic stability is retained (Harding, 2004).

The concept of cryopreservation comprises few correlated disciplines of physiological to cryophysical. Water content in the sample and the type of cryoprotectant usage are the top influencer of survival conjointly with physiological factors (Reed, 2008). Re-establishment of shoot cultures of small, cryopreserved samples may take several weeks and is plant specific. It also takes several months to years to become fully grown and ready to be acclimatised (Kaczmarczyk et al., 2012). Initially, cryopreservation was used for suspension and callus culture, now been widely used for organised tissues (Reed, 2008). There are many methods of cryopreservation available. Some of them are encapsulation-dehydration, encapsulation-vitrification, droplet-vitrification, slow-cooling, or controlled rate cooling.

Successful cryopreservation of plant depends on ‘vitrification’, the concretion of liquid without solidification (Plate 2.2). This comprises a “glassy state” as the system is amorphous and lacks organised structure but possesses stable mechanical and physical properties (Fuller et al., 2004). Cell viscosity (more solute) determines the water vitrification in biological systems. Increased viscosity prevents the accumulation of water molecules from forming ice.

The vitrified state is called metastable meaning that it can relatively easily convert back to its original state. There are many ways to achieve vitrification, but all will result in increased solute concentration at the end. Vitrification is used to support cryopreserving multiplex, diverse tissues for which it is tough to achieve optimum cooling rates. Plant materials may be preconditioned on special media or with specific growth conditions, precultured with cryoprotective chemicals just before cryopreservation, and protected with vitrification solutions before plunging in techniques, so the ice phases of water are avoided.

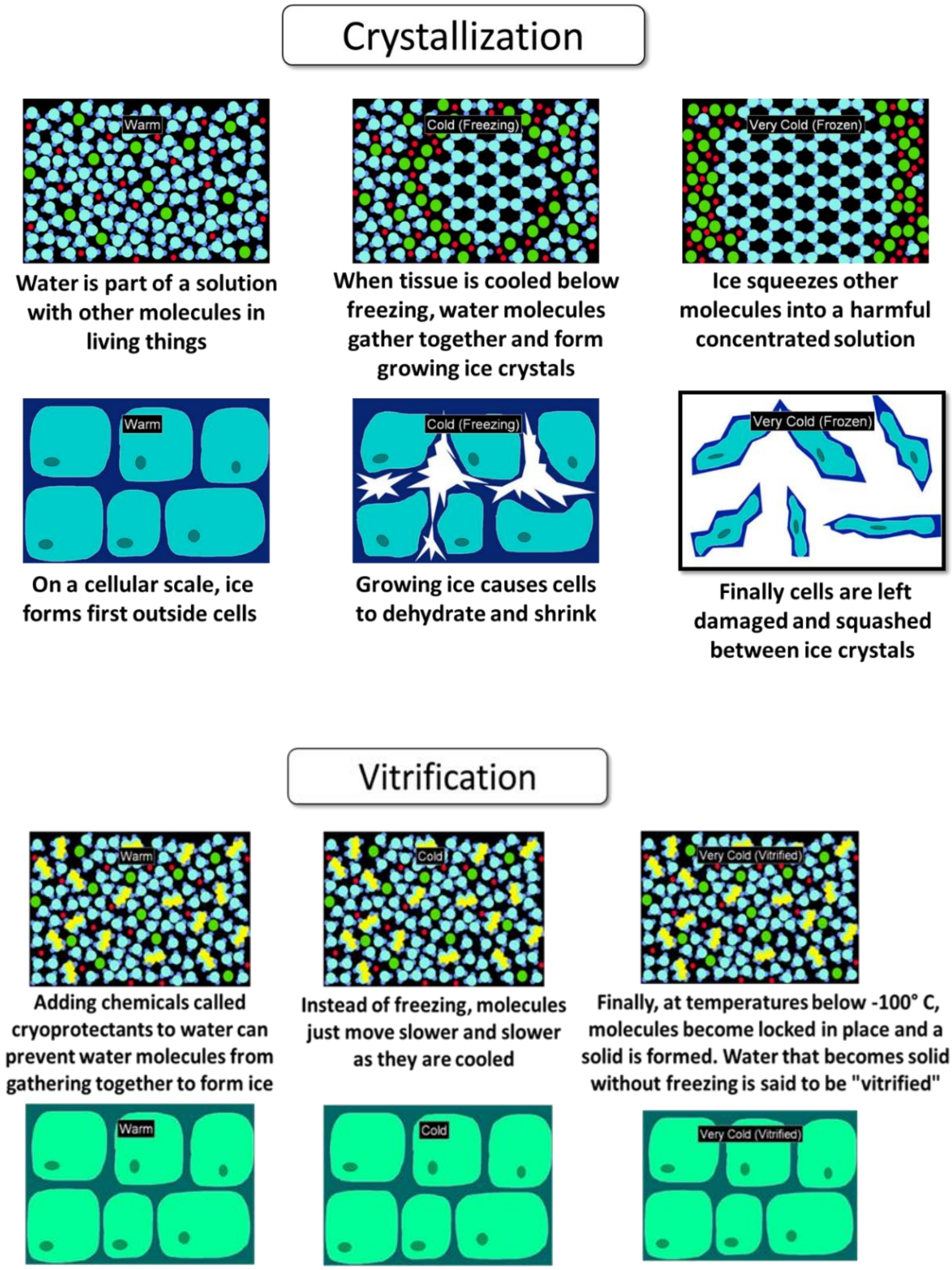

Plate 2.2: Comparison of crystallization and vitrification

(Source: https://www.alcor.org/library/what-is-vitrification/)

2.5.1 Cryo-plate method

Aluminium cryo-plate (Plate 2.3) facilitates the cryopreservation process by dehydrating explants by two different protocols: vitrification and dehydration. In the vitrification protocol, various types of vitrification solutions are used, and this protocol is termed as V cryo-plate method. However, the dehydration protocol uses simple air-dehydration and is termed D cryo-plate method (Yamamoto et al., 2011; Niino et al., 2013). Cryo-plate method was developed to simplify the cryopreservation process and aids the semi-skilled workers to perform efficiently (Yamamoto et al. 2012).

The cryo-plate method is considered a convenient cryopreservation method compared to others due to its simplicity and user-friendly. V cryo-plate method is a result of a combination of encapsulation-vitrification and droplet-vitrification methods. V cryo-plate method was newly invented to preserve different plant varieties in cold conditions 9 years ago by Yamamoto et al. (2011). This latest method is a new vitrification style whereby the explants are fixed in calcium alginate on aluminium cryo-plate wells before osmoprotectant and dehydration steps (da Silva et al., 2015).

Aluminium plates used in this method are thicker than the aluminium foil used in the droplet-vitrification method. This method ensures very high warming and cooling rates of explants, which results in low freezing damage (Yamamoto et al., 2011; Niino et al., 2013; Rafique et al., 2015). This property can be linked to the fact that aluminium plates have better thermal conductivity than aluminium foils. According to Thammasiri et al. (2020), the cooling and warming rate of aluminium foil is approximately 4,000°C/min and 3,000°C/min respectively; while aluminum plate has a cooling rate of 5,000 °C/min and a warming rate around 4,500 °C/min. Due to this feature, cryo-plate results in a very high regrowth rate in explant after cryopreservation (Niino et al., 2013).

A wide-scale of studies is being carried out lately on the efficiency of V cryo-plate techniques. Root tips of *Passiflora pohlii* Mast. (Passifloraceae), were selected as the starting material of cryopreservation in the experiment by Simão et al. (2018). In this study, they used V cryo-plate method using two plant vitrification solutions, PVS2 and PVS3. 45 mins immersion in PVS2 is chosen as the best condition as it yields 79% of the recovery. Another study by Pettinelli et al. (2020), shows that a 100% viability result was observed in somatic embryos immersed in PVS2 for 15 minutes. In this experiment, they used 0.5M sucrose to dehydrate the explant, 3% sodium alginate, and 1.2M of sucrose in unloading solution.

The aluminium cryo-plate that has been used in this study was modified and bigger than the one readily available in the market. It has been designed to be more significant to ease the embedment of larger samples in the cryo-plate wells.

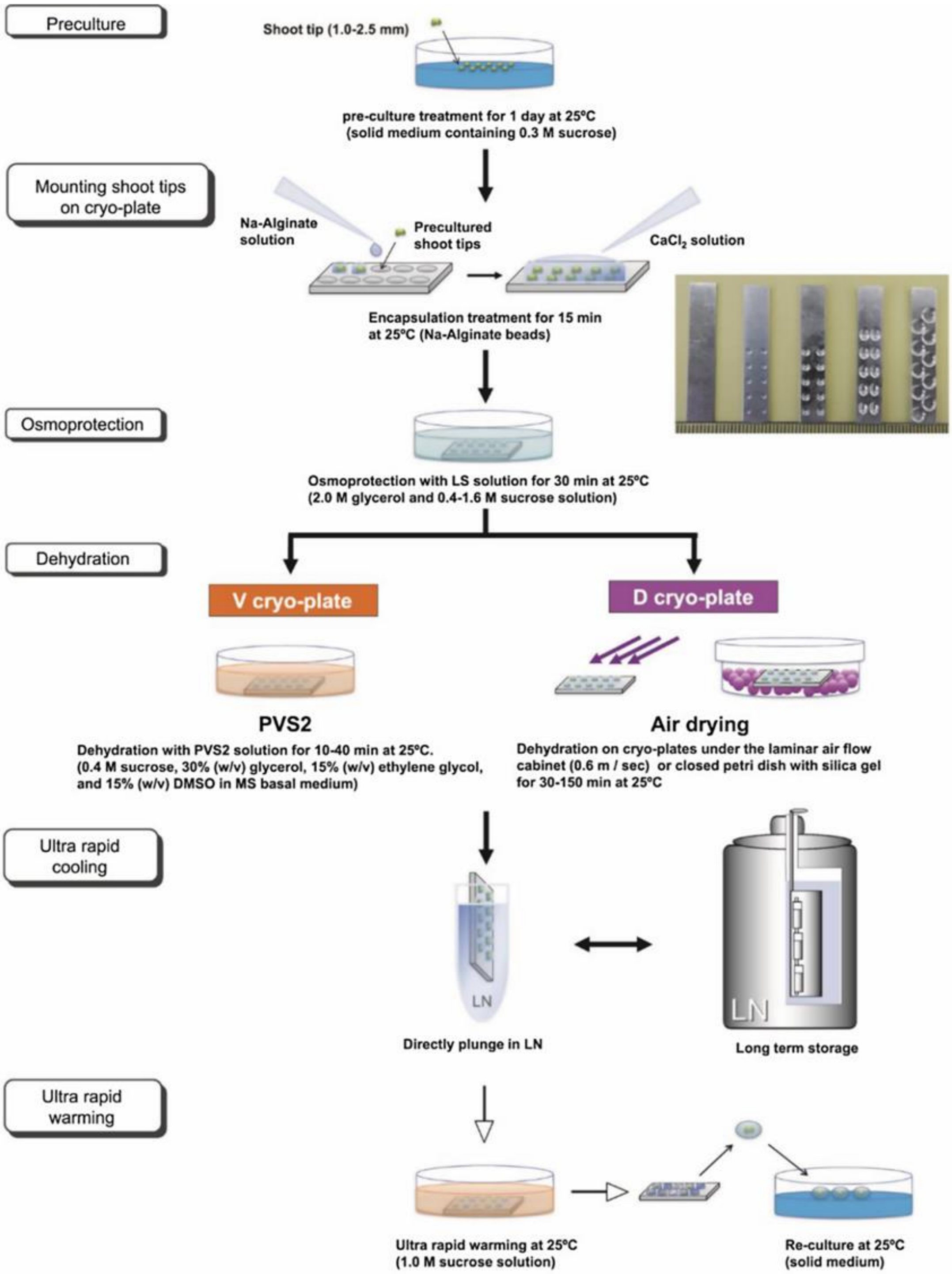

Plate 2.3: General procedure of cryo-plate cryopreservation method. (Tanaka et al., 2018)

2.6 Orchid cryopreservation

There are studies been published about cryopreservation on orchids using various cryopreservation methods. Different parts of the orchids been used as explants for cryopreservation process, and varying degree of success was obtained. Cryopreservation initially started from the dormant bud method. Dormant buds will be collected during winter and used as the explants, usually been dehydrated at low temperatures (-10 to -30°C) before transferring them in liquid nitrogen (-196°C). Although there are few literatures on the dormant bud cryopreservation method, none were reported on orchids.

Cryopreservation then emerged from the dormant bud method to the slow freezing method. A programmable freezing controller was needed to achieve the goal in this method. This controller was controlled at a rate of 0.5–2 °C/min and can reach up to -40°C depends on the plant material been used before plunging in LN. Despite the popularity of this method during the 1980-1990s, it is time-consuming and expensive.

After that, the vitrification method was born and evolved into the latest ones such as encapsulation-vitrification, encapsulation-dehydration, droplet-vitrification, and finally the cryo-plate method. Vitrification is a method developed to increase the cooling and warming rate's efficiency and ensure that all parts of cells and tissues of the explant are in a glass state. Both encapsulation-dehydration and encapsulation-vitrification methods are developed from artificial seed creation that explants are encapsulated in alginate beads, precultured with high sucrose, desiccated or vitrified, and then plunged into liquid nitrogen (Matsumo & Sakai, 1995, Hirai & Sakai, 1999, Sakai et al., 2000, Matsumo, 2017).

Protocorms-like bodies (PLBs) of *Vanda lilacina Teijsm. & Binn* was successfully cryopreserved using D cryo-plate and V cryo-plate methods. In this study, three PLBs were embedded in wells of a cryo-plate filled with 2% Na-alginate solution (2 % Na-alginate in ½ MS medium solution). Covered with 50 mM $CaCl_2$ solution for 30 min at room temperature

(25±2 °C) for polymerisation. Immersion in loading solution (LS) (2 M glycerol and 0.4 M sucrose in ½ MS medium solution) for 30 min, PVS2 solution (30 % (w/v) glycerol + 15 % (w/v) ethylene glycol + 15 % (w/v) DMSO in ½ MS medium solution) for 0, 20, 40, 60, and 120 min for dehydration was involved. The cryo-plate put into 1.8 ml uncapped cryotubes and then directly plugged into LN for 1 h. For D cryo-plate PLBs were treated with silica gel method 0, 0.5, 1, 1.5, 2, 2.5, and 3 h. 20min duration in PVS2 was chosen as best for V cryo-plate and 1hr desiccation was chosen for D cryo-plate. The survival rate for D cryo-plate and V cryo-plate was 83.87% and 33.33%, respectively (Imsomboon et al., 2020).

Other than that, D cryo-plate and V cryo-plate cryopreservation methods were applied to *D. cruentum* Rchb. f. seeds. In this experiment, the seed viability was tested by TTC solution, and the result was up to 93.8%. For the cryo-plate technique, seeds were encapsulated over the cryo-plate using 2% (w/v) sodium alginate and polymerised with 100 mM $CaCl_2$. Encapsulated seeds were desiccated using a laminar airflow and PVS2 solution treatment with the same exposure time (0, 30, 60, 90, and 120 min). After cryopreservation, seeds were cultured on an agar medium. As a result, the D cryo-plate technique with 60 min of dehydration time gave the highest germination (68.9 %) and regrowth (57.8 %) (Prasongsom et al., 2020).

2.7. Factors involved in optimisation for cryopreservation by vitrification-based methods

A complete cryopreservation method consists of several steps involved, such as preculture, osmoprotection, dehydration, ultra-cooling, rewarming, and recovery. Not all plant species will give the same response to a particular treatment. Hence, the optimisation of the steps involved in cryopreservation is crucial to achieve the highest recovery rate.

2.7.1 Choices of plant material for cryopreservation

Choosing the right plant material is considered very important in cryopreservation. Stress accumulatively builds up from one step to another throughout the cryopreservation procedure. The tolerance and sensitivity of a plant material become the primary determinant of the success rate of cryopreservation. Moreover, the physiological condition of explants tends to influence the success of cryopreservation. Explants should compose of actively dividing meristematic cells. Thus, explants are generally chosen from actively growing mother plants (Engelmann, 2004).

Cells which have dense cytoplasm and tiny vacuole are observed to survive freezing. Furthermore, the reason to use meristematic explants is to avoid any variation (Kulus & Zalewska, 2014). For instance, protocorm-like (PLB) of *Cymbidium* orchid evinced a high percentage of plant regrowth and finally converted to a complete plantlet (Gogoi et al., 2013). Other than that, a study on *Platycodon grandiflorum* shows that axillary buds were used as the explants in V cryoplate cryopreservation method (Matsumo et al., 2020), and shoot tips of blueberry were isolated and used for cryopreservation and seems to yield a 100% survival rate (Dhungana et al., 2017).

Table 2.1: Successful cryopreservation on different plant species using various cryopreservation methods.

Species/hybrid	Plant material	Cryopreservation method	Regrowth rate (%)	References
Apple cultivar (Pinova)	Axillary buds	Droplet-vitrification	46.7	Condello et al., 2011
Ascocenda Wangsa *Gold*	Protocorm-like bodies	Vitrification	53.3	Rajasegar et al., 2015
Caladenia latifolia	Protocorms	Vitrification	96	Watanawikkit et al., 2012
Cannabis sativa L.	Axillary buds	V-Cryoplate Droplet-Vitrification	47	Lata et al., 2019
Catasetum atratum	Seeds	Droplet- vitrification	67.7	Prenzier et al., 2018
Cymbidium finlaysonianum	Matured seed	Vitrification	82	Hirano et al., 2011
Cymbidium goeringii	Matured seed	Vitrification	32	Hirano et al., 2011
Cymbidium macrorhizon	Matured seed	Vitrification	76	Hirano et al., 2011
D. chrysanthum Wall. ex Lindl.	Protocorm-like bodies	Encapsulation–vitrification	59.9	Mohanty et al., 2013

Dendrobium nobile	Protocorm-like bodies	Encapsulation–vitrification	75.9	Mohanty et al., 2012
Dendrobium hybrid	Protocorms	Droplet-vitrification	14	Galdiano et al., 2012
Dendrobium hybrid	Matured seed	Vitrification	79	Galdiano et al., 2012
Diospyros kaki Thunb. ‘ Saijo’	Shoot tips	D cryo-plate	84	Matsumo et al., 2015
Hypericum richeri ssp. transsilvanicum	Axillary buds	Droplet-vitrification	71	Coste et al., 2012
Vitis spp.	Shoot tips	Droplet-vitrification	50.5	Bi et al., 2018
Sugarcane	Shoot tips	V Cryo-plate	100	Rafique et al., 2015
Perilla frutescens L. Britton	Nodes	V Cryo-plate	80	Matsumo et al., 2014

2.7.1(a) Axillary bud as plant material for cryopreservation

The starting material used for cryopreservation in this experiment is the axillary bud (Plate 2.4). Nodes are the points where the leaves and flowers grow out of a stem. However, internodes are the intervening length of a stem. The elongation of internodal cells entirely determines the elongation of the stem. Bulbs and rosette plants tend to form when these internodal cells fail to elongate.

Generally, during this process, small groups of meristematic cells accumulate at the axils (the angle between the stem and leaf primordium) of the leaf or point of leaf stalk connected to the stem. These meristematic parts of a stem are referred to as axillary buds. Axillary buds could turn into either branches or flowers. The most common stem that being witnessed is the stem above ground. But there are also stems that grow underground. The common potato is a popular example of an underground stem (Hopkins & Hüner, 2004).

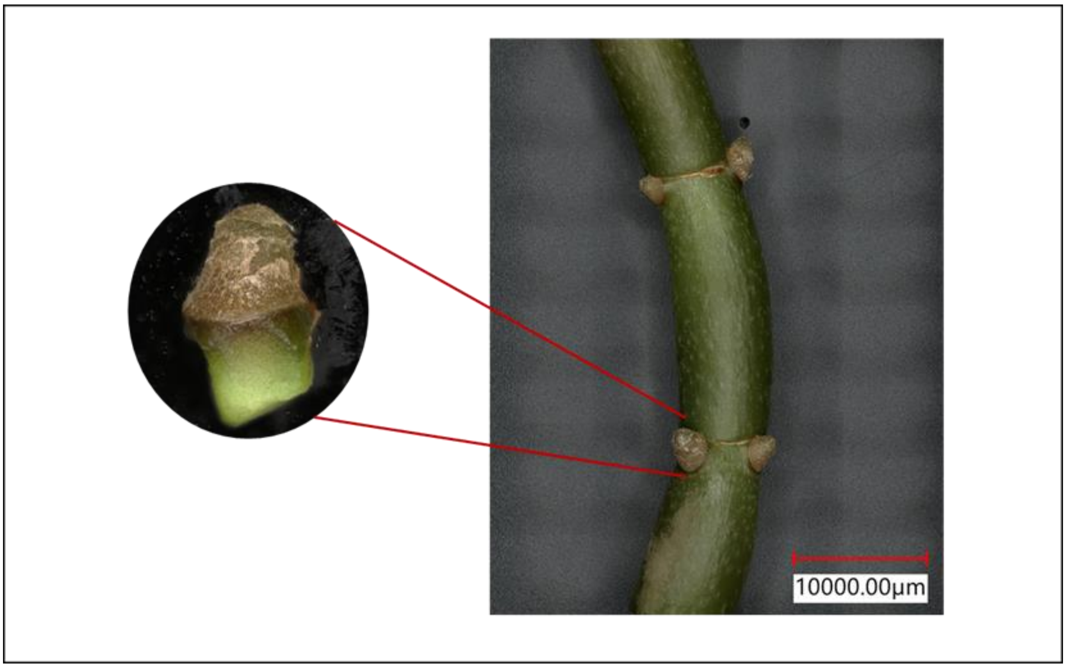

Plate 2.4: Axillary bud of *L. discolor* used for cryopreservation. Scale bar represents 10000µm