

**VITRIFICATION-BASED CRYOPRESERVATION OF  
*VANDA COERULEA* GRIFF. EX LINDL.**

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Thesis  
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OF *VANDA COERULEA* GRIFF. EX LINDL.**

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**ABSTRACT**

*Vanda coerulea* is a rare Thai orchid that faces problems in conservation attempts that is desired in this research. The effects of media (modified Vacin and Went 1949 (VW), Murashige and Skoog 1962 (MS)), sucrose concentrations (0-30 g/l) and plant growth regulators (BA, TDZ and NAA) were studied on micropropagation of *Vanda coerulea* from shoot tips. Shoot tips cultured on VW agar medium supplemented with 10 g/l sucrose showed higher shoot and root formation than those cultured on MS medium. Addition of 1 mg/l BA to VW agar medium was found to be the best for induction of shoots from shoot tips. The combination of 0.5 mg/l NAA and 2 mg/l TDZ was found to be the best for root formation from shoots. Survival rate of plantlets cultured in the greenhouse was 100%. There was no difference in morphology and similar patterns of ploidy level of stock plants and regenerated plants.

Seeds of *Vanda coerulea* were successfully cryopreserved using direct plunging into liquid nitrogen, vitrification, encapsulation-dehydration and encapsulation-vitrification. The manipulation of encapsulated seeds was much easier than vitrification. Protocorms, shoot tips and protocorm-like bodies of *Vanda coerulea* were successfully cryopreserved by encapsulation-dehydration in combination with loading solution, slow freezing and droplet-vitrification, respectively. The highest regrowth of cryopreserved protocorms was 40% with 35% water content after 8 h dehydration. The survival rate of cryopreserved shoot tips and cryopreserved protocorm-like bodies without exposing to PVS2 solution was 20% and 5 %, respectively. The RAPD marker was able to be efficiently used for estimating the genetic variation of plantlets. The same RAPD patterns of non-cryopreserved and cryopreserved protocorm-like bodies were observed. The size and sequences of the *trnL* (UAA) region of cpDNA for non-cryopreserved and cryopreserved protocorm-like bodies were not different. There was no difference in morphology and similar patterns of ploidy levels of non-cryopreserved and cryopreserved plantlets. The successful development of the methods will lead to more successful preservation of *Vanda coerulea*.

**KEY WORDS:** *VANDA COERULEA* / MICROPROPAGATION / CRYOPRESERVATION / VITRIFICATION / RAPD

181 pp.

การเก็บรักษาเชื้อพันธุ้กล้วยไม้พุ่มที่อุณหภูมิต่ำกว่าจุดเยือกแข็ง โดยวิธี Vitrification  
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#### บทคัดย่อ

การศึกษาผลของสูตรอาหาร (สูตรดัดแปลง Vacin and Went 1949 (VW), Murashige and Skoog 1962 (MS)) และความเข้มข้นของน้ำตาลซูโครส (0-30 กรัมต่อลิตร) ต่อการขยายพันธุ์ของกล้วยไม้พุ่มจากปลายยอด ผลการทดลองพบว่าเมื่อเลี้ยงปลายยอดบนอาหารสูตรดัดแปลง VW ที่เติมน้ำตาลซูโครส 10 กรัมต่อลิตร สามารถให้จำนวนยอดและรากเฉลี่ยสูงกว่าเมื่อเลี้ยงบนอาหารสูตร MS การศึกษาผลของสารควบคุมการเจริญเติบโต (BA TDZ และ NAA) ต่อการชักนำให้ปลายยอดเกิดยอดและรากของกล้วยไม้พุ่ม พบว่าเมื่อเลี้ยงปลายยอดบนอาหารที่เติม BA ความเข้มข้น 1 มิลลิกรัมต่อลิตร ให้จำนวนยอดเฉลี่ยสูงสุด และอาหารที่เติม NAA ความเข้มข้น 0.5 มิลลิกรัมต่อลิตร ร่วมกับ TDZ ความเข้มข้น 2 มิลลิกรัมต่อลิตร ให้จำนวนยอดที่ผลิตรากและจำนวนรากเฉลี่ยสูงสุด อัตราการรอดชีวิตของต้นกล้วยไม้พุ่มเมื่อเลี้ยงในสภาพโรงเรือนเฉลี่ยร้อยละ 100 การขยายพันธุ์ต้นกล้วยไม้พุ่มจากปลายยอดไม่พบการเปลี่ยนแปลงทางสัณฐานวิทยาและการเปลี่ยนแปลงชุดของโครโมโซม

เมล็ดกล้วยไม้พุ่มสามารถเก็บรักษาโดยวิธีการเก็บในไนโตรเจนเหลวโดยตรง vitrification, encapsulation-dehydration และ encapsulation-vitrification การทดลองพบว่าวิธีการเก็บรักษามะล็ดในรูปแบบเมล็ดเทียม (encapsulation) ง่ายกว่าการเก็บรักษาโดยวิธี vitrification การเก็บรักษาโปรโตคอร์มของกล้วยไม้พุ่มโดยวิธี encapsulation-dehydration ร่วมกับสารละลาย loading พบว่าโปรโตคอร์มสามารถเจริญเป็นต้นกล้าได้สูงสุดเฉลี่ยร้อยละ 40 เมื่อใช้ลมเป่าเป็นเวลา 8 ชั่วโมง ซึ่งมีปริมาณน้ำในเมล็ดร้อยละ 35 การเก็บรักษาปลายยอดโดยวิธี slow freezing และการเก็บรักษา protocorm-like bodies โดยวิธี droplet-vitrification โดยไม่ได้ใส่สารละลาย PVS2 พบว่าอัตราการรอดชีวิตสูงสุดเฉลี่ยร้อยละ 20 และ 5 ตามลำดับ การเก็บรักษา protocorm-like bodies ในไนโตรเจนเหลวไม่พบการเปลี่ยนแปลงทางลักษณะทางพันธุกรรมเมื่อทดสอบด้วยเทคนิค RAPD และไม่พบว่ามี การเปลี่ยนแปลงขนาดและลำดับเบสบริเวณ *trnL* (UAA) ของ cpDNA ชุดของโครโมโซมและสัณฐานวิทยาเมื่อเปรียบเทียบกับ protocorm-like bodies ที่ไม่ได้เก็บรักษาในไนโตรเจนเหลว

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## LIST OF ABBREVIATIONS

### Abbreviation or symbol

ANOVA	analysis of variance
ABA	abscisic acid
AFLP	amplified fragment length polymorphisms
BA	6-benzylaminopurine
°C	degree Celsius
°C/min	degree Celsius per minute
cm	centimeter
cpDNA	chloroplast DNA
CRD	Completely Randomized Design
d	day
2,4-D	2,4-dichlorophenoxy acetic acid
DMSO	dimethylsulphoxide
EG	ethylene glycol
e.g.	example gratia (Latin), for example
et al.	et alli (Latin), and others
<i>etc.</i>	et cetera (Latin), other things
g	gram
g/g	gram per gram
°F	degree Fahrenheit
FCM	flow cytometry
g/l	gram per liter
GA <sub>3</sub>	gibberellic acid
h	hour
H3	Hyonex
IAA	indole acetic acid
IBA	indole butaric acid

**LIST OF ABBREVIATIONS (continued)**

## Abbreviation or symbol

KC	Knudson
km	kilometer
KN	kinetin
LN	liquid nitrogen
LS	loading solution
LSD	least significant difference
M	Molar
m	meter
mg	milligram
mg/l	milligram per liter
min	minute
ml	milliliter
ml/l	milliliter per liter
mm	millimeter
mM	millimolar
MS	Murashige and Skoog
NAA	$\alpha$ -naphthalene acetic acid
ND	New Dogashima
NP	New <i>Phalaenopsis</i>
PEG	poly ethylene glycol
PLBs	protocorm-like bodies
PVS1	plant vitrification solution formula 1
PVS2	plant vitrification solution formula 2
PVS3	plant vitrification solution formula 3
PVS4	plant vitrification solution formula 4
RAPD	random amplified polymorphic
rpm	round per minute

**LIST OF ABBREVIATIONS (continued)**

## Abbreviation or symbol

sec	second
TDZ	thidiazuron
Td	devitrification temperature
Tg	glass transition temperature
Tm	melting temperature
μl	microliter
μM	micromolar
v	voltage
v/v	volume per volume
VW	Vacin and Went
w/v	weight per volume
w/w	weight per weight
ZA	zeatin riboside
%	percentage

## CHAPTER I

### INTRODUCTION

Thailand is located in the Southeast Asia with suitable climate for the growth of tropical orchids and has about 1,125 species distributed all over the country (164). Thai orchids are famous for their beauty and distinct features and are in high demand all over the world. The flowers are highly adapted for attracting, deceiving, and manipulating insects to achieve pollination. Many of these are very beautiful. Few of them are selected and hybridized with other species, resulting in many nice hybrids (74). One of the outstanding Thai orchid species of Thailand is *Vanda coerulea*, the blue flowered orchid. *Vanda coerulea* is an important parent in *Vanda* hybridization.

Thailand has a long history of orchid trade, especially for export. Thai orchids make up for cut-flower and plant exports. Thailand exported orchid plants of about 34,846,242 plants in 2007, including *Dendrobium* (48%), *Phalaenopsis* (31%), *Vanda* (7%), *Oncidium* (3%), *Cattleya* (2%), Mokara (2%) and others (7%). Value for exported Thai orchids is estimated 422,778,181 baht in 2007. Thailand exported *Vanda* plants with quantities varying from 717,958 plants in 2006 to 881,634 plants in 2007. Value for *Vanda* plant exports is estimated 28,540,820 baht in 2006 and 30,444,168 baht in 2007 and value for *Vanda* cut-flower exports is estimated 2,016,751 baht in 2007 (1).

Most orchids in the world are listed under Convention on International Trade of Endangered Species of Wild Fauna and Flora (CITES) Appendix II. *Vanda coerulea* was included in Appendix I of the Convention since 1979 and then was transferred from Appendix I to Appendix II in 2005. Appendix II plants are considered endangered, and as such, the international trade is regulated by CITES and trade in the taxa would require a permit or certificate. *Vanda coerulea* has become endangered because of wild orchid trade, deforestation and environmental changes, although it was transferred to Appendix II. Therefore, micropropagation and conservation of *Vanda coerulea* are urgently needed.

Cryopreservation using liquid nitrogen (LN) (-196°C) is an important technique for long-term preservation without genetic alteration and the system does not use expensive facilities. Cryopreservation techniques are based on vitrification, cell dehydration is performed before freezing by slow freezing, exposing to vitrification solution or air desiccation, and then rapid cooling. Vitrification-based procedures are vitrification, encapsulation-dehydration, encapsulation-vitrification and droplet-vitrification. Parts of *Vanda coerulea* plants will be used for cryopreservation such as seeds, protocorms, shoot tips and protocorms-like bodies. Seeds of orchids are very small and can tolerate to cold and suitable for long-term preservation. Protocorms are very small organs which can easily develop into whole plants. Shoot tips were the explants selected for cryopreservation because they are programmed to directly develop into shoots without intermediate callus formation. The successful cryopreservation is not only that explants can start regrowth after cryopreservation but also that the explants have retained the same genotypes as they had before cryopreservation. Random amplified polymorphic DNA (RAPD) marker is one of the technique to examine the genetic stability in recovered plantlets from cryopreservation.

### **Objectives**

1. To study the effects of media, sucrose concentration, 6-benzylaminopurine (BA),  $\alpha$ -naphthaleneacetic acid (NAA) and thidiazuron (TDZ) on micropropagation of shoot tips of *Vanda coerulea*.
2. To develop the method for cryopreservation of seeds, protocorms, shoot tips and protocorm-like bodies of *Vanda coerulea*.
3. To study the genetic stability in recovered plantlets after cryopreservation.

## **CHAPTER 2**

### **LITERATURE REVIEW**

Orchids (Orchidaceae family) are the largest and most diverse of the flowering plant families, with 25,000-30,000 species (32). Orchids have diverse habit with variously modified vegetative and floral structure, forms and colors with varying requirements of light, temperature and nutrition. They may be either terrestrial (growing rooted in the soil), epiphytes (growing on trees), lithophytes (growing on rocks) or saprophytes (growing on decaying organic matter). The stem of an orchid can grow in one of these two ways: monopodial (single stemmed) or sympodial (many stemmed cluster). They grow from the Arctic to the Equator and south except Antarctica. The great majority are to be found in the tropics, mostly Asia, South America and Central America (130).

#### **2.1 The characteristics of orchids**

Orchids are distinct from other flowering plants because (28):

1. The stamens and style (male and female parts) are fused together in one structure known as a column.
2. There are three petals and three petal-like sepals.
3. Usually has one different petal that forms a lip or labellum making the flower bilaterally symmetrical.
4. The pollen is usually bound together in a few large masses known as pollinia.
5. The flower stem twists around during development so that the lip is down (resupination).
6. The seeds are very tiny (0.05 to 6.0 mm in length) and numerous.
7. Upon germination the embryo forms a tubercle (protocorm).
8. Under natural conditions most orchids will germinate only when a symbiosis with a fungus has been established.

## 2.2 Orchids in Thailand

Thailand is located in the Southeast Asia with suitable climate for the growth of tropical orchids. Orchids are found in all different habitats ranging from hill evergreen forest at 2,565 m in the north to sea level in the peninsular about 1,125 species (164). Some are found in all regions and some are rare and endemic found only in certain regions. Among these, 80% are epiphytic and most of the rest are terrestrial and only few species are saprophytic orchids (112). Some of the important genera are *Dendrobium* spp., *Bulbophyllum* spp., *Eria* spp., *Habenaria* spp., *Coelogyne* spp., *Cymbidium* spp., *Paphiopedilum* spp., *Vanda* spp., *Aerides* spp., *Rhynchostylis* spp. and *Spathoglottis* spp.

*Vanda* species and hybrids have been very popular among hobbyists as well as commercial growers, particularly in the warmer areas of the world, including Thailand, Singapore, Hawaii and southern Florida. The genus *Vanda* comprises 30 to 40 species, distributed from the Himalayas through Southeast Asia to New Guinea and northern Australia. About 12 species occur in Thailand including *Vanda coerulea* (Queen of Vandas). They can be classified into three groups on the basis of their leaves: terete, semiterete and flat or strap (74).

## 2.3 *Vanda coerulea* Griff. ex Lindl.

*Vanda coerulea* Griff. ex Lindl. is native to north-east India where it grows at elevations ranging from 2,500 to 4,000 feet in the Himalayan foothills. Botanist William Griffith's description of this unusual blue-flowered species in 1847 sparked much excitement amongst orchid enthusiasts and hybridizers, who were eager to add this rare plant to their collections (145).

*Vanda coerulea* is the most popular *Vanda* species of Thailand. The name *Vanda* is derived from the Sanskrit name for the species *Vanda tessellata*. It is distributed Northeast India, South China, Myanmar and Thailand. In Thailand, *Vanda coerulea* has a scattered distribution over 500 km range in western (Kanchanaburi) to northern (Chiang Mai) province. *Vanda coerulea* known as the blue flower orchid is epiphytic orchid. The climate in their natural habitats is cool and moist. *Vanda coerulea* will tolerate intermediate to cool temperature but will also do well in warm

temperature. Plants are suited for cultivation in Chiang Mai, which promotes floral initiation and development (74).

Taxonomy (144):

Kingdom	:	Plantae
Division	:	Magnoliophyta
Class	:	Liliopsida
Order	:	Asparagales
Family	:	Orchidaceae
Subfamily	:	Epidendroideae
Tribe	:	Vandae
Subtribe	:	Sarcanthinae
Genus	:	<i>Vanda</i>
Species	:	<i>coerulea</i>

The strap leaves of *Vanda coerulea* are short and thick about 6 inches long and 1 inch wide (Figure 1). The tip is unequally notched. The long ascending inflorescence carries from 10 to 15 flowers. The flowers are often 3-4 inches across. There is much variation in color from blue-tinted white to deep blue and bluish lavender to pink. The petals are usually twisted at the base, exposing the back of the petals. The dark-blue lip is small with narrow, curved side lobes. The spur is short. Flowers are obtained throughout the year, but usually the peak season is in August (74).

*Vanda coerulea* was included in Appendix I since 1979 and then transferred from Appendix I to Appendix II in 2005. *Vanda coerulea* is an important parent in *Vanda* hybridization.

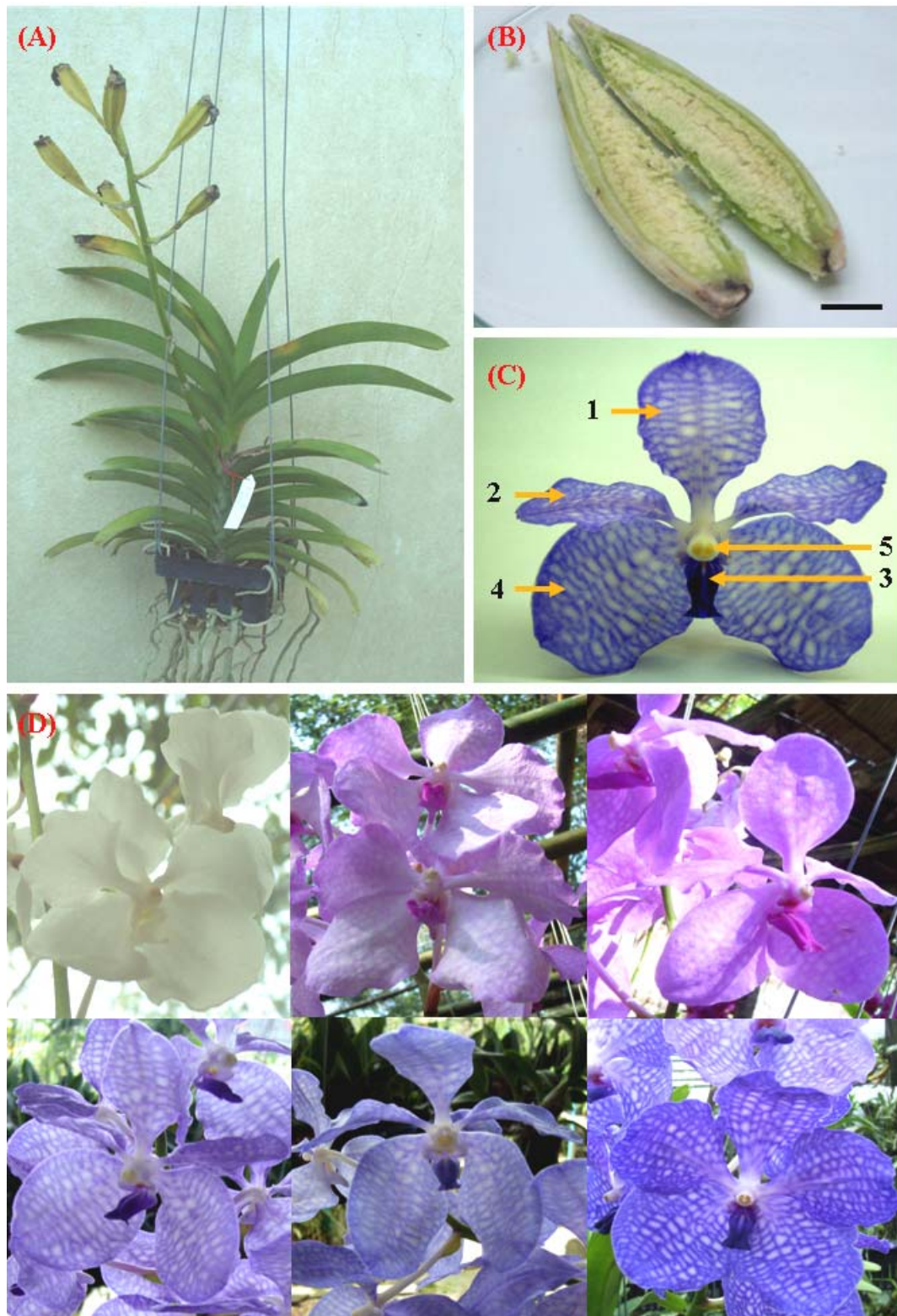


Figure 1. Characteristics of *Vanda coerulea* Griff. ex Lindl. (A) Plant and fruits. (B) Mature fruit and seeds. (C) Parts of flower: 1: dorsal sepal, 2: petals, 3: lip, 4: lateral sepals, 5: column. (D) Flowers. Scale bar: B = 1 cm.

## 2.4 Conservation of orchids

The two basic approaches for conservation of plant genetic resources are in situ and ex situ conservation. The Convention on Biological Diversity (1992) provides the following definitions for these categories: in situ conservation means the conservation of ecosystems and natural habitats and the maintenance and recovery of viable populations of species in their natural surroundings. Ex situ conservation means the conservation of components of biological diversity outside their natural habitats. It involves removing the plant genetic resources from their natural habitat and placing them under artificial storage conditions. In situ conservation includes forests and national parks, while ex situ conservation includes seed storage, in vitro storage, DNA storage, pollen storage, field gene banks, greenhouse, and botanical garden. Methods are necessary for a safe, efficient and cost-effective conservation (34).

Many orchid species in the world risk extinction because of environmental changes including heat, flood and drought stresses. Deforestation and wild orchid trade are human behavior. Thai orchid species are famous for their beauty and distinct features and are in high demand all over the world. Orchids are left in the wild. In this way, the plants slowly become destroyed. Some orchid species have become extinct because buyers often do not know how to look after them and the orchids often die thereafter.

In orchids, they can maintain in their natural habitat (in situ conservation) and under artificial conditions (ex situ conservation). In situ conservation is the best way to conserve the genetic diversity of orchid germplasm; however, it is very difficult to maintain orchids for a long period and it requires proper management. Preservation only in field collection (in growing condition) is risky, as valuable germplasm can be lost because of pest, disease, biotic and abiotic stress and is usually costly due to labor and land uses. In vitro culture, the development and growth of orchids on the medium may take a long time and must be subcultured several times. The maintenance of orchids in vitro is quite expensive, laborious, accumulation of somaclonal variation and losing materials by contamination (119). The orchid seeds are orthodox seeds which can store under room temperature or in the refrigerator but the viability of seeds reduces. To avoid this, the cryopreservation of seeds could be a feasible way of preserving their genetic diversity.

## 2.5 Methods for orchid propagation

Orchid seeds are very small, extremely tiny, and almost dust-like and produced in great numbers. Most range in length from 0.05 mm to 6.0 mm. Number of seeds per fruit (pod) may contain up to four million seeds and low as 20-50 seeds and seed weights extend from 0.31  $\mu\text{g}$  to 24  $\mu\text{g}$  (4). They can float in the air for long periods. The seeds contain a small embryo, lack endosperm to enable the seed germination and lack enzyme to metabolize polysaccharides but utilize lipid as a major nutrient source and the embryo also lacks enzyme to convert lipid to soluble sugar (95). Due to the lack of enzyme, in order to germinate under natural conditions, orchid seeds require a symbiotic relationship with a mycorrhizal fungus (135). This fungus digest soil organic matters, such as cellulose and converts it into simple sugar and provides nutrients, minerals and water which become available to support orchid seed germination and embryo growth (134, 159). Carbon and other nutrients are translocated from the fungi to the orchid cells by digestion of the pelotons (dense coil of mycelium) (155). Germination in association with mycorrhizal fungus is known as symbiotic germination and is used the most commonly for terrestrial orchids. Shimura and Koda (155) reported that inoculation of fungus to the seeds of *Cypripedium macranthos* var. *rebunense* at 4°C induced seed germination. The process of seed germination is quite complicated. Seed germinates into protocorm, a specific tuber-shaped microorganism formed from the embryo cell and then meristems and rhizoids appear on the protocorm, their development results in a juvenile shoot and give a whole plant (119).

However, germination rates of orchid seeds were often low and seedling death was common under normal condition. Knudson (79) demonstrated that it was possible to germinate orchid seed in vitro in the absence of the mycorrhizal fungus but by nutrient solution produced externally upon digestion of the fungus. Knudson (80) successfully germinated seeds of several epiphytic orchid genera in the nutrient medium contained a suitable soluble carbon source from sucrose. Germination of orchid seeds without mycorrhizal fungus is known as asymbiotic germination.

Chou and Chang (27) reported that germination rate of *Anoectochilus formosanus* and *Haemaria discolor* on Murashige and Skoog (MS) medium were 70% higher but germination of symbiotic seeds with *Rhizoctonia* spp. had more than

80% after 50 d of culture. Asymbiotic and symbiotic germination occurred after the uptake of water, and the seed coat was ruptured by the enlargement of embryo and then the papilla, protocorm and apical meristem appeared. In the symbiotic germinated embryos, the fungal hyphae penetrated the protocorm and formed pelotons.

## **2.6 Tissue culture of orchids**

Plant tissue culture is a useful technique for ex situ conservation. The techniques used include seed germination, micropropagation, meristem culture and callus culture grown in sterile culture. In vitro culture has proved useful with many species of plant which are difficult to propagate using conventional techniques (40). Tissue culture is potentially useful technique for micropropagation of orchid because the traditional asexual propagation of orchids is extremely slow. Tissue culture was first used to produce *Cymbidium* orchids by Georges Morel in 1960. Tissue culture have been extensively exploited, not only for rapid and large scale propagation of orchids but also for their ex situ conservation.

Tissue culture methods for the regeneration of orchids were introduced by several workers using various plant parts and culture media. The need for mass propagation of selected elite genotype has led to development of several in vitro methods including culture of flower stalk-tips (20, 169), shoot tips (69, 75, 137, 168), root tips (198) and leaves (113, 153, 202). The meristem is the central portion of a rapidly growing shoot, bud or other parts of the plant where cell division is taking place. Meristems are formed during embryogenesis and consist of group undifferentiated cells that will initiate organ primordial during plant life. The apical shoot meristem developed to a complete shoot and the apical root meristem is the origin of root. The callus grows in a nutrient to make the cells proliferate but prevent forming leaves and roots. When working with plant conservation, it is generally considered to avoid callus culture as this can result in somaclonal variation, i.e. genetic variation in plants derived from somatic tissue (40). In vitro of shoot tip culture can have major advantages over callus culture such as, shoot production is reliable and consistent, multiplication rates can be three-fold to eight-fold a month, plants produced by shoot culture are usually true-to-type and uniform.

## 2.7 Factors affecting in vitro culture of orchids

### 2.7.1 Culture media

There are many different media used for orchid culture such as, MS (1962) (110), VW (Vacin and Went, 1949) (182), KC (Knudson C, 1946) (81), ND (New Dogashima, 1993) (168). They are different in their components. Optimization of medium composition was an important approach to fasten the micropropagation, improve the quality of regenerated plantlets and callus culture, adventitious shoots or protocorm-like bodies. Lakshmanan et al. (84) reported that the three basal media (VW, KC and MS) were used. VW and KC were found to be equally suitable for thin section culture from shoot tip of *Aranda Deborah*. 80% of the thin section explants produced protocorm-like bodies (PLBs) when cultured on VW or KC medium supplemented with 20% (v/v) coconut water and developed into plantlets on VW agar medium supplemented with 10% (v/v) coconut water and 0.5 g/l activated charcoal. Ket et al. (75) reported the three basal media (MS, KC, and H3 (Hyonex)), MS and H3 were found more suitable for shoot tip culture of *Anoectochilus formosanus* than KC medium. They used H3 medium because it is cheaper than MS medium and commercially available. Kalimuthu et al. (73) reported that in vitro regeneration multiplication and rooting of plantlets were achieved from the immature seeds of *Oncidium* on MS medium supplemented with 2 mg/l BA. Chen and Chang (20) reported that high frequency somatic embryogenesis and plant regeneration from flower stalk-bud of *Oncidium* were cultured on ½ strength macro- and micro-elements of MS medium supplemented with NAA in combination with TDZ. Tokuhara and Mii (168) reported that shoot tip explants from flower stalk buds of *Phalaenopsis* and *Doritaenopsis* were cultured on ½ MS medium showed necrosis. However explants on ND medium remained mostly green without necrosis. Protocorm-like body formation on ND medium supplemented with 0.1 mg/l NAA in combination with 1 mg/l BA was also higher than that on ½ MS medium. Protocorm-like bodies developed into plantlets on ND medium without plant growth regulator. This methodology could be used on a commercial scale for vegetative propagation of *Phalaenopsis* and *Doritaenopsis*.

### 2.7.2 Carbon source

The sources of carbon are a very important component in in vitro culture media (68). Carbon sources are added to the culture media because of the light energy deficiency and low CO<sub>2</sub> concentration present in in vitro conditions (83). Plant cultures in vitro often show a low photosynthesis rate and incomplete autotrophy (39). Sugar acts as a carbon and energy source and also acts as an osmotic regulator in the induction medium (64). Sucrose is the most frequently used sugar but glucose, fructose, sorbitol, maltose and other sugars are also used (67, 68). Sucrose concentrations of 20 and 30 g/l are the most commonly used in orchid tissue culture. Greater increase in plant height and high seedling multiplication of *Dendrobium nobile* were observed in the 60 g/l sucrose without the addition of plant hormone (39). Trehalose has been used on protocorm-like body proliferation (90). Concentration of sucrose may affect on somatic embryogenesis (22). Increasing the sucrose concentration up to 20 g/l had no significant effect on protocorm-like body formation, whereas at 30 g/l it decreased the percentage of explants and protocorm-like body formation of *Aranda Deborah* using thin section culture (84). Liu et al. (90) reported that protocorm-like bodies from flower stalk-bud of *Phalaenopsis* and *Doritaenopsis* cultured on New *Phalaenopsis* (NP) solid medium (63) containing trehalose showed higher proliferation rate than on NP medium containing sucrose. In liquid culture system, both KC and NP media containing trehalose showed better results for protocorm-like body proliferation than sucrose containing media. Tokuhara and Mii (169) reported that embryogenesis calluses were induced from *Phalaenopsis* shoot tip explants excised from flower stalk buds by cultured on ND medium supplemented with 0.5 µM NAA in combination with 4.4 µM BA and 10 g/l sucrose. The browning of protocorms could be reduced by decreasing the sucrose concentration and most protocorms remained green and grew into plantlets on media supplemented 10 g/l sucrose. A high concentration of sucrose might act as an osmotic stress or to inhibit chlorophyll formation to induce embryogenic callus formation.

### 2.7.3 Plant growth regulators

Two main groups of plant growth regulator are used in media for orchid culture. These are cytokinins and auxins (23, 198). Cytokinins are normally used for their ability to induce either shoot proliferation by breaking dormancy in lateral buds

or adventitious meristem (shoot formation) such as BA (116, 168, 169), TDZ (23, 24, 137) and *etc.* Auxins have two major roles in plant tissue culture. Firstly, they can be used to induce root formation such as indole acetic acid (IAA), indole butaric acid (IBA). Secondly, they can induce callus formation such as 2,4-dichlorophenoxy acetic acid (2,4-D) and NAA (20, 23).

Nasiruddin et al. (113) reported that protocorm-like bodies and plantlet regeneration from leaf of *Dendrobium formosum* were cultured on MS medium supplemented with 2.5 mg/l BA in combination with 1-2 mg/l NAA. The fastest callusing and the amount were the greatest with 1 mg/l 2,4-D. Silva et al. (156) reported that protocorm-like bodies were induced from shoot tips, cell or tissue suspension culture of *Cymbidium* cultured on VW medium supplemented with 0.1 mg/l NAA in combination with 0.1 mg/l kinetin, 2 g/l tryptone and 20% sucrose but protocorm-like bodies formation did not occurred from leaf tip or sheath, or from the distal ends of root. However, the combination of 1 mg/l BA and NAA (1-2 mg/l) increased necrosis (137). The VW medium supplemented with 0.1 mg/l NAA in combination with 0.01 mg/l TDZ was optimal for callus formation (60).

TDZ is a substituted phenyl urea with cytokinin-like activity. It is useful for plant regeneration of several species through organogenesis and promotes shoot formation in in vitro culture of several orchid species (62). TDZ alone was efficient to induce direct somatic embryogenesis from leaf explants but there was not effective in induction of somatic embryogenesis from root-derived of *Oncidium* (20, 21, 23). TDZ (0.23  $\mu$ M–11.35  $\mu$ M) is proved useful in multiplying plant production of *Doritaenopsis* and *Phalaenopsis*. TDZ alone was more efficient than BA in orchids (36). Adventitious shoots were formed from leaf explants of *Phalaenopsis* cultured on MS ( $\frac{1}{2}$  strength macro and full-strength micro-elements) in dark and shoot bud formation from leaf segment explants on medium supplemented with 0.45  $\mu$ M TDZ (168).

TDZ has been shown to be more effective than BA or kinetin (KN) even at extremely low concentration (62). It can stimulate shoot multiplication either alone or in combination with other growth regulators (20). TDZ either alone or in combination with IAA induced high frequency of shoot regeneration (33). The combination of 0.1 mg/l NAA and 0.1-3 mg/l TDZ promoted embryo formation from callus culture

(20). TDZ alone or in combination with 2,4-D were found to be effective in adventitious shoot induction from leaf explants (23). Similarly, Chang and Chang (18) found that TDZ in combination with 2,4-D were required for callus induction in *Cymbidium* and *Oncidium* (21). However, Huang et al. (61) reported that TDZ inhibited shoot proliferation and rooting in *Paphiopedilum*.

Nayak et al. (116) reported that BA at 11.0  $\mu\text{M}$  on MS medium was optimal for *Dendrobium nobile* while zeatin riboside (ZA) at 14.0  $\mu\text{M}$  was the most effective for protocorm-like bodies formation of *Dendrobium aloifolium*. Shoot and root development were reduced while proliferation of protocorm-like bodies increased with increasing concentration of TDZ. Chen and Piluek (24) reported that TDZ was more effective than BA in stimulating the axillary bud from the flower stalk of *Phalaenopsis* on VW agar medium supplemented with 15% coconut water. Sheelavanthmath et al. (153) reported that among various cytokinins test (BA, KN and TDZ), BA is found to be more efficient in protocorm-like bodies induction from leaf explants of *Aerides crispum* regenerated protocorm-like bodies on the MS medium. The combination of NAA and BA has induced protocorm-like body regeneration; however, results were very low compared to BA alone. Nayak et al. (115) reported that of the three cytokinins (BA, KN and TDZ), TDZ was the most effective in inducing shoot buds from the foliar explants of *Acampe praemorsa*. TDZ was more effective at lower concentration. Disadvantage of TDZ in regeneration is the difficulty in elongation and rooting of the regenerated shoots. This problem was overcome by transferring regenerated shoots to MS medium supplemented with BA and NAA. Higher concentration of NAA (5 mg/l) suppressed shoot bud regeneration.

It has been considered that the use of plant growth regulator may cause somaclonal variation in various plant species. Ket et al. (75) reported that an optimal concentration of TDZ (1-2 mg/l) or BA (1 mg/l) developed an averaged 52 shoots/explant but increasing concentration of cytokinins often led to lower proliferation rate and stunted growth. Higher concentration of TDZ is sometime associated with morphological abnormalities and inhibited the shoot growth (62).

#### **2.7.4. Non-defined organic additives**

Many media used with orchids and other plants contain components of which the exact chemical composition is unknown and variable including yeast extract,

coconut water, banana pulp (30, 36) and potato extract. They have been shown to promote plant growth in vitro culture. Mature seed of several *Ophrys* species were cultured on a coconut milk-enriched or a pineapple-enriched medium induced germination (78). Coconut water contained substantial amount of auxin and cytokinin. Coconut water has been successfully used in the propagation of many orchids such as *Cymbidium* (60), *Aerides crispum* (153), *Dendrobium fimbriatum* (137) and *Aranda Deborah* (84). The supplementation of 200 ml/l coconut water in VW medium without plant growth regulator produced many protocorm-like bodies from callus somatic embryo structures in *Cymbidium*. Roy and Banerjee (137) reported that callus and plant regeneration from shoot tip explants of *Dendrobium fimbriatum* were successful when cultured on KC medium with 10% coconut water, 0.5 mg/l NAA in combination with 1 mg/l BA. Chen and Chang (22) reported that direct embryo formation from leaf tip segments of *Oncidium* on ½ MS medium supplemented with 10-20 g/l sucrose, 170 mg/l NaH<sub>2</sub>PO<sub>4</sub> and 0.5 g/l peptone gave the highest embryogenic response on leaf tips.

#### **2.7.5. Activated charcoal**

Some explants excrete waste products into medium and so they accumulate and can become toxic to the plant. They also often cause a darkening around browning of medium around the cut end of explants and inhibit the growth of plant. There were reported that activated charcoal can absorb the inhibitory substances. Activated charcoal has not only good adsorption properties but also creates partial darkness. Activated charcoal in medium enhanced the induction of roots (14). Effect of charcoal on shoot proliferation is that it improves aeration and adsorbs ethylene which can inhibit growth and proliferation (35). Ket et al. (75) reported that the addition of activated charcoal to the H3 medium induced shoot growth and elongate of *Anoectochilus formosanus*. Production of multiple shoots was dependent on the amount of activated charcoal in medium. Adding BA to medium containing activated charcoal had a little effect on shoot growth.

### **2.8 Micropropagation of *Vanda coerulea***

Having a monopodial growth habit, *Vanda coerulea* rarely produces offshoots in nature. Traditionally they are vegetatively propagated by cutting but the cloning

rate is extremely slow. Orchids can be rapidly proliferated by protocorm-like body formation and multiplication by shoot tip culture. The excision of shoot tip causes the loss of the source plants. Thus, flower stalks are good choice for clonal propagation because they do not damage the plants and more easily sterilized (24).

Micropropagation of *Vanda coerulea* has been reported by Mote (108), Nath et al. (114), Seeni and Latha (150) and Malabadi et al. (93). Seeni and Latha (150) reported that leaf base of seedling of *Vanda coerulea* cultured on medium Mitra et al. (107) supplemented with 3% sucrose, 10% coconut water, 500 mg/l casein hydrolysate and a combination of 8.8  $\mu\text{M}$  BA and 4.1  $\mu\text{M}$  NAA induced higher frequency formation of maximum number of protocorm-like bodies (10-15 PLBs) and shoots (4-7 shoots) and shoot buds (3-12 shoot buds) were occurred from shoot tip (0.5-0.8 cm) in 12 weeks. Shoot formation from protocorm-like bodies occurred on medium supplemented with 1.08  $\mu\text{M}$  NAA, 30% coconut water and 35 g/l banana pulp and root formation occurred on medium supplemented with 1.08  $\mu\text{M}$  NAA and 35 g/l banana pulp. Survival rate was 70-80% in alien forest habitats. All protocols lack reliability and gave low plant regeneration from *Vanda coerulea*, thus Malabadi et al. (93) reported that protocorm-like bodies or shoot bud proliferation was observed when thin shoot tip section of *Vanda coerulea* on VW medium supplemented with 11.35  $\mu\text{M}$  TDZ and shoots produced roots when cultured on  $\frac{1}{2}$  VW medium supplemented with 11.42  $\mu\text{M}$  IAA. The well rooted shoots were transferred to pots containing charcoal chips, coconut husk and broken tiles (2:2:1) and a 98% survival rate was achieved.

## **2.9 Cryopreservation**

### **2.9.1 Principle of Cryopreservation**

Cryopreservation is the process of freezing living materials to keep it safe. This is normally done at temperature of liquid nitrogen (LN) (-196°C or -320°F). At this temperature, physical and metabolic cellular processes are effectively stopped and the living tissue is in a state of suspended animation free from pathogens and the risk of genetic drift. When required, it can be recovered and grown to regenerate a whole plant (138).

Cryopreservation is a valuable technology for the best and most cost-effective, sustained long-term conservation of plant genetic material. Cryopreservation demands the least space and reduces susceptibility to disease, mutation and environmental condition. A variety of plant materials can be used including cells in tissue culture (callus, cell suspension), pollen, seeds, embryos, buds, twigs and meristem tissue. For cryopreservation of an endangered species, shoot apices have the advantage over other tissue as seeds as they allow for the conservation of genetically stable tissues, facilitating the conservation of elite clonal lines, essential for future reintroduction programs (138).

### **2.9.2 Crystallization**

Molecules in liquid undergo random, Brownian motion. For freezing, liquids can be supercooled to several degrees below the melting point of the solid phase and will freeze only upon the spontaneous formation of, or addition of, a substance that acts as a catalyst for the liquid-phase transition. Catalysts for the water-ice phase transition are referred to as ice nuclei. There are two types of ice nucleation: homogeneous (ice crystal growth is only molecule of water) and heterogenous (ice crystal growth is composed of both water molecules and other kinds of molecules) nucleation. In homogeneous nucleation, the nuclei are formed spontaneously in the liquid (pure water) without intervention of foreign bodies at a very low temperature approaching  $-40^{\circ}\text{C}$ . The freezing process involves two different phenomena, formation of ice nuclei and growth of crystal unit (141).

### **2.9.3. Vitrification**

At very low temperature, the viscosity of aqueous solutions rises and molecular diffusion is reduced. If a liquid is cooled sufficiently quickly, the viscosity can become so large that molecular diffusion is effectively halted and the probability of nuclei formation becomes negligible. The sample is then become glass of which process is called vitrification. Vitrification can be defined as the physical process of transition of an aqueous solution into an amorphous phase or glass, while avoiding the formation of crystalline ice. In cryopreservation, the usual goal is to achieve intracellular vitrification while avoiding intracellular ice formation and membrane damage (141).

## **2.9.4 Factors for a cell to vitrify**

### **2.9.4.1 Cooling and thawing**

For pure water, it can be made to vitrify, if cooled at rate of 10 million of degree Celsius per second. Water can also vitrify, if mixed with cryoprotectants. For aqueous solutions of cryoprotectants, cooling rate of about 0.1-10°C/sec is sufficient to achieve vitrification. More rapid freezing rates can be obtained in two means: (i) freezing in partially solidified nitrogen which has a temperature of about -208°C (instead of -196°C in case of LN), and (ii) a closer contact between the tissue and cooling agent. Cooling rate of 6°C/sec is normally obtained by plunging plant materials enclosed in a cryotube into LN. Higher cooling rate can be obtained by enclosing the meristems in semen straw, resulting in cooling rate of 60°C/sec. Droplet freezing method, the plant materials are placed on aluminium foil strips that are directly plunged into LN, giving rise to cooling rate of 130°C/sec (173).

A highly concentrated plant vitrification solution formula 2 (PVS2) supercooled below -70 °C and finally become vitrified at about -115°C (T<sub>g</sub>: glass transition temperature). Benito et al. (11) reported that PVS2 solution has a T<sub>g</sub> of approximate -120°C using different scanning calorimeters. Glass transition occurred during subsequent slow warming followed by exothermic devitrification (crystallization) (T<sub>d</sub>: devitrification temperature) at about -75°C and endothermic melting (T<sub>m</sub>: melting temperature) at about -36°C. The crystallization during the warming process can be prevented, if warming occurs rapidly.

Carlo et al. (17) reported that cryopreserved shoot tips of plum showed the best regrowth when cryovials were quickly warmed at 40°C, corresponding to a warming rate of 180°C/min. Lambardi et al. (86) reported that thawing the temperature at 45°C (corresponding to a warming rate of about 200°C/min) resulted in the highest regrowth rate of embryogenic callus when compared with temperature at 30, 35, 40 and 50°C. Warming was carried out in 43°C water for 1 min, followed by 22°C water for 10 sec which improved the recovery of ash shoot tips (148). Rapid warming in water bath at 37-40°C for 1-2 min protects frozen shoot tips, the damaging effect of ice crystal formation in the cell, which may occur during slow warming. Fast thawing yields better results than slow thawing (147).

### **2.9.4.2 Dehydration**

Plant cells consist of approximately 95% water. When the temperature is lowered below 0°C, water will usually turn into ice. Formation of intracellular ice in plant cells is mostly lethal (160) and needs to be avoided, at least in most cells, for successful cryopreservation. By dehydrating the cells, the chance of formation of ice crystals in the cells can be strongly reduced. Most of cells (callus, cell suspension, embryos and shoot tips) in cryopreservation contain high amounts of cellular water and are thus extremely sensitive to freezing injury. In vitrification-based procedures, cell dehydration is performed before freezing to avoid intracellular ice-crystal formation. For dehydration, the following techniques are applied:

#### **2.9.4.2.1 Air drying**

Samples are dried by the sterile air-flow of a laminar air-flow cabinet or with silica gel in desiccators. There is not any control of temperature and air humidity, both strongly influencing the evaporation rate.

#### **2.9.4.2.2 Freeze dehydration**

Dehydration by slow freezing minimizes intracellular ice formation and protects against cryoinjury (103). Generally, cooling rates of 0.5 or 2°C/min down to approximately -35°C, depending on the type and state of plant material are applied. Taylor and Fletcher (163) suggested that reducing cooling rate allows sufficient time for osmotic equilibrium. Zhang et al. (205) suggested that cooling rate, holding temperature and holding time had effects on the survival of cryopreserved *Laminaria japonica*. So, an optimum freeze-dehydration has to be achieved by combining an appropriate cooling rate with holding temperature and time. After the slow freezing steps, the cells are sufficiently dehydrated, and then immersed in LN. By rapid immersion into LN, the cells were completely vitrified without crystallization and stored without cryoinjury.

Slow cooling are generally obtained using computer-driven cooling devices, stirred methanol baths and propanol container held at -70°C. However, slow freezing procedure with a slow cooling rate can be achieved with simple cooling in a laboratory freezer rather than a programmable freezer (82). Wu et al. (195) reported that an addition freeze-induced dehydration during slow freezing (0°C to -40°C; 0.2°C/min) was thus necessary to decrease bead moisture content before immersion of

encapsulated shoot tips of kiwi in LN. Axillary buds of *Betula pendula* were frozen at the rate of 0.17°C/min down to -38°C using a PL-KRYO 560-16 kryosave controlled rate freezer before storage in LN (139).

#### **2.9.4.2.3 Cryoprotective substances**

Cryoprotective substances that reduce the injury of cells during freezing and thawing are usually separated into two classes, namely non-penetrating cryoprotectants and penetrating cryoprotectants, based on their ability to diffuse across cell membrane. Non-penetrating cryoprotectants cannot enter the cells to act outside the cells whereas penetrating cryoprotectants are able to move across cell membranes (102).

##### **2.9.4.2.3.1 Non-penetrating cryoprotectants**

Non-penetrating cryoprotective agents are sugars, sugar alcohols and high molecular weight polymer (poly ethylene glycol, PEG). A sugar treatment of tissues results in a slow reduction of the moisture content due to osmosis, leading to a more concentrated protoplasmatic solution. Moreover, sugars are also known to play an important role in the preservation of the membrane and protein structure during dehydration (123). Crowe et al. (29) found that soluble sugar can maintain the liquid crystalline state of the membrane bilayer and can stabilize proteins under extreme dry condition. Thus, they protect the structural integrity of membrane by preventing membrane fusion, phase transition and phase separation. Sorbitol is a non-penetrating cryoprotectant that lowers the cellular water content and reduces the rate of initial ice crystallisation during freezing (103).

##### **2.9.4.2.3.2 Penetrating cryoprotectants**

Penetrating cryoprotective agents are demethylsulphoxide (DMSO), glycerol, ethylene glycol (EG). Glycerol is antifreeze; it reduces ice formation and lower freezing point (10). Ethylene glycol is used with water as automobile antifreeze (10). For many applications, DMSO is preferred because of its extreme rapid penetration into the cells (10). When DMSO can be more toxic at higher temperature (9), glycerol or amino acids are applied.

The addition of cryoprotective solute is very effective in reducing the rate at which a solution must be cooled to avoid nucleation and the growth of ice nuclei. Vitrification solutions are complex mixtures of cryoprotectants, which have been

formulated for their ability to vitrify during cooling. Most vitrification solution employed for plant materials have been elaborated by Sakai's, Steponkus's and Towill's group (Table 1).

Table 1. Compositions of the various vitrification solutions.

Solution	Composition	Reference
PVS1	22% glycerol + 15% EG + 15% PEG + 7% DMSO + 0.5 M sorbitol	Uragami et al. (180)
PVS2	30% glycerol + 15% DMSO + 15% EG in medium with 0.4 M sucrose	Sakai et al. (139)
PVS3	50% glycerol + 50% sucrose in medium	Nishizawa et al. (121)
PVS4	35% glycerol + 20% EG + 20.5% sucrose	Sakai et al.(142)
Steponkus	50% EG + 15% sorbitol + 6% bovine serum albumin (BSA) in medium with 0.4 M sucrose	Langis and Steponkus (87)
Towill	35% EG + 1 M DMSO + 10% PEG in medium with 0.4 M sucrose	Towill (170)

PVS2 is the most commonly employed for cryopreservation of plant species, has a total molarity of 7.8 M and is highly toxic. The injurious effects that might occur during the dehydration process were eliminated or reduced by optimizing the time of exposure to the PVS2 treatment (9, 94, 185). Dehydration of samples at 0°C instead of room temperature reduced the toxicity of the vitrification solution and increases the potential period of exposure to vitrification solution (142).

Direct exposure of shoot tips to the high concentration of PVS2 solution might cause a sudden, extreme change in osmotic pressure, leading to rapid and excessive dehydration of the explant cells. The loading solution (LS), a mixture of 2 M glycerol and 0.4 M sucrose, may overcome this problem by slowly dehydrating the explants, thus conditioning the cells for the subsequent treatment with the PVS2 solution. Therefore, the dehydration tolerance of cells appeared to be enhanced by the loading treatment. Until now, the effect of loading solution is not very well understood. It was observed that during loading, cells dehydrate and plasmolyse and little

permeation of glycerol take place into the cytosol and increase dehydration tolerance through the modification of cell structures and the stabilization of membranes (100). The use of glycerol seems to be effective in inducing dehydration tolerance of shoot tips prior to incubation in PVS2 solution (148). Short-term treatment with loading solution may be appropriated for the cryoprotection of suspension cells that are sensitive to long-term preculture.

The use of vitrification solution containing combination of DMSO, EG, PEG, glycerol and sucrose may minimize the detrimental effects of DMSO exposure. In addition, the much shorter exposure duration may also have reduced the possible mutagenic effect of DMSO (176, 177). Häggman et al. (49) reported that PGD solution (10% PEG 6000, 10% glucose and 10% DMSO) was better regrowth of cryopreserved embryogenic culture of Scots pine than DMSO and PGD II (2.5% PEG 6000, 2% glucose and 2.5% DMSO on ice for 15 min followed by 5% PEG 6000, 4% glucose and 5% DMSO). It has been applied for *Abies cephalonica* (5) and apical of *Populus tremula* L. X *Populus tremuloides* Michx (72).

#### **2.9.4.2.4 Adaptive metabolism (hardening)**

Hardening is a process that increase plants to survive under environmental stress. This is triggered by reduction in temperature and shortening of day length. Hardening can result in a considerable increase of proteins, sugars, proline and glycine betaine which will participate in the increase of osmotic value of the cell solutes. Ramirez et al. (133) reported that the cold-hardening treatment at 4°C for 4 weeks was necessary to improve survival after cryopreservation using encapsulation-dehydration of strawberry shoot tips. Rynänen and Aronen (139) reported that cold hardening of donor shoots of *Betula pendula* at 5°C under short day-length (8/16 h light/dark) for 28 d is based on the improved protocol developed for the slow-cooling cryopreservation method. Cold-hardening at 5°C under short day-length (8/16 h light/dark) more than 30 d improved the survival levels of cryopreserved buds of mat rush (*Juncus* Spp.) by vitrification (117). Zhao et al. (206) found that apple plantlets were cold-hardened for 21 d at 5°C enhanced survival rate of cryopreserved shoot tips. However, cold-hardening of shoot tip of potato at 4°C for 7 d did not increase plant recovery after cryopreservation (50).

The successful of cryopreservation is dehydration tolerance. This tolerance can be induced by cryoprotective substances and also induced by adaptive metabolism. Towill et al. (174) reported that shoot tips of *Arabidopsis thaliana* can be successful cryopreserved using either PVS2 solution or PVS3 solution (Plant vitrification solution formula 3) (60 min at 24°C) as cryoprotectants prior to rapidly cooling in LN using foil strips. Cold acclimation at 4°C with an 8 h photoperiod for 8 d or 18 d improved regrowth of cryopreserved shoot tips. In addition, shoot tips could be cryopreserved using a two-step cooling (cooling rate at 0.3°C/min from 0°C to -50°C, then plunged into LN) procedure with polyethylene glycol-glucose-dimethyl sulfoxide as a cryoprotectant. Benito et al. (11) reported that a two step cold-hardening was used to further increase tolerance to dehydration in PVS2 solution, in the first step, explants were cultured at low temperature (10°C) to achieve the elongation of axillary bud and second step, excised apices were precultured at 3°C using a medium with increase sucrose concentration (0.15 M).

### **2.9.5 Cryopreservation injury**

The water-rich cell in a plant will not survive exposure to the temperature of LN. When the temperature is lowered below 0°C, water will usually turn into ice. Formation of intracellular ice in plant cells is mostly lethal (160). The injury to the cells results from the freeze-thaw cycle. Slow cooling and warming, plant cells may be damaged as a result of (i) rupture during freezing and contraction, (ii) irreversible osmotic contraction, and (iii) damage produced by severe dehydration. During freezing and thawing, some cells rupture, in the liquids that are the major molecular component of cell membrane will undergo phase transition from liquid crystalline to gel phase will cause leakage and cell damage (15). Coexistence of the phases has been associated with reduced semipermeability. Membranes may undergo topological changes. Low temperature may also result in inactivation of proteins that are sensitive to cold. A dehydration step results in concentration of solute in cells and in strong plasmolysis of cell to avoid the formation of intracellular ice crystals. Removal of water can lead to solute effect in the cell, such as pH changes, increased electrolyte concentrations and macromolecular interaction (171). Loss of membrane semipermeability in the freezing induced dehydrated state. Stress to the cells caused

by freezing and thawing may result in the formation of free radicals that cause damage, for example by lipid peroxidation, denaturation of protein and mutation in DNA (9, 13, 16, 158). Factors affecting cryoinjury in the plant cells are cooling rate, tissue temperature, time and temperature. Mikula et al. (106) reported that suspension cell of *Gentiana tibetica* were precultured with 0.4 M sorbitol for 4 weeks shown replacement of large vacuoles by numerous small ones, condensation of cytoplasm, accumulation of starch and fragmentation of endoplasmic reticulum. Treatment with PVS2 led to degradation of starch, mitochondria had spherical shapes with sparse cristae. After freezing, it was found that membrane and nuclei degradation, and cell destruction. Reversible changes after freezing were observed in tissue treated with vitrification solution. In vitrification method, cell organelles appeared normal as early as 5 h after thawing.

In nature, many cellular are change during cold acclimation. Protection mechanisms include an increase number of hydrated phospholipids in the polar head groups on the surface of plasma membranes, accumulation of soluble carbohydrates in cytoplasm, accumulation of anti-freeze proteins in the sap of the apoplastics space (47).

## **2.10 Factors for successful cryopreservation of plant cells**

### **2.10.1 Start materials**

One important parameter for successful cryopreservation was the selection of explants at an optimal development stage. Undifferentiated cell suspensions, which consist of large vacuolated cells, are prone to severe cryoinjury compared with embryogenic cultures and apical organs which contain small cytoplasmic-rich meristematic, the highest cryotolerance is obtained when samples are collected during their exponential growth phase (187). The highest growth rate of mango embryogenic was observed when cultured in liquid medium for 20 d and were selected for cryopreservation (197).

Wagner et al. (186) reported that the size of the plant tissue may also affect the proportion of residue freezable water and lead to freezing injury. Think et al. (167) suggested that the optimal meristems type for cryopreservation, example apical dome partially covered by one to two leaves primordial. In case of garlic, low survival was

observed when inflorescences were too young or too old. The large size of bulbils may have slowed down dehydration and penetration of cryoprotectants (77). Yoon et al. (201) reported that the optimal size of shoot tips was 1.5-2.0 mm and 1.0-1.5 mm and the optimum subculture duration was 7 and 5 weeks for cryopreservation of potato var. Dejima and STN13, respectively. Baek et al. (6) reported that smaller apices of garlic (1.5 or 3 mm in diameter) displayed higher regeneration than large ones (4.5 mm in diameter).

### **2.10.2 Preculture**

Preculture of plant materials in media containing sugars enhanced the tolerance of explants against the treatment with the PVS2 solution and improved regrowth after cryopreservation. Sucrose has an osmotic dehydration to reduce water content in plant cells and it is able to enter the cells. The accumulation of soluble sugars in cells is thought to protect proteins and membranes from damaging effects of dehydration and freezing in LN (104, 123). For many plant species, preculture alone appears to be insufficient to produce a high level of cryopreserved plants following cryopreservation. Thus, an effective osmoprotective treatment appears to be essential for improving cryopreserved plants. Conditioning of plants through cold acclimation or sugar preculture of tissue on the medium containing 0.1 up to 1 M during 1-5 d are often applied to improve dehydration tolerance. Sucrose becomes toxic when relatively high concentrations, generally above 1.0 M, are employed during pretreatment and its toxicity increases in line with the duration of preculture (197). Halmagyi et al. (50) studied the effect of preculture in various sugars and sugar alcohols including sucrose, glucose, mannitol and sorbitol on the recovery of potato from cryopreserved shoot tips. They found that lower recovery was obtained when glucose and mannitol were used. Chmielarz et al. (26) reported that embryogenic calli were precultured with sucrose in solid medium was significantly higher than those precultured in high concentration of sucrose solution, followed by glycerol. Chen and Wang (25) reported that the highest survival rate was achieved in the double preculture treatment where carrot cells were precultured first in the medium containing 0.175 M sucrose for 1-3 d, and then in the medium containing 0.4 M sorbitol for 4 d. The best recovery of cryopreserved mango embryogenic culture was obtained with 24 h pretreatment on medium containing 0.5 M sucrose, desiccation

with PVS3 solution down to 30-50% moisture content (197). Preculture in glycerol gave better the recovery than preculture on DMSO containing medium (121).

A preculture with abscisic acid (ABA) induced proline accumulation and could induce some level of tolerance to vitrification. However, the use of an even higher concentration of ABA ( $10^{-4}$  M) did not increase survival of non-cryopreserved and cryopreserved shoot tips after short or long period treatment (183).

### **2.10.3 Cryopreservation techniques**

There are two cryopreservation techniques: (i) classical techniques involved freeze-induced dehydration and (ii) new techniques are based on vitrification. These techniques use a dehydration step to avoid intracellular ice crystal formation.

#### **2.10.3.1 Classical cryopreservation techniques**

Classical cryopreservation techniques involved slow cooling down to a defined prefreezing temperature, followed by directly plunged into LN (34). During slow cooling, the cells and external medium initially supercool, followed by ice formation in the medium. The cell membrane acts as a physical barrier and prevents the ice from the cell interior and the cells remain unfrozen but supercooled. As the temperature is decreased, an increasing amount of the concentration of intracellular solutes occurs. Since cells remain supercooled and their aqueous vapour pressure exceeds that of the frozen external compartment, cells equilibrate by loss of water to external ice. Depending upon the rate of cooling and the prefreezing temperature, different amounts of water will leave the cell before the intracellular content solidified. In optimal conditions, most or all intracellular freezable water is removed, thus reducing or avoiding detrimental intracellular ice formation upon subsequent immersion of samples in LN. However, freeze-induced dehydration can incur different damaging events owing to the concentration of intracellular salts and changes in the cell membrane. Warming should be rapid to avoid devitrification. However, classical cryopreservation techniques required the use of sophisticated and expensive programmable freezers or laboratory freezer to adjust the temperature (34).

The slow freezing method was developed by Withers and King in 1980. In this method, plant cells dehydrated with cryoprotectant were slowly prefrozen to about  $-40^{\circ}\text{C}$  to  $-100^{\circ}\text{C}$  prior to being immersed in LN. There are two factors that are known to affect the viability of frozen cells; (i) is the combination of cryoprotectants and (ii)

is a freezing program that permit extracellular freezing and the occurrence of protective dehydration.

The highest survival rate of plant cell cultures follow a two-step procedure when they were precultured with sugar and polyols, treated with cryoprotectants, combined with slow cooling (1-2°C/min) of cells to subzero temperature (-30°C to -100°C) before plunging into LN (103). Menges and Murray (103) reported that *Arabidopsis* suspension were precultured in 0.5 M sorbitol, and then treated with 5% DMSO at 4°C for 1 h, stored in freezer at -80°C. A controlled slow freezing rate of 0.5°C/min during storage at -80°C/min for 5.5 h prior to transfer into LN resulted in optimum viability of up to 90%.

Gale et al. (44) reported that the immature zygotic embryos of *Picea sitchensis* were precultured with 0.4 M sorbitol, and then dehydrated with 5% DMSO on ice for 30 min and then placed in Freezer KRYO. The cooling program was initiated with 10 min hold at 0°C to allow equilibrate and a first cooling ramp at the rate of 0.5°C/min to -15°C followed by a 15 min to allow ice nucleation and a second ramp at rate of 0.5°C/min to -50°C for 10 min to transfer of vial to LN. They suggested that controlled cooling required the incorporation of an intermediate ramp and hold to ensure ice nucleation before cooling to a terminal transfer temperature followed by immersion in LN. Zhang et al. (205) reported that a process of cooling to -60°C at a rate of 1°C/min and holding at this temperature for 40 min was optimal, resulting in 59% survival of cryopreserved gametophyte of *Laminaria japonica*. They suggested that cooling rate, holding temperature and holding time had the effect on the survival of cryopreserved *Laminaria japonica*. Salaj et al. (144) reported that embryogenic cell of *Pinus nigra* were precultured with 18% sucrose and 7.5% DMSO and then were stored in deep freezer until the temperature reached -40°C before plunging into LN improved survival rate after cryopreservation. Zhao et al. (206) reported that shoot tip of apple cryopreservation by droplet freezing were precultured with 1% DMSO at 5°C, treated with 0.3 M sucrose and 15% DMSO for 75 min, then frozen to -40°C at 0.2°C/min in droplets before immersion in LN. There was more than 70% survival.

Kobayashi et al. (82) reported that tobacco BY-2 cells were successfully cryopreserved by a simple slow freezing method using an encapsulation technique.

Beads were precultured with LS for 45 min, transferred to freezer at -30°C stored for 2 h and then immersed in LN. However, Lynch et al. (92) reported that no variable of encapsulated shoot tips of *Rosa multiflora* was held at 20°C for 10 min, reduced to -35°C at -1°C/min using a controlled rate freezer.

### **2.10.3.2 New cryopreservation techniques**

New cryopreservation techniques are based on the phenomenon of vitrification. In vitrification-based procedure, cells are dehydrated by exposure to concentrated cryoprotectant or air desiccation before freezing followed by rapid cooling to avoid the formation of intracellular ice (8, 71). Seven different vitrification-based procedures can be identified: (i) vitrification, (ii) encapsulation-dehydration, (iii) encapsulation-vitrification, (iv) desiccation, (v) pregrowth, (vi) pregrowth-desiccation and (vii) droplet freezing (34, 143).

#### **2.10.3.2.1 Vitrification technique**

Vitrification technique involves treatment of plant tissues with highly concentrated vitrification solution in order to induce dehydration of the tissues to avoid intracellular ice-crystal formation during cooling and warming (140, 179). It is considered that there are two important points which are necessary for the plant materials to be successfully vitrified: (i) rapid freezing rates (ii) a concentrated cellular solution (124).

The key to successful cryopreservation by complete vitrification is necessary to carefully control the procedures for dehydration and cryoprotectant permeable and to prevent injury by chemical toxicity or excess osmotic stress during dehydration. Thus, optimizing the time of exposure to PVS2 solution is the most important for producing a high level of cryopreserved plants following vitrification (117). The exposure duration of plant cells to the vitrification solution is critical for their survival, because over dehydration may result in cell injury by chemical toxicity and excess osmotic stress of cryoprotectants and intracellular ice formation during cooling. The suitable dehydration duration was related to several factors, including the nature and the size of the sample, the composition and loading procedure of cryoprotectants (25). Matsumoto et al. (101) found that two-step dehydration procedure (50% PVS2 solution for 30 min at 0°C followed by PVS2 solution for 50

min at 0°C) by vitrification method gave a higher recovery of cryopreserved shoot tips of grape over that of the one-step procedure.

The optimum exposure time is size dependent and species specific. Lambardi et al. (86) reported that the development stage of embryogenic callus was shown to play an important role. Torpedo stage gave the highest survival after cryopreservation by vitrification. Sensitive of the various cryoprotectants might be different according to the stage of embryos development, and might be specific to organs and tissue. March et al. (96) reported that no regrowth of cryopreserved embryogenic callus when dehydrated with 5% DMSO and 2% proline and PVS2 solution. PVS2 solution was not effective for the regrowth of cryopreserved embryogenic callus.

#### **2.10.3.2.2 Encapsulation-dehydration technique**

Encapsulation-dehydration technique is based on the technology developed for the production of artificial seeds. It was developed for shoot tips of *Solanum* (37). In this technique, explants are encapsulated in alginate beads, precultured with high sucrose, desiccated by air-drying in a laminar air-flow cabinet or with silica gel, and then plunged into LN (99, 142).

Wang et al. (190) reported that comparison between the effects of dehydration with silica gel and by air drying on cryopreserved shoot tips of *Vitis vinifera*, survival rate was dependent on water content, not on dehydration method. Several studies using the encapsulation-dehydration technique have indicated that the best water content for sample survival after LN exposure ranged from 15% to 25% (154, 179, 180). Ryyänen and Aronen (139) successfully cryopreserved shoot tip of strawberry using encapsulation–dehydration technique together with vitrification technique. This method reduced the time needed for cryogenic procedure by eliminating the precultured with 0.8 M sucrose for 19 h prior to dehydration, as required by the conventional procedure. Glycerol has been used in encapsulation in combination with sucrose to increase dehydration tolerance and encapsulated beads were still flaccid which may confirm dehydration and fast recovery growth in wasabi apices, chrysanthemum apices and mint apices (142).

Compared to other vitrification techniques, the manipulation of encapsulated explants by this technique is easy (57), and non-toxic cryoprotectants are applied to protect during dehydration (118). However, the problems of encapsulation-

dehydration technique are the lower rate of regrowth, later recovery growth after cryopreservation and a longer dehydration procedure than vitrification (142). Sakai et al. (142) developed the encapsulation-dehydration technique. In this protocol, two processes of encapsulation and osmoprotection with loading solution were combined into one step and the osmoprotection was carried out simultaneously during the encapsulation process. This protocol reduced the dehydration time and produced higher rate of recovery growth in wasabi (98), chrysanthemum and mint (142) than those cryopreserved by the conventional encapsulation-dehydration.

#### **2.10.3.2.3 Encapsulation-vitrification technique**

Encapsulation-vitrification technique is a combination of encapsulation and vitrification technique. The explants are encapsulated in alginate bead, and then subjected to freezing by vitrification (187). Yamuna et al. (199) reported that the highest of cryopreserved shoot tips of ginger was 66% when precultured with 0.1 M sucrose for 24 h following 0.3 M sucrose for 16 h, then treated with 2 M glycerol and 1.6 M sucrose for 3 h and dehydrated with PVS2 solution.

#### **2.10.3.2.4 Desiccation technique**

Desiccation technique consists of dehydration explants by air-drying in a laminar air-flow cabinet or with silica gel, and then freezing them rapidly by direct immersion in LN. A double stress from desiccation and freezing is imposed on seeds during cryopreservation, the intensities of which depend on the moisture content level (192). They reported that developing maize embryo suffered cryoinjury if they were plunged in LN at their initial moisture contents, which were always greater than or close to 50% but they could survive when dried to moisture content below 13% prior to freezing. Moisture content is the most important factor affecting the ability of germplasm to be store in LN. Optimal survival rates are generally obtained when the water content of the explants are 10-20% (fresh weight basis). Lambardi et al. (85) reported that in *Citrus*, seed dehydration to less than 20% water content prior to storage in LN is fundamental for embryo survival. Seo et al. (151) reported that the highest regrowth rate of cryopreserved shoot tips of *Paeonia lactiflora* was obtained from desiccation of shoot tips by air-drying under a laminar air-flow cabinet for 5 h. Perán et al. (128) reported that encapsulated excise axes of *Ekebergia capensis* (recalcitrant seeds) were dried either rapidly by flash drying, or slowly drying. Flash

drying was achieved by placing naked axes on grid in a 250 ml jar over activated silica gel, water content decreased from 2.0 g/g to 0.4 g/g within 20 min. Slowly drying of encapsulated axes was achieved by placing beads on filter paper strips in 100 ml glass jars containing 50 g of activated silica gel, it took 3 h to reduce water content. They suggested that flash dried axes produced both shoots and roots on recovery medium supplemented with BA, but axes dried slowly in an alginate bead failed to produce shoots.

#### **2.10.3.2.5 Droplet-freezing technique**

Droplet-vitrification technique, a combination of droplet-freezing and solution-based vitrification consists in cooling sample in droplets of vitrification solution, then placed on aluminium foil strip in minute droplet of vitrification solution and then frozen by rapidly immersion in LN (143). Rapid warming by dipping the aluminium foil strips in pre-heated unloading (0.8 M sucrose) (76). During the cooling and warming procedures, rapid heat transfer is needed to avoid freezing injury (2, 76, 201). Aluminium foil has an efficient thermal conductivity, resulting in quick and uniform heat distribution among tissue (50, 76, 201). A high warming rate should be employed to avoid recrystallization of intracellular ice or addition cell dehydration by extracellular ice (76). This technique gave higher survival than droplet-freezing or vitrification alone. Panis et al. (124) reported that shoot tips from rooted in vitro plants of *Musa* and *Enset* were successfully cryopreserved with droplet-vitrification technique. They were precultured with sucrose, treated with LS for 7 h and then dehydrated with PVS2 solution for 30-50 min at 0°C and proved to be essential for high regeneration rate. Wagner et al. (186) reported potato apices cryopreservation by droplet freezing. They were treated with 10% DMSO for 2 h in MS medium supplemented with zeatin, transferred to 2.5 µl droplets placed on strips of aluminium foil, and then were plunged into LN. After several year storage in LN cryopreserved shoot tips were cultured in recovery medium. Halmagyi et al. (50) reported that the combined vitrification-droplet method can be used as an effective and useful technique for cryopreservation of potato shoot tips and *Rosa* shoot tips (51). Shoot tips of *Rosa* were precultured in MS medium containing 0.1 to 1 M sucrose for 24 and 48 h, and dehydrated with PVS2 solution for 10-30 min at room temperature. After thawing, cryopreserved shoot tips resumed growth within 10 d. Kim et al. (77)

reported that unripe inflorescences were successfully cryopreserved with droplet-vitrification technique. They were precultured on MS medium containing 0.3 M sucrose, treated with LS for 50 min and then dehydrated with PVS3 solution. After thawing, cryopreserved explants were treated with unloading solution (0.8 M sucrose). It can result in the regenerated of virus-free or a little infected material.

#### **2.10.4 Post-thaw treatment**

After warming, the highly concentrated vitrification solution must be removed by placing the cells or tissue into an elevated osmotic solution, for example 1.2 M sucrose or sorbitol, before transferring the tissues to recovery medium. This is done to avoid rapid water uptake into the cells while still allowing some of vitrification solution to diffuse out (172). The efficiency of the unloading solution is influenced by its osmolarity (osmotic stress and efficiency of unloading) and by the duration of its application (accumulation of osmotic stress and amount of cryoprotectants effluxed) (76). Kim et al. (76) reported that cryopreserved shoot tips of potato were washed in 0.8 M sucrose resulted in higher survival in comparison with 0.3 M or 1.2 M sucrose. Chen and Wang (25) reported that survival of cryopreserved carrot cells unloading in medium containing 1.2 M sorbitol was higher than 0.4 M but did not differ significantly with that of 2.0 M sorbitol. Therefore, 1.2 M was considered as a suitable sorbitol concentration in the unloading medium.

In the recovery phase, the ability of plant tissues to grow into healthy, genetically identical, variable plants after LN immersion is vitally important for cryopreservation to be effectively applied for conservation (176). Two major concerns exist for the use of cryopreservation for routine genebank management. Firstly, cryopreserved plants may occur somaclonal variation or organogenesis during recovery growth. Secondly, over a long period of storage in LN, cryopreserved apices would show deterioration and a loss of viability from recrystallization during rewarming led to cell damage in those cells that still contain freezable water or other effects (186). The lower regeneration rate of the bigger explants might be attributed to a less efficient heat exchange during cooling as well as thawing and to the presence of more vacuolated and thus more hydrated cells which have a higher chance to form ice crystal (124).

Plant materials after cryopreservation may have some special requirements for their survival and for the regeneration of plantlets when post-cultured. Plant species respond to in vitro manipulation of culture media. Halmagyi et al. (50) reported that recovery of shoot tips of potato after cryopreservation was affected by the dimension of explants. The highest shoot development after LN exposure occurred from 3-4 mm long apices. The low recovery rates in shoot tips with 5-6 mm could be due to insufficient dehydration of the explants prior to freezing, with consequences for the vitrification process, ice crystals may have formed during freezing and warming. However, the dehydration duration of treatment with PVS2 solution has affected on the recovery of cryopreserved shoot tips, with survival highest after 10 and 20 min dehydration for small and large shoot tips, respectively.

The composition of the recovery medium and plant growth regulators in the recovery medium plays an important role in the recovery of cryopreserved plant tissues. The development of callus on shoot tips after cryopreservation is generally regarded as undesirable, since it is an induction of cryoinjury and is associated with somaclonal variation (190). Wang et al. (190) suggested that the presence of cytokinins in the recovery medium is essential to induce and accelerate shoot tip regrowth, but concentration has to be carefully selected in order to avoid callus formation because it can cause somaclonal variation. Similarly, Wang et al. (188) suggested that an appropriate medium must meet the requirements for high survival and fast regrowth of cryopreserved shoot tip without callus formation. Turner et al. (176) reported that the application of combination of cytokinin and gibbellelic acid (GA<sub>3</sub>) in recovery medium appears to be more beneficial for plant growth and development after LN immersion for *Anigozanthos viridis*. Any stress and damage occurs during both preculture and freeze-thaw cycle, which may alter the levels and proportions of endogenous hormone in shoot tips, may cause a difference between non-cryopreseved and cryopreserved shoot tips. The optimal BA concentration for recovery of cryopreserved shoot tips may also differ among different cryogenic procedures (190).

Wang et al. (188) reported that the addition of 1 and 2  $\mu$ M BA to the recovery medium (MS medium) increased survival of cryopreserved shoot tips of grapevine and citrus by vitrification, encapsulation-dehydration, respectively. Low survival of

non-cryopreserved and cryopreserved shoot tips was observed with BA-free recovery medium. Seo et al. (151) reported that regrowth of cryopreserved shoot tips of *Paeonia lactiflora* was promoted in MS medium supplemented with 1 mg/l GA<sub>3</sub> in combination with 0.5 mg/l BA and the elongated shoots produced roots on a ¼ MS medium supplemented with 0.1 mg/l NAA. Shoot regeneration from cryopreserved shoot tips of potato were cultured on ½ MS medium supplemented with 0.4 mg/l GA<sub>3</sub>, 0.5 mg/l zeatin and 0.2 mg/l IAA and 30g/l sucrose (50). Halmagyi and Pinker (51) reported that the highest number of cryopreserved shoot tips of *Rosa* was observed when cultured on MS medium supplemented with 2.5 mg/l thiamine, 0.2 mg/l biotin, 0.2 mg/l pyridoxine, 0.08 M sucrose, 0.25 mg/l BA and 0.5 mg/l GA<sub>3</sub> within 3 weeks. Chmielarz et al. (26) reported that cryopreserved embryogenic calli formed somatic embryos and shoot produced root on ½ WPM medium supplemented with 2.9 µM IAA and 15 g/l sucrose. However, Sanayaima et al. (147) reported that cryopreserved shoot tips of *Crateva nurvala* were first cultured on MS medium supplemented with 0.1 mg/l BA and finally for elongation, were transferred to 0.5 mg/l BA. In the case of LN treated shoot tips, the original green color of the shoot tips was visible immediately after thawing, but was lost within 24-48 h. At the end of 1 week, some of explants turned brown.

#### **2.10.5 Condition for successful cryopreservation**

The condition for successful cryopreservation is not only that the cryopreserved explants can start regrowth after thawing, but also that cryopreserved explants has retained the same characteristics as it had before cryopreservation. Many reports have shown that cell and organ cultures regenerated after cryopreservation retain characteristics identical to those of the original cell line with respect to cytogenetic properties, biochemical properties, DNA polymorphism and transgene expression.

Molecular markers can be used for assaying the genetic fidelity, but they should preferably be used together with other approaches, such as morphological, cytological observation, isozyme (149). Chromosome number stability and mitotic activity of cryopreserved meristems of *Hypericum perforatum L.* was determined by chromosome counting. The mitotic index of the control did not significantly differ from that of the treated samples (181). The use of rDNA (ribosomal gene) probes as

tools assess stability and the possible detrimental effects of slow growth, somaclonal variation, cryoprotectants, and freezing injury (53).

The somatic embryo morphology is a stable feature and the embryogenic cell lines of *Pinus nigra* retain this characteristic also after cryopreservation (144). Zhang et al. (205) reported that gametophyte of *Laminaria japonica* after thawing appeared dark brown and lost their chlorophyll when they were changed from continuous to 12 h light/12 h dark that gave rise to saprophytes and remained the total potential of normal gametophyte. Kobayashi et al. (82) reported that morphology of cryopreserved tobacco BY-2 cells on LS medium was not different from the original cell culture.

Wang et al. (191) reported that prolong storage of shoot tips of papaya by vitrification in LN up to 2 years had no effect on regeneration. Mandal and Sharma (94) reported that the cryopreserved shoot tips of *Dioscorea deltoidea* maintained their viability and an unaltered level of regeneration capability after up to one year of storage in LN. Wagner et al. (186) success in cryopreservation of shoot tips of potato by droplet freezing is the recovery of the whole plant after a period of storage in LN (4 years).

One important aspect of cryopreservation is the genetic fidelity of plants recovered from cryogenic. The genetic stability for cryopreserved material is of the uppermost importance for all the applications of cryopreservation. The fact that cryoprotective chemicals, like DMSO, are known as mutagens, also the necessity of study the genetic stability of vitrified material treated with highly concentrated solution of cryoprotectants. It has been suggested that 2-10% DMSO solution involved in generating genetic or epigenetic change not only in microorganism and animal cell but also in higher plants. The mutagenic potential of DMSO most probably consists of several factors. It affects the metabolic functions of cells by changing their membrane permeability and fluidity, by interfering with enzyme system and by altering O<sub>2</sub> uptake (43). On the other hand, DMSO inhibits DNA synthesis, alters the secondary structure of DNA and RNA and causes scission in DNA and alteration in folded genome (43). In dividing plant cells, DMSO treatment induces the decrease of mitotic index and mitotic irregularities, such as numerous chromatin bridges, laggards, metaphase and multipolar anaphases and binucleate cells (56).

The genetic stability of cryopreserved material is being increasingly studied using molecular marker (55), e.g. amplified fragment length polymorphisms (AFLP) (52, 176) and RAPD (149). Harding and Benson (54) reported that nuclear DNA fragments and the chloroplast genome using DNA-DNA hybridization are stable in potato plants regenerated from cryopreserved shoot tips.

RAPD and AFLP are the two most widely used molecular markers. They are highly polymorphic and can cover the whole genome. RAPD is an easily performed molecular technique and differences in RAPD fragments denote alteration of the genome (89). They found that no detectable difference were found in soluble protein and POD isozyme electrophoresis band, RAPD (fifteen random primers) and AFLP (six primer pairs) fragments between control and cryopreserved shoots of apple.

The RAPD markers were chosen for monitoring the genetic stability after cryopreservation, because they provide an easy, fast and relatively cheap way of randomly screening large part of genome (5). DMSO and two mixtures of PEG, glucose and DMSO (PGD I and PDG II) were used as cryoprotectants in treatment with and without storage in LN. There was no effect of cryopreservation on the genetic fidelity of PGD treated somatic embryo cultures of Scots pine (49). Genetic stability was reported after RAPD analysis of the genomic DNA of *cryopreserved* apical *Abies cephalonica* (72), embryogenic of white spruce (31), callus of rice (109), shoot tips of *Paeonia lactiflora* (151) and shoot tips of ginger (199).

Jokipii et al. (72) reported that no genetic change were observed in hybrid aspen stored in LN or when DMSO was used in the PGD (10% PEG 6000, 10% glucose and 10% DMSO) cryoprotectant mixtures. RAPD analysis with ten RAPD primers (OPC-01, OPD-14, -15, OPE-05, -06, -09, -10, -17, OPF-03 and -06) assays the genetic fidelity of the cryostored material was maintained during both slow cooling and vitrification method. Martin et al. (97) reported that genetic stability of *Dendranthema grandiflora* Tzvelev shoot apices after cryopreservation by vitrification and encapsulation-dehydration by using RAPD markers (eight primers: OPO-05, -07, -10, -15, -18, -20, OPF-01 and -10) found that only one cryopreserved regenerate from encapsulation–dehydration method showed a different band pattern. Ryyänen and Aronen (139) reported that the genetic stability of birch plants regenerated following tissue culture, vitrification and cryostorage was used by

comparing their RAPD profiles with the markers amplified from the original donor trees. The RAPD profiles were generated with repeated amplification using ten arbitrary 10-mers (OPE-06, -17, -19, OPF-03, -04, -06, -08, -12, -13 and -15), results showed that no changes were visible in the morphology and RAPD profiles of the regenerated plants in the greenhouse. Seo et al. (151) reported that no different RAPD fragments were detected between non-cryopreserved and cryopreserved shoot tips of *Paeonia lactiflora* using six random primers (OPB-13, -14, -15, -16, -17 and -19). Zhai et al. (203) reported that RAPD markers were employed to test the genetic stability of grape and kiwi in vitro plantlets from cryopreserved shoot tips. Polymorphic RAPD markers cover approximately 0.004-0.06% of the grape genome and 0.005-0.07% of the kiwi genome.

Chloroplast DNA (cpDNA) variation has been used extensively to investigate phylogenetic relationships among plants (45, 200). Previous studies based on restriction analysis and/or gene sequences suggest that chloroplast genes have slow rates of nucleotide substitution with an average synonymous rate almost half that of plant nuclear DNA (193, 207). However, non-coding regions of cpDNA exhibit faster mutation rates, especially through the addition and/or deletion of short sequences (207). Conceivably, such regions may potentially complement more slowly evolving coding cpDNA sequences by providing a greater number of genetic markers, especially at the level of infrageneric comparisons. One region shown to be particularly useful at detecting species level variation is the *trnL* (UAA) non-coding region of cpDNA intron (41, 46, 162). Molecular techniques consider the analysis of isolate double strand DNA in contrast to epigenetic. The epigenetic molecular mechanisms do not involve the primary DNA sequence but they affect the inheritance of characters which are largely DNA-protein interactions at the higher order levels of nucleosome and nucleosomal arrays within chromatin structure. DNA-protein interactions are effected by histone chemical modification (acetylation, methylation and phosphorylation). Epigenetic variation in chromatin and DNA methylation of gene sequence has been found in plant after cryopreservation, resulting in alteration pattern of gene expression, where the possibility exists for plant to alter their phenotypic characters (55). Any genetic stability assessment must also include physiological and the most importantly, morphology of plant regeneration (203).

Table 2. Successful cryopreservation of plant cultured cells and organs.

Species/Materials/ Pregrowth	Method	Preculture/Dehydration	Desiccation/ LN treatment	Viability	Reference	
<i>Prunus domestica</i> shoot tips (in vitro, 1 mm)	vitrification	0.09 M sucrose; 4°C; 2 d	direct plunging	57%	Carlo et al. (17)	
			slow cooling; 0.5°C/min up to 45°C; direct plunging	23.8%		
			Two-step cooling; -160°C; 25 min; direct plunging	33%		
			direct plunging	47.5%		
<i>Wasabia japonica</i> (wasabi) shoot tips (in vitro, 1 mm) ½ MS + 0.3 M sucrose; 1 d; 20°C	encapsulation-vitrification	LS; 1 h; 25°C PVS2; 3 h; 0°C 0.5 M sucrose; 2 d	silica gel; 4 h; direct plunging	4.2%	Sakai et al. (142)	
			encapsulation-dehydration	direct plunging		96%
				silica gel; 7 h; direct plunging		75%
	vitrification	LS; 20 min; 25°C PVS2; 10 min; 25°C beads; 0.4 M sucrose 0.8 M sucrose; 16 h; 20°C	silica gel; 4-5 h; direct plunging	90%		
			encapsulation-dehydration	direct plunging		83%
				beads; 2 M glycerol + 0.8 M sucrose; 1 h; 25°C; 0.3 M sucrose; 16 h		76%
<i>Dioscorea deltoidea</i> shoot tips (in vitro; 0.8-1.2 mm)	encapsulation-dehydration	bead; 3% calcium alginate + 0.4 M sucrose 0.75 M sucrose; 3 d	direct plunging	83%	Mandal and Sharma (94)	
			air-flow; 4 h; 25% water content, direct plunging	76%		

Table 2. Successful cryopreservation of plant cultured cells and organs (continued).

Species/Materials/ Pregrowth	Method	Preculture/Dehydration	Desiccation/ LN treatment	Viability	Reference
<i>Chrysanthemum morifolium</i> shoot tips (in vitro, 1-1.5 mm) MS + 0.3 M sucrose; 3 d; 5°C; dark	vitrification	LS; 20 min; 25°C PVS2; 20 min; 25°C	direct plunging	85%	Sakai et al. (142)
	encapsulation-dehydration	beads; 0.4 M sucrose 0.8 M sucrose; 16 h; 20°C	silica gel; 9 h; direct plunging	20%	
	new encapsulation- dehydration	beads; 2 M glycerol + 0.8 M sucrose; 1 h; 25°C 0.3 M sucrose; 16 h	silica gel; 3 h; direct plunging	85%	
<i>Mentha spicata</i> apical meristem MS + 0.3 M sucrose; 3 weeks; 5°C; 12 h photoperiod	encapsulation-vitrification	beads; 0.4 M sucrose LS; 1 h; 25°C PVS2; 3 h; 0°C	direct plunging	87%	Mikula (105)
	encapsulation-dehydration	beads; 0.4 M sucrose 0.8 M sucrose; 16 h; 20°C	silica gel; 2 h; direct plunging	51%	
	new encapsulation- dehydration	beads; 2 M glycerol + 0.8 M sucrose; 1 h; 25°C 0.3 M sucrose; 16 h	silica gel; 3 h; direct plunging	61%	
<i>Gentiana tibetica</i> embryogenic cell suspension 6% sucrose	slow freezing	0.4 M sorbitol; 2 d + 0.08 M DMSO; 1 h; on ice	slow freezing; 0°C to -40°C; 1°C/min; -150°C; 10°C/min; direct plunging	3%	Mikula (105)
	vitrification	LS; 20 min; 22°C PVS2; 2.5 h; 0°C	direct plunging	85%	
	encapsulation	bead; 60 g/l sucrose	air-flow; 4 h; 30% water content; direct plunging	70%	

Table 2. Successful cryopreservation of plant cultured cells and organs (continued).

Species/Materials/ Pregrowth	Method	Preculture/Dehydration	Desiccation/ LN treatment	Viability	Reference
<i>Musa</i> spp. meristem 0.3 M sucrose; 2 weeks	simple-freezing (2-3 mm meristem)		direct plunging	12.5%	Agrawal et al. (2)
	vitrification (1-1.5 mm meristem)	LS; 20 min; room T PVS2; 20 min; 0°C	direct plunging	39.2%	
	vitrification (0.5 mm meristems)	LS; 20 min; room T PVS2; 20 min; 0°C	direct plunging	42.8%	
	fast-freeze/fast-thaw (1-1.5 mm meristem)	LS; 20 min; room T PVS2; 150 min; 0°C	droplet freezing; direct plunging	51.8%	
<i>Fraxinus excelsior</i> shoot tips (in vitro; 1 mm) 22°C; 8 h photoperiod + 0°C; 16 h; dark; 7 d 0.8 M glycerol; 2 d	vitrification	2 M glycerol; 20 min; 22°C PVS2; 25 min; 0°C	direct plunging	73%	Schoenwe iss et al. (148)
	encapsulation-dehydration	bead; 0.75 M sucrose	silica gel; 5 h; 21% water content; direct plunging	0	
22°C; 8 h photoperiod + 0°C; 16 h; dark; 7 d	encapsulation-vitrification	bead; 2 M glycerol + 0.4 M sucrose PVS2; 3 h; on ice	direct plunging	16%	

Table 2. Successful cryopreservation of plant cultured cells and organs (continued).

Species/Materials/ Pregrowth	Method	Preculture/Dehydration	Desiccation/ LN treatment	Viability	Reference
<i>Malus domestica</i> shoot tips (in vivo; 2 mm) MS + 0.3 M sucrose; 3 d; 5°C; dark	two-step freezing	50% glycerol + 50% sucrose; 1 h; 0°C	slow cooling; 0°C to -10°C; 15 min; -10°C to -40°C; direct plunging direct plunging	83%	Wu et al. (195)
	vitrification	50% glycerol + 50% sucrose (PVS3); 80 min; 25°C	direct plunging	60%	
	encapsulation-dehydration	bead: 3% alginate + 0.5 M sucrose 0.1 M sucrose; 1 d + 0.3 M sucrose; 1 d + 0.7 M sucrose; 1 d + 1 M sucrose; 1 d; 5°C	air-flow; 4 h; 30% water content; direct plunging	33%	
<i>Mangifera indica</i> (mango) embryogenic mass (in vitro; 3-5 mm) 0.5 M sucrose; 24 h; 25°C	vitrification	50% glycerol + 50% sucrose (PVS3); 20 min	35% water content; direct plunging	94.3%	Wu et al. (197)
	encapsulation-dehydration	bead: 4% alginate + 0.5 M sucrose	silica gel; 6 h; 41% water content; direct plunging	0	
	pregrowth/dehydration		silica gel; 1 h; 59% water content; direct plunging	8.3%	

## 2.11 Cryopreservation of orchids

### 2.11.1 Cryopreservation of seed orchids

Orchids can maintain in their natural habitat (in situ conservation) and unnatural conditions (ex situ conservation). Under normal condition, orchid seeds require the symbiosis with fungi for growth and development (7, 27, 56). The development and growth of orchids in vitro on the medium may take a long time and must be subcultured several times. The maintenance of orchids in vitro is quite expensive, laborious, accumulation of somaclonal variation and losing materials by contamination (119). The orchid seeds can store under room temperature or in the refrigerator but the viability of seeds reduces and hardly stored for a long time. To avoid this, the cryopreservation of seeds could be a feasible way of preserving their genetic diversity. However, up to now there have been only a few reports on orchid cryopreservation.

For successful cryopreservation, water content in seeds is an important factor. Wang et al. (189) reported that freshly harvested four-month-old seeds of *Dendrobium candidum* contained about 43% water content and did not survive expose to LN. The highest survival rate (95%) could be achieved by drying seeds to water content ranging from 8 to 19%. Seed growth was lower in samples dried to water content less than 12%. There is optimum water content for conservation between 12 and 19%. Hirano et al. (58) reported that germination rate of immature seeds of *Bletilla striata* increased when water content in seeds decreased. Water content of immature seeds decreased with increasing time after pollination was 84%, 57%, and 33% in seeds of 2, 3, and 4 months after pollination and germination rate was 0.2, 25 and 98%, respectively. The moisture content of orchid mature seed is usually less than 13% that can storage by direct plunging into LN without any treatments (without dehydration and vitrification process).

Pritchard et al. (131) reported that seeds of *Eulophia gonychila*, *Dactylorhiza anosmun*, *Dactylorhiza fuchsia*, *Dactylorhiza majalis* and *Paphiopedilum rothschildianum* could storage at -196°C. It has been reported that seeds of *Bratonia* (129), immature seeds of *Bletilla striata* (58) and seeds of rare tropical orchids (119) could be frozen by direct plunging into LN without any treatments. Batty et al. (7) reported that seeds of rare and endangered Western Australian terrestrial (*Caladenia*,

*Diuris*, *Pterostylis* and *Thelymitra*) dried over silica gel for 24 h and plunged into LN exhibited a further increase in germination levels. The germination of seeds after storage in LN was higher than storage at 4, 18 or 22°C. Mycorrhizal fungi promoted the germination and growth of orchids preserved in LN. Popov et al. (129) reported that cryopreservation did not inhibit the germination rate of seeds of the hybrid *Bratonia* and there were no significant differences in leaf number and shoot length between juvenile plants derived from non-cryopreserved and cryopreserved seed. Liquid Morel medium was the best preferable for protocorm multiplication and liquid MS medium with half-strength macronutrient was the best one for the development of juvenile plants of the hybrid *Bratonia*. The storage of seeds in LN had no negative effect on growth and development of protocorms and juvenile plants of rare tropical orchids (119, 120).

Vitrification method has been applied to some orchid species such as, immature seeds of *Bletilla striata* (58) and *Ponerorchis graminifolia* var. *suzukiana* (59), seeds of *Doritis pulcherrima* (165), zygotic embryos of *Bletilla striata* (66), mature seeds of *Dendrobium* hybrid (186) and seeds of *Vanda coerulea* (166). Cells were precultured with high concentration of sucrose acted to protect cells from dehydration injury and to tolerate desiccation and freezing (104, 194). During preculture, cellular changes might have occurred such as induction of endogenous abscisic acid synthesis, stabilization of the membranes and gene expressions necessary for adapting to the high osmotic condition. Ishikawa et al. (66) reported that precultured with 0.3 M sucrose for 3 d improved survival rate of cryopreserved zygotic embryo of *Bletilla striata* and immature seeds of *Bletilla striata* (58) and *Ponerorchis graminifolia* (59). The loading solution was very efficient in inducing dehydration and freezing tolerance in tissues (140). The germination rate of cryopreserved zygotic embryo of *Bletilla striata* (66), immature seeds of *Bletilla striata* (58), and immature seeds of *Ponerorchis graminifolia* var *suzukiana* (59) was higher than those of cryopreserved seeds without LS. The highest germination rate of cryopreserved seeds of *Doritis pulcherrima* was 62% when dehydrated with PVS2 solution at  $25 \pm 2^\circ\text{C}$  for 50 min. The incubation time and temperature in PVS2 solution has the effect on germination rate of cryopreserved seeds because over-exposure to PVS2 solution can cause chemical toxicity. Dehydration time of explants

at 0°C reduces the toxicity of vitrification solution and increase exposure time to PVS2 solution (142). For the cryopreservation of zygotic embryo of *Bletilla striata*, a 3 to 5 h at 0°C incubation of PVS2 solution was necessary (66). Dehydration with PVS2 solution at 0°C for 60 min was necessary for immature seeds of *Bletilla striata* (58), and immature seeds of *Ponerorchis graminifolia* var *suzukiana* (59), respectively. Hirano et al. (58) using flow cytometry examined the ploidy level. They reported that nuclei samples isolated from the leaves of control plantlets and plantlets regenerated from immature seed which were cryopreserved with preculture and vitrification showed the same peak of relative DNA content and no change in the ploidy level was observed.

Vendrame et al. (184) reported that mature seeds of *Dendrobium* hybrid exposed to PVS2 solution at ice temperature from 1 to 3 h prior to LN exhibited significantly higher germination (50%) that seeds exposed to PVS2 solution at room temperature for the same time prior. No abnormalities, nutritional deficiencies, or disease were observed in developed seedling. 100% survival for all hybrids was observed for 6-8 months in the greenhouse.

In encapsulation-dehydration method, germination rate of cryopreserved seeds depended on water content and duration time of the dehydration. Wood et al. (194) reported cryopreserved seeds of *Dactylorhiza fuchsii* and *Anacamptis morio* encapsulated in alginate beads with *Ceratobasidium cornigerum*. Beads were precultured with 0.7 M sucrose for 18 h and then dehydrated by sterile air-flow cabinet, reduced bead moisture content from 90% to 20%, viability of the seed and the fungus remained uncharged during 30 day storage in LN and germinating seeds produced both epidermal hairs (rhizoids) and shoot. Flachsland et al. (42) reported moisture content of encapsulated bead of *Oncidium bifolium* precultured with sucrose dropped from 72% to 13.3% after 6 h dehydration with silica gel and 4.8% of cryopreserved seeds produced complete plants.

### **2.11.2 Cryopreservation of protocorms, protocorm-like bodies and cell suspension of orchids**

Protocorms and protocorm-like bodies have the potential to regenerate to the whole plants, and represent thus a source of material for germplasm conservation. Only very few cases of orchid has been successfully survived following freezing and

storage in LN. Sucrose has an osmotic dehydration to reduce water content in plant cells. The accumulation of soluble sugars in cells is thought to protect proteins and membranes from damaging effects of dehydration and freezing in LN (104). The loading solution was very efficient in inducing dehydration and freezing tolerance in tissues (140).

In vitrification method, Wang et al. (189) reported that protocorms formed by seeds of *Dendrobium candidum* can be cryopreserved. Survival rate of 88% were achieved when protocorms were cultured for 3 weeks in media containing abscisic acid, dehydrated with PVS2 solution for 15 min.

Tsukazaki et al. (175) cryopreserved cell suspension of *Doritaenopsis* by vitrification. Both LS for 15 min at room temperature and PVS2 solution for 1-3 h on ice were important for the cryopreservation of *Doritaenopsis*. Protocorm like-bodies were induced from cryopreserved cells without morphological variation.

Nikishina et al. (120) cryopreserved protocorms of *Dactylorhiza fuchsii* by slow freezing and vitrification method. In slow freezing, GSD solution (15% glycerol, 10% sucrose, and 7% DMSO) was used as a cryoprotectant while during vitrification, protocorms were treated with LS and PVS2 solution. Cryopreserved protocorms did not survive by slow freezing method but 9% of cryopreserved protocorms survived by vitrification method.

In encapsulation-dehydration method, Miao et al. (104) reported that preculture of protocorm-like bodies of *Oncidium* with high concentration of sucrose and glycerol changed the cellular state so that these cells could tolerate desiccation and freezing. Without preculture, cellular structure of protocorm-like bodies were damaged after desiccation. During preculture, numerous starch grains accumulated and occupied a large portion of the cytoplasm, decreasing the volume of vacuoles that associated with the decrease in water content of cells which cells easy to vitrify and survival in LN.

Flachsland et al. (42) cryopreserved protocorms of *Oncidium bifolium* Sims by encapsulation-dehydration with silica gel. The results showed that survival of cryopreserved protocorms was 80% after 7 h dehydration (21.2% moisture content). Regrowth rate following encapsulation-dehydration is dependent on the amount of water present in the cells. The water within encapsulated beads was removed by both

osmotic dehydration and sterile air-flow. If the cells are not sufficiently dehydrated, intracellular ice will be formed. The duration of dehydration was an important factor for protocorms forming plantlets after cryopreservation. The suitable water content for sample survival after LN exposure ranged from 15% to 25% (154, 180).

### **2.11.3 Cryopreservation of orchid shoot tips and shoot primordia**

Up to now, there are two reports on cryopreservation of orchid shoot tips and shoot primordia. Shoot tip is the suitable material for conservation but it is difficult to cryopreservation because it has high water content and is very sensitive to desiccation and freezing. Na et al. (111) reported successful cryopreservation of shoot primordia from shoot tips of *Vanda pumila*. Shoot primordia were precultured in 1 mg/l ABA for 3 d and then desiccated under 40-45% relative humidity down to 24% relative water content. The survival rate of cryopreserved shoot primordia was 65% when cultured in B5 medium supplemented with 0.02 mg/l BA. The regrowth of cryopreserved shoot primordia displayed no abnormalities in chromosome numbers.

Lurswijidjarus and Thammasiri (91) reported the successful cryopreservation of shoot tips of *Dendrobium Walter Oumae* by encapsulation/dehydration. The highest survival was 13.33 % (2,3,5 Triphenyltetrazolium chloride, TTC test) when encapsulated beads were precultured on 0.3 M sucrose for 2 d, and then dehydrated in the sterile air-flow cabinet for 6-8 h. Cryopreserved shoot tips could develop directly into complete plantlets on the modified VW medium without protocorm-like body formation and they had normal morphology.

## CHAPTER 3

### MATERIALS AND METHODS

#### 3.1 Chemicals and reagents

All chemicals and reagents used for this study were analytical grade and molecular grade. They were purchased from Fluka (Switzerland), Mallinckrodt (USA), Merck (Germany) and Sigma (USA).

#### 3.2 Micropropagation of *Vanda coerulea* from shoot tips

##### 3.2.1 Plant materials

Mature fruits from selfing of *Vanda coerulea*, harvested 7 months old, were used for this study. Fruits were cleaned by getting rid of dead tissue, wiped with 70% ethyl alcohol and then brought inside a laminar air-flow cabinet, soaked in 95% ethyl alcohol for 1 min and flamed with lamp until the flame stopped. Fruits were cut and seeds were removed from the fruits and then were sown on modified VW agar medium supplemented with 150 ml/l coconut water, 100 g/l banana, 10 g/l sucrose, 0.7 g/l activated charcoal, 8 g/l agar and pH at 5.2. They were cultured at  $25 \pm 3^\circ\text{C}$  under illumination of about  $37 \mu\text{mol.m}^{-2}.\text{s}^{-1}$  provided by fluorescent tubes (Phillip, Thailand) for 16 h/d. The plantlets were used for these experiments.

##### 3.2.2 Study the effects of media and sucrose concentrations on shoot and root formation

For shoots and roots formation different media, modified VW and MS (Appendix A) agar and liquid medium were used as basal medium. Shoot tips of 2-3 mm were excised under stereo microscope from 2-3 cm long plantlets and placed on the medium (Figure 2). Various sucrose concentrations at 0, 5, 10, 15, 20 and 30 g/l were used as carbon sources. Shoot tips were cultured under the same condition as described above.

### **3.2.3 Study the effects of BA, NAA and TDZ on shoot and root formation**

Based on the results of the experiments, the most suitable medium and sucrose concentration were selected, and used for further investigation. The effects of BA, NAA and TDZ alone or in combination with NAA were investigated. Shoot tips of 2-3 mm were excised under stereo microscope from 2-3 cm long plantlets and placed on the medium supplemented with BA at concentration of 1, 2 and 3 mg/l individually and combination with NAA at 0.5 and 1 mg/l. TDZ was used at concentration of 0.1, 1, 2 and 4 mg/l individually and combination with NAA at 0.5 and 1 mg/l. Shoot tips were cultured under the same condition as described above.

### **3.2.4 Plant regeneration and field transfer**

After 6 months of culture, shoots produced roots were separated and then cultured on modified VW agar medium supplemented with 150 ml/l coconut water, 100 g/l banana, 10 g/l sucrose, 0.7 g/l activated charcoal, 8 g/l agar and pH at 5.2 without plant growth regulator at  $25 \pm 3^\circ\text{C}$  under illumination of about  $37 \mu\text{mol. m}^{-2}.\text{s}^{-1}$  provided by fluorescent tubes (Phillip, Thailand) for 16 h/d. The plantlets with 2-3 cm in height, 2-3 leaves and 2-3 roots were removed from bottles. The rooted shoots were washed in water and transplanted into pots on the osmunda in the greenhouse (Figure 2). Ten plantlets were planted in each pot. The survival rate was recorded after 3 months of culture in the greenhouse.

### **3.2.5 Ploidy stability analysis using flow cytometry (FCM)**

To analyze their ploidy stability, nuclei were isolated by chopping the leaves (0.5 x 0.5 cm) of plantlets developed from all treatments in 200  $\mu\text{l}$  of nucleic acid extraction buffer following the addition of 1 ml DAPI solution (10 mM Tris, 50 mM sodium citrate, 2 mM  $\text{MgCl}_2$ , 0.1% (w/v) polyvinyl pyrrolidone (PVP), 0.1% (v/v) Triton X-100, 2 mg/l 4,6-diamidino-2-phenylindole (DAPI), pH at 7.5). After chopping the leaves, the suspension was filtered through 20  $\mu\text{m}$  mesh size nylon filter. Nuclei were analyzed with a flow cytometer (Partec Cell Analyzer, Germany) (58).

### **3.2.6 Data collection and statistical analysis**

After 3 months of culture, survival rate, number of shoot buds, number of shoots, shoots produced roots (%), number of roots, plantlet height and size of protocorm-like bodies were recorded. Twenty explants were used for each treatment. The experiments were replicated two times and the mean standard errors of the results

were calculated. Experiments were organized according to a completely randomized design (CRD). Data were subjected to analysis of variance (ANOVA) and the means were compared using the least significant difference (LSD) test.

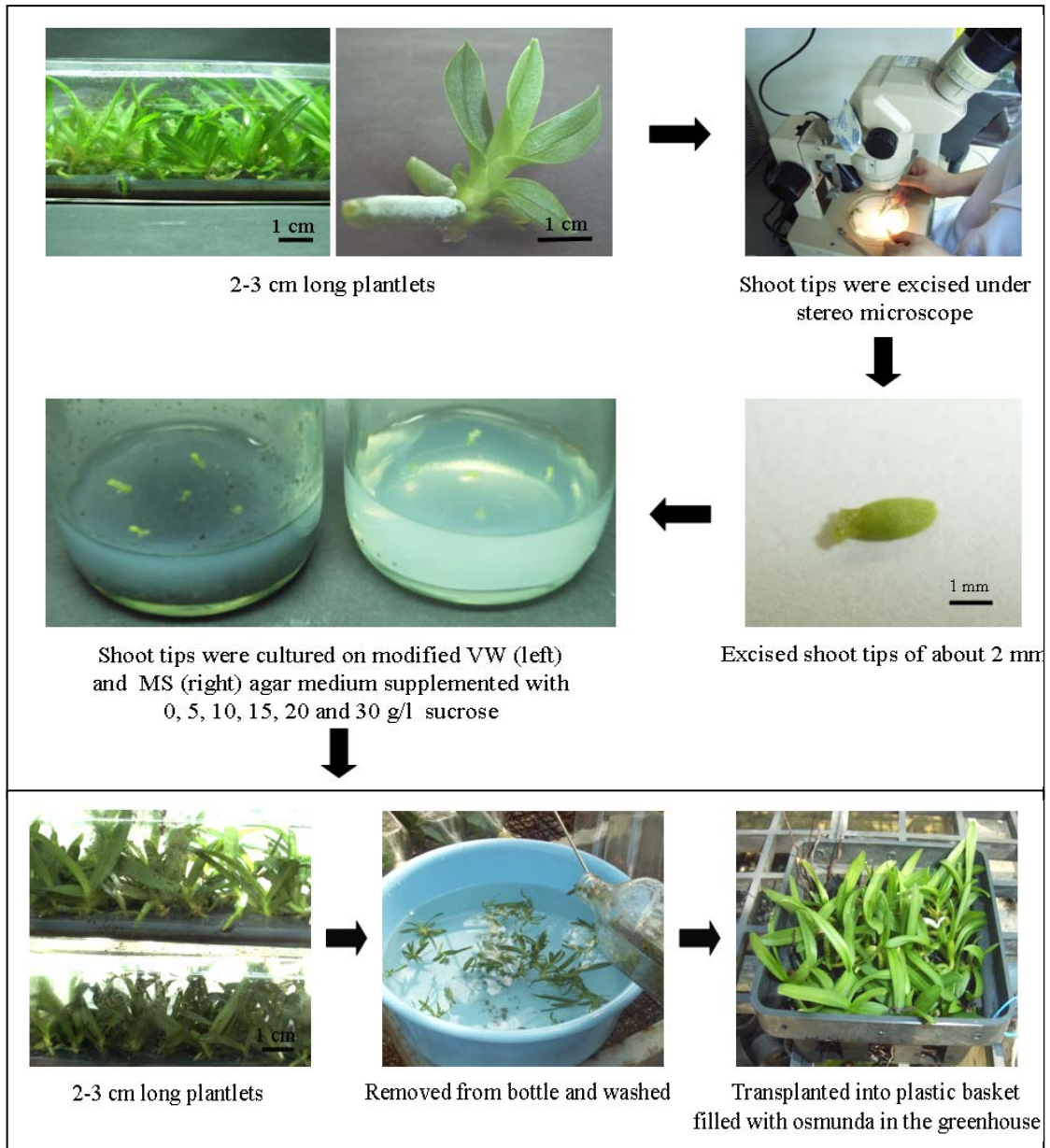


Figure 2. Established micropropagation protocol of *Vanda coerulea* from shoot tips.

### **3.3 Cryopreservation of seeds**

#### **3.3.1 Plant materials**

Mature fruits from selfing of *Vanda coerulea*, harvested 7 months old, were used for this study. Fruits were cleaned by getting rid of dead tissue, wiped with 70% ethyl alcohol and then brought inside a laminar air-flow cabinet, soaked in 95% ethyl alcohol for 1 min and flamed with lamp until the flame stopped. Fruits were cut and seeds were removed from the fruits and used for these experiments.

#### **3.3.2 Cryopreservation methods**

##### **3.3.2.1 Directly plunged into liquid nitrogen method**

The seeds were transferred to 2.0 ml cryotubes, then directly plunged into LN (Figure 3). After 1 d, the cryotubes were rapidly warmed in a water bath at 40°C for 2 min, then seeds (approximately 100-200 seeds) were sown on modified VW agar medium supplemented with 150 ml/l coconut water, 100 g/l banana, 0.7 g/l activated charcoal, 8 g/l agar (pH at 5.2), then cultured at  $25 \pm 3^\circ\text{C}$  under illumination of about  $37 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  provided by fluorescent tubes (Phillip, Thailand) for 16 h/d.

##### **3.3.2.2 Vitrification method**

The seeds without preculture were transferred to 2.0 ml cryotubes containing 1.5 ml LS (Appendix B) for 15 min at  $25 \pm 3^\circ\text{C}$  or without LS, then dehydrated with PVS2 solution (140) which contained 30% (w/v) glycerol, 15% (w/v) ethylene glycol and 15% (w/v) DMSO in VW liquid medium with 0.4 M sucrose, pH at 5.2 (Appendix B) on ice for 0-140 min. After dehydration, the seeds were directly plunged into LN (Figure 4). After 1 d, the cryotubes were rapidly warmed in a water bath at 40°C for 2 min. PVS2 solution was removed and replaced by adding 1.5 ml of VW liquid medium supplemented with 1.2 M sucrose for 20 min, then the seeds (about 100-200 seeds) were cultured on modified VW agar medium as described above. As control, seeds without preculture were treated with or without LS, and then dehydration with PVS2 solution for 0-140 min but non-frozen, then treated with 1.2 M sucrose were added to each tube and hold for 20 min, then cryopreserved seeds were cultured on modified VW agar medium. Approximately 100 seeds were tested for each of four replicates. The experiment was repeated two times.

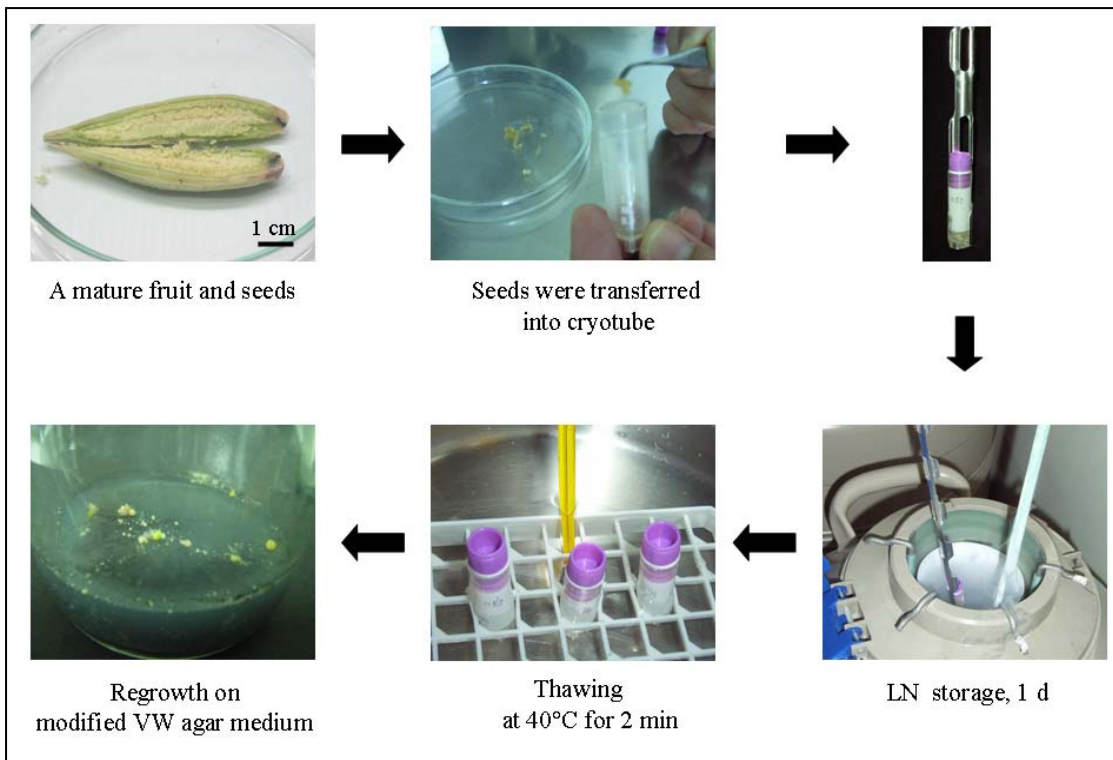


Figure 3. Established protocol for cryopreservation of seeds by directly plunged into liquid nitrogen method.

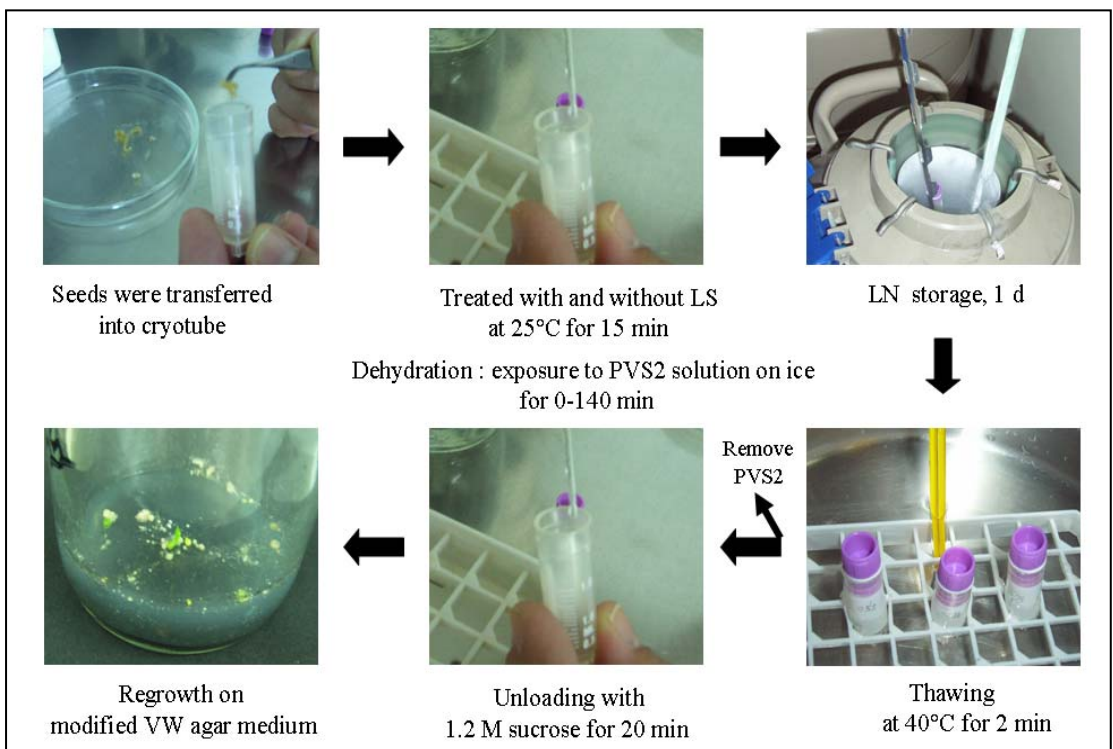


Figure 4. Established protocol for cryopreservation of seeds by vitrification method.

### **3.3.2.3 Encapsulation-dehydration method**

The seeds were suspended in VW medium with 3% Na-alginate containing 0.4 M sucrose (Appendix B). The mixture including seeds was dispensed into 100 mM CaCl<sub>2</sub> solution containing 0.4 M sucrose for 1 h at 25 ± 3°C. The beads (about 3 mm diameter) were precultured in VW liquid medium supplemented with 0.5 and 0.7 M sucrose for 18 h on a shaker (110 rpm) at 25 ± 3°C (Figure 5), and then subjected to dehydration in the laminar air-flow cabinet at 25 ± 3°C for 0-8 h. After dehydration, about 10 dried beads were placed in a 2.0 ml cryotube, then directly plunged into LN. After 1 d, the cryotubes were rapidly warmed in a water bath at 40°C for 2 min. Cryopreserved seeds were plunged from cryotube into 1.5 ml of VW liquid medium supplemented with 1.2 M sucrose for 20 min (Figure 5), then cryopreserved beads were cultured on modified VW agar medium as described above. As a control, precultured beads were dehydrated in the laminar air-flow cabinet but non-frozen, then treated with 1.2 M sucrose in the same way as described above. Each bead contained 11 seeds. Approximately 10 beads were tested for each of four replicates.

### **3.3.2.4 Encapsulation-vitrification method**

The seeds were suspended in VW liquid medium with 3% Na-alginate containing 0.4 M sucrose. The mixture including seeds was dispensed into 100 mM CaCl<sub>2</sub> solution and 0.4 M sucrose for 1 h at 25 ± 3°C. Encapsulated seeds (about 3 mm in diameter) were precultured in VW liquid medium supplemented with 0.5 and 0.7 M sucrose for 18 h on a shaker (110 rpm) at 25 ± 3°C. The precultured beads were treated with LS for 45 min at 25 ± 3°C, then dehydrated with PVS2 solution on ice for 0-240 min. After dehydration, about 10 dried beads were suspended in 1.5 ml PVS2 solution in a 2.0 ml cryotube, then directly plunged into LN. After 1 d, the cryotubes were rapidly warmed in a water bath at 40°C for 2 min (Figure 6). PVS2 solution was removed and replaced by adding 1.5 ml of VW liquid medium supplemented with 1.2 M sucrose for 20 min, and then cryopreserved seeds were cultured on modified VW agar medium as described above. As a control, precultured beads were treated with LS and dehydrated with PVS2 solution but non-frozen, then treated with 1.2 M sucrose in the same way as described above.

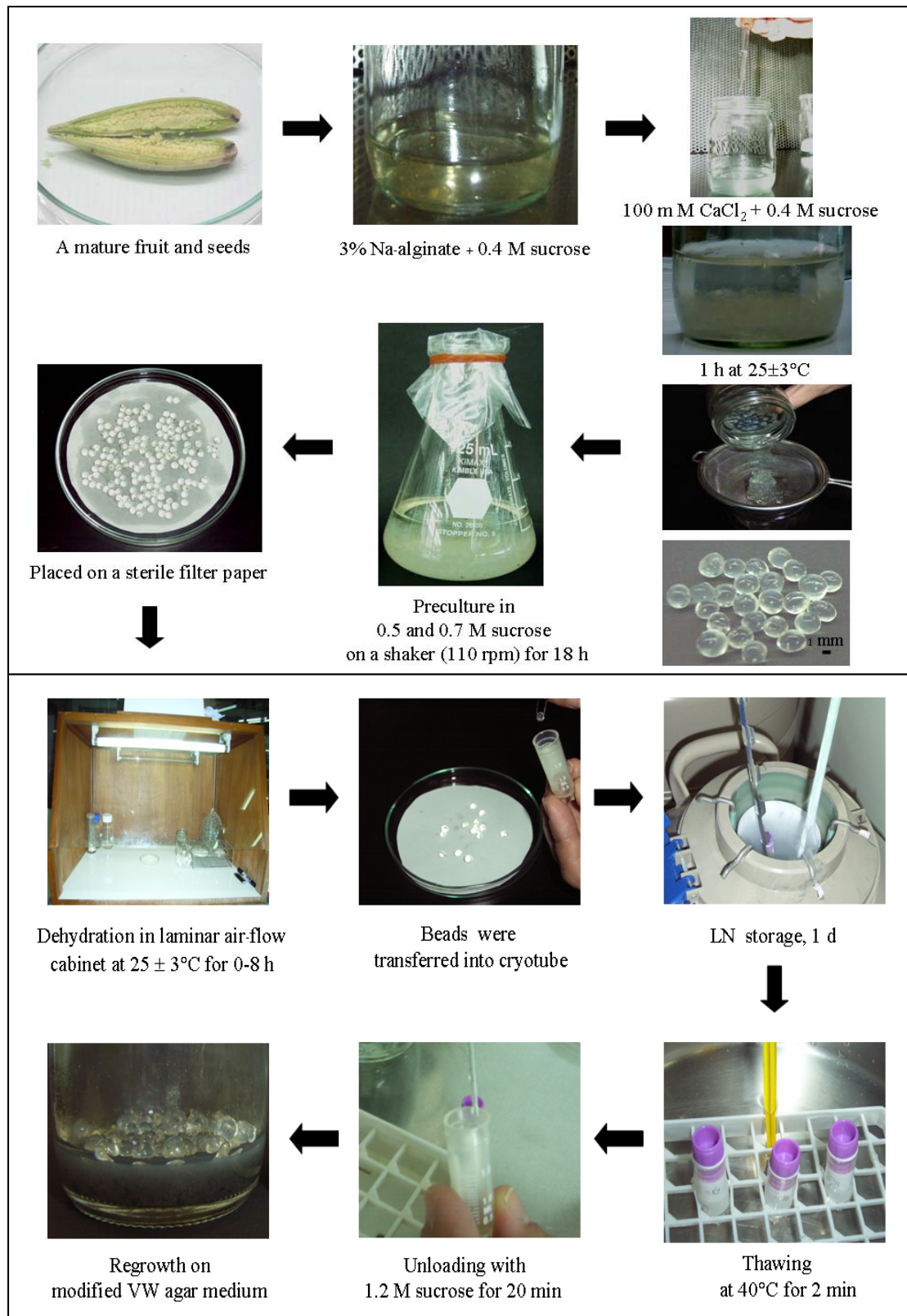


Figure 5. Established protocol for cryopreservation of seeds by encapsulation-dehydration method.

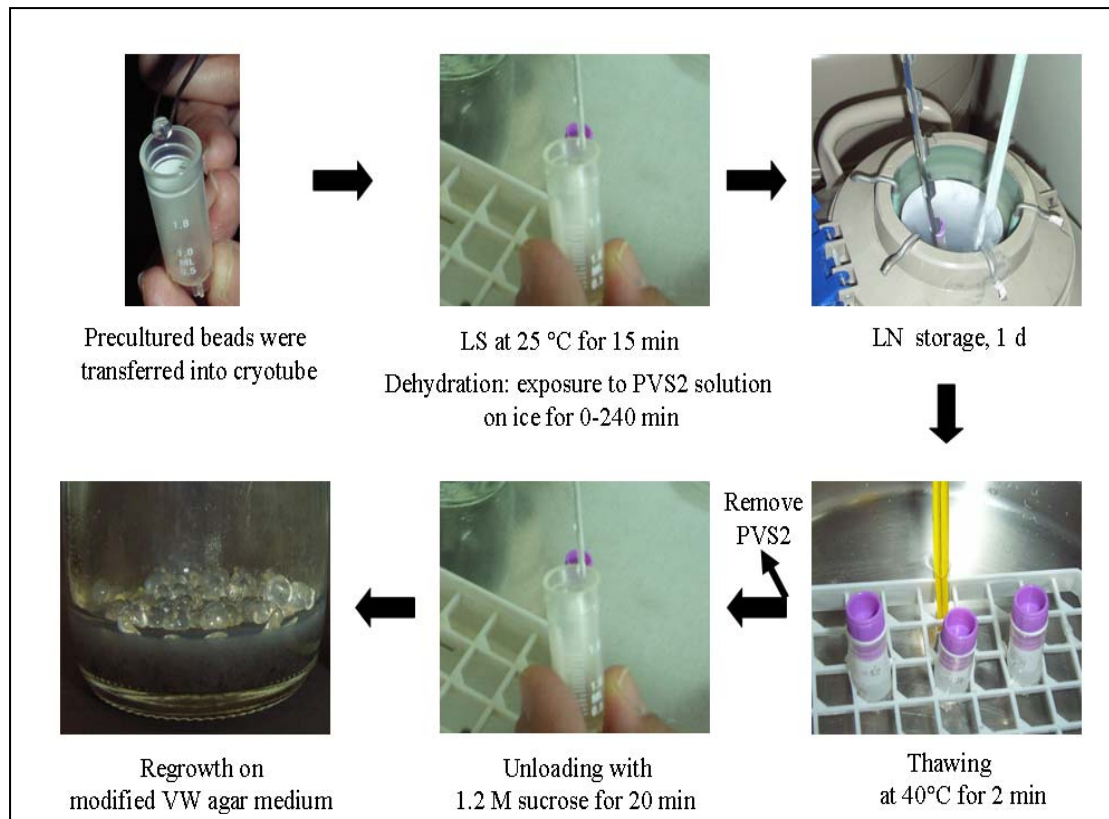


Figure 6. Established protocol for cryopreservation of seeds by encapsulation-vitrification method.

### 3.3.3 Water content determination

Water content was determined for seeds and encapsulated seeds following 0.5 and 0.7 M sucrose incubation and dehydration in the laminar air-flow cabinet for 0-8 h. Seeds and encapsulated seeds dry weight were measured after 2 h drying in the oven at 130°C (12). The percentage of water content was calculated on the basis of the fresh weight. Five beads were tested for each of four replicates.

### 3.3.4 Germination rate

In directly plunged into liquid nitrogen and vitrification methods, germination rate was calculated on the basis of the protocorms or plantlets developed from seeds after 3 months of culture on modified VW agar medium.

In encapsulation-vitrification and encapsulation-dehydration, germination rate was calculated on the basis of the protocorms or plantlets from encapsulated seed

(only one protocorm or plantlet/bead) and the number of protocorms in cryopreserved seeds (50 beads) were counted under the stereo microscope.

### **3.3.5 Ploidy stability analysis using flow cytometry**

Nuclei were isolated by chopping the leaves of 11-month-old plantlets developed from non-cryopreserved and cryopreserved seeds. Nuclei were analyzed in the same way as described in 3.2.5.

### **3.3.6 Statistical analysis**

Germination of non-cryopreserved and cryopreserved seeds was subjected to ANOVA and the means were compared using LSD test.

## **3.4 Cryopreservation of protocorms**

### **3.4.1 Plant materials**

Seeds of *Vanda coerulea* were sown on modified VW agar medium. They were cultured at  $25 \pm 3^\circ\text{C}$  under illumination of about  $37 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  provided by fluorescent tubes (Phillip, Thailand) for 16 h/d. Seeds germinated and developed into green protocorms within 30 d after sowing. After 70 d of sowing, protocorms (about 2 mm in diameter) were used for this experiment (Figure 7).

### **3.4.2 Encapsulation-dehydration method**

Protocorms were selected and encapsulated in an alginate matrix composed of 2% Na-alginate, 2 M glycerol plus 0.4 M sucrose (LS) in modified VW liquid medium, pH at 5.2. Drops of this solution, were dispensed with a pipette into 100 mM  $\text{CaCl}_2$  solution supplemented with LS, and kept for 1 h at  $25 \pm 3^\circ\text{C}$  (Figure 7). Encapsulated protocorms (about 4 mm in diameter) were then removed from the solution and their surfaces were dried with filter paper. Encapsulated protocorms were precultured in modified VW liquid medium supplemented with 0.7 M sucrose on a shaker (110 rpm) at  $25 \pm 3^\circ\text{C}$  for 20 h.

To dehydrate the beads, they were placed on a sterile filter paper in open Petri dishes (9 cm in diameter) and then exposed to sterile air-flow at 0.5 inches/water column from the laminar air-flow cabinet at  $25 \pm 3^\circ\text{C}$  for 0-10 h. After dehydration, about 10 dried beads were placed in a 2.0 ml cryotube, then directly plunged into LN for 1 d. The cryotubes were taken out of LN tank and rapidly warmed in a water bath at  $40^\circ\text{C}$  for 2 min. Cryopreserved protocorms were placed in 1.5 ml of modified VW

liquid medium supplemented with 1.2 M sucrose for 20 min, then cryopreserved protocorms were cultured on modified VW agar medium as described above. For control, precultured beads were exposed to sterile air-flow at 0.5 inches/water column from the laminar air-flow cabinet at  $25 \pm 3^\circ\text{C}$  for 0-10 h, then treated with 1.2 M sucrose for 20 min. Ten beads were used for each treatment with four replicates.

### 3.4.3 Water content determination

Water content was determined for encapsulated protocorms following 0.7 M sucrose incubation and dehydration in the laminar air-flow cabinet for 0-10 h. Dry weight of the beads were measured after drying in the oven at  $130^\circ\text{C}$  for 2 h (12). The percentage of water content was calculated on the basis of the fresh weight.

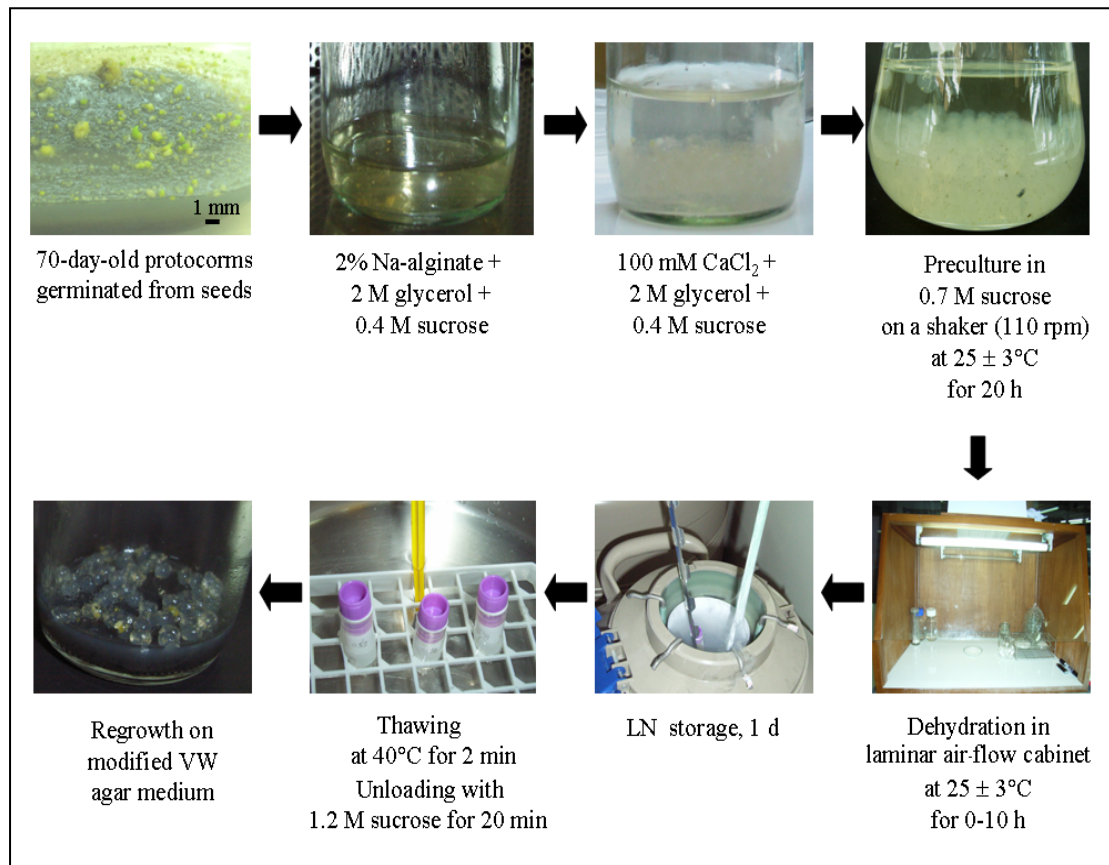


Figure 7. Established protocol for cryopreservation of protocorms by encapsulation-dehydration method.

#### **3.4.4 Regrowth rate**

After 3 months of culture, regrowth rate was calculated on the basis of protocorms forming plantlets from encapsulated protocorms.

#### **3.4.5 Morphological study**

The plantlets with well-developed shoots and roots were selected and then washed with water to remove the culture medium and were transplanted into pots in the greenhouse. After 0, 5 and 15 months of culture in the greenhouse, the survival rate, plantlet height, root length, number of roots/plantlet and number of leaves/plantlet were recorded.

#### **3.4.6 Ploidy stability analysis using flow cytometry**

Nuclei were isolated by chopping the leaves of 11-month-old plantlets developed from non-cryopreserved and cryopreserved protocorms. Nuclei were analyzed in the same way as described in 3.2.5.

#### **3.4.7 Statistical analysis**

Protocorms forming plantlets of non-cryopreserved and cryopreserved protocorms were subjected to ANOVA and the means were compared using LSD test.

### **3.5 Cryopreservation of shoot tips**

#### **3.5.1 Plant materials**

Plantlets of *Vanda coerulea* were cultured on ND solid medium consisted of 20 g/l sucrose, 0.7 g/l activated charcoal, 0.25% gelrite, pH at 5.4. Cultures were maintained at 25°C under illumination provided at an intensity of 62  $\mu\text{mol. m}^{-2}. \text{s}^{-1}$  for 16 h/d. Shoot tips 2 mm long were excised under stereo microscope from 2-3 cm long plantlets (21-day-old plantlets) (Figure 8).

#### **3.5.2 Slow freezing method**

Shoot tips were precultured on ND solid medium supplemented with 0.3 M sucrose for 1 d. Following preculture, shoot tips were treated with LS for 15 min at 25°C and then dehydrated with PVS2 solution on ice for 0, 20, 40 and 60 min, then PVS2 solution were removed from cryotubes. The cryotubes were placed into a cryofreezing- container and stored at -80°C for 6 h and then cryotubes were plunged into LN (Figure 8). Shoot tips were stored in LN for 1 d, the cryotubes were rapidly warmed in a water bath at 40°C for 1 min. After thawing, cryopreserved shoot tips

were incubated in ND liquid medium supplemented with 1.2 M sucrose for 20 min at 25°C and then put on a sterile filter paper for 1 h and then cultured on ND solid medium under illumination provided at an intensity of 62  $\mu\text{mol. m}^{-2}. \text{s}^{-1}$  for 16 h/d at 25°C.

### 3.5.3 Survival rate

After 2 months of culture, survival rate was calculated on the basis of plantlet or protocorm-like body from non-cryopreserved and cryopreserved shoot tips.

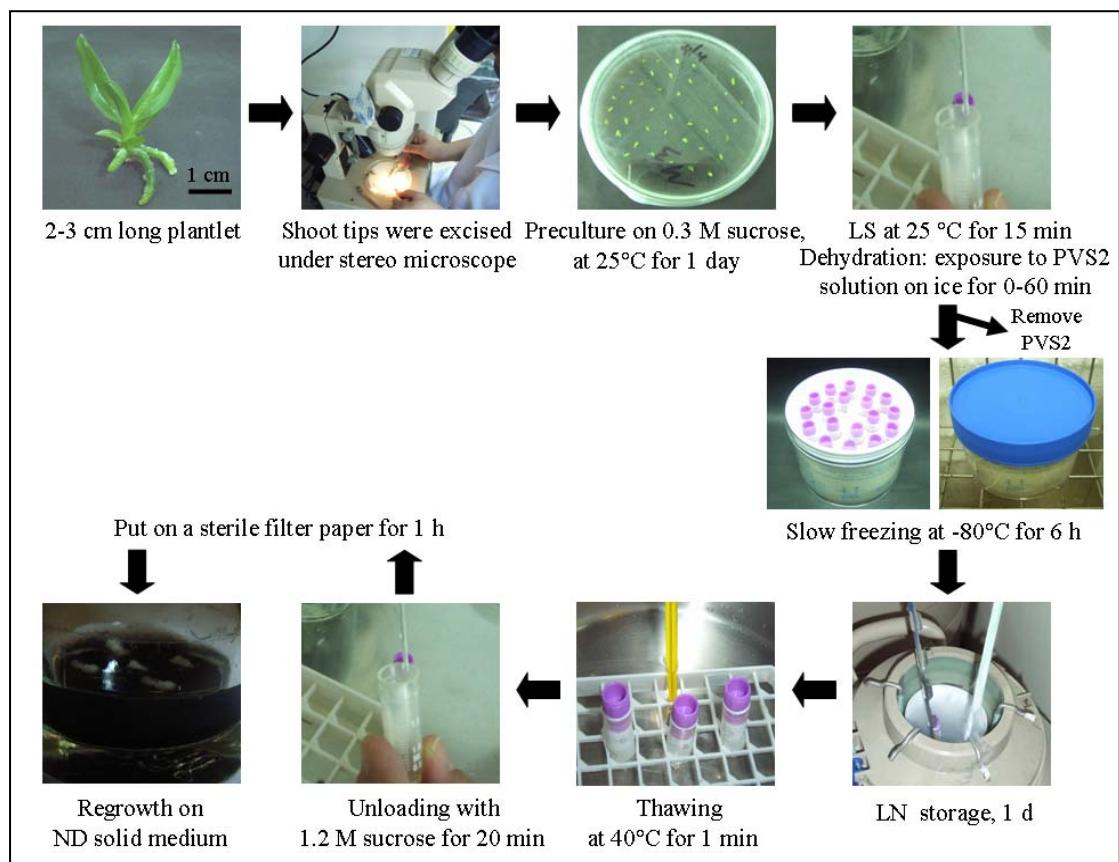


Figure 8. Established protocol for cryopreservation of shoot tips by slow freezing method.

### **3.5.4 Study the effect of maltose concentrations on protocorm-like body and shoot formation from cryopreserved shoot tips**

Protocorm-like bodies were cultured in ND liquid medium supplemented with 1 mg/l BA in combination with 0.5 mg/l NAA. Various maltose concentrations at 0, 10, 20, 30 and 40 g/l were used as carbon sources. After 3 months of culture, the fresh weight and morphogenesis were recorded.

After 3 months of culture, shoots were separated and then cultured on ND solid and modified VW agar medium supplemented with 1 mg/l BA and 10 g/l sucrose. After 4 months of culture, the survival rate was recorded.

### **3.5.5 Ploidy stability analysis using flow cytometry**

Nuclei were isolated by chopping the leaves of 4-month-old plantlets developed from non-cryopreserved and cryopreserved shoot tips with a razor blade in a Petri dish containing 1 ml of ice-cold Tris-MgCl<sub>2</sub> buffer. The suspension of nuclei then filtered through a 40 µm nylon filter and 50 µg/ml propidium iodide and 50 µg/ml RNase were added to the samples to stain the DNA. Samples were analysed within a few minutes to one hour (Appendix C).

### **3.5.6 Statistical analysis**

Plantlets or protocorm-like bodies of non-cryopreserved and cryopreserved shoot tips were subjected to ANOVA and the means were compared using LSD test.

## **3.6 Cryopreservation of protocorm-like bodies**

### **3.6.1 Plant materials**

Protocorm-like bodies were originated from one protocorm germinated from one seed in ND liquid medium supplemented with 1 mg/l BA in combination with 0.5 mg/l NAA and 30 g/l maltose, pH at 5.4. Cultures were maintained in 15 ml ND liquid medium on shaker (110 rpm) under illumination provided at an intensity of 62 µmol. m<sup>-2</sup>. s<sup>-1</sup> for 16 h/d at 25°C for 3 months. Protocorm-like bodies were used for these experiments.

### **3.6.2 Droplet-vitrification method**

Protocorm-like bodies (3 mm) were precultured in ND liquid medium supplemented with 0, 0.1, 0.3, 0.5, 0.7 and 1.0 M sucrose for 1 d on a shaker (110 rpm) at 25°C. Following preculture, protocorm-like bodies were treated with LS

at 25°C for 15 min, and then dehydrated with PVS2 solution for 0, 10, 20, 30, 40, 60 and 80 min at 25°C. A few minutes before plunging into LN, placed 10 drops 2 µl of PVS2 solution on a sterile aluminium foil strip (7 x 20 mm in length), then put one protocorm-like body in each of ten PVS2 drops (Figure 9). After dehydration with PVS2 solution for the mentioned time, the aluminium foil with protocorm-like bodies was quickly transferred with forceps to 1.8 ml cryotube fill with LN. After 1 h of storage in LN, taken off aluminium foil strip from cryotube and put into liquid ND medium supplemented with 1.2 M sucrose for 20 min, then protocorm-like bodies were transferred into 0.5 M sucrose for 10 min, and then cultured on ND solid medium supplemented with 1 mg/l BA in combination with 0.5 mg/l NAA and 30 g/l maltose, pH at 5.4 for 1 month. Ten protocorm-like bodies were tested for each of four replicates.

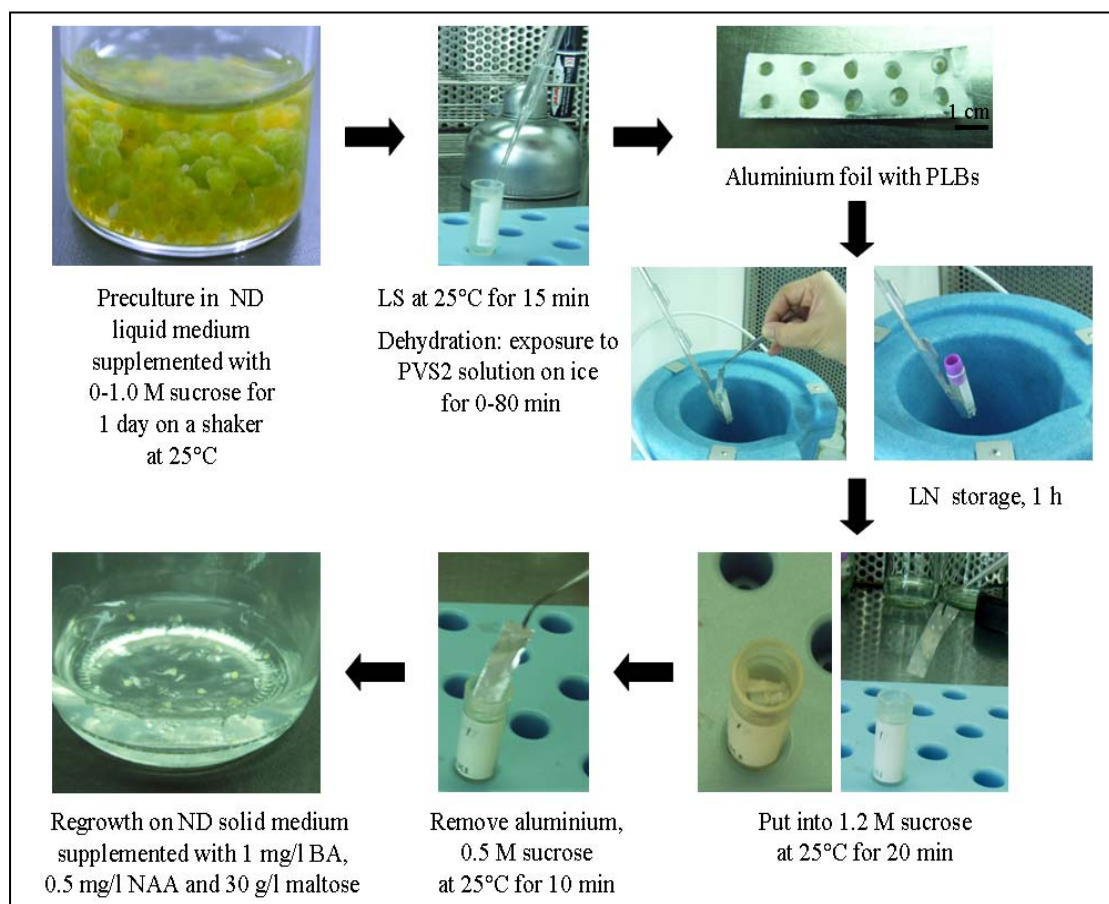


Figure 9. Established protocol for cryopreservation of protocorm-like bodies by droplet-vitrification method.

### **3.6.3 Survival rate**

After 1 month of culture, survival rate was calculated on the basis of protocorm-like bodies from non-cryopreserved and cryopreserved protocorm-like bodies and then were cultured in ND liquid medium supplemented with 1 mg/l BA in combination with 0.5 mg/l NAA and 10 g/l maltose, pH at 5.4 for shoot formation.

### **3.6.4 Ploidy stability analysis using flow cytometry**

Nuclei were isolated by chopping the leaves of 4-month-old plantlets developed from non-cryopreserved and cryopreserved protocorm like-bodies. Nuclei were analyzed in the same way as described in 3.5.5.

## **3.7 Study the genetic variation of plantlets from seeds of *Vanda coerulea* using RAPD markers**

### **3.7.1 DNA extraction**

Genomic DNA was extracted from leaves of plantlets germinated from seeds of *Vanda coerulea*. 0.2 g of young leaves from 10 plantlets were crushed with 150  $\mu$ l 20 mM Tris-HCl pH at 8.0 and 20  $\mu$ l 10% SDS and then incubated at 70°C in a water bath for 15 min. The sample was mixed with 110  $\mu$ l NH<sub>4</sub>OAC, incubated on ice for 30 min, centrifuged at 15,000 rpm at 4°C for 10 min, and the supernatant was then collected in a new tube. DNA was precipitated from supernatant by mixing with 0.4 ml isopropanol. Following precipitation, DNA was incubated at room temperature for 10 min, centrifuged at 15,000 rpm at room temperature for 10 min, and the supernatant was then discarded. DNA pellet was washed with 70% ethanol and centrifuged at 15,000 rpm at 4°C for 10 min. DNA was dried in air and resuspended in 20  $\mu$ l TE buffer solution.

After DNA was extracted, DNA was checked by electrophoresis in 1.5% agarose gel in TBE buffer at 100 V, stained with ethidium bromide and photographed under UV light with Polaroid film.

### **3.7.2 RAPD analysis of genomic DNA**

Eighteen arbitrary 10-base primers (OPC-01, -02, -03, -04, -05, -06, -07, -08, -09, -10, OPE-01, -02, OPK-01, -02, -03, -04, -05 and -06) (Table 3) from Operon Technologies (USA) were used for PCR amplification. PCR reaction was performed in a volume of 25  $\mu$ l. The reaction mixture contained 2.5  $\mu$ l of 10X buffer, 2  $\mu$ l of 2.5 mM of dNTPs, 0.125  $\mu$ l of 5U/ $\mu$ l Taq polymerase, 1  $\mu$ l of 20  $\mu$ M primers, 1  $\mu$ l of 1/20 template DNA and 18.375  $\mu$ l of distilled water.

Amplification was performed in a Perkin-Elmer Thermalcycler programmed for 35 cycles as follows: preliminary denaturation of DNA at 94°C for 2 min followed by 35 cycles of 1 min denaturation at 94°C, 1 min annealing at 44°C, 2 min extension at 72°C which is followed by final extension cycle of 10 min at 72°C and then cooling down to 4°C. The amplification products were resolved by electrophoresis on 1.5% agarose gel in TBE buffer at 100 V, stained with ethidium bromide and photographed under UV light with Polaroid film. The size of the amplification products was estimated from the  $\Phi$ X174DNA-*Hae*III.

### **3.7.3 RAPD data analysis**

RAPD bands were observed as presence (1) or absence (0) of patterns of plantlets germinated from seeds.

Table 3. Sequences of RAPD primers used in this experiment.

Name of primer	Primer sequence (5'–3' )
OPC-01	5'–TTCGAGCCAG–3'
OPC-02	5'–GTGAGGCGTC–3'
OPC-03	5'–GGGGGTCTTT–3'
OPC-04	5'–CCGCATATAC–3'
OPC-05	5'–GATGACCGCC–3'
OPC-06	5'–GAACGGACTC–3'
OPC-07	5'–GTCCCGACGA–3'
OPC-08	5'–TGGACCGGTG–3'
OPC-09	5'–CTCACCGTCC–3'
OPC-10	5'–TGTCTGGGTG–3'
OPE-01	5'–CCCAAGGTCC–3'
OPE-02	5'–GGTGCGGGAA–3'
OPK-01	5'– CATTGAGCC–3'
OPK-02	5'– GTCTCCGCAA–3'
OPK-03	5'– CCAGCTTAGG–3'
OPK-04	5'– CCGCCCAAAC–3'
OPK-05	5'– TCTGTGAGG–3'
OPK-06	5'– CACCTTTCCC–3'

### **3.8 Universal primers c and d for amplification of the *trnL* (UAA) region of cpDNA**

#### **3.8.1 Study the optimum annealing temperature for PCR amplification of the *trnL* (UAA) region of cpDNA for *Vanda coerulea***

##### **3.8.1.1 Plant materials and DNA extraction**

Plantlets of *Vanda coerulea* from Kanchanaburi, Chiang Mai and Bangkok province were used in this study. Total DNA was extracted from leaves of plantlets from *Vanda coerulea* in the same way as described in 3.7.1.

##### **3.8.1.2 PCR amplification of the *trnL* (UAA) region**

The *trnL* (UAA) non-coding region of cpDNA for *Vanda coerulea* was amplified with universal primers c (5'-CGAAATCGGTAGACGCTACG-3') and d (5'-GGGGGATAGAGGGACTTGAAC-3') (Figure 10). Two universal primers c and d are from Taberlet et al. (161). PCR amplification were performed in a volume of 25  $\mu$ l containing 2.5  $\mu$ l of 10X buffer, 2  $\mu$ l of 2.5 mM of dNTPs, 0.125  $\mu$ l of 5U/ $\mu$ l Taq polymerase, 1  $\mu$ l of 20  $\mu$ M primers, 1  $\mu$ l of 1/20 template DNA and 18.375  $\mu$ l of distilled water.

Amplification was performed in a Perkin-Elmer Thermalcycler programmed for 35 cycles as follows: preliminary denaturation of DNA at 94°C for 2 min followed by 35 cycles of 1 min denaturation at 94°C, 1 min annealing at 49.2 to 55.8°C (49.2, 49.3, 49.7, 50.3, 51.0, 51.9, 52.7, 53.6, 54.4, 55.0, 55.5 and 55.8°C), 2 min extension at 72°C which is followed by final extension cycle of 7 min at 72°C and then cooling down to 4°C. Amplification products were visualized by electrophoresis on 1.5% agarose gel, as 3.3.2. The size of the amplification products was estimated from the standard marker  $\Phi$ X174DNA-*Hae*III.

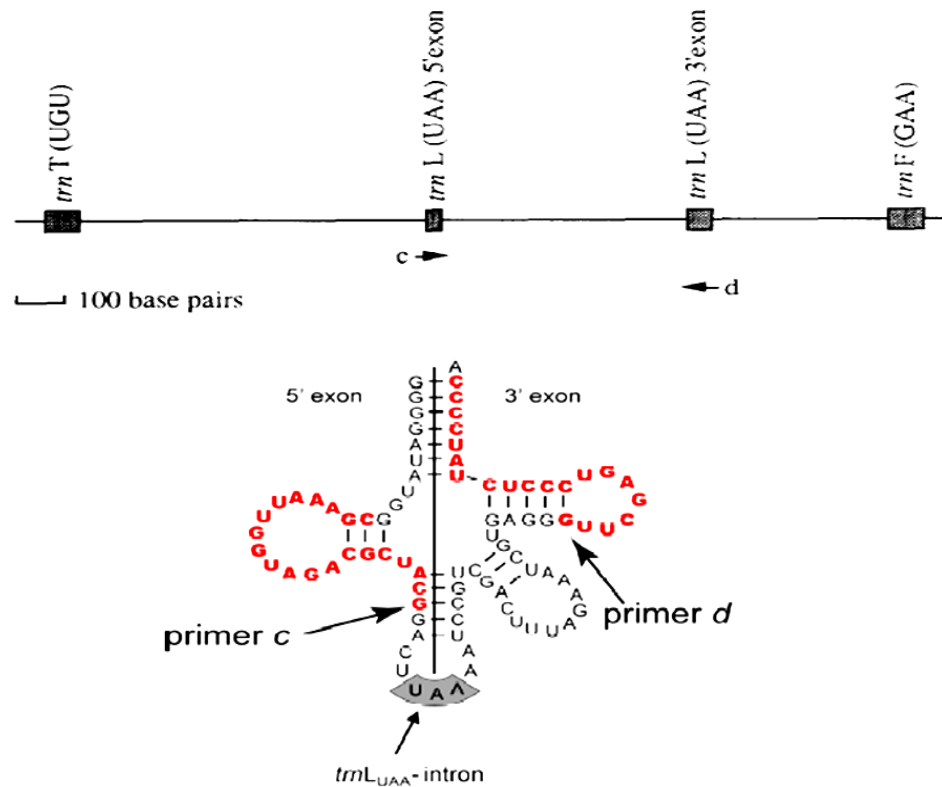


Figure 10. Position and direction of universal primers c and d used to amplify the *trnL* (UAA) non-coding region of cpDNA (161, 162).

### 3.8.2 Study the size of the *trnL* (UAA) region

#### 3.8.2.1 Plant materials

Plantlets of *Vanda coerulea* from Kanchanaburi province, *Grammatophyllum speciosum*, *Dendrobium aphyllum*, *Dendrobium lindleyi* from Thailand, *Vanda tricolor* from Botanical garden of Tsukuba, *Bletilla striata* from Botanical garden of Toyama, and DNA of *Oryza sativa* from Chiba University, Japan were used in this experiment.

#### 3.8.2.2 DNA extraction

Total DNA was extracted from leaves of plantlets from *Vanda coerulea*, *Vanda tricolor*, *Dendrobium aphyllum*, *Dendrobium lindleyi*, *Bletilla striata*, and *Grammatophyllum speciosum* in the same way as described in 3.7.1

### 3.8.2.3 PCR amplification of the *trnL* (UAA) region

Based on the results of 3.8.1.2, the optimum annealing temperature was selected, and used for further investigation. Two universal primers c and d for amplification of the *trnL* (UAA) region were used. PCR amplifications were done in the same way as 3.8.1.2.

Amplification was performed in a Perkin-Elmer Thermalcycler programmed for 35 cycles as follows: preliminary denaturation of DNA at 94°C for 2 min followed by 35 cycles of 1 min denaturation at 94°C, 1 min annealing at 55°C, and 2 min extension at 72°C; 7 min at 72°C; and holding at 4°C. After PCR amplification, PCR products were run by electrophoresis on 1.5% (w/v) agarose gel, as 3.8.1.2.

## 3.8.3 Study the sequences of the *trnL* (UAA) region of cpDNA for *Vanda coerulea*

### 3.8.3.1 DNA extraction and amplification

Total DNA was extracted from leaves of plantlets from *Vanda coerulea* in the same way as described in 3.7.1. The *trnL* (UAA) region was amplified with universal primers c and d. Amplification reactions were done in the same way as described in 3.8.1.2. Sequences were obtained with the universal primers c and d designed for the *trnL* (UAA) region (161, 162).

### 3.8.3.2 Recovery PCR products and DNA sequencing

After PCR amplification, PCR products were separated by electrophoresis in 0.7% agarose gel (for recovery) in TBE buffer at 180 V, stained with new ethidium bromide for 5 min, then transferred to distill water for 5 min and then excised from gel under UV light (Figure 11. A-B), then transferred gel into filter tube, centrifuged at 10,000 rpm 4°C for 10 min. The sample was mixed with 40 µl of 3 M NH<sub>4</sub>OAC, pH at 5.2 and 800 µl of ethanol (cool), centrifuged at 15,000 rpm at 4°C for 10 min, and the supernatant was then discarded. DNA pellet was washed with 70% ethanol and centrifuged at dissolved at 15,000 rpm at 4°C for 10 min. DNA was dried in air and resuspended in 20 µl TE buffer solution. After DNA was extracted, DNA was checked by electrophoresis in 1.5% agarose gel in TBE buffer at 100 V, stained with

ethidium bromide and photographed under UV light with camera. DNA was sequenced of the *trnL* (UAA) region of cpDNA for *Vanda coerulea*.

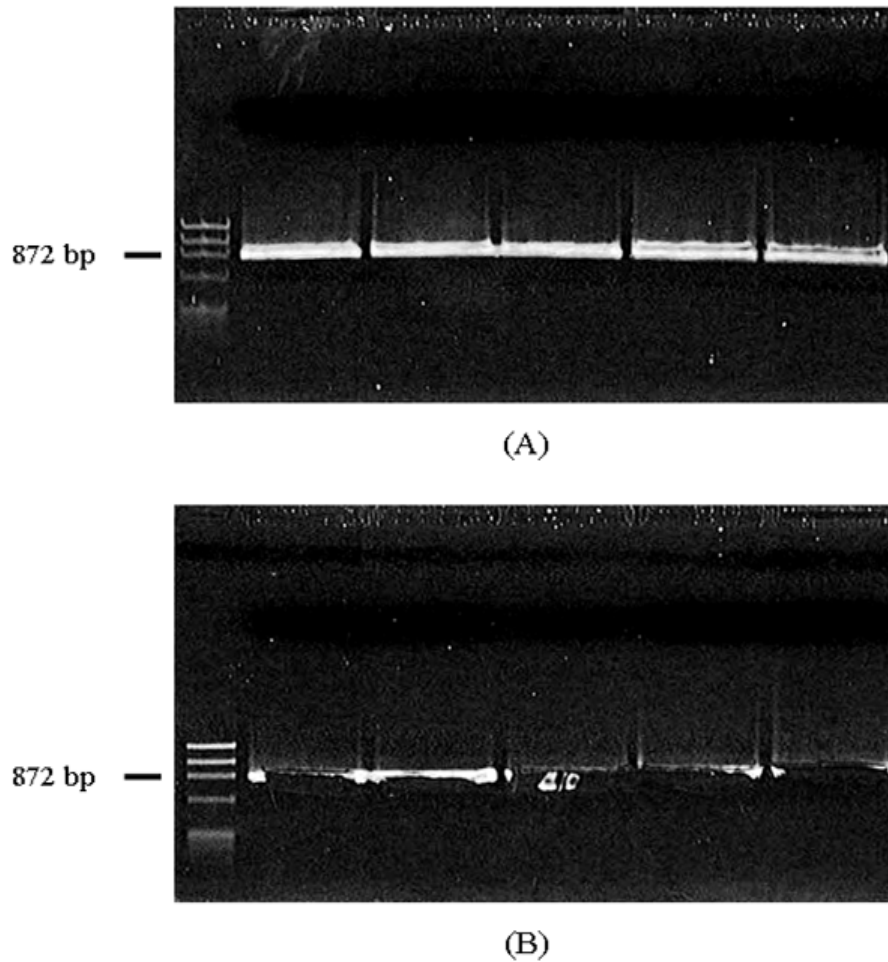


Figure 11. PCR products of two universal primers c and d for the *trnL* (UAA) region of cpDNA for *Vanda coerulea* in 0.7% agarose gel (A) and excised from gel under UV light for recovery PCR products (B).

### **3.9 Study the genetic stability after cryopreservation**

#### **3.9.1 DNA extraction and RAPD analysis**

DNA was extracted from non-cryopreserved and cryopreserved protocorm like-bodies in the same way as described in 3.7.1. Sixty arbitrary 10-base primers (OPC-01-20, OPE-01-20 and OPK-01-20) from Operon Technologies (USA) (Table 4) were used for PCR amplification. PCR reaction mixture and DNA amplification were performed in the same way as described in 3.7.2.

#### **3.9.2 Comparison the size and sequences of the *trnL* (UAA) region of cpDNA between non-cryopreserved and cryopreserved protocorm-like bodies**

DNA was extracted from non-cryopreserved and cryopreserved protocorm-like bodies. PCRs were done with combination of the universal primers c and d for amplification of the *trnL* (UAA) region in the same way as described in 3.8.2. Sequences were obtained with the primers c and d for the *trnL* (UAA) region. Recovery PCR products and sequencing of the *trnL* (UAA) region were done in the same way as described in 3.8.3.

Table 4. Sequences of RAPD primers used in this study.

Name of primer	Primer sequence (5' -3' )
OPC-01	5'-TTCGAGCCAG-3'
OPC-02	5'-GTGAGGCGTC-3'
OPC-03	5'-GGGGGTCTTT-3'
OPC-04	5'-CCGCATATAC-3'
OPC-05	5'-GATGACCGCC-3'
OPC-06	5'-GAACGGACTC-3'
OPC-07	5'-GTCCCGACGA-3'
OPC-08	5'-TGGACCGGTG-3'
OPC-09	5'-CTCACCGTCC-3'
OPC-10	5'-TGTCTGGGTG-3'
OPC-11	5'-AAAGCTGCGG-3'
OPC-12	5'-TGTCATCCCC-3'
OPC-13	5'-AAGCCTCGTC-3'
OPC-14	5'-TGCGTGCTTG-3'
OPC-15	5'-GACGGATCAG-3'
OPC-16	5'-CACACTCCAG-3'
OPC-17	5'-TTCCCCCAG-3'
OPC-18	5'-TGAGTGGGTG-3'
OPC-19	5'-GTTGCCAGCC-3'
OPC-20	5'-ACTTCGCCAC-3'

Table 4. Sequences of RAPD primers used in this study (continued).

Name of primer	Primer sequence (5'–3')
OPE-01	5'–CCCAAGGTCC–3'
OPE-02	5'–GGTGCGGGAA–3'
OPE-03	5'–CCAGATGCAC–3'
OPE-04	5'–GTGACATGCC–3'
OPE-05	5'–TCAGGGAGGT–3'
OPE-06	5'–AAGACCCCTC–3'
OPE-07	5'–AGATGCAGCC–3'
OPE-08	5'–TCACCACGGT–3'
OPE-09	5'–CTTCACCCGA–3'
OPE-10	5'–CACCAGGTGA–3'
OPE-11	5'–GAGTCTCAGG–3'
OPE-12	5'–TTATCGCCCC–3'
OPE-13	5'–CCCGATTTCGG–3'
OPE-14	5'–TGCGGCTGAG–3'
OPE-15	5'–ACGCACAACC–3'
OPE-16	5'–GGTGACTGTG–3'
OPE-17	5'–CTACTGCCGT–3'
OPE-18	5'–GGACTGCAGA–3'
OPE-19	5'–ACGGCGTATG–3'
OPE-20	5'–AACGGTGACC–3'

Table 4. Sequences of RAPD primers used in this study (continued).

Name of primer	Primer sequence (5'-3')
OPK-01	5'- CATTTCGAGCC-3'
OPK-02	5'- GTCTCCGCAA-3'
OPK-03	5'- CCAGCTTAGG-3'
OPK-04	5'- CCGCCCAAAC-3'
OPK-05	5'- TCTGTCGAGG-3'
OPK-06	5'- CACCTTTCCC-3'
OPK-07	5'- AGCGAGCAAG-3'
OPK-08	5'- GAACACTGGG-3'
OPK-09	5'- CCCTACCGAC-3'
OPK-10	5'- GTGCAACGTG-3'
OPK-11	5'- AATGCCCCAG-3'
OPK-12	5'- TGGCCCTCAC-3'
OPK-13	5'- GGTTGTACCC-3'
OPK-14	5'- CCCGCTACAC-3'
OPK-15	5'- CTCCTGCCAA-3'
OPK-16	5'- GAGCGTCGAA-3'
OPK-17	5'- CCCAGCTGTG-3'
OPK-18	5'- CCTAGTCGAG-3'
OPK-19	5'- CACAGGCGGA-3'
OPK-20	5'- GTGTCGCGAG-3'

## CHAPTER IV

### RESULTS

#### 4.1 Micropropagation of *Vanda coerulea*

##### 4.1.1 Seed germination, protocorm formation and plantlet development

Seeds germinated into green protocorms on modified VW agar medium supplemented with 150 ml/l coconut water, 100 g/l banana, 10 g/l sucrose, 0.7 g/l activated charcoal, 8 g/l agar and pH at 5.2 30 d after sowing (Figure 12. A). Protocorms developed into shoots (Figure 12. B) and roots 50 d after sowing (Figure 12. C). The plantlets were transferred into the same medium and plantlets are 2 cm in height 120 d after sowing (Figure 12. D).

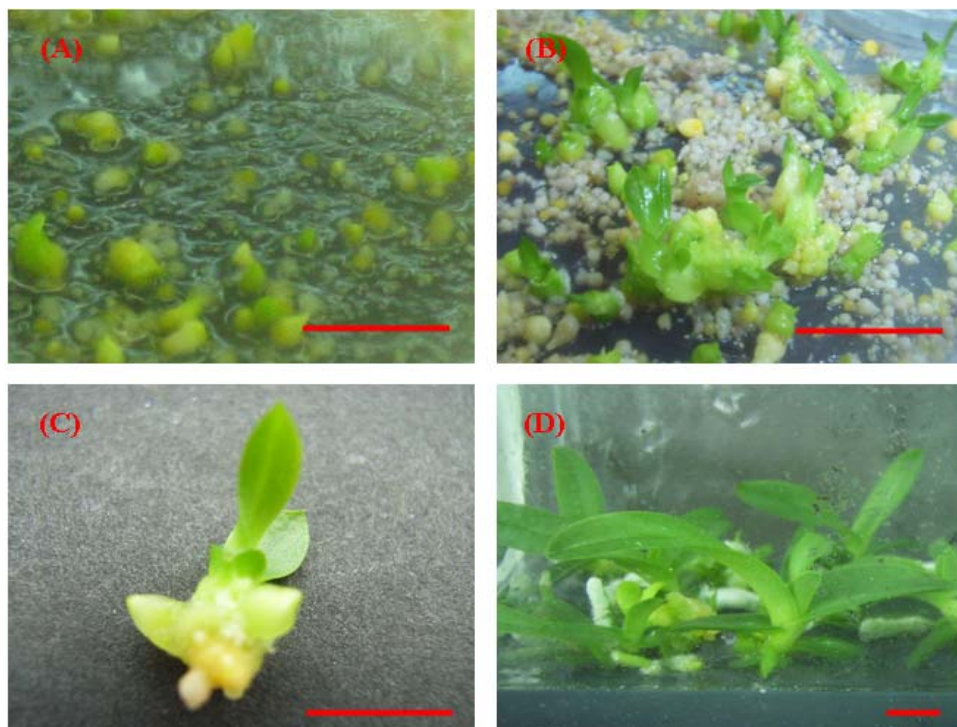


Figure 12. Development of plantlets from seeds of *Vanda coerulea* germinated on modified VW agar medium. (A) Seeds germinated into protocorms 30 d after sowing. (B-C) Protocorms developed into shoots and roots 50 d after sowing. (D) Plantlets gave 1-2 cm long 120 d after sowing. Scale bar: A-D = 0.5 cm.

#### **4.1.2 Effects of media and sucrose concentrations on shoot and root formation**

The media and concentration of sucrose were tested for their effects on shoot and root formation from shoot tips (Table 5). Shoot tips were excised from the plantlets and then cultured on modified VW or MS agar medium or liquid medium supplemented with various sucrose concentrations (0-30 g/l).

The shoot tips can be grown on modified VW or MS agar medium. Significant differences were observed between media type and concentrations of sucrose for survival rate, number of shoots, size of protocorm-like bodies (Table 5), root formation, number of roots and plantlet height (Table 6).

After 3 months of culture, the survival rate of shoot tips cultured on modified VW or MS agar medium was about 50-87% and less than 50%, respectively. The highest number of shoots (2 shoots/explant), shoot produced roots (100%), number of roots (2 roots/shoot) and plantlet height (0.8 cm.) were observed on modified VW agar medium supplemented with 10 g/l sucrose (Table 6). The size of protocorm-like bodies was increased when concentration of sucrose decreased in MS agar medium. Shoot tips grown on modified VW agar medium did not form protocorm-like body at all sucrose concentrations.

Effect of sucrose concentrations was observed on the root formation. Rooting occurred on modified VW agar medium with and without sucrose (Table 6). Whereas, plantlets grew on MS agar medium without sucrose did not produce any roots. Modified VW agar medium supplemented with 150 ml/l coconut water, 100 g/l banana and 0.7 g/l activated charcoal induced root formation. The results showed that the color of MS agar medium changed from white color to brown color after culturing plantlets for 3 months but it was not found in modified VW agar medium. The results showed that activated charcoal adsorbed phenolic compound on modified VW agar medium. After 2 weeks of culture in liquid medium, shoot tip changed from green color to brown color and died when cultured in the liquid medium supplemented with 10-30 g/l sucrose. Protocorm-like body formation was found in the MS liquid medium without sucrose.

Table 5. Effects of media and concentrations of sucrose on shoots and protocorm-like body formation from shoot tips of *Vanda coerulea* after 3 months of culture.

Medium	Sucrose (g/l)	Survival rate (%)	Number of shoots per explant	Size of protocorm-like bodies (cm)
VW	0	56.67 ± 9.20 <sup>b</sup>	1.12 ± 0.12 <sup>b</sup>	0 <sup>e</sup>
	5	50.00 ± 9.28 <sup>bc</sup>	1.00 ± 0.00 <sup>b</sup>	0 <sup>e</sup>
	10	53.33 ± 9.26 <sup>b</sup>	2.00 ± 0.06 <sup>a</sup>	0 <sup>e</sup>
	15	86.67 ± 6.31 <sup>a</sup>	1.00 ± 0.00 <sup>b</sup>	0 <sup>e</sup>
	20	80.00 ± 7.43 <sup>a</sup>	1.29 ± 0.14 <sup>ab</sup>	0 <sup>e</sup>
	30	86.67 ± 6.31 <sup>a</sup>	1.00 ± 0.00 <sup>b</sup>	0 <sup>e</sup>
MS	0	26.67 ± 8.21 <sup>cd</sup>	0.25 ± 0.16 <sup>c</sup>	0.65 ± 0.15 <sup>a</sup>
	5	40.00 ± 9.10 <sup>c</sup>	0.92 ± 0.08 <sup>b</sup>	0.06 ± 0.06 <sup>d</sup>
	10	33.33 ± 8.75 <sup>c</sup>	0.80 ± 0.13 <sup>b</sup>	0.15 ± 0.11 <sup>c</sup>
	15	16.67 ± 6.92 <sup>d</sup>	1.00 ± 0.32 <sup>b</sup>	0.30 ± 0.30 <sup>b</sup>
	20	26.67 ± 8.21 <sup>cd</sup>	1.38 ± 0.26 <sup>ab</sup>	0 <sup>e</sup>
	30	26.67 ± 8.21 <sup>cd</sup>	1.38 ± 0.26 <sup>ab</sup>	0 <sup>e</sup>

Data based on 20 explants per treatment (The experiment was replicated two times).  
Mean ± Standard Error.

Similar letters within columns mean no significant difference at  $P \leq 0.05$  by LSD test.

Table 6. Effects of media and concentrations of sucrose on root formation and growth of plantlet from shoot tips of *Vanda coerulea* after 3 months of culture.

Medium	Sucrose (g/l)	Root formation (%)	Number of roots per explant	Number of leaves per explant	Plantlets height (cm)
VW	0	88.24 ± 8.05 <sup>ab</sup>	2.00 ± 0.27 <sup>a</sup>	3.12 ± 0.17 <sup>a</sup>	0.46 ± 0.05 <sup>b</sup>
	5	73.33 ± 11.8 <sup>b</sup>	1.80 ± 0.35 <sup>ab</sup>	2.67 ± 0.27 <sup>ab</sup>	0.45 ± 0.06 <sup>b</sup>
	10	100.0 ± 0.00 <sup>a</sup>	2.25 ± 0.31 <sup>a</sup>	2.94 ± 0.14 <sup>a</sup>	0.77 ± 0.08 <sup>a</sup>
	15	96.15 ± 3.85 <sup>ab</sup>	2.08 ± 0.21 <sup>a</sup>	2.88 ± 0.17 <sup>a</sup>	0.66 ± 0.06 <sup>ab</sup>
	20	75.00 ± 9.03 <sup>b</sup>	1.79 ± 0.28 <sup>ab</sup>	2.33 ± 0.17 <sup>ab</sup>	0.53 ± 0.06 <sup>ab</sup>
	30	61.54 ± 9.73 <sup>b</sup>	1.19 ± 0.25 <sup>b</sup>	1.88 ± 0.23 <sup>b</sup>	0.41 ± 0.04 <sup>b</sup>
MS	0	0 <sup>d</sup>	0 <sup>c</sup>	0.50 ± 0.33 <sup>c</sup>	0.08 ± 0.05 <sup>d</sup>
	5	83.33 ± 11.2 <sup>ab</sup>	1.08 ± 0.26 <sup>b</sup>	2.58 ± 0.31 <sup>ab</sup>	0.33 ± 0.04 <sup>c</sup>
	10	70.00 ± 15.3 <sup>b</sup>	1.20 ± 0.33 <sup>b</sup>	2.80 ± 0.49 <sup>a</sup>	0.40 ± 0.09 <sup>bc</sup>
	15	40.00 ± 24.5 <sup>c</sup>	0.80 ± 0.58 <sup>b</sup>	2.60 ± 0.93 <sup>ab</sup>	0.46 ± 0.16 <sup>b</sup>
	20	75.00 ± 16.4 <sup>b</sup>	1.75 ± 0.45 <sup>ab</sup>	3.25 ± 0.31 <sup>a</sup>	0.56 ± 0.09 <sup>ab</sup>
	30	75.00 ± 16.4 <sup>b</sup>	1.75 ± 0.45 <sup>ab</sup>	3.25 ± 0.31 <sup>a</sup>	0.56 ± 0.09 <sup>ab</sup>

Data based on 20 explants per treatment (The experiment was replicated two times). Mean ± Standard Error.

Similar letters within columns mean no significant difference at  $P \leq 0.05$  by LSD test.

#### **4.1.3 Effects of BA, NAA and TDZ on shoot and root formation**

Based on the results of previous experiment, shoot tips were cultured on modified VW agar medium supplemented with BA, NAA and TDZ alone or in combination with NAA, 150 ml/l coconut water, 100 g/l banana, 10 g/l sucrose, 0.7 g/l activated charcoal, 8 g/l agar and pH at 5.2. Significant differences were observed between concentration of BA, NAA and TDZ used alone or in combination with NAA for survival rate, number of shoot buds, number of shoots (Table 7), root formation, number of roots, number of leaf and plantlet height (Table 8).

The highest survival rate (98 %) was observed when shoot tips were cultured on modified VW agar medium supplemented with 1 mg/l BA in combination with 0.5 mg/l NAA (Table 7). Modified VW agar medium supplemented with NAA and TDZ alone and TDZ in combination with NAA have induced shoot buds from shoot tips after 3 months of culture but frequency of shoot buds and shoot formation were less than compared to medium supplemented with BA (Figure 13. A). No significant difference of shoot formation when shoot tips were cultured on modified VW medium supplemented with 1 mg/l BA and 2 mg/l TDZ in combination with 0.5 mg/l NAA but the highest shoot buds (8.7 shoot buds/explant) (Figure 13. B) and number of shoots (5.3 shoots/explant) (Figure 13. C) were observed on modified VW agar medium supplemented with 1 mg/l BA.

It was observed that BA concentration above 3 mg/l reduced shoot buds and shoot formation (Table 7). The combination of BA with NAA inhibited shoots formation but increased shoot buds formation compared to BA alone. These results showed that higher TDZ concentration reduced shoot generation. A lower concentration of TDZ (0.1 mg/l) induced high frequency of shoot regeneration (3.8 shoots/explant). The combination of 0.5 mg/l NAA with 2 mg/l TDZ induced higher formation of shoots (4.9 shoots/explant) than TDZ or NAA alone. The highest plantlets (0.5 cm) were observed on modified VW agar medium without plant growth regulator and the medium supplemented with 0.5 mg/l NAA.

TDZ can be used at a lower concentration than BA. TDZ produced shoots at concentration below 1 mg/l. In the rooting, shoots produced roots on modified VW agar medium in all treatments (Table 8). The highest shoot produced roots (62.2%) was observed when shoot tips were cultured on modified VW medium supplemented

with 0.5 mg/l NAA in combination with 2 mg/l TDZ, number of roots/explant was 3 roots/shoot (Figure 13. C-F). These results showed that activated charcoal was added to modified VW agar medium, it induced root formation without adding plant growth regulator (control group).

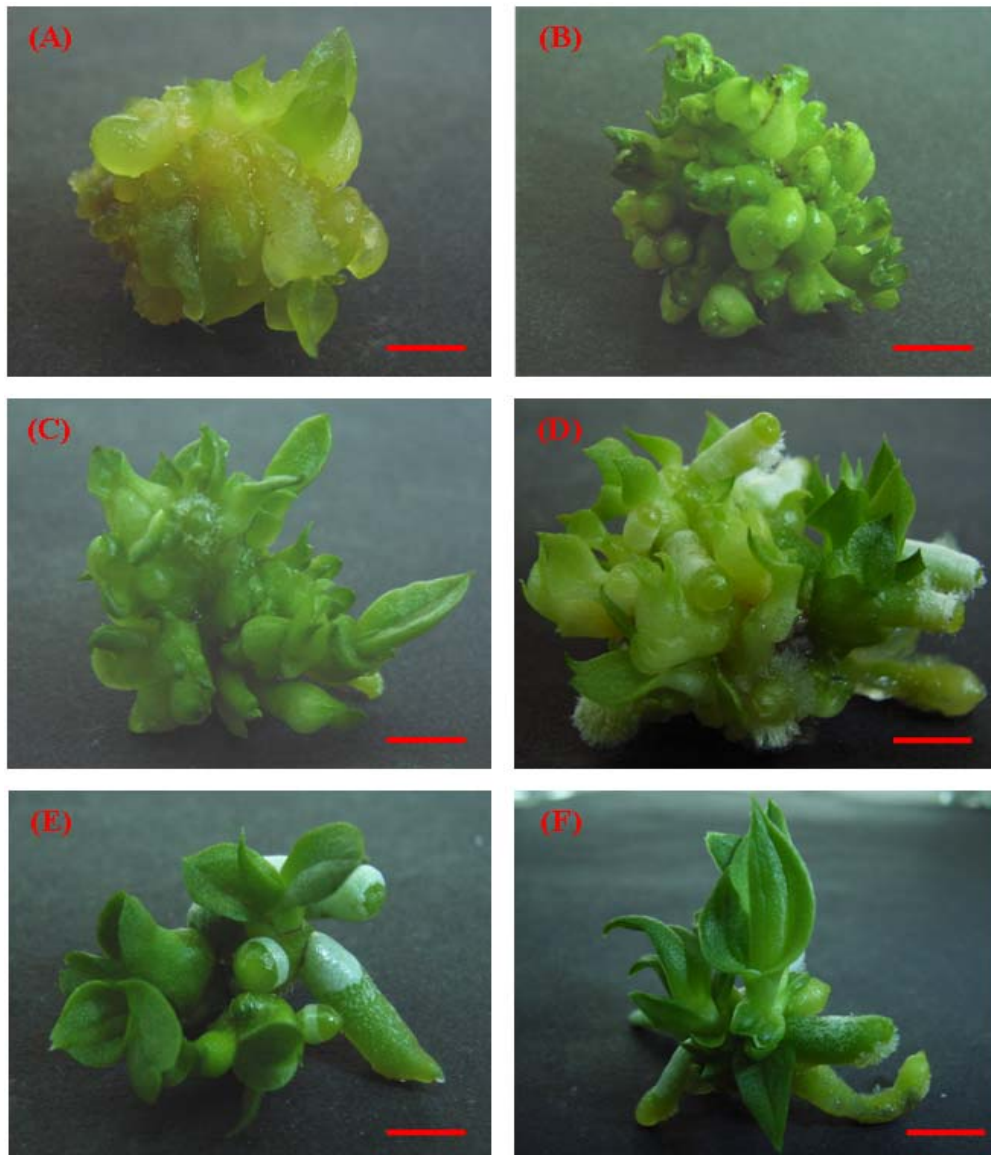


Figure 13. Development of plantlets of *Vanda coerulea* from shoot tips. (A) Shoots formed shoot buds after 4 weeks, (B) 6 weeks and (C) 8 weeks of culture on modified VW agar medium supplemented with 1 mg/l BA. (D) Roots induced from shoot tips were cultured on modified VW agar medium supplemented with 0.5 mg/l NAA in combination with 2 mg/l TDZ after 9 weeks, (E) 10 weeks and (F) 12 weeks of culture. Scale bar: A-F = 1 cm.

Table 7. Effects of BA, NAA and TDZ on survival rate and shoot formation from shoot tips of *Vanda coerulea* on modified VW agar medium after 3 months of culture.

Plant growth regulator (mg/l)			Survival rate (%)	Number of shoot buds per explant	Number of shoots per explant
BA	NAA	TDZ			
-	-	-	75.00 ± 6.93 <sup>b</sup>	2.20 ± 1.21 <sup>bc</sup>	1.97 ± 0.32 <sup>c</sup>
1.0	-	-	75.00 ± 6.93 <sup>b</sup>	8.73 ± 3.89 <sup>a</sup>	5.27 ± 0.95 <sup>a</sup>
3.0	-	-	67.50 ± 7.50 <sup>d</sup>	0.93 ± 0.76 <sup>cd</sup>	3.74 ± 1.08 <sup>ab</sup>
5.0	-	-	90.00 ± 4.80 <sup>ab</sup>	0.67 ± 0.40 <sup>cd</sup>	2.17 ± 0.38 <sup>bc</sup>
-	0.5	-	95.00 ± 3.49 <sup>a</sup>	0.87 ± 0.56 <sup>cd</sup>	2.45 ± 0.31 <sup>b</sup>
-	1.0	-	75.00 ± 6.93 <sup>b</sup>	0.67 ± 0.46 <sup>d</sup>	2.20 ± 0.38 <sup>b</sup>
-	-	0.1	87.50 ± 5.30 <sup>b</sup>	1.94 ± 0.97 <sup>c</sup>	3.80 ± 0.53 <sup>ab</sup>
-	-	1.0	70.00 ± 7.34 <sup>c</sup>	1.36 ± 0.70 <sup>c</sup>	2.32 ± 0.35 <sup>b</sup>
-	-	2.0	70.00 ± 7.34 <sup>c</sup>	0.64 ± 0.32 <sup>d</sup>	2.79 ± 0.56 <sup>b</sup>
-	-	4.0	70.00 ± 7.34 <sup>c</sup>	0.18 ± 0.18 <sup>e</sup>	1.71 ± 0.22 <sup>c</sup>
1.0	0.5	-	97.50 ± 2.50 <sup>a</sup>	0.36 ± 0.25 <sup>e</sup>	2.10 ± 0.30 <sup>bc</sup>
3.0	0.5	-	80.00 ± 6.41 <sup>b</sup>	3.50 ± 1.93 <sup>b</sup>	2.72 ± 0.41 <sup>b</sup>
5.0	0.5	-	75.00 ± 6.93 <sup>b</sup>	3.17 ± 1.86 <sup>b</sup>	2.87 ± 0.28 <sup>b</sup>
1.0	1.0	-	75.00 ± 6.93 <sup>b</sup>	3.47 ± 2.04 <sup>b</sup>	2.37 ± 0.39 <sup>b</sup>
3.0	1.0	-	80.00 ± 6.41 <sup>b</sup>	1.28 ± 0.85 <sup>c</sup>	2.56 ± 0.40 <sup>b</sup>
5.0	1.0	-	77.50 ± 6.69 <sup>b</sup>	3.13 ± 1.57 <sup>b</sup>	2.77 ± 0.36 <sup>b</sup>
-	0.5	0.1	72.50 ± 7.15 <sup>bc</sup>	2.24 ± 1.12 <sup>bc</sup>	2.76 ± 0.37 <sup>b</sup>
-	0.5	1.0	72.50 ± 7.15 <sup>bc</sup>	0.41 ± 0.41 <sup>d</sup>	1.76 ± 0.32 <sup>c</sup>
-	0.5	2.0	92.50 ± 4.22 <sup>ab</sup>	1.57 ± 0.67 <sup>c</sup>	4.89 ± 0.87 <sup>a</sup>
-	0.5	4.0	87.50 ± 5.30 <sup>b</sup>	0.71 ± 0.71 <sup>cd</sup>	1.43 ± 0.17 <sup>c</sup>
-	1.0	0.1	80.00 ± 6.41 <sup>b</sup>	0.28 ± 0.23 <sup>e</sup>	2.47 ± 0.40 <sup>b</sup>
-	1.0	1.0	82.50 ± 6.10 <sup>b</sup>	0.27 ± 0.22 <sup>e</sup>	1.91 ± 0.36 <sup>c</sup>
-	1.0	2.0	67.50 ± 7.50 <sup>d</sup>	1.04 ± 0.66 <sup>c</sup>	2.48 ± 0.48 <sup>b</sup>
-	1.0	4.0	85.00 ± 5.72 <sup>b</sup>	0.59 ± 0.37 <sup>d</sup>	2.29 ± 0.47 <sup>b</sup>

Data based on 20 explants per treatment (The experiment was replicated two times). Mean ± Standard Error.

Similar letters within columns mean no significant difference at  $P \leq 0.05$  by LSD test.

Table 8. Effects of BA, NAA and TDZ on root formation and growth of plantlets from shoot tips of *Vanda coerulea* after 3 months of culture.

Plant growth regulator (mg/l)			Root formation (%)	Number of roots per explant	Number of leaf per explant	Plantlet height (cm)
BA	NAA	TDZ				
-	-	-	20.00 ± 7.43 <sup>d</sup>	0.80 ± 0.30 <sup>c</sup>	3.00 ± 0.12 <sup>a</sup>	0.45 ± 0.02 <sup>ab</sup>
1.0	-	-	46.67 ± 9.26 <sup>b</sup>	1.43 ± 0.34 <sup>b</sup>	2.97 ± 0.11 <sup>a</sup>	0.36 ± 0.02 <sup>c</sup>
3.0	-	-	37.04 ± 9.47 <sup>c</sup>	0.70 ± 0.19 <sup>c</sup>	2.96 ± 0.11 <sup>a</sup>	0.40 ± 0.02 <sup>b</sup>
5.0	-	-	19.44 ± 6.69 <sup>d</sup>	0.78 ± 0.46 <sup>c</sup>	2.92 ± 0.13 <sup>a</sup>	0.44 ± 0.02 <sup>b</sup>
-	0.5	-	21.05 ± 6.70 <sup>d</sup>	1.03 ± 0.40 <sup>c</sup>	2.82 ± 0.13 <sup>ab</sup>	0.49 ± 0.07 <sup>a</sup>
-	1.0	-	13.33 ± 6.31 <sup>de</sup>	0.77 ± 0.54 <sup>c</sup>	2.97 ± 0.12 <sup>a</sup>	0.44 ± 0.02 <sup>b</sup>
-	-	0.1	28.57 ± 7.75 <sup>cd</sup>	0.97 ± 0.36 <sup>c</sup>	2.57 ± 0.18 <sup>b</sup>	0.46 ± 0.03 <sup>ab</sup>
-	-	1.0	21.43 ± 7.90 <sup>d</sup>	0.43 ± 0.19 <sup>cd</sup>	2.89 ± 0.14 <sup>ab</sup>	0.44 ± 0.02 <sup>b</sup>
-	-	2.0	28.57 ± 8.69 <sup>cd</sup>	0.96 ± 0.40 <sup>c</sup>	2.86 ± 0.14 <sup>ab</sup>	0.43 ± 0.02 <sup>b</sup>
-	-	4.0	21.43 ± 7.90 <sup>d</sup>	0.75 ± 0.34 <sup>c</sup>	2.86 ± 0.12 <sup>ab</sup>	0.46 ± 0.02 <sup>ab</sup>
1.0	0.5	-	10.26 ± 4.92 <sup>e</sup>	1.26 ± 0.41 <sup>bc</sup>	2.97 ± 0.10 <sup>a</sup>	0.43 ± 0.01 <sup>b</sup>
3.0	0.5	-	37.50 ± 8.70 <sup>c</sup>	1.00 ± 0.27 <sup>c</sup>	2.78 ± 0.11 <sup>ab</sup>	0.40 ± 0.02 <sup>b</sup>
5.0	0.5	-	50.00 ± 9.29 <sup>ab</sup>	1.47 ± 0.32 <sup>b</sup>	2.63 ± 0.12 <sup>b</sup>	0.40 ± 0.02 <sup>b</sup>
1.0	1.0	-	53.33 ± 9.26 <sup>ab</sup>	1.57 ± 0.40 <sup>ab</sup>	2.80 ± 0.13 <sup>ab</sup>	0.42 ± 0.02 <sup>b</sup>
3.0	1.0	-	28.13 ± 8.08 <sup>cd</sup>	0.75 ± 0.24 <sup>c</sup>	2.59 ± 0.11 <sup>b</sup>	0.43 ± 0.02 <sup>b</sup>
5.0	1.0	-	41.94 ± 9.01 <sup>bc</sup>	1.10 ± 0.30 <sup>bc</sup>	2.65 ± 0.09 <sup>b</sup>	0.41 ± 0.02 <sup>b</sup>
-	0.5	0.1	48.28 ± 9.44 <sup>b</sup>	1.14 ± 0.32 <sup>bc</sup>	2.69 ± 0.09 <sup>b</sup>	0.44 ± 0.02 <sup>b</sup>
-	0.5	1.0	37.93 ± 9.17 <sup>c</sup>	0.90 ± 0.27 <sup>c</sup>	2.76 ± 0.12 <sup>ab</sup>	0.46 ± 0.02 <sup>ab</sup>
-	0.5	2.0	62.16 ± 8.08 <sup>a</sup>	2.49 ± 0.53 <sup>a</sup>	2.54 ± 0.11 <sup>b</sup>	0.38 ± 0.02 <sup>c</sup>
-	0.5	4.0	11.43 ± 5.46 <sup>de</sup>	0.17 ± 0.09 <sup>d</sup>	2.66 ± 0.10 <sup>b</sup>	0.48 ± 0.01 <sup>a</sup>
-	1.0	0.1	21.88 ± 7.43 <sup>d</sup>	0.44 ± 0.21 <sup>cd</sup>	2.50 ± 0.12 <sup>b</sup>	0.43 ± 0.02 <sup>b</sup>
-	1.0	1.0	30.30 ± 8.12 <sup>cd</sup>	0.73 ± 0.22 <sup>c</sup>	2.79 ± 0.10 <sup>ab</sup>	0.46 ± 0.02 <sup>ab</sup>
-	1.0	2.0	44.44 ± 9.75 <sup>bc</sup>	0.85 ± 0.22 <sup>c</sup>	2.67 ± 0.12 <sup>b</sup>	0.44 ± 0.02 <sup>b</sup>
-	1.0	4.0	38.24 ± 8.46 <sup>c</sup>	0.82 ± 0.23 <sup>c</sup>	2.35 ± 0.15 <sup>c</sup>	0.37 ± 0.03 <sup>c</sup>

Data based on 20 explants per treatment (The experiment was replicated two times). Mean ± Standard Error.

Similar letters within columns mean no significant difference at  $P \leq 0.05$  by LSD test.

#### 4.1.4 Plant regeneration and ploidy levels stability

After 3 months of culture, shoots produced roots, they were separated and cultured on modified VW agar medium without plant growth regulator. The size of shoots increased after 1 month of culture and plantlets with well developed roots after 3 months of culture (Figure 14. A-B). The plantlets with 2-3 cm in height, 2-3 leaves and 2-3 roots were removed from the bottle. The rooted shoots were washed in water and transplanted into pots filled with osmunda. Survival rate of plantlets after 3 months of culture in the greenhouse was 100% (Figure 14. C). Similar patterns of ploidy levels were observed by flow cytometry from the leaves of both stock plants and regenerated plants (Figure 15. A-B).



Figure 14. Development of plantlets on modified VW agar medium without plant growth regulator. (A-B) After 2 months of culture. (C) 3-month-old plantlets in the greenhouse. Scale bar: A-C = 1 cm.

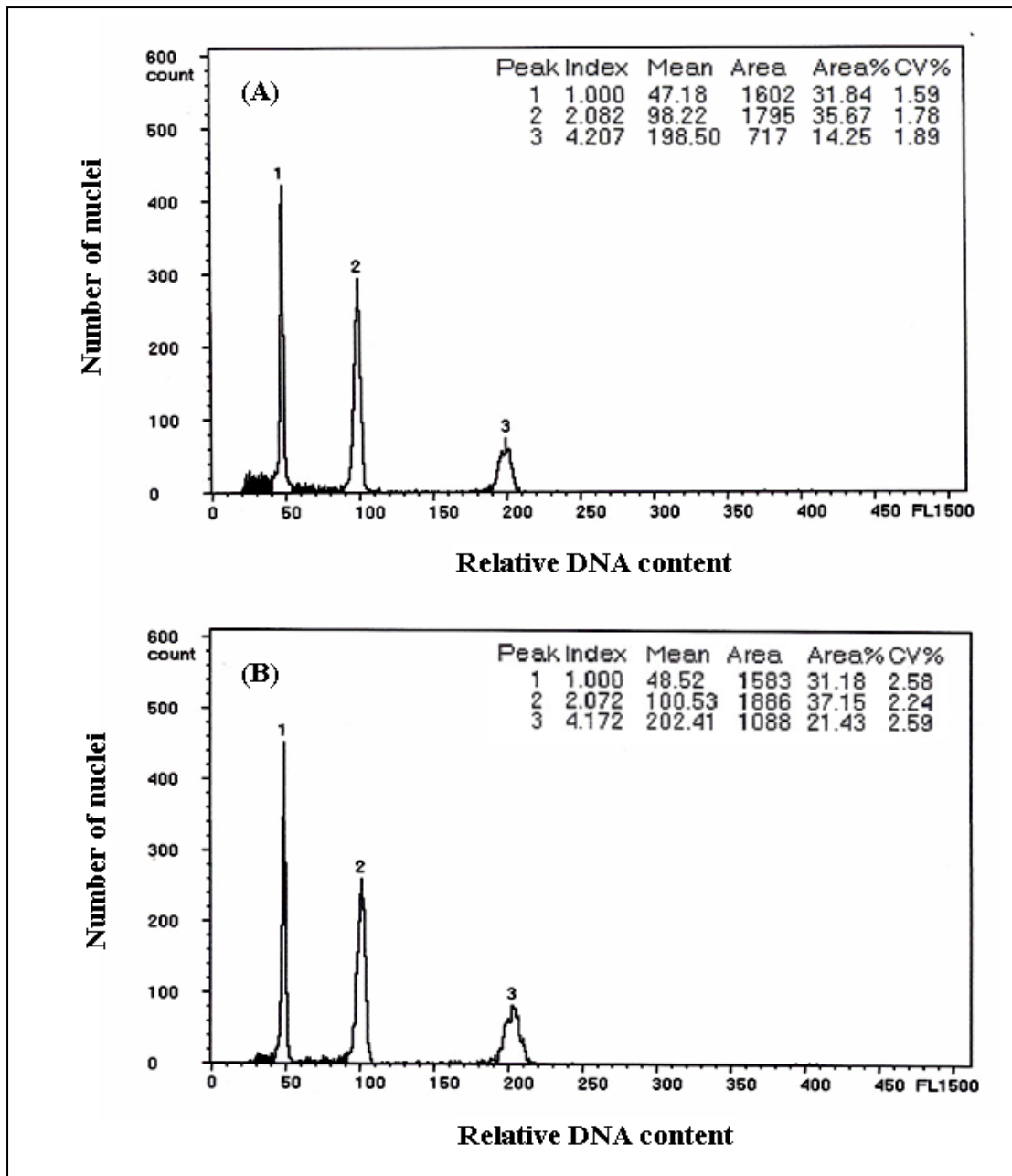


Figure 15. Histogram showing ploidy levels in leaves of *Vanda coerulea* from 6-month-old stock plants (A) and regenerated plants (B).

## **4.2 Cryopreservation of seeds**

### **4.2.1 Cryopreservation of seeds by directly plunged into liquid nitrogen**

The water content of the seeds of *Vanda coerulea* was 56%. When the seeds without any treatments, such as preculture, loading treatment and dehydration with PVS2 solution, subjected into LN, germination rate of cryopreserved seeds was 17%. The germination rate of non-cryopreserved seeds (control) was 97%. The development of protocorms from seeds exposed to LN was not different from the control.

### **4.2.2 Cryopreservation of seeds by vitrification method**

When the seeds without preculture were treated with or without LS for 15 min at  $25 \pm 3^\circ\text{C}$ , then dehydrated with PVS2 solution for 0-140 min with ice at  $0^\circ\text{C}$ , the highest germination rate of cryopreserved seeds was 47% when seeds were treated with LS, and then dehydrated with PVS2 solution for 40 min (Figure 16). The germination rate of cryopreserved seeds without loading treatment was 38% after dehydration with PVS2 solution for 20 min. The germination rate of the non-cryopreserved seeds and cryopreserved seeds decreased when increased with exposure time to PVS2 solution for 60-140 min. The germination rate of cryopreserved seeds with loading treatment was significantly different from the seeds without loading treatment.

After cryopreservation, cryopreserved seeds (Figure 17. A) developed into protocorms (Figure 17. B). Protocorms developed into shoots and roots after 3 months (Figure 17. C). Normal plantlets were observed after 9 months of culture on modified VW agar medium (Figure 17. E). Some protocorms developed into multiple shoots (8 shoots/protocorm) (This data were calculated from 30 protocorms) (Figure 17. D). After 11 months of culture, normal plantlets were transferred to the greenhouse (Figure 17. F).

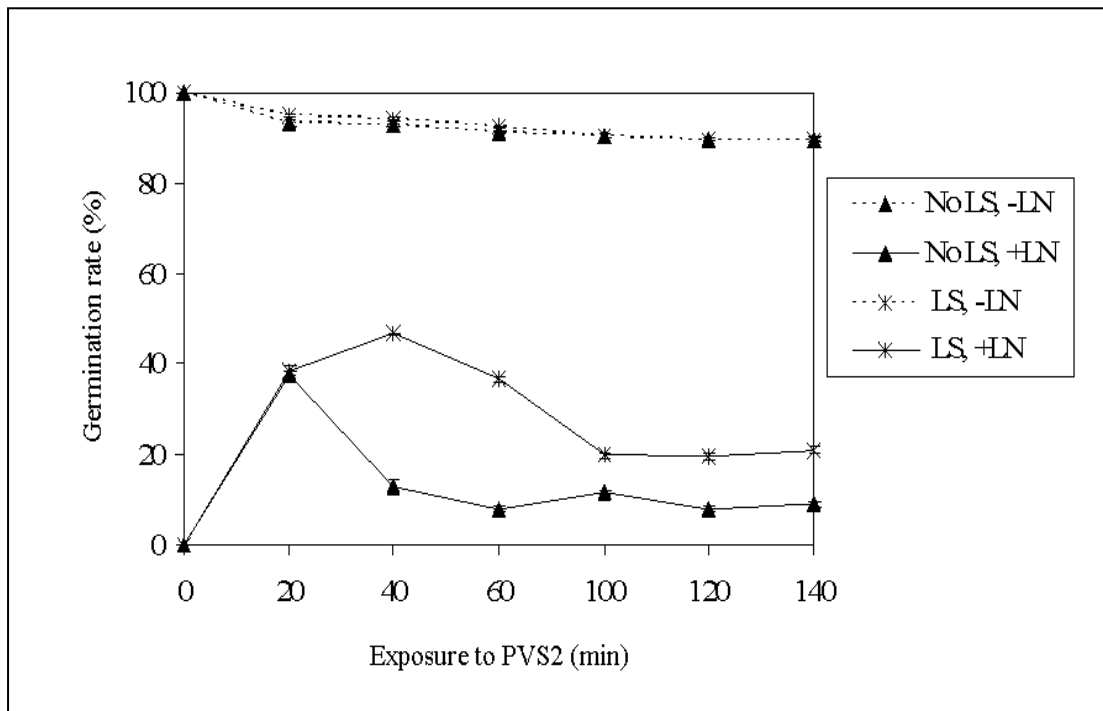


Figure 16. Effects of loading solution and dehydration with PVS2 solution on germination rate of non-cryopreserved seeds (-LN) and cryopreserved seeds (+LN) of *Vanda coerulea* after cryopreservation by vitrification method. Bars represent standard errors.

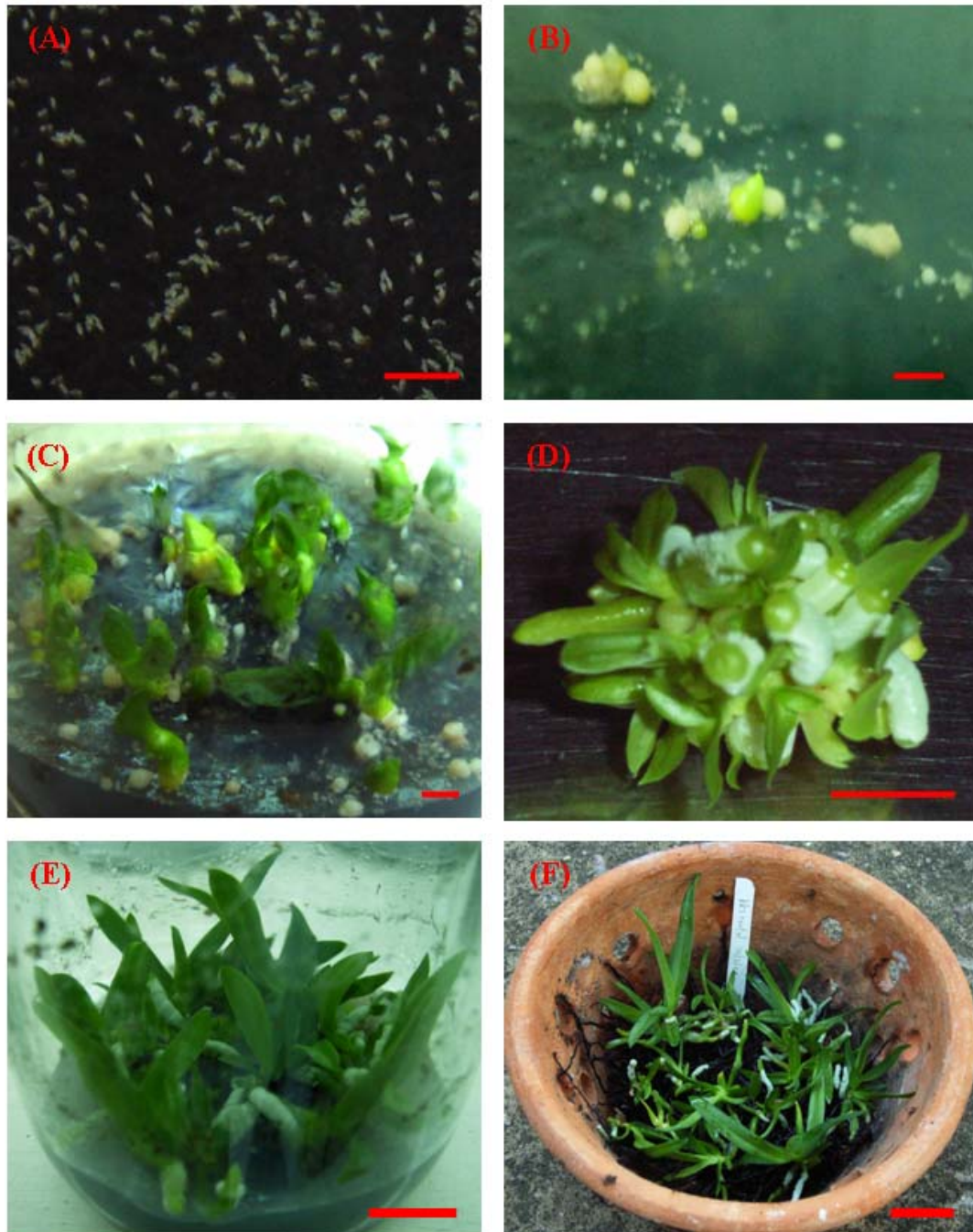


Figure 17. Germination and development of seeds of *Vanda coerulea* after cryopreservation by vitrification method. (A) Seeds of *Vanda coerulea*. (B) 45-day-old protocorms. (C) 3-month-old plantlets. (D) 7-month-old multiple shoots from one protocorm. (E) 9-month-old plantlets. (F) Plantlets derived from cryopreserved seeds after 11 months of culture on modified VW agar medium. Scale bar: A-C = 1 mm, D = 0.5 cm, E-F = 1 cm.

### 4.2.3 Cryopreservation of seeds by encapsulation-dehydration method

When encapsulated seeds were precultured in the modified VW liquid medium supplemented with 0.5 and 0.7 M sucrose for 18 h on a shaker (110 rpm) at  $25 \pm 3^\circ\text{C}$ , the water content of precultured seeds dropped from 81% to 25% and 77% to 22% after 6 h dehydration for 0.5 and 0.7 M sucrose in the preculture medium, respectively (Figure 18).

Germination rate was calculated on the basis of the protocorms or plantlets from encapsulated seed (only one protocorm or plantlet/bead). Germination rate of cryopreserved seeds was not significantly different between precultured in 0.5 and 0.7 M sucrose for 18 h. Germination of cryopreserved seeds was detected when water content of the preculture seeds reached 53% and 68% after 3 and 2 h for 0.5 and 0.7 M sucrose in the preculture medium, respectively (Figure 18). The highest germination rate of cryopreserved seeds developed into protocorms was 100% at 25% and 22% water content for 0.5 and 0.7 M sucrose in the preculture medium after 6 h dehydration, respectively (Figure 19).

One bead contained about 11 seeds (This data was calculated from 50 beads). In this result showed that cryopreserved seeds in one bead developed into protocorms were about 5.5 protocorms. Cryopreserved seeds by encapsulation-dehydration gave higher than those of the seeds by vitrification method because germination rate of cryopreserved seeds by encapsulation-dehydration was calculated on the basis of the protocorms or plantlets from encapsulated bead (only one protocorm or plantlet/bead).

Cryopreservation of seeds by encapsulation-dehydration method did not affect the germination of seeds. Cryopreserved seeds germinated like non-cryopreserved seeds. At 1 month after thawing, protocorms (yellow and green spots) were observed on cryopreserved seeds (Figure 20. A-B) and developed into normal plantlets after 8 months of culture on modified VW agar medium (Figure 20. C).

A protocorm from cryopreserved seed developed into only one shoot (Figure 20. D) and multiple shoots (Figure 20. E). After 11 months of culture, normal plantlets from non-cryopreserved and cryopreserved seeds were transferred to the greenhouse (Figure 20. F). There was no difference in morphology of cryopreserved and non-cryopreserved plantlets.

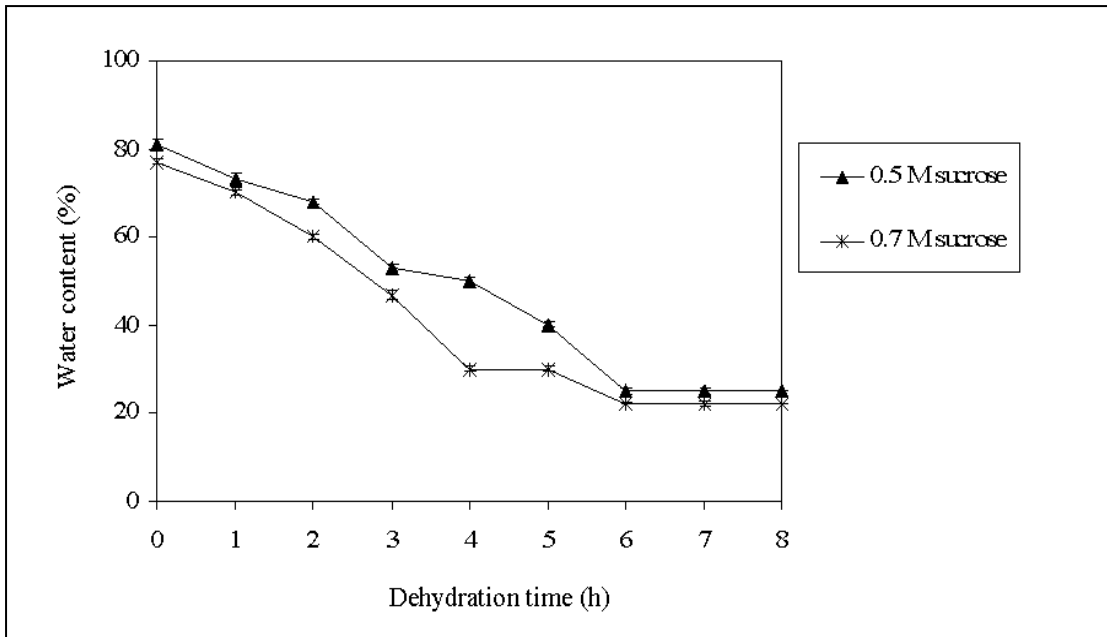


Figure 18. Change in water content of encapsulated seeds of *Vanda coerulea* precultured in modified VW liquid medium supplemented with 0.5 and 0.7 M sucrose for 18 h, then dehydration in the laminar air-flow cabinet for 0-8 h. Bars represent standard errors.

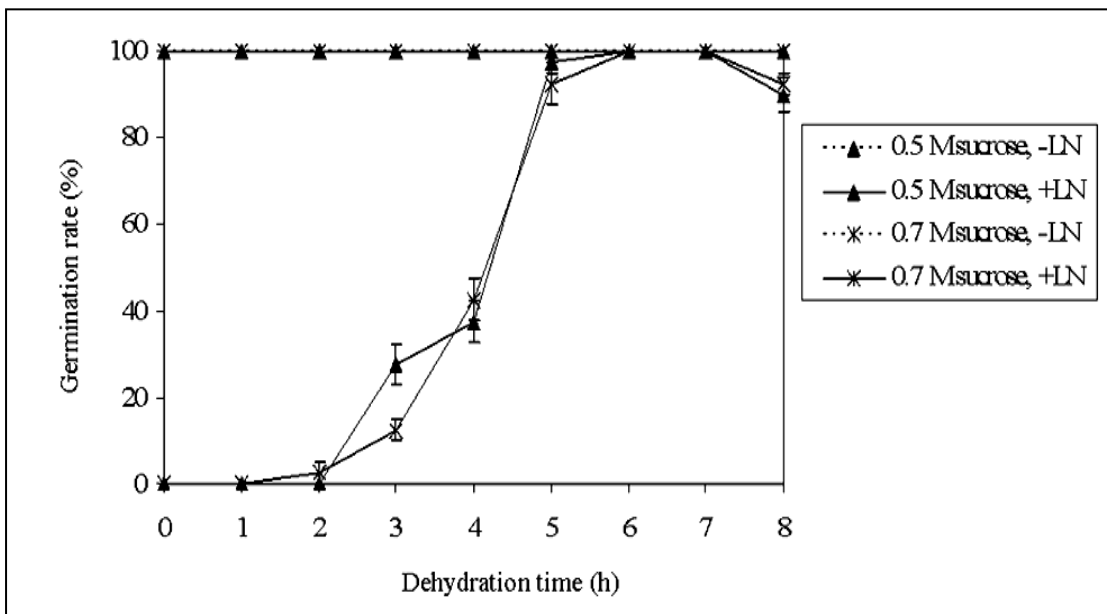


Figure 19. Effect of dehydration in the laminar air-flow cabinet on germination rate of non-cryopreserved seeds (-LN) and cryopreserved seeds (+LN) of *Vanda coerulea* after cryopreservation by encapsulation-dehydration method. Bars represent standard errors.

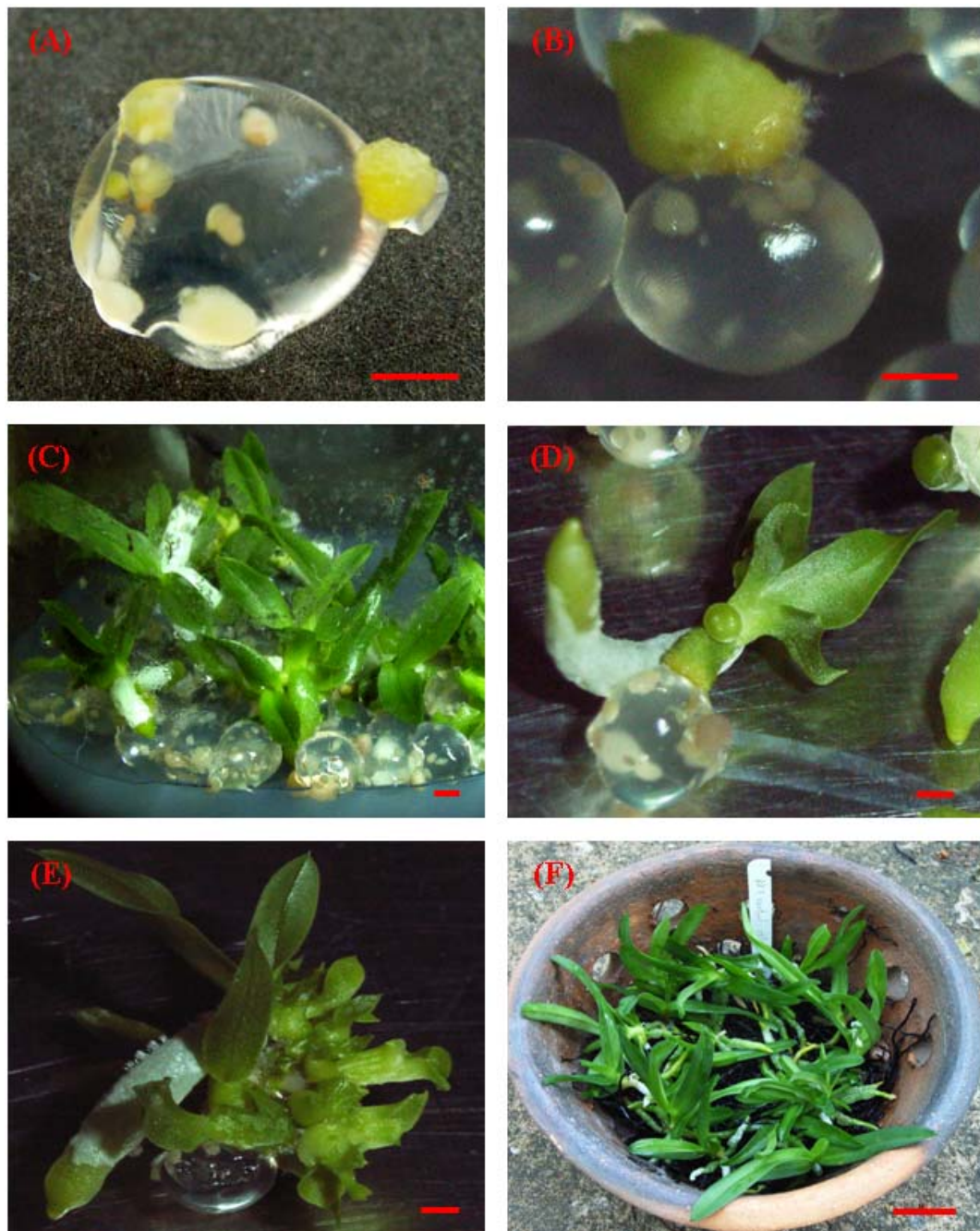


Figure 20. Germination and development of seeds of *Vanda coerulea* after cryopreservation by encapsulation-dehydration method. (A) 1-month-old protocorms. (B) 2-month-old protocorms. (C) 8-month-old plantlets. (D) A 9-month-old plantlet from a cryopreserved seed. (E) 9-month-old multiple shoots from one protocorm. (F) 11-month-old plantlets were transferred to the greenhouse. Scale bar: A-E = 1 mm, F = 1 cm.

#### 4.2.4 Cryopreservation of seeds by encapsulation-vitrification method

With encapsulation-vitrification method, the effect of dehydration time with PVS2 solution at 0°C on germination rate was investigated. Each bead contained 11 seeds. Germination rate was calculated on the basis of the protocorms or plantlets from encapsulated seed (only one protocorm or plantlet/bead).

The germination rate of cryopreserved seeds into protocorms increased after dehydration with PVS2 solution for 30 min (Figure 21). The highest germination rate of cryopreserved seeds into protocorms was 83% and 75% for 0.5 and 0.7 M sucrose in the preculture medium which dehydrated with PVS2 solution for 150 min, respectively and then decreased. Germination rate of cryopreserved seeds was not significantly different between precultured in 0.5 and 0.7 M sucrose for 18 h. After non-cryopreserved seeds were dehydrated with PVS2 solution for 120 min, germination rate was decreased (Figure 21). Germination of cryopreserved seeds by encapsulation-vitrification method was not different from those of cryopreserved seeds by encapsulation-dehydration method.

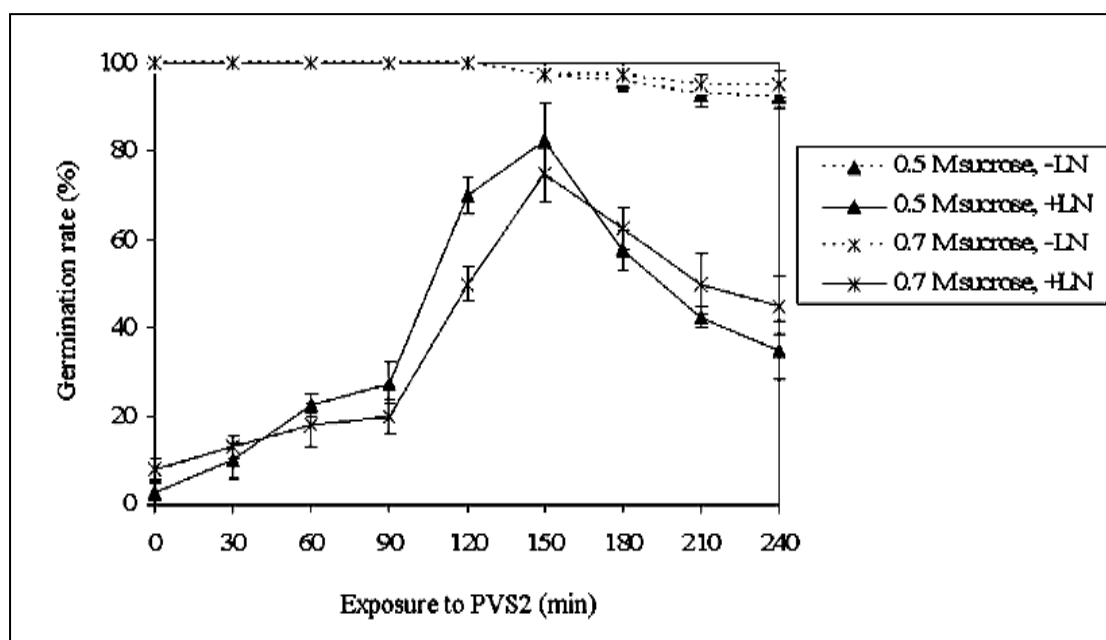


Figure 21. Effect of dehydration with PVS2 solution on germination rate of non-cryopreserved seeds (-LN) and cryopreserved seeds (+LN) of *Vanda coerulea* after cryopreservation by encapsulation-vitrification method. Bars represent standard errors.

Nuclei were isolated from leaves of *Vanda coerulea* from 11-month-old non-cryopreserved plantlets and cryopreserved plantlets which showed similar patterns of ploidy levels observed by flow cytometry (Figure 22. A-B).

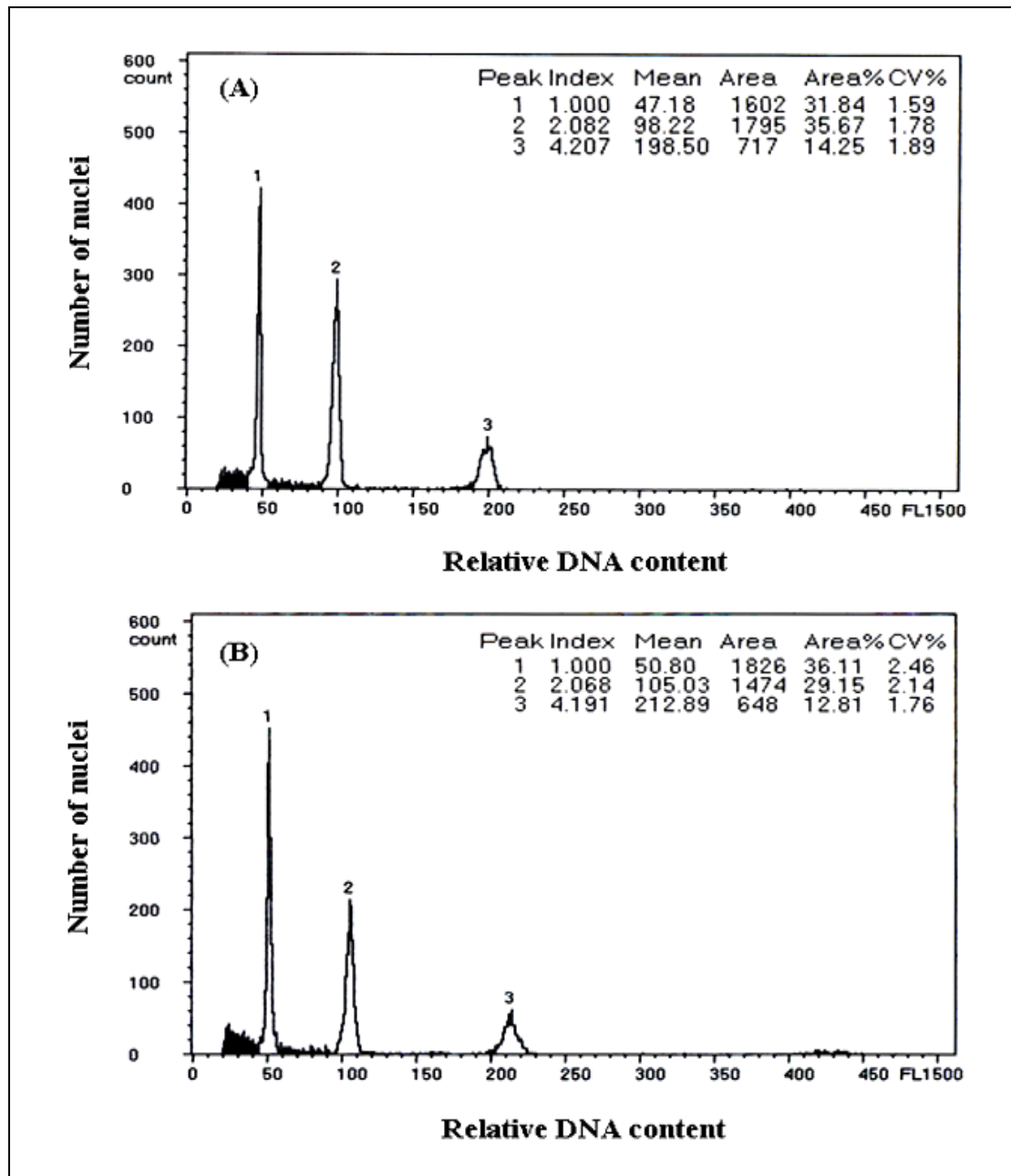


Figure 22. Histogram showing ploidy levels in leaves of *Vanda coerulea* from 11-month-old non-cryopreserved plantlets (A) and cryopreserved plantlets (B) after cryopreservation.

### 4.3 Cryopreservation of protocorms by encapsulation-dehydration method

Cryopreservation of protocorms of *Vanda coerulea* was successfully achieved by encapsulation-dehydration in combination with loading solution. During the encapsulation process, protocorms were osmoprotected with a mixture of 2 M glycerol plus 0.4 M sucrose (LS), then precultured in 0.7 M sucrose for 20 h prior to sterile air-flow dehydration.

Regrowth rate following encapsulation-dehydration is dependent on the amount of water presented in the cells. The water within encapsulated protocorms was removed by both osmotic dehydration and sterile air-flow. If the cells are not sufficiently dehydrated, intracellular ice will be formed. The duration of dehydration was an important factor for protocorm forming plantlets after cryopreservation. The water content of encapsulated protocorms of *Vanda coerulea* was 88.6% (Figure 23). After preculture in 0.7 M sucrose on a shaker (110 rpm) at  $25 \pm 3^\circ\text{C}$  for 20 h, the initial water content of precultured beads was 77% on fresh weight basis. During dehydration, the water content changed. The longer the duration of the dehydration, the lower the water content (Figure 23). The water content of precultured beads rapidly decreased to 51% within the first 6 h dehydration, then decreased slowly to 25% after 10 h dehydration (Figure 23) and the white color of precultured beads changed to brown color (Figure 25. A).

Protocorms forming plantlets before and after freezing are indicated in Figure 24. The reduction in water content affected the regrowth rate of protocorms. Regrowth of both non-cryopreserved and cryopreserved protocorms changed with the water content of the precultured beads during dehydration for 0-10 h (Figure 23). With the decrease in water content, regrowth before freezing decreased about 61% with the water contents ranging from 77% to 48% at 5 h dehydration then decreased to 43% at 25% water content at 10 h dehydration (Figure 23).

Protocorms forming plantlets of cryopreserved beads (1.3%) were detected (Figure 24) when water content of the preculture beads reached 56% after 3 h dehydration (Figure 23). The highest regrowth rate of cryopreserved protocorms was 40% at 35% water content after 8 h dehydration and then decreased to 34% at 25% water content at 10 h dehydration (Figure 24). Protocorms forming plantlets were not significantly different between non-cryopreserved and cryopreserved protocorms at 8 h dehydration.

After 20 d of culture, green protocorms were observed in crypreserved beads (Figure 25. B). These developed a shoot after 3 months (Figure 25. C). These shoots were removed from precultured beads and then planted on fresh modified VW agar medium.

After 4 months of culture, plantlets developed shoots and roots. They were selected and then washed in water to remove the culture medium and were transplanted into pots filled with osmunda in the greenhouse. After 0, 5 and 15 months of culture in the greenhouse, there were no significant difference in survival rate, plantlet height, root length, number of roots and number of leaves between plantlets from non-cryopreserved and cryopreserved protocorms (Table 9). No morphological variation between non-cryopreserved and cryopreserved plantlets was detected (Figure 25. D-F).

Flow cytometry analysis was applied to study the ploidy stability of *Vanda coerulea* 11-month-old from non-cryopreserved protocorms and cryopreserved protocorms. Nuclei were isolated from leaves of non-cryopreserved plantlets and cryopreserved plantlets which showed similar patterns of ploidy levels (Figure 26. A-B).

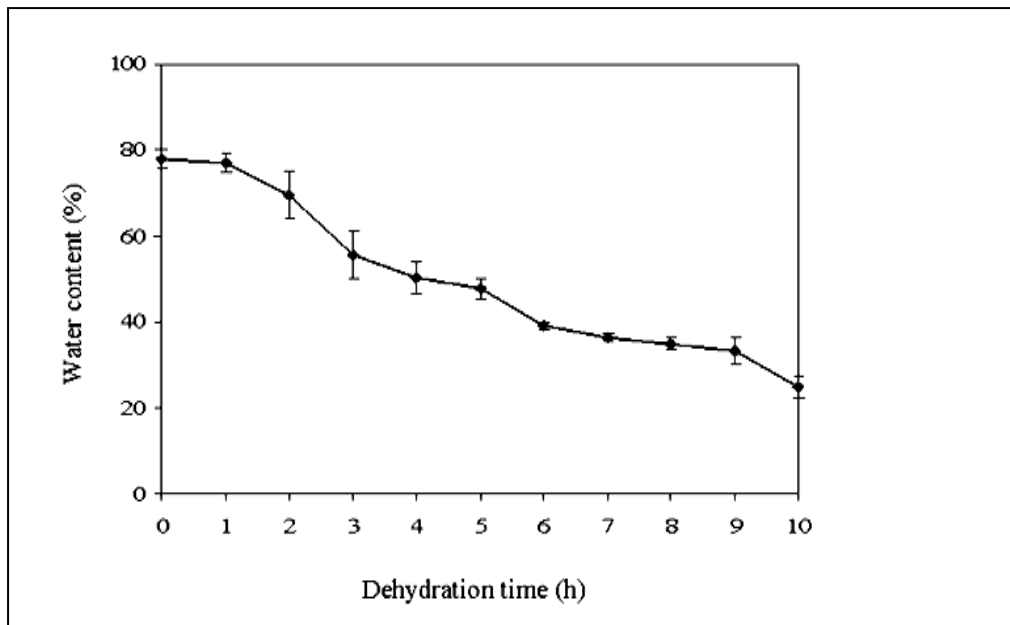


Figure 23. Effect of dehydration time on the water content of encapsulated beads of *Vanda coerulea* protocorms precultured in modified VW liquid medium supplemented with 0.7 M sucrose for 20 h, then dehydration in the laminar air-flow cabinet for 0-10 h based on fresh weight. Bars represent standard errors.

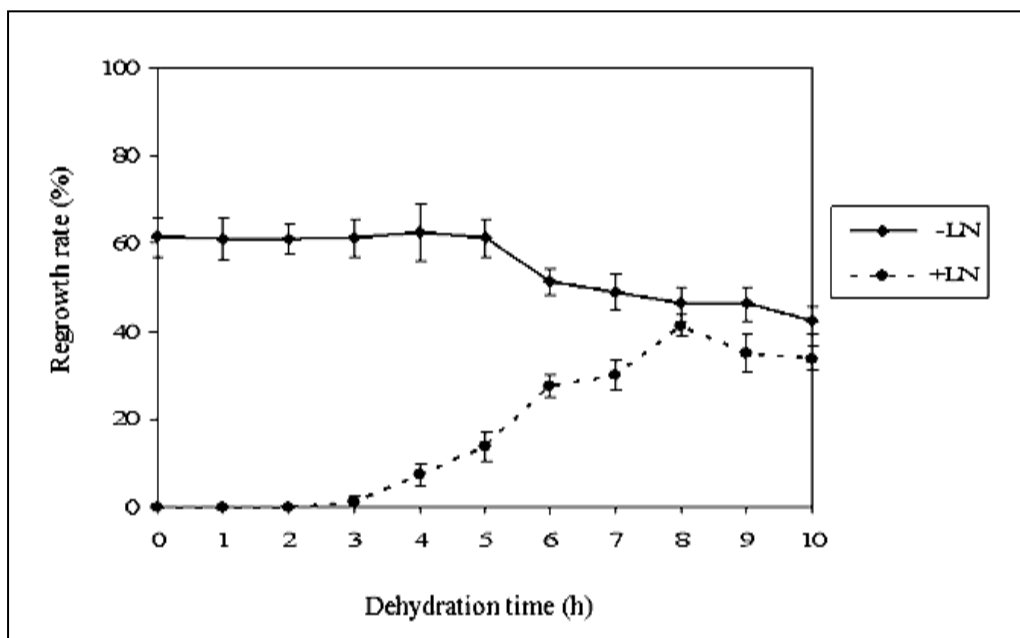


Figure 24. Effect of dehydration time in the laminar air-flow cabinet on regrowth rate of non-cryopreserved protocorms (-LN) and cryopreserved protocorms (+LN) of *Vanda coerulea* after cryopreservation by encapsulation-dehydration combination with loading solution. Bars represent standard errors.

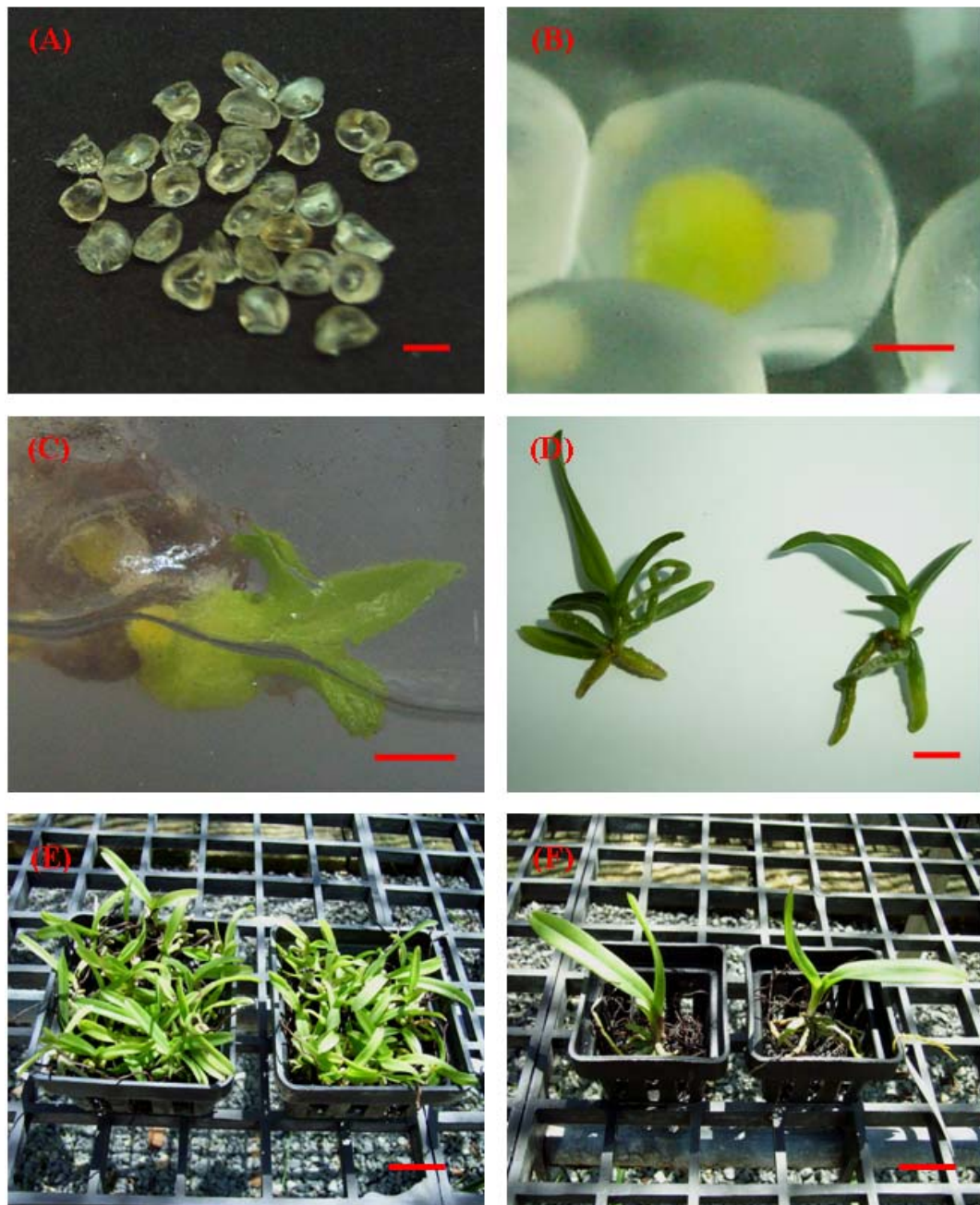


Figure 25. Regrowth of *Vanda coerulea* protocorms after cryopreservation by encapsulation-dehydration in combination with loading solution. (A) Precultured beads after dehydration with sterile air-flow dehydration for 10 h. (B) Cryopreserved protocorms after 20 d. (C) 3 months of culture on modified VW agar medium. (D) Plantlets after 8 months of culture on modified VW agar medium (left: non-cryopreserved plantlets, right: cryopreserved plantlet). (E) Plantlets derived from cryopreserved protocorms after 5 months and (F) 15 months of culture in the greenhouse. Scale bar: A-C = 1 mm, D = 0.5 cm, E-F = 1 cm.

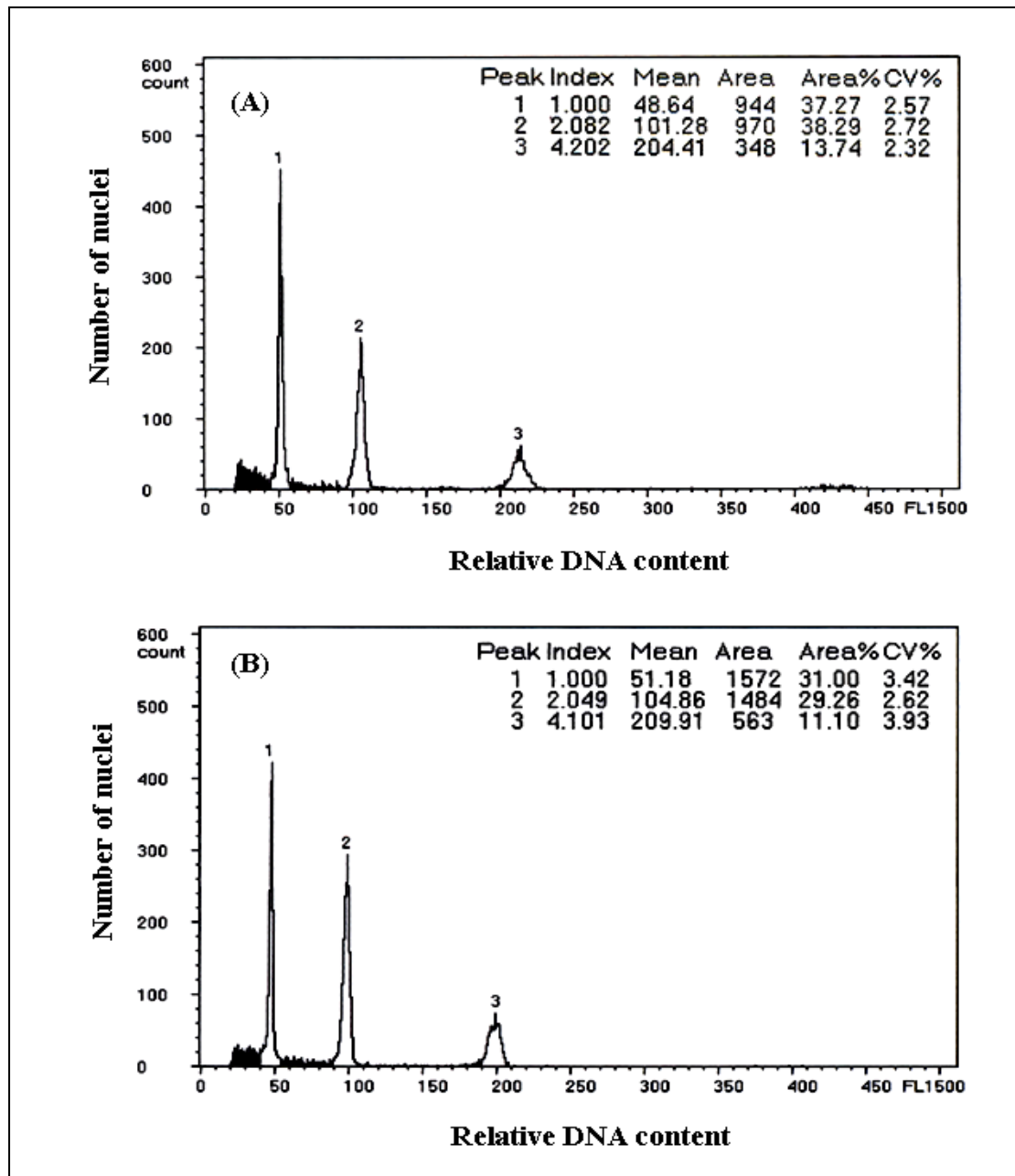


Figure 26. Histogram showing ploidy levels in leaves of *Vanda coerulea* from 11-month-old non-cryopreserved plantlets (A) and cryopreserved plantlets (B) after cryopreservation of protocorms by encapsulation-dehydration in combination with loading solution.

Table 9. Development of non-cryopreserved plantlets (-LN) and cryopreserved plantlets (+LN) of *Vanda coerulea* protocorms in the greenhouse.

Time (month)		Survival rate (%)	Plantlet height (cm)	Root length (cm)	Number of roots/plantlets	Number of leaves/plantlets	Leaf color
0	-LN	100 ± 0.0	2.5 ± 0.2	1.3 ± 0.1	3.6 ± 0.2	5.1 ± 0.3	Dark green
	+LN	100 ± 0.0	2.7 ± 0.1	1.4 ± 0.1	3.7 ± 0.4	4.8 ± 0.3	Dark green
5	-LN	100 ± 0.0	6.9 ± 0.4	2.1 ± 0.1	6.5 ± 0.4	4.2 ± 0.2	Green
	+LN	100 ± 0.0	5.7 ± 0.4	1.7 ± 0.2	6.8 ± 0.4	4.1 ± 0.2	Green
15	-LN	66.7 ± 14	8.5 ± 0.9	2.4 ± 0.2	9.3 ± 0.6	4.8 ± 0.3	Green
	+LN	75.0 ± 13	7.4 ± 0.8	3.0 ± 0.4	8.9 ± 0.7	4.3 ± 0.4	Green

Data based on 20 plantlets per treatment (The experiments were replicated two times).

Mean ± Standard Error.

Similar letters within columns mean no significant difference at  $P \leq 0.05$  by LSD test.

#### 4.4 Cryopreservation of shoot tips

##### 4.4.1 Cryopreservation of shoot tips by slow freezing method

Shoot tips of *Vanda coerulea* were precultured with 0.3 M sucrose for 1 d, treated with LS for 15 min at 25°C and then dehydrated with PVS2 solution with ice for 0, 20, 40 and 60 min, then removed PVS2 solution from cryotubes followed by a slow freezing in a cryofreezing-container during storage at -80°C for 6 h, then directly plunged into LN. Results showed that survival rate of non-cryopreserved and cryopreserved shoot tips without dehydration with PVS2 solution was about 60% and 20%, respectively (Table 10). No survival rate of cryopreserved shoot tip was observed when dehydrated with PVS2 solution. The results showed that PVS2 solution has effect on survival of shoot tips.

Table 10. Effect of dehydration with PVS2 solution on survival rate of non-cryopreserved shoot tips (-LN) and cryopreserved shoot tips (+LN) of *Vanda coerulea* after cryopreservation by slow freezing method.

Exposure time to PVS2 (min)	Survival rate (%)	
	-LN	+LN
0	60 ± 23.3 <sup>a</sup>	20 ± 20 <sup>a</sup>
20	40 ± 24.5 <sup>b</sup>	0 <sup>b</sup>
40	40 ± 24.5 <sup>b</sup>	0 <sup>b</sup>
60	20 ± 20.0 <sup>c</sup>	0 <sup>b</sup>

Data based on 20 explants per treatment (The experiment was replicated two times).  
Mean ± Standard Error.

Similar letters within columns mean no significant difference at  $P \leq 0.05$  by LSD test.

After 2 months of culture on ND solid medium supplemented with 1 mg/l BA and 20 g/l sucrose, the growth characteristic of cryopreserved shoot tips were compared with non-cryopreserved shoot tips. The results showed that non-cryopreserved shoot tips developed into plantlets but cryopreserved shoot tips developed into callus (Figure 27). Callus did not develop from shoot tip but it developed from root bud (Figure 27. A-B). Callus from cryopreserved shoot tips were transferred into ND liquid medium supplemented with 1 mg/l BA in combination with 0.5 mg/l NAA and 30 g/l maltose, pH at 5.4 for protocorm-like body formation (Figure 27. C-D).

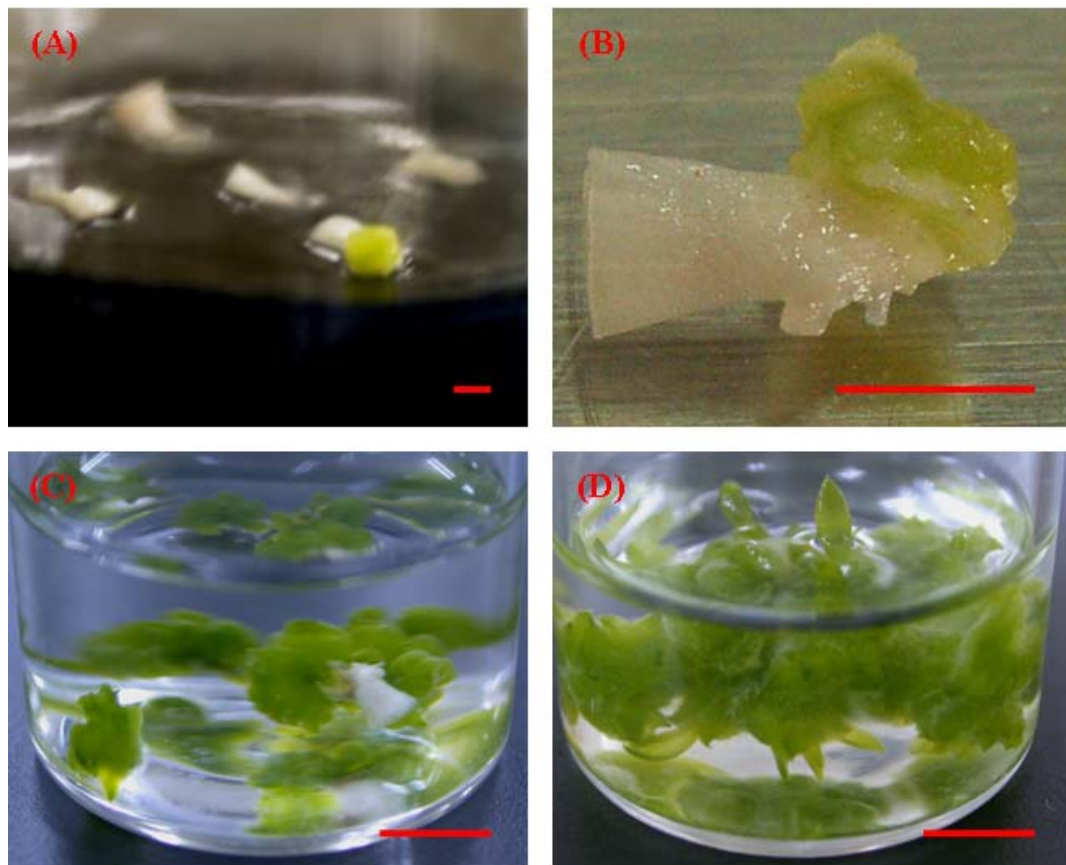


Figure 27. Development of cryopreserved shoot tips after cryopreservation by slow freezing method. (A-B) Callus formation on cryopreserved shoot tip after 2 months of culture. (C) Protocorm-like body formation from callus in ND liquid medium supplemented with 1 mg/l BA in combination with 0.5 mg/l NAA and 30 g/l maltose after 1 month and (D) 3 months of culture. Scale bar: A-D = 1 mm, E-F = 1 cm.

#### **4.4.2 Effect of maltose concentrations on protocorm-like body and shoot formation from cryopreserved shoot tips**

For protocorm-like body and shoot formation, concentration of maltose was tested the effect on shoot formation from protocorm-like bodies.

Protocorm-like bodies from cryopreserved shoot tips were cultured in ND liquid medium supplemented with 0, 10, 20, 30 and 40 g/l maltose, pH at 5.4 on a shaker (110 rpm) for shoot formation. After 3 months of culture, the fresh weight of protocorm-like bodies increased as the concentrations of maltose increased (Figure 28). Protocorm-like body proliferation was observed in ND liquid medium with and without maltose. The formation and proliferation of protocorm-like bodies were the most suitable in ND liquid medium supplemented with 20 g/l maltose (7.36 g fresh weight) (Table 11).

Protocorm-like body proliferation and shoot formation were observed when cultured in ND liquid medium supplemented with 10, 20, 30 and 40 g/l maltose. Protocorm-like bodies can grow in ND liquid medium without maltose and any carbon source but they cannot form shoots (Figure 28. A). Protocorm-like bodies were cultured in ND liquid medium supplemented with 20, 30 and 40 g/l maltose gave yellow color (Figure 28. C-E) but in ND liquid medium supplemented with 10 g/l maltose gave green color (Figure 28. B).

Formation of green shoots was the most suitable in ND liquid medium supplemented with 10 g/l maltose. Shoot formation from protocorm-like bodies cultured in ND liquid medium supplemented with 30 and 40 g/l maltose gave browning of shoots (Figure 28. C-D).

Table 11. Effect of maltose concentrations on protocorm-like body formation and proliferation and shoot formation from cryopreserved shoot tips in ND liquid medium supplemented with 1 mg/l BA in combination with 0.5 mg/l NAA after 3 months of culture.

Maltose concentration (g/l)	Fresh weight of protocorm-like bodies (g)	Morphogenesis
0	1.31 ± 0.13 <sup>c</sup>	protocorm-like body formation
10	4.46 ± 0.78 <sup>b</sup>	protocorm-like body and green shoot formation
20	7.36 ± 0.82 <sup>a</sup>	protocorm-like body formation
30	7.12 ± 1.10 <sup>a</sup>	protocorm-like body and brown shoot formation
40	6.76 ± 1.10 <sup>a</sup>	protocorm-like body and brown shoot formation

Data based on 50 protocorm-like bodies per treatment after 3 months of culture in ND liquid medium supplemented with 1 mg/l BA in combination with 0.5 mg/l NAA.

(The experiment was replicated two times).

Mean ± Standard Error.

Similar letters within columns mean no significant difference at  $P \leq 0.05$  by LSD test.

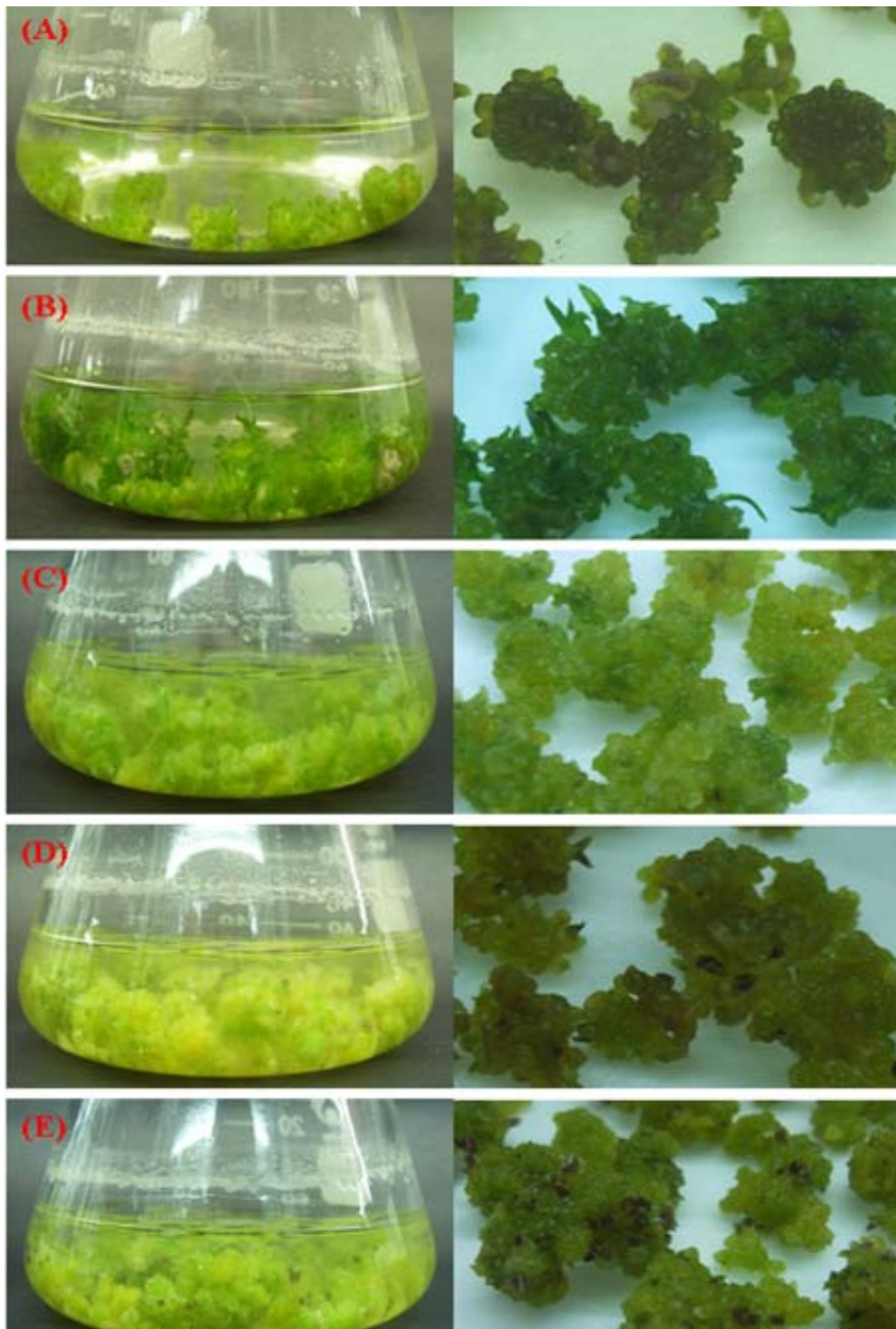


Figure 28. Protocorm-like body formation and proliferation and shoot formation from cryopreserved shoot tips in ND liquid medium supplemented with 1 mg/l BA in combination with 0.5 mg/l NAA and various maltose concentrations. (A) 0, (B) 10, (C) 20, (D) 30 and (E) 40 g/l maltose, pH at 5.4 on a shaker (110 rpm) after 3 months of culture. Scale bar: A- F = 1 cm.

#### 4.4.3 Plant regeneration

Shoot formation from protocorm-like bodies cultured in ND liquid medium supplemented with 10 g/l maltose were transferred to modified VW agar medium and ND solid medium supplemented with 1 mg/l BA. After 1 month of culture, green shoots did not survive on ND solid medium (Figure 29. A), they became brown color and then died. Shoots formed multiple shoots and roots on modified VW agar medium (Figure 29. B). No morphological variation between plantlets from non-cryopreserved and cryopreserved plantlets was detected (Figure 29. C).

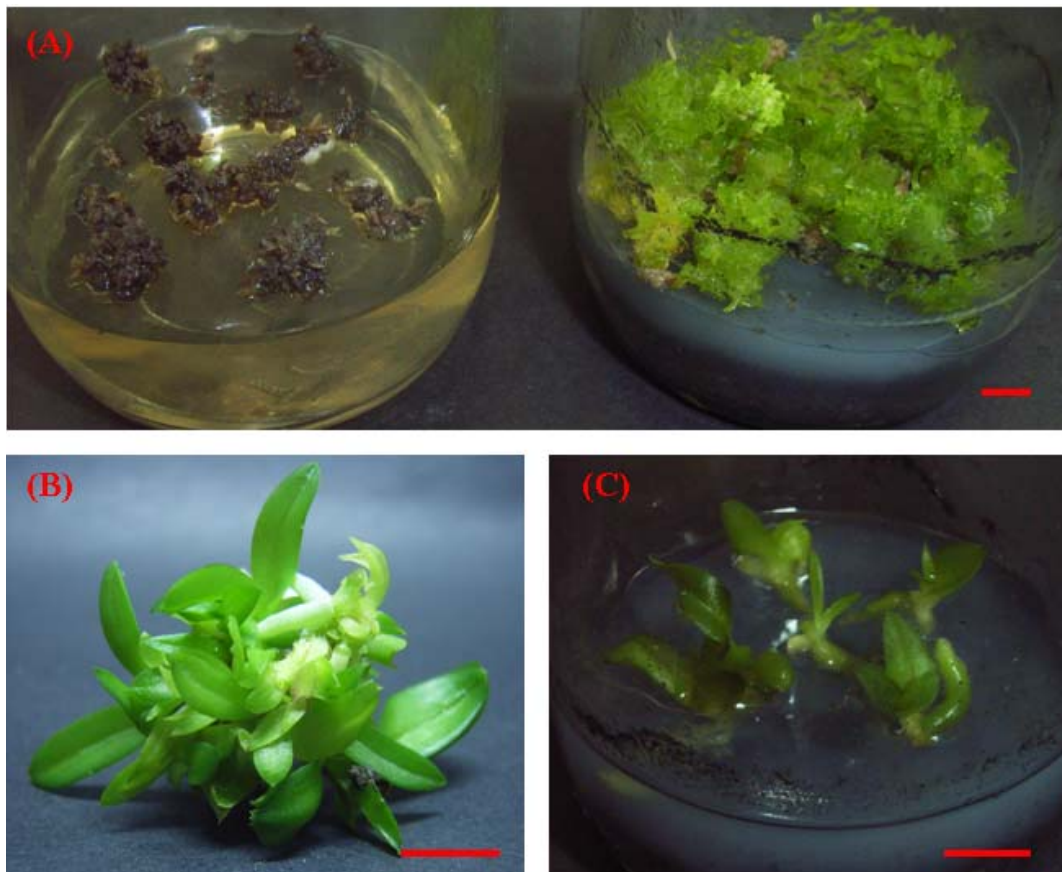


Figure 29. Plant regeneration from protocorm-like bodies developed from cryopreserved shoot tips. (A) Multiple shoots formation from protocorm-like bodies after 1 month of culture on ND (left) and modified VW agar medium (right) supplemented with 1 mg/l BA. (B) Root formation from shoots after 3 months of culture on modified VW agar medium. (C) Normal plantlets from cryopreserved shoot tips. Scale bar: A-B = 1 cm, C = 0.5 cm.

Flow cytometry analysis was applied to study the ploidy stability of *Vanda coerulea* 4-month-old from non-cryopreserved and cryopreserved plantlets. Nuclei were isolated from leaves of non-cryopreserved and cryopreserved plantlets which showed similar patterns of ploidy levels (Figure 30. A-B).

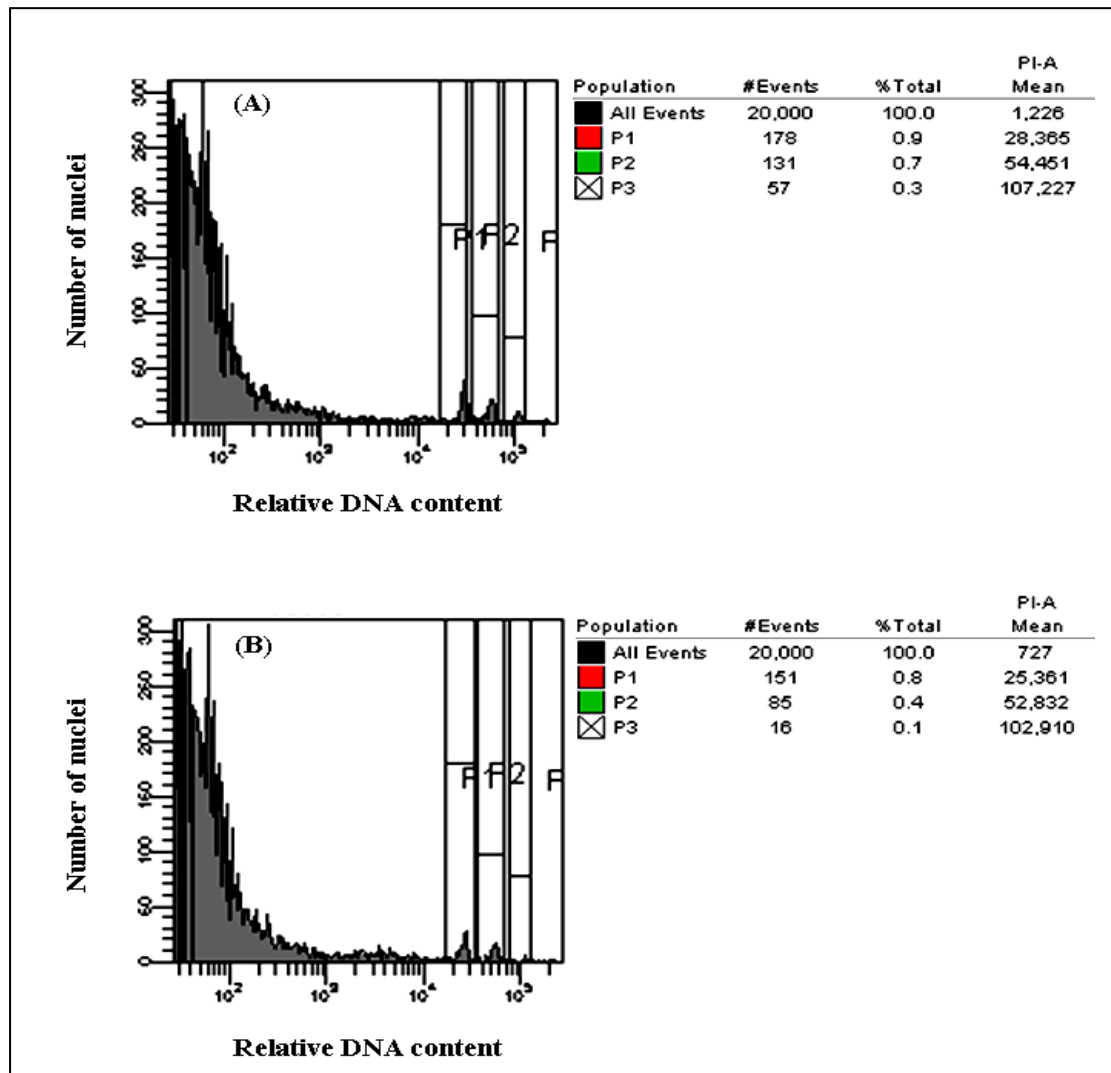


Figure 30. Histogram showing ploidy levels in leaves of *Vanda coerulea* from 4-month-old non-cryopreserved plantlets (A) and cryopreserved plantlets (B) after cryopreservation of shoot tips by slow freezing method.

#### **4.5 Cryopreservation of protocorm-like bodies by droplet-vitrification method**

Protocorm-like bodies were precultured in ND liquid medium supplemented with 0, 0.1, 0.3, 0.5, 0.7 and 1.0 M sucrose for 1 d, treated with LS for 15 min at 25°C and then dehydrated with PVS2 solution for 0-80 min at 25°C, then cryopreserved by droplet-vitrification method. After 1 month of culture, there was no survival of non-cryopreserved protocorm-like bodies after dehydration with PVS2 solution. Survival rate of non-cryopreserved and cryopreserved protocorm-like bodies were precultured in 0.3 M sucrose without dehydration with PVS2 solution was about 75% and 5%, respectively.

Survival rate of cryopreserved protocorm-like bodies was about 5% when precultured in 0.5 M sucrose, and then dehydrated with PVS2 solution for 30 min and without preculture but dehydrated with PVS2 solution for 30 min (Figure 31. A).

After 2 months of culture, non-cryopreserved and cryopreserved protocorm-like bodies were transferred in ND liquid medium supplemented with 1 mg/l BA in combination with 0.5 mg/l NAA and 30 g/l maltose, pH at 5.4. The size and number of protocorm-like bodies increased after 1 and 2 months of culture (Figure 31. B-D). Shoot formation was observed from protocorm-like bodies when cultured in ND liquid medium supplemented with 1 mg/l BA in combination with 0.5 mg/l NAA and 10 g/l maltose (Figure 31. E-F). After 3 months of culture, shoots were transferred to modified VW agar medium. Shoots produced roots after 2 months of culture. No morphological variation between plantlets from non-cryopreserved and cryopreserved protocorm-like bodies was detected

Nuclei were isolated from leaves of non-cryopreserved and cryopreserved plantlets which showed similar patterns of ploidy levels by using flow cytometry analysis (Figure 32. A-B).

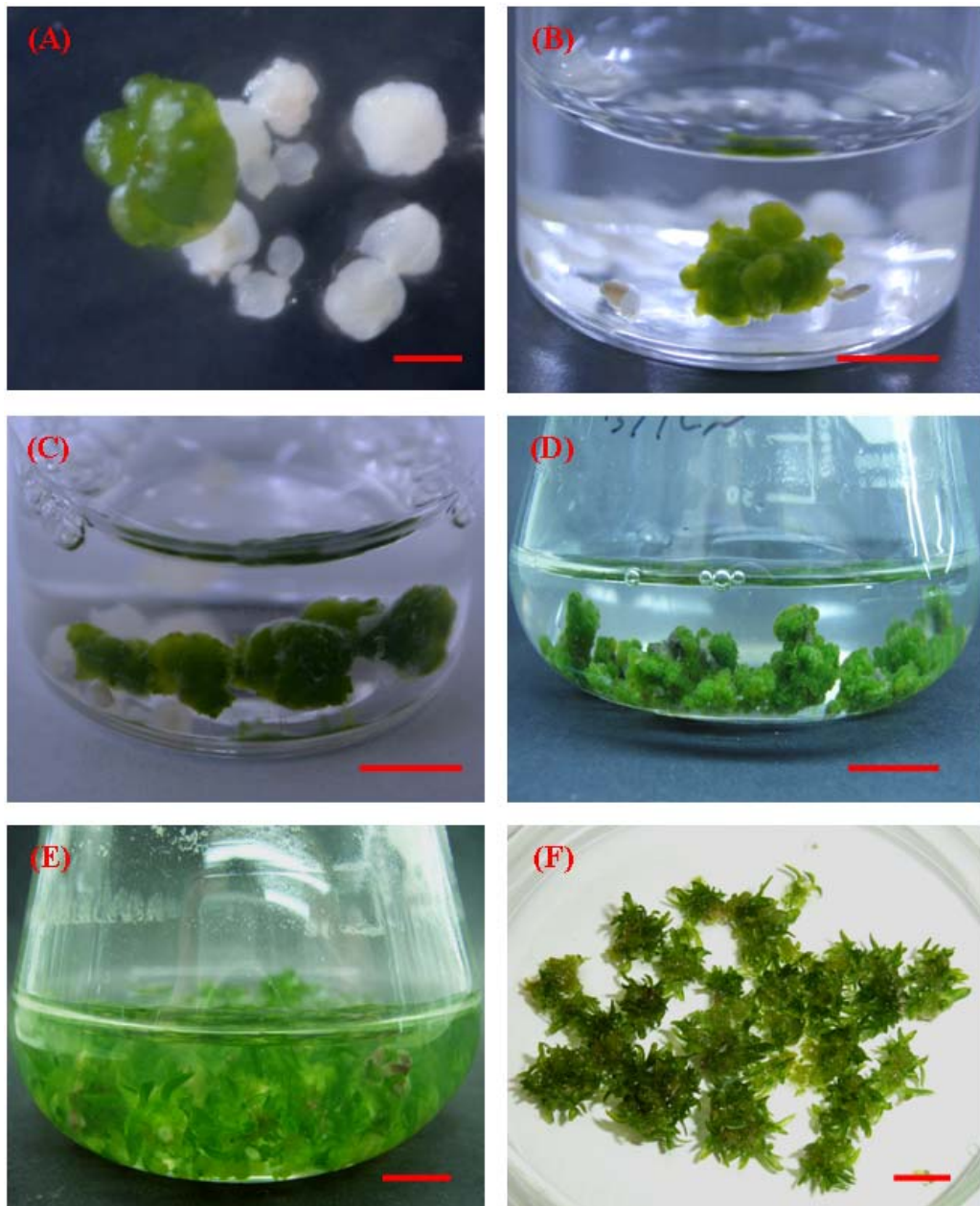


Figure 31. Development of protocorm-like bodies after cryopreservation by droplet-vitification method. (A) Survival of protocorm-like bodies on ND solid medium supplemented with 1 mg/l BA in combination with 0.5 mg/l NAA and 30 g/l maltose after 2 months of cryopreservation. (B) Protocorm-like body proliferation in ND liquid medium supplemented with 1 mg/l BA in combination with 0.5 mg/l NAA and 30 g/l maltose after 20 d, (C) 1 month and (D) 2 months of culture. (E-F) Shoot formation after 3 months of culture in ND liquid medium supplemented with 1 mg/l BA in combination with 0.5 mg/l NAA and 10 g/l maltose. Scale bar: A-F = 1 cm.

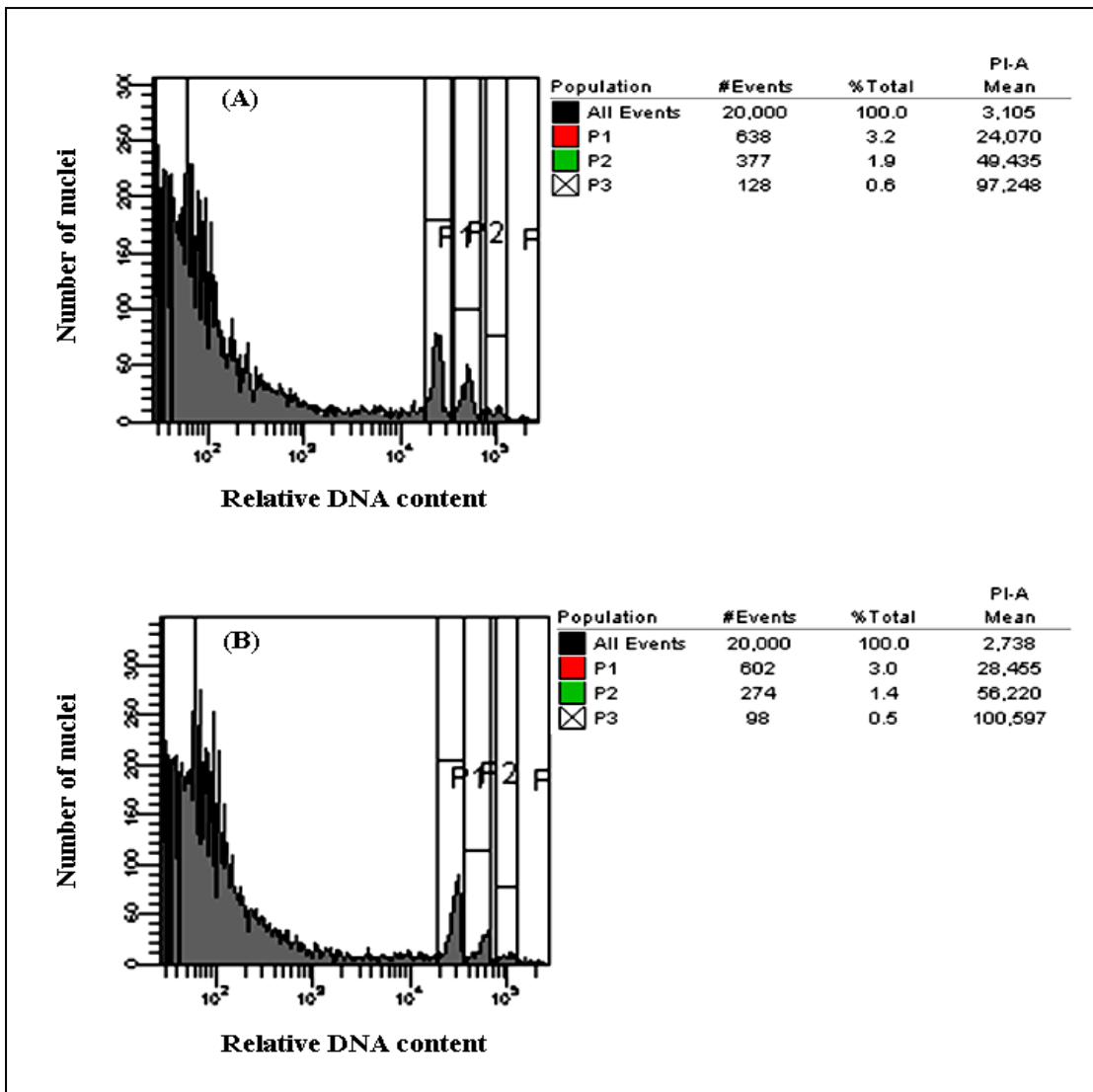


Figure 32. Histogram showing ploidy levels in leaves of *Vanda coerulea* from 4-month-old non-cryopreserved plantlets (A) and cryopreserved plantlets (B) after cryopreservation of protocorm-like bodies by droplet-vitrification method.

#### 4.6 Study the genetic variation of plantlets from seeds of *Vanda coerulea* using RAPD markers

The results showed that DNA can be isolated from leaves of ten plantlets germinated from seeds of *Vanda coerulea* (Figure 33).

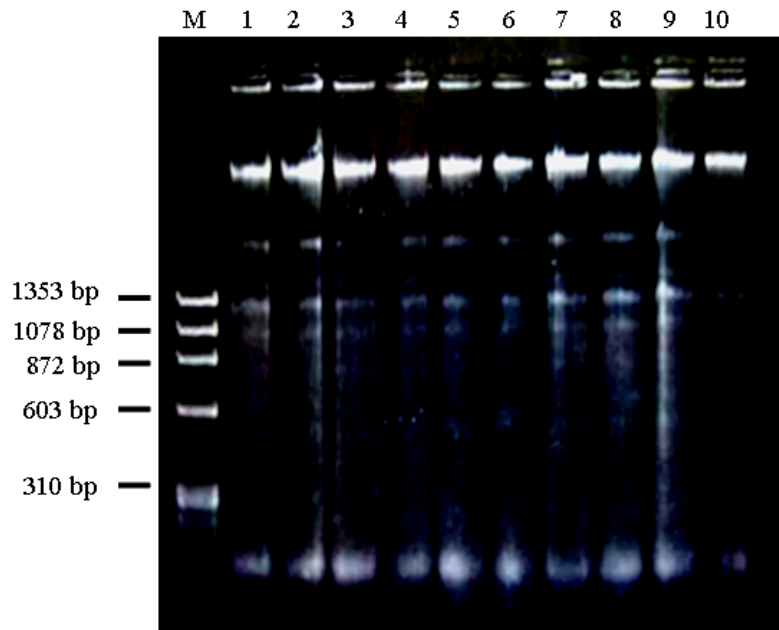


Figure 33. Total DNA extracted from the leaves of ten plantlets germinated from seeds of *Vanda coerulea*.

M: Molecular weight marker ( $\Phi$ X174DNA-*Hae*III)

Lane 1, 2, 3, 4, 5, 6, 7, 8, 9, 10: plantlets

The RAPD markers were efficiently used for estimating the genetic variation of ten plantlets. Out of eighteen 10-base primers used in this study, the same RAPD patterns of ten plantlets were observed when primer OPC-06 was used, whereas primers OPC-01, -02, -04, -05, -07, -08, -09, -10, OPE-01, -02, OPK-01, -02, -04 and -05, gave different RAPD patterns of ten plantlets (Figure 34, Table 12). Three primers (OPC-03, OPK-03 and OPK-06) failed to amplify DNA.

For the ten plantlets of *Vanda coerulea* tested, the eighteen primers produced 916 fragments. The size of the amplified DNA fragments ranged from 310 bp to above 1353 bp (Figure 34). The number of bands produced by each primer varied from 0 to 11.8, with an average of 5.09 bands per primer. The highest number of bands (12.1) was produced by primer OPE-02 and the lowest number of bands (0) was produced by primer OPC-03, OPK-03 and OPK-06. The number of bands produced by primers OPC-01-10, OPE-01-02 and OPK-01, -02, -04, -05 with an average of 5.3, 9.8 and 3.1 bands, respectively.

The primers OPK-01, -02, -04 and -05 did not yield good amplified product, although primers OPK-01, -02, -04 and -05 and template DNA were increased in a volume from 1  $\mu$ l to 1.5  $\mu$ l, whereas the primers OPC-01-10 and OPE-01-02 gave good amplified product for *Vanda coerulea* in a volume of 1.5  $\mu$ l. These results showed that ten plantlets germinated from seeds of *Vanda coerulea* had genetic diversity by RAPD analysis (Table 13).

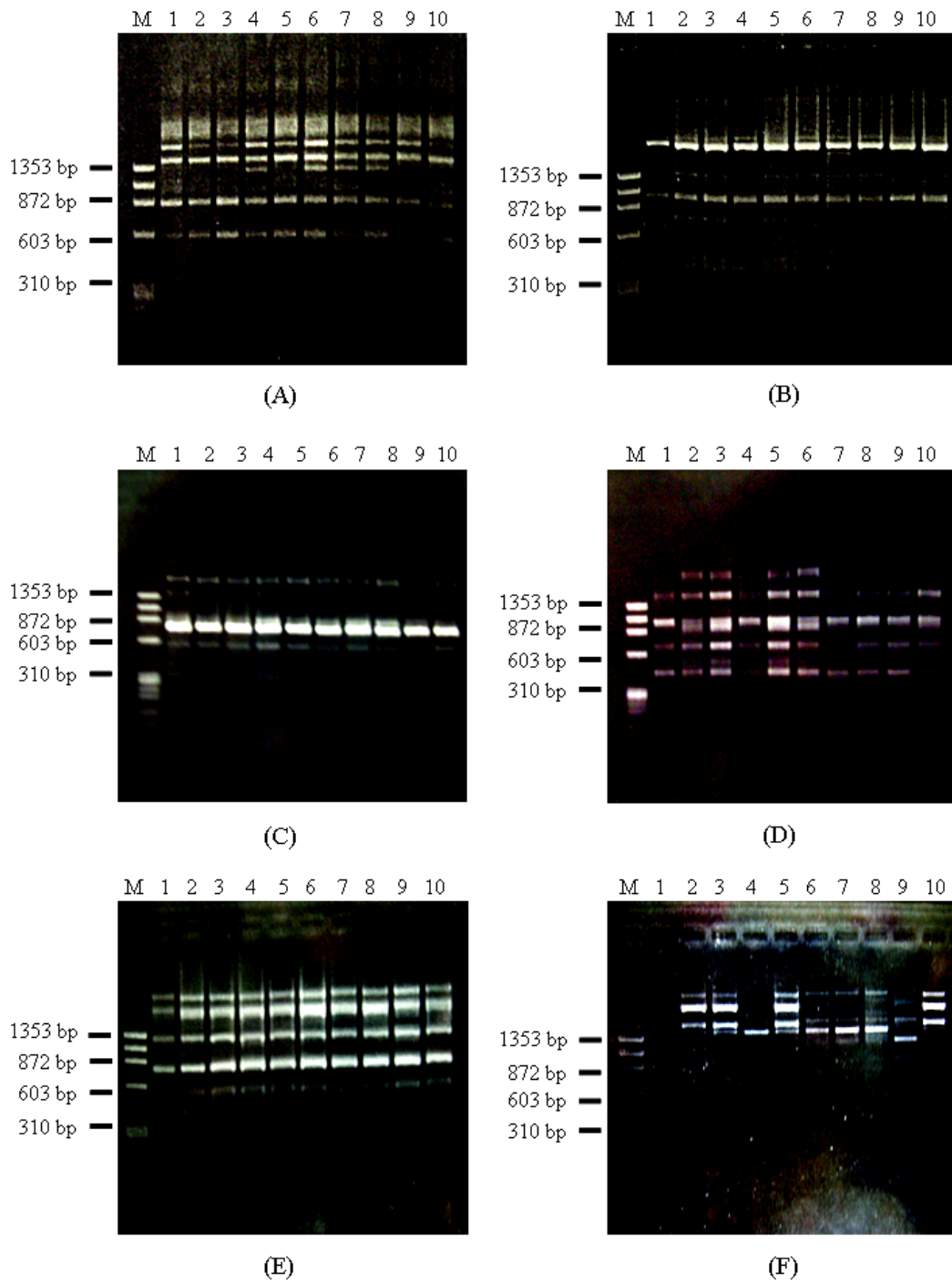


Figure 34. The RAPD patterns generated by primers OPC-07 (A), OPC-08 (B), OPE-01 (C), OPE-02 (D), OPK-02 (E) and OPK-04 (F) of leaves of ten plantlets germinated from seeds of *Vanda coerulea*.

Lane M : Molecular weight marker ( $\Phi$ X174DNA-*Hae*III)

Lane 1-10 : plantlets.

Table 12. The primers produced RAPD patterns for ten plantlets germinated from seeds of *Vanda coerulea*.

Primer	Plantlets showing the same RAPD pattern	Number of total bands	Total number of bands per each primer	Average number of band per each primer	Average number of band per primer
OPC-01	1 and 9	5	66	6.6	5.26
	2, 3, 4, 5, 6, 7, 8 and 10	7			
OPC-02	1, 9	13	117	11.7	
	2, 3, 5 and 10	12			
	4	10			
	6, 7 and 8	11			
OPC-03	-	0	0	0	
OPC-04	1 and 9	11	130	13	
	2, 3 and 10	13			
	4	11			
	5	14			
	6	13			
	7 and 8	11			
	9	9			
OPC-05	1, 4, 6, 7, 8 and 9	3	34	3.4	
	2, 3, 5 and 10	4			
OPC-06	1, 2, 3, 4, 5, 7, 8, 9 and 10	2	18	1.8	
	6	0			
OPC-07	1 and 9	5	50	5	
	2, 3, 5 and 10	4			
	4, 6, 7 and 8	6			

Table 12. The primers produced RAPD patterns for ten plantlets germinated from seeds of *Vanda coerulea* (continued).

Primer	Plantlets showing the same RAPD pattern	Number of total bands	Total number of bands per each primer	Average number of band per each primer	Average number of band per primer
OPC-08	1 and 9	5	50	5	9.8
	2, 3 and 5	4			
	4, 7 and 10	6			
	6 and 8	5			
OPC-09	1 and 9	4	35	3.5	
	2, 3, 4, 5 and 10	3			
	6, 7 and 8	4			
OPC-10	1, 7, 9 and 10	1	26	2.6	
	2, 3 and 5	4			
	4	6			
	6	4			
	8	0			
OPE-01	1	8	75	7.5	
	2, 3, 5 and 10	8			
	4 and 6	8			
	7	9			
	8	10			
OPE-02	1, 9 and 10	11	121	12.1	
	2, 3 and 5	13			
	4	13			
	6	12			
	7	12			

Table 12 The primers produced RAPD patterns for ten plantlets germinated from seeds of *Vanda coerulea* (continued).

Primer	Plantlets showing the same RAPD pattern	Number of total bands	Total number of bands per each primer	Average number of band per each primer	Average number of band per primer
OPK-01	1 and 9	8	79	7.9	3.23
	2, 3, 5 and 10	9			
	4	6			
	6, 7 and 8	7			
OPK-02	1	5	41	4.1	
	2, 3, 5 and 10	4			
	4	3			
	6, 7 and 8	4			
	9	5			
OPK-03	-	0	0	0	
OPK-04	1	5	59	5.9	
	2, 3, 4, 5, 6, 7, 8, 9 and 10	6			
OPK-05	1 and 5	0	15	1.5	
	2	1			
	4, 6, 7, 8 and 9	2			
	3 and 10	2			
OPK-06	-	0	0	0	







Table 13. DNA polymorphism of *Vanda coerulea* in RADP analysis (continued).

(1 : presence, 0 : lack of polymorphism products)

Primer	Band No.	Plantlet No.									
		1	2	3	4	5	6	7	8	9	10
OPK-04	111	1	1	1	1	1	1	1	1	1	1
	112	1	1	1	1	1	1	1	1	1	1
	113	0	1	1	1	1	1	1	1	1	1
	114	1	1	1	1	1	1	1	1	1	1
	115	1	1	1	1	1	1	1	1	1	1
	116	1	1	1	1	1	1	1	1	1	1
	117	0	0	0	1	0	1	1	1	1	0
	118	0	1	1	1	0	1	1	1	1	1
	119	0	0	0	0	0	0	0	0	0	1
OPK-05	120	0	0	0	1	0	1	1	1	1	0
	121	0	1	1	1	0	1	1	1	1	1
	122	0	0	1	0	0	0	0	0	0	1

#### **4.7 Universal primers for amplification of the *trnL* (UAA) region of cpDNA for *Vanda coerulea*, some orchid species and *Oryza sativa***

##### **4.7.1 Study the optimum annealing temperature for amplification of the *trnL* (UAA) region of cpDNA for *Vanda coerulea***

Two universal primers c and d for amplification of the *trnL* (UAA) region of cpDNA for *Vanda coerulea* via the PCR have been design.

Figure 35 showed the PCR products of the universal primers c and d for amplification of the *trnL* (UAA) region of cpDNA for *Vanda coerulea*. Optimum annealing temperature was studied. Out of the 12 temperatures (49.2, 49.3, 49.7, 50.3, 51.0, 51.9, 52.7, 53.6, 54.4, 55.0, 55.5 and 55.8°C) of annealing for 1 min were used in this study. Eight temperatures (49.2-53.6°C) failed to amplify DNA, the number of bands were two bands (Figure 35). Four temperatures (54.4, 55.0, 55.5 and 55.8°C) produced one band pattern of the *trnL* (UAA) region of cpDNA for *Vanda coerulea*. The size of amplified fragments was 872 bp.

In this study, the optimum annealing temperature for amplification of the *trnL* (UAA) region of cpDNA for *Vanda coerulea* was 55.0°C for 1 min.

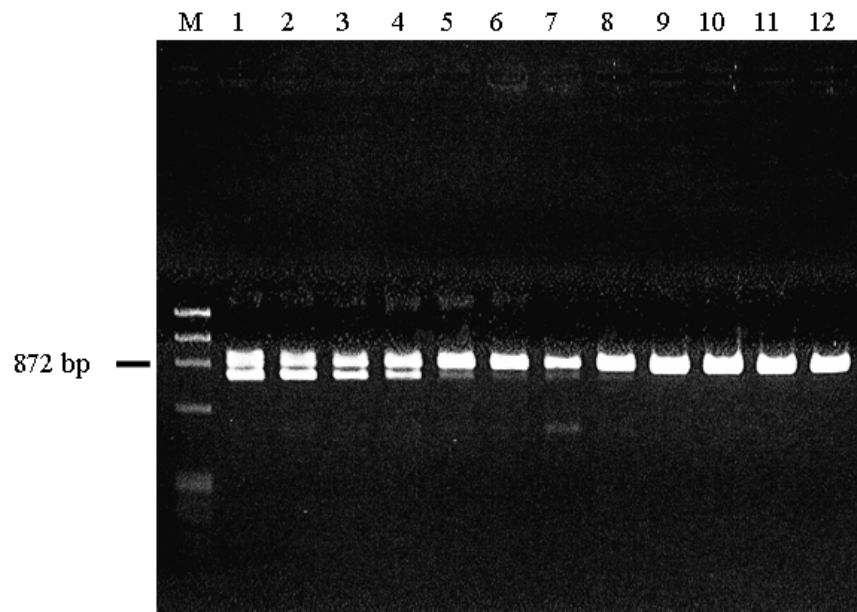


Figure 35. Electrophoresis of PCR products in 1.5% agarose gel showed the successful amplification of primers c and d for the *trnL* (UAA) region of cpDNA for leaves of *Vanda coerulea*.

Lane M : Molecular weight marker ( $\Phi$ X174DNA-*Hae*III)

Lane 1 : 49.2°C

Lane 2 : 49.3°C

Lane 3 : 49.7°C

Lane 4 : 50.3°C

Lane 5 : 51.0°C

Lane 6 : 51.9°C

Lane 7 : 52.7°C

Lane 8 : 53.6°C

Lane 9 : 54.4°C

Lane 10 : 55.0°C

Lane 11 : 55.5°C

Lane 12 : 55.8°C

#### 4.7.2 Study the size of the *trnL* (UAA) region of cpDNA for *Vanda coerulea*, some orchid species and *Oryza sativa*

Two universal primers c and d amplification of *trnL* (UAA) region of cpDNA via PCR were tested for leaves of plantlets of *Vanda coerulea* from Kanchanaburi, Chiang Mai and Bangkok provinces. The results showed the same size of fragment of the *trnL* (UAA) region for plantlets of *Vanda coerulea* from different areas (Figure 36). The size of amplified fragments was 872 bp.

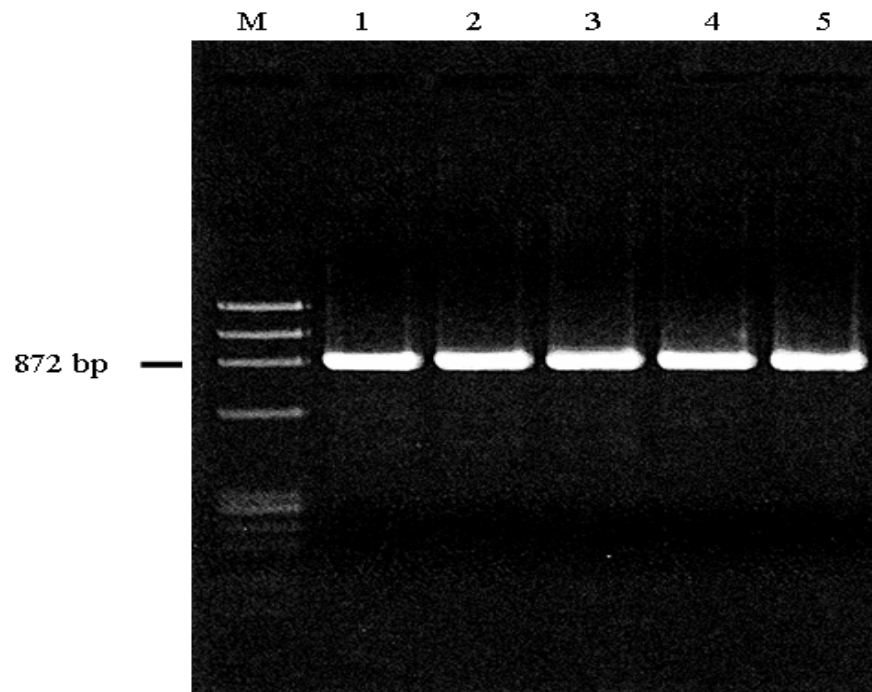


Figure 36. Electrophoresis of PCR products in 1.5% agarose gel showed the successful amplification of primers c and d for the *trnL* (UAA) region of cpDNA for plantlets of *Vanda coerulea* from different areas.

Lane M : Molecular weight marker ( $\Phi$ X174DNA-*Hae*III)

Lane 1 : *Vanda coerulea* from Kanchanaburi

Lane 2 : *Vanda coerulea* from Chiang Mai

Lane 3 : *Vanda coerulea* from Chiang Mai

Lane 4 : *Vanda coerulea* from Chiang Mai

Lane 5 : *Vanda coerulea* from Bangkok

DNA was isolated from leaves of *Dendrobium aphyllum*, *Dendrobium lindleyi*, *Grammatophyllum speciosum* from Thailand, *Vanda tricolor* from Botanical garden of Tsukuba and *Bletilla striata* from Botanical garden of Toyama (Figure 37). Concentration of DNA from *Vanda tricolor*, *Dendrobium aphyllum*, *Dendrobium lindleyi*, *Bletilla striata*, *Grammatophyllum speciosum* was about 15, 15, 30, 15 and 60 ng/ $\mu$ l, respectively.

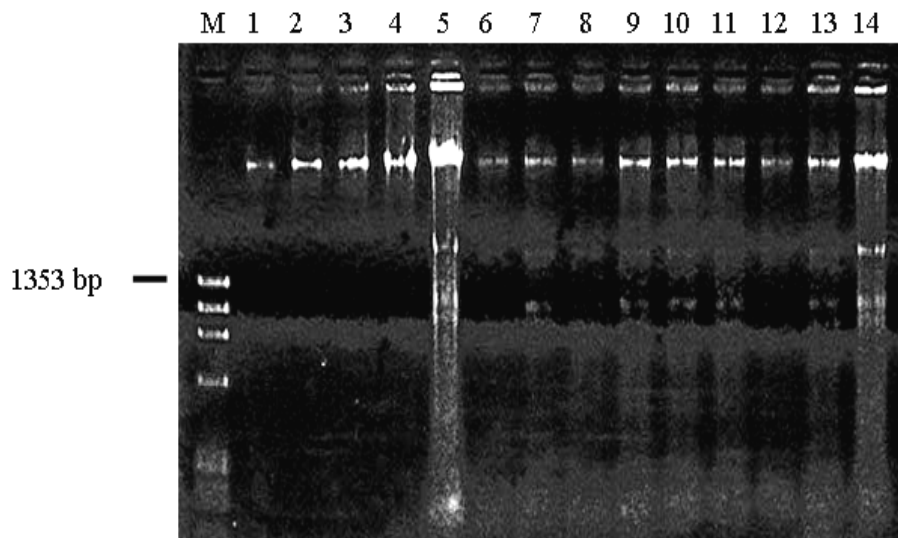


Figure 37. Electrophoresis of DNA products of some orchid species in 1.5% agarose gel.

Lane M: Molecular weight marker ( $\Phi$ X174DNA-*Hae*III).

Lane 1 : 15 ng/ $\mu$ l lamda DNA

Lane 2 : 30 ng/ $\mu$ l lamda DNA

Lane 3 : 60 ng/ $\mu$ l lamda DNA

Lane 4 : 120 ng/ $\mu$ l lamda DNA

Lane 5-6 : DNA from *Vanda tricolor*

Lane 7-8 : DNA from *Dendrobium aphyllum*

Lane 9-10 : DNA from *Dendrobium lindleyi*

Lane 11-12 : DNA from *Bletilla striata*

Lane 13-14 : DNA from *Grammatophyllum speciosum*

Two universal primers c and d amplification of *trnL* (UAA) region via PCR were tested for leaves of *Vanda coerulea*, *Vanda tricolor*, *Dendrobium aphyllum*, *Dendrobium lindleyi*, *Grammatophyllum speciosum*, *Bletilla striata*, and *Oryza sativa*. The primers c and d worked for *Oryza sativa* and some orchid species were tested. Figure 38 showed the variation in their size of the *trnL* (UAA) region of cpDNA.

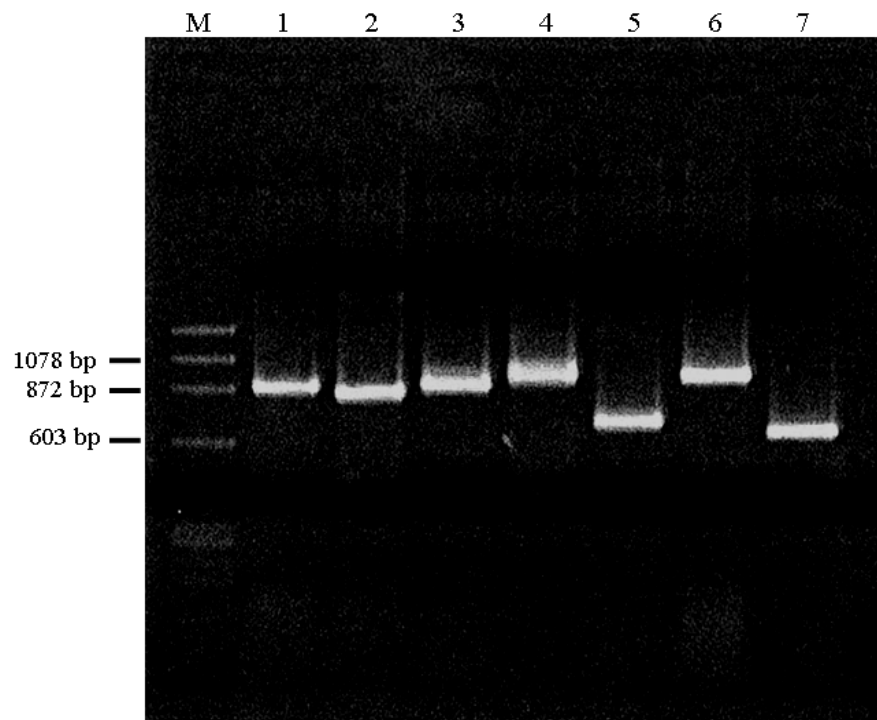


Figure 38. Electrophoresis of PCR products in 1.5% agarose gel showed the successful amplification of primers c and d for the *trnL* (UAA) region.

Lane M : Molecular weight marker ( $\Phi$ X174DNA-*Hae*III)

Lane 1 : *Vanda coerulea*

Lane 2 : *Vanda tricolor*

Lane 3 : *Dendrobium aphyllum*

Lane 4 : *Dendrobium lindleyi*

Lane 5 : *Bletilla striata*

Lane 6 : *Oryza sativa*

Lane 7 : *Grammatophyllum speciosum*

#### 4.7.3 Study the sequences of the *trnL* (UAA) region of cpDNA for *Vanda coerulea*

DNA was extracted from leaves of plantlets from *Vanda coerulea*. PCR was done with combination of two universal primers c and d for the *trnL* (UAA) region. PCR products were recovery and then sequencing of the *trnL* (UAA) region.

Figure 39 showed the sequences of the *trnL* (UAA) region of cpDNA for *Vanda coerulea*. The length of the sequences of the *trnL* (UAA) region amplified with primers c and d was 876 bp.

```
GGGGATAGAGGGACTTGAACCCTCACGATTGAAAAATTTCGACGGATTTTC
CTCTTACTATAAATTTTCATTGTTGTCGGTATTGACATGTAAAATGGGACTC
TCTCTTTATTCTCGTACGATTCAATTTCAAAAAGATCTATCAAAAATTCTGG
AATGAATAATTTGATTATTGAATATTCGAATTCTATTCTTTTTTCAACTCA
ATTGGAATTGATTCACAATAACTCTTCAATTTTTCATATATCTTTTTGATCT
CTATCATTCTAATGAAAAAGAATAGAAAAATAGGGTTTCTTATAGATCC
TTATCTCATATATCTCATATTAATAATAGAATCATATTAATAATAGAATCA
TATTAATAATAGAATATAATATTAATTAATAATATAATATTAATAGAATAA
TAGAAAGAAAGAGAAGATTTTTTATTTTCATATAAAAATTTCAAAAATTCAT
ATATAAATATATATAAAAATTCATATATAAATATATATAAATATATAGAG
ATACAGAAGAGATACAGAATAGAATTCGATATAAGATTTGGGTTGTGATT
AATCGTTTGCTATGTCAGTATTTATACGTACGTCTTAGGTATATAAGACGT
ATCCTTTCTGTCATTTTGATAGAAGTCTTTTAGCTACTAACGTAACGTAAT
CAATTTTCATTCGTTAGAACAGCTTCCATTGAGTCTCTGCACCTATCCCTTTT
TTATTCTTATTTTTTCATCTTTCATCGTTTTTCTCTCGAAACAAAGATTTGGC
TCAGGATTGCCCTTTTTTAGTTCCAGGGTTTCTCTGAATTTGGAAGTTACC
ACTTAGCAGGTTTCCATACCAAGGCTCAATCCAATCAAGTCCGTAGCGTN
CTACCGATTTTCG
```

Figure 39. The sequences of the *trnL* (UAA) region of cpDNA for *Vanda coerulea*.

## **4.8 Study the genetic stability after cryopreservation**

### **4.8.1 RAPD analysis**

DNA can be isolated from non-cryopreserved and cryopreserved protocorm-like bodies of *Vanda coerulea*. The RAPD marker has efficiently used for estimating the genetic variation of non-cryopreserved and cryopreserved protocorm-like bodies.

The genetic fidelity of protocorm-like bodies was tested by comparing RAPD profiles generated with sixty arbitrary 10-mers. Thirteen of sixty 10-mer primers (OPC-03, -16, -17, OPE-03, -04, -05, -07, -10, -13, -17, OPK-03, -05 and -18) failed to amplify DNA (Figure 40-42, Table 14-16). The other forty-seven primers (OPC-01, -02, 03, -04, -05, -06, -07, -08, -09, -10, -11, -12, -13, -14, -15, -16, -18, -19, -20, OPE-01, -02, -06, -08, -09, -11, -12, -14, -15, -16, -17, -18, -19, -20, OPK-01, -02, -04, -K07, -08, -09, -10, -11, -12, -13, -14, -15, -16, -17, -19 and -20) yielded a total of 233 bands.

DNA fragments varied in numbers and size depending on the primer used. The size of the amplified DNA bands ranged from 310 bp to above 1353 bp. The number of amplified fragments varied from 0 to 10 per primer, with an average of 3.88 fragments per primer.

The highest number of bands (10) was produced by primer OPE-20. The total number of bands was produced by primers OPC-01 to 20, OPE-01 to 20 and OPK-01 to 20 with an average of 72, 69 and 92 bands, respectively. The number of bands produced by each primer (OPC-01-20, OPE-01-20 and OPK-01-20) with an average of 3.6, 3.45 and 4.6 bands/primer, respectively.

There was no difference in the banding pattern of non-cryopreserved and cryopreserved protocorm-like bodies. No genetic variability of non-cryopreserved and cryopreserved protocorm-like bodies. The RAPDs markers provide an easy, fast and relatively cheap way of randomly screening large part of the genome.

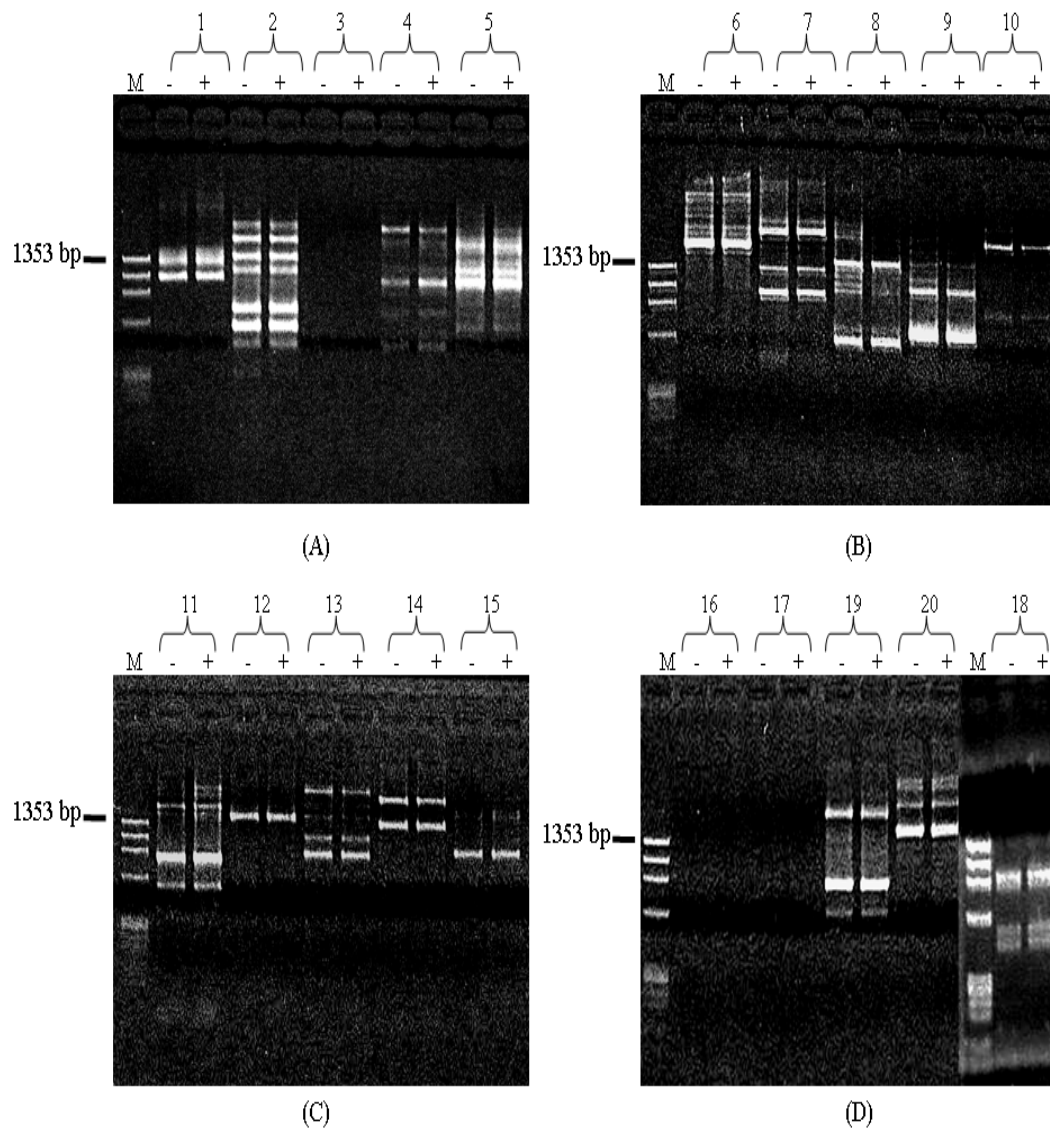


Figure 40. The RAPD patterns of non-cryopreserved protocorm-like bodies (-) and cryopreserved protocorm-like bodies (+) of *Vanda coerulea* generated by primers OPC-01 to 20.

Lane M : Molecular weight marker ( $\Phi$ X174DNA-*Hae*III)

(A) : primers OPC-01, -02, -03, -04, -05

(B) : primers OPC-06, -07, -08, -09, -10

(C) : primers OPC-11, -12, -13, -14, -15

(D) : primers OPC-16, -17, -19, -20, -18

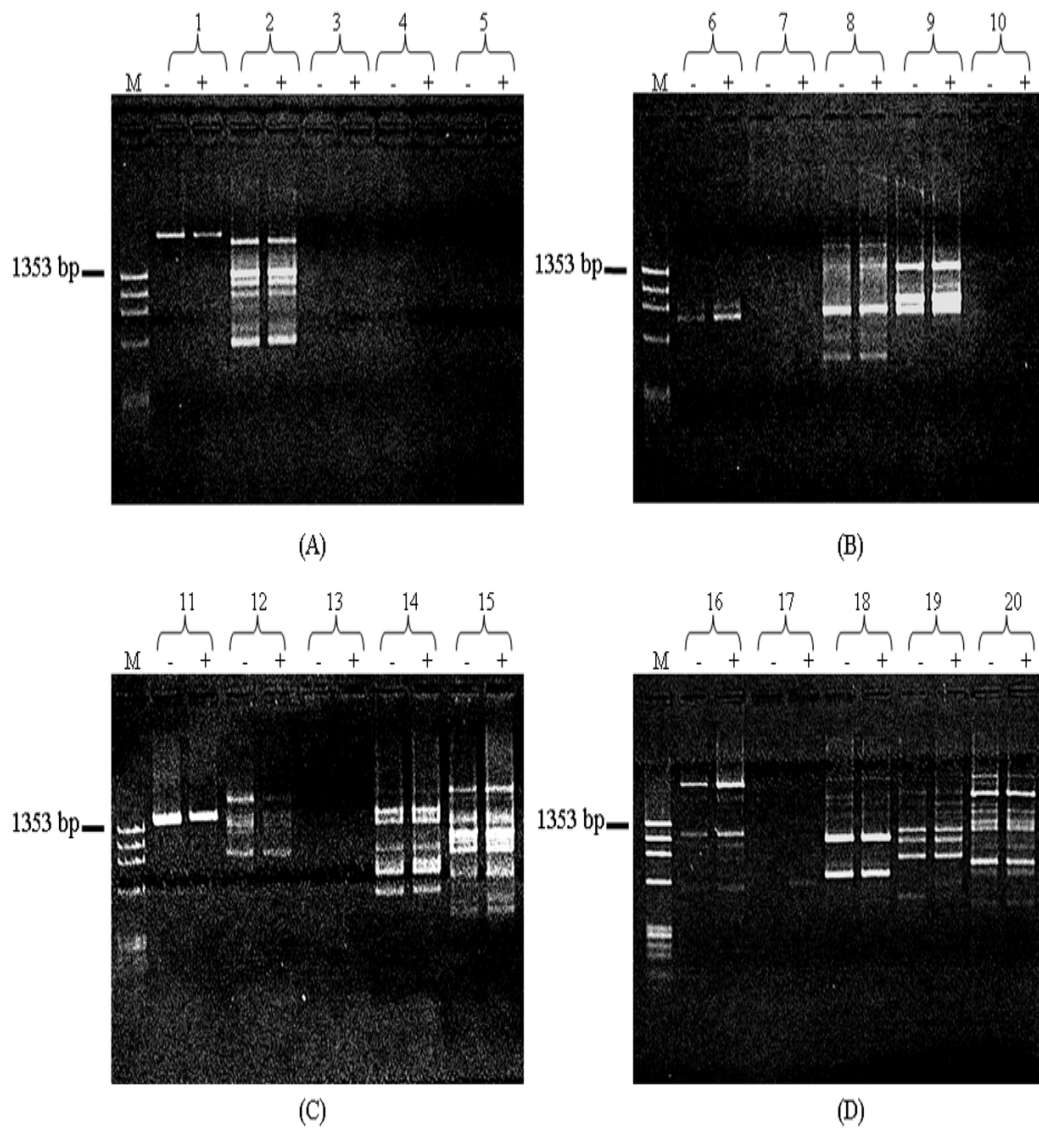


Figure 41. The RAPD patterns of non-cryopreserved protocorm-like bodies (-) and cryopreserved protocorm-like bodies (+) of *Vanda coerulea* generated by primers OPE-01 to 20.

Lane M : Molecular weight marker ( $\Phi$ X174DNA-*Hae*III)

(A) : primers OPE-01, -02, -03, -04, -05

(B) : primers OPE-06, -07, -08, -09, -10

(C) : primers OPE-11, -12, -13, -14, -15

(D) : primers OPE-16, -17, -18, -19, -20

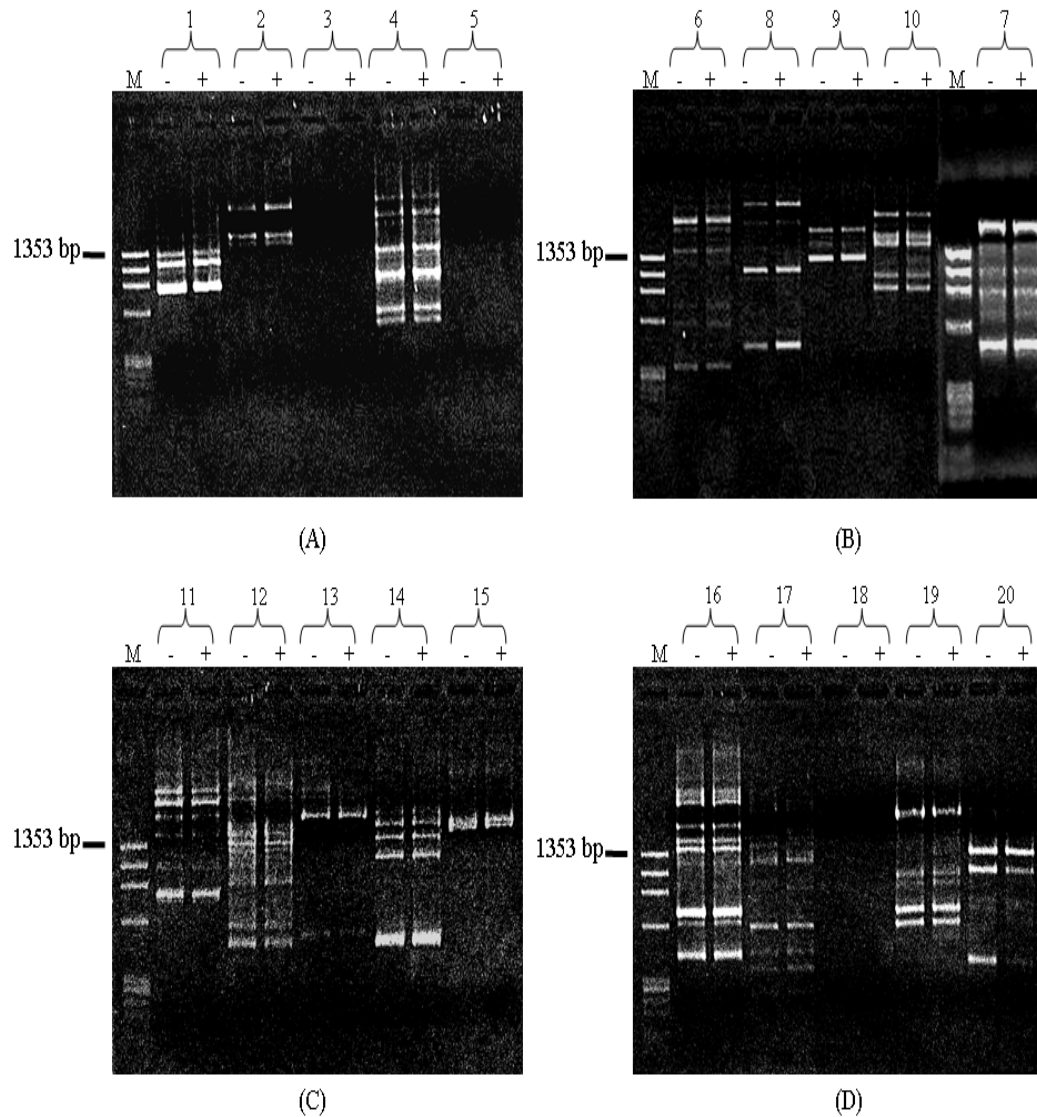


Figure 42. The RAPD patterns of non-cryopreserved protocorm-like bodies (-) and cryopreserved protocorm-like bodies (+) of *Vanda coerulea* generated by primers OPK-01 to 20.

Lane M : Molecular weight marker ( $\Phi$ X174DNA-*Hae*III )

- (A) : primers OPK-01, -02, -03, -04, -05
- (B) : primers OPK-06, -08, -09, -10, -07
- (C) : primers OPK-11, -12, -13, -14, -15
- (D) : primers OPK-16, -17, -18, -19, -20

Table 14. Sequences of the selected primers OPC-01-20 used in RAPD analysis and number of generated RAPD markers.

Primer	Number of bands	Total number of bands per primers	Average number of band per primer
OPC-01	2	72	3.6
OPC-02	7		
OPC-03	0		
OPC-04	5		
OPC-05	4		
OPC-06	7		
OPC-07	6		
OPC-08	9		
OPC-09	4		
OPC-10	3		
OPC-11	6		
OPC-12	1		
OPC-13	4		
OPC-14	2		
OPC-15	3		
OPC-16	0		
OPC-17	0		
OPC-18	2		
OPC-19	3		
OPC-20	4		

Table 15. Sequences of the selected primers OPE-01-20 used in RAPD analysis and number of generated RAPD markers.

Primer	Number of bands	Total number of bands per primer	Average number of band per primer
OPE-01	2	69	3.45
OPE-02	6		
OPE-03	0		
OPE-04	0		
OPE-05	0		
OPE-06	2		
OPE-07	0		
OPE-08	6		
OPE-09	3		
OPE-10	0		
OPE-11	2		
OPE-12	4		
OPE-13	0		
OPE-14	6		
OPE-15	9		
OPE-16	5		
OPE-17	0		
OPE-18	7		
OPE-19	7		
OPE-20	10		

Table 16. Sequences of the selected primers OPK-01-20 used in RAPD analysis and number of generated RAPD markers.

Primer	Number of bands	Total number of bands per primers	Average number of band per primer
OPK-01	4	92	4.6
OPK-02	3		
OPK-03	0		
OPK-04	7		
OPK-05	0		
OPK-06	9		
OPK-07	5		
OPK-08	4		
OPK-09	3		
OPK-10	6		
OPK-11	7		
OPK-12	9		
OPK-13	3		
OPK-14	5		
OPK-15	2		
OPK-16	8		
OPK-17	9		
OPK-18	0		
OPK-19	5		
OPK-20	3		

#### 4.8.2 Comparison the size of the *trnL* (UAA) region of cpDNA between non-cryopreserved and cryopreserved protocorm-like bodies of *Vanda coerulea*

Two universal primers c and d amplification of the *trnL* (UAA) region via PCR were tested for non-cryopreserved and cryopreserved protocorm-like bodies of *Vanda coerulea* after cryopreservation by droplet-vitrification method. The result showed the same size of the *trnL* (UAA) region of cpDNA for non-cryopreserved and cryopreserved protocorm-like bodies of *Vanda coerulea* (Figure 43). The size of amplified fragment was 872 bp.

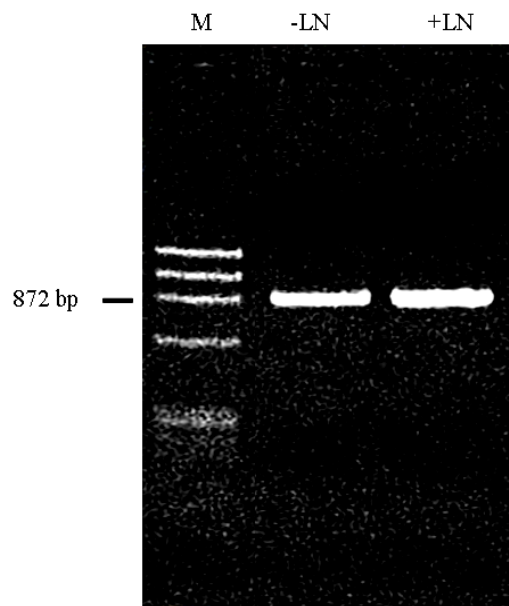


Figure 43. Electrophoresis of PCR products in 1.5% agarose gel showed the successful amplification of universal primers c and d for the *trnL* (UAA) region for non-cryopreserved protocorm-like bodies (-LN) and cryopreserved protocorm-like bodies (+LN) of *Vanda coerulea*.

Lane M : Molecular weight marker ( $\Phi$ X174DNA-*Hae*III).

### 4.8.3 Comparison the sequences of the *trnL* (UAA) region of cpDNA between non-cryopreserved and cryopreserved protocorm-like bodies

Two universal primers c and d amplification of the *trnL* (UAA) region via PCR tested of non-cryopreserved and cryopreserved protocorm-like bodies of *Vanda coerulea* have been design. The result showed that the sequences of the *trnL* (UAA) region of cpDNA for non-cryopreserved and cryopreserved protocorm-like bodies (Figure 44) did not different. No genetic variability of non-cryopreserved and cryopreserved protocorm-like bodies after cryopreservation by droplet-vitrification.

Length: 876

Identity: 876/876 (100.0%)

Similarity: 876/876 (100.0%)

Gaps: 0/876 (0.0%)

Score: 4716.0

```

-LN      1 GGGGATAGAGGGACTTGAACCCTCACGATTGAAAAATTCGACGGATTTTC      50
          |||
+LN      1 GGGGATAGAGGGACTTGAACCCTCACGATTGAAAAATTCGACGGATTTTC      50

-LN     51 CTCTTACTATAAATTTTCATTGTTGTTCGGTATTGACATGTAAAATGGGACT     100
          |||
+LN     51 CTCTTACTATAAATTTTCATTGTTGTTCGGTATTGACATGTAAAATGGGACT     100

-LN    101 CTCTCTTTATTCTCGTACGATTCAATTTCAAAGATCTATCAAAAATTCT     150
          |||
+LN    101 CTCTCTTTATTCTCGTACGATTCAATTTCAAAGATCTATCAAAAATTCT     150

-LN    151 GGAATGAATAAATTTGATTATTGAATATTCGAATTCTATCTTTTTTCAAC     200
          |||
+LN    151 GGAATGAATAAATTTGATTATTGAATATTCGAATTCTATCTTTTTTCAAC     200

-LN    201 TTCAATTGGAATTGATTCACAATAACTCTTCAATTTTTCATATATCTTTT     250
          |||
+LN    201 TTCAATTGGAATTGATTCACAATAACTCTTCAATTTTTCATATATCTTTT     250

-LN    251 TGATCTCTATCATTCTAATGAAAAAGAATAGAAAAATAGGGTTTCTTAT     300
          |||
+LN    251 TGATCTCTATCATTCTAATGAAAAAGAATAGAAAAATAGGGTTTCTTAT     300

-LN    301 AGATCCTTATCTCATATATCTCATATTAATAATAGAATCATATTAATAAT     350
          |||
+LN    301 AGATCCTTATCTCATATATCTCATATTAATAATAGAATCATATTAATAAT     350

-LN    351 AGAATCATATTAATAATAGAAATATAATATTAATTAATAATATAATATTA     400
          |||
+LN    351 AGAATCATATTAATAATAGAAATATAATATTAATTAATAATATAATATTA     400

-LN    401 TAGAATAATAGAAAGAAAGAGAAGATTTTATTTTCATATAAAAATTTC     450
          |||
+LN    401 TAGAATAATAGAAAGAAAGAGAAGATTTTATTTTCATATAAAAATTTC     450
    
```



## CHAPTER V

### DISCUSSION

#### 5.1 Micropropagation of *Vanda coerulea*

There are many different media used for orchid culture such as, MS, VW, KC and ND. They are different in their components. Optimization of medium composition was an important approach to fasten the micropropagation, improve the quality of regenerated plantlets and culture the callus, adventitious shoots or protocorm-like bodies. Lakshmanan et al. (84) reported that the three basal media (VW, KC and MS) used, VW and KC were found to be equally suitable for thin section culture from shoot tip of *Aranda Deborah*. The source of carbon is a very important component in in vitro culture media. Carbon sources are added to the culture medium because of the light energy deficiency and low CO<sub>2</sub> concentration present in in vitro conditions. Plants cultures in vitro often show a low photosynthetic rate and incomplete autotrophy (39). Sugar acts as a carbon and energy source and also acts as an osmotic regulator in the induction medium. Sucrose is the most frequently used sugar but glucose, fructose, sorbitol, maltose and other sugars are also used. Sucrose is commonly used in tissue culture media. Sucrose concentrations of 20-30 g/l are the most commonly used in orchid tissue. The results found that shoot can be regenerated from shoot tips on the modified VW and MS agar medium. The modified VW medium supplemented with high sucrose concentration (30 g/l) was not produced high number of new shoots. Similarly was reported by Shatnawi et al (152) on in vitro proliferation and rooting of *Pyrus syriaca*. In addition, high concentration of sucrose on MS agar medium increased shoot produced roots and the number of roots. Faria et al. (39) reported that the presence of 60 g/l sucrose in the medium was the most efficient treatment for increasing height and fresh weight of *Dendrobium nobile* in vitro culture. Ishii et al. (65) observed that the presence of sucrose in the culture medium caused protocorm formation in *Phalaenopsis*. The highest of root formation was observed on the modified VW agar medium supplemented with

10 g/l sucrose. The root formation was inhibited on modified VW agar medium supplemented with high sucrose concentration. Shatnawi et al. (152) reported that increased sucrose or fructose concentration was inhibitory to root formation of *Pyrus syriaca*. In addition, root formation on MS agar medium required sucrose. In this results found that addition of 150 ml/l coconut water and 100 g/l banana in the modified VW medium induced shoot and root formation in vitro culture (control group). Similarity is supported by the result of Sinha and Roy (157). The addition of activated charcoal on modified VW agar medium was not necessary to increase sucrose or plant growth regulator for rooting. This is similar to a previous study by Gubbuk and Pekmezci (48) on shoot multiplication and rooting of some new Banana.

Two main groups of plant growth regulator are used in media for orchid culture. These are cytokinins and auxins (23, 198). Cytokinins (BA and TDZ) and auxin (NAA) and their influenced concentration significantly affected shoot and root formation. Low concentration of cytokinin increased shoot buds and shoot formation, but very high concentration decreased significantly shoot and root formation. TDZ is a substituted phenyl urea with cytokinin-like activity. It is useful for plant regeneration of several species through organogenesis and promotes shoot formation in in vitro culture of several orchid species (62). TDZ alone was efficient to induce direct somatic embryogenesis from leaf explants but there was not effective in induction of somatic embryogenesis from root-derived of *Oncidium* (20, 21, 23). The highest shoot buds and shoot formation were found on modified VW medium supplemented with 1 mg/l BA. In addition, the combination of BA and NAA decreased shoot formation but increased shoot bud formation compared to BA alone. Previously, Seeni and Latha (150) reported that the combination of 8.8  $\mu\text{M}$  BA and 4.1  $\mu\text{M}$  NAA induced shoot formation from leaf of *Vanda coerulea* and the highest percentage of seeds of *Vanda teres* germinated into protocorm on the modified VW agar medium supplemented with 1 mg/l BA combination with 0.5 mg/l NAA (157). The results suggested that a lower dose of TDZ (0.1 mg/l) induced high shoot formation from shoot tips. Higher TDZ concentration reduced shoot formation, as has been reported for *Doritaenopsis* hybrid (125), *Cybidium sinense* (19). Similar response was observed by Seeni and Latha (150). TDZ alone was efficient to induce direct somatic embryogenesis from leaf explants but there was not effective in

response was observed by Seeni and Latha (150). TDZ alone was efficient to induce direct somatic embryogenesis from leaf explants but there was not effective in induction of somatic embryogenesis from root-derived of *Oncidium* (20, 21, 23). Using 0.5 mg/l NAA in combination with 2 mg/l TDZ induced higher frequency of shoot formation than TDZ alone. TDZ alone or in combination with auxins has been used to induce embryogenesis in *Cymbidium ensifolium* (18), *Oncidium* (20), protocorm-like body in *Doritaenopsis* (125) and *Doritaenopsis* hybrid (126). The effect of TDZ on shoot formation is similar to those observed in *Cybidium*, *Doritaenopsis*, *Epidendrum radicans* (19, 20, 125, 126). Comparing the effects of BA and TDZ at different levels on shoot formation, it was found that TDZ can be used at a much lower concentration than BA. A similar result was obtained by Park et al. (125). In this study found that not only shoots could be promoted from shoot tips, but also roots were formed simultaneously from shoots in all treatments. Contrastingly, Malabadi et al. (93) reported that shoots of *Vanda coerulea* produced roots on half VW medium supplemented with IAA, IBA and NAA, and root of *Dendrobium formosum* was not found without BA and NAA (113). The combination of NAA and BA has induced protocorm-like body regeneration; however, results were very low compared to BA alone. Nayak et al. (115) reported that of the three cytokinins (BA, KN and TDZ), TDZ was the most effective in inducing shoot buds from the foliar explants of *Acampe praemorsa*. TDZ was more effective at lower concentration. Disadvantage of TDZ in regeneration is the difficulty in elongation and rooting of the regenerated shoots. This problem was overcome by transferring regenerated shoots to MS medium supplemented with BA in combination with NAA. Higher concentration of NAA (5 mg/l) suppressed shoot bud regeneration. Activated charcoal in medium enhanced the induction of roots (14). Effect of activated charcoal on shoot proliferation is that it improves aeration and adsorbs ethylene which can inhibit growth and proliferation (35). Ket et al. (75) reported that the adding activated charcoal to the H3 medium induced shoot growth and elongation. Production of multiple shoots was dependent on the amount of activated charcoal in medium. Survival rate was about 100% in the greenhouse after 3 months of culture. Similar patterns of ploidy levels were observed by flow cytometry from the leaves of both stock plants and regenerated plants.

## 5.2 Cryopreservation of seeds

For cryopreservation of *Vanda coerulea*, there is only one report on seed cryopreservation by vitrification method (166). This study showed that different methods can be used to cryopreserve seeds of *Vanda coerulea*. Seeds of *Vanda coerulea* can be tolerant to dehydration and suitable for cryopreservation. For successful cryopreservation, the water content of seeds is an important factor (58). The water content of seeds of *Vanda coerulea* was 56%. When the seeds were directly plunged into LN, the germination rate was lower than those of the control seeds. Hirano et al. (58) reported that water content of immature seeds of *Bletilla striata* was 33% which gave the highest germination rate.

With vitrification method, when seeds without preculture were treated with LS, then dehydrated with PVS2 solution on ice at 0°C for 40 min, germination rate (47%) of cryopreserved seeds was higher than those of cryopreserved seeds without loading treatment. The loading solution was very efficient in inducing dehydration and freezing tolerance in tissues (140) such as BY-2 suspension cell of tobacco (82), zygotic embryo of *Bletilla striata* (66), immature seeds of *Bletilla striata* (58), and immature seeds *Ponerorchis graminifolia* var *suzukiana* (59). The incubation time and temperature in PVS2 solution has the effect on germination rate of cryopreserved seeds because over-exposure to PVS2 solution can cause chemical toxicity. Dehydration time of explants at 0°C reduces the toxicity of vitrification solution and increases exposure time to PVS2 solution (142).

In encapsulation-dehydration method, germination rate of cryopreserved seeds depended on water content and duration time of the dehydration (194). 100% germination rate of cryopreserved seeds was obtained when water content of precultured beads was 25% and 22% for 0.5 and 0.7 M sucrose in the preculture medium after 6 h dehydration, respectively. Pretreatment with high concentration of sucrose protected cells from dehydration injury and to tolerate desiccation and freezing (104, 194).

Encapsulated seeds were treated with LS which reduced the toxicity of PVS2 solution (187). Germination rate of cryopreserved seeds by encapsulation-vitrification method was lower than those of encapsulation-dehydration method because PVS2 solution can cause toxicity (187). Cryopreserved seeds by encapsulation-dehydration

gave higher than those of the seeds by vitrification method because germination rate of cryopreserved seeds by encapsulation-dehydration was calculated on the basis of protocorms or plantlets from encapsulated bead (only one protocorm or plantlet/bead).

In this study, protocorms developed into one shoot/protocorm and multiple shoots (8 shoots/protocorm). Morphologies of plantlets germinated from non-cryopreserved and cryopreserved seeds were normal. The storage of seeds in LN had no effect on the growth and development of protocorms and plantlets (119, 129). The different methods for cryopreservation in this study did not have the effect on the ploidy levels of *Vanda coerulea* seeds.

### 5.3 Cryopreservation of protocorms

Cryopreservation of protocorms of *Vanda coerulea* was successfully achieved by encapsulation-dehydration in combination with loading solution. During the encapsulation process, protocorms were osmoprotected with a mixture of 2 M glycerol plus 0.4 M sucrose, then precultured in 0.7 M sucrose for 20 h prior to dehydration with sterile air-flow. The loading solution was very efficient in inducing dehydration and freezing tolerance in tissues (67, 82, 98, 99). Glycerol has been used in encapsulation in combination with sucrose to increase dehydration tolerance and encapsulated beads were still flaccid which may confirm dehydration and fast recovery growth in wasabi apices, chrysanthemum apices and mint apices (142). Preculture is usually essential for high levels of survival in cryopreserved explants (38, 104). Encapsulated beads were precultured in 0.7 M sucrose. Preculture results in the accumulation of sugars and increase the stability of membrane. Sucrose acts to protect cells from dehydration injury and to tolerate desiccation and freezing (38, 104).

Regrowth rate following encapsulation-dehydration is dependent on the amount of water presented in the cells. The water within encapsulated beads was removed by both osmotic dehydration and sterile air-flow. If the cells are not sufficiently dehydrated, intracellular ice will be formed. The duration of dehydration was an important factor for protocorms forming plantlets after cryopreservation. The suitable water content for sample survival after LN exposure ranged from 15% to 25% (104, 154).

This is the first report of successful cryopreservation of protocorms of *Vanda coerulea* by encapsulation-dehydration in combination with loading solution. The manipulation of the beads involved a longer dehydration process, but the method was much easier than the vitrification method and avoided the use of toxic vitrification solution (140).

#### 5.4 Cryopreservation of shoot tips

Shoot tips were the explants selected for cryopreservation because they are programmed to directly develop into shoots without intermediate callus formation. However, when compared to other plant materials, the shoot tip survival after cryopreservation is very low because shoot tips contain many types of tissues which dehydration and freezing tolerance are different among tissues. Each tissue requires different optimal treatments for cryopreservation.

The slow freezing method was applied for cryopreservation of shoot tips of *Vanda coerulea*. Plant cells dehydrated with cryoprotectant are slowly prefrozen to about  $-40^{\circ}\text{C}$  to  $-100^{\circ}\text{C}$  prior to being immersed in LN. There are two factors that are known to affect the viability of frozen cells; (i) is the combination of cryoprotectants and (ii) is a freezing programme that permit extracellular freezing and the occurrence of protective dehydration. Zhang et al. (205) suggested that cooling rate, holding temperature and holding time had effects on the survival of cryopreserved *Laminaria japonica*. So, an optimum free-dehydration has to be achieved by combining an appropriate cooling rate with holding temperature and holding time. After the slow freezing steps, cells were sufficiently dehydrated, and then immersed in LN. By rapid immersion into LN, the cells were completely vitrified without crystallization and stored without cryoinjury.

In this study, survival rate of cryopreserved shoot tips of *Vanda coerulea* was 20% when precultured on 0.3 M sucrose for 1 d, treated with LS without dehydration with PVS2 solution and then stored in freezer at  $-80^{\circ}\text{C}$  for 6 h before plunging into LN. No survival rate of cryopreserved shoot tip was observed when dehydrated with PVS2 solution. PVS2 solution has the effect on survival rate of cryopreserved shoot tips because over-exposure to PVS2 can cause chemical toxicity (142). PVS2 solution

has to penetrate into plant cells, the effect of PVS2 solution depended on the temperature during incubation and size of tissue or explant.

Menges and Murray (103) reported that *Arabidopsis* suspension were precultured in 0.5 M sorbitol, and then treated with 5% DMSO at 4°C for 1 h, stored in freezer at -80°C. A controlled slow freezing rate of 0.5°C/min during storage at -80°C/min for 5.5 h prior to transfer into LN resulted in optimum viability of up to 90%. Zhang et al. (205) reported that a process of cooling to -60°C at a rate of 1°C/min and holding at this temperature for 40 min was optimal, resulting in 59% survival of cryopreserved gametophyte of *Laminaria japonica*. They suggested that cooling rate, holding temperature and holding time had the effect on the survival of cryopreserved *Laminaria japonica*.

The growth characteristic of cryopreserved shoot tips was compared with non-cryopreserved shoot tips. The results showed that non-cryopreserved shoot tips developed into plantlets but cryopreserved shoot tips developed into callus. Callus did not develop from shoot tips but it developed from root buds. In the recovery phase, the ability of plant tissues to grow into healthy, genetically identical, variable plants after LN immersion is vitally important for cryopreservation to be effectively applied for conservation (176). The development of callus on shoot tips after cryopreservation is generally regarded as undesirable, since it is an induction of cryojury and is associated with somaclonal variation (190). Wang et al. (190) suggested that the presence of cytokinins in the recovery medium is essential to induce and accelerate shoot tip regrowth, but concentration has to be carefully selected in order to avoid callus formation because it can cause somaclonal variation. Similarly, Wang et al. (188) suggested that an appropriate medium must meet the requirements for high survival and fast regrowth of cryopreserved shoot tip without callus formation.

Protocorm-like body formation was induced when callus from cryopreserved shoot tip was cultured in ND liquid medium supplemented with 1 mg/l BA in combination with 0.5 mg/l NAA and 30 g/l maltose. Protocorm-like bodies formed shoots when cultured in ND liquid medium supplemented with 1 mg/l BA in combination with 0.5 mg/l NAA and, 10, 30 and 40 g/l maltose. However, 10 g/l maltose gave the highest number of shoots. Concentration of maltose had the effect on

protocorm-like body and shoot formation. Protocorm-like bodies formed shoots in medium supplemented with 30 and 40 g/l maltose but they gave browning of shoots. Trehalose has been used on protocorm-like body proliferation (90). Chen and Chang (22) reported that concentration of sucrose may affect on somatic embryogenesis. Increasing the sucrose concentration up to 20 g/l had no significant effect on protocorm-like body formation, whereas at 30 g/l, it decreased the percentage of explants and protocorm-like body formation of *Aranda* Deborah using thin section culture (84). Liu et al. (90) reported that in liquid culture system, both KC and NP media containing trehalose showed better results for protocorm-like body proliferation than sucrose containing media. Tokuhara and Mii (169) reported that embryogenesis calluses were induced from *Phalaenopsis* shoot tip explants excised from flower stalk buds by cultured on ND medium supplemented with 0.5  $\mu$ M NAA, 4.4  $\mu$ M BA and 29.2 mM sucrose. The browning of protocorms could be reduced by decreasing the sucrose concentration and most protocorms remained green and grew into plantlets on media supplemented 29.2 mM sucrose. A high concentration of sucrose might act as an osmotic stress or to inhibit chlorophyll formation to induce embryogenic callus formation.

When transferred shoots to ND solid medium, they died but shoots produced root on modified VW agar medium. The modified VW agar medium supplemented with activated charcoal which enhanced the induction of roots (14). Effect of activated charcoal on shoot proliferation is that it improves aeration and adsorbs ethylene which can inhibit growth and proliferation (35). No morphological variation between non-cryopreserved and cryopreserved plantlets developed from callus was detected and did not have the effect on the ploidy levels of *Vanda coerulea*.

### **5.5 Cryopreservation of protocorm-like bodies**

Protocorms and protocorm-like bodies have the potential to regenerate plants, and represent thus a source of material for germplasm conservation. Only very few cases of orchid has been successfully survived following freezing and stored in LN such as, protocorm-like bodies of *Oncidium* (104).

This study represents the successful cryopreservation of protocorm-like bodies using droplet-vitrification method. Droplet-vitrification technique, a combination of

droplet-freezing and solution-based vitrification consists in cooling sample in droplets of vitrification solution, then placed on aluminium foil strip in minute droplet of vitrification solution and then frozen by rapidly immersion in LN (143). Rapid warming by dipping the aluminium foil strips in pre-heated unloading (76). The highest germination rate of these plant materials was observed when samples were placed on aluminium foil and then directly plunged into LN for freezing and directly put in 1.2 M sucrose (unloading) during warming at 25°C. During the cooling and warming procedures, rapid heat transfer is needed to avoid freezing injury (2, 76, 165). Rapid warming, aluminium foil with samples were directly plunged into unloading solution which was faster than thawing LN in water bath at 40°C and adding unloading solution into cryotubes. Aluminium foil has an efficient thermal conductivity, resulting in good heat distribution among tissue (50, 76).

Preculture with high concentration of sucrose enhanced the tolerance of protocorm-like bodies against the treatment with the PVS2 solution and improved regrowth of cryopreserved protocorms-like bodies. Sucrose has an osmotic dehydration to reduce water content in plant cells and it is able to enter the cells. The accumulation of soluble sugars in cells is thought to protect proteins and membranes from damaging effects of dehydration and freezing in LN (104). The loading solution was very efficient in inducing dehydration and freezing tolerance in plant tissue (143).

The results showed that survival rate of non-cryopreserved and cryopreserved protocorm-like bodies was 75% and 5%, respectively when precultured in 0.3 M sucrose for 1 d without dehydration with PVS2 solution. Survival rate of cryopreserved protocorm-like bodies was about 5% when precultured in 0.5 M sucrose, and then dehydrated with PVS2 solution for 30 min and without preculture but dehydrated with PVS2 solution for 30 min. The incubation time in PVS2 solution affected the survival rate of protocorm-like bodies, because over exposure to PVS2 solution can cause chemical toxicity. PVS2 solution has to penetrate into plant cells, the effect of PVS2 solution depended on the temperature during incubation and size of tissue or explant. Exposure time to PVS2 solution at room temperature (25°C) for 20 min was sufficient (50, 76). In this study, PVS2 solution affected on survival rate of non-cryopreserved and cryopreserved protocorm-like bodies.

### **5.6 Genetic variation of plantlets from seeds of *Vanda coerulea* using RAPD markers**

The RAPD markers were efficiently used for estimating the genetic variation of plantlets of *Vanda coerulea*. The size of the amplified DNA fragments ranged from 310 bp to above 1353 bp. Lim et al. (88) reported RAPD analysis of some species in the genus *Vanda* (Orchidaceae). RAPD is an easy and cost-effective profiling assay based on PCR with arbitrary primers that by amplifying a set of DNA segments randomly distributed throughout the genome, can detect genetic polymorphisms. Nevertheless, RAPD makers have some limitations concerning reproducibility and an uncertain homology of co-migrating fragments in gel electrophoresis (136). These limitations can be minimized by carefully adjusting the reaction and detection condition (122).

### **5.7 Universal primers for amplification of the *trnL* (UAA) region of cpDNA for *Vanda coerulea*, some orchid species and *Oryza sativa***

cpDNA variation has been used extensively to investigate phylogenetic relationships among plants (45, 200) hybrid zones but restriction analysis and/or gene sequences suggest that chloroplast genes have slow rates of nucleotide substitution with an average synonymous rate almost half that of plant nuclear DNA (193). However, non-coding regions of cpDNA display the highest frequency of mutation, exhibit faster mutation rates, especially through the addition and/or deletion of short sequences (207). One region shown to be particularly useful at detecting species level variation is the *trnL* (UAA) cpDNA intron (41, 46, 132, 162). The whole *trnL* intron has many advantages: the primers are highly conserved and the amplification system is very robust (161). Taberlet et al. (162) studied the universal primers a, b, c, d, e and f for amplification three non-coding regions.

In this study, the *trnL* (UAA) non-coding region of cpDNA for *Vanda coerulea* was amplified with universal primers c and d (162). Out of the 12 temperatures (49.2, 49.3, 49.7, 50.3, 51.0, 51.9, 52.7, 53.6, 54.4, 55.0, 55.5 and 55.8°C) of annealing for 1 min were used in this study. Eight temperatures (49.2-53.6°C) failed to amplify DNA, the number of bands were two bands. Four temperatures (54.4, 55.0, 55.5 and 55.8°C) produced one band pattern of the *trnL*

(UAA) region of cpDNA for *Vanda coerulea*. The size of amplified fragment was 872 bp. The optimum annealing temperature for amplification of the *trnL* (UAA) region of cpDNA for *Vanda coerulea* was 55.0°C. Similarly, Paulsrud and Lindblad (127) reported that an annealing temperature for amplification of the *trnL* (UAA) region of cpDNA for *Nostoc* symbionts in some lichens was 55.0°C for 1 min. Taberlet et al. (162) reported that an annealing temperature for amplification three non-coding regions: (i) *trnL* (UGU) and the *trnL* (UAA), (ii) the *trnL* (UAA) intron and (iii) another intergenic spacer between the *trnL* (UAA) 3' exon and *trnF* (GAA) with the universal primers a, b, c, d, e and f was 50-55°C for 1 min. Procaccini et al. (132) reported the amplification of the *trnL* (UAA) chloroplast intron with primers C, C1, C2, D, D1, D2, D3, and D4 for seagrass were carried out using 94°C for 15 min, followed by 35 cycles (1.5 min at 94°C, 1 min at 55°C, 3 min at 72°C) and 7 min extension at 72°C. Saltonstall (146) reported that non-coding region of cpDNA in the grasses was amplified with primers using 56°C annealing for 1 min. James and Schmidt (70) reported that the amplification of the wheat and rice within the *trnL* (UAA) region using primer WT-1F and PL-2D and RE-2F and PL-2D, respectively, an annealing temperature was 58°C for 1 min.

There is no difference the size of the *trnL* (UAA) region of cpDNA for plantlets of *Vanda coerulea* from Kanchanaburi, Chiang Mai and Bangkok province. However, result showed the variation in size of the *trnL* (UAA) region of cpDNA for *Vanda coerulea*, *Vanda tricolor*, *Dendrobium aphyllum*, *Dendrobium lindleyi*, *Bletilla striata*, *Grammatophyllum speciosum* and *Oryza sativa*. The sequences of the *trnL* (UAA) region of cpDNA for *Vanda coerulea* was 876 bp. Procaccini et al. (132) reported the size of the tRNA<sup>Leu</sup> (UAA) intron ranged from 547 bp in *Triglochin maritimum* to 723 bp in *Halophila stipulacea*. James and Schmidt (70) reported that size of the tRNA (*trnL*) intron for wheat, peanut, soybean, potato and canola were about 662, 642, 584, 571 and 387 bp, respectively.

### 5.8 Genetic stability after cryopreservation

The successful of the conservation of plant genetic resource is not only to store germplasm but also to minimize variations to the lowest variable level during conservation. One important aspect of cryopreservation is the genetic fidelity of plants recovered from cryopreservation.

The genetic fidelity of *Vanda coerulea* was demonstrably maintained after storage in LN at the morphological level and molecular marker. Molecular markers are a more direct way to examine genetic variable at the DNA level.

RAPD markers have been also used successfully to test genetic stability of shoot tips of *Dioscorea floribunda* (3), hybrid aspen (*Populus tremula* L. X *Populus tremuloides* Michx.) (72), meristem-tips of *Melia azedarach* (149) were successfully cryopreserved, RAPD markers showed no genetic variation. Zhang et al. (204) tested plantlets regenerated from shoot tips of *Amorphophallus* cryopreserved by vitrification using RAPD markers and did not find any DNA alteration. No genetic changes were observed in RAPD profile of cryopreserved embryogenic cultures of Scot pine (*Pinus sylvestris* L.) (49). Similarly, Liu et al. (89) found that no different RAPD fragments were detected between non-cryopreserved and cryopreserved shoots using fifteen random primers.

When assayed by RAPD profiles, a widely used cryoprotect, DMSO was shown to cause considerable genetic variation in the DMSO-treated but non-frozen embryogenic cultures of *Abies cephalonica* (5). In this study, sixty 10-base primers were selected to amplify the DNA of non-cryopreserved and cryopreserved protocorm-like bodies using RAPD markers. Primers OPC-01 to 20, OPE-01 to 20 and OPK-01 to 20 produced 233 electrophoresis bands, no differences were found in comparison with the non-cryopreserved protocorm-like bodies. Zhai et al. (203) found that RAPD profiles observed were similar between non-cryopreserved and cryopreserved shoot tips of grape and kiwi. Jokipii et al. (72) reported that no change of genetic were observed in hybrid aspen stored in LN or when DMSO was used in the PGD (10% PEG 6000, 10% glucose and 10% DMSO) cryoprotectant mixtures. RAPD analysis with ten RAPD primers (OPC-01, OPD-14, -15, OPE-05, -06, -09, -10, -17, OPF-03 and -06) assays the genetic fidelity of the cryostored material was maintained during both slow cooling and vitrification method. Thus, the RAPD

marker is a fast, simple and efficient genetic stability evaluation method that can be used rapidly after cryopreservation. However, Zhai et al. (203) reported that RAPD markers were employed to test the genetic stability of grape and kiwi in vitro plantlets from cryopreserved shoot tips. Polymorphic RAPD markers cover approximately 0.004-0.06% of the grape genome and 0.005-0.07% of the kiwi genome.

In this study the *trnL* (UAA) region of cpDNA was selected for tested genetic stability after cryopreservation of protocorm-like bodies by droplet-vitrification method. The size of amplified fragment of the *trnL* (UAA) region of cpDNA for non-cryopreserved and cryopreserved protocorm-like bodies of *Vanda coerulea* was 872 bp. The sequences of the *trnL* (UAA) region of cpDNA for non-cryopreserved protocorm-like bodies of *Vanda coerulea* was 876 bp, no differences were found in comparison with cryopreserved protocorm-like bodies of *Vanda coerulea*. Molecular techniques consider the analysis of isolate double strand DNA in contrast to epigenetic. The epigenetic molecular mechanisms do not involve the primary DNA sequence but they affect the inheritance of characters which are largely DNA-protein interactions at the higher order levels of nucleosome and nucleosomal arrays within chromatin structure. DNA-protein interactions are effected by histone chemical modification (acetylation, methylation and phosphorylation). Epigenetic variation in chromatin and DNA methylation of gene sequence has been found in plant after cryopreservation, resulting in alteration pattern of gene expression, where the possibility exists for plant to alter their phenotypic characters (55). Any genetic stability assessment should not be based only on molecular studies but must also include physiological and most importantly morphological examination of plant regeneration (203). In this study showed that no morphological variation between plantlets from non-cryopreserved and cryopreserved plantlets was detected. Nuclei were isolated from leaves of non-cryopreserved and cryopreserved plantlets showed similar patterns of ploidy levels by using flow cytometry analysis.

## **CHEPTER VI**

### **CONCLUSIONS**

#### **6.1 Micropropagation of *Vanda coerulea***

6.1.1 The survival rate of shoot tips cultured on modified VW agar medium was higher than MS agar medium.

6.1.2 Rooting occurred on modified VW agar medium with and without sucrose but plantlets did not produce any root on MS agar medium without sucrose.

6.1.3 The highest number of shoots and roots were observed on modified VW agar medium supplemented with 10 g/l sucrose.

6.1.4 The highest survival rate (98%) was observed for shoot tips grown on modified VW agar medium with 1 mg/l BA in combination with 0.5 mg/l NAA.

6.1.5 The highest shoot buds (8.7 shoot buds/explant) and number of shoots (5.3 shoots/explant) were observed on modified VW agar medium supplemented with 1 mg/l BA.

6.1.6 The highest shoot produced roots (62%) and number of roots (3 roots/explant) were observed on modified VW agar medium supplemented with 0.5 mg/l NAA in combination with 2 mg/l TDZ.

6.1.7 There was no difference in morphology and similar patterns of ploidy levels were observed by flow cytometry from the leaves of both stock plants and regenerated plants.

6.1.8 Survival rate of plantlets cultured on osmunda in the greenhouse for 3 months was 100%.

#### **6.2 Cryopreservation of *Vanda coerulea***

6.2.1 This is the first report of successful cryopreservation of seeds of *Vanda coerulea* by encapsulation-dehydration and encapsulation-vitrification method. One encapsulated bead contained about 11 seeds, thus germination rate of cryopreserved

seeds by encapsulation-dehydration and encapsulation-vitrification methods gave higher than those of the seeds by vitrification method. The manipulation of the beads was a longer dehydration process but it was much easier than vitrification method because seeds of *Vanda coerulea* are very small which are difficult for handling, preculture and loading treatments.

6.2.2 Some *Vanda coerulea* fruits have small amount of seeds in addition to low seed fertility. Therefore, cryopreserving protocorms have some advantages over seeds because we can estimate regrowth of successful cryopreserved protocorms and it is much easier to handle protocorms than seeds in cryopreservation protocol. This is the first report of successful cryopreservation of protocorms of *Vanda coerulea* by encapsulation-dehydration in combination with loading solution. Regrowth rate following encapsulation-dehydration is dependent on the amount of water presented in the cells. The highest regrowth rate of cryopreserved protocorms was 40% at 35% water content after 8 h dehydration

6.2.3 The survival rate of cryopreserved shoot tips after cryopreservation by slow freezing method was 20% without dehydration with PVS2 solution. PVS2 solution has the effect on survival rate of cryopreserved shoot tips because over-exposure to PVS2 solution can cause chemical toxicity. Protocorm-like body proliferation from cryopreserved shoot tips were observed in ND liquid medium supplemented with 1 mg/l BA in combination with 0.5 mg/l NAA and 30 g/l maltose. Protocorm-like bodies formed shoots in ND liquid medium supplemented with 1 mg/l BA in combination with 0.5 mg/l NAA and 10 g/l maltose. Shoots produced roots on modified VW agar medium.

6.2.4 Survival rate cryopreserved protocorm-like bodies after cryopreservation by droplet-vitrification method was 5% when precultured in 0.3 M sucrose for 1 d without dehydration with PVS2 solution, precultured in 0.5 M sucrose, and then dehydrated with PVS2 solution for 30 min and without preculture but dehydrated with PVS2 solution for 30 min.

6.2.5 Similar patterns of ploidy levels were observed by flow cytometry from the leaves of both non-cryopreserved and cryopreserved plantlets.

6.2.6 There was no difference in morphology of non-cryopreserved plantlets and cryopreserved plantlets.

### **6.3 Genetic stability after cryopreservation**

6.3.1 RAPD marker has efficiently used for estimating the genetic variation of plantlets of *Vanda coerulea*.

6.3.2 The *trnL* (UAA) region of cpDNA for *Vanda coerulea* was amplified with universal primers c and d. The annealing at 55°C for 1 min was selected for PCR amplification. The size of amplified fragment was 872 bp. The sequences of the *trnL* (UAA) region of cpDNA for *Vanda coerulea* were 876 bp.

6.3.4 The out of sixty 10-base primers used for estimating the genetic variation of protocorm-like bodies after cryopreservation by droplet-vitrification method. The same RAPD patterns of non-cryopreserved and cryopreserved protocorm-like bodies were observed.

6.3.5 The *trnL* (UAA) region of cpDNA for *Vanda coerulea* was selected for testing genetic stability after cryopreservation. The size and sequences of the *trnL* (UAA) region of cpDNA for non-cryopreserved and cryopreserved protocorm-like bodies were not different. The size of amplified fragment was 872 bp. The sequences of the *trnL* (UAA) region were 876 bp.

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## **APPENDIX**

**APPENDIX A****Media for micropropagation and cryopreservation**

## 1. Components of Modified Vancin and Went (VW, 1949) medium.

Components	mg/l
Stock I	
KNO <sub>3</sub>	525
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	500
KH <sub>2</sub> PO <sub>4</sub>	250
MnSO <sub>4</sub> .H <sub>2</sub> O	5.7
Stock II	
MgSO <sub>4</sub> .7H <sub>2</sub> O	250
Stock III	
FeSO <sub>4</sub> .7H <sub>2</sub> O	27.85
Na <sub>2</sub> EDTA	37.25
Ca <sub>3</sub> (PO <sub>4</sub> ) <sub>2</sub>	200
Sucrose	10,000-20,000
Agar	8,000
Activated charcoal	0.7 g
Banana	100 g
Coconut water	150 ml
pH 5.2	

Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub> were dissolved in 10 ml of 1 N HCl.

## 2. Components of New Dogashima (ND, 1993) medium.

Components	mg/l
Macro elements	
NH <sub>4</sub> NO <sub>3</sub>	480
KNO <sub>3</sub>	200
Ca(NO <sub>3</sub> ) <sub>2</sub> .4H <sub>2</sub> O	470
KCl	150
MgSO <sub>4</sub> .7H <sub>2</sub> O	250
KH <sub>2</sub> PO <sub>4</sub>	550
Micro elements	
MnSO <sub>4</sub> .4H <sub>2</sub> O	3
ZnSO <sub>4</sub> .7H <sub>2</sub> O	0.5
H <sub>3</sub> BO <sub>3</sub>	0.5
CuSO <sub>4</sub> .5H <sub>2</sub> O	0.025
Na <sub>2</sub> MoO <sub>4</sub> .2H <sub>2</sub> O	0.025
CoCl <sub>2</sub> .6H <sub>2</sub> O	0.025
Concentrated H <sub>2</sub> SO <sub>4</sub>	0.5 µl
Organic compounds	
Myo-Inositol	100
Nicotinic acid	1
Pyridoxine hydrochloride	1
Thiamine hydrochloride	1
Calcium pantothenate	1
Adenine	1
L-Cysteine	1
D-Biotin	0.1
Sucrose	10,000
Gel rite	0.25%
pH 5.4	

## 3. Components of Murashige and Skoog (MS, 1962) medium.

Components	mg/l
Macro elements	
NH <sub>4</sub> NO <sub>3</sub>	1,650
KNO <sub>3</sub>	1,900
CaCl <sub>2</sub> .2H <sub>2</sub> O	440
MgSO <sub>4</sub> .7H <sub>2</sub> O	370
KH <sub>2</sub> PO <sub>4</sub>	170
Micro elements	
H <sub>3</sub> BO <sub>3</sub>	6.2
MnSO <sub>4</sub> .4H <sub>2</sub> O	22.3
ZnSO <sub>4</sub> .7H <sub>2</sub> O	22.3
KI	0.83
Na <sub>2</sub> MoO <sub>4</sub> .2H <sub>2</sub> O	0.25
CuSO <sub>4</sub> .5H <sub>2</sub> O	0.025
CoCl <sub>2</sub> .6H <sub>2</sub> O	0.025
Na <sub>2</sub> EDTA	37.25
FeSO <sub>4</sub> .7H <sub>2</sub> O	27.85
Organic compounds	
Myo-Inositol	100
Glycine	2.0
Nicotinic acid	0.5
Pyridoxine hydrochloride	0.1
Thiamine hydrochloride	0.1
Sucrose	30,000
Agar	8,000
pH 5.8	

## APPENDIX B

### Solution for cryopreservation

#### 1. Loading solution (LS)

Glycerol	2	M
Sucrose	0.4	M

Dissolved glycerol in VW (1949) or ND liquid medium supplemented with 0.4 M sucrose. After dissolving, sterilized in an autoclave at 121°C for 15 min and kept at 4°C.

#### 2. Plant Vitrification Solution formula 2 (PVS2)

Glycerol	30%	(w/v)
Ethylene glycol (EG)	15%	(w/v)
Dimethyl sulphoxide (DMSO)	15%	(w/v)
Sucrose	0.4	M

Dissolved glycerol, ethylene glycol and dimethyl sulphoxide in VW or ND liquid medium supplemented with 0.4 M sucrose. After dissolving, sterilized in an autoclave at 121°C for 15 min and kept at 4°C.

#### 3. Na-alginate solution for seed cryopreservation

Na-alginate	3%	(w/v)
Sucrose	0.4	M

Slowly and carefully added 3% Na-alginate in VW liquid medium supplemented with 0.4 M sucrose due to low solubility. After mixing homogeneous, sterilized in an autoclave at 121°C for 15 min and kept at 4°C.

**4. Na-alginate solution for protocorm cryopreservation**

Na-alginate	2%	(w/v)
Glycerol	2	M
Sucrose	0.4	M

Slowly and carefully added 2% Na-alginate in VW liquid medium supplemented with 2 M glycerol and 0.4 M sucrose due to low solubility. After mixing homogeneous, sterilized in an autoclave at 121°C for 15 min and kept at 4°C.

**5. Calcium chloride solution for seed cryopreservation**

CaCl <sub>2</sub>	100	mM
Sucrose	0.4	M

Dissolved CaCl<sub>2</sub> in VW liquid medium supplemented with 0.4 M sucrose. After dissolving, sterilized in an autoclave at 121°C for 15 min and kept at 4°C.

**6. Calcium chloride solution for protocorm cryopreservation**

CaCl <sub>2</sub>	100	mM
Glycerol	2	M
Sucrose	0.4	M

Dissolved CaCl<sub>2</sub> in VW liquid medium supplemented with 2 M glycerol and 0.4 M sucrose due to low solubility. After mixing homogeneous, sterilized in an autoclave at 121°C for 15 min and kept at 4°C.

**APPENDIX C****Solution for flow cytometry analysis****1. Propidium iodide stock solution**

1 mg/ml propidium iodide 50 mg

- Dissolve in 50 ml H<sub>2</sub>O
- Filter through 0.22 µm filter to remove small particles
- Store at -20°C in 0.5 ml aliquots

**2. RNase stock solution**

1 mg/ml RNase (IIA Sigma) 25 mg

- Dissolve in 50 ml H<sub>2</sub>O
- Filter through 0.22 µm filter to remove small particles
- Heat to 90°C for 15 min to inactivate DNase
- Store at -20°C in 0.5 ml aliquots

**3. Tris-MgCl<sub>2</sub> buffer stock solution**

0.2 M Tris 484 g

4 mM MgCl<sub>2</sub>.6H<sub>2</sub>O 162.64 mg

0.5% Trion X-100 1 ml

- Adjust volume 200 ml
- Adjust pH to 7.5
- Filter through 0.22 µm filter to remove small particles
- Store at 4°C

**APPENDIX D**  
**Data of cryopreservation**

Table 17. Effects of loading solution and dehydration with PVS2 solution on germination rate of non-cryopreserved seeds (-LN) and cryopreserved seeds (+LN) of *Vanda coerulea* after cryopreservation by vitrification method. Seeds without preculture, were treated with or without loading solution for 15 min at  $25 \pm 3^\circ\text{C}$  before dehydration with PVS2 solution on ice for 0-140 min, followed by immersion into LN.

Exposure time to PVS2 (min)	Without loading solution		Loading solution	
	-LN	+LN	-LN	+LN
20	93.3 $\pm$ 0.89 <sup>a</sup>	37.7 $\pm$ 0.67 <sup>a</sup>	95.0 $\pm$ 0.58 <sup>a</sup>	38.7 $\pm$ 0.88 <sup>b</sup>
40	93.0 $\pm$ 0.58 <sup>a</sup>	13.0 $\pm$ 1.53 <sup>b</sup>	94.3 $\pm$ 0.33 <sup>a</sup>	46.7 $\pm$ 0.33 <sup>a</sup>
60	91.3 $\pm$ 0.33 <sup>a</sup>	8.0 $\pm$ 0.58 <sup>c</sup>	92.3 $\pm$ 0.33 <sup>a</sup>	36.7 $\pm$ 0.67 <sup>b</sup>
100	90.3 $\pm$ 0.33 <sup>a</sup>	11.7 $\pm$ 0.33 <sup>b</sup>	90.3 $\pm$ 0.33 <sup>a</sup>	20.0 $\pm$ 0.58 <sup>c</sup>
120	89.7 $\pm$ 0.33 <sup>a</sup>	8.0 $\pm$ 0.58 <sup>c</sup>	89.7 $\pm$ 0.33 <sup>a</sup>	19.7 $\pm$ 0.88 <sup>c</sup>
140	89.7 $\pm$ 0.67 <sup>a</sup>	9.0 $\pm$ 0.58 <sup>c</sup>	89.7 $\pm$ 0.33 <sup>a</sup>	21.0 $\pm$ 0.58 <sup>c</sup>

Data based on 100 seeds per treatment (The experiment was replicated four times).  
Mean  $\pm$  Standard Error.

Similar letters within columns mean no significant difference at  $P \leq 0.05$  by LSD test.

Table 18. Change in water content of encapsulated seeds of *Vanda coerulea* precultured in the modified VW liquid medium supplemented with 0.5 and 0.7 M sucrose for 18 h, then dehydration in the laminar air-flow cabinet for 0-8 h.

Dehydration time (h)	Water content (%)	
	0.5 M sucrose	0.7 M sucrose
0	81 ± 1.2 <sup>a</sup>	77 ± 0.6 <sup>a</sup>
1	73 ± 1.7 <sup>b</sup>	70 ± 0.6 <sup>b</sup>
2	68 ± 0.6 <sup>c</sup>	60 ± 0.6 <sup>c</sup>
3	53 ± 0.6 <sup>d</sup>	46.7 ± 1.0 <sup>d</sup>
4	50 ± 0.6 <sup>d</sup>	30 ± 0.6 <sup>e</sup>
5	40 ± 0.6 <sup>e</sup>	30 ± 0.1 <sup>e</sup>
6	25 ± 0.6 <sup>f</sup>	22.2 ± 0.6 <sup>f</sup>
7	25 ± 0.6 <sup>f</sup>	22.2 ± 0.6 <sup>f</sup>
8	25 ± 0.0 <sup>f</sup>	22.2 ± 0.0 <sup>f</sup>

Data based on 10 beads per treatment (The experiment was replicated four times).

Mean ± Standard Error.

Similar letters within columns mean no significant difference at  $P \leq 0.05$  by LSD test.

Table 19. Effect of dehydration in the laminar air-flow cabinet on germination rate of non-cryopreserved seeds (-LN) and cryopreserved seeds (+LN) of *Vanda coerulea* after cryopreservation by encapsulation-dehydration method. Encapsulated seeds were precultured in the modified VW liquid medium supplemented with 0.5 and 0.7 M sucrose for 18 h, then dehydration in the laminar air-flow cabinet for 0-8 h, followed by immersion into liquid nitrogen.

Exposure time to PVS2 (min)	Germination rate (%)			
	0.5 M sucrose		0.7 M sucrose	
	- LN	+LN	-LN	+LN
0	100 ± 0.0 <sup>a</sup>	2.5 ± 2.5 <sup>g</sup>	100 ± 0.0 <sup>a</sup>	7.5 ± 2.5 <sup>e</sup>
30	100 ± 0.0 <sup>a</sup>	10.0 ± 4.1 <sup>f</sup>	100 ± 0.0 <sup>a</sup>	12.5 ± 2.5 <sup>e</sup>
60	100 ± 0.0 <sup>a</sup>	22.5 ± 2.5 <sup>e</sup>	100 ± 0.0 <sup>a</sup>	17.5 ± 4.8 <sup>d</sup>
90	100 ± 0.0 <sup>a</sup>	27.5 ± 4.8 <sup>e</sup>	100 ± 0.0 <sup>a</sup>	20.0 ± 4.1 <sup>d</sup>
120	100 ± 0.0 <sup>a</sup>	70.0 ± 4.1 <sup>b</sup>	100 ± 0.0 <sup>a</sup>	50.0 ± 4.1 <sup>c</sup>
150	97.5 ± 2.5 <sup>a</sup>	82.5 ± 8.5 <sup>a</sup>	97.5 ± 2.5 <sup>a</sup>	75.0 ± 6.5 <sup>a</sup>
180	97.5 ± 2.5 <sup>a</sup>	57.5 ± 4.8 <sup>c</sup>	97.5 ± 2.5 <sup>a</sup>	62.5 ± 4.7 <sup>b</sup>
210	95.0 ± 2.9 <sup>a</sup>	42.5 ± 2.5 <sup>d</sup>	95.0 ± 2.9 <sup>a</sup>	50.0 ± 7.1 <sup>c</sup>
240	92.5 ± 2.5 <sup>a</sup>	35.0 ± 6.5 <sup>d</sup>	95.0 ± 2.9 <sup>a</sup>	45.0 ± 6.5 <sup>c</sup>

Data based on 10 beads per treatment (The experiment was replicated four times).  
Mean ± Standard Error.

Similar letters within columns mean no significant difference at  $P \leq 0.05$  by LSD test.

Table 20. Effect of dehydration with PVS2 solution on germination rate of non-cryopreserved seeds (-LN) and cryopreserved seeds (+LN) of *Vanda coerulea* after cryopreservation by encapsulation-vitrification method. Encapsulated seeds were precultured in the modified VW liquid medium supplemented with 0.5 and 0.7 M sucrose for 18 h, then treated with loading solution for 15 min at  $25 \pm 3^\circ\text{C}$  before dehydration with PVS2 solution on ice at  $0^\circ\text{C}$  for 0-240 min, followed by immersion into LN.

Dehydration time (h)	Germination rate (%)			
	0.5 M sucrose		0.7 M sucrose	
	-LN	+LN	-LN	+LN
0	$100 \pm 0.0^a$	$0^e$	$100 \pm 0.0^a$	$0^e$
1	$100 \pm 0.0^a$	$0^e$	$100 \pm 0.0^a$	$0^e$
2	$100 \pm 0.0^a$	$0^e$	$100 \pm 0.0^a$	$2.5 \pm 2.50^d$
3	$100 \pm 0.0^a$	$27.5 \pm 4.79^d$	$100 \pm 0.0^a$	$12.5 \pm 2.50^c$
4	$100 \pm 0.0^a$	$37.5 \pm 4.79^c$	$100 \pm 0.0^a$	$42.5 \pm 4.79^b$
5	$100 \pm 0.0^a$	$97.5 \pm 2.50^a$	$100 \pm 0.0^a$	$92.5 \pm 4.79^a$
6	$100 \pm 0.0^a$	$100 \pm 0.00^a$	$100 \pm 0.0^a$	$100 \pm 0.00^a$
7	$100 \pm 0.0^a$	$100 \pm 0.00^a$	$100 \pm 0.0^a$	$100 \pm 0.00^a$
8	$100 \pm 0.0^a$	$90.0 \pm 4.08^a$	$100 \pm 0.0^a$	$92.5 \pm 2.50^a$

Data based on 10 beads per treatment (The experiment was replicated four times).

Mean  $\pm$  Standard Error.

Similar letters within columns mean no significant difference at  $P \leq 0.05$  by LSD test.

Table 21. Effect of dehydration time on the water content of encapsulated beads of *Vanda coerulea* protocorms precultured in modified VW liquid medium supplemented with 0.7 M sucrose at  $25 \pm 3^\circ\text{C}$  for 20 h, dehydration in the laminar air-flow cabinet for 0-10 h based on fresh weight.

Dehydration time (h)	water content (%)
0	$78.0 \pm 2.2$
1	$76.9 \pm 2.2$
2	$69.5 \pm 5.5$
3	$55.6 \pm 5.6$
4	$50.3 \pm 3.6$
5	$47.7 \pm 2.3$
6	$39.2 \pm 1.0$
7	$36.4 \pm 1.0$
8	$34.9 \pm 1.5$
9	$33.2 \pm 3.2$
10	$24.8 \pm 2.5$

Data based on 10 beads per treatment (The experiment was replicated four times).  
Mean  $\pm$  Standard Error.

Table 22. Effect of dehydration time in the laminar air-flow cabinet on regrowth rate of non-cryopreserved protocorms (-LN) and cryopreserved protocorms (+LN) after cryopreservation by encapsulation-dehydration combination with loading solution. Encapsulated protocorms were precultured in modified VW liquid medium supplemented with 0.7 M sucrose at  $25 \pm 3^\circ\text{C}$  for 20 h, then dehydrated in the laminar air-flow cabinet for 0-10 h, followed by immersion into LN.

Dehydration time (h)	Regrowth rate (%)	
	-LN	+LN
0	$61.5 \pm 4.5^a$	$0^e$
1	$61.0 \pm 4.8^a$	$0^e$
2	$61.0 \pm 3.3^a$	$0^e$
3	$61.3 \pm 4.4^a$	$1.3 \pm 1.3^d$
4	$62.5 \pm 6.5^a$	$7.5 \pm 2.5^{cd}$
5	$61.3 \pm 4.4^a$	$13.8 \pm 3.2^c$
6	$51.3 \pm 3.0^b$	$27.5 \pm 2.5^b$
7	$48.8 \pm 4.0^b$	$30.0 \pm 3.3^b$
8	$46.3 \pm 3.8^{bc}$	$41.3 \pm 2.3^a$
9	$46.3 \pm 3.8^{bc}$	$35.0 \pm 4.2^{ab}$
10	$42.5 \pm 3.1^c$	$33.8 \pm 3.1^{ab}$

Data based on 10 beads per treatment (The experiment was replicated four times). Mean  $\pm$  Standard Error.

Similar letters within columns mean no significant difference at  $P \leq 0.05$  by LSD test.

## APPENDIX E

### The sequences of the *trnL* (UAA) for non- cryopreserved and cryopreserved protocorm-like bodies of *Vanda coerulea* after cryopreservation by droplet-vitrification method

GGGGATAGAGGGACTTGAACCCTCACGATTGAAAAATTCGACGGATTTTCCTCTTACTATA  
 AATTTCAATTGTTGTCGGTATTGACATGTAATAATGGGACTCTCTCTTTATTCTCGTACGATTC  
 AATTTCAAAGATCTATCAAAAATTCTGGAATGAATAATTTGATTATTGAATATTCGAATT  
 CTATTCCTTTTTCAACTTCAATTGGAATTGATTCACAATAACTCTTCAATTTTTCATATATCT  
 TTTTGATCTCTATCATTCTAATGAAAAAGAATAGAAAAATAGGGTTTCTTATAGATCCTT  
 ATCTCATATATCTCATATTAATAATAGAATCATATTAATAATAGAATCATATTAATAATAG  
 AATATAATATTAATTAATAATATAATATTAATAGAATAATAGAAAGAAAGAGAAGATTTT  
 TATTTTCATATAAAAATTTCAAATTCATATATAAATATATATAAAAATTCATATATAAATA  
 TATATAAATATATAGAGATACAGAAGAGATACAGAATAGAATTCGATATAAGATTTGGGT  
 TGTGATTAATCGTTTGCTATGTCAGTATTTATACGTACGTCTTAGGTATATAAGACGTATCC  
 TTTCTGTCATTTTGATAGAAGTCTTTTAGCTACTAACGTAACGTAATCAATTTTCATTCGTTA  
 GAACAGCTTCCATTGAGTCTCTGCACCTATCCCTTTTTTATTCTTATTTTTCATCTTTCATCG  
 TTTTCTCTCGAAACAAAGATTTGGCTCAGGATTGCCCTTTTTTAGTTCCAGGGTTTCTCTG  
 AATTTGGAAGTTACCACTTAGCAGGTTTCCATACCAAGGCTCAATCCAATCAAGTCCGTAG  
 CGTNCTACCGATTTTCG

Figure 45. The sequences of the *trnL* (UAA) region of cpDNA for non-cryopreserved protocorm-like bodies of *Vanda coerulea*.

GGGGATAGAGGGACTTGAACCCTCACGATTGAAAAATTCGACGGATTTTCCTCTTACTATA  
 AATTTCAATTGTTGTCGGTATTGACATGTAATAATGGGACTCTCTCTTTATTCTCGTACGATTC  
 AATTTCAAAGATCTATCAAAAATTCTGGAATGAATAATTTGATTATTGAATATTCGAATT  
 CTATTCCTTTTTCAACTTCAATTGGAATTGATTCACAATAACTCTTCAATTTTTCATATATCT  
 TTTTGATCTCTATCATTCTAATGAAAAAGAATAGAAAAATAGGGTTTCTTATAGATCCTT  
 ATCTCATATATCTCATATTAATAATAGAATCATATTAATAATAGAATCATATTAATAATAG  
 AATATAATATTAATTAATAATATAATATTAATAGAATAATAGAAAGAAAGAGAAGATTTT  
 TATTTTCATATAAAAATTTCAAATTCATATATAAATATATATAAAAATTCATATATAAATA  
 TATATAAATATATAGAGATACAGAAGAGATACAGAATAGAATTCGATATAAGATTTGGGT  
 TGTGATTAATCGTTTGCTATGTCAGTATTTATACGTACGTCTTAGGTATATAAGACGTATCC  
 TTTCTGTCATTTTGATAGAAGTCTTTTAGCTACTAACGTAACGTAATCAATTTTCATTCGTTA  
 GAACAGCTTCCATTGAGTCTCTGCACCTATCCCTTTTTTATTCTTATTTTTCATCTTTCATCG  
 TTTTCTCTCGAAACAAAGATTTGGCTCAGGATTGCCCTTTTTTAGTTCCAGGGTTTCTCTG  
 AATTTGGAAGTTACCACTTAGCAGGTTTCCATACCAAGGCTCAATCCAATCAAGTCCGTAG  
 CGTNCTACCGATTTTCG

Figure 46. The sequences of the *trnL* (UAA) region of cpDNA for cryopreserved protocorm-like bodies of *Vanda coerulea* after cryopreservation by droplet-vitrification method.

The sequences of the *trnL* (UAA) region of cpDNA for *Vanda coerulea* were closed relationships with *Ascocentrum ampullaceum*, *Neofinetia falcata* and *Vanda coerulescens*, respectively.

**APPENDIX F****Presentation and Publication**

1. Nipawan Jitsopakul, Kanchit Thammasiri and Keiko Ishikawa. 2008. Cryopreservation of *Vanda coerulea* protocorms by encapsulation-dehydration method. CryoLetters (in press).
2. Nipawan Jitsopakul, Keiko Ishikawa and Kanchit Thammasiri. 2006. Comparative study of seed cryopreservation of *Vanda coerulea* Griff. ex Lindl., an endangered Thai orchid species. The 2<sup>nd</sup> International Symposium on Diversity and Conservation of Asian Orchids. 3<sup>rd</sup> December 2006. Tsukuba Botanical Garden, National Science Museum, Tsukuba, Japan.
3. Nipawan Jitsopakul, Keiko Ishikawa and Kanchit Thammasiri. 2007. Cryopreservation of *Vanda coerulea* protocorms by encapsulation-dehydration method. The 33<sup>rd</sup> congress on science and technology of Thailand (STT33). 8-20 October, 2007. Walailak University, Nakhon Si Thammarat, Thailand.
4. Nipawan Jitsopakul, Kanchit Thammasiri, Tomohisa Yukawa and Keiko Ishikawa. Effect of cryopreservation on seeds germination and protocorms development of *Vanda tricolor*. The 3<sup>rd</sup> International Symposium on Diversity and Conservation of Asian Orchids. 15<sup>th</sup> December 2007. Tsukuba Botanical Garden, National Science Museum, Tsukuba, Japan.

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