CRYOPRESERVATION OF *DENDROBIUM CRUENTUM* RCHB. F.

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Thesis

Entitled

CRYOPRESERVATION OF

***DENDROBIUM CRUENTUM* RCHB. F.**

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CRYOPRESERVATION OF DENDROBIUM CRUENTUM RCHB. F.

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ABSTRACT

Dendrobium cruentum Rchb. f. is a wild Thai orchid species that faces extinction. Due to the loss of natural habitats and the difficulty of its cultivation, practical conservation methods should be established. Cryopreservation, an important method for long-term storage of biological germplasm under ultra low temperature without deterioration, was studied for *Dendrobium cruentum* Rchb. f.

The seeds of *Dendrobium cruentum* Rchb.f. at varying periods of 2, 3 and 4 months after pollination were cryopreserved by vitrification. The seeds were exposed to plant vitrification solution 2 (PVS2) for 0-90 min at 25±2℃ before being plunged into liquid nitrogen (LN). The water content of the seeds decreased from 81.6%FW to 13.1%FW as the seed pods matured. After cryopreservation, the germination rates of the seeds preserved at 2, 3 and 4 months after pollination were 0%, 13% and 32%, respectively. Maturity of seeds was an important factor for successful cryopreservation. After 6 months culturing, plantlets were transferred to the saranhouse. There was no significant difference in growth between control and cryopreserved plantlets.

Protocorms of *Dendrobium cruentum* Rchb. f. were cryopreserved. With the vitrification method, protocorms were precultured on modified Vacin and Went (1949) medium supplemented with 0.06 (control), 0.3, 0.5, and 0.7 M sucrose for 1-5 d and then exposed to Loading Solution, and PVS2 for 0-420 min at 0±2℃ before being plunged into LN. In encapsulation-dehydration method, protocorms were encapsulated in calcium alginate before preculturing in sucrose supplemented medium and dehydration for 0-10h by sterile air from a laminar air-flow cabinet and then being plunged into LN. After cryopreservation, the survival rate of protocorms at water content of 65%FW, diameter 0.1-0.2mm was 33% (vitrification), 27% (encapsulation-dehydration). However, protocorms at water content of 87%FW, diameter 0.2-0.5mm did not survive. After 6 months culturing, plantlets were transferred to the saranhouse. There was no significant difference in growth between control and cryopreserved plantlets.

In vitro protocorm-like bodies (PLBs) and shoot tips of *Dendrobium cruentum* Rchb. f. cryopreserved by vitrification, encapsulation-dehydration and encapsulation-vitrification had no survival after preculturing with high sucrose concentration medium. PLBs and shoot tips had high water content. Preconditioning by cold acclimation, ABA and glycerol before preculturing had no effect on acquiring dehydration tolerance.

KEY WORDS : CRYOPRESERVATION / *DENDROBIUM CRUENTUM* / VITRIFICATION / ENCAPSULATION / DEHYDRATION

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LIST OF ABBREVIATIONS

Abbreviation or symbol	
ANOVA	Analysis of variance
ABA	Abscisic acid
℃	degree Celsius
cm	centimeter
d	day
DMSO	Dimethylsulphoxide
DW	Dry weight base
EG	Ethylene glycol
e.g.	exampli gratia (Latin), for example
et al.	et alli (Latin), and others
etc.	et cetera (Latin), other things
Fig.	Figure
FW	Fresh weight base
g	gram
g/l	gram per liter
h	hours
LN	Liquid Nitrogen
LS	Loading Solution
m	meter
MAP	months after pollination
mg	milligram
min	minute
ml	milliliter
mm	millimeter
M	Molar
MS	Murashige and Skoog

LIST OF ABBREVIATIONS (Continued)

Abbreviation or symbol	
No.	Number
PEG	poly ethylene glycol
PLBs	protocorm like bodies
PVP	poly vinyl pyrrolidone
PVS2	Plant vitrification solution 2
%	percentage
RH	relative humidity
s	second
S.D.	standard deviation
sp.	species
rpm	round per minute
TDZ	thidazuron
TTC	Triphenyl tetrazolium chloride
VW	Vacin and Went
w/v	weight per volume
w/w	weight per weight
μ m	micrometer
μ mol	micromole

CHAPTER 1
INTRODUCTION

1.1 Motivation

In Thailand, about 1,200 orchid species exist, and orchids are major economic crops of Thailand. Wild orchids differ distinctly from cultivated orchids, particularly in size, shape, color, vigor and resistance to diseases and insects. Breeders have improved and altered those features by bringing in some specimens from the wild to cultivate. So, it can be clearly seen that wild orchids are the source of essential features and play an important role in the continuing development and maintenance of outstanding hybrids. But many of wild orchids face extinction caused by environmental destruction and over gathering. Therefore, Thai orchid conservation is urgently needed.

Cryopreservation of orchids is one of the high potential methods for orchid conservation because it needs little space, is of low cost and maintenance and can be easily transported. But before preserving in liquid nitrogen (LN), plant materials require optimal treatments to stand dehydration and ultra-low temperature. The treatments decide survival rate after cryopreservation. In this thesis, we aim to find optimal treatments for Wild Thai orchid, *Dendrobium cruentum* Rchb.f

1.2 Problem statement

Cryopreservation of shoot tips of tropical plants had been more difficult than those of temperature plants until vitrification method was developed. In 1997, application of vitrification method, some kinds of tropical plants succeeded in cryopreservation. Thus optimal treatments make it possible to give plant materials dehydration tolerance.

Which organ, tissue and cell of orchids should be cryopreserved, depends on the purpose of conservation. Some orchids have difficulty in cultivation at artificial condition, greenhouse or tissue culture laboratories. Only conservation of species, seeds, protocorms, protocorm-like bodies, (PLBs) are considered. For conservation of species that take long time to germinate, protocorms and PLBs are better materials rather than seeds. For conservation of useful genes, shoot tips are suitable, because shoot recovery without intermediate callus formation is essential for clonal conservation.

Generally, the lower water content of plant materials is, the higher the survival rate after cryopreservation is. So optimal treatments before plunging into LN should be different among plant materials. Concretely, treatments with high concentration of cryoprotectants, sugar, ABA, alcohol *etc*. are the main technique. Finding optimal concentration, exposure time, and suitable temperature are the keys for successful cryopreservation.

1.3 Objectives of Thesis

The objectives of this thesis are:

1. To apply vitrification method to cryopreservation of seeds, protocorms, PLBs, and shoot tips of *Dendrobium cruentum* Rchb.f.
2. To find optimal treatments before plunging into LN.
3. To compare regrowth between vitrification method and encapsulation-dehydration method and investigate the influence of cryoprocedures on regrowth and morphology of seedlings.

1.4 Thesis outline

This thesis organized into nine chapters as follows:

Chapter 1: Introduction

This chapter describes the motivation, the problem statement, the objectives and the outline of the thesis.

Chapter 2: Literature Review

This chapter provides the necessary background concepts of orchids, conservation, and cryopreservation. In addition, some related research works are discussed.

Chapter 3: Materials and Methods

This chapter explains what plant resources we used, and how we carry out the experiments with figures.

Chapter 4: Results

This chapter shows our sober results with tables and figures.

Chapter 5: Discussion

This chapter provides consideration of our results and discuss hypothesis comparing other related works.

Chapter 6: Conclusions

This chapter summarizes our research.

CHAPTER 2
LITERATURE REVIEW

2.1 Background of orchids

2.1.1 The growth habits of orchids

Orchidaceae distributes in the world except for desert and polar area. In the family, there are about 750 genera and 20,000-25,000 species. The onset of orchid ancestors is about 60 million years ago. The morphological characters of orchids are varied to adapt to different environments. Professor Dressler(1), who is a famous orchid taxonomist, reported that the orchids are much more diverse in the tropical belt than they are to the north or south. For example, 8,266 species (306 genera) are seen in tropical America, and 6,800 species (250 genera) are seen in tropical Asia, although 153 species (26 genera) are seen in North America.

From ecological characters, *Orchidaceae* can be separated into terrestrial orchids and epiphytic orchids. There are several important differences between growing on the ground and on the trees, some are favorable for orchid growth and some are not. 80 % of orchids are epiphytic and often seen in tropical rain forests. The reasons why there are so many epiphytic species are that in rainforests, height of trees is 50 - 80 m, so light do not reach on the ground. The second is that orchid seeds are easy scattered by wind. The last reason is that canopy is easy for pollinators to visit orchid flowers. Growth in the tree tops has its unfavorable aspects, too, the most serious of which is probably the lack of water. But in rain forests, epiphytic orchids would acquire enough water from rain and atmosphere.

2.1.2 The characteristics of orchid family

Orchid seeds consist of a thickened testa, enclosing an embryo of about 100 cells. The seeds have tiny (200 to 1,700 μm) undifferentiated embryos lacking a cotyledon and endosperm.(2) The embryo has a round or spherical form. The process of seed germination is quite complicated. The embryo imbibes water via the testa, and becomes swollen. After cell division has begun, the embryo cracks out of the seed coat. A protocorm like structure is formed from the clump of cells, and on this, a shoot meristem can be distinguished. Under natural environmental conditions, the growth of protocorm depends on the symbiosis with certain endomycorrhyzal fungi. Later on, meristems and rhizoids appear on the protocorm; their development results in a juvenile shoot and subsequently gives rise to a whole plant (Fig.2-1).

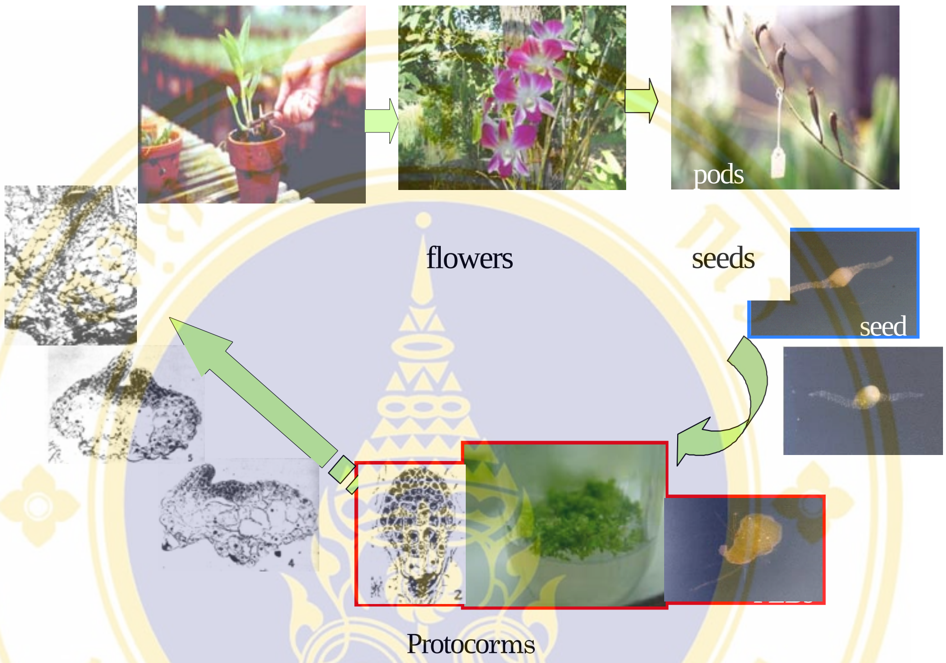


Figure 2-1. Life cycle of orchids.

Orchidaceae is distinct from other flowering plants because:

- The stamens and style are fused together in one structure known as a column.
- There are 3 petals and 3 petal-like sepals.
- Usually one different petal forms a lip or labellum making the flower bilaterally symmetrical.
- The pollen is usually bound together in a few large masses known as pollinia.
- The flower stem twists around during development so that the lip is down (resupination).
- The seeds are tiny and numerous.
- Upon germination, the embryo forms a tubercle (protocorm).
- Under natural conditions, most orchids will germinate only when a symbiosis with a fungus has been established. The most important fungi that have symbiotic relationships with orchids are in the genus *Rhizoctonia*. In the case of tropical orchid germination, it appears that the role of the fungus is not pronounced. While Knudson showed that germination was

possible on a simple medium containing suitable minerals and sugars, in the absence of a fungus.(3)

2.1.3 *Dendrobium cruentum* Rchb. f.

Dendrobium cruentum (Fig.2-2) is one of an epiphytic wild orchid originated from southwestern or peninsular Thailand. The taxonomical and biological description is reported below:

Habitat: in open forests at low elevations on small trees.

Characters: Stem length 25-30cm. Leaves oblong. Flower fragrance, 4-6cm diameter.(4) Sepals and Petals pale green with dark green veins. Lip creamy white flushed with brilliant red. Flowering period: year around.(5) *Dendrobium cruentum* belongs to *Dendrobium* Sw. Section *Formosae*. Yukawa(6) reported details about the phylogenetic status of the species. Jones *et al*.(7) reported nuclear DNA content by using flow cytometry.

Suitable culture condition: Temperature 27-30 ℃ day / 21-22 ℃ night, RH : 80-85 % humidity.

Dendrobium cruentum also has a commercial value, because there are many species, Dawn Maree (*D. cruentum*×*D. formosum*)(Fig.2-3), Green Lantern (*D.* Dawn Maree ×*D. cruentum*)(Fig.2-4), Frosty Dawn (Dawn Maree ×*D.* Lime Frost)(Fig.2-5) that come from *Dendrobium cruentum*. In developed countries, these hybrids are traded at around 17 US dollars per 1 pot.

Figure 2-2. *Dendrobium cruentum* Rchb. f.

Figure 2-3. Dawn Maree.

Figure 2-4. Green Lantern.

Figure 2-5. Frosty Dawn

2.2 Conservation of orchids

From the ecological aspects, *Dendrobium cruentum* is in the face of extinction. In 2004, this species is listed in the IUCN CITES Appendix(I) as "World Endangered Orchid Species". The CITES offer that all wild orchid species should not be moved to other nations for commercial purposes. At the 9th orchid world conference in 1978, it is proposed that all government should cooperate for research and conservation of *Orchidaceae*. Now, there are many organizations to protect nature. IPGRI (International Plant Genetic Resources Institute) proposes that ecological environment and genetic diversity especially in endangered orchid species should be maintained. Now, Thailand has about 1200 orchid species,(8) but most of them are endangered..

2.2.1 *In situ* conservation

The two basic approaches to conservation of plant genetic resources are *in situ* and *ex situ* conservation. The Convention on Biological Diversity (UNCED 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 and, in the case of domesticates or cultivated species, in the surroundings where they have developed their distinctive properties. *Ex situ* conservation means the conservation of components of biological diversity outside their natural habitats. The two basic conservation strategies can be further subdivided into several specific techniques. *Ex situ* conservation includes cryopreservation (conservation under ultra low temperature), seed bank, *in vitro* storage, DNA storage, pollen storage, field gene banks and botanical gardens, while the *in situ* approach encompasses genetic reserves, on-farm and home garden conservation.

2.2.2 *Ex situ* conservation

For ideal conservation of plants species, *in situ* conservation is better because plants can be conserved in natural habitats. The definition of national parks and conservation forests is that a tract of land declared public property by a national government with a view to its preservation and development for purposes of recreation and culture. The ecosystem, vegetation and evolution of all plant species can be conserved. But it is very difficult to keep natural habitats and ecosystem away from destruction. Koopowitz(9) reported that 55 tropical orchids species per year have been extinguished with loss of tropical rain forests. And it is said that 5,477 tropical orchids species have already been vanished. To change this current, saving natural habitats is the first priority. Actually, development and environmental preservation of tropical rain forests can not cooperate easily, so *ex situ* conservation is essential.

As aspect from management, *ex situ* conservation is better than *in situ* conservation because it is possible to set plant species under control. Moreover, *in situ* conservation may not be adequate to preserve the genetic diversity represented by the thousands of plant species that have arisen in the natural evolutionary process. *In situ* conservation must be supplemented by extensive *ex situ* conservation. Botanical gardens, seed banks, and clonal germplasm repositories are known as *ex situ* conservation.

Seeds have traditionally been grouped into two groups according to their physiological storage potential. Generally maintaining seed longevity requires dryness of seeds (2-5 % moisture content) and low temperature conservation with low moisture content. Roberts(10) gave the definition, orthodox (or ordinary) seeds, to this kind of seeds. While, some kinds of seeds lose their vigor by exposing dryness or low temperature, the definition is recalcitrant seeds. Recalcitrant seeds maintain high moisture content at maturity (> 30-50 %) and are sensitive to desiccation below 12-30 %, depending on species. They have a short storage potential.

Orchid seeds are known as orthodox seeds. Pritchard *et al*.(11,12) succeeded in long-term orchid seeds storage by drying orchid seeds and conserving under low temperature. Roberts(10) set the numerical formula for predicting orthodox seed longevity based on the fact that viability of orthodox seeds is prolonged in a predictable manner by moisture reduction and reduction in storage temperature. According to this, cryopreservation would succeed longer term conservation of orchid seeds.

Another advantage of cryopreservation is cost effectiveness. Falk(13) compared the costs of seed banks (-18 ℃) and cryopreservation of onion seeds and reported that seed banks required renewing seeds each 25 years. Wood *et al*.(14) reported that cryopreserved seeds of two orchid species showed higher survival rate than seeds stored at -20 and -70 ℃.

There is no report how much cost is required for cryopreservation and seed banks of orchids. Anyway, under previous seed bank conditions(> -20 ℃), renewing orchid seeds would be unavoidable. Because even dried seeds can not stop metabolism, so deterioration, e.g.

decrease of enzyme activity, accumulation of detrimental substances, occurred.(15) Millennium Seed Bank Project supported by royal botanic garden Kew considers cryopreservation as one of tool for seed bank.

Other *ex situ* conservation technique is tissue culture, *in vitro* conservation. It dose not require sophisticated equipments and special skills rather than cryopresrevation. In spite of advantages, *in vitro* conservation can be only short – medium term conservation. Problems arising from the loss of vigor of plantlets, progressive changes of the genome, microbial contamination, and requiring human hands for maintaining valuable *in vitro* collections, can not be ignored. Especially genetic change through somatic embryogenesis is a problem for conserving useful genotype. Thus, there is need for methods of conserving useful genotypes and phenotypes before these are lost as a consequence of maintaining the cultures by repeated subculture.

2.3 Cryopreservation

2.3.1 Principles of cryopreservation

Cryopreservation is literally "preservation in the frozen state". Plant germplasms put into cryotubes are preserved in LN. LN (-196 ℃) is the most common use rather than solid carbon dioxide (-79 ℃) and deep freezers (around -80 ℃). Cryopreservation is based on the reduction and subsequent interruption of metabolic functions and deterioration of biological materials by the decrease of the temperature to the level of that of LN, while viability is maintained.(16) Under low temperature, the viscosity of water becomes high, and the movement of water molecule is restricted. Water molecules are classified into three groups depending on speed of rotary motion. Water molecules rotating at 10^{-6} -10^{-7} s, directly combine with nucleic acid, protein and cytoplasm. These molecules does not freeze under -196 ℃. Water molecules rotating at 10^{-12} s, enhance metabolism under optimum temperature, but under low temperature, this molecules change to solid (formation of ice). Water molecules rotating at 10^{-9}s, work as buffer.(17) Crowe *et al*.(18) reported that 95% of water present in plant tissues can freeze under -196 ℃. Converting to ice during cryopreservation causes irreversible damage.

2.3.2 Cryopreservation injury

The water rich cells like most cells in a plant, will not survive after exposure to the temperature of LN. Upon cooling and thawing, lipids in membrane undergo phase transition from liquid-crystalline to gel-phase. Co-existence of liquid-crystalline and gel-phase in a membrane will cause leakage and therefore cell damage.(19)

Below 0 ℃, formation of intracellular ice in plant cells is mostly lethal,(20) and needs to be avoided. Because too much dehydration of plant cells and formation of large ice nuclei can destroy plant cells. In most cells, dehydration of the cells can decrease the chance of formation of ice crystals.

A dehydration step before plunging into LN, results in concentration of solutes in cells and in strong plasmolysis of cells. Removal of water can lead to "solute effects" in the cells, such as pH changes, increased electrolyte concentrations and macromolecular interactions.(21) Too much and rapid dehydration is also lethal damage to plant cells.

Stress to the cells caused by dehydration, freezing and thawing, may result in the formation of free radicals. These compounds can cause further damage, for example by lipid peroxidation, denaturation of proteins and mutations in DNA.(22,23)

2.3.3 Factors for successful cryopreservation

2.3.3.1 Speed of cooling and thawing

For successful cryopreservation, the most important factor is to avoid ice crystallization during freezing and thawing process, because ice crystal destroys plant cells mechanically. To avoid ice crystallization, plant materials should be dehydrated before plunging into LN until most of the freezable water is removed, but unless causing lethal dehydration. Usually water in plant cells make ice at around -30 ℃. Around -30 ℃, ice nuclei begins to generate and the rate of generation increases until around -70 ℃. While the growth rate of ice crystal begins to increase around -5 ℃, and keeps increase until -20 ℃ (Fig.2-6). At lower temperature, the number of ice crystal is much, but the size of ice crystal is smaller. The machinery damage caused by smaller ice crystal is less serious than that of bigger one. So freezing and thawing process require so high speed to pass immediately through temperature zone that permits ice crystal to grow.

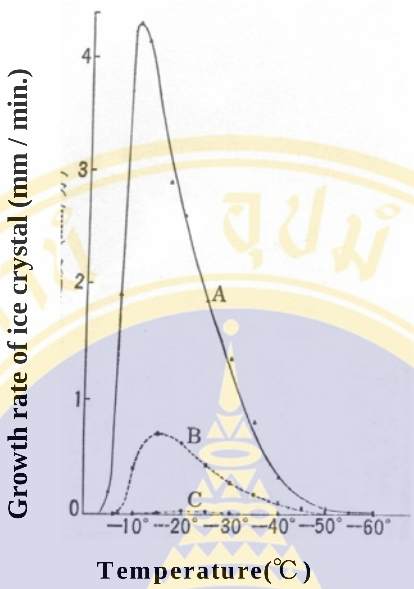


Figure 2-6. The effect of concentration of solution on the growth rate of ice crystal.(24)

A: 42.5% PVP solution, B: 50.0% PVP solution,

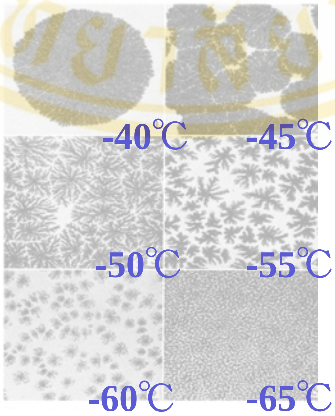


Figure 2-7. Ice crystal formation of 50% PVP solution.(25)

2.3.3.2 Cryoprotectants

Most of plant materials contain high amounts of cellular water and are sensitive to freezing injury, so they have to be dehydrated artificially to protect them from the damages. The techniques employed and the physical mechanisms upon which they are based are different in new and classical cryopreservation techniques. New cryopreservation techniques are based on vitrification, whereas classical techniques involve freeze – induced dehydration.(26)

The term "vitrification" means glass states of water molecule. Especially in cryopreservation protocol, "vitrification" is the process that water inside plant cell is replaced with high molecular solution and dehydrated by osmotic pressure of high molecular solution. Replacing freezable water by high molecular solution and Reducing freezable water by dehydration suppress growth of ice crystallization.(24) This mechanism is explained that viscosity of water in plant cells becomes higher, and some composition; for example, glycerol can combine to water molecule. Since the discovery of that cells treated with high molecular solutions survive exposure to LN, variations on solution composition have been developed for plant cells.(27) These cryoprotectants consist of poly alcohols, glycerol, propylene glycol, PEG, DMSO, sugars etc. These cryoprotectants are classified based on their permeability to plant materials. DMSO, glycerol, EG and sucrose have been classified as penetrating since they permeate the cell wall and plasmalemma, but do not have time to penetrate the cytosol and that they have only an osmotic action.(28)

Recently the mechanism of cryoprotectant has been understood. Turner *et al.*(29) researched the survival rate by using several cryoprotectants and proposed that the mode of action of poly alcohols is not based on molarity, but on the total number of OH groups present in the medium, and the orientation of OH groups is determining factor in effective cryopreservation. Volk *et al.*(30) revealed the role of PVS2, (31) the most popular cryoprotectants, by quantifying how much enthalpy of melting transitions decreased and amount of unfrozen water increased in PVS2. Sakai *et al.*(31) showed that the transition temperature from water to ice of PVS2 is around -100℃, and glass transition temperature from ice to glass is around -115 ℃. Thus, as a result of vitrification, it is possible to make the temperature zone that can make ice crystal shorter (Fig.2-8). But any high concentrations of cryoprotectants give chemical damage to plant cells. PVS2 has a total molarity of 7.8 M. It is highly toxic and the duration of contact with plant materials has to be determined very precisely.(32) Lowering temperature during dehydration treatment can relieve too rapid dehydration and can broaden the range of treatment duration. Suzuki *et al.*(33) reported that cryoprotectants of impermeable sugar group lost their cryoprotective effect at high concentration, which may due to severe dehydration and cell damage occurred in hypertonic solution. DMSO shows high cryoprotective effect due to high permeability to plant cells, while it may affect scientists' health. These should be taken attention on when cryoprotectants are selected.

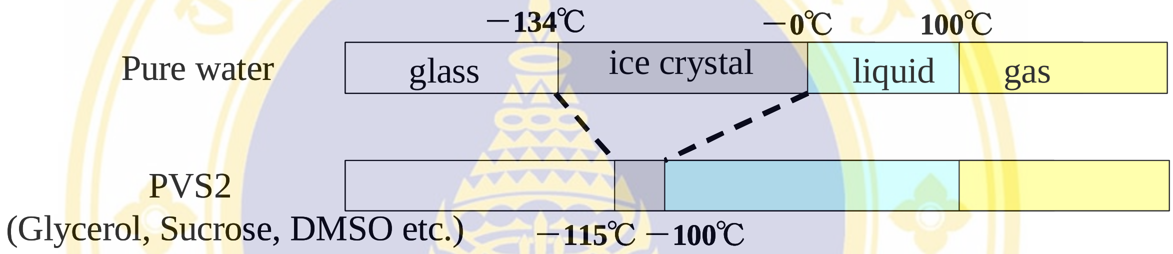


Figure 2-8. Transition temperature from liquid to ice and from ice to glass of pure water and PVS2.

2.3.3.3 Pretreatments

Some plant germplasm does not require dehydration process; for example, pollen and orthodox seeds, because naturally they are adapted to extreme desiccation during mature development and much free water does not exist in such plant cells. Desiccation tolerance to these conditions correlates with a number of physiological and molecular changes such as changes in membrane composition,(34,35) accumulation of small organic molecules,(36) and certain proteins.(37) For example, desiccation tolerance of orthodox seed axes is known to be correlated with increases in soluble sugars. Soluble sugars play a major role in preventing fusion, phase transitions and most likely also phase separations.(38,39) However, recalcitrant seeds, plant cell suspensions and calli *etc.* are particularly sensitive to a water loss which can result in a series of damaging. So before starting vitrification process, it is necessary to give desiccation tolerance recalcitrant plant germplasm. The capacity of plant cells to adapt desiccation stress can be employed in pretreatments for cryopreservation procedure.

Most common pretreatments for cryopreservation are preculture or several days-weeks on medium with high concentrations of sugars, poly alcohols, glycerol or ABA. Whether sugar uptakes from medium is apoplastic or symplastic, is unclear, however starch accumulation in plants at the end of the pretreatment reflects that, probably, a large quantity of sugars was absorbed and metabolized.(40) Sucrose is well-known for its implication in desiccation tolerance of plant tissues and could act either by replacing the water molecules involved in the structural maintenance of macromolecules, or by inducing vitrification of the intracellular medium.(18,36,41) Other studies have shown that as the crystallisable water is removed from the high sucrose pretreated polyembryonic culture, their survival after cryopreservation increased.(42)

2.3.4 Cryopreservation techniques

2.3.4.1 Vitrification

Vitrification involves treatment of plant materials with cryoprotective substances, dehydration with cryoprotectants, rapid freezing and thawing, removal of cryoprotectants and recovery. Basic practical protocol is as follows:

1. Preculture: culturing plant materials on medium with high concentration of sugar, poly alcohol or ABA.
2. Dehydration with cryoprotectants: If plant materials are sensitive to dehydration, stepwise method is useful, in which the concentration of cryoprotectants increases gradually.
3. Plunging into LN.
4. Thawing: warming rapidly plant materials rapidly in water bath at 37- 40 ℃ for 1-2 min to prevent ice formation.
5. Unloading: plunging plant materials into 1.0-1.2 M solution for 20 min before culture to avoid sudden change of osmotic pressure.
6. Culture: culturing plant materials in laboratory.

2.3.4.2 Encapsulation - dehydration

For small plant materials, such as shoot tips, encapsulation methods (encapsulation - vitrification, encapsulation - dehydration) are often used. In these methods, plant materials are suspended in inorganic medium supplemented with 2.5 – 3.0 % (w/v) Na-alginate, and then dropped into medium supplemented with 0.1 M $CaCl_2$. Finally it looks like a bead. The main advantages of encapsulation is to provide much greater ease of handling a large number of shoot tips, because shoot tips is too small (0.5-2.0 mm). Usually shoot tips are sensitive to dehydration. Encapsulation can relieve rapid water loss caused by vitrification / air current of a laminar air-flow cabinet or with silica gel down to a water content around 20 %FW.

Basic practical protocol of encapsulation-dehydration is as follows:

1. Encapsulation: encapsulating plant materials with Na-alginate / $CaCl_2$ solution.
2. Preculture: culturing beads on medium with high concentration of sugar, sugar alcohol or ABA.
3. Dehydration: drying beads under laminar air-flow / silica gel.
4. Same as vitrification method, protocol 6.

2.3.4.3 Encapsulation - vitrification

Encapsulation - vitrification is a combination of encapsulation and vitrification procedures, where samples are encapsulated in alginate beads, then subjected to freezing by vitrification.

Until now, other new methods, such as new vitrification,(43) desiccation, (44) pregrowth,(45) droplet,(46) *etc.* have been developed. But mostly these are based on mentioned methods.

2.3.4.4 Classical cryopreservation

Classical cryopreservation method involves slow cooling down to a defined prefreezing temperature, followed by rapid immersion in LN. With temperature reduction during slow cooling, plant materials 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 seedling the cell interior and the cells remain unfrozen but supercooled. As the temperature is further decreased, as increasing amount of the extracelluar solution is converted into ice, thus resulting in the concentration of intracellular solutions. Since cells remain supercooled and their aqueous vapor pressure exceeds that of the frozen external compartment, cells equilibrate by loss of water to external ice. Depending on the rate of cooling and the prefreezing temperature, different amounts of water will leave the cells before the intracellular contents solidify. In optimal conditions, most or all intracellular freezable water is removed, thus reducing or avoiding detrimental intracellular ice formation on subsequent immersion of plant materials in LN. However, classical cryopreservation needs precise adjustment of temperature, so sophisticated and expensive programmable freezers are required.

2.3.5 Cryopreservation of orchids

2.3.5.1 Cryopreservation of orchid seeds

Nikishina *et al.*(47) reported that naked seeds of some tropical terrestrial orchid species showed survival after plunging into LN without any treatments (neither vitrification nor dehydration process). The moisture content is an important factor. The majority of investigators apply deep-freezing to seeds with moisture content of less than 13 %FW. Only scarce work dealt with direct freezing of orchid seeds. For example, Pritchard(48) succeeded in cryopreservation of seeds of 11 orchid species, and the seed germinabilities did not change after cryopreservation for the most species examined. In this report, the moisture content in seeds did not exceed 13% in most cases, except for freshly harvested seeds of *E.cochleata* (drying at 95℃ until accomplished to be constant weight). It seems that the problem of freezing is the presence of unbound water in seeds reducing germinabilities and forming ice crystal, not absolute moisture content.

When the moisture content exceeds the threshold level, cryoprotectants treatments are useful. For successful cryopreservation, most of orchid seeds should take pretreatment (preculture, vitrification, or dehydration).

Thammasiri(8,49) succeeded in cryopreservation of orchid seeds by vitrification. He reported 4 species by vitrification method. *Dendrobium chrysotoxum* (germination rate before immerging LN was 99 %, germination rate after immerging LN was 99 %, and optimum exposure time to PVS2 was 50 min), *Dendrobium draconis* (100 %, 95 %, 30 min), *Doritis pulcherrima* (91 %, 63 %, 50 min), and *Rhynchostylis coelestis* (90 %, 85 %, 50 min). These 4 species were treated with PVS2. Vitrification with PVS2 is useful, but too exceed exposure may cause lethal desiccation. It is required to find optimum exposure time to PVS2 for each species. Moreover, he showed the morphological differences between PVS2 treated seeds and untreated seeds. After immerging LN, seeds untreated with PVS2 shrunk. While, seeds treated with PVS2 showed same shape and size as naked seeds.

Hirano *et al.*(50) reported the effect of preculture and vitrification before plunging into LN on survival rate. For *Bletilla striata* immature seeds, preculture with high contents of sucrose (0.3 M) for 3 days before vitrification increased the survival rate after cryopreservation. However, the difference was not statistically significant for mature zygotic embryos of the same species (51). Hirano *et al.*(52) reported that preculture expanded the range of optimum exposure time to PVS2 for *Ponerorchis graminifolia* var. suzukiana immature seeds. With preculture, immature seeds can sustain exposure to PVS2 for long time and show high survival rate. They also showed the change of water contents of seeds during maturation.

The water contents of seeds decrease as seeds developed. Vacuolation, which has been observed in the young embryo cells in orchids, disappears as approaching maturity. Thus, reduced vacuolation and, consequently, a lower level of bulk water in the embryo cells that accompany seed maturation, prevented lethal freezing in the seeds. Moreover it is known that composition changes in the cytoplasm underlie the increase in viscosity and that increasing cytoplasmic viscosity correlates with desiccation tolerance.

Orchid seeds usually have dormancy before germination. During dormancy, seeds stop their growth development and result in sustaining unsuitable environmental condition for germination. Seed dormancy is accomplished by desiccation during mature. Some physiological mechanisms precede desiccation; for example, acquiring desiccation tolerant, accumulation of late embryogenesis abundant protein, LEA, and activation of genes that induce ABA.

During preculture process, important cellular changes might have occurred such as induction of endogenous ABA synthesis, stabilization of membranes (38) and gene expressions necessary for adapting to the high osmotic conditions.(53)

Mature seeds originally have desiccation tolerance so it is possible for some orchid species to survive after cryopreservation without preculture to acquire dehydration tolerance. Cryopreservation of immature seeds can be successful, if they are treated with preculture under optimum conditions.

Finally, We introduce the cryopreservation of orchid seeds with fungus. For germination, orchid seeds require help from fungus, because there is not enough nutrition in orchid seeds to germinate. Nutrition is supplied from endophytic fungus, usually from basidomycete family.

This requirement indicates how important it is to consider methodologies for *ex situ* conservation of both the fungus and orchid seeds. Wood *et al*.(14) examined the effects of moisture content and temperature on the survival of encapsulated *Dactylorhiza fuchsia* (common spotted orchid) and *Anacamptis morio* (green-winged orchid) seeds and *Ceratobasidium cornigerum* (fungi). Encapsulated seeds with fungi were pretreated and then were dried before low temperature conservation. When they were dried to 20 %FW and stored under -196 ℃, the survival rate after 30d store was higher than when they were dried to over 20%FW or stored for 30d under 16, -20, and -70 ℃. From the aspect of *ex situ* conservation, the protocol of seeds cryopreservation with fungi should be improved, as well as conducting the research on other species.

2.3.5.2 Cryopreservation of orchid protocorm-like bodies (PLBs)

There are some successful reports of PLBs cryopreservation. Wang *et al*.(54) succeeded in *Dendrobium candidum* PLBs cryopreservation by air-drying methods and vitrification methods. In air-drying methods, they examined the effects of preculture with medium supplemented with ABA and water contents on survival rate. When water content of PLBs was desiccated to 0.1～ 0.5 g/gDW(= 11 ～33 %FW), there was no reduction in survival rate. It was reasonable to assume that intracellular freezing was avoided in *D.candidum* PLBs at this water contents during cryopreservation. However, when PLBs are not dehydrated sufficiently, freezing injury can occur due to intracellular ice formation. On the other hand, when over-dehydrated, the osmotic stress can damage the cells. In this report, desiccation process took 3 days to reach water content of 0.5 g/gDW. The accumulation of soluble sugar mainly occurred at early desiccation stage (at water content of about 7.2 g/gDW). Pretreatment with medium supplemented with ABA increased the accumulation of soluble sugar, and resulted in increasing desiccation tolerance. Whereas, the accumulation of heat-stable proteins happened at relatively lower water contents. It began to increase at water content of 2.0 g/gDW of PLBs and reached a maximum at 1.0 g/gDW. Finally, accumulation of 2 dehydrins took place in PLBs only when the water content dropped to 1.0 g/gDW.

Exogenous ABA enhances the accumulation of soluble sugars, heat-stable proteins and dehydrins in PLBs. Dehydrin in the vicinity of the plasma membrane might play a role in preventing the destabilization of the membrane during dehydration and freezing conditions. It is also suggested that interactions between sugars and heat stable proteins might play a role in improving the dehydration tolerance of plant cells.

It is known that slow drying significantly increases desiccation tolerant of somatic embryos and immature zygotic embryos or seeds.(55) Slow drying was believed to allow plant tissues to acquire desiccation tolerant during the period of prolonged slow dehydration. By contrast, slow drying was found to be harmful to desiccation tolerance of many recalcitrant (desiccation- sensitive) seeds and embryos. More recently, it was reported that there is actually an optimal drying rate for a plant tissue to achieve its maximum desiccation tolerance.(56) Therefore, drying rate appears to modify significantly the ability of plant tissues to sustain dehydration stress. Wang *et al*.(57) examined the relationship between dehydration tolerant and drying rate and the effect of ABA pretreatment in *Spathoglottis plicata* PLBs.

PLBs pretreated with ABA changed components.(58) The accumulation of soluble sugar and ions decreased osmotic potential of PLBs. This decreased drying rate of PLBs even under severe drying conditions and then critical water content of PLBs became lower compared to untreated PLBs with ABA under the same drying conditions. ABA enhanced synthesis of dehydrin. Slow drying also induced synthesis of dehydrins with / without ABA treatment.(58)

Pretreatments for cryopreservation, medium supplemented with ABA or / and high concentration of sucrose are used. The mechanism of ABA is described above. Wang *et al*.(58) examined the difference of mechanism to induce dehydration tolerant by abscisic acid and sucrose in *Spathoglottis plicata* PLBs. They reported that ABA affected drying rate and significantly enhanced the synthesis of dehydrins, but dehydrin protein disappear after removing PLBs from ABA. Only slow drying without ABA also enhanced the degradation of several high molecular weight proteins and the synthesis of dehydrins. While the increase in dehydration tolerance by sucrose treatment, did not depend on drying rate. The amount of dehydrin proteins induced by sucrose, were very low during treatment but increase rapidly upon drying. Main effect of sucrose treatment is accumulation of soluble sugar in PLBs.(58) It is known that soluble sugars has been implicated to protect membranes through water replacement and to protect the cytoplasm by transiting them into glassy state.

2.3.5.3 Cryopreservation of orchid shoot tips, and shoot primodia

Unfortunately, there are very few successful reports about cryopreservation of orchid shoot tips although shoot tips are the best plant materials for conserving particular useful genotypes. The reason why cryopreservation of orchid shoot tips is difficult is that shoot tip is composed of several kinds of tissues. Each tissue has optimum condition for pretreatment, exposure time to vitrification solution, or desiccation time. Second, shoot tips are too sensitive to desiccation and freezing. Usually shoot tips, especially meristemic tissue have higher water contents than other parts of axes. Na *et al*.(59) reported successful cryopreservation of *Vanda pumila* shoot primordia from shoot apices of cultured protocorms. Shoot primordial was pretreated with medium supplemented with ABA and then dried under 45 % relative humidity, (RH) condition. Wanna *et al*.(60) reported successful cryopreservation of *Dendrobium* Walter Oumae shoot tips by encapsulation-desiccation method. Encapsulated shoot tips were precultured with medium supplemented with high concentration of sucrose.

There are so many successful reports of cryopreservation of shoot tips, for example; strawberry (*Fragaria*×*ananassa* Duch.),(61) garlic (*Allium sativum* L.),(62) Yam (*Dioscorea* soecies),(63) wasabi (*Wasabi japonica*)(64) and so on. However, it is difficult to gain regeneration of cryopreserved orchid shoot tips. Although the result of TTC test shows high survival rate, shoot tips can not regrow after cryopreservation. Some hypothesis are assumed that TTC test does not reflect survival, culture conditions after cryopreservation are not suitable, the damage from cryopreservation begins gradually, the vigor of shoot tips drops during culture, there is practical problems by researchers, or more improved protocol is required. It is necessary to conduct the research more about physiological characters of orchid shoot tips, tissue culture technique, and cryopreservation protocol.

CHAPTER 3
MATERIALS AND METHODS

3.1 Chemicals and reagents

All chemicals and reagents used throughout this study were analytical grade and they were purchased from Fluka (Switzerland), Merck (Germany), Mallinckrodt (USA), and Sigma (USA). All of them were Abscisic acid (ABA), Activated charcoal, Ammonium sulphate, Calcium chloride, Calcium phosphate, Clorox, Dimethylsulphoxide (DMSO), Disodium ethylenediaminetetraacetate (EDTA), Poly ethylene glycol (PEG), Ferrous sulphate, Glycerol, Magnesium sulphate, Manganese sulphate, Potassium hydrogen phosphate, Potassium nitrate, Sodium alginic acid, Hydrochloric acid, Potassium hydroxide, Agar, Ethanol, 2,3,5-triphenyl tetrazolium chloride.

3.2 Cryopreservation of seeds

3.2.1 Plant materials for cryopreservation of seeds

6 seed pods (2,3,4 months after self-pollination, MAP) of *Dendrobium cruentum* Rchb. f. were harvested from Buncha Hongsanyatham's orchid farm located in Nakornpratom, or from saranhouse of the Institute of Science and Technology for Research and Development, Salaya campus, Mahidol University, Thailand. The length, weight of each seed pods, and the water content of seeds were measured.

3.2.2 Cryopreservation of seeds by vitrification

Harvested seed pods were cleaned and washed with running tap water for a few minutes. Subsequently, the pods were brought inside a laminar air-flow cabinet, soaked in 95 % ethyl alcohol and flamed with a lamp until the flame stopped. The pods were then cut on a sterile Petri dish and the seeds were taken out. For each treatment, approximately 4 mg of seeds were put in a 2 ml cryotube. 0.5 ml of PVS2 (31, Appendix 5) which is composed of 30 %(w/v) glycerol, 15 %(w/v) ethylene glycol, 15 %(w/v) dimethylsulphoxide, was added into the cryotubes. Vitrification was carried out at 25±2 ℃ for 0,10,20,30,40,50,60,70,80 and 90 min before plunging into LN.

3.2.3 Recovery of cryopreserved seeds

The cryotubes were taken out from LN after 1 day and warmed rapidly in a water bath at 40 ℃ for 2 min. The PVS2 solution was replaced by 0.5 ml of 1.2 M sucrose in modified VW(65, Appendix 1) liquid medium in which the seeds were kept at 25±2 ℃ for 20 min. Seeds from each cryotube were cultured on VW agar medium to check survival. Survival was assessed by counting the number of seeds that germinate 3 months after starting regrowth cultures. After checking germination rate, seeds were subcultured on VW modified agar medium with banana and activated charcoal for 3 months, and then transferred in saranhouse.

3.3 Cryopreservation of protocorms

3.3.1 Plant materials for cryopreservation of protocorms

Seed pods (3,4 MAP) of *Dendrobium cruentum* Rchb. f. were harvested from saranhouse of the Institute of Science and Technology for Research and Development, Salaya campus, Mahidol University, Thailand. Seeds were sowed on modified VW agar medium. 1 month after germination, protocorms were formed. The water content of protocorms was measured before cryopreservation treatment.

3.3.2 Preculture

For the effect of sucrose preculture, protocorms were cultured in modified VW liquid medium supplemented with 0.06 (control), 0.3, 0.5, and 0.7 M sucrose for 1, 2, and 3 d, rotating at 110 rpm. Other conditions are described in 3.7.

3.3.3 Cryopreservation of protocorms by vitrification

After preculture in medium supplemented with high concentration of sucrose, 0.3 g of protocorms were placed in 2.0 ml cryotubes. 1.5 ml of Loading Solution (LS, Appendix 4) (31) was added into cryotubes and kept at 25±2 ℃ for 20 min. The LS contained 2 M glycerol plus 0.4 M sucrose in modified VW liquid medium. After removing LS, 1.5 ml of PVS2 was added into the cryotubes. Vitrification was carried out at 0±2 ℃ for 0,60,120,180,240,300,360, and 420 min before plunging into LN.

3.3.4 Cryopreservation of protocorms by encapsulation - dehydration

After preculture in medium supplemented with high concentration of sucrose, protocorms were suspended in calcium free modified VW liquid medium supplemented with 3 %(w/v) sodium alginate and 0.4 M sucrose (Appendix 2). The protocorms with suspension was dropped into modified liquid VW medium with 0.1 M $CaCl_2$ (Appendix 3). After 30 min, beads (4-5 mm diameter) containing 1 protocorm per bead was formed. After removal of extra medium on surface of beads by absorbing with cotton wool, about 70 beads were placed on a 15 cm diameter Petri dish, and put at the laminar air-flow cabinet. Beads were dehydrated for 0,1, 2,3,4,5,6,7,8,9 and 10 h before plunging into LN.

3.3.5 Recovery of cryopreserved protocorms

The cryotubes were taken out from LN after 1d and warmed rapidly in a water bath at 40 ℃ for 2 min. The PVS2 solution was replaced by 0.5 ml of 1.2 M sucrose in modified VW(65, Appendix 1) liquid medium in which the seeds were kept at 25±2 ℃ for 20 min. Protocorms from each cryotube were cultured on VW agar medium to check survival. Survival was assessed by counting the number of green colored protocorms that germinated 2 months after starting regrowth culture.

3.4 Cryopreservation of *in vitro* protocorm-like bodies (PLBs)

3.4.1 Plant materials for cryopreservation of *in vitro* PLBs

Young shoots were cultured from seeds. Harvested seed pods were cleaned and washed with running tap water for a few minutes. Subsequently, the pods were brought inside a laminar air-flow cabinet, soaked in 95 % ethyl alcohol and flamed with a lamp until the flame stopped. The pods were then cut on a sterile Petri dish and the seeds were cultured on modified VW medium. After 1 month, protocorms were formed and then subcultured on modified VW agar medium supplemented with 1 %(w/v) banana and 0.5 g/l activated charcoal. Young shoots were obtained from protocorms. Apical shoot tips (1-3 mm in length), comprising the meristematic dome with one or two unfolded leaves, are excised under a stereo microscope from 3 to 5 cm long orchid plantlets. Shoot tips were also excised from axillary buds and used the same as apical shoot tips. This process was made under a laminar air-flow cabinet with 0.2 µm HEPA filter. For inducing PLBs, excised shoot tips were subcultured in modified VW liquid medium at

110 rpm. For mass propagation, PLBs were subcultured on modified VW solid medium supplemented with 9.0 μM of thidiazuron, (TDZ).

3.4.2 Preculture

PLBs were cultured on VW agar medium supplemented with 1.5 %(w/v) agar and 0, 0.1, 1.0 ppm ABA for two weeks. The cultivation container was wadded with cotton wool cap. After that, PLBs were transferred to VW liquid medium supplemented with 0.06, 0.3, 0.5, and 0.7 M sucrose and 0, 0.1, and 1.0 ppm ABA for 2 d.

3.4.3 Cryopreservation of *in vitro* PLBs by vitrification

PLBs were separated into two groups depending on their diameter. For vitrification method, PLBs that had diameter less than 2.0 mm were used. Other conditions followed the same as protocorms (3.3.3).

3.4.4 Cryopreservation of *in vitro* PLBs by encapsulation-dehydration

Experiment 1

PLBs were separated into two groups depending on their diameter. For encapsulation-dehydration method, PLBs that had diameter less than 2.0 mm were used. Other conditions followed the same as protocorms (3.3.4).

Experiment 2

PLBs were separated into two groups depending on their diameter, one group was less than 2.0mm diameter, another group was more than 2.1 mm to less than 3.5mm diameter. PLBs were cultured on VW agar medium supplemented with 0.4 M sucrose and 0.1 M glycerol over night under dark condition. After that, encapsulated PLBs were precultured in VW liquid medium supplemented with 0.3 M sucrose for 3 d. Other condition followed the same as protocorms (3.3.4).

3.4.5 Recovery of cryopreserved *in vitro* PLBs

Followed the same as protocorms (3.3.5).

3.5 Cryopreservation of *in vitro* shoot tips

3.5.1 Plant materials for cryopreservation of *in vitro* shoot tips

Young shoots were cultured from seeds. Harvested seed pods were cleaned and washed with running tap water for a few minutes. Subsequently, the pods were brought inside a laminar air-flow cabinet, soaked in 95 % ethyl alcohol and flamed with a lamp until the flame stopped. The pods were then cut on a sterile Petri dish and the seeds were cultured on modified VW agar medium. After 1 month, protocorms were formed and then subcultured on modified VW agar medium supplemented with 1 %(w/v) banana and 0.5 g/l activated charcoal. Young shoots were obtained from protocorms. Apical shoot tips (1-3 mm in length), comprising the meristematic dome with one or two unfolded leaves, are excised under a stereo microscope from 3 to 5 cm long orchid plantlets. Axillary buds also excised and used the same as apical shoot tips. This process was made under a laminar air-flow cabinet with 0.2 μm HEPA filter.

3.5.2 Cold acclimation and Preculture

Experiment 1

For the effect of sucrose preculture, excised shoot tips were cultured on modified VW agar medium supplemented with 0.06 (control), 0.3, 0.5, and 0.7 M sucrose for 1, 2, and 3 d..

Experiment 2

Excised shoot tips were cultured on VW liquid medium for 2 d and then transferred to VW agar medium for 2 d. Both medium were supplemented with 0.06 M sucrose, 0.1 ppm NAA, and 1 ppm BA. For the effect of sucrose preculture, cultured shoot tips were transferred VW agar medium supplemented with 0.3 M sucrose, 0.1 ppm NAA, and 1 ppm BA for 2 d.

Experiment 3

For the effect of cold acclimation, *in vitro* plantlets from 3 to 5 cm long were transferred to a refrigerator at 15±2 ℃ during night (dark condition) from 3:00 p.m. to 9:00 a.m. (18 h) and 25±2 ℃ during day from 9:00 a.m. to 3:00 p.m. (6 h). Some *in vitro* plantlets were transferred to a refrigerator at 15±2 ℃ during night (dark condition) for all day. Illumination of day was about 37 μ mol.m^{-2}.s-^{1} provided by F30/GRO(GE) fluorescent tubes. Cold acclimation treatment continued for 5 d.

After cold acclimation, shoot tips were excised from plantlets and cultured on VW agar medium supplemented with 0.06 (control), or 0.3 M sucrose, 0.1 ppm NAA, and 1 ppm BA for 2 d. During preculture, excised shoot tips were transferred to refrigerator at 15± 2 ℃ during night (dark condition).

3.5.3 Cryopreservation of *in vitro* shoot tips by vitrification

After preculture in medium supplemented with high concentration of sucrose, 15 of shoot tips were placed in each 2.0 ml cryotube. 1.5 ml of Loading Solution(LS, Appendix 4) (31) was added into cryotubes and kept at 25±2 ℃ for 20 min. The LS contained 2M glycerol plus 0.4 M sucrose in modified VW liquid medium. After removing LS, 1.5 ml of PVS2 was added into the cryotubes. Vitrification was carried out at 25±2 ℃ for 0,20,40,60, 80 and 100 min before plunging into LN.

3.5.4 Cryopreservation of *in vitro* shoot tips by encapsulation-dehydration

After preculture in medium supplemented with high concentration of sucrose, shoot tips were suspended in calcium free modified VW liquid medium supplemented with 3 %(w/v) sodium alginate and 0.4 M sucrose (Appendix 2). The shoot tips with suspension were dropped into modified liquid VW medium with 0.1 M $CaCl_2$ (Appendix 3). After 30 min, beads (4-5 mm diameter) containing 1 shoot tip per bead was formed. After removal of extra medium on surface of beads by absorbing with cotton wool, about 70 beads were placed on a 15 cm diameter Petri dish, and dehydrated for 0,1,2,3,4,5,6,7,8,9 and 10 h before plunging into LN.

3.5.5 Cryopreservation of *in vitro* shoot tips by encapsulation-vitrification

After preculture in medium supplemented with high concentration of sucrose, shoot tips were suspended in calcium free modified VW liquid medium supplemented with 3 %(w/v) sodium alginate and 0.4 M sucrose (Appendix 2). The shoot tips with suspension were dropped into modified liquid VW medium with 0.1 M $CaCl_2$ (Appendix 3). After 30 min, beads (4-5 mm diameter) containing 1 shoot tip per bead was formed. After removal of extra medium on surface of beads by absorbing with cotton wool, beads were placed in 2.0 ml cryotubes. 1.5 ml of Loading Solution(LS, Appendix 4) (31) was added into cryotubes and kept at 25±2 ℃ for 20 min. The LS contained 2M glycerol plus 0.4 M sucrose in modified VW liquid medium. After removing LS, 1.5 ml of PVS2 was added into the cryotubes. Vitrification was carried out at 25 ±2 ℃ for 0,20,40,60,80,100,120,140,160,180,200,220 and 240 min before plunging into LN.

3.5.6 Recovery of cryopreserved *in vitro* shoot tips

Thawing, rehydration process were followed the same as protocorms (3.3.5).

Experiment 1

Shoot tips from each cryotube were cultured on VW agar medium

Experiment 2,3

Shoot tips from each cryotube were cultured on VW agar medium supplemented with 0.1 ppm NAA and 1 ppm BA.

Survival was assessed by counting the number of green elongated shoot 2 months after starting regrowth cultures. The TTC test was also applied for 1, 2 d after starting regrowth culture.

3.6 Determination of water content

Two independent samples were weighted and then placed in hot air oven at 130 ℃ for 3 h until dry weight does not change. The water content was expressed on a fresh weight (FW) or dry weight (DW) basis as follows:

%FW = Weight (before desiccation − after desiccation) / Weight before desiccation × 100

g/gDW = Weight (before desiccation − after desiccation) / Weight after desiccation

3.7 Conditions during culture

The cultures were incubated at 25±2 ℃ under illumination of about 37 μmol.m^{-2}.s-^{1} provided by F30/GRO(GE) fluorescent tubes for 16 h per d.

3.8 Statistical analysis

Germination rate of seeds and survival rate of protocorms, PLBs, shoot tips were subjected to analysis of variance (ANOVA), and the means were compared by the least significant differences (LSD) test.

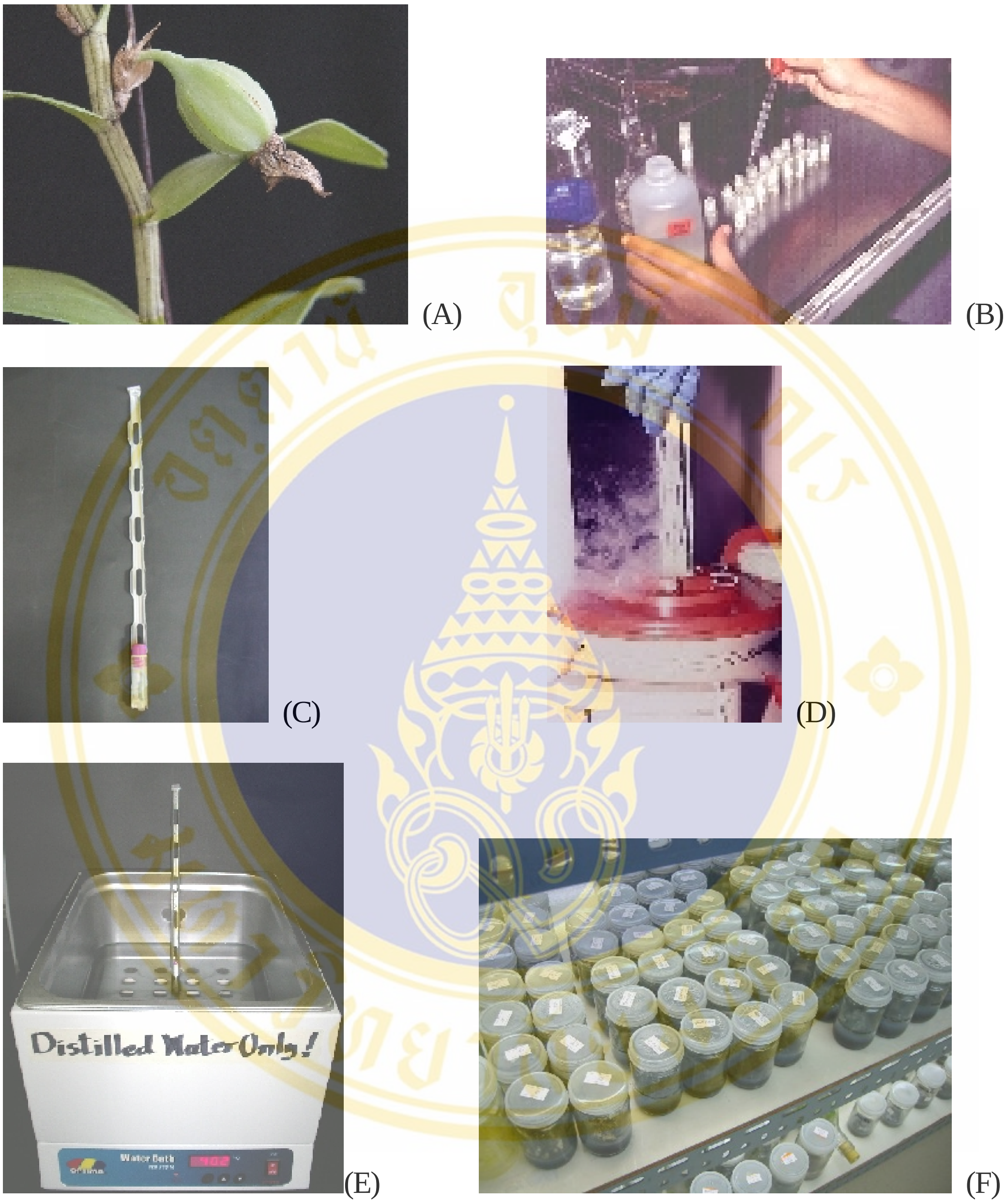


Figure 3-1. Procedure of seed cryopreservation.

(A) Seed pod

(B) Adding PVS2 into cryotubes

(C) A cryotube attached to a cryocane

(D) Plunging into LN

(E) Thawing cryotubes in warm water bath

(F) Regrowth culture

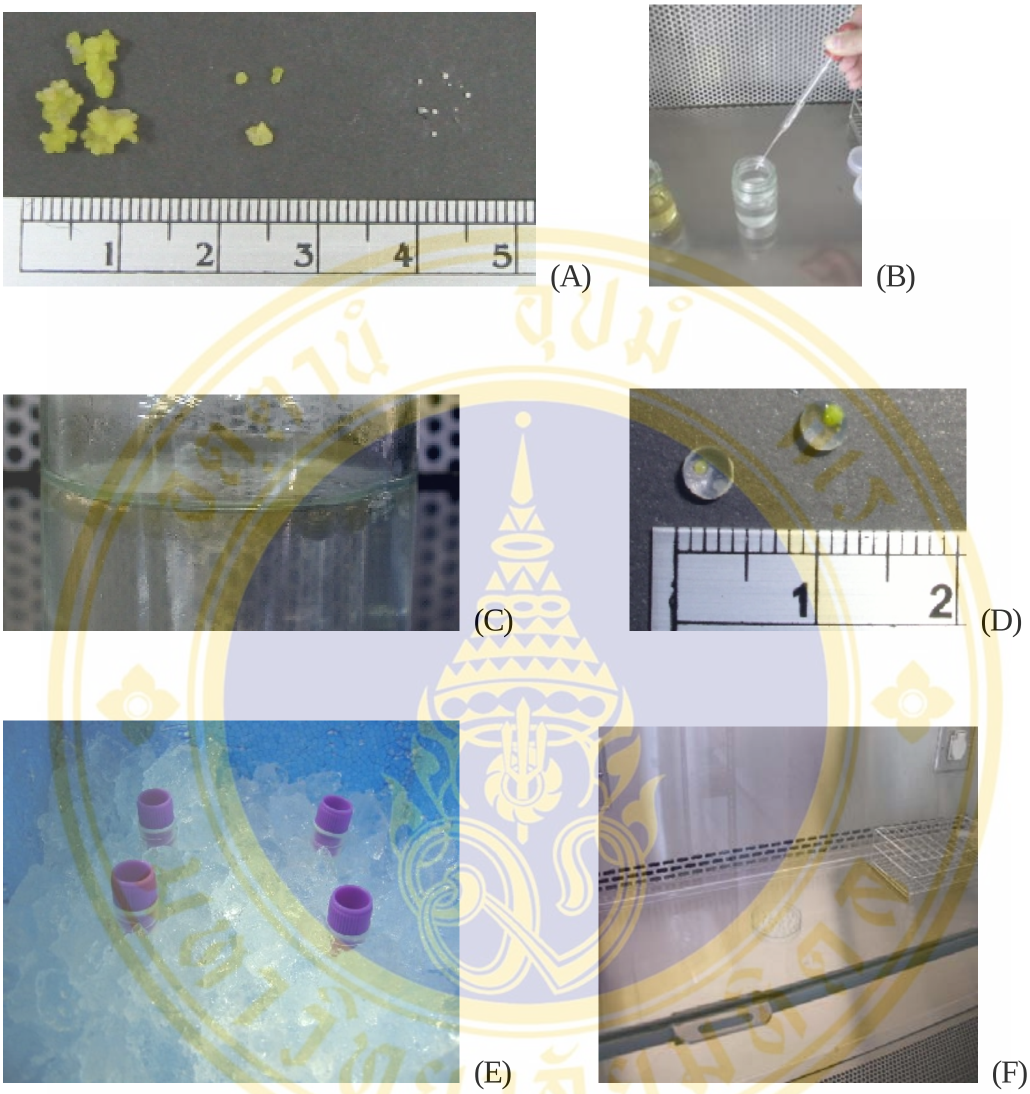


Figure 3-2. Procedure of protocorms and PLBs cryopreservation.

(A) PLBs varied in size

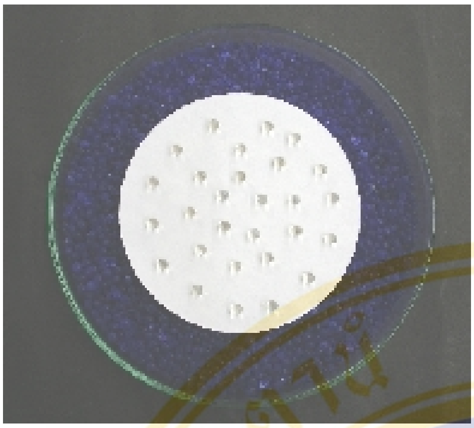
(B) Dropping Na-Alginate solution containing protocorm or PLBs into 0.1M $CaCl_2$ solution

(C) Beads formed in 0.1M $CaCl_2$ solution

(D) Beads containing one protocorm, or PLBs per each bead

(E) Cryotubes with beads and PVS2 inside at 0 ± 2 ℃

(F) Dehydration beads under a laminar air-flow cabinet



(G)

Figure 3-2.(Continued)

(G) Dehydration beads with silica gel

CHAPTER 4
RESULTS

4.1 Results of cryopreservation of seeds of *Dendrobium cruentum* Rchb. f.

Table 4-1 and Fig.4-1 showed the characters of each seed pod and seeds of *Dendrobium cruentum* Rchb. f. Seeds from seed pods harvested 4 months after pollination, MAP, were yellow, and low water content (less than 15.0 %FW). While seeds from seed pod harvested 3 MAP, slightly higher water content (25.0 %FW), and seeds from seed pods harvested 2 MAP, were white, and high water content (about 80.0 %FW).

Table 4-1. Characters of seed pods and seeds of *Dendrobium cruentum* Rchb. f.

	Pot size (l×w) (cm)	Pod weight (g)	Seed FW (%)	Seed DW (g/g)	Seed color	Months after pollination (date of harvest)
Seed pod 1	2.94×1.76	2.31	15.0	0.18	Yellow	4 (Nov.28th, 2004)
Seed pod 2	2.40×1.74	2.34	10.2	0.11	Yellow	4 (Mar.20th, 2006)
Seed pod 3	2.58×1.85	3.34	14.2	0.16	Yellow	4 (Mar.20th, 2006)
Seed pod 4	2.39×1.63	1.90	25.0	0.33	Yellow	3 (Mar.28th, 2006)
Seed pod 5	2.86×2.05	2.20	83.3	5.00	White	2 (Mar.28th, 2006)
Seed pod 6	2.89×2.11	3.16	79.9	3.98	White	2 (Mar.28th, 2006)

Seed pod 1, 2 were harvested from Buncha Hongsanyatham's orchid farm.

Seed pod 3-6 were harvested from saranhouse of the Institute of Science and Technology for Research and Development, Salaya campus, Mahidol University.

(A)

(B)

(C)

Figure 4-1. Seeds from mature and immature seed pods.

(A) A seed pod harvested 4 months after pollination.

(B) Mature seeds, yellow color, from a seed pod harvested 4 months after pollination.

(C) Immature seeds, white color, from a seed pod harvested 2 months after pollination.

For successful cryopreservation, it is important to assess the water content of seeds. The seeds of *Dendrobium cruentum* used in this thesis showed a steady decrease in water content with increasing time after pollination: averagely 82 %FW, 25 %FW and 13 %FW in the seeds of 2, 3 and 4 MAP, respectively (Table 4-2). When these seeds were directly plunged into LN without exposure to PVS2, there was no survival. Even 4 MAP seeds had not acquired any ability to survive the frozen state (Table 4-2).

Seeds were exposed to PVS2 at 25±2 ℃ before plunging into LN. 3 months after starting regrowth culture, germination rate was counted. Seeds at low water content (4 MAP) showed high germination rate (30-34 %, Table 4-2, Fig.4-2) after cryopreservation. Seeds at high water content (3 MAP) decreased germination rate to 13 % (Table 4-2, Fig.4-2).

Germination of 2 MAP seeds was not observed with / without plunging into LN. These control seeds had no germination (Table 4-2, Fig.4-2). TTC test showed no survival of these control seeds at 3 months after starting regrowth culture. Immature seeds of *Dendrobium cruentum* might not have germinability or lose vigor under unsuitable conditions for immature seed germination, such as, medium components, light and so on.

Table 4-2. Germination rate and water content of seeds.

Treatment	Germination rate (%)		
	Age of seeds (months after pollination)		
	2	3	4
Control seeds	0	75b	92a
Direct plunge into LN	0	0	0
Vitrification with PVS2	0	13d	32c
Water content of seeds FW (%)	81.6	25.0	13.1
Water content of seeds DW (g/g)	4.49	0.33	0.15

Data represents mean of 3 replicates for 4 MAP seeds, 1 replicate for 3 MAP, and 2 replicates for 2 MAP. Values followed by the same letter are not significantly different at the 0.05 level by the least significant different test (LSD).

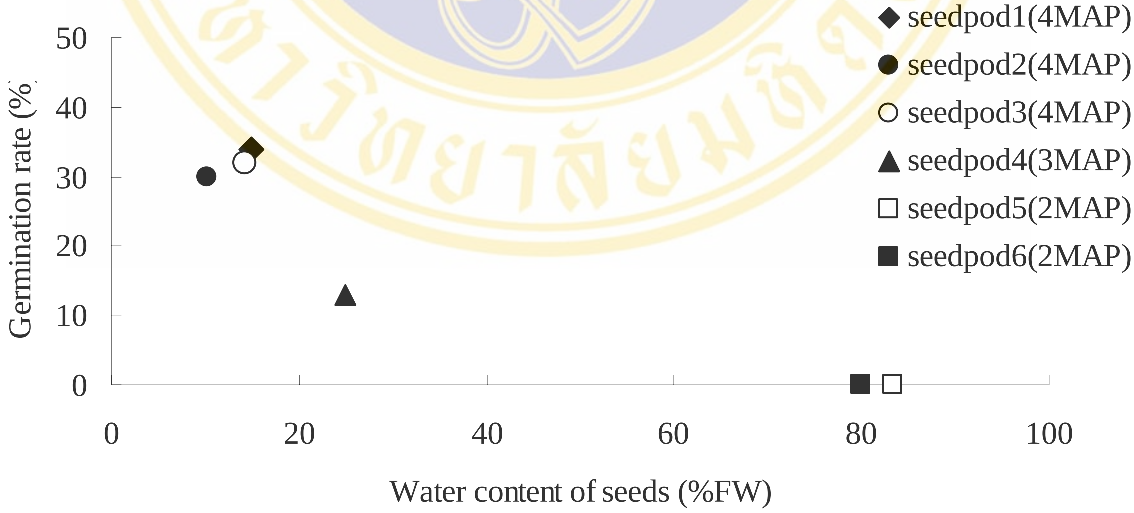


Figure 4-2. Effect of water content of seeds on germination rate after seeds cryopreservation.

Optimizing exposure time to PVS2 is an important step for cryopreservation by vitrification. PVS2 is potentially harmful due to phytotoxicity or osmotic stress damage.(66) The optimal exposure time to PVS2 was different among seed pods. There was no relationship between water content of seeds and optimal exposure time to PVS2 (Table 4-3,4,5,6,7,8).

The effect of exposure time to PVS2 solution with and without cooling to -196℃ (LN) is shown in Table 4-3,4,5,6,7, and 8. Exposure time to PVS2 solution of 0 min before plunging into LN resulted in no survival. As exposure time was extended to 10 - 50 min, survival rate increased to maximum of 34%. Longer exposure times resulted in decrease of survival.

Control seeds from seed pod 2 and 3 showed high germinability (100 %). When those seeds were exposed to PVS2 for 90 min without cooling to -196℃ (LN), the germination rate was still high (85-93 %). While control seeds from other seed pod 1 and 4 showed slightly low germination rate (75 %), showed significant drop with longer exposure to PVS2 without cooling to -196℃ (LN). Low vigor seeds might not stand long exposure to PVS2.

Table 4-3. Germination rate of seeds from seed pod 1 (4MAP), at 3 months after starting regrowth culture.

exposure time to PVS2 (min)	LN	germination rate(%)
0	+	0
10	+	contamination
20	+	8
30	+	34
40	+	0
50	+	2
60	+	5
70	+	0
80	+	0
90	+	0
0	−	61
10	−	37
20	−	71
30	−	75
40	−	76
50	−	4
60	−	41
70	−	15
80	−	55
90	−	15
control seeds		76

+LN means plant materials were treated with PVS2 and then plunged into LN.

−LN means that plant materials were treated with PVS2 and then hydrated with 1.2 M sucrose without plunging into LN.

Germination rate of control seeds was 76 %.

Replicate was 1 due to a little amount of seeds.

Table 4-4. Germination rate of seeds from seed pod 2 (4MAP), at 3 months after starting regrowth culture.

exposure time to PVS2 (min)	LN	germination rate(%)	S.D.
0	＋	0	0
10	＋	30	27
20	＋	20	9
30	＋	13	4
40	＋	6	5
50	＋	6	2
60	＋	0	0
70	＋	0	1
80	＋	0	0
90	＋	13	4
0	－	100	0
10	－	100	0
20	－	100	0
30	－	100	0
40	－	100	0
50	－	87	4
60	－	89	6
70	－	57	20
80	－	83	6
90	－	85	5
control seeds		100	0

＋LN means plant materials were treated with PVS2 and then plunged into LN.

－LN means that plant materials were treated with PVS2 and then hydrated with 1.2 M sucrose without plunging into LN.

Germination rate of control seeds was 100 %.

Replicate was 3.

Table 4-5. Germination rate of seeds from seed pod 3 (4MAP), at 3 months after starting regrowth culture.

exposure time to PVS2 (min)	LN	germination rate(%)	S.D.
0	+	0	0
10	+	15	3
20	+	11	0
30	+	11	2
40	+	contamination	
50	+	32	4
60	+	1	1
70	+	19	3
80	+	contamination	
90	+	0	1
0	−	100	0
10	−	100	0
20	−	100	0
30	−	100	0
40	−	100	0
50	−	100	0
60	−	100	0
70	−	100	0
80	−	93	2
90	−	93	9
control seeds		100	0

+LN means plant materials were treated with PVS2 and then plunged into LN.

−LN means that plant materials were treated with PVS2 and then hydrated with 1.2 M sucrose without plunging into LN.

Germination rate of naked seeds was 100 %.

Replicate was 3.

Table 4-6. Germination rate of seeds from seed pod 4 (3MAP), at 3 months after starting regrowth culture.

exposure time to PVS2 (min)	LN	germination rate(%)	S.D.
0	+	0	0
10	+	contamination	
20	+	13	15
30	+	contamination	
40	+	5	5
50	+	3	2
60	+	2	2
70	+	1	0
80	+	contamination	
90	+	9	5
0	−	contamination	
10	−	59	15
20	−	36	9
30	−	26	3
40	−	7	2
50	−	25	4
60	−	35	4
70	−	contamination	
80	−	35	6
90	−	0	1
control seeds		75	0

+LN means plant materials were treated with PVS2 and then plunged into LN.

−LN means that plant materials were treated with PVS2 and then hydrated with 1.2 M sucrose without plunging into LN.

Germination rate of naked seeds was 75 %.

Replicate was 3.

Table 4-7. Germination rate of seeds from seed pod 5 (2MAP), at 3 months after starting regrowth culture.

exposure time to PVS2 (min)	LN	germination rate(%)	S.D.
0	+	0	0
10	+	0	0
20	+	0	0
30	+	0	0
40	+	0	0
50	+	0	0
60	+	0	0
70	+	0	0
80	+	0	0
90	+	0	0
0	−	0	0
10	−	0	0
20	−	0	0
30	−	0	0
40	−	0	0
50	−	0	0
60	−	0	0
70	−	0	0
80	−	0	0
90	−	0	0
control seeds		0	0

+LN means plant materials were treated with PVS2 and then plunged into LN.

−LN means that plant materials were treated with PVS2 and then hydrated with 1.2 M sucrose without plunging into LN.

Germination rate of naked seeds was 0 %.

Replicate was 3.

Table 4-8. Germination rate of seeds from seed pod 6 (2MAP), at 3 months after starting regrowth culture.

exposure time to PVS2 (min)	LN	germination rate(%)	S.D.
0	＋	0	0
10	＋	0	0
20	＋	0	0
30	＋	0	0
40	＋	0	0
50	＋	0	0
60	＋	0	0
70	＋	0	0
80	＋	0	0
90	＋	0	0
0	－	0	0
10	－	0	0
20	－	0	0
30	－	0	0
40	－	0	0
50	－	0	0
60	－	0	0
70	－	0	0
80	－	0	0
90	－	0	0
control seeds		0	0

＋LN means plant materials were treated with PVS2 and then plunged into LN.

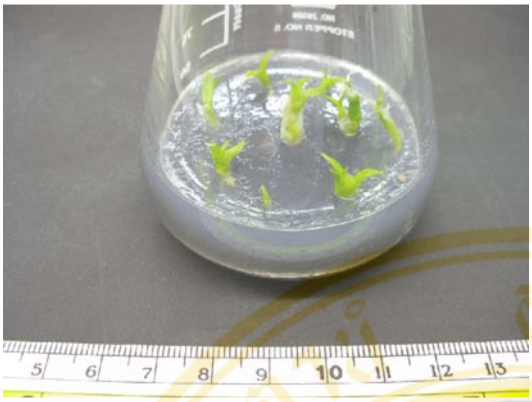
－LN means that plant materials were treated with PVS2 and then hydrated with 1.2 M sucrose without plunging into LN.

Germination rate of naked seeds was 0 %.

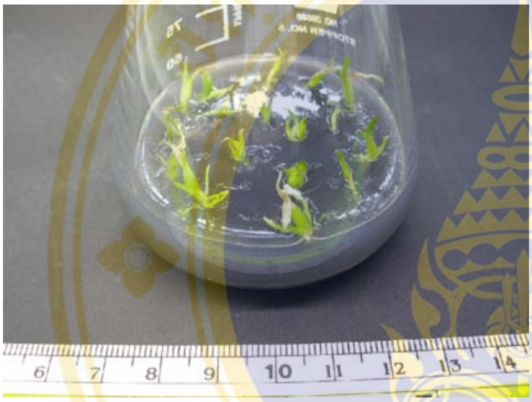
Replicate was 3.

Seeds treated with cryogenic procedures developed into normal protocorms (Fig.4-3) after 3-month culture on VW modified agar medium. Then, protocorms were tranfered on VW modified agar medium supplemented with banana and activated charcoal. 3 months after further cultivation, plantlets (Fig.4-4) were transferred to saranhouse (Fig.4-5) after 4 weeks acclimatization.

Figure 4-3. Protocorms derived from seeds, 3 months after starting regrowth culture.
Left: Protocorms from control seeds.
Center: Protocorms from seeds treated with PVS2.
Right: Protocorms from seeds treated with PVS2 and then plunged into LN.



(A)



(B)

Figure 4-4. Plantlets totally 6 months after starting regrowth culture.

(A) Plantlets from control seeds.

(B) Plantlets from seeds treated with PVS2 and then plunged into LN.


(A)


(B)


(C)

Figure 4-5. Plantlets 2 months after starting culture in the saranhouse.

(A) Plantlets from control.

(B) Plantlets from seeds treated with PVS2.

(C) Plantlets from seeds treated with PVS2 and then plunged into LN.

4.2 Results of cryopreservation of protocorms of *Dendrobium cruentum* Rchb. f.

1 month after sowing seeds from mature seed pods, protocorms were formed on VW agar medium (Fig.4-6). Protocorms were precultured in VW liquid medium supplemented with 0.06, 0.3, 0.5, and 0.7 M sucrose concentration. After preculture, we examined vitrification and encapsulation - dehydration. The process of exposure to PVS2 was done at 0±2 ℃ to avoid severe toxicity of PVS2.

Figure 4-6. Protocorms.

4.2.1 Vitrification

Experiment 1

The diameter of protocorms was 0.1-0.2 mm. The water content of protocorms was 65 %FW (= 2.0 g/gDW). Protocorms precultured in VW liquid medium supplemented with 0.06, 0.3, 0.5, and 0.7 M sucrose for 1 d and without plunging into LN, showed survival (Table 4-9, Fig.4-7). When exposure time to PVS2 was longer, survival rate of protocorms without LN decreased. However, protocorms had no survival after plunging into LN (Table 4-9, Fig.4-7). From these results, dehydration of protocorms was not enough to avoid lethal freezing damage and to survive after plunging into LN, although protocorms precultured for 1 d acquired dehydration tolerance to PVS2.

Protocorms precultured for 2 d had survival after plunging into LN (Table 4-10, Fig.4-7). Protocorms precultured in medium supplemented with 0.3 M sucrose for 2 d and then exposed to PVS2 for 240 min showed the highest survival rate of 33 %. Protocorms precultured in medium supplemented with 0.7 M sucrose for 2 d and then exposed to PVS2 for 120 min also showed high survival rate of 24 %.

Protocorms precultured for 5 d had no survival with / without plunging into LN (Table 4-11, Fig.4-7). Long preculture duration may cause severe dehydration. Five days of preculture in medium supplemented with 0.5 M sucrose might remove essential water resulting in lethal damage.

Protocorms precultured in medium supplemented with 0.06 M for 5 d without LN, decreased survival rate, compared to that of 1, 2 d preculture. In our experiment, liquid medium was used for preculture of protocorms to equally expose all dimensions of protocorms to medium. While water uptake by protocorms from medium was increased, water content of protocorms raised. This might be the reason why protocorms precultured in liquid medium with 0.06 M sucrose decrease survival rate, when preculture duration was prolonged.

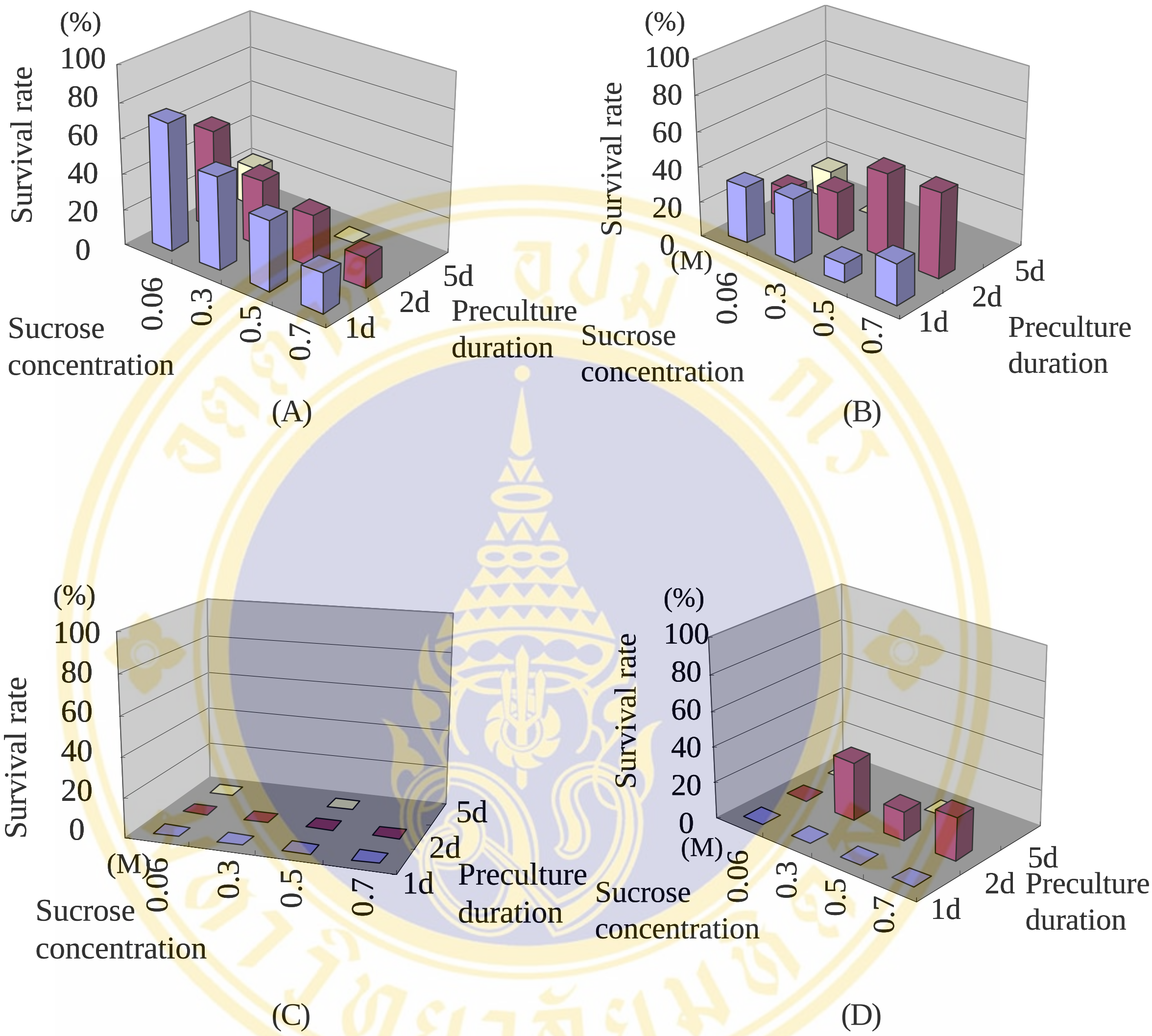


Figure 4-7. Survival rate of protocorms

(A) Survival rate after preculture.

(B) Survival rate after preculture and exposure to PVS2.

(C) Survival rate after preculture and plunging into LN without exposure to PVS2.

(D) Survival rate after preculture, exposure to PVS2 and then plunging into LN.

Table 4-9. Survival rate of protocorms precultured for 1 d.

preculture		exposure time to PVS2 (min)	+LN	−LN
sucrose (M)	duration (d)		survival rate (%)	survival rate (%)
0.06	1	0	0	72
		60	0	32
		120	0	18
		180	0	7
		240	0	0
0.3	1	0	0	52
		60	0	36
		120	0	12
		180	0	8
		240	0	0
0.5	1	0	0	39
		60	0	11
		120	0	7
		180	0	0
		240	0	2
0.7	1	0	0	22
		60	0	16
		120	0	12
		180	0	14
		240	0	23

Preculture was done in VW liquid medium supplemented with 0.06, 0.3, 0.5, and 0.7 M sucrose for 1d.

+LN means plant materials were treated with LS, PVS2 and then plunged into LN.

−LN means plant materials were treated with LS, PVS2 and then hydrated with 1.2 M sucrose without plunging into LN.

Replicate was 1.

Water content of protocorms was 65 %FW (= 2.0 g/gDW), diameter was 0.1-0.2 mm.

Table 4-10. Survival rate of protocorms precultured for 2 d.

preculture		exposure time to PVS2 (min)	＋LN	－LN
sucrose (M)	duration (d)		survival rate (%)	survival rate (%)
0.06	2	0	0	56
		60	0	18
		120	0	10
		180	0	6
		240	0	0
0.3	2	0	0	38
		60	0	27
		120	12	23
		180	0	16
		240	33	25
0.5	2	0	0	29
		60	0	25
		120	0	16
		180	contamination	48
		240	16	36
0.7	2	0	0	17
		60	0	26
		120	24	48
		180	15	30
		240	11	26

Preculture was done in VW liquid medium supplemented with 0.06, 0.3, 0.5, and 0.7 M sucrose for 2 d.

＋LN means plant materials were treated with LS, PVS2 and then plunged into LN.

－LN means plant materials were treated with LS, PVS2 and then hydrated with 1.2 M sucrose without plunging into LN.

Replicate was 1.

Water content of protocorms was 65 %FW (= 2.0 g/gDW), diameter was 0.1-0.2 mm.

Table 4-11. Survival rate of protocorms precultured for 5 d.

preculture		exposure time to PVS2 (min)	+LN	−LN
sucrose (M)	duration (d)		survival rate (%)	survival rate (%)
0.06	5	0	0	23
		60	0	16
		120	0	6
		180	0	0
		240	0	0
0.5	5	0	0	0
		60	0	0
		120	0	0
		180	0	0
		240	0	0

Preculture was done in VW liquid medium supplemented with 0.06, and 0.5 M sucrose for 5d.

+LN means plant materials were treated with LS, PVS2 and then plunged into LN.

−LN means plant materials were treated with LS, PVS2 and then hydrated with 1.2 M sucrose without plunging into LN.

Replicate was 1.

Water content of protocorms was 65 %FW (= 2.0 g/gDW), diameter was 0.1-0.2 mm.

Experiment 2

From the results of experiment 1, preculture for 2 d and longer exposure time to PVS2 (120 – 240 min) seemed to be suitable for cryopreservation. So in experiment 2, we concentrated on preculture for 2 d and expanded the exposure time to PVS2 from 0 to 360 min. Moreover, preculture for 3 d was examined to investigate whether it was possible to shorten optimal PVS2 exposure time.

The diameter of protocorms was 0.2-0.5 mm. The water content of protocorms was 87 %FW (= 6.7 g/gDW). Compared to experiment 1, water content of protocorms was very high and protocorms had large volume.

No survival was found in experiment 2 after plunging into LN (Table 4-12,13). Compared survival rate without plunging into LN in experiment 2 to that of experiment 1, protocorms at high water content and large volume reduced dehydration tolerance to PVS2.

Table 4-12. Survival rate of protocorms precultured for 2 d.

preculture		exposure time to PVS2 (min)	+LN		−LN	
sucrose (M)	duration (d)		survival rate (%)	S.D.	survival rate (%)	S.D.
0.06	2	0	0	0	57	12
		60	0	0	33	7
		120	0	0	3	1
		180	0	0	6	4
		240	0	0	4	2
		300	0	0	7	3
		360	0	0	6	2
0.3	2	0	0	0	24	16
		60	0	0	1	2
		120	0	0	4	4
		180	0	0	5	3
		240	0	0	contamination	
		300	0	0	2	1
		360	0	0	20	8
0.5	2	0	0	0	23	14
		60	0	0	20	5
		120	0	0	10	2
		180	0	0	29	0
		240	0	0	1	2
		300	0	0	15	13
		360	0	0	22	7
0.7	2	0	0	0	52	9
		60	0	0	contamination	
		120	0	0	8	2
		180	0	0	11	3
		240	0	0	22	11
		300	0	0	1	1
		360	0	0	14	11

Preculture was done in VW liquid medium supplemented with 0.06, 0.3, 0.5, and 0.7 M sucrose for 2 d.

+LN means plant materials were treated with LS, PVS2 and then plunged into LN.

−LN means plant materials were treated with LS, PVS2 and then hydrated with 1.2 M sucrose without plunging into LN. Replicate was 3.

Water content of protocorms was 87 %FW (= 6.7 g/gDW), diameter was 0.2-0.5 mm.

Table 4-13. Survival rate of protocorms precultured for 3 d.

preculture		exposure time to PVS2 (min)	+LN		−LN	
sucrose (M)	duration (d)		survival rate (%)	S.D.	survival rate (%)	S.D.
0.06	3	0	0	0	16	9
		60	0	0	8	6
		120	0	0	3	2
		180	0	0	0	0
		240	0	0	0	0
		300	0	0	0	0
		360	0	0	0	0
0.3	3	0	0	0	14	16
		60	0	0	1	2
		120	0	0	contamination	
		180	0	0	4	1
		240	0	0	contamination	
		300	0	0	3	5
		360	0	0	0	0
0.5	3	0	0	0	21	4
		60	0	0	contamination	
		120	0	0	7	2
		180	0	0	0	0
		240	0	0	22	17
		300	0	0	11	3
		360	0	0	contamination	
0.7	3	0	0	0	33	10
		60	0	0	contamination	
		120	0	0	contamination	
		180	0	0	contamination	
		240	0	0	33	1
		300	0	0	contamination	
		360	0	0	contamination	

Preculture was done in VW liquid medium supplemented with 0.06, 0.3, 0.5, and 0.7 M sucrose for 2d.

+LN means plant materials were treated with LS, PVS2 and then plunged into LN.

−LN means plant materials were treated with LS, PVS2 and then hydrated with 1.2 M sucrose without plunging into LN. Replicate was 3.

Water content of protocorms was 87 %FW (= 6.7 g/gDW), diameter was 0.2-0.5 mm.

4.2.2 Encapsulation - dehydration

Experiment 1

The diameter of protocorms was 0.1-0.2 mm. The water content of protocorms was 65 %FW (= 2.0 g/gDW).

Protocorms precultured in VW liquid medium supplemented with 0.7 M sucrose for 1 d and then dehydrated for 5 h showed survival rate of 10 % after plunging into LN (Table 4-14).

Protocorms precultured in VW liquid medium supplemented with 0.7 M sucrose for 2 d and then dehydrated for 4 h showed the highest survival rate of 27 %, for 9 h showed survival rate of 20 % after plunging into LN (Table 4-15).

For 5 d preculture, no survival was found after plunging into LN (Table 4-16).

Table 4-14. Survival rate of protocorms precultured for 1 d.

preculture			water	water	+LN	−LN
sucrose (M)	duration (d)	dehydration time (h)	content (%FW)	content (g/gDW)	survival rate (%)	survival rate (%)
0.3	1	0	61.0	1.56	0	55
		1	53.0	1.13	0	52
		2	49.4	0.98	0	33
		3	52.4	1.10	0	22
		4	33.4	0.50	0	38
		5	21.8	0.28	0	26
		6	29.2	0.41	0	29
		7	24.3	0.32	0	16
		8	19.9	0.25	0	0
		9	16.7	0.20	0	0
		10	13.7	0.16	0	0
0.5	1	0	64.0	1.78	0	59
		1	59.0	1.44	0	57
		2	56.7	1.31	0	43
		3	54.0	1.17	0	48
		4	41.4	0.71	0	26
		5	41.8	0.72	0	24
		6	26.7	0.36	0	18
		7	26.0	0.35	0	13
		8	23.4	0.31	0	0
		9	15.1	0.18	0	0
		10	13.4	0.15	0	0
0.7	1	0	63.5	1.74	0	51
		1	50.0	1.00	0	46
		2	48.0	0.92	0	36
		3	54.6	1.20	0	41
		4	39.3	0.65	0	14
		5	32.5	0.48	10	29
		6	32.0	0.47	0	20
		7	19.1	0.24	0	0
		8	17.3	0.21	0	3
		9	15.1	0.18	0	16
		10	13.2	0.15	0	0

Preculture was done in VW liquid medium supplemented with 0.3, 0.5, and 0.7 M sucrose for 1 d.

Replicate was 1. Water content of protocorms was 65 %FW (= 2.0 g/gDW), diameter was 0.1-0.2 mm.

Table 4-15. Survival rate of protocorms precultured for 2 d.

preculture					+LN	−LN
sucrose (M)	duration (d)	dehydration time (h)	water content (%FW)	water content (g/gDW)	survival rate (%)	survival rate (%)
0.3	2	0	64.6	1.82	0	49
		1	53.1	1.13	contamination	52
		2	53.3	1.14	contamination	55
		3	26.5	0.36	0	23
		4	27.4	0.38	0	31
		5	21.0	0.27	0	19
		6	16.6	0.20	0	21
		7	15.0	0.18	0	4
		8	13.1	0.15	0	1
		9	13.8	0.16	0	0
		10	12.2	0.14	0	0
0.5	2	0	68.3	2.16	0	68
		1	46.2	0.86	0	40
		2	40.6	0.68	0	46
		3	26.5	0.36	0	31
		4	19.9	0.25	0	26
		5	19.1	0.24	0	8
		6	15.5	0.18	0	12
		7	14.8	0.17	10	25
		8	14.2	0.17	0	11
		9	14.4	0.17	0	0
		10	12.7	0.15	0	0
0.7	2	0	68.6	2.18	0	67
		1	48.5	0.94	0	51
		2	41.2	0.70	0	53
		3	26.2	0.35	0	19
		4	25.5	0.34	27	35
		5	19.2	0.24	0	30
		6	18.6	0.23	0	16
		7	15.9	0.19	contamination	8
		8	13.6	0.16	0	12
		9	13.9	0.16	20	31
		10	13.2	0.15	0	2

Preculture was done in VW liquid medium supplemented with 0.3, 0.5, and 0.7 M sucrose for 2 d.

Replicate was 1. Water content of protocorms was 65 %FW (= 2.0 g/gDW), diameter was 0.1-0.2 mm.

Table 4-16. Survival rate of protocorms precultured for 5 d.

preculture					+LN	−LN
sucrose (M)	duration (d)	dehydration time (h)	water content (%FW)	water content (g/gDW)	survival rate (%)	survival rate (%)
0.5	5	0	69.5	2.28	0	51
		1	52.6	1.11	0	29
		2	37.2	0.59	0	13
		3	31.2	0.45	0	26
		4	27.2	0.37	0	4
		5	21.8	0.28	0	9
		6	17.8	0.22	0	0
		7	15.6	0.18	0	0
		8	14.8	0.17	0	0
		9	14.5	0.17	0	0
		10	12.8	0.15	0	0

Preculture was done in VW liquid medium supplemented with 0.5 M sucrose for 5 d.

Replicate was 1.

Water content of protocorms was 65 %FW (= 2.0 g/gDW), diameter was 0.1-0.2 mm.

Experiment 2

From the result of experiment 1, Preculture for 2 d seemed to be suitable duration for encapsulation - dehydration of protocorms. In experiment 2, we focused on 2 d preculture.

The diameter of protocorms was 0.2-0.5 mm. The water content of protocorms was 87 %FW (= 6.7 g/gDW). Compared to experiment 1, water content of protocorms was very high and protocorms had large volume.

No survival was found in experiment 2 after plunging into LN (Table 4-17). High water content and / or large volume might be reasons for the failure of encapsulation – dehydration application.

Table 4-17. Survival rate of protocorms precultured for 2 d.

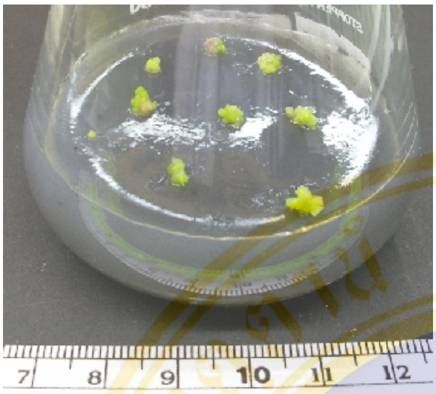
preculture					+LN	−LN	
sucrose (M)	duration (d)	dehydration time (h)	water content (%FW)	water content (g/gDW)	survival rate (%)	survival rate (%)	
0.3	2	0	82.6	4.74	0	90	
		1	77.4	3.42	0	44	
		2	66.9	2.03	0	36	
		3	48.9	0.96	0	contamination	
		4	20.8	0.26	0	52	
		5	18.3	0.22	0	57	
		6	12.7	0.15	0	36	
		7	17.8	0.22	0	contamination	
		8	18.0	0.22	0	18	
		9	16.2	0.19	0	6	
		10	16.2	0.19	0	0	
0.5	2	0	72.1	2.58	0	40	
		1	71.8	2.55	0	43	
		2	65.1	1.87	0	9	
		3	56.0	1.27	0	19	
		4	23.1	0.30	0	contamination	
		5	18.5	0.23	0	19	
		6	18.1	0.22	0	contamination	
		7	16.6	0.20	0	contamination	
		8	15.5	0.18	0	22	
		9	17.0	0.21	0	contamination	
		10	14.6	0.17	0	0	
0.7	2	0	78.2	3.59	0	80	
		1	65.2	1.87	0	contamination	
		2	61.1	1.57	0	21	
		3	36.8	0.58	0	55	
		4	20.9	0.26	0	contamination	
		5	22.4	0.29	0	56	
		6	18.0	0.22	0	43	※
		7	17.2	0.21	0	10	※
		8	16.9	0.20	0	contamination	
		9	17.4	0.21	0	29	
		10	15.4	0.18	0	0	

Preculture was done in VW liquid medium supplemented with 0.3, 0.5, and 0.7 M sucrose for 2 d.

Replicate was 1. Water content of protocorms was 87 %FW (= 6.7 g/gDW), diameter was 0.2-0.5 mm.

※ Protocorms changed to albino 3 months after starting regrowth culture.

Protocorms treated with cryogenic procedures developed into normal plantlets (Fig.4-8) after 3 months culture on VW modified agar medium supplemented with banana and activated charcoal. 3 months after further cultivation, plantlets (Fig.4-9) were transferred to saranhouse (Fig.4-10) after 4 weeks acclimatization.


(A)


(B)

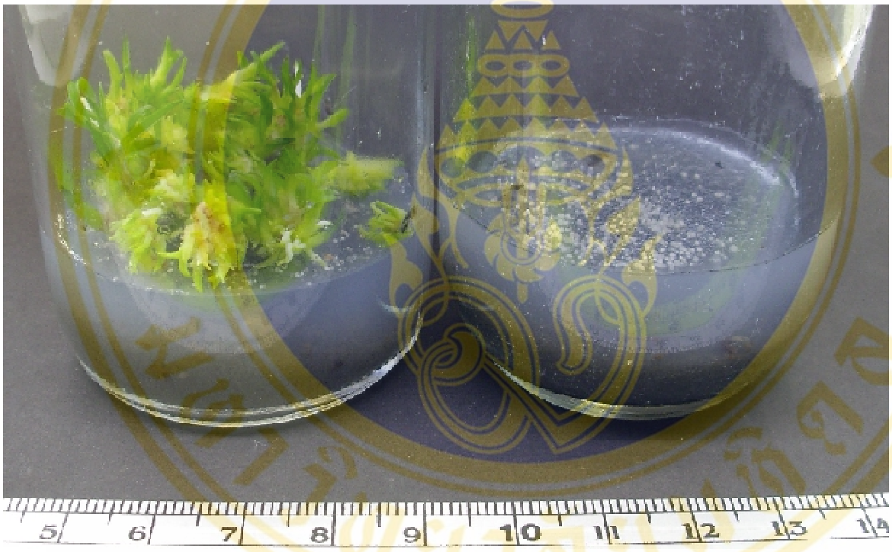

(C)

Figure 4-8. Plantlets derived from cryopreserved protocorms.

(A) 1 month after starting regrowth culture.

(B) 2 months after starting regrowth culture.

(C) 3 months after starting regrowth culture. Left: Plantlets derived from cryopreserved protocorms. Right: Protocorms that were no alive after plunging into LN

Figure 4-9. Plantlets derived from cryopreserved protocorms, 6 months after starting regrowth.

Figure 4-10. Plantlets 2 months after starting culture in the saranhouse.

4.3 Results of cryopreservation of PLBs of *Dendrobium cruentum* Rchb. f.

PLBs, obtained by propagation from shoot tips, were used for cryopreservation. As evidence from the results of 4.2, water content of protocorms seemed to be an important factor for successful cryopreservation. However, PLBs propagated in liquid medium could not be avoidable to have high water content and large volume. It might cause freezing damage by the existence of bulk water, delay of penetration of cryoprotectants, and delay of cooling speed. Therefore, PLBs were transferred from liquid medium to agar medium supplemented with 1.5 % (w/v) agar, two weeks before preculture with high concentration of sucrose medium. Moreover, to increase gas exchange and emission of water vapor out into the air, cotton wool cap were used. It resulted in decrease of water content from 90.0 %FW (= 9.3 g/gDW) to 73.8 %FW (= 2.8 g/gDW) (Table 4-18). When duration of culture on high agar medium was prolonged to 2 months, water content of PLBs maintained at about 70 %FW.

Wang *et al*.(54) reported the effect of ABA on increasing dehydration tolerance and succeeded in *Dendrobium candidum* PLBs cryopreservation by air-drying method and vitrification method. Pretreatment with medium supplemented with ABA increase the accumulation of soluble sugar, and resulted in increasing desiccation tolerance. So, the effect of preconditioning with ABA on survival rate was examined.

To investigate the effect of diameter of PLBs, PLBs were separated depending on diameter, one group was less than 2.0 mm diameter, another group was more than 2.1 mm to less than 3.5 mm.

Table 4-18. Effect of medium condition and cap on water content of PLBs.

medium		%FW	g/gDW
VW liquid medium	plastic seal	90.0	9.3
VW agar medium	plastic cap	83.3	5.0
VW agar medium	cotton cap	73.8	2.8

VW agar medium supplemented with 1.5 %(w/v) agar.

Despite of preconditioning on medium supplemented with high agar concentration and ABA, no survival was found with or without plunging into LN.

After preconditioning, PLBs transferred on VW agar medium started regrowth without any necrosis. However, PLBs precultured with high sucrose medium could not start regrowth. Preconditioning was not optimal for PLBs to acquire dehydration tolerance for preculture (table4-19). Data in details were shown in table 4-20,21,22.

Table 4-19. Summary of results of PLBs.

Preconditioning on VW agar medium							Preculture in VW liquid medium				Cryopreservation		
Agar %(w/v)	RH (%)	ABA (ppm)	Sucrose (M)	Glycerol (M)	duration (d)	survival	Sucrose (M)	ABA (ppm)	duration (d)	survival		survival +LN	survival −LN
1.5	34	0	0.06	0	14	○	0.06 0.3 0.5 0.7	0	2	○ × × ×	Vitrification	× × × ×	× × × ×
		0.1	0.06	0	14	○	0.06 0.3 0.5 0.7	0	2	○ × × ×	Vitrification	× × × ×	× × × ×
		1	0.06	0	14	○	0.06 0.3 0.5 0.7	0	2	○ × × ×	Vitrification	× × × ×	× × × ×
		10	0.06	0	14	×	—	—	—	—	—	—	—
1.5	34	1	0.06	0	14	○	0.7	0 0.1 1 10	2	× × × ×	Encapsulation-dehydration	× × × ×	× × × ×
1.5	34	0	0.4	0.1	3	○	0.3	0	3	×	Encapsulation-dehydration	×	×

Survival ○ means PLBs showed regrowth.

Survival × means PLBs showed no survival.

Table 4-20. Survival rate of PLBs preconditioned with ABA, precultured for 2 d and cryopreserved by vitrification.

preculture		exposure time to PVS2 (min)	+LN	−LN
sucrose (M)	duration (d)		survival rate (%)	survival rate (%)
0.06	2	0	0	0
		60	0	0
		120	0	0
		180	0	0
		240	0	0
0.3	2	0	0	0
		60	0	0
		120	0	0
		180	0	0
		240	0	0
0.5	2	0	0	0
		60	0	0
		120	0	0
		180	0	0
		240	0	0
0.7	2	0	0	0
		60	0	0
		120	0	0
		180	0	0
		240	0	0

Preconditioning was done on VW agar medium supplemented with 1.5 %(w/v) agar and 0, 0.1, and 1.0 ppm ABA.

Preculture was done in VW liquid medium supplemented with 0.06, 0.3, 0.5, and 0.7 M sucrose for 2 d.

+LN means plant materials were treated with LS, PVS2 and then plunged into LN.

−LN means plant materials were treated with LS, PVS2 and then hydrated with 1.2 M sucrose without plunging into LN.

Replicate was 1.

From the result of protocorms (Table 4-15), preculture on medium supplemented with 0.7 M sucrose concentration for 2 d was optimal preculture condition for protocorms. So, for PLBs, this preculture condition following preconditioning with ABA was only applied. Since preconditioning with maximam 1.0 ppm ABA was not enough, 10.0 ppm ABA for preconditioning was investigated. After preconditioning, PLBs transferred on VW agar medium started regrowth without any necrosis, except for PLBs treated with 10.0 ppm of ABA. They changed the color brown and died during two weeks preconditioning.

The effect of ABA during preculture on survival rate was examined. PLBs treated with 1.0 ppm ABA were precultured on VW agar medium supplemented 0.7 M sucrose and 0, 0.1, 1.0, and 10.0 ppm ABA before cryopreservation. However, no survival was found with / without plunging into LN (Table 4-21).

Table 4-21. Survival rate of PLBs preconditioned with ABA, precultured for 2 d and cyopreserved by encapsulation - dehydration.

preculture			dehydration time (h)	water content (%FW)	water content (g/gDW)	+LN	−LN
sucrose (M)	ABA (ppm)	duration (d)				survival rate (%)	survival rate (%)
0.7	0 / 0.1 / 1.0 / 10.0	2	0	69.7	2.30	0	0
			1	66.7	2.00	0	0
			2	64.4	1.81	0	0
			3	58.0	1.38	0	0
			4	55.6	1.25	0	0
			5	47.4	0.90	0	0
			6	42.4	0.74	0	0
			7	31.0	0.45	0	0
			8	20.0	0.25	0	0
			9	23.1	0.30	0	0
			10	19.2	0.24	0	0

Preconditioning was done on VW agar medium supplemented with 1.5 %(w/v) agar and 0, 0.1, 1.0 ppm ABA.

Preculture was done in VW liquid medium supplemented with 0.7 M sucrose and 0, 0.1, 1.0, and 10.0 ppm ABA for 2 d.

Replicate was 1.

The effect of glycerol and sucrose during preconditioning on survival rate was examined.

No survival was found with / without plunging into LN (Table 4-22). PLBs preconditioned without preculture were transferred on VW agar medium, and then started regrowth. However, preconditioned and then precultured PLBs changed the color brown and died after transferred on VW agar medium.

Table 4-22. Survival rate of PLBs preconditioned with sucrose and glycerol, precultured for 3 d and cryopreserved by encapsulation - dehydration.

PLBs diameter (mm)	preculture sucrose (M)	preculture duration (d)	dehydration time (h)	water content (%FW)	water content (g/gDW)	+LN survival rate (%)	−LN survival rate (%)
1.5-2.0	0.3	3	0	73.9	3.8	0	0
			1	66.7	3.0	0	0
			2	50.0	2.0	0	0
			3	40.0	1.7	0	0
			4	30.0	1.4	0	0
			5	20.0	1.3	0	0
			6	25.0	1.3	0	0
			7	16.7	1.2	0	0
			8	12.5	1.1	0	0
2.0-3.5	0.3	3	0	78.1	4.6	0	0
			1	68.8	3.2	0	0
			2	46.7	1.9	0	0
			3	46.7	1.9	0	0
			4	46.7	1.9	0	0
			5	28.6	1.4	0	0
			6	25.0	1.3	0	0
			7	15.4	1.2	0	0

Preconditioning was done on VW agar medium supplemented with 0.4 M sucrose and 0.1 M glycerol over night under dark condition.

Preculture was done in VW liquid medium supplemented with 0.3 M sucrose and for 3 d.

Replicate was 2.

4.4 Results of cryopreservation of shoot tips of *Dendrobium cruentum* Rchb. f.

Experiment 1

Shoot tips treated with PVS2 after preculture on VW agar medium supplemented with 0.06, 0.3, 0.5, and 0.7 M sucrose for 1, 2, and 3 d had no survival with / without plunging into LN. Only shoot tips without any treatment (control) showed survival of 33 % at 2 months after starting regrowth culture (Table 4-23). However, some shoot tips changed the color brown and then died 3 months after starting regrowth culture. Finally, the survival rate decreased to 13 %.

Table 4-23. Survival rate of shoot tips cryopreserved by vitrification.

preculture				2 months after starting regrowth culture	3 months after starting regrowth culture
sucrose (M)	duration (d)	exposure time to PVS2 (min)	LN	survival rate(%)	survival rate(%)
0.06	1	0 - 100	+ / —	0	0
	2	0 - 100	+ / —	0	0
	3	0 - 100	+ / —	0	0
0.3	1	0 - 100	+ / —	0	0
	2	0 - 100	+ / —	0	0
	3	0 - 100	+ / —	0	0
0.5	1	0 - 100	+ / —	0	0
	2	0 - 100	+ / —	0	0
	3	0 - 100	+ / —	0	0
0.7	1	0 - 100	+ / —	0	0
	2	0 - 100	+ / —	0	0
	3	0 - 100	+ / —	0	0
Control shoot tips				33	13

Exposure time to PVS2 was 0,20,40,60, 80 and 100 min.

＋LN means plant materials were treated with PVS2 and then plunged into LN.

－LN means plant materials were treated with PVS2 and then hydrated with 1.2 M sucrose without plunging into LN.

Survival rate of control shoot tips decreased from 33 % to 13 % during 2 to 3 months after starting regrowth culture.

Replicate was 1.

n =15.

The results indicated that excised shoot tips were difficult to start growth on VW agar medium at our culture conditions. So, the effect of phytohormone to enhance shooting of orchids, ascorbic acid to avoid deterioration, and activated charcoal to improve aeration and to absorb toxic substances were examined. Not only modified VW medium but Murashige and Skoog (MS)(Appendix 7) medium were applied. Moreover, to avoid quick dehydration, we compared the effect of liquid medium and agar medium for the first two weeks of culture.

When suitable medium for regrowth of excised shoot tips was examined, it seemed that modified VW agar medium supplemented with 0.1 ppm NAA and 1 ppm BA should be chosen (Table 4-24).

VW medium is widely used for shoot tip culture, clonal propagation culture, *etc*. of *Dendrobium*. MS medium is also popularly used for orchid tissue culture. For *Dendrobium*, MS medium is often used for clonal propagation from mature leaves, micropropagation through pseudobulb segments, initial culture and multiple shoot production for some species. However this medium was not suitable for *Dendrobium cruentum*.

In experiment 2 and 3, 0.1 ppm NAA and 1 ppm BA were added to modified VW agar medium for cold acclimation, preculture, and regrowth.

Table 4-24. Effect of medium components on shooting rate.

	medium 1	medium 2	NAA (ppm)	BA (ppm)	Ascorbic acid (ppm)	Activated charcoal (g/l)	shooting rate (%)
VW	liquid	agar	0	0	0	0	60
VW	liquid	agar	0.1	1	0	0	80
VW	agar	agar	0.1	1	0	0	40
VW	liquid	agar	0.1	1	1	0	40
VW	liquid	agar	0.1	1	0	1	70
MS	liquid	agar	0.1	1	0	0	0
MS	liquid	agar	0.1	1	1	0	0
MS	liquid	agar	0.1	1	0	1	0

VW means modified VW medium.

MS means Murashige and Skoog medium.

Excised shoot tips were cultured in / on medium 1 for two weeks. After two weeks culture, shoot tips were transferred to medium 2.

Each 3 weeks, medium 2 was renewed.

Shooting rate was counted 1 month after starting culture.

Replicate was 1 time.

n =15.

Experiment 2

First of all, excised shoot tips were cultured in VW liquid medium for 2 d to avoid quick dehydration by exposing naked shoot tips to the air. After that, shoot tips were transferred on VW agar medium for 2 d, to be accustomed to room temperature and to avoid sudden dehydration by following preculture on high concentration of sucrose. Control shoot tips that were continued to culture on VW agar medium showed survival rate of 60 %, 2 months after starting regrowth culture.

Other shoot tips were treated by vitrification with PVS2, encapsulation-dehydration, encapsulation-vitrification. However, there was no survival. Some shoot tips changed the color from green to brown due to necrosis. Other shoot tips were green, but no shooting was found during 2 months after starting regrowth culture.

To identify reasons of this failure to shooting, we applied TTC test to check survival rate. 1 d after starting regrowth culture, almost all of shoot tips dyed red. However, 3 d after starting regrowth culture, all shoot tips did not dye that means no alive in TTC test (Fig.4-11). Although shoot tips looked green by human eyes, TTC test revealed that they were already not alive.

Figure 4-11. TTC test for checking survival of shoot tips.

Left: shoot tips, 1d after starting regrowth culture, dyed red by TTC test.

Right: shoot tips, 3 d after starting regrowth culture, did not dye by TTC test.

Table 4-25. Effect of precultures to avoid quick dehydration on survival rate of shoot tips.

treatment	preculture 1 sucrose (M)	preculture 1 duration (d)	preculture 2 sucrose (M)	preculture 2 duration (d)	encapsulation	exposure time to PVS2 (min)	dehydration time (h)	LN	survival rate (%)
vitrification	−	−	−	−	−	0 - 100	−	+ / −	0
	−	−	0.3	2	−	0 - 100	−	+ / −	0
	0.029	4	0.3	2	−	0 - 100	−	+ / −	0
encapsulation dehydration	−	−	−	−	+	−	0-10	+ / −	0
	−	−	0.3	2	+	−	0-10	+ / −	0
	0.029	4	0.3	2	+	−	0-10	+ / −	0
encapsulation vitrification	−	−	−	−	+	0 - 240	−	+ / −	0
	−	−	0.3	2	+	0 - 240	−	+ / −	0
	0.029	4	0.3	2	+	0 - 240	−	+ / −	0
control	−	−	−	−	−	−	−	−	60

Excised shoot tips were cultured in VW liquid medium for 2 d, and then transferred on VW agar medium for 2 d.

After total 4 days culture, shoot tips were transferred on VW agar medium supplemented with 0.3 M sucrose for 2 d.

Control shoot tips were continued to culturte on VW agar medium.

All medium were supplemented with 0.1 ppm NAA and 1 ppm BA.

Survival rate was counted 2 months after starting regrowth culture.

Replicate was 1.

n =15.

Experiment 3

In vitro plantlets were subjected to cold acclimation for 5 d. And then shoot tips were excised for preculture on VW agar medium supplemented with 0.3 M sucrose for 2 d. During preculture, shoot tips were subjected to cold acclimation.

Shoot tips were treated by vitrification with PVS2, encapsulation-dehydration, encapsulation-vitrification. However, there was no survival. Some shoot tips changed the color from green to brown due to necrosis. Other shoot tips were green, but no shooting was found during 2 months after starting regrowth culture.

Table 4-26. Effect of cold acclimation on survival rate of shoot tips.

treatment	cold acclimation 9:00-15:00 (℃)	cold acclimation 15:00-9:00 (℃)	preculture 9:00-15:00 (℃)	preculture 15:00-9:00 (℃)	sucrose (M)	encapsulation	exposure time to PVS2 (min)	dehydration time (h)	LN	survival rate (%)
vitrification	25	25	—	—	—	—	0 - 100	—	+/ —	0
	25	25	15	15	0.3	—	0 - 100	—	+/ —	0
	25	15	15	15	0.3	—	0 - 100	—	+/ —	0
	15	15	15	15	0.3	—	0 - 100	—	+/ —	0
encapsulation dehydration	25	25	—	—	—	+		0-10	+/ —	0
	25	25	15	15	0.3	+		0-10	+/ —	0
	25	15	15	15	0.3	+		0-10	+/ —	0
	15	15	15	15	0.3	+		0-10	+/ —	0
encapsulation vitrification	25	25	—	—	—	+	0 - 240		+/ —	0
	25	25	15	15	0.3	+	0 - 240		+/ —	0
	25	15	15	15	0.3	+	0 - 240		+/ —	0
	15	15	15	15	0.3	+	0 - 240		+/ —	0
control	25	25	—	—	—	—	—	—	—	60

The glass containers in which *in vitro* plantlets were cultured, were put into refrigerator for cold acclimation at 15±2 ℃, dark condition during night time or all through 5 d.

After cold acclimation, shoot tips were excised and then precultured on VW agar medium supplemented with 0.3 M sucrose for 2 d at 15±2 ℃ under dark condition.

All medium except for plantlets culture, were supplemented with 0.1 ppm NAA and 1 ppm BA.

Survival rate was counted 2 months after starting regrowth culture.

Replicate was 1.

n =15.

CHAPTER 5
DISCUSSION

5.1 Cryopreservation of seeds of *Dendrobium cruentum* Rchb. f.

Seed cryopreservation is one of the useful methods for orchid conservation because of its cost effectiveness and requirement of small space and human management.

Dendrobium cruentum Rchb. f. is listed in the IUCN CITES Appendix(I) as "World Endangered Orchid Species", but seed grown examples are commercially available. *Dendrobium* species closely allied to *Dendrobium cruentum*, like the equally beautiful *D. bellatulum*, difficult to cultivate successfully.(67) These are reasons why establishment of cryopreservation method of *Dendrobium cruentum* are needed immediately.

This thesis first reports the success of seed cryopreservation of *Dendrobium cruentum* Rchb. f. by vitrification with PVS2. We examined the effect of water content of seeds on cryopreservation by different seed maturity. As seeds were mature, water content of seeds decreased and resulted in increasing survival rate. In orchids, the content of various reserve materials in embryo change after fertilization, and starch-filled amyloplasts found in the young embryo cells disappear by the time the seeds ripe, (68,69) whereas lipids begin to accumulate after the cells have ceased to divide. Compositional changes in the cytoplasmic viscosity correlates with desiccation tolerance.(70) These are good accord with the experimental result of mature seeds cryopreservation of *Dendrobium cruentum*.

Seeds from some kinds of orchids acquire strong dormancy with maturity. Sometimes, these kinds of seeds take more than two years to germinate. This character is not comfortable for seed banks. Hirano *et al*.(50) succeeded in cryopreservation of immature orchid seeds by applying preculture seeds on medium supplemented with high concentration of sucrose in advance of exposure to PVS2. However, for extremely immature seeds that had too high water content (more than 80 %FW), preculture lowered germination rate. When severe preculture, too much concentration of sucrose or too long duration, would cause dramatic change in plant materials, plant materials lose vigor. Biochemical and ultrastructural observations indicate that desiccation during the early intolerant developmental stages of seed development drastically reduces the metabolic and cellular integrity of the axis. During the desiccation-tolerant stage of seed development such perturbations do not occur.(71) Fortunately, seeds of *Dendrobium cruentum* do not seem to have dormancy for germination. For these reasons, mature seeds should be chosen for cryopreservation in case of *Dendrobium cruentum*. When immature seeds are used, suitable condition of preculture must be selected.

Nikishina *et al*.(47) reported that tropical orchid seeds that water content of less than 13 % showed survival after directly plunging into LN without PVS2. However, seeds of *Dendrobium cruentum* had no survival after directly plunging into LN, although 4MAP seeds of *Dendrobium cruentum* had water content of 13 %FW. Not only water content but also accumulation of substances in seeds that enhances acquiring dehydration tolerance. *e.g*., LEA, might be correlated with germination rate after cryopreservation.

Our thesis is the first time to succeed in seed cryopreservation of *Dendrobium cruentum* by PVS2 treatment. Compared to other reports of tropical orchid seeds,(8,47,48,49) germination rate of *Dendrobium cruentum* after cryopreservation by vitrification with PVS2 was low. In this thesis, decrease of germination rate after exposure to PVS2 was found. *Dendrobium cruentum* might be more sensitive to PVS2 than other species. If so, other methods that can avoid damage by PVS2 might have effect on improving germination rate after cryopreservation.

Successful cryopreservation of plant materials by vitrification is a balance between adequate dehydration by PVS2 and prevention of damage by osmotic stress and chemical phytotoxicity. A delay in PVS2 toxicity between no cryopreserved seeds and cryopreserved seeds was found in our results. Cryopreserved seeds became sensitive to PVS2 at early exposure time, while no cryopreserved seeds can tolerate longer exposure to PVS2. A reason for this disparity could be that viability of no cryopreserved seeds depends solely on toxicity of PVS2, while that of cryopreserved seeds depend on both toxicity and crystallization.(66)

Thomashow(72) has recently reviewed freezing tolerance mechanism and suggests several possibilities for conferring natural freeze-resistance in plants.

- Prevention of freeze-induced protein denaturation.
- Prevention of molecules precipitating.
- Prevention of intracellular ice formation.
- Stabilization of membrane.
- Fatty acid desaturation.
- Stabilization of membranes by hydrophilic polypeptides.

- Expression of specific cold-tolerance genes.
- Accumulation of sucrose and other simple sugars.

Recent studies have suggested that treatments with sugar or ABA enhance activation of these cold tolerance genes and acquiring freezing tolerance.(57,58) The applications of preculture with high concentration of sugar, treatment with loading solution before exposure to PVS2 or the usage of other cryoprotectants also have potential to increase germination rate after cryopreservation. Although we examined only the effect of PVS2 because of limit number of seeds, further more suitable condition of vitrification composed of preculture, loading solution, and cryoprotectants might be found in future.

Finally it should be noted that seed characteristics affect whether cryopreservation will succeed or not. Optimal condition of cryopreservation would be changed by which seed lots are used in the experiment. In this thesis, only water content of seeds, months after pollination, and germination rate of control seeds are considered to be important factors. Unfortunately, an accumulation of research of wild orchid seeds has not been developed rather than that of commercial crops. Moreover, the varieties of characters, *e.g.*, terrestrial, epiphytic, temperate, and tropical orchids, make it difficult to unify approach to orchid seeds. The further knowledge and investigation in this area will contribute toward successful cryopreservation of orchid seeds.

5.2 Cryopreservation of protocorms and PLBs of *Dendrobium cruentum* Rchb. f.

The earliest stage of orchid "seedling" development is known as a protocorm. Protocorm is defined as the small spherical tuber-like bodies formed by germinating orchid seeds and has a morphological state that lies between undifferentiated embryo and a shoot.(73) PLBs are the proper term for the structures that resemble protocorms and are formed by tissue explants and / or callus *in vitro*.(73) Root hairs are produced from the lower portions of protocorm and PLBs. A growing point forms on the upper surface of the protocorm and PLBs, and a leafy shoot may be found. Once shoot and root are formed, physiological characteristics change. In our experiment, protocorms and PLBs before formation of shoot and root were used.

The advantage of cryopreservation of protocorms and PLBs is that we can save time before germination after starting regrowth culture. Some orchid seeds have strong dormancy for germination. Those seeds take more than two years to germinate. Moreover, protocorms and PLBs are the resource of large–scale propagation of orchid. However compared to seeds, protocorms and PLBs have lost desiccation tolerance, so pretreatment and immersion in mild cryoprotectant, LS before exposure to PVS2 or dehydration by air were applied for acquiring dehydration tolerance.

In this thesis, water content and size of plant materials were very important in virtification by PVS2 and encapsulation - dehydration methods. Protocorms at water content of 65 %FW (= 2.0 g/gDW) and 0.1-0.2 mm diameter, succeeded in cryopreservation, although protocorms at water content of 87 %FW (= 6.5 g/gDW) and 0.2-0.5 mm diameter, and PLBs at water content of 74 %FW (= 2.8 g/gDW) and about 2.0 mm diameter had no survival after cryopreservation. A number of experiments have shown that plant cells, which can tolerate the dehydration step of cryopreservation, can also survive the rapid freezing step.(54,74)

Before freezing, protocorms at any water content and size showed some survival. After freezing, however, protocorms at water content of 87 %FW (= 6.5 g/gDW) and 0.2-0.5 mm diameter had no survival. When protocorms have too much water in the cells, freezing injury can occur due to intracellular ice formation. Lower volume of bulk water in plant cells has advantage to avoid freezing injury. On the other hand, when over dehydrated, the osmotic stress can be damaging. Bian *et al*.(74) reported that when PLBs were dehydrated to water content of 0.1 g/gDW, there were no reduction of survival rate after cryopreservation. PLBs that withstood such severe desiccation also survived cryopreservation. Therefore, how to enhance the tolerance to dehydration in plant cells is critical to cryopreservation.

In our experiment, we applied preculture in medium supplemented with high sucrose concentration. Preculture with 0.3, 0.5 and 0.7 M sucrose medium for 2 d were useful for cryopreservation of protocorms at water content of 65 %FW (= 2.0 g/gDW) and 0.1-0.2 mm diameter. For successful cryopreservation of protocorms and PLBs at higher water content and larger size, other effective procedures should be conducted.

To reduce of water content, Preconditioning plant materials before preculture was applied. By culture on high agar medium with well porous cap to increase emission of water vapor, PLBs reduced water content from 90.0 %FW (= 9.3 g/gDW) to 73.8 %FW (= 2.8 g/gDW). Solubilized agar forms a gel that can bind water (the higher the agar concentration, the stronger that water is bound), and absorb compounds. Medium with high concentration of agar limits the uptake of water and other compounds by plants.

For further effect, 0.1, 1.0, and 10.0 ppm ABA were added into preconditioning and preculture medium. It has been widely established that ABA plays an important role in mediating responses to water deficiency.(75) A preculture with ABA induce proline accumulation and could induce some levels of tolerance to vitrification. Tetteroo *et al*.(76) were able to induce desiccation tolerance in carrot somatic embryos by treatment with ABA. Lee *et al*.(77) could increase freezing tolerance of potato cells by ABA treatment. Na *et al*.(59) succeeded in cryopreservation of tissue-cultured shoot promordia from shoot apices of cultured protocorms of orchids by 1.0 ppm ABA preculture for 3 d. On the other hand, Bian *et al*.(74) reported that exogenous ABA was able to induce the accumulation of sugars, heat-stable proteins and dehydrins The two dehydrins and other heat-stable protein were not detectable after removal of exogenous ABA but reappeared when PLBs were desiccated to water contents below 1.0 g/gDW of PLBs. The increased accumulation of soluble sugars in ABA treated samples was associated with increased dehydration tolerance (survival rate before freezing).

However, in our experiment, exogenous ABA did not have effect on acquiring dehydration tolerance to stand dehydration by PVS2 and encapsulation dehydration. When PLBs were exposed to PVS2 very short time, or dehydrated for short time, there was no survival before freezing. For PLBs exposed to PVS2 for 0 min, rehydrating solution, VW liquid medium supplemented 1.2 M sucrose might cause dehydration. It seemed that the water content of PLBs in our experiment was too high for ABA treatment to induce dehydration tolerance.

Bian *et al*.(74), and Dussert *et al*.(78) succeeded in decreasing water content of plant materials by exposure to lower RH atmosphere for some days. Shimonishi *et al*.(79) succeeded in cryopreservation of melon (*Cucumis melo* L.) embryos by dramatically decreasing water content from more than 90 %FW to 11 %FW. They applied ABA treatment before exposure embryos to lower RH atmoshphere for 7d and then directly plunged into LN. For plant materials that have too high water content, such a slowly desiccation method can be useful method. In our experiment, the application of slowly desiccation until plant materials can stand should have been done before preculture.

Finally, preconditioning by other cryoprotectant, glycerol before preculture was examined. Glycerol is one component of PVS2, and it has high potential of cryoprotectant for plant cells. Matsumoto *et al*.(80) reported that encapsulated lily meristems precultured with a mixture of high concentration of sucrose and glycerol produced considerable high shoot recovery rather than preculture only with sucrose. While a mixture of sucrose and EG or DMSO produced toxic effects during the dehydration. It is considered that glycerol contributes to minimize the injurious membrane changes resulting from severe dehydration. However, there was no survival before / after freezing.

Size of protocorms and PLBs also should be considered, because it affects on dehydration speed and cooling rate. Plant materials that have large size require long time to dehydrate, and effect of cryoprotectants goes on slowly, because surface area exposed to air or cryoprotectants is small. When inner cells are dehydrated enough to stand cryopreservation, outer cells might be damaged by too much dehydration. And cooling rate of plant materials with large size decreases when plant materials are plunged into LN. Slow cooling in vitrification method and encapsulation – dehydration method stimulates ice formation in plant cells. So protocorms and PLBs with small size should be used for successful cryopreservation.

5.3 Cryopreservation of *in vitro* shoot tips of *Dendrobium cruentum* Rchb. f.

The recent progress of cryopreservation techniques has considerable contributed to the improvement of post-thaw recovery in the last few years. Compared to other plant materials, the shoot recovery rate after cryopreservation is very low. Because shoot tips contain many types of tissues, which dehydration and freezing tolerance are different between tissues. Each tissue requires different optimal process for cryopreservation.(81)

However, cryopreservation of shoot tips is a useful method for conservation of genotypes, because shoot recovery without intermediate callus formation is essential for clonal conservation of germplasm.

Vitrification method by PVS2, encapsulation – dehydration method, and encapsulation - vitrification method were examined. There was no survival of shoot tips before and after freezing. Shoot tips could not stand dehydration by preculture on medium supplemented with high sucrose concentration.

Takagi (81) reported that adequate management of whole culture systems is essential to induce favorable physiological conditions for micropropagation of shoot tips from donor plants, effective preculturing of dissected shoot tips and vigorous recovery of shoot tips without intermediate callus formation. It is well-known that cold acclimation (0-5 ℃) is effective for cryopreservation of temperate crops although there is little effective for tropical plants. Adaptation of cold acclimation in tropical plants has not been examined extensively. In our experiment, donor plants were exposed to 15 ℃ due to limit of the equipment. The effect of cold acclimation on survival rate could not be investigated because shoot tips died after preculture.

Overall plant quality and uniformity of the explant also influence survival. Several studies have noted that shoot tips from vigorous plants give better results, and a system whereby nodal sections are cultured for a few days in advance of harvesting the shoot tips has been described for several species. This culture allows for development of the axillary bud into a growing apical shoot tip. In some species there appears to be a differential response of axillary and apical shoot tips to some stages of cryopreservation. Light treatment and temperature condition also affects survival, but it is uncertain whether there is a general phenomenon over species.

In case of orchids, it should be considered that origin of species, temperature or tropical, affects its tolerance to dehydration and low temperature. Species derived from temperature zone seems to be more tolerate to dehydration and low temperature than tropical species. Because a tropical species does not need tolerance to dehydration and to low temperature in natural habitats. So more complicated treatment might be essential for cryopreservation of shoot tips of tropical plants.

Until now, there are some reports for cryopreservation of shoot tips. However most studies have been limited in approach and treatment application, *e.g.*, pregrowth of meristem and / or donor plants on sucrose enriched medium, preculture on sucrose, ABA, cryoprotectants enriched medium before dehydration. The lack of understanding of what makes shoot tips more cryopreservable is apparent.

In my experiment, *in vitro* shoot tips can not survive after cryopreservation with preculture on sucrose enriched medium or cold acclimation. Experimental treatment and other culture conditions were followed a successful report of cryopreservation of *Dendrobium* shoot tips,(60) but no survival was found in *Dendrobium cruentum*. The reasons of no survival were difference in tolerance between species, and / or unwritten condition of donor plants, culture conditions, and experimental protocol.

Totally 20 species and cultivars of tropical plants, *e.g.*, taro, banana, pineapple and orchids, were succeeded in cryopreservation using vitrification with only slight modifications of the technique and preculture on sucrose enriched medium. From these facts, the main reason of no survival in my thesis might be condition of donor. *In vitro* shoot tips were excised from donor plants acquired by micropropagation for long time. It is well known that repetition of micropropagation may decrease vigor of plants. Moreover, a culture container of donor plants had much water dew inside. It means that relative humidity inside container is almost 100 %. Such a condition cause high water content of plant material and no tolerate to dehydration.

Successful cryopreservation is not only determined by the cryopreservation procedure itself, but also by the condition of plant material. Thus, for successful cryopreservation, excised shoot tips must be a physical state suitable for the acquisition of osmotolerance and the production of vigorous growth recovery.

Similar to PLBs, suitable preconditioning method to stand dehydration before cryopreservation should be studied. The key of successful preconditioning is how to control water content and to acquire dehydration tolerance. For further development of cryopreservation of shoot tips of tropical plants, studies on preconditioning for the induction of dehydration tolerance, seem to be most important.

CHAPTER 6
CONCLUSIONS

The objectives of this thesis were to develop a reliable method for cryopreservation of seeds, protocorms, PLBs and *in vitro* shoot tips of *Dendrobium cruentum* Rchb. f. and to investigate the influence of cryopreservation on regrowth and morphology of seedling. From all of the results in this study, it was concluded follows:

1. The water content of seeds decreased from 81.6 %FW to 13.1 %FW during mature process of seeds.
2. Seeds of 4 months after pollination had low water content (13.1 %FW) showed high germination rate after cryopreservation by vitrification with PVS2.
3. As the increase of water content of seeds, the germination rate after cryopreservation decreased.
4. Seeds that had high germinability can tolerate longer exposure to PVS2.
5. Many factors of cryopreservation from vitrification technique, such as PVS2, LN did not affect regrowth and morphological characteristics of recovered seedling of *Dendrobium cruentum* Rchb. f.
6. Protocorms at water content of 65 %FW (= 2.0 g/gDW) and 0.1-0.2 mm diameter succeeded in cryopreservation by vitrification and encapsulation dehydration method with preculture in medium supplemented with 0.3, 0.5, and 0.7 M sucrose for 2 d.
7. Protocorms at water content of 87 %FW (= 6.5 g/gDW) and 0.2-0.5 mm diameter had no survival after cryopreservation. Water content of protocorms is the key factor of successful cryopreservation.
8. PLBs at water content of 74 %FW (= 2.8 g/gDW) and about 2.0 mm diameter had no survival after preculture with high sucrose concentration medium. Preconditioning with ABA or glycerol had no effect to acquire dehydration tolerance.

9. There was no survival of shoot tips after preculture with high sucrose concentration medium.

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APPENDIX

1. Modified Vacin and Went (VW) medium

Components	mg/l		g/l (for stock)
KNO_3	525	Stock A (×100)	52.5
KH_2PO_4	250		25
$(NH_2)SO_4$	500		50
$MnSO_4 \cdot H_2O$	5.7		0.57
$MgSO_4 \cdot 7H_2O$	250	Stock B (×100)	25
$FeSO_4 \cdot 7H_2O$	27.85	Stock C (×100)	2.785
Na_2EDTA	37.25		3.725
$Ca_3(PO_4)_2$	200		
Sucrose	10000 - 20000		
Agar	0 - 8000		
Cocunut milk	150 ml		

pH 5.2

$Ca_3(PO_4)_2$ is resolved in HCl. Components except for $Ca_3(PO_4)_2$, sucrose, coconut milk, agar can be preserved as premixed stock A-C. Each reagent is resolved in distilled water separately, and measure up to 1000 ml. Stock should be kept at 4 ℃ refrigerator under dark condition. For culture of seedling, 1 % (w/v) banana and 0.5 g/l activated charcoal are added.

2. Alginate solution

Na-alginate 3 % (w/v)

Sucrose 0.4 M

Calcium – free modified VW medium

Slowly and carefully add Na-alginate to calcium – free modified VW medium supplemented with 0.4 M sucrose. Autoclaving at 121℃ for 15 min and store at 4 ℃ for encapsulation.

3. Calcium chloride solution

Addition of 0.1 M $CaCl_2$ in modified VW liquid medium supplemented with 0.4 M sucrose. Adjust pH to 5.2 and autoclaving at 121 ℃ for 15 min and store at 4 ℃ for encapsulation.

4. Loading solution (LS)

Glycerol 2.0 M

Sucrose 0.4 M

2.0 M glycerol was dissolved with VW liquid medium supplemented with 0.4 M sucrose. Autoclaving at 121 ℃ for 15 min and store at 4 ℃.

5. Plant vitrification solution 2 (PVS2)

Glycerol 30% (w/v)

Ethlene glycol (EG) 15 % (w/v)

Dimethyl sulphoxide (DMSO) 15 % (w/v)

Sucrose 0.4 M

A mixture of glycerol, EG and DMSO were dissolved with VW liquid medium supplemented with 0.4 M sucrose (pH 5.8). Autoclaving at 121 ℃ for 15 min and store at 4 ℃.

6. TTC solution

Na_2HPO_4 0.05M

KH_2PO_4 0.05M

2,3,5 – triphenyltetrazolium chloride 0.8 % (w/v)

Dissolved 0.8 % (w/v) 2,3,5 - triphenyltetrazolium chloride in sodium phosphate buffer (which was composed of 0.05 M Na_2HPO_4 and 0.05 M KH_2PO_4 in the water). Adjust pH to 7.5 and store in a dark bottle at 4 ℃ for TTC staining.

7. Murashige and Skoog (MS) medium

Components	mg/l
NH_4NO_3	1650
KNO_3	1900
KH_2PO_4	170
H_3BO_3	6.2
$MnSO_4 \cdot 4H_2O$	22.3
$ZnSO_4 \cdot 4H_2O$	8.6
KI	0.83
$Na_2 \cdot MoO_4 \cdot 2H_2O$	0.25
$CuSO_4 \cdot 5H_2O$	0.025
$CoCl_2 \cdot 6H_2O$	0.025
$CaCl_2 \cdot 2H_2O$	440
$MgSO_4 \cdot 7H_2O$	370
FeSO4 · 7H2O	27.8
Na_2EDTA	37.3
Myoinositol	100
Nicotinic acid	0.5
Pyridoxine Hydrochloride	0.5
Thiamine Hydrochloride	0.1
Glycine	2

BIOGRAPHY

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