DEVELOPMENT OF CRYOPRESERVATION PROTOCOLS FOR *PAPHIOPEDILUM ROTHSCHILDIANUM* AND *PHALAENOPSIS GIGANTEA* FOR LONG-TERM GERMPLASM CONSERVATION PROGRAM

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THESIS SUBMITTED IN FULFILLMENT FOR THE DEGREE OF MASTER OF SCIENCE

BIOTECHNOLOGY RESEARCH INSTITUTE
UNIVERSITI MALAYSIA SABAH
2008

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DEGREE : **MASTER OF SCIENCE (PLANT BIOTECHNOLOGY)**

VIVA DATE : **18 DECEMBER 2008**

DECLARED BY

1. **SUPERVISOR**
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Signature

DECLARATION

I hereby declare that the material in this dissertation is my own except for quotations, excerpts, equations, summaries and references, which have been duly acknowledged.

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ACKNOWLEDGEMENT

First of all, I would like to express my thanksgiving to the Lord Jesus Christ Who strengthen me to complete this project. I also wish to express my sincere appreciation and gratitude to Dr. Zaleha Abdul Aziz, my supervisor, for her encouragements, guidance, helps, advices and patience throughout my Master study.

Especially thanks to Wei Jing Jing, undergraduate student, who assisted me on some parts of my study. I would like to appreciate to the lab assistants, Radizah, Doreen, Christina, Richard and Vidarita, for their assistance and technical support. I also thank Dr. Mariam and Kenneth, who gave a lot of suggestions and opinions.

I would like to express my gratitude to all the staffs in Poring Orchid Conservation Center and Kinabalu Park for their help in providing *P. rothschildianum* and *P. gigantea* capsules. I also want to thank our government for their financial support for this project. My appreciation also goes to Biotechnology Research Institute, University Malaysia Sabah for providing me a comfortable environment and partnership throughout this project.

My deepest gratitude also goes to my family members. My parents who given me fully support for my study in UMS. Especially thanks for my elder sister, Koo Gaik Kee, who have given me financial support throughout my Master study. I also want to express my gratitude to my church members, who always support me by prayers.

ABSTRACT

DEVELOPMENT OF CRYOPRESERVATION PROTOCOLS FOR *PAPHIOPEDILUM ROTHSCHILDIANUM* AND *PHALAENOPSIS GIGANTEA* FOR LONG-TERM GERMPLASM CONSERVATION PROGRAM

Paphiopedilum rothschildianum and *Phalaenopsis gigantea* are two endangered orchids of Sabah. Both are currently protected and listed under the Convention in Trade on Endangered Species of Flora and Fauna (CITIES). Cryopreservation is an effective method for long term storage of plant materials. Hence, development of cryopreservation protocols for these two species will facilitate the long term conservation programmes of these orchids. In this study, protocorms and seeds were used as the explants. Preculture conditions, preculture durations and combinations of loading solution were evaluated for cryopreservation of protocorms of both species. The effect of PVS2 incubation period (0-90 min) on the viability of protocorms was evaluated. Protocorms were precultured in liquid medium supplemented with different concentrations of sucrose (0-0.5M) and precultured at different period (0-7 days). Different combinations of glycerol (0-2.5M) and sucrose (0-0.5M) were evaluated for loading solution. Protocorms of *P. rothschildianum* were used in developing encapsulation dehydration and encapsulation vitrification method. The viability of protocorms was determined using 2,3,5-triphenyl tetrazolium chloride (TTC) test. For the development of cryopreservation protocol of *P. rothschildianum* seeds, incubation time (0-5 hours) in PVS2 solution at 0^{o}C, preculture conditions (0-0.5M sucrose), preculture durations (1-7 days) and storage durations in liquid nitrogen (2-24 weeks) were evaluated. The viability of the seeds was determined using TTC test and the germination ability was recorded. Protocorms of *P. rothschildianum* precultured in MS medium supplemented with 0.25M sucrose for 3 days gave the highest viability value while protocorms of *P. gigantea* in XER medium supplemented with 0.25M sucrose for 5 days showed the highest viability value prior to liquid nitrogen storage. Loading solution comprised of 0.5M glycerol and 0.4M sucrose showed the highest viability value for protocorms of *P. rothschildianum* while the combination of 0.5M glycerol and 0.2M sucrose resulted in the highest viability value for *P. gigantea*. Protocorms showed low viability in all incubation periods. Protocorms failed to grow even after two months on recovery media after cryopreservation. No regrowth of protocorms were observed after cryopreservation by both encapsulation-dehydration and encapsulation-vitrification methods. Seeds that were dehydrated with PVS2 solution for 5 hours gave the highest seed germination percentage (30%). Seeds precultured on medium supplemented with 0.4M sucrose and precultured for 7 days resulted in the highest seed germination percentages, which was 30% and 63%, respectively. No significant difference in seed germination was observed when seeds were stored in liquid nitrogen for 2 to 24 weeks.

Key words: Paphiopedilum rothschildianum, Phalaenopsis gigantea, vitrification, encapsulation dehydration, encapsulation vitrification

ABSTRAK

Paphiopedilum rothschildianum dan Phalaenopsis gigantea merupakan dua orchid spesies yang terancam di Sabah. Kedua-dua spesies orkid ini telah dilindungi dan disenaraikan bawah Convention in Trade on Endangered Species of Flora and Fauna (CITES). Pengawetan-krio merupakan suatu kaedah yang berkesan untuk menyimpan bahan tumbuhan dalam jangka masa panjang. Oleh itu, perkembangan sistem pengawetan-krio bagi kedua-dua spesies ini akan memudahkan program pemeliharaan secara jangka masa panjang. Protokom dan biji benih digunakan dalam kajian ini. . Kesan bagi tempoh pengeraman dalam larutan vitrifikasi tumbuhan 2 (PVS2)(0-90min) telah dikaji. Kesan prakultur, tempoh prakultur dan gabungan larutan 'loading' dinilaikan untuk pengawetan-krio bagi protokom dua spesies orkid tersebut. Protokom diprakultur dalam media cecair yang dibekal dengan kepekatan sukrosa yang berlainan (0-0.5M) dan diprakultur pada tempoh yang berbeza (0-7 hari). Gabungan gliserol (0-2.5M) dan sukrosa (0-0.5M) yang berbeza telah dinilaikan bagi larutan 'loading'. Protokom P. rothschildianum digunakan dalam mengaji pengawetan-krio secara 'encapsulation dehydration' dan 'encapsulation vitrification'. Viabiliti bagi protokom dikaji dengan menggunakan 2,3,5-triphenyl tetrazolium chloride (TTC). Untuk perkembangan protokol pengawetan-krio bagi biji benih P. roschildianum, tempoh pengeraman dalam larutan PVS2 pada 0^0C (0-5 jam), kesan prakultur (0-0.5M sukrosa), tempoh prakultur (1-7 hari) dan tempoh penyimpanan dalam cecair nitrogen (2-24 minggu) telah dikaji. Viabiliti biji benih dikaji dengan TTC dan peratus percambahan. Protokom P. rothschildianum diprakultur dalam media cecair MS yang dibekal dengan 0.25M sukrosa selama 3 hari menunjukkan nilai viabiliti yang tertinggi manakala protokom P. gigantea dalam media cecair XER dibekal dengan 0.25M sukrosa selama 5 hari menunjukkan nilai viabiliti tertinggi sebelum disimpan dalam cecair nitrogen. Larutan 'loading' yang mengandungi 0.5M gliserol dan 0.4M sukrosa menunjukkan nilai viabiliti yang tertinggi bagi protokom P. rothschildianum manakala kombinasi 0.5M gliserol dan 0.2M sukrosa menunjukkan nilai viability yang tertinggi bagi protokom P. gigantea Protokom bagi dua spesies orkid tersebut menunjukkan viabiliti yang rendah dalam semua tempoh pengeraman yang dikaji. Protokom tidak menumbuh semula selepas pengawet-krio walaupun telah dikultur dua bulan. Tiada pertumbuhan semula bagi protokom yang diawet-krio dengan menggunakan kedua-dua cara tersebut. Biji benih yang dikeringkan dengan larutan PVS2 selama 5 jam menunjukkan peratus percambahan yang tertinggi (30%). Biji benih yang dikultur dalam media yang dibekal dengan 0.4M sukrosa dan selama 7 hari menunjukkan peratus percambahan yang tertinggi masing-msasing pada 30% dan 39%. Didapati bahawa peratus percambahan tidak berubah apabila biji nenih disimpan dalan cecair nitrogen selam 2 hingga 24 minggu.

Kata kunci: Paphiopedilum rothschildianum, Phalaenopsis gigantea, vitrifikasi, encapsulation dehydration, encapsulation vitrification

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LIST OF ABBREVIATIONS

BGCI	Botanic Gardens Conservation International
CITES	Convention on International Trade in Endangered Species
IPGRI	International Plant Genetic Resources Institute
LN	Liquid nitrogen
MS	Murashi and Skoog Medium, 1962
PVS2	Plant Vitrification Solution 2
PVS4	Plant Vitrification Solution 4
TTC	2,3,5-Tritetrazolium chloride
USDA	United States Department of Agriculture
XER	Experimental Ernst Robert Medium
ABA	Abscisic acid

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CHAPTER 1

INTRODUCTION

The orchids are classified under the Orchidaceae with over 25,000 known species and with an estimation of 5,000 species waiting to be discovered (McGough *et al.*, 2006). Orchids are now the second most popular potted floriculture crop with wholesale prices estimated at $128 million in the year 2004 (USDA, 2005) and have became popular houseplants.

Most of the orchid species are at risk of extinction as the results of habitat destruction caused by human activities such as logging, mining and urban development (Hágster and Dumont, 1996). It was been facing the stress of over collection of wild population for illegal trade (Sivasithamparam *et al.*, 2002). McGough *et al.* (2006) reported that over-collection of species that are important in trade can lead to the extinction of the species in the wild within a few years of discovery.

Many efforts have been done to conserve orchid species using biotechnological approaches. The Tropical Botanic Gardens and Research Institute in Kerala, India studied the *in vitro* conservation of 30 orchids from the conservation hotspot of Western Ghats (Sarasan *et al.*, 2006). Stewart and Kane (2006) had successfully developed some effective *in vitro* seed germination and seedling development media for *Habenaria macroceratitis*, which is a rare Florida terrestrial orchid.

Several species of orchids that had been cryopreserved include *Spathoglottis plicata* (Wang *et al.*, 2003), *Vanda pumila* (Na and Kondo, 1996), *Bletilla striata* (Ishikawa *et al.*, 1997; Hirano *et al.*, 2005), *Doritaenopsis* (Tsukazaki *et al.*, 2000) and *Dendrobium* Walter Oumae (Lurswijidjarus and Thammasiri, 2004). The materials used in cryopreservation included protocorms, shoot apices, zygotic embryos, suspension cells, immature seeds and shoot tips.

In Sabah, Malaysia, *Paphiopedilum rothschildianum* and *Phalaenopsis gigantea* are the two species of endangered orchids. Their number in the wild is reducing due to several reasons such as forest fires and illegal collection for the horticultural trade (Cribb, 1998). Cribb (1997) stated that one of the three sites on the lower slopes around Mount Kinabalu, where *P. rothschildianum* could be found, was totally destroyed by logging, mining and shifting agriculture.

Since the population of both *Paphiopedilum rothschildianum* and *Phalaenopsis gigantea* is reducing in the wild, there is a need to develop an alternative conservation strategy to protect these two species. This study is about developing cryopreservation protocols for *P. rothschildianum* and *P. gigantea* to address small portion of this problem.

There were two parts in this study. The first part was the development of cryopreservation protocols for protocorms whereas the second part focuses on the development of cryopreservation procedure for *P. rothschildianum* seeds. In the beginning of this project, only protocorms were available. It was because the flowering season for *P. rothschildianum* was in March and November. The capsule could only be collected after 6 months of pollination. Seeds of *P. gigantea* were not studied in this project because there was no flowering in the year 2005-2006. Thus, the seeds of *P. gigantea* were not available throughout the project.

The objectives of this study are:

(i) to develop the vitrification protocol for protocorms of *P. rothschildianum* and *Phalaenopsis gigantea* by optimizing preculture condition, preculture duration, loading solution, vitrification solution and PVS2 incubation duration.

(ii) to evaluate encapsulation-dehydration and encapsulation-vitrification method for *P. rothschildianum* protocorms.

(iii) to develop the vitrification protocol for seeds of *P. rothschildianum* by optimizing preculture condition, preculture duration and PVS2 incubation duration.

(iv) to evaluate the effect of long-term storage of *P. rothschildianum* seeds.

(v) to evaluate the applicability of optimized vitrification protocol for *Paphiopedilum rothschildianum* seeds.

CHAPTER 2

LITERATURE REVIEW

2.1 Orchid

Orchids are members of the plant family Orchidaceae. It is one of the largest families of flowering plants, which consists of 700 to 800 genera, 17,500 to 35,000 species (Dressler, 1993), and more than 70,000 ornamental hybrids (Bukhov *et al.*, 2006). Orchids are categorized into two main groups, namely terrestrials and epiphytes. Terrestrials orchids typically occur in temperate regions whereas epiphytes orchids mostly can be found in tropical regions (Sivasithamparam *et al.*, 2002).

The flowers of orchids are diverse in size, shape, colour, structure, number and fragrance. Thus, orchids are highly demanded in the cut-flower industry and ornamental potted plants. According to the United States Department of Agriculture (USDA, 2005), orchids are the second most popular potted floriculture crop in 2004 with whole prices estimated at $128 million (Kauth *et al.*, 2005).

Despite of the contribution in horticulture trade, orchids are also used for therapeutic purposes. For example, the stems of *Dendrobium officinale* have been used as a traditional Chinese tonic medicine (Su and Yang, 2006). Its main function is to nourish kidney and promote the production of body fluid, resist cancer and prolong life (The Pharmacopoeia Commission of People's Republic of China (PRC), 2000).

Because of the high demand in horticulture trade as well as therapeutic purposes, many orchids are facing collection stress. Most of the orchids are currently at risk of extinction as the results of human activities, which include habitat destruction and extraction of wild plant for trade (Sivasithamparam *et al.*, 2002). Hágster and Dumont (1996) characterized the factors that have been identified to cause orchids species being threatened into two categories. The first factor is habitat destruction, modification and fragmentation that include longing, agriculture and plantations, urban development and mining. The second factor is due to collecting for horticulture trade, amateur collection and consumable orchids.

2.1.1 Orchid Seeds and Their Development

Orchid seeds are unique and they are termed as 'dust seeds' which mean that the seeds are small measuring 200 to 1700μm (Rasmussen, 1995; Szendrak, 1997). Orchid seeds are characterized as being very light, produced in large numbers and enclosed within capsules (Figure 2.1). These characteristics not only facilitate dispersion around the parent plant but also over a wider area (Nakamura and Hamada, 1978; Rasmussen, 1995; Arditti and Abdul Ghani, 2000).

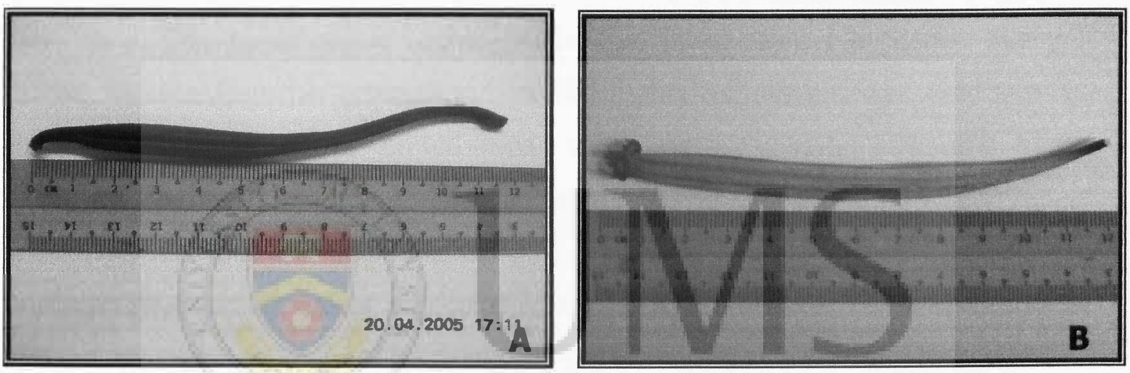

Figure 2.1: Capsule of orchids. A): Capsule of *Paphiopedilum rothschildianum* and, B): Capsule of *Phalaenopsis gigantea*.

Clifford and Smith (1969) had classified orchid seeds into five general shapes, namely shape one to five (Figure 2.2). Shape one and five are the most common shape whereas shape four is relatively rare. Different morphology and classes of orchid seeds are showed on Appendix D.

An orchid seed consists of a testa surrounding a tiny embryo in the globular stage. The seeds do not contain endosperm and have limited nutrient (Arditti, 1992). Thus, only 2-5% of seeds could be germinated in their natural environment (Nikishina *et al.*, 2006).