IN VITRO CONSERVATION AND MICROPROPAGATION OF *DENDROBIUM* SPP. AND *EULOPHIA SPECTABILIS*

CHUTIMA JAIPHET

A Thesis Submitted to University of Phayao

in Partial Fulfillment of the Requirements

for the Master of Science Degree in Biotechnology

May 2012

IN VITRO CONSERVATION AND MICROPROPAGATION OF *DENDROBIUM* SPP. AND *EULOPHIA SPECTABILIS*

CHUTIMA JAIPHET

A Thesis Submitted to University of Phayao

in Partial Fulfillment of the Requirements

for the Master of Science Degree in Biotechnology

May 2012

ACKNOWLEDGEMENT

I would like to express my sincere thank to my advisor, assistant professor Dr.Niramol Rangsayatorn, for her kind encouragement and valuable advice during this study.

I also appreciate the assistance of my examining committee members. All the supports from Plant Genetic Conservation Project under the Royal Initiative of Her Royal Highness Princess Maha Chakri Sirindhorn are highly acknowledged.

Many thanks extend to my friends and staff at Biology laboratory for their cheerfulness and kindness assistance, as well as thanks the School of Science, University of Phayao for permitting this study program.

I am very grateful to my beloved father and mother and my sister for their understanding, encouragement and help during many years of my study.

Chutima Jaiphet

ชื่อเรื่อง การอนุรักษ์และขยายพันธุ์กล้วยไม้สกุลหวายและว่านหัวครู ในสภาพปลอดเชื้อ

ผู้วิจัย ชุติมา ใจเพ็ชร

ประธานที่ปรึกษา ผู้ช่วยศาสตราจารย์ ดร.นิรมล รังสยาธร

กรรมการที่ปรึกษา ดร.วนิดา แซ่จึง

ประเภทสารนิพนธ์ วิทยานิพนธ์ วท.ม. สาขาวิชาเทคโนโลยีชีวภาพ, มหาวิทยาลัยพะเยา, 2555

คำสำคัญ การขยายพันธุ์ด้วยวิธีจุลภาค การอนุรักษ์ในสภาพปลอดเชื้อ กล้วยไม้สกุลหวาย ว่านหัวครู

บทคัดย่อ

การศึกษาการขยายพันธุ์เพื่อเพิ่มจำนวนอย่างรวดเร็วของเอื้องกิ่งดำ (*Dendrobium gratiosissimum*) เอื้องช้างน้าว (*D. pulchellum*) เอื้องแซะภูกระดึง (*D.christyanum*) และว่านหัวครู (*Eulophia spectabilis*) ด้วยเทคนิค thin cell layers (TCLs) ในสภาพหลอดทดลอง โดยนำโปรโตคอร์มอายุ 4 สัปดาห์ ผ่าแบ่งครึ่งและแต่ละครึ่งถือว่าเป็นตัวอย่างพืช จากนั้นนำตัวอย่างพืชเพาะเลี้ยงบนอาหารสูตร MS (Murashige and Skoog) ที่เติม N^6–benzyl adenine (BA), kinetin (Kn) และ **α**–naphthaleneacetic acid (NAA) ที่ความเข้มข้นต่างกัน ร่วมกับน้ำตาลซูโครส 20 กรัมต่อลิตร พบว่า ตัวอย่างพืชของเอื้องช้างน้าวและเอื้องแซะภูกระดึงไม่สามารถเกิด protocorm like bodies (PLBs) ได้เมื่อเพาะเลี้ยงบนอาหาร MS ที่ปราศจากสารควบคุมการเจริญเติบโต ส่วนตัวอย่างพืชของเอื้องกิ่งดำและว่านหัวครูสามารถพัฒนาเป็น PLBs ภายใน 3 ถึง 4 สัปดาห์ บนอาหาร MS ที่เติมสารควบคุมการเจริญเติบโต สำหรับการชักนำให้เกิด PLBs ของเอื้องกิ่งดำ เอื้องแซะภูกระดึง และว่านหัวครู มีประสิทธิภาพมากที่สุดบนอาหารที่เติม Kn ความเข้มข้น 2 มิลลิกรัมต่อลิตร ขณะที่อาหารที่เติม BA ความเข้มข้น 1 มิลลิกรัมต่อลิตรเหมาะสมสำหรับการชักนำให้เกิด PLBs ในเอื้องช้างน้าว จากนั้นอนุบาลต้นพืชที่เกิดขึ้นใหม่และย้ายสู่โรงเรือน

การศึกษาการอนุรักษ์เอื้องช้างน้าวและว่านหัวครูในสภาพหลอดทดลองภายใต้สภาวะชะลอการเจริญ โดยเพาะเลี้ยงเมล็ดบนอาหารสูตร MS ที่เติมน้ำตาล 20 กรัมต่อลิตร แล้วย้ายโปรโตคอร์มลงบนอาหารชะลอการเจริญ พบว่าสามารถอนุรักษ์ต้นอ่อนเอื้องช้างน้าวในสภาพหลอดทดลองที่อุณหภูมิ 25 องศาเซลเซียส เป็นระยะเวลา 5 เดือน โดยเลี้ยงในอาหารสูตร 1/4 MS ที่เติมซูโครส 10–20 กรัมต่อลิตร และว่านหัวครูเป็นระยะเวลา 6 เดือน โดยปราศจากการ

ย้ายเลี้ยงในอาหาร 1/8MS ที่เติมซูโครส 10 กรัมต่อลิตร ซึ่งต้นพืชมีการเจริญเติบโตช้ามากและมีการพัฒนาไปเป็นยอดน้อย การเพาะเลี้ยงไม่พบการปนเปื้อน จากนั้นย้ายต้นพืชที่เก็บรักษาไว้ลงบนอาหาร MS ที่เติม BA ความเข้มข้น 1 มิลลิกรัมต่อลิตร และน้ำตาล 10 กรัมต่อลิตร เพื่อเพิ่มจำนวน พบว่า ต้นพืชไม่มีการสูญเสียการเจริญเป็นต้นใหม่และไม่มีการเปลี่ยนแปลงลักษณะทางสัณฐานวิทยา

Title *IN VITRO* CONSERVATION AND MICROPROPAGATION OF *DENDROBIUM* SPP. AND *EULOPHIA SPECTABILIS*

Author Chutima Jaiphet

Advisor Assistant Professor Dr.Niramol Rangsayatorn

Co-advisor Dr.Wanida Saejung

Academic Paper Thesis M.S. in Biotechnology,
University of Phayao, 2012

Keywords micropropagation, *in vitro* conservation, *Dendrobium*, *Eulophia spectabilis*

ABSTRACT

A rapid and mass propagation of *Dendrobium gratiosissimum*, *D.pulchellum*, *D.christyanum* and *Eulophia spectabilis* using thin cell layers (TCLs) were studied. Four-week-old protocorms were segmented into two halves and each half was considered as a TCLs explant. The TCLs explants were cultured on MS (Murashige and Skoog) medium supplemented with different combinations of N^6-benzyl adenine (BA), kinetin (Kn), α-naphthaleneacetic acid (NAA) and 20 g/L sucrose. MS medium without growth regulators failed to induce protocorm like bodies (PLBs) formation in *D.pulchellum* and *D.christyanum*. The TCLs explants of *D.gratiosissimum* and *E.spectabilis* could developed PLBs within 3 to 4 weeks. For induction of PLBs, 2.0 mg/L Kn was the most effective for *D.gratiosissimum*, *D.christyanum* and *E.spectabilis*, while 1.0 mg/L BA was optimum for *D.pulchellum*. Regenerated plants were successfully acclimatized and eventually transferred to the nursery.

In vitro conservation under minimal growth conservation of *D.pulchellum* and *E.spectabilis* was also studied. Seed were cultured on MS medium supplemented with 20 g/L sucrose; then protocorms were transferred to minimal growth medium. *In vitro* culture could effectively be conserved *D.pulchellum* at 25±2°C for 5 when cultured on 1/4 MS supplemented with sucrose 10-20 g/l. *E.spectabilis could be cultured on* 1/8 MS supplemented with sucrose 10 g/l. The microplants on those medium exhibited very slow growth and rarely developed another shoot. The culture was also free of contamination. The conserved

microplants were subsequently recovered on MS medium and multiplied normally in MS medium with 1.0 mg/L BA and 10 g/L sucrose. They could regrowth microplants without losing regeneration capacity and without apparent morphological changes.

ABBREVIATIONS

ABA	=	abscisic acid
BA	=	N^6–benzyl adenine
BAP	=	N^6–benzylaminopurine
cm	=	centimeter
CPPU	=	N–(2–chloro–4–pyridyl)–N'–phenylurea or forchlofenuron
CW	=	coconut water
DMSO	=	*Dymethil sulfoxide*
DMAP	=	dimethylaminopurine
2, 4–D	=	2, 4–dichlorophexyacetic acid
GA_3	=	Gibberellic acid
H3	=	hyponex
IAA	=	indoleacetic acid
IBA	=	indole–3–butyric acid
KC	=	Knudson C medium
Kn	=	6–furfurylaminopurine or kinetin
lTCL	=	longitudinally thin cell layer
mg/L	=	milligrams per liter
MS	=	Msrashige and Skoog
NAA	=	α–naphthaleneacetic acid
PGRs	=	plant growth regulators
PLBs	=	protocorm–like bodies
TCL	=	thin cell layer
TDZ	=	thidiazuron
tTCL	=	transversally thin cell layer

LIST OF CONTENTS

Chapter		Page
I	**INTRODUCTION**	1
	Statement of purpose	1
	Objectives of the study	2
	Expected outputs of the study	2
II	**LITERATURE REVIEW**	3
	Dendrobium	3
	Eulophia	4
	Plant tissue cultures	4
	Media components	4
	Plant growth regulators	6
	Micropropagation	7
	Tissue explants	8
	Thin Cell Layer (TCL)	10
	Slow growth	14
III	**RESEARCH METHODOLOGY**	18
	Part I: Thin cell layers (TCLs)	18
	Part II: *In vitro* conservation under minimal growth conservation	20
IV	**RESULTS**	22
	Part I: Thin cell layers (TCLs)	22
	Part II: *In vitro* conservation under minimal growth conservation	34

LIST OF CONTENTS (CONT.)

Chapter	Page
V DISCUSSIONS AND CONCLUSIONS	40
Part I: Thin cell layers (TCLs)	40
Part II: *In vitro* conservation under minimal growth conservation	43
BIBLIOGRAPHY	45
BIOGRAPHY	53

LIST OF TABLES

Table		Page
1	Combination of growth regulators	19
2	Combinations of minimal growth medium and sucrose concentrations	20
3	Effect of growth regulators on in vitro protocorm–like bodies (PLBs) induction in thin cell layers section of *D. gratiosissimum*	23
4	Effect of growth regulators on in vitro protocorm–like bodies (PLBs) induction in thin cell layers section of *D. pulchellum*	26
5	Effect of growth regulators on in vitro protocorm–like bodies (PLBs) induction in thin cell layers section of *D. christyanum*	29
6	Effect of growth regulators on in vitro protocorm–like bodies (PLBs) induction in thin cell layers section of *E. spectabilis*	32
7	Effects of various media components on *in vitro* conservation of *D. pulchellum*	35
8	Effects of various media components on *in vitro* conservation of *E. spectabilis*	38

LIST OF FIGURES

Figure **Page**

1 Protocorm–like body (PLB) induction and plant regeneration in *D. gratiosissim* through thin cell layers culture 24

2 Protocorm–like body (PLB) induction and plant regeneration in *D. pulchellum* through thin cell layers culture 27

3 Protocorm–like body (PLB) induction and plant regeneration in *D. christyanum* through thin cell layers culture 30

4 Protocorm–like body (PLB) induction and plant regeneration in *E. spectabilis* through thin cell layers culture 33

5 *In vit*ro conservation of *D. pulchellum* 36

6 Three–month–old microplants on slow growth medium of *E. spectabilis* 39

CHAPTER I

INTRODUCTION

Statement of purpose

Thailand, rich in biodiversity of both botanical and horticultural interests is known as a major center for orchid cultivation and research during the past 40 years. Recently, orchids are found 170 genera and 1,230 species, in which 150 species are considered endemic to the country. Among these, eighty percentages are epiphytic orchid and most of the rest are terrestrial orchid (Nanakorn and Indharamusika, 1998). Orchids, especially wild orchids are well known as important ornamental plants in Thailand, and it has a high economical valve in markets. Unfortunately, orchid seed germination in the nature is very low and differs from that of other seeds because of the absence of endo-sperm, radicle, and leaf rudiments. Moreover, the orchids natural habitat has been heavily disturbed by forest degradation, over-exploitation for commercial and agricultural practices. The future of Thai orchids should be considered and managed to ensure a balance between conservation and demand (Maneerattanarungroj, Bunnag and Monthatong, 2007).

Recent advances in biotechnology for orchids have had a great impact on plant cultivation, such as tissue culture techniques for orchid propagation. It can be utilized for orchid's improvement and supply the huge demand for planting materials. Micropropagation of plants through tissue culture has become a significant and informative technique to reproduce and make the availability of crops, orchids and ornamental plants that are, otherwise, difficult to traditionally propagate by seed. Moreover, vegetative propagation of orchids is a complex process, which involves the environmental and physiological changes that may get influenced by the internal and external signaling factor (Kannan, 2009). The cytokinines, benzyladenine (BA), kinetin (Kn), auxin and α-naphthaleneacetic acid (NAA) are involved in that process (Roy and Banerjee, 2003; Nayak, et al., 2002; Rangsayatorn, 2009). *In vitro* propagation techniques has been studied in various orchids for example *Cymbidium* (Kannan, 2009), *Phalaenopsis* (Duan and Yazawa, 1995) and *Dendrobium* (Roy and Banerjee, 2003; Rangsayatorn, 2009).

In vitro storage of plant germplasm is an alternative way to maintain a gene bank of plant that is threatened sustainable utilization. Several *in vitro* plant cell and tissue culture techniques have been used, including micropropagation and conservation attempts. Various *in vitro* conservation methods have been recently developed such as cryopreservation, alginate encapsulation and slow growth. Slow growth has been widely used to reduce growth and prolong subculture intervals. Standard culture condition can be utilized for slow growth storage of some species. The environmental condition and the component of the culture medium were modified in many species (Fijoo and Iglesias, 1998; Onay, et al., 2003; Prosorn and Kanchanapoom, 2006). The successful implementation of minimal growth technology requires the establishment of specific protocols for each species of orchid under condition.

In this study, the effect of different sucrose and medium concentration on slow growth of various orchids (*Dendrobium pulchellum* and *Eulophia spectabilis*) were observed, for *in vitro* conservation. Micropropagation of orchids (*D. gratiosissimum*, *D. pulchellum*, *D. christyanum* and *E. spectabilis*) was also studied with thin cell layer (TCL) technique. The TCL was culture method for rapid mass micropropagation of Thai orchids.

Objectives of the study

1. To study the thin cell layer (TCL) culture method for rapid mass micropropagation of Thai orchids

2. To investigate a protocol for *in vitro* conservation of Thai orchid, using slow growth technique

Expected outputs of the study

1. The efficient thin cell layer (TCL) culture will be used for micropropagation of Thai orchids.

2. The effective slow growth technique will be applied for *in vitro* conservation of Thai orchids.

CHAPTER II

LITERATURE REVIEW

Dendrobium

Wood (2006) reported that *Dendrobium*, an epidendroid orchid is one of the largest genus in the orchid family, about 1,100 species. At least 300 species have been cultivated. The dendrobium flowers have constant and distinctive features. There are a mentum, naked pollinia and rostellum incorporating a sac of glue. The spurlike mentum is formed by the lateral sepals, column foot and lip, which may be variably conjoined. In several section of *Dendrobium* with large menta, these components fuse distally to form a true spur. The dendrobiinae have four polllinia, arranged within the anther in two pair side by side, each pair in separate cavity or receptacle. These pollinia, lacking appendages (caudicles or stipes), are called "naked".

D. christyanum

It has pseudobuls rathers. The inflorescences spikes can support as many as 4 flowers. The diameter of flower is 5 cm. They are excellent drainage and judicious watering, but much reduces in winter. They are distributed in Thailand, Vietnam and China at elevations of 850 to 1200 m (Wood, 2006).

D. gratiosissimum

The color of flower is several whitish with purple-edged tepals, yellow and white line-lip. The diameter of flower is 5.7 cm. They are blooming winter to spring. They are distributed in the Eastern Himalayas, India, Myanmar, Thailand, China, Laos and Vietnam (Wood, 2006).

D. pulchellum

The inflorescences are very short and support 1 to 2 flowers. The diameter of flower is 1.5 to 2.5 cm. The color of flower is yellowish or white, may have dark purple stripes. It has imported occasional plant from Thailand with greater succulence and lack of purple pigmentation in plant or flower, which seem generally "different". They are blooming also during dry seasons without storms reducing temperature. They are distributed in Bangladesh,

India, Myanmar, Thailand, Malaysia, Singapore and Vietnam, from sea level to 1,500 m (Wood, 2006).

Eulophia

Orchids in this genus are usually terrestrial. They are distributed in shady rainforests, in open scrub, woodland in the tropics and subtropics of Asia, Africa and Australia. The inflorescence arises from the base. It grows into a raceme. The inflorescences on the species with non–branching spikes can support as many as 50 flowers, but in species with branching inflorescences, up to 150 blooms can occur per spike. The sepals and the petals are alike. The lip usually has three lobes. As for most orchids, there are two pollinia for each flower (Seidenfaden, 1983).

Plant tissue cultures

Illg (1991) reported that plant cell and tissue were a simple technique that utilizes either single plant cells, groups of unorganized cells (callues), organized tissues or organ put in culture, under controlled sterile condition. Plant cell shall divide indefinitely in appropriate culture medium and in many cases (but not in all species) and can be induced to regenerate into the whole plants, so that thousands of clones can be produced from a single plant. It is commonly used for rapid clonal propagation of superior genetic material, with an immediate effect on plant improvements. This technique also allows virus–free plants producing and cryopreservation of germoplasm. Another very important application of plant tissue culture is in the field of genetics and plant breeding.

Media components

Macroelements

Macroelements are added for plant cell and tissue include calcium (Ca), magnesium (Mg), nitrogen (N), phosphorus (P), potassium (K) and sulfur (S). Depending on the medium, several salts may be used to supply each mineral. The combinations of salts in each medium are designed to provide an appropriate balance of nutrients in proper concentrations (Arditti, 2008).

Microelements

Culture media is variable widely in the use and content of micro– or minor element. The reasons for this are: utilization of existing formulations, imprecise information regarding the requirements of orchids and the presence of many of these elements as impurities in other media components (Arditti, 2008).

Sugar

Sucrose is the most commonly used in orchid culture media as an energy source. When sterilized by autoclaving some of it hydrolyzes into the component molecules fructose and glucose. Therefore, seeds and tissues are usually cultured in a mixture of fructose, glucose and sucrose. There is some evidence to suggest, at least in respect to the seeds of several species, that cane sucrose is preferable to that obtained from beets. However, some orchid seeds and explants may grow better on fructose (Arditti, 2008). In recent years, the nutritional and osmotic role which in plant physiology is usually attributed to sucrose gradually changing due to many new findings which indicate that sucrose in plants plays an important regulatory role (Dragan and Branka, 1999).

Khan, et al. (2006) studied the effect of sucrose and growth regulators on the micropropagation. Early, mid and late maturing sugarcane clone were obtained under different concentration of commercial sugar and plant growth regulators. Late maturing sugarcane clone AEC82–223 did not produce any auxillary shoot at 6% commercial sugar concentration. In contrast, multiplication rate was higher in early and mid maturing sugarcane clone NIA–2004, BL4 and NIA–98, respectively. Apical meristems were cultured on modified

Murashige and Skoog 1962 (MS) medium containing different concentration of auxins and cytokinins. An optimal multiplication was observed on M4 [1.0 mg/L indoleacetic acid (IAA) + 1.0 mg/L N^6–benzylaminopurine (BAP) + 0.1 mg/L kinetin (Kn)] and M5 [0.1 mg/L Kn + 1.5 mg/L BAP + 1.5 mg/L IAA)]. Maximum numbers of shoot were observed in BL4 followed by NIA–2004 when 0.1 mg/L of BAP and IAA were applied with low concentration of 0.1 mg/L Kn. Shoot elongation multiplication was improved on media devoid of BAP (MS + 2.0 mg/L Kn + 2.0 mg/L IBA + 2.0 mg/L IAA). Best rooting was observed on media containing MS + 1.0 mg/L IBA + 6% sucrose.

pH

The term pH is indicative of the alkalinity or acidity of a medium. The pH of culture media should be as indicated in each method, determined experimentally, or adjusted to 4.8–6.0. Solid media may not solidify if the pH is much below 4.0 or higher than 8.0. Growth may be inhibited if the pH is lower than 4.0, higher than 8.0, or inappropriate for the plant being cultured.

Sanavy and Moeini (2003) studied the effect of different pH levels of medium on growth and rooting of single node resulted from potato meristem culture on MS medium. Solid MS medium with 0.25 mg/L Gibberellic acid (GA_3), 0.01 mg/L NAA, 0.2 mg/L calcium pantothenate, 30 g/L sucrose and 7 g/L agar showed significant differences between different pH levels in respect of its ability to induction of rooting and shooting in plantlets produced from the single node of two cultivar potato (*Solanum tuberosum*) varieties after subjecting them with thermotherapy. Overall pH 5.5 was the best for all the traits. Low and high levels of pH from 5.5 were found to reduce the growth and rooting of single nodes. The reduction was more pronounced at low levels than high levels of pH.

Plant growth regulators

Plant growth regulators are organic compounds naturally synthesized in higher plants. They also include synthetic compounds, which influence growth and development. They are usually active at different sites in the plants, apart from where they are produced and are only present and active in very small quantities (George, et al., 2008). Auxin was the first plant hormone. The word auxin is derived from a Greek word that means "to increase". The first auxin was discovered by biologists who were looking for the factor that regulated the growth is rate of stem tissue. Auxin regulates many aspects of plant growth and development. It is possible that auxin is essential for plant life. In addition to the regulation of cell elongation, auxin has been implicated in the control of the phototropism, inhibition of abscission zone formation, inhibition of lateral bud development, vascular tissue differentiation, vascular cambium cell division, maintenance of tissue polarity and leaf blade expansion (Fosket, 1990). Aditti and Ernst (1993) said the most commonly used auxins in orchid tissue culture media. The naturally is occurred IAA and the three synthetics NAA, indolebutyric acid (IBA),

and 2,4–dichlorophexyacetic acid (2, 4–D). Other auxins and occasionally auxin–amino acid conjugates are also used in some media. Both the auxin and the concentration being used are usually a result of trial error. Moreover, it is the best not to make changes, substitution without careful thought and good reason. It is necessary to keep in mind that auxins may differ from each other both qualitatively and quantitatively when changes are made. This means that the effects of one auxin on a certain species may different from those of other auxins and on other orchids.

Cytokinins regulate many aspects of plant growth and development. Cell division is tightly regulated in higher plant. It occurs only in specialized regions and tissue, such as meristems, developing embryos, leaf primordial, the vascular cambium, and a few other tissues (Fosket, 1990). In addition cytokinin also mediates the response to variable extrinsic factor, such as light condition in the shoot, availability of nutrients and water in the root, and has a role in the response to biotic and abiotic stress (Werner, 2009). Naturally occurring cytokinins are a large group of structurally related compounds. Aditti and Ernst (1993) said the synthetic compounds kinetin, benzyl adenine (BAP, BA), dimethylaminopurine (DMAP) and the naturally occurring zeatin are used most commonly in orchid culture media. As with auxin, the choice of cytokinin and their concentration is based on empirical finding. Changes should be avoided. The effects of different cytokinins and their concentration differ like those of auxins.

Micropropagation

Micropropagation of orchid was first proposed in 1968 and defined from organs, tissue and cells for the asexual production of plantlets other means of asexual propagation. This term should never be used to describe *in vitro* seed germination as is being done in the literature. Orchids micropropagation did not originate suddenly and de novo in the mind of one person despite a self serving effort to create such an impression. The root of orchid micropropagation is intertwined with the history of tissue culture but they also have other origins. The history of plant hormones will also be presented because these substances are of critical importance to the culture *in vitro* of plant cells, tissue and organs as well as to the differentiation of cultured plantlets (Arditti, 2008).

Leng and Keng (2004) studied a micropropagation method for *Orthosiphon stamineus*, using stem nodal segments. The highest number of regenerated shoot was obtained on MS medium supplemented with 6.7 µM BA with the formation of an average of 6.1 shoots per explant over a period of 4 week. The number of shoots increased with longer culture duration on proliferation medium. Multiple shoots which were maintained on the proliferation medium for 6 week had the highest proliferation rate. Separation of multiple shoot and culturing in larger flasks significantly promoted the growth and formation of plantlets. All the *in vitro* plantlets survived when transferred to the field and showed no significant morphological differences from the mother plants.

Sopalum, Thammasiri and Ishikawa (2010) were studied micropropagation of the Thai orchid *Grammatophyllum speciosum* Blume. This is the first report of an established protocol for microprapagation of *G.speciosum*. Protocorm–like bodies (PLBs) were induced from shoot tips of *G.speciosum*. The highest frequency of PLBs (93%) were observed on explants incubated on 1/2 MS liquid medium containing 2% (w/v) sucrose without any plant growth regulators (PGRs). Tests with different carbon sources compared to sucrose revealed that maltose promoted the highest relative growth of *G. speciosum* PLBs (7–fold increase), while trehalose and sucrose yielded 5–fold and 4–fold increases, respectively. In 1/2 MS liquid medium, addition of 15 mg/L of chitosan rate in solid culture was significantly lower than that in liquid culture.

Tissue explants

The selection of specific tissue as primary explants depends on the ultimate goal of tissue culture. The response of a tissue or explant to *in vitro* condition may vary widely between families, genera, species, hybrids, clones and genotypes, and even within the same genotype grown under different environmental conditions. There may also be endogenous physiological rhythms in plants that undergo periodic fluctuations which can play critical roles in the establishment of successful cultures. Endogenous cycles may play critical roles in the establishment of protoplast cultures from various tissues of plants (Arditti, 2008).

Shoot tip

Shoot tip can be utilized as primary explants for the establishment of callus or for mass clonal propagation. Techniques for excision vary slightly with the growth from of the shoot. Generally, meristems are located at the tips of shoots or buds protected by sheathing petioles, leaves, or scales. The shoot tips are sterile and the protective structures maintain their sterility. These structures also protect the shoots form surface sterilants. The shoot tips of *Paphiopedilum* sterilization. This is one reason for the limited success in the culture of shoot tips from mature plants of this genus. *In vitro* grown seedlings are easier to culture because they do not require surface sterilization.

Roy and Banerjee (2003) were studied induction of callus and plant regeneration from shoot tip explants of *Dendrobium fimbriaum* Lindl. var. oculatum HK. f. on modified nutrient solution of Knudson's C, supplemented with 10% (v/v) coconut water, 0.5 mg/L niacin, 0.5 mg/L pyridoxine HCl, 0.1 mg/L thiamine HCl and different concentrations of BAP and NAA in factorial combination. After 2 weeks, the explants produced a compact mass of translucent embryogenic callus. Application both PGRs was essential for the induction of callus. Optimum callusing was recorded in the presence of 0.5 mg/L NAA and 1 mg/L BAP (66.7%). Following transfer to the PGR–free medium, the callus further proliferated with side–by–side regeneration of PLBs. These PLBs readily germinated into well–rooted plantlets in the same medium.

Košir, Škof and Luthar (2004) reported that direct shoot regeneration from node of *Phalaenopsis* orchids. Nodes with dormant buds from flower stalks of orchid *Phalaenopsis* sp. were plated on six culture media. The composition of medium A was the same as P 6793 (Sigma) commercial medium. Medium B contained macro and microelement of MS medium; other components used were as described in Tisserat and Jones (1999). Medium D was composed of B5 medium's macro element, MS medium's microelement and other components from the medium described by Hinnen, et al. (1989) but without banana homogenate. Medium C was the same as medium D except for the addition of hormone BAP. Medium E and F were composed according to Arditti and Ernst (1993) except for the gelling agent (medium E) alone or gelling agent and carbon source (medium F) combined (sucrose instead of glucose). The composition of the culture media affected the induction, regeneration, number

and form of *Phalaenopsis* regenerants. Direct shoot was to obtain regeneration without callus formation on all media except for medium F. Medium A, supplemented with 2 mg/L of BAP and 0.5 mg/L of NAA was found to be the most appropriate of all the media used for rapid micropropagation of a large number of vegetative shoots (multiplication factor 8.35 per node) without roots 160 days after inoculation. Medium B, supplemented with 4.41 mg/L BAP and 1 mg/L NAA less appropriate for vegetative shoot production (multiplication factor 2.08 per node) also because the regenerants formation. Vegetative regenerants with a regenerative shoot also formed. This type of regenerant could be major commercial interest, since period until flowering would be much shortened. Dormant bud induction was poor on medium E (23% regeneration rate) and there was lower (1.18 per node). Most vegetative regenerants were ready for acclimatization after 90 days of subcultutivation on medium D, which also proved to be a successful subcultivativation medium for regenerants formed on media A, B and C.

Ket, et al. (2004) reported that the shoot tip explants of jewel orchid (*Anoectochilus formousanus*) produce multiple shoot when cultured on Hyponex (H3) media supplemented with 1 mg/L benzyladenine or 1–2 mg/L thidiazuron (TDZ). Addition of activated charcoal (1 g/dm^3) to the TDZ containing medium promoted multiple shoot formation (11.1 shoots per explant).

Thin Cell Layer (TCL)

TCL system consists of explants of a small size excised from different plant organs (stems, leaves, floral inflorescences, flower primordial or floral organs, cotyledons, hypo–/epicoty apical zone or embryo), either longitudinally (lTCL), or transversally (tTCL). lTCL contain only one tissue type, such as a monolayer of epidermal cells, whereas tTCLs include a small number of cells from different tissue types: epidermal, cortical, cambium, perivascular and medullar tissue, parenchyma cells (Tran Than Van, 1980, pp.291–311).

For another TCL method, Tran Thanh Van (1973) studied since the inception of the term and concept of a TCL in tobacco (*Nicotiana tabacum*). It has become the model system on which all other TCL studies are based. In 1986, Tran Than Van (pp. 316–335) studied four morphogenic program; direct flower formation, direct root formation, direct bud

formation, and callus without organogenesis from tobacco (*N. tabacum* Wisc.38). TCLs, were excised from floral ramifications.

TCL technology originated almost 30 years ago with the controlled development of flowers, roots, shoots and somatic embryos on tobacco pedicel longitudinal TCLs. Since then TCLs have been successfully used in the micropropagation of many ornamental plant species whose previous *in vitro* regeneration was not successful using conventional methods (Silva, 2003). In the present, The TCL method has already been used to regenerate of many plant species such as ornamental plant, crop, leguminous and orchids.

Le, et al. (1999) reported that often regeneration in orchids successful using protocorm, i.e. the juvenile stage. In order to produce directly shoots via bud regeneration both rapidly and with a high frequency, a tTCL explants (0.3–0.5 mm) excised along the stem from the basis to the shoot tip of one–year–old plantlets were cultured on MS medium supplemented with different combination of BAP, NAA, TDZ and 3% sucrose. The optimal combination for maximal bud regeneration was 3 µM BAP and 3 µM TDZ, giving rise to 11.7 buds per explant. Roots were obtained with 10 µM forchlofenuron (CPPU) and 1% sucrose. The *in vitro* plants (>3 cm long) obtained 4 to 6 weeks after the tTCLs culture were transferred to the greenhouse; their morphology was normal. Efficient micropropagation of direct production of shoots without passing through protocorm stage of orchid species can be achieved using the TCL method.

Le, Nhut and Tran Thanh Van (1999) have reported tTCL (0.2–0.4 mm thick) excised from the pseudo–bulblets of *Lilium longgiflorum* culture. The explants were placed on the MS medium supplemented with 3% sucrose and concentration of CPPU varying from 0.1 to 10 µM. Buds were obtained from the tTCL pseudo–bulblet explants cultured in the presence of CPPU in the light but not in the dark. The number of buds formed per tTCL was the highest with 1 or 3 µM CPPU (16.8±1.1 and 16.2±1.6 buds per tTCL, respectively). A combination of sucrose (from 1% to 12%) and CPPU produced per tTCL was the highest when 1 or 3% sucrose was combination with transferred to soil after rooting with 10 µM IBA and normal plants were produced.

Carvalho, et al. (2000) has reported rapid and high frequency direct shoot regeneration (without intermediate callus) of the commercially important common bean, *Phaseolus vulgaris* L., using the tTCL method. The pretreatment of seed with 10 µM TDZ significantly increased bud regeneration frequency on tTCL explants. After 2 weeks of culture of tTCLs on 10 µM TDZ followed by a reduction in the TDZ concentration (1 µM) was needed to achieve optimal bud induction and further development of the neo–formed buds. An incubation period greater than 2 weeks of tTCLs with 10 µM TDZ resulted in inhibitory effects on the development of shoots and roots. Shoot development was enhanced by 10 µM BAP and 10 µM $AgNO_3$ leading to 100% true to type fertile plants.

Nhut, et al. (2001) have reviewed thin cell layer culture system in Lilium. They demonstrated the effectiveness of using specific organ sources in conjunction with the TCL system to establish successful organogenic and somatic embryogenic pathways in *Lilium longiflorum*. TCLs derived from different explant sources, such as receptacle, leaf, young stem, stem node, bulblets, pseudo–bulblets, shoot and somatic embryocould are manipulated to from different organs. Furthermore, the choice of plant growth regulator and medium additives such as activated charcoal and sucrose strongly affect the success of process. They further highlight that transient transgene expression derived from either particle bombardment or agro–infection is both strong and repeatable, as a result of direct exposure of competent cells to the gene introduction method, resulting in a reduction of escapes. The ability to manipulate and control organogenesis through a TCL system, coupled with repeatable, efficient transformation opens up the possibility of micropropagation members of this genus with new and available characteristics, production superior plants of high quality.

Nhut, et al. (2007) reported that an unique procedure for the mass shoot propagation of *Gerbera* using receptacle tTCL culture. Genotype, flower bud age, explants size, position of receptacle tTCL and culture media were found effect on the success of culture. Ten interspecific crosses of *Gerbera* showed different shoot regeneration rates and callus induction via receptacle tTCL culture; all of which had shoot regeneration rates higher than 57%. Flower buds collected on the 10^{th} day resulted in 91% shoot regeneration after 6 weeks of culture on basal MS medium supplemented with 0.02 mg/L TDZ, 0.8 mg/L adenine

and 10% (v/v) coconut water (CW). This was significantly higher than those from flower bud on the 7th and 14th days (22% and 54%), respectively. Shoot regeneration rate was the highest (94–100%) in the middle layers of the receptacle. For mass shoot propagation, shoot clusters were subcultured on half–strength MS medium supplemented with 0.5 mg/L IBA, 0.5 mg/L BA and 2.0 mg/L Kn after every 4 weeks. Plantlets formed when single shoots were culture on half–strength MS medium containing 1 mg/l IBA. All plantlets acclimatized well in the greenhouse.

Rangsayatorn (2009) performed micropropagation of *D. draconis* Rchb.f. from thin cross section culture. The TCS explants were excised along the stem from the base to shoot tip of 6–month–old plantlets and cultured on MS medium supplemented with 20 g/L sucrose and different concentration of BA, Kn and NAA, either individually or in combination. PLBs were directly induced from the TCS explants and completely developed into shoots within 6–7 weeks. The optimal growth regulators combination for maximal PLB development was 2 mg/L BA and 0.1 mg/L NAA, giving rise to 68% of responding explants with an average 11 PLBs per explant. Shoot development was best achieved on MS medium containing sucrose and coconut water. Plantlets, 6–8 cm height, were transplanted into coconut husk peat with 92% survival rate in a nursery.

Vyas, et al. (2010) studied micropropagation of *Cymbidium* Sleeping Nymph through PLBs production by TCL culture. The tTCLs of PLBs of two stages (30 and 60 days) of *Cymbidium* Sleeping Nymph were used as explants to induce PLBs by using CW as a natural additive. Knudson C (KC) medium supplemented with5% (v/v) CW was induced and average of 5 PLBs per responding tTCL of 30 day old PLBs with 83% of responding tTCLs. A low percentage of responding tTCL were observed in 60 day old PLBs tTCLs. Anatomical and confocal microscopic studies traced the origin of PLBs to subepidermal layers of the tTCL. A significantly high percentage of shoot regeneration was obtained from PLBs formed on 1–10% (v/v) CW from tTCLs of 30 day old PLBs in comparison to PLBs induced on control after first subculture on KC medium (without CW). The induced PLBs regenerated into plantlets with velamenous roots and these plantlets were transferred to greenhouse conditions on cocopeat: perlite (9:1) with nearly 100% survival. Post–transfer performance of the plantlets was monitored. The results suggest tTCLs as potential explants (with

respect to economy of precious hybrid materials) which can overcome the slow growth of hybrid PLBs and CW as a single natural additive for the mass multiplication of commercially important orchids.

Slow growth

In vitro culture is now widely used for industrial propagation in agriculture and horticulture. This technique will make a rapid survey of the various methods set up for the conservation of plant organs produced *in vitro*. The techniques are different depending on the storage duration requested. For short to medium term conservation, growth reduction will be sought in order to increase the intervals between subcultures (Engelmann, 1991). Prolonged maintenance of *in vitro* materials was to develop optimum and efficient preservation of terrestrial orchids. The main approaches to *in vitro* conservation involve either growth limitations or plantlets differentiation. In spite of, the advantages offered by the latter method for long term conservation, recent technological advances employ the minimal or slow growth strategies for tissue culture conservation of vegetative propagated potato, enset and vanilla (Sakar and Naik, 1998).

Desbrunais, Noirot and Charrier (1992) involved slow growth condition the storage of coffee shoots cultured *in vitro*. Reduced concentrations of sucrose in medium containing half–strength MS nutrient salts, decreased culture temperature and genotypic effects have been examined. This study is discovery that after six months, low sucrose concentration reduced microcutting growth, rooting and survival rate.

Conservation of two species of the genus *Vitis* for 14 months without renewal of the culture medium was reported by Galzy and Compan (1998). This work should help to define culture condition for damage free long term storage of two varieties of vine which are genetically remote from each other.

Dragan and Branka (1999) demonstrated that in *Dracaena fragrans*, increased sucrose nutrition induced two distinct morphogenetic responses, branching of adventitious roots and formation of axillary buds.

Watt, et al. (2000) presented protocols for the medium term storage of shoot culture under minimal growth conditions. Because of axillary buds encapsulated in alginate are

ideally suitable for manipulate and require less storage space than shoot culture, a preliminary investigation was also undertaken using such explants.

Negash, et al. (2001) did conservation of enset clone. It is traditionally carried out *in situ* in farmer's backyards. However, in recent decades many valuable clones have been lost as a result of diseases (e.g. bacterial wilt disease) and unfavorable environmental condition. A small number of enset accession collected by the Ethiopian Biodiversity Institute is currently conserved in field genebanks. No alternative storage strategy has so far been developed.

Nassar (2003) studied involves investigation of slow growth condition for storage of *Coffea arabica* germplasm encapsulated in calcium alginate beads. Water or half strength MS nutrient salt in combination with reduced temperature, low sucrose concentration with or without the addition of abscisic acid (ABA) as a growth retardant were examined. Viability when encapsulated shoot bubs were retrieved from storage condition was evaluated. Substrate containing growth regulator free 1/2 MS salts, 1% sucrose and 10 mg/L ABA was found to be the most effective in suppressing growth of encapsulated *C. arabica* shoot buds. The most suitable temperature for storage with all substrates tested was 20°C. However, if ABA is added to the substrate, a good storage is also possible at 15°C. Storage at 25°C was not recommended since buds germinated during storage and showed low percent of conservation into plantlets. The approach of the present investigation may be helpful for preservation of *C. arabica* germplasm.

The culture of *Drosophyllum lusitanicum* were initiated *in vitro* as described earlier and were multiplied and maintained on MS medium supplemented with 20 g/dm sucrose and 0.91 µM zeatin at an interval of 6 weeks (Gonçalves and Romano, 2005). In 2007 Gonçalves and Romano were studied a protocol for minimal growth conservation of *D. lusitanicum in vitro*. Double node cutting were maintained for 4, 8 and 12 months at 5 or 25°C in the dark. The effects of sucrose either alone at 5, 20, 30, 40 and 60 g/dm or at 20, 40 and 60 g/dm in combination with 20 g/dm mannitol, on survival under minimum growth at 54°C for 8 months on MS medium supplemented with 60 g/dm sucrose, 20 g/dm mannitol and 0.91 µM zeatin. Following extended conservation, the cultures could be successfully regenerated into new shoot.

Promsorn and Kanchanapoom (2006) showed that apical and lateral bud were suitable starting materials. MS medium supplemented with 44 µM BA and incubated at 25±2 °C with a 16 h photoperiod for 12 weeks was suitable for micropropagation of "Kluai Hin" since 21.22 shoots per explant were obtained. Whereas, explants were cultured on MS medium supplemented with TDZ differentiated to clusters. The storage of shoots over cotton saturated with 10 g/L sucrose and water at 25 °C and a 16 h photoperiod could extend the survival time for 6 months. Surviving shoots were transferred to MS medium without plant growth regulators and then rooted normally. Rooted shoots, after acclimatization with vermiculite, had 100% survival when transplanted in the field.

Morata, Arrillaga and Segura (2006) have developed protocols for short term storage of microcuttings of *Cedrus libani* and *Cedrus atlantica* under minimal growth condition. Microcutting could be stored effectively up to 6 months at 4ºC and reduced light intensity, provided that they were grown on a diluted modified MS medium. The addition of 6% mannition to the storage media affected negatively survival and multiplication capacity of the cultures.

In 2006, Keller reported that, in living plant collections, vegetative propagated accessions are outstanding material with respect to vulnerability and labour amount. This is also true for the main vegetative material, held in the Institute of Plant Genetic and Crop Plant Research (IPK), Gatersleben. A survey of the preservation of potato, garlic and other alliums, mint and yam is given. More than 630 accessions are in slow growth conditions. Amongst them, 99 clones for garlic and 35 of shallot have been tested to be virus–free. Cryopreservation is routinely applied for potato using the droplet method. The cryo–collection contains more than 1,000 accessions, a part of which has been integrated from another collection, formerly established at Braunschweig. Cryopreservation of garlic has been used to store the accession of the European core collection. Cryopreservation is successful in three *Dioscores* species using a combination of the original vitrification with the droplet method. Investigations before, during and after cryopreservation were included in these activities.

Budiarto (2009) reported that the medium term of *in vitro* conservation for chrysanthemum were successfully conducted up to 12 months using 1/2 MS, *Dymethil sulfoxide*

(DMSO) 2.5%, 4% sucrose and modified Hyponex, 4% sucrose without any significant differences and decreases in viability when transferred in to shoot induction media. In the absent of sucrose, however, the plantlet survival rate decreased on the media of modified Hyponex after 8 months of storage.

CHAPTER III

RESEARCH METHODOLOGY

Part I: Thin cell layers (TCLs)

Aseptic protocorms and shoots with 1 to 2 nodes were used as explant source. They were raised from mature seeds of *D. gratiosissimum* and *E. spectabilis* on MS medium supplemented with 20 g/L sucrose.

D. pulchellum and *D. christyanum*, obtained from Maejo University as a gift, were germinated from mature seeds on Vacin and Went (VW). Four–week–old protocorms (about 1 to 2 mm in diameter) were segmented into two halves and each half and considered as a TCLs explant. The shoot was excised transversely using a sharp surgical blade into approximately 0.3 to 0.5 mm thickness from the base to the shoot tip. The TCLs explants were cultured on MS medium supplemented with 20 g/L sucrose and different concentration of BA, Kn and NAA, either individually or in factorial combination (Table 1). The pH of the culture medium was adjusted to 5.8±0.05 after addition of 7 g/L agar. Twenty five milliliters of the media were dispensed into a glass culture bottle. The media was autoclaved at 121°C for 20 min. In all the treatments, subculturing of aseptic shoot tip were incubated at 25±2°C under cool white fluorescent tube providing 40 µmol/m^2/s illuminations with 12 h photoperiod. The experimental units were assigned to randomized complete block design with 6 replicates (culture bottle) and 10 explants per replicate. Visual observation was carried out weekly; the number of protocorm–like bodies (PLBs) from each responding explants were counted at the protocorm stage (average about 10 weeks of culture). The data on different morphogenetic responses was subjected to analysis of variance (ANOVA). Mean values were compared using Duncan's multiple range test of 95% confidence interval.

Table 1 Combination of growth regulators

Growth regulator (mg/l)		
BA	Kn	NAA
Control*		
1.0		0
2.0		0
5.0		0
1.0		0.5
2.0		0.5
5.0		0.5
1.0		1.0
2.0		1.0
5.0		1.0
	1.0	0
	2.0	0
	5.0	0
	1.0	0.5
	2.0	0.5
	5.0	0.5
	1.0	1.0
	2.0	1.0
	5.0	1.0

Note: * is No growth regulator

Part II: *In vitro* conservation under minimal growth conservation

This part was carried out to investigate the effects of sucrose and culture medium on *in vitro* conservation under minimal growth conservation. The study composed of two stages as follows:

Stage 1 minimal growth conservation

Stage 2 regrowth ability of microplants

Minimal growth conservation

In vitro minimal growth conditions, mature seeds of *D. darconis*, *D. pulchellum* and *E. spectabilis*, were cultured on MS medium containing 20 g/L sucrose. The medium was solidified with 7 g/L agar and the pH was adjusted to 5.8±0.05 before autoclaving at 121°C for 20 min. The culture was incubated in a growth room at 25±2 °C under cool white fluorescent lamp providing 40 µmol/m^2/s with a 12 h photoperiod. After 8 weeks of germination, small protocorms (about 4 mm in diameter) were used for the conservation experiments. For minimal growth experiments, the MS basal medium was used in 1/8, 1/4, 1/2 and full strength in combination with a carbon source, sucrose at different concentrations (Table 2).

Table 2 Combination of minimal growth medium and sucrose concentrations

Medium concentration	Sucrose concentration (g/L)		
	0	10	20
1/8	T1	T2	T3
1/4	T4	T5	T6
1/2	T7	T8	T9
Full MS	T10	T11	T12

Note: T is treatment

Regrowth ability of microplants

For recovery, the microplants from all treatments were transfered to MS supplemented with 10 g/L sucrose. A surviving microplant capable of resuming growth at the 3 months after recovery was recored. Data were expressed at the percentage.

Statistical analysis

Data obtained were statistically analyzed by using analysis of variance (ANOVA). By using Duncan's multiple range test program.

CHAPTER IV

RESULTS

Part I: Thin cell layers (TCLs)

D. gratiosissimum

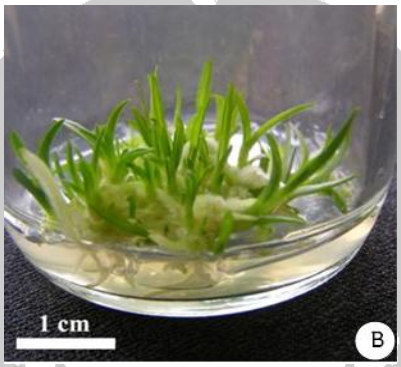
Explants turned light after incubate on MS medium contains various growth regulators for 7 days. The explants had small greenish–yellow protuberances after 3–4 weeks of culture (Figure 1A). Effect of growth regulators on percentage explants with PLBs of *D. gratiosissimum* on MS medium after 3 months of culture is shown in Table 3. The percentage of explants with regenerated protocorms of *D. gratiosissimum* was ranged from 61 to 83 %, with the average number of PLBs per responsive explants from 6 to 18. MS medium supplemented with 2.0 mg/L BA and 0.5 mg/L NAA in combination increased in PLBs forming percentage. While medium containing 2.0 mg/L Kn alone also increased in PLBs forming percentage, and the explants developed on average 18 PLBs per protocorm explant. Of two cytokinins tested, Kn was more effective than BA in PLBs induction. The frequency of responsive protocorm explants were multiplication with increasing concentration of Kn from 0 to 2.0 mg/L and decreased when above 2.0 mg/L. They regenerated PLBs were transferred to growth regulator–free MS medium. Gradually formed shoots for another 2 to 3 weeks, then plantlet formation was successfully achieved (Figure 1B). Plantlets with well–developed roots were planted in pots containing equal volumes of coconut husk peat and perlite (Figure 1C). The pots were covered with a transparent plastic bag to maintain a high humidity during acclimatization for 3 to 4 weeks before transfer to a nursery.

Table 3 Effect of growth regulators on in vitro protocorm–like bodies (PLBs) induction in thin cell layers section of *D. gratiosissimum*

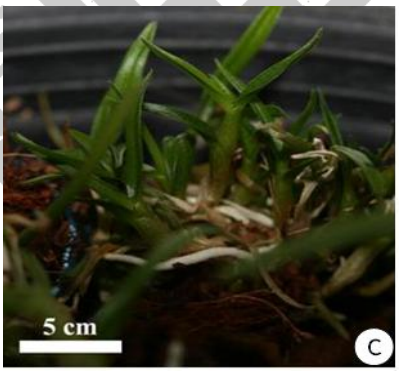
Growth regulators (mg/L)			Explants with PLBs (%)	No. of protocorms/ Responding explant
BA	Kn	NAA		
Control			39	3±1.8^{e}
1.0		0	68	11±2.0^{bc}
2.0		0	72	8±1.2^{c}
5.0		0	76	9±0.9^{c}
1.0		0.5	74	13±17^{b}
2.0		0.5	83	10±0.8^{c}
5.0		0.5	61	6±1.8^{d}
1.0		1.0	72	13±0.9^{b}
2.0		1.0	78	11±0.2^{bc}
5.0		1.0	76	12±1.9^{bc}
	1.0	0	79	12±0.6^{bc}
	2.0	0	83	18±1.5^{a}
	5.0	0	71	19±1.1^{a}
	1.0	0.5	81	14±0.8^{b}
	2.0	0.5	78	14±1.9^{b}
	5.0	0.5	72	10±1.3^{c}
	1.0	1	81	12±2.1^{bc}
	2.0	1	76	15±1.8^{b}
	5.0	1	68	11±0.8^{bc}

Note: Values within the column followed by different letters are significantly different at P≤0.05 by Duncan's multiple range test.

(A) Initiation of PLBs on the protocorm sections



(B) Well–rooted and developed plantlets from PLBs



(C) acclimatization of the plantlets

Figure 1 Protocorm–like body (PLB) induction and plant regeneration in *D. gratiosissim* through thin cell layers culture

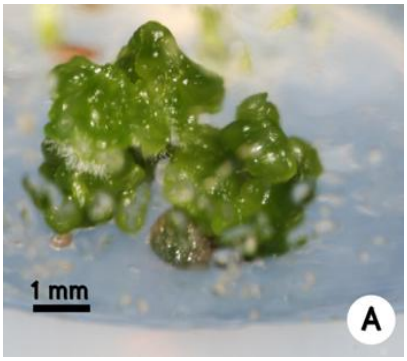
D. pulchellum

The response of the TCL explants of *D. pulchellum* to various concentrations of cytokinin and/or auxin is shown in Table 4. Initially, the TCL explants were extremely slow growth and browning after transferred to the medium test 2 weeks. The basal medium without PGRs was used as a control. The TCL explants from protocorm showed their first response by swelling, becoming dark green and the PLBs appeared as small protuberances on the surfaces of the TCL explants within 4 to 5 weeks of culture (Figure 2A). A significant in the number of PLBs per responding was observed in all cultures and compared with control. The highest frequency of responsive protocorm explants was observed in 1.0 mg/L BA combination with 0.5 mg/L NAA, with the average of 63 PLBs per protocorm explant. Among treatments, 1.0 mg/L BA in combination with 0.5 mg/L NAA proved to be more effective than 2.0 mg/L BA alone in PLBs induction from protocorm explants. In the presence of cytokinin alone, the best response was recorded on the medium containing 2.0 mg/L BA, with 66% PLBs of the explants and average 34 PLBs per protocorm explants. Of two cytokinins tested, BA was more effective than Kn in PLBs induction. After 3 months, these shoots were transferred to fresh MS medium without PGRs and cultured for 2 months. The multiple shoots thus obtained from multiple shoot experiments. Roots originating from shoots were thick, long and easy to handle (Figure 2B). When rooted plantlets reached 2 to 3 cm in height, they were transferred to pots containing equal volumes of coconut husk peat. The pots were covered with a transparent plastic bag to maintain a high humidity during acclimatization for 4 to 6 weeks before transfer to a nursery (Figure 2C).

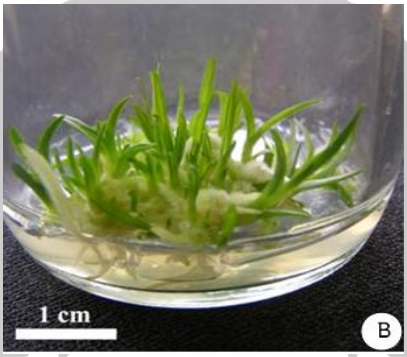
Table 4 Effect of growth regulators on in vitro protocorm–like bodies (PLBs) induction in thin cell layers section of *D. pulchellum*

Growth regulators (mg/L)			Explants with PLBs	No. of protocorms/
BA	Kn	NAA	(%)	Responding explant
Control			0	0±0.0[d]
1.0		0	52	31±6.6[abcd]
2.0		0	66	34±10.0[abcd]
5.0		0	50	36±6.7[abcd]
1.0		0.5	34	63±25.7[a]
2.0		0.5	36	43±5.8[abc]
5.0		0.5	52	47±16.1[abc]
1.0		1.0	22	12±6.0[cd]
2.0		1.0	30	42±8.2[abc]
5.0		1.0	46	50±14.4[ab]
	1.0	0	18	12±6.6[cd]
	2.0	0	46	43±10.8[abc]
	5.0	0	30	13±6.1[bcd]
	1.0	0.5	46	41±8.5[abc]
	2.0	0.5	40	25±5.9[bcd]
	5.0	0.5	14	13±12.6[bcd]
	1.0	1	22	35±14.4[abcd]
	2.0	1	34	23±6.4[bcd]
	5.0	1	8	3±2.0[d]

Note: Values within the column followed by different letters are significantly different at P≤0.05 by Duncan's multiple range test.



(A) PLBs protuberances on the surfaces



(B) Well–rooted and developed plantlets from PLBs

(C) transplanted in pots

Figure 2 Protocorm–like body (PLB) induction and plant regeneration in *D. pulchellum* through thin cell layers culture

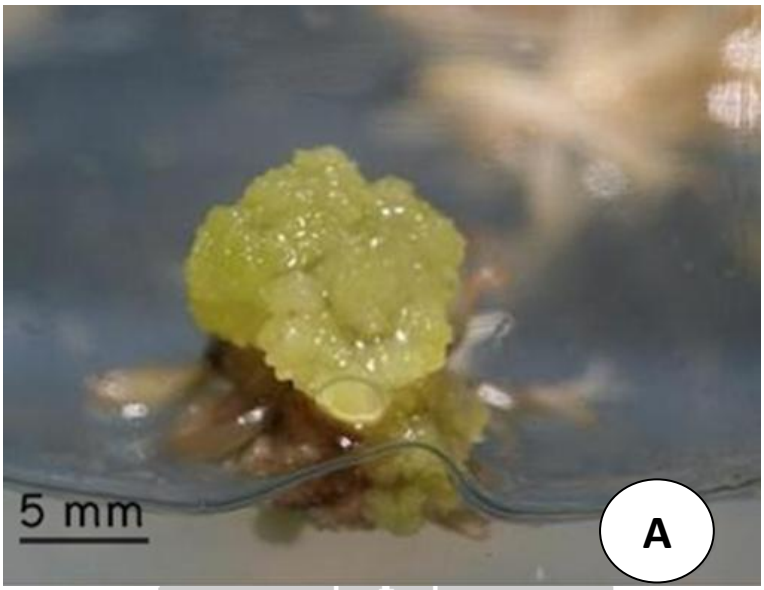
D. christyanum

The TCL explants from protocorm were cultured on media containing different combination of BA, Kn and for PLBs induction. The basal medium without PGRs was used as a control. After transferred to the medium, the explants cultured failed to show any response; even though, they remained green up to 1 to 2 weeks (Figure 3A). Yellow and PLBs formed from the explants becoming dark green within 5 weeks except the control. The TCL explants cultured on the MS medium without PGRs failed to turn brown within 10 days and subsequently died. A significant increase ($P \leq 0.05$) in number of PLBs per explants was observed in all cultures in comparison to control (Table 5). BA was less effective for PLBs development in comparison to Kn. The frequency of responsive protocorm explant were multiplication with increasing concentration of Kn 0 to 2.0 mg/L and decreased when above 2.0 mg/L. Incorporation of NAA at 1.0 mg/L into medium was essential for enhancing the percentage of TCL explants producing PLBs. The best response was recorded for the medium containing 2.0 mg/L Kn in combination with 1.0 mg/L NAA. It could induce PLBs in 68.3% of the explants within 12 weeks of culture and the average number of PLBs per explant was 33. The PLBs could be subcultured on growth regulator–free medium and exhibited fast proliferation and further development. After 3 to 4 weeks, small round PLBs differentiated into mature PLBs with the profusion of leaves on the apical region (Figure 3B).

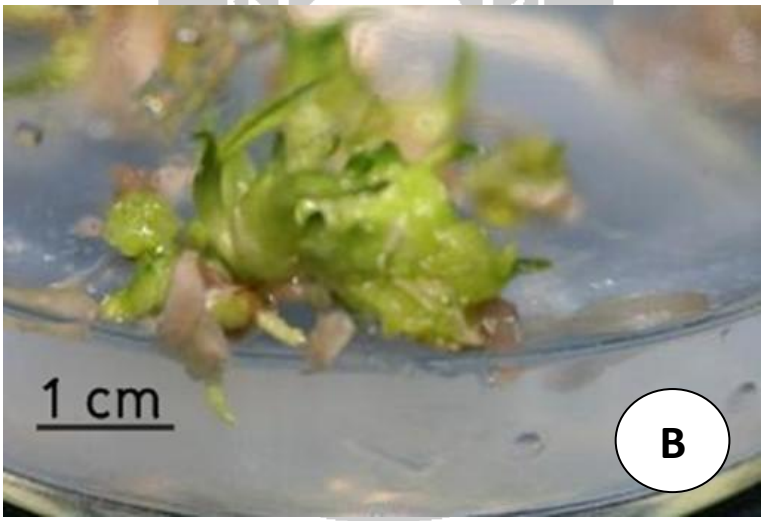
Table 5 Effect of growth regulators on in vitro protocorm–like bodies (PLBs) induction in thin cell layers section of *D. christyanum*

Growth regulators (mg/L)			Explants with PLBs (%)	No. of protocorms/ Responding explant
BA	Kn	NAA		
Control			0	0 ± 0.0^{f}
1.0		0	46.7	18 ± 6.1^{cdef}
2.0		0	40	15 ± 7.1^{cdef}
5.0		0	50	18 ± 2.9^{cdef}
1.0		0.5	50	28 ± 3.8^{abcde}
2.0		0.5	50	29 ± 10.4^{abcde}
5.0		0.5	60	30 ± 5.2^{abcd}
1.0		1.0	31.7	14 ± 3.4^{cdef}
2.0		1.0	66.7	42 ± 5.6^{ab}
5.0		1.0	41.7	22 ± 5.3^{bcde}
	1.0	0	53.3	22 ± 7.1^{bcde}
	2.0	0	35	18 ± 4.4^{cdef}
	5.0	0	40	14 ± 4.8^{cdef}
	1.0	0.5	55	18 ± 3.2^{cdef}
	2.0	0.5	61.7	44 ± 13.9^{a}
	5.0	0.5	26.7	8 ± 1.5^{ef}
	1.0	1	40	9 ± 2.9^{def}
	2.0	1	68.3	33 ± 7.8^{abc}
	5.0	1	35	11 ± 4.1^{def}

Note: Values within the column followed by different letters are significantly different $P\leq0.05$ by Duncan's multiple range test.



(A) PLBs protuberances on the surfaces



(B) Well–rooted and developed plantlets from PLBs

Figure 3 Protocorm–like body (PLB) induction and plant regeneration in *D. christyanum* through thin cell layers culture

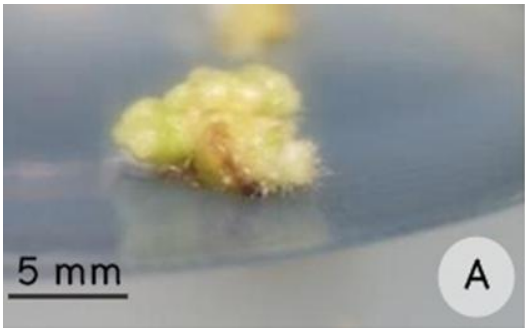
E. spectabilis

In this study, BA and Kn were tested alone or in combination with NAA. The effect of BA and Kn at different concentration on PLBs induction was assessed on *E. spectabilis* TCLs cultured on MS medium (Figure 4A). Formation of PLBs could be observed on growth regulator–free medium, but explants responded at a low frequency with very few PLBs per explants. The frequency of responsive protocorm explant increased with increasing concentration of Kn 0 to 2.0 mg/L and decreased when Kn concertration above 2.0 mg/L. The best response was recorded for the medium containing 2.0 mg/L Kn, on which 68% of the explants developed on average 12 PLBs per explants within 12 weeks. The PLBs per explant was significantly as compared to control (Table 6). After the end of each culture period, the regenerated PBLs were transferred to growth regulator free MS medium (Figure 4B). Tuberization could be observed from an external change. For tuber growth, it was found after the tuber primordial emerged. It continued to grow straight down into the medium. After culturing for 3 weeks, the tuber had an appropriate length, and began to markedly increase its width. Some plantlet produced new tuber primordial after culturing 6 weeks. A marked increase in shoot, leaf and root lengths were observed in the plants. The plantlets developed 3 to 4 leaves and 4 to 6 roots. The width of tuber was 0.5 cm. Regenerated plantlets with well developed roots (3 to 6 cm height) were planted in pots containing coconut husk peat and soil (1:2) (Figure 4C). The pots were covered with a transparent plastic bag in order to maintain a high humidity during acclimatization for 4 weeks before transfer to a nursery.

Table 6 Effect of growth regulators on in vitro protocorm–like bodies (PLBs) induction in thin cell layers section of *E. spectabilis*

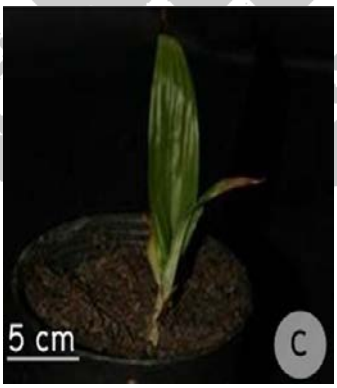
Growth regulators (mg/L)			Explants with PLBs (%)	No. of protocorms/ Responding explant
BA	Kn	NAA		
Control			28	3±0.9^{d}
1.0		0	24	2±1.3^{d}
2.0		0	24	3±2.1^{d}
5.0		0	40	1±0.6^{d}
1.0		0.5	32	4±1.7^{bcd}
2.0		0.5	28	4±1.3^{cd}
5.0		0.5	28	6±2.7^{bcd}
1.0		1.0	36	2±0.8^{d}
2.0		1.0	32	3±1.2^{d}
5.0		1.0	36	5±1.7^{bcd}
	1.0	0	56	23±4.4^{a}
	2.0	0	68	12±2.7^{b}
	5.0	0	60	9±3.3^{bcd}
	1.0	0.5	48	20±3.7^{a}
	2.0	0.5	56	12±2.5^{bc}
	5.0	0.5	52	5±1.2^{bcd}
	1.0	1	60	8±2.4^{bcd}
	2.0	1	52	11±4.1^{bc}
	5.0	1	48	5±1.2^{bcd}

Note: Values within the column followed by different letters are significantly different at P≤0.05 by Duncan's multiple range test.



(A) PLBs protuberances on the surfaces

(B) Well–rooted and developed plantlets from PLBs



(C) Acclimatization of the plantlets

Figure 4 Protocorm–like body (PLB) induction and plant regeneration in *E. spectabilis* through thin cell layers culture

Part II: *In vitro* conservation under minimal growth conservation

D. pulchellum

The results were extending the subculture interval from normal (2–3 months) to a much longer period in *D. pulchellum.* There was a significantly effect of MS nutrient medium on average height of microplants. MS medium, when used in full strength, supported normal growth and development of microplants (Figure 5A). After 3 months of storage, an average height of microplant was 0.33 to 1.32 cm. The microplant required subculture at 3 months intervals and these microplants remained green or pale green during storage. The results clearly exhibited that a reduction of nutrients to low levels (1/4 and 1/8 strength) induced slow growth. Subsequently, the culture could be maintained for 5 months without subculture (Figure 5B). Microplants maintained in 1/4 strength MS medium showed reduced growth with maximum survival (80%). The percentage of survival decreased to 70 when sucrose concentration increased to 10 and 20 g/L.

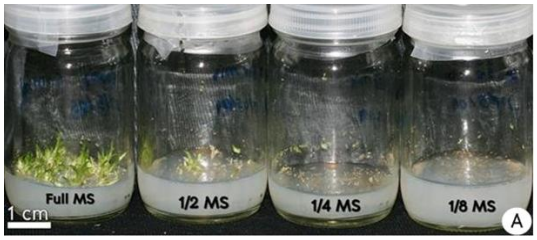
After all the storage period, the microplants turned greenish–yellow. An average height of microplants was reduced when microplants were cultured on 1/4 and 1/8 strength MS medium. An important difference between 1/4 and 1/8 strength MS medium that was not only average height of microplants but also morphological changes. Morphological abnormalities frequently developed in microplant conserved on 1/8 strength MS medium. The study ended after 5 months of storage. The result showed that the subculture duration of 3 months under normal culture condition could be extended to 5 months by reducing the content of nutrients.

The conserved microplants were transferred to strength MS medium supplemented 10 g/L sucrose and 1 mg/L BA for retrieval of normal shoot and their multiplication. The microplants conserved on 1/4 strength MS medium without sucrose could demonstrate regrowth and multiplied well with the considerable increase in average height of microplant (Figure 5C). The conserved microplants with all the newly formed shoot and root were cultured into regrowth medium and the development of these shoot and were normal. The microplant successfully regrew and reached 1.67 cm in height with 2 to 5 leaves within 3 months. The recovered microplants multiplied normally, without losing regeneration capacity and without apparent morphological changes.

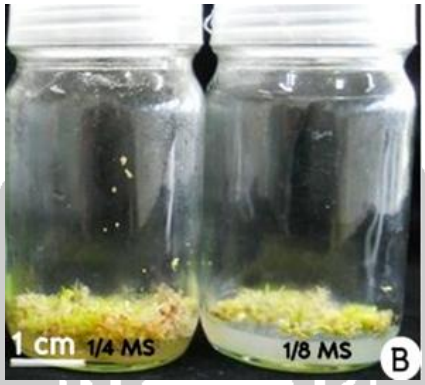
Table 7 Effects of various media components on *in vitro* conservation of *D. pulchellum*

Basal medium	Sucrose (g/L)	Microplant height (cm)	Survival (%)	Storage period (months)	Microplant height after 3 months recovery (cm)
1/8 MS	0	0.22±0.75^{c}	50	5	0.53±0.04^{b}
	10	0.27±0.25^{bc}	50	5	0.46±0.06^{b}
	20	0.27±0.25^{bc}	60	5	0.63±0.9^{ab}
1/4 MS	0	0.27±0.25^{bc}	80	5	0.87±0.17^{a}
	10	0.22±0.25^{c}	70	5	0.57±0.06^{b}
	20	0.27±0.25^{bc}	70	5	0.52±0.07^{b}
1/2 MS	0	0.55±0.2^{bc}	50	3	0.45±0.05^{b}
	10	0.45±0.1^{bc}	80	3	0.63±0.08^{ab}
	20	0.37±0.35^{bc}	70	3	0.65±0.13^{ab}
Full MS	0	0.33±0.15^{bc}	0	3	0.0±0.0^{c}
	10	0.62±0.1^{ab}	80	3	0.6±0.08^{ab}
	20	1.3±0.27^{a}	100	3	0.61±0.07^{ab}

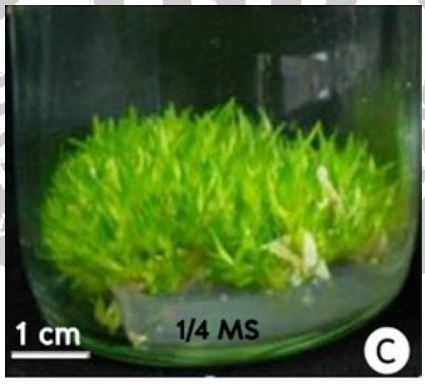
Note: Values within the column followed by different letters are significantly different at P≤0.05 by Duncan's multiple range test.



(A) Three–mont–old microplants on slow growth medium



(B) Five–month–old microplants on slow growth medium



(C) Recovery of microplants, conserved on 1/4 strength MS for 5 months on full strength MS

Figure 5 *In vitro* conservation of *D. pulchellum*

E. spectabilis

The objective of the minimal growth procedure of *E. spectabilis* was to extend the subculture interval from the normal (1–2 months) to a much longer period. Analyses of variance showed that MS nutrient medium had a strong effect on growth of *E. spectabilis*. MS medium, when used in 1/4 strength and 1/8 strength, supported normal growth and development of micropants (Figure 6). An average height of microplant was 5.5 cm after 3 months of storage on 1/8 strength MS medium and these microplant required subculture at 3 months intervals.

The maximum survival percentage was found when cultures were stored for 6 months at 1/8 strength MS combination 10 g/L sucrose (90%). The microplant was not survival when these microplants were cultured on 1/4 strength, 1/8 strength and full strength MS medium without sucrose. The microplant could not only grow that very slowly on low nutrient medium and rarely developed and additional shoot. An increase of the sucrose concentration from 10 to 20 g/L also reduced average height of microplants (Table 8). In general, for media without sucrose i.e. without any osmotic microplants growth was relatively poor after all the storage period. This factor reflected the effect heights of microplant were linked to growth reduced and eventually death. Healthy microplants with vigorous growth and without symptom or other apparent abnormalities were observed after the recovery. The results clearly exhibited that a reduction of nutrients to low levels (1/8 strength) induced slow growth and subsequently the culture could be maintained for a period 6 months without subculture.

The conserved microplants were transferred to full strength MS medium supplemented 10 g/L sucrose and 1 mg/L BA for retrieval of normal shoot and their multiplication. The microplant conserved on 1/8 strength and full strength MS medium supplemented both 10 and 20 g/L sucrose could demonstrate regrowth and multiplied well with the considerable increase in shoot and root. The microplant successfully regrew and reached 5.7 cm in height with 4 to 6 leaves within 3 months. Growth recovery of the microplant conserved on 1/8 strength MS showed a significant in comparison with those conserved on full strength MS medium.

Table 8 Effects of various media components on *in vitro* conservation of *E. spectabilis*

Basal medium	Sucrose (g/L)	Microplant height (cm)	Survival (%)	Storage period (months)	Microplant height after 3 months recovery (cm)
1/8 MS	0	0.0±0.0^{f}	0	6	0.0±0.0^{c}
	10	5.5±0.35^{a}	90	6	5.7±0.25^{a}
	20	1.5±0.2^{de}	70	6	3.85±1.15^{ab}
1/4 MS	0	1.0±0.0^{ef}	50	6	0.0±0.0^{c}
	10	3.5±0.5^{bc}	70	6	4.05±0.7^{ab}
	20	4.6±0.4^{ab}	80	6	5.05±1.05^{ab}
1/2 MS	0	0.0±0.0^{f}	0	3	2.0±0.4^{bc}
	10	4.0±0.7^{b}	80	3	3.7±0.76^{ab}
	20	4.3±0.14^{b}	80	3	3.9±1.1^{ab}
Full MS	0	0.0±0.0^{f}	0	3	0.0±00^{c}
	10	1.0±0.0^{ef}	80	3	5.2±1.0^{ab}
	20	2.5±0.2^{bc}	100	3	3.26±0.53^{ab}

Note: Values within the column followed by different letters are significantly different at P≤0.05 by Duncan's multiple range test.

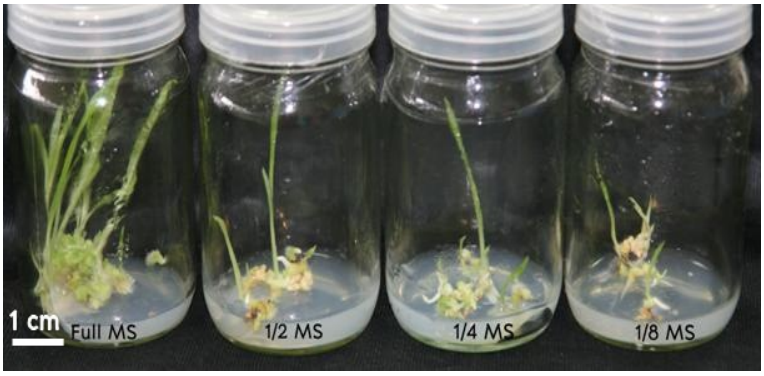


Figure 6 Three–month–old microplants on slow growth medium of *E.spectabilis*

CHAPTER V

DISCUSSIONS AND CONCLUSIONS

Part I: Thin cell layers (TCLs)

The TCL system as originally developed by Tran Thanh Van Thi and Chlyah (1974) for tobacco (*N. tabacum*) has such properties. Advantages of TCLs for the study of transformation have already been outlined by Tran Thanh Van, et al. (1990). Therefore, one advantage of the TCL system is to produce a high frequency of PLBs, shoot regeneration and to reduce time interval required in several orchid species e.g. *Rhychostylis gigantean* (Le, et al., 1999) *Cymbidium aloifolium* and *Dendrobium nobile* (Nayak, et al., 2002) and *Cymbidium* Sleeping Nymph (Vyas, et al., 2010). The objective of the present investigation was to use the TCL system for the *in vitro* propagation of four orchid species (*D. gratiosissimum*, *D. pulchellum*, *D. christyanum* and *E. spectabilis*). The results demonstrated the morphogenic potential of the TCL explants of four orchid species. The TCLs system could produce more than 30 PLBs per explants for *D. pulchellum* and *D. christyanum*. Moreover the frequency of responsive protocorm was higher than 60% in all species. This technique has been proved to be superior to other *in vitro* methods using various plant parts as explants. It has tested in a number of orchid species (Lakshmanan, et al., 1995; Malabadi, et al., 2004; Rangsayatorn, 2009). The efficiency of TCL culture is very high compared to the conventional technique of tissue culture which has been attributed by Rout, et al. (2006). Recently thin cross section (TCSs) of actively growing parts such as shoots, leaves, inflorescence stalks and developing PLBs have been successfully used by some workers for plantlet regeneration in a few orchids e.g. *D. moschatum* (Kanjilal, et al., 1999), *D. nobile* (Malabadi, et al., 2005) and *Phalaenopsis ambilis* (Sinha, et al., 2007). Lakshmanan, et al. (1995) was found to have a high regenerative capacity of commercial orchids by TSC system. Using TCLs more than 80,000 plantlets can be produced from thin section obtained from a single shoot tip in a year as compared to about 11,000 plantlets produced by the conventional shoot tip method. The TCSs were obtained from the PLBs developed *in vitro* which made it easier to obtain explants in an aseptic condition without damaging the natural resource. It could be a

starting point for a method which could be used to propagate mature plants (Kanjilal, et al., 1999). The PLBs have been used as explant source for the TCS method for other orchid species. This method could be accelerating propagation *in vitro* without the danger of undesirable mutations (Begum, et al., 1994; Ishii, et al., 1998). In this study, TCSs were taken from 4 weeks old protocorm for regeneration because at this stage the protocorm are composed of high frequency meristematic cells. Besides PGRs have a major role in regeneration shoots from different explants of orchids. Lakshmanan, et al. (1995) and Teixeira da Silva, et al. (2006) reported that thin section possibly ease the diffusion of nutrients and growth promoting substances at the site of regeneration and elimination correlative control imposed by other tissues. Most orchid tissue requires auxins, cytokinins and other PGRs for growth, the formation of PLBs, proliferation and plantlet development. Where both of these PGRs are present the ratio between them are critically important. For example, auxins induce root initiation at high levels, the elongation of roots at low concentrations and stimulate shoot elongation whereas cytokinins promote cell division (Arditti, 2008). The requirement of growth regulators also varies from species to species and explants to explants (Nayak, et al., 2002). Among the regulators, cytokinins like BA, Kn, etc. were found to be most effective in regenerating plantlets in a number of orchid (Rangsayatorn, 2009).

In this study, it was found that BA at 1.0 to 2.0 mg/L was most effective in regenerating PLBs from TCL explants of *D. pulchellum* and Kn at 1.0 to 2.0 mg/L was most suitable for *D. gratiosissimum*, *D. christyanum* and *E. spectabilis*. The superiority of BA in promoting PLB induction and *in vitro* morphogenesis was also observed in different orchids e.g. *D. nobile* and *C. aloifolium* (Nayak, et al., 1997), *R. gigantean* (Le, et al., 1999a) and *D. draconis* (Rangsayatorn, 2009). The result of *D. pulchellum* showed the frequency of responsive protocorm of the TCL explants culture on MS medium supplemented with 1.0 mg/L BA in combination with 0.5 mg/L NAA were increased. Similarly, Begum, et al. (1994) were induced embryogenic callus from thin section of PLBs with the help of NAA in *Cymbidium*. On the contrary, BA has only small effect on the regeneration process from TCL explants of *D. gratiosissimum*, *D. christyanum* and *E. spectabilis*. The result clearly showed that high concentration of Kn at 1.0 to 2.0 mg/L stimulated to PLBs proliferated from TCL explants. Similar results were also reported for *Brassocattleya* (Kako, 1973) and *Eulophia hormusjii*

(Vij, et al., 1989). However, increasing concentration of cytokinins above the optimum level led to lower proliferation rate of embryo (Rangsayatorn, 2009).

Two main groups of PGRs, cytokinins (BA and Kn) and auxin (NAA) are commonly used in orchid culture media. Their concentration significantly affected shoot and root formation. This study showed that BA alone or Kn alone can decrease the number of PLBs per explant. The combination of NAA and BA or Kn can promote PLBs formation. However, the increase of NAA, BA or Kn concentration could reduce the number of PLBs per explant. For example, Vij, et al. (1989) reported that 1.0 mg/L Kn in combination with 1.0 mg/L NAA induced shoot bud from rhizome segments of *E. hormusjii.* According to Nayak, et al. (1997), lower BA and NAA ratios in the culture medium could induce PLB formation in *Acampe praemorsa*, but the induction was very low when compared with the use of BA alone. Thus many orchid species require different auxins and/or cytokinins for neoformation of PLBs and plantlet development. The ratio of auxin and cytokinin of PLB formation depends upon the species and explant tissue studied (Arditti, 2008).

In conclusion, PGRs (BA, Kn and NAA) had an effect on PLBs formation of D. *gratiosissimum*, *D. pulchellum*, *D. christyanum* and *E. spectabilis* from TCL system. The number of PLBs per explant was more pronounced in BA treatments for *D. pulchellum*. On the contrary, the number of PLBs per explant was pronounced in Kn treatments fo *D. gratiosissimum*, *D. christyanum* and *E. spectabilis*. In addition, NAA at 0.5 to 1.0 mg/L in combination with cytokinins could increase PLBs per explant. No phenotypic variation was observed among the regenerants in all species. Rapid and efficient multiplication rate, rooting and successful transfer of plantlets to the nursery make this protocol suitable for large scale multiplication as well as *ex situ* conservation of these commercially important orchid species.

Part II: *In vitro* conservation under minimal growth conservation

Carbohydrate source is considered to have prime importance in *in vitro* morphogenesis (Romano, et al., 1995). Most orchid seed and tissue culture medium require the addition of sugar as source of carbon (Arditti, 2008). Nevertheless, the widespread exploitation of micropropagation is restricted by production cost, especially media costs, by contamination and also losses due to the low survival rates during acclimatization (Leifert, et al., 1994; Mitra, et al., 1998). Several workers had successfully demonstrated the use of minimal growth medium for the conservation of germplasm e.g. *Cedrus atlantica* and *C. libani* (Morrata and Arrillaga, 2006), *Coffea* spp. (Desbrunais, et al., 1991), *Musa* (Banerjee and De Langhe, 1985), *Vanilla* (Divakaran, et al., 2006) and *Drosophyllum lusitanicum* (Gonçalves and Romano, 2007). The effect of different minimal growth medium and sucrose concentration was studied in *D. pulchellum* and *E. spectabilis* for conservation and propagation. After 5 months of storage, the microplant height decreases with an increase in sucrose concentration. Moreover, Mitra, et al. (1998) reported that sucrose–free cultures have been established under CO_2 enrichment. In addition, the higher survival rate of the plantlets developed on sucrose–free medium as depicted by Kozai, et al. (1997). It is due to the high photosynthetic competence acquired by the uptake of mineral elements due to increased transpirational activities during the culture on sucrose–free medium. Similarly, Martin and Pradeep (2003) reported that half strength MS and sugar–free medium was the best for *in vitro* conservation and the storage of *Ipsea malabarica* on this medium for 27 months. In this study, the data showed that the microplant height increased with an increasing in sucrose concentration when sucrose concentration was over 10 g/L in *E. spectabilis* after all the storage periods. The effect of sucrose concentration on microplant high in *E. spectabilis* similar with other plants such as in *I. malabarica* (Martin and Pradeep, 2003) and *D. nobile* (Fabiana et al., 2004). Chen, et al. (2003) reported that sucrose may act as a carbon source and as an osmotic regulator. Moreover, sucrose seems to be the most critical stimulus for tuber formation in potato (*Solanum tuberosum*). It may be essential as an osmoticum and an energy source. At higher concentration may have a role as a signal for microtuber formation as reported by Wang and Hu (1982) and Khuri and Moorby (1995). The optimal sucrose concentration in the medium should be high enough to satisfy the basic energy requirement for plant growth and low enough not

to impose any negative osmotic effect on shoot elongation. The microplants had been conserved for 5 months (*D. pulchellum*) and 6 months (*E. spectabilis*) rapidly multiplied on regrowth medium under normal growth condition. Those which were visually scored viable after incubation under slow growth condition showed a full regeneration capacity. The optimal minimal growth medium and sucrose concentration for microplant high depends on species. For example, a significant effect of sucrose concentration on plant height and high seedling multiplication of *D. nobile* were observed in 60 g/L sucrose treatment (Fabiana, et al., 2004).

In conclusion, the results showed that *D. pulchellum* and *E. spectabilis* can be successfully maintained for several months by *in vitro* slow growth preservation technique. The properly medium for in vitro conservation of *D. pulchellum* for 5 months without sub culturing was 1/4 MS supplemented with sucrose 10–20 g/l. While, *E. spectabilis* could be conserve for 6 months on 1/8 MS supplemented with sucrose 10 g/lL. After regrowth, *D. pulchellum* and *E. spectabilis* could healthy grow with no morphological abnormalities observed. The present study suggests an efficient and easy to handle protocol for micropropagation of orchids. Therefore, this technique could be useful for *in vitro* slow growth collection as duplicate storage technique with cheaply cost and least risk of genetic instability.

BIBLIOGRAPHY

BIBLIOGRAPHY

Arditti J. and Erant R. (1993). **Micropropagation of orchids**. New York: Wiley.

Arditti J. (2008). **Micropropagation of orchids** (2nd ed.). United Kingdom: Blackwell publishing.

Banerjee N. and De Langhe E. A. (1985). A tissue technique for rapid clonal propagation and storage under minimal growth conditions of Musa (banana and plantain). **Plant Cell Reports**, 4, 351–354.

Begum A. A., Tamaki M. and Kako S. (1994). Formation of protocorm–like bodies (PLBs) and shoot development through *in vitro* culture of outer tissue of *Cymbidium* PLB. **Journal of the Japanese Society for Horticultural Science**, 3(63), 663–673.

Budiarto K. (2009). Alternative in vitro media for medium–term conservation of chrysanthemum (*Dendranthema Grandiflora* Tvelve). **Jurnal Bumi Lestari**, 1(9), 48–53.

Carvalho C. de M. H., Le B. V., Zuily–Fodil Y., Pham Thi A. T. and Thanh Van K. T., (2000). Efficient whole plant regeneration of common bean (Phaseolus vulgaris L.) using thin–cell–layer culture and silver nitrate. **Plant science**, 159, 223–232.

Chen Y., Lin S., Duguid S. D., Dribnenki P. and Kenaschuk, E. O. (2003). Effect of sucrose concentration on elongation of shoots from flax anther culture. **Plant Cell Tissue and Organ Culture**, 2(72), 181–183.

Desbrunais A. B., Noirot M. and Charrier A. (1992). Slow growth *in vitro* conservation of coffee (*Coffea* spp.). **Plant Cell, Tissue and Organ Culture**, 31, 105–110.

Divakaran M., Babu K. N. and Peter K. V. (2006). Conservation of *Vanilla* species, *in vitro*. **Scientia Horticulturae**, 110, 175–180.

Dragan V. V. and Branka S. V. (1999). Hormone–like effect of sucrose in plant *in vitro* cultures. **Phyton (Austria) Secial issue: Plant Physiology**, 3(39), 57–60.

Duan J. X. and Yazawa S. (1995). Floral induction and development in *Phalaenopsis in vitro*. **Plant Cell Tissue and Organ Culture**, 43, 71–78.

Fariana R. T. de, Rodrigues F. N., Oliveira L. do V. R and Muller C. (2004). In vitro *Dendrobium* nobile plant growth and rooting in different sucrose concentrations. **Horticultural Brasileira, Brasilia**, 4(22), 780–783.

Feijoo M. C. and Iglesias I. (1998). Multiplication of an endangered plant: *Gentiana lutea* L. sub sp. *Aurantiaca* Lainz, using in vitro culture. **Plant Tissue Cult Biotechnol**, 4(87), 87–94.

Fosket D. E. (1994). **Plant Growth and Develop**. San Diego: Academic Press.

George E. F., Hall M. A. and Klerk G. J. (2008). **Plant Propagation by Tissue Culture** (3nd ed.). Netherlands: Springer.

Gonçalves, S. and Romano, A. (2005). Micropropagation of *Drosophyllum lusitanicum* (Dewy pine), an endangered West Mediterranean endemic insectivorous plant. Biodiversity and Conservation. **Biodiversity and Conservation,** 5(14), 1071–1081.

Gonçalves S. and Romano A. (2007). *In vitro* minimum growth for conservation of *Drosophyllum lusitanicum*. **Plant Biology**, 51, 795–798.

Ishii Y., Takamura T., Goi M. and Tanaka M. (1998). Callus induction and somatic embryogenesis of Phalaenopsis. **Plant Cell Report**, 17, 446–450.

Jaime A., Teixeira da Silva and Tanaka M. (2006). Multiple regeneration pathways via thin cell layers in Hybrid *Cymbidium* (Orchidaceae). **Plant Growth Regulation**, 25, 203–210.

Khan I. A., Dahot M. U., Yasmin S., Khatri A., Seema N. and Naqvi M. H. (2006). Effect of sucrose and growth regulators on the micropropagation of sugarcane clones. **Pakistan Journal of Botany**, 4(38), 961–967.

Kako S. (1973). Clonal propagation of *Cattleya* through shoot meristem culture. **Japan Agricultural Research Quarterly**, 7, 109–115.

Kanjilal B., De Sarkar D., Mitra J. and Datta K. B. (1999). Stem disc culture: development of a rapid mass propagation method for *Dendrobium moschatum* (Buch.–Ham) Swartz–an endangered orchid. **Current Science**, 77, 497–500.

Kannan N. (2009). An *in vitro* study on micropropagation of *Cymbidium* orchids. **Current Biotica**, 3(2), 244–250.

Keller E. R. J., Senula A., Leunufna S. and Grube M. (2006). Slow growth storage and cryopreservation–tool to facilitate germplasm maintenance of vegetatively propagated crops in living plant collections. **International Journal of Refrigeration**, 29, 411–417.

Ket N. V., Hahn E. J., Park S. Y., Chakrabarty D. and Paek K. Y. (2004). Micropropagation of an endangered orchid *Anoectochilus formosanus*. **Biologia Plantarum**, 3(48), 339–344.

Khuri S. and Moorby J. (1995). Investigations into the role of sucrose in potato *cv.* Estimate microtubers production *in vitro*. **Annales Botanici Fennici**, (75), 295–303.

Koši P., Škof S. and Luthar Z. (2004). Direct shoot regeneration from nodes of Phalaenopsis orchids. **Acta agriculturae slovenica**, 2(83), 233–242.

Kozai T. (1991). Photoautotrophic micropropagation. **In Vitro Cellular&Developmental Biology–Plant**, 271, 47–51.

Kozai T., Kubota C. and Jeong B.R. (1997). Environmental control for the large–scale production of plants through *in vitro* techiques. **Plant Cell Tissue Organ Culture**, 51, 49–56.

Lakshmanan P., Loh C. S. and Goh C. J. (1995). An *in vitro* method for rapid regeneration of monopodial orchid hybrid *Arada* "Deborah" using thin section culture. **Plant Cell Reports**, 14, 510–514.

Lakshmanan P., Lee C. L., Loh C. S. and Goh C. J. (1995). *In vitro* propagation of commercial orchids. An assessment of current methodology and development of a novel approach–thin cross section culture. (In. Islam A.S., Ed.), **Plant Tissue Culture**, oxford–IBN, New Delhi, India, 42–49.

Le B. V., Phuong N., Hong L. and Tran Thanh Van K. (1999). High frequency shoot regeneration from *Rhychostylis gigantean* (orchidaceae) using thin cell layers. **Plant Growth Regulation**, 28, 179–185.

Le B. V., Nhut D. and Tran Thanh Van K. (1999). Plant production via shoot regeneration from thin cell layer pseudo–bulblet expiants of *Lilium longgiflorum* in vitro. **Académie des sciences/Elsevier**, 4(322), 303–310.

Leifert C., Marris C. and Waites W. M. (1994). Microbial contaminants and pathogens of plant tissue culture. **Critical Reviews in Plant Sciences**, 13, 139–183.

Malabadi R. B., Mulgund G. S. and Natraja K. (2004). Efficient regeneration of *Vanda coerulea*, an endangered orchid using thidiazuron. **Plant Cell Tissue Organ Culture**, 76, 289–293.

Malabadi R. B., Mulgund G. S. and Kallappa N. (2005). Micropropagation of *Dendrobium nobile* from shoot tip section. **Journal of Plant Physiology**, 162, 473–478.

Maneerattanarungroj P., Bunnag S. and Monthatong M. (2007). In vitro Conservation of Cleisostoma areitinum (Rchb.f.) Garay, Rare Thai Orchid Species by an Encapsulation–Dehydration Method. **Asian Journal of Plant Sciences**, 6(8), 1235–1240.

Martin K. P. and Pradeep A. K. (2003). Simple strategy for the *in vitro* conservation of *Ipsea malabarica* an endemic and endangered orchid of the Western Ghats of Kerala, India. **Plant Cell, Tissue and Organ Culture**, 74, 197–200.

Martin K. P. (2004). In vitro propagation of the herbal spice Eryngium foetidum L. on sucrose–added and sucrose–free medium without growth regulators and CO_2 enrichment. **Scientia Horticulturae**, 102, 277–282.

Martin K. P. and Madassery J. (2006). Rapid in vitro propagation of Dendrobium hybrids through direct shoot formation from foliar explants, and protocorm–like bodies. **Scientia Horticulturae**, 108, 95–99.

Mitra A., Dey S. and Sawarka S. K. (1998).Photoautotrophic in vitro multiplication of *Dendrobium* under CO_2 enrichment. **Plant Biology**, 41, 145–148.

Morrata B. R. and Arrillaga I. (2006). In vitro storage of cedar shoot cultures under minimal growth conditions. **Plant Cell Reports**, 25, 636–642.

Murashige, T. and F. Skoog. (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. **Physiologia Plantarum**, 15, 473–497.

Nanakorn W. and Indharamusika S. (1999). **Ex situ Conservation of Native Thai Orchids1at1Queen1Sirikit1Botanic1Garden,** form http://www.iupac.org/symposia/proccedings/phuket97/nanakorn.html

Nassar A. H. (2003). Slow growth storage of encapsulated germplasm of Coffea Arabica L. **International Journal of Agriculture & Biology**, 4(5), 517–520.

Nayak N. P., Sahoo S., Patnaik S. and Rath S. P. (1997). In vitro propagation of three epiphytic orchids, *Cymbidium aloifolium* (L.) Sw., *Dendrobium aphyllum* (Robx.) Fisch. And *Dendrobium moschstum* (Buch–Ham) Sw. throughthaidiazuron–induced high frequency shoot proliferation. **Scientia Horticulturae**, 71, 243–250.

Nayak N. P., Sahoo S., Patnaik S. and Rath S. P. (2002). Establishment of thin cross section (TCS) culture method foe rapid micropropagation of *Cymbidium aloifolium* (L.) Sw. and *Dendrobium nobile* Lindl. (Orchidaceae). **Scientia Horticulturae**, 94, 107–116.

Negash A., Puite K., Schaart J., Visser B. and Krens F. (2000). In vitro regeneration and micro–propagation of enset from Southwestern Ethiopia. **Plant Cell, Tissue and Organ Culture**, 62, 153–158.

Nhut D. T., Le B. V. and Teixeira da Silva, J. A. (2001). Thin cell layer culture system in *Lilium*: regeneration and transformation perspectives. **In Vitro Cellular & Developmental Biology–Plant.** 37(516), 516–523.

Nhut D.T. An T. T. T., Huong N. T. D., Don N. T., Hai N. T., Thinen N. Q. and Va N. H. (2007). Effect of genotype, explant size, position, and culture medium on shoot generation of Gerbera jamesonii by receptacle transverse thin cell layer culture. **Scientia Horticulturae**, 111, 146–151.

Onay A.,Işkalan C., Adyaman F., Pirinç V., Tilkat E. and Başaran D. (2003). *In vitro* Micrografting of Pistachio (*Pistacia vera* L.). **Plant Cell, Tissue and Organ Culture**, 77, 215–219.

Promsorn N. and Kanchanapoon K. (2006). Conservation and Micropropagation of Musa balbisiana "Kluai Hin". **BRT 2006 Research Reports**, 127–130.

Rangsayatorn N. (2009). Micropropagation of *Dendrobium draconis* Rchb.f. from thin cross–section culture. **Scientia Horticulturae**, 122, 662–665.

Robert D. L. and Dixon K. W. (2008). Orchids, **Current Biology**, 18(8), R325–R329.

Romano A., Noronha C. and Martins–Loucao M. A. (1995). Role of carbohydrates In micropropagation of cork oak. **Plant Cell Tissue Organ Culture**, 40, 159–167.

Rout G. R., Mohapatra A. and Mohan Jain S. (2006). Tissue culture of ornamental potplant: acritical review on present scenario and future prospect. **Biotechnol. Advances.**, 24, 531–560.

Roy J. and Banerjee N. (2003). Induction of callus and plant regeneration from shoot–tip explants of Dendrobium fimbriatum Lindl. var. Oculatum Hk. f. **Scientia Horticulturae**, 97, 333–340.

Sanavy S. A. M. and Moeini M. J. (2003). Effects of different pH levels of medium on growth and rooting of single node resulted from potato meristem culture. **Plant Tissue Culture**, 2(13), 151–154.

Sarkar D. and Naik P. S. (1998). Factors affecting minimal growth conservation of potato microplants *in vitro*. **Euphytica**, 102, 275–280.

Sinha P., Hakim M. K. and Alam m. F. (2007). Effecent micropropagation of *Phalaenopsis amabilis* (L) BL. cv. "Cool Breeze" using inflorescence axis thin section as explants. **Propagation Ornamental Plants**, 7, 9–15.

Sopalum K., Thammasiri K. and Ishikawa K. (2010). Micropropagation of the Thai orchid *Grammatophyllum speciosum* blume. **Plant Cell, Tissue and Organ Culture**, 10, 143–150.

Teixeira da Silva J. A. (2003). Thin Cell Layer technology in ornamental plant micropropagation and biotechnology. **African Journal of Biotechnology**, 2(12), 638–691.

Teixeira da Silva, J. A., Singh N. and Tanaka M. (2006). Priming biotic factors for optimal protocorm–like body and callus induction in hybirid *Cymbidium* (Orchidaceae) and assessment of cytogenetic stability in regenerated plants. **Plant Cell Tissue Org.**, 8, 135–144.

Tran Thanh Van M., Thi D.N. and Chlyah A. (1974). Regulation of organogenesis in small explants of superficial tissue of Nicotina tobacum L.. **Planta**, 119, 149–159.

Tran Thanh Van K., Richard L., Gendy C. A. (1990). **An experimental model for the analysis of plant cell differentiation: thin cell layer.** Concept, strategy, methods, records and potential. In: Durzan D. and Rodriguez R. (Eds.) NATO Biotechnology series, New York: Plenum press, 215–224.

Vij S. P., Sood A. and Pathak P. (1989). On the utility of rhizome segments in micropropagation *Eulophia hormusjii* Duth. **Journal Orchid Society**, 3, 41–45.

Vyas S., Guha S., Kapoor P. and Rao I. U. (2010). Micropropgation of Cymbidium Sleeping Nymph through protocorm–like bodies production by thin cell layer culture. **Scientia Horticulturae**, 123, 551–557.

Wang P. and Hu C. (1982). *In vitro* tuberization and virus free seed–potato production in Taiwan. **American Potato Journal**, 59, 33–37.

Watt M. P.,Thokoane N. L., Mycock D. and Blakeway F. (2000). *In vitro* storage of *Eucalyptus gradis* germplasm under minimal growth conditions. **Plant Cell, Tissue and Organ Culture**, 61, 161–164.

Werner T. and Schmulling T. (2009). Cytokinin action in plant development. **Current Opinion in Plant Biology**, 12, 527–538.

Westcott R. J. (1981). Tissue culture storage of potato germplasm. I. Minimal growth storage. **Potato Research**, 24, 331–342.

Wood H. P. (2006). **The Dendrobiums**. Porland: Timber Press.

BIOGRAPHY

BIOGRAPHY

Name Surname Chutima Jaiphet

Date of Birth June 11, 1985

Place of Birth Phichit Province, Thailand

Address 80/1 Moo 4 Dongtakhop, Taphanhin, Phichit Province, Thailand 66110

Education Background

Year 2007 B.S. (Biology), Naresuan University.

Publications

Jaiphet C. and Rangsayatorn N. (2010). Micropropagation of a rare orchid *Dendrobium gratiosissimum* using thin cell layers. **ISHS Acta Horticulturae**. 878, 185–190.

Rangsayatorn N. and Jaiphet C. (2010). *In vitro* conservation of *Dendrobium draconis* under minimal growth conditions. **ISHS Acta Horticulturae**. 878, 179–184.