Growth and Genetic Variation of Lady's Slipper Orchid: *Paphiopedium callosum* var. *sublaeve*

Nararatn Wattanapan

A Thesis Submitted in Fulfillment of the Requirements for the Degree of

Doctor of Philosophy in Biology

Prince of Songkla University

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I hereby certify that this work has not been accepted in substance for any other degree, and is not being currently submitted in candidature for any degree.

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ชื่อวิทยานิพนธ์ การเจริญเติบโตและการแปรผันทางพันธุกรรมของกล้วยไม้รองเท้านารีม่วงสงขลา (*Paphiopedilum callosum* var. *sublaeve*)

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บทคัดย่อ

การศึกษาการงอกและการเจริญของเมล็ดกล้วยไม้รองเท้านารีม่วงสงขลา (*Paphiopedilum callosum* var. *sublaeve*) โดยการใช้สารจากธรรมชาติ คือ กรดไฮยาลูโรนิก (HA) และไคโตซาน พบว่า อาหารวุ้นสูตรดัดแปลงวาซินและเวนท์ (MVW) ที่มีไคโตซานความเข้มข้น 1.0 มก/ล เป็นอาหารที่มีความเหมาะสมต่อการเจริญของโพรโทคอร์มมากที่สุดโดยให้โพรโทคอร์มที่แข็งแรง และมีอัตราการงอกสูงสุดในระยะ C (1.93±0.35%) ส่วนการเพาะเลี้ยงบนอาหารวุ้นสูตร MMS ที่มี HA ความเข้มข้น 1.0 มก/ล พบว่ามีการเกิดยอดสูงสุด (3.22±0.36 ยอด) และมีอัตราการเกิดยอดสูงสุด (100%)

การขยายพันธุ์ในหลอดทดลองของรองเท้านารีม่วงสงขลาโดยใช้เทคนิค transverse thin cell layer (tTCL) พบว่าช่วงเวลาที่เหมาะสมในการปรับสภาพในน้ำกลั่น สำหรับการงอกของเมล็ดในหลอดทดลองคือ 2 สัปดาห์ก่อนการย้ายลงอาหารวุ้นความเข้มข้นลดลงครึ่งหนึ่งของสูตรมูราชิกิและสกู๊ค (1/2MS) ปลายยอดที่ได้จากการเพาะเลี้ยงด้วยระบบ tTCL บนอาหารวุ้นสูตร MVW ที่เติม Thidiazuron (TDZ) 1.0 มก/ล เป็นเวลา 8 สัปดาห์ให้การสร้างโพรโทคอร์มไลค์บอดี้ที่งอกใหม่สูงสุด 46.67±6.67 เปอร์เซ็นต์ ให้การเกิดยอด 40.00±5.16 67 เปอร์เซ็นต์ การเกิดราก 30.00±12.38 เปอร์เซ็นต์ และอัตราการรอดชีวิต 70.00±4.47 67 เปอร์เซ็นต์ สำหรับผลการศึกษาความผันแปรทาง

ชีวโมเลกุลโดยเทคนิค Random amplification of polymorphic DNA (RAPD) ด้วยไพรเมอร์จำนวน 10 ไพรเมอร์ ไม่พบความแปรผันทางพันธุกรรมระหว่างการเกิดต้นอ่อนที่ได้จากการชักนำและต้นแม่

จากการเก็บตัวอย่างพืชจากแหล่งต่างๆ ได้แก่ ชายแดนมาเลเซีย ชายแดนกัมพูชา และอำเภอรัตภูมิ จังหวัดสงขลา มาระบุชนิดโดยใช้ลักษณะทางสัณฐานอ้างอิงจากรายงานของ Atwood (1984) เพื่อศึกษาความสัมพันธ์ของรองเท้านารีม่วงสงขลากับชนิดพืชที่มีสายสัมพันธ์ใกล้ชิดกันบางชนิดใน หมู่ *Barbata* คือ *P. barbatum*, *P. callosum* var. *callosum* และ *P. callosum* var. *potentianum* โดยใช้ทั้งลักษณะสัณฐานและการวิเคราะห์โดยวิธี RAPD พบว่า ตัวอย่างพืชเหล่านี้สามารถแบ่งได้เป็น 3 กลุ่มคือ 1) *P. barbatum* 2) *P. callosum* var. *callosum* และ *P. callosum* var. *sublaeve* และ 3) *P. callosum* var. *popentianum*

การศึกษาในครั้งนี้สามารถยืนยันได้ว่า การใช้สารธรรมชาติที่จำเพาะและการเพาะเลี้ยงด้วยวิธี tTCL สามารถนำไปประยุกต์ใช้สำหรับการขยายพันธุ์พืชในสภาพหลอดทดลองของ *P. callosum* var. *sublaeve* ได้ และความสัมพันธ์ของ *P. callosum* var. *sublaeve* กับกลุ่มพืชชนิดอื่นที่มีสายสัมพันธ์ใกล้เคียงกันยังแสดงให้เห็นว่า *P. callosum* var. *sublaeve* มีทั้งลักษณะทางสัณฐานและความสัมพันธ์เชิงวิวัฒนาการความใกล้ชิดกับ *P. callosum* var. *callosum* มากกว่าชนิดอื่น

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ABSTRACT

The study of asymbiotic seed germination and growth of endangered lady's slipper orchid: *Paphiopedilum callosum* var. *sublaeve* was firstly undertaken by using natural additives, hyaluronic acid (HA) and chitosan. The modified Vacin and Went (MVW) medium supplemented with 1.0 mg/L chitosan was the most suitable medium for vigorous protocorms protocorm development and the highest seed germination percentage at stage C (1.93±0.35%). Meanwhile, explants were cultured on the modified Murashige and Skoog (MMS) medium containing 1.0 mg/L HA gave the highest number of shoots (3.22±0.36 shoots) and the maximum shoot formation rate (100%).

The *in vitro* propagation of *P. callosum* var. *sublaeve* seedlings was then conducted through transverse thin cell layer (tTCL) culture system. The results showed that the optimal period of its pretreatment process in distilled water to promote seed germination *in vitro* was two-week before being transferred to 1/2MS solidified medium. Shoot tip-derived tTCL explants cultured on MVW solidified medium containing 1.0 mg/L Thidiazuron (TDZ) for 8 weeks provided the highest percentage of regenerated protocorm-like bodies (46.67±6.67), shoot formation (40.00±5.16), root formation (30.00±12.38) and survival rate (70.00±4.47). According to the Random amplification of polymorphic DNA (RAPD) technique with 10 primers, no genetic variation was detected between the regenerated plantlets and their own mother plants.

Plant samples collected from various sources, namely, border of Malaysia, border of Cambodia, Rattaphum district, Songkhla province, and border of Vietnam were identified by Atwood (1984) to investigate the relationship of *P. callosum* var. *sublaeve* with some closed relative plants in section *Barbata*, namely *P.*

barbatum, *P. callosum* var. *callosum* and *P. callosum* var. *potentianum* by using both morphological characteristics and RAPD analysis. These plants were separated into three groups, 1) *P. barbatum*, 2) *P. callosum* var. *callosum* and *P. callosum* var. *sublaeve* and 3) *P. callosum* var. *popentianum*.

This study confirmed that the specific natural additives and the tTCL culture system can be greatly applied for the *in vitro* propagation of *P. callosum* var. *sublaeve*, and the relationship of this species with other closed relative plants revealed that *P. callosum* var. *sublaeve* was morphologically and phylogenetically closer to *P. callosum* var. *callosum* than other candidate species.

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Nararatn Wattanapan

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LISTS OF ABBREVIATIONS

AC	=	Activated charcoal
Ab	=	Abaxial epidermis
Ad	=	Adaxial epidermis
ANOVA	=	Analysis of variance
BA	=	6-Benzyladenine
BAP	=	6-Benzylaminopurine
CBP	=	Cytokinin-binding protein
Chl		Chlorophyll
CITES	=	Convention on International Trade in Endangered Species
CW	=	Coconut water
CRD	=	Completely randomized design
CuTab	=	Cuticle thickness of abaxial
CuTad	=	Cuticle thickness of adaxial
Cu	=	Cuticle
DAP	=	Days after pollination
DMRT	=	Duncan's multiple range test
DW	=	Distilled water
EPA	=	Environmental Protection Agency
FAAII	=	Formalin-aceto-alcohol
GAG	=	Glycosaminoglycans
GC	=	Guanine-cytosine
HA	=	Hyaluronic acid
IAA	=	Indol-3-acetic acid
ISSR	=	Inter-simple sequence repeat
ITS	=	Nuclear ribosome internal transcribed spacer
JA	=	Jasmonic acid
KC	=	Knudson's C

LISTS OF ABBREVIATIONS (CONTINUED)

Lab	=	Long of abaxial
Lad	=	Long of adaxial
LL	=	Length of leaf
LP	=	Leaf primodium
M	=	Mesophyll cells
MMS	=	Modified Murashige and Skoog
MT	=	Mesophyll thickness
MVW	=	Modified Vacin and Went
NaOCl	=	Sodium hypochlorite
NAA	=	1-Naphthaleneacetic acid
PCR	=	Polymerase chain reaction
PLBs	=	Protocorm- like bodies
PT	=	Palisade tissue
RAPD	=	Random amplified polymorphic DNA
S	=	Stoma
SA	=	Stomatal area
SA	=	Salicylic acid
SAM	=	Shoot apical meristem
SI	=	Stomatal index
SL	=	Stomatal length
ST	=	Spongy tissue
SV	=	Seed viability
TAE	=	Tris-acetate-EDTA
TBO	=	Toluidine blue O
TCL	=	Thin cell layer
TDZ	=	Thidiazuron

LISTS OF ABBREVIATIONS (CONTINUED)

TDZ	=	Thidiazuron
TTC	=	2,3,5-Triphenyltetrazolium chloride
VB	=	Vascular bundle
VW	=	Vacin and Went medium
Wab	=	Wide of abaxial
Wad	=	Wide of adaxial
Wl	=	Width of leaf

CHAPTER 1

INTRODUCTION

1.1 Introduction

Paphiopedilum (Orchidaceae) is commonly known as 'Lady's slipper orchid' because of its modified lip, which is pouch shape, giving the appearance that resemble to a lady's slipper (Zeng et al., 2015). This orchid appears to be one of the most popular commercially available orchids among Orchidaceae due to the variety of shapes, sizes, and colors of flowers (Ng et al., 2010). As a result, many orchid species are globally threatened by over collection from the natural forest habitat which was also ruined for horticultural purpose. Consequently, members of *Paphiopedilum* orchids have been indicated as endangered plants by the Convention on International Trade in Endangered Species of Wild Fauna and Flora-CITES (Pedersen et al., 2011). *Paphiopedilum callosum* var. *sublaeve* is known as 'Rongthao Nari Muang Songkhla' that mainly distributes in Ranong, Phangnga, Krabi and Songkhla province (Pedersen et al., 2011). Moreover, the genus *Paphiopedilum* was composed of 3 subgenera: *Parvisepalum, Brachypetalum* and *Paphiopedilum*. The subgenus *Paphiopedilum* was composed of 5 sections, namely, *Coryopedilum, Pardalopetalum, Cochlopetalum*, *Paphiopedilum* and *Barbata* (Cribb, 1998). However, some plants in section *Barbata*, such as *P. callosum* (including 3 varieties) and *P. barbatum* were still controversial and required the supporting data to clarify their characteristics.

This study (Figure 6) was divided into the following 3 reasons: Firstly, the information concerning reproductive biology and methods of *in vitro* propagation are thus of great importance for the conservation and the commercial production (Lee, 2006). However, the establishment of protocols for *in vitro* orchid seed culture is species-specific and depends on several factors including capsule maturity, components of culture media, light and temperature culture conditions (Avila-Diaz et al., 2009). To enhance the seed germination and development, many complex organic additives, such as coconut water, banana, potato extract raisins, apple, tomato and V-8

juices have been added to the culture media (Kananont et al., 2010). Chitosan was reported to enhance the growth of *Dendrobium* without increasing the detectable levels of somaclonal variation (Pornpienpakdee et al., 2010) besides hyaluronic acid could enhance both PLBs and shoot formation of *Cymbidium* orchid (Nahar et al., 2011). The management for *ex situ* conservation of *P. callosum* var. *sublaeve* has been interested, the natural substances incorporating into the culture medium was investigated in order to minimize the genetic variation. Secondly, an efficient method for *in vitro* propagation of a number of this orchid species has been created. For the purposes of propagation and preservation of *P. callosum* var. *sublaeve,* it is substantial to prove a competent system for *in vitro* propagation via thin cell layers (TCLs) technique. To sum up, this study was designed to investigate the capacity to induce plantlets via seed culture and protocorm-like bodies (PLBs) derived from shoot tip TCL explants. In addition, the obtained protocorms, PLBs and subsequent plantlets were histologically observed and their genetic variations by molecular technique were also evaluated. Finally, since slipper orchids are difficult to identify without their flowers, the original methods of classification namely; the morphological and physiological systems, should be supported by molecular technique (Trung et al., 2013). Molecular data are likely to play a very important role in determining the genetic relationship among plants and providing the novel genetic classification that often coincides with primary taxonomy (Jobst et al., 1998). Therefore, the relationships in section *Barbata,* namely *P. barbatum, P. callosum* var. *sublaeve, P. callosum* var. *callosum* and *P. callosum* var. *potentianum* were investigated by using morphological characteristics and RAPD marker analysis.

1.2 Review of Literature

Orchidaceae is the one of the largest families of angiosperms composing of about 779 genera (Cribb, 1998). The Cypripedioideae is a subfamily of the Orchidaceae having various names, such as the lady's slipper orchid, the lady slippers or the slipper orchids (Atwood, 1984). They have unique characters which easy to recognize, such as a synsepal, a saccate labellum, a conspicuous staminode and two fertile stamens (Atwood, 1984). The growth patterns of the slipper orchids were

divided into two types; (1) some species have thin, plicate leaves distributed on elongate stems (*Cypripedium and Selenipedium*), and (2) some have thick, coriaceous leaves forming basal, distichous rosettes (*Phragmipedium* and *Paphiopedilum*) (Atwood, 1984). *Paphiopedilum* were conducted into the lowest 60 species and the supremacy at 80 species (Atwood, 1984).

1.2.1 The genus *Paphiopedilum*

This genus was divided into three subgenera, *Pavisepalum*, *Brachypetalum* and *Paphiopedilum*. The subgenus *Paphiopedilum* was separated into five sections, *Coryopedilum, Pardalopetalum, Cochlopetalum*, *Paphiopedilum* and *Barbata* (Cribb, 1998). The latter section was comprised of 27 species distinguished by their usually one-flowered inflorescences and tessellated leaves. Moreover, their lips have protuberant incurved side-lobes, usually warted and their petals are spotted or warted in most of species (Cribb, 1998). The relationships within section *Barbata* are distinct and the species form a very natural group (Cribb, 1998). The section *Barbata* has large genomes having widely ranged and highly chromosome number (2n=28-42) (Cox et al., 1998). This reason gives rise to the raise recombination affected their genetic variation, centric fission and fusion. In addition, the slipper orchids in this section were rapidly adapted to environment (forest floors) (Atwood, 1984; Cox et al., 1998).

The lists of species in section *Barbata* (Cribb, 1998).

-*Paphiopedilum acmodontum*

-*Paphiopedilum appletonianum*

-*Paphiopedilum argus*

-*Paphiopedilum barbatum*

-*Paphiopedilum bougainvilleanum*

-*Paphiopedilum bullenianum*

-*Paphiopedilum callosum*

-*Paphiopedilum ciliolare*

-*Paphiopedilum dayanum*

-*Paphiopedilum fowliei*

-*Paphiopedilum hennisianum*

-*Paphiopedilum hookerae*

-*Paphiopedilum javanicum*

-*Paphiopedilum lawrenceanum*

-*Paphiopedilum mastersianum*

-*Paphiopedilum papuanum*

-*Paphiopedilum purpuratum*

-*Paphiopedilum sangii*

-*Paphiopedilum schoseri*

-*Paphiopedilum sukhakulii*

-*Paphiopedilum superbiens*

-*Paphiopedilum tonsum*

-*Paphiopedilum urbanianum*

-*Paphiopedilum venustum*

-*Paphiopedilum violascens*

-*Paphiopedilum wardii*

-*Paphiopedilum wentworthianum*

A. Scientific classification of *P. callosum* var. *sublaeve*

Kingdom: Plantae

Division: Magnoliophyta

Class: Liliopsida

Order: Asparagales

Family: Orchidaceae

Subfamily: Cypripedioideae

Genus: *Paphiopedilum*

Subgenus: *Paphiopedilum*

Section: *Barbata*

Species: *Paphiopedilum callosum*

(Cribb, 1998)

B. Characteristics of *P. callosum*

P. callosum is a native plant of Southeast Asia where they appear in extensively dispersed localities in Thailand, Cambodia, Laos and Vietnam (Cribb, 1998). They are terrestrial herbs having 3-5 narrow elliptic leaves (oblong-elliptic or obovate) and acute apex is tridenticulate. The leaves are 10-20 cm long and 3.2-4.8 cm wide, ciliate at base, tessellated pale and dark above, sometimes purple at base on lower surface.

This species has 1- (or rarely 2-) flowers having purple and 12-25 cm long of peduncle. The bract is ovate to elliptic, acute to subacute, 1.5-2 cm wide, 1.5-2.8 cm long, green color, sometimes marked with purple ciliate. The flower is huge, 8-11 cm across. The sepals are white flushed with purple in lower half, veined with purple and green. The petals are white to yellow-green with a purple apical third, spotted with maroon on upper margin and sometimes in basal half. The lip is green and heavily flushed deep maroon. The length of lip is 2.5-4.4 cm, the width is 2-2.5 cm. They have warts on incurved side-lobes. The staminode is lunate, the length is 11 mm, the width is 7 mm, and has apical lateral teeth falcate, acute to obuse. The staminode is pale green with darker veins, flushed purple. The length of pedicel and ovary are 3-6.5 cm which is green, flushed with purple, purple-pubescent. The dorsal sepal is broadly ovate to subcircular, apiculate and the length is 4-5.5 cm, the width is 4.2-6 cm with recurved ciliate margins. The synsepal is concave, elliptic or lanceolate, acute, the length is 2.7-3.2 cm, and the width is 1.6-2.5 cm. The petals are sometimes reflexed, subsigmoid, ligulate, obtuse or round at apex. The length is 4.6-6.8 cm, the width is 1.2-1.8 cm, and has maroon ciliate. The chromosome number has been found as 2n=32 (Cribb, 1998).

This species is nearly allied to *P. barbatum* which is native to Malaysia. However, some characters of them, such as size of dorsal sepal, size of petal are different (Cribb, 1998). The species of *P. callosum* comprises of 3 varieties

namely, *P. callosum* var. *callosum* (Figure 1), *P. callosum* var. *sublaeve* (Figure 2), and *P. callosum* var. *potentianum* (Figure 3) (Pedersen et al., 2011).

There are few differences of characteristics among varieties of *P. callosum* species. *Paphiopedilum callosum* var. *callosum* is a typical variety which has petals with warts on the upper margin. The width of dorsal sepals is 4.2-6 cm. The petal has more than 3.7 times as long as wide, and subsigmoid. They distribute in Thailand (Northern; Chaing Mai, Tak, Notherneastern; Loei, Southeastern; Trat), Cambodia and Laos (Pedersen et al., 2011).

Figure 1. Flower of *Paphiopedilum callosum* var. *callosum*.
source: Cribb (1998).

Meanwhile, *P. callosum* var. *sublaeve* also has the petals with warts on the upper margin, but the width of dorsal sepals is only 3-4.1 cm. The petal has more than 3.4 times as long as wide, and hardly subsigmoid. The distribution is in Thailand (Peninsular; Ranong, Phangnga, Krabi and Songkhla), Northwest Peninsular Malaysia (Pedersen et al., 2011).

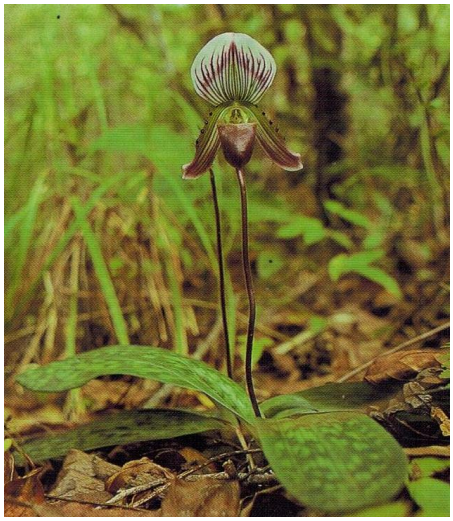

Figure 2. Plant of *Paphiopedilum callosum* var. *sublaeve*.
source: Cribb (1998).

In addition, *P. callosum* var. *potentianum* has petals without warts on the upper margin and the size of dorsal sepal is about 4 cm wide, 2.6 cm long which is narrower than var. *callosum* and var. *sublaeve* (Pedersen et al., 2011). The distribution is endemic and unknown of occurrence in Thailand (Pedersen et al., 2011).

Figure 3. Flower of *Paphiopedilum callosum* var. *potentianum*.
source: Cribb (1998).

1.2.2 Seed germination and protocorm induction

The specific characteristic of orchid seed is the lack of storage organs desired for germination and seedling development (Pant, 2013). Vellupilla et al. (1997) reported that the tropical epiphytic seed orchids divided the cell at the chalazal end of the globular embryo during germination after one week on culture medium. Moreover, the reserved protein was consumed within six days of germination.

Factors affecting *in vitro* seed germination of *Paphiopedilum* were reported, for instance, seed maturity and seed pretreatment (Tay et al., 1988; Lee, 2007; Long, 2010), medium types (Stimart and Ascher, 1981; Pierik et al., 1988; Long et al., 2010), organic nutrient additives (Pierik et al., 1988; Raknim, 2007; Long et al., 2010), plant growth regulators (Wattanawikkit et al., 2011) and light culture conditions (Stimart and Ascher, 1981; Tay et al., 1988; Pierik et al., 1988).

Many types of media have been used for the axenic germination of both terrestrial and epiphytic orchids. However, none of these media is universal. The commonly nutrient media for orchid seeds culture were proposed many reports. Zhang et al. (2015) proposed that the basal medium for seed germination and protocorm development of *Paphiopedilum armeniacum* S.C. Chen et F. Y. Liu was the fraction one eight-strength Murashige and Skoog (1/8MS). Prasongsom et al. (2016) reported that the modified Vacin and Went (MVW) liquid medium provided the highest PLBs number and survival rate of *Rhynchostylis gigantean* (Lindl.) Ridl. Moreover, Abraham et al. (2012) presented that MS medium assisted the maximum seed germination and this medium supplemented with 3 mg/L BA and 0.5 mg/L NAA also gave the highest seedling growth of *Coelogyne nervosa* A. Rich.

1.2.3 Thin Cell Layer (TCL) technique, micropropagation for *ex situ* conservation and variation analysis by RAPD

During the last 50 years, the tissue culture technology has been widely utilized for many purposes; for instance, the rapid and large scale propagation of orchids and their *ex situ* conservation (Behera et al., 2012). Different protocols have been developed through *in vitro* culture of various parts including shoot tip (Huang et al., 2001), leaf (Chen et al., 2004), inflorescence axis and flower bud (Chugh et al.,

2009), rhizome (Sheelavantmath et al., 2000), root (Chugh et al., 2009) and micropropagation via TCL technique (Rout et al., 2006).

TCL system consists of explants of a small size from different plant organs excised either longitudinally (lTCL, containing one tissue type) or transversely (tTCL, containing small number of cell from different tissue types) (Vyas et al., 2010). The advantage of the tTCL system in orchid is to produce a high frequency of shoot regeneration and to reduce the time interval required. The single tTCL potentially produced more than 80,000 plantlets in a year, while the conventional shoot tip method produced only 11,000 plantlets. (Teixeira da Silva et al., 2007). This technique has been successfully employed in the secondary PLB induction from the primary PLBs of *Cymbidium* Sleeping Nymph (Vyas et al., 2010), PLB induction from shoot tip of *Aranda deborah* (Lakshmanan et al., 1995) and PLB from thin leaf section of *Doritaenopsis* hybrid (Park et al., 2002), PLBs from stem segments of *Dendrobium draconis* Rchb. F. (Rangsayatorn, 2009) and *D. Candidum* Wall Ex Lindl. Zhao et al. (2007) revealed that adventitious buds could be induced from tTCL of stem segment of *Dendrobium candidum* Wall Ex Linld. There are many studies of thin cell layer technology, such as trimming pattern (Jala and Balla, 2011) additional of plant growth regulators (Nayak et al., 2002). In addition, Rout et al. (2006) confirmed that the efficiency of the TCL is very high, when compared to the conventional technique of tissue culture (Chugh et al., 2009). This method could not induce the genetic variation of *in vitro* plantlets of hybrid *Cymbidium* Twilight Moon 'Day Light' received from secondary PLB induction (Teixeira da Silva et al., 2006).

Molecular analysis has played an essential role in determining the genetic relationship among many plants (Jobst et al., 1998). RAPD analysis is the one of molecular marker that is a fingerprinting method using short, random, oligonucleotide primers to search for variation in the entire genomic DNA (Williums et al., 1990; Khosravi et al., 2009). The RAPD analysis is usually utilized in many orchid researches, such as *Phalaenopsis* (Epidendroideae: Orchidaceae) (Niknejad et al., 2009), *Cypripedium macranthos* var. *rebunense* (Jo et al., 2005), *Paphiopedilum* and *Phragmepedium* (Chung et al., 2006). The major advantage of RAPD assay is that there is no requirement for DNA sequence information. The process of RAPD assay is also rapid and simple to operate without using the fluorescence of

radioactivity (Williums et al., 1990; Tingey and del Tufo, 1998). Moreover, RAPD is a powerful tool to estimate the range of genetic variability and it is useful to evolve conservation strategies of particular species (Khasim and Ramesh, 2010).

1.2.4 Organic additives and genetic variation

A large number of complicated additives; for example, coconut water (CW), banana pulp, peptone, tomato juice, slap honey and beef extract can be very effective in supporting undefined mixture of organic nutrients and growth factors. However, some organic additives inhibited growth of PLBs and seedlings (Arditti, 1967; Akter et al., 2008). Coconut water was the most often utilized in the orchid protocorm production. Piria et al. (2008) reported that CW induced cell division in non-dividing cells and promoted morphogenesis and mass multiplication of protocorms in orchids. CW was also found to be the best organic additive for the proliferation of *Dendrobium* Alya Pink PLBs (Nambiar et al., 2012). It can easily be supplemented in orchid media without loss of activity from autoclaving or by exposure to ordinary laboratory conditions for limited periods of time (Chugh et al., 2009). Banana pulp was a rich source of natural cytokinins which inhibited culture initiation but promoted differentiation and growth of shoots at later stages (Chugh et al., 2009).

These organic additives helped in producing more PLBs, shoots and leaves of *Dendrobium* orchid (Akter et al., 2007), increased the size of date palm (*Phoenix dactylifera* L.) cv. Khanezi somatic embryos (Al-Khateeb, 2008), and also promoted growth and development of asymbiotic seeds and regeneration of *Cymbidium findlaysonianum* Lindl. plantlets (Tawaro et al., 2008). Al-Khateeb (2008) revealed that the organic additives having sugar and other nutrients (proteins, lipids and minerals) could be added into the medium.

Furthermore, inducible defense responses are triggered by following recognition of a range of chemical factors termed ‘elicitors’, such as jasmonic acid (JA), salicylic acid (SA), hyaluronic acid (HA) and chitosan. Elicitors could be used as enhancers of plant-secondary-metabolite synthesis and could play an important role in biosynthetic pathways (Angelova et al., 2006).

Chitosan is described as a family of linear polysaccharides consisting of varying amounts of β-(1,4) linked residues of *N*-acetyl-2 amino-2-deoxy-D-glucose (denoted in this review as A residues) and 2-amino-2-deoxy-D glucose residues (denoted in this review as D residues) (Figure 4) (Aranaz et al., 2009). In agriculture, there is a worldwide trend to use chitosan as an alternative compound because of its fungicidal effects and elicitation of defense mechanisms in many plant tissues (Terry and Joyce, 2004; Uthairatanakij et al., 2007). Moreover, chitosan is reported to activate the synthesis and accumulation of a series of PR-proteins and defense-related proteins among phenylalanine ammonia-lyase (PAL) and peroxidase (PRX) (Hadrami et al., 2010). The chitosan is biodegradable and has low potential toxicity. This is caused of the USA Environmental Protection Agency (EPA) inferred that chitosan is safe to people, pets, wildlife, and the environment (Uthairatanakit et al., 2007). It was also found to be non-toxic when fed to mice, rats, and rabbits (Uthairatanakij et al., 2007). Moreover, chitosan has recently been reported to act as a plant growth promoter in some plant species including orchids (Uthairatanakij et al., 2007).

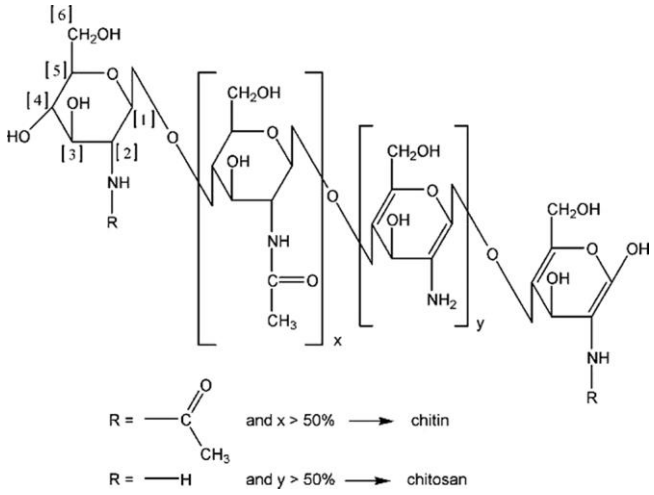

Figure 4. Structure of chitin and chitosan (Zargar et al., 2015).

Chitosan has been shown to enhance plant growth in *Dendrobium* without increasing the detectable levels of somaclonal variation (Pornpienpakdee et al., 2010). The effect of chitosan on the morphological characteristics of the adaxial leaf side showed that all chitosan treatments caused a lower stomata number, but a larger stomata size compared to the control (Obsuwan et al., 2010). Chitosan

significantly enhanced growth factors in terms of the average values of flower-stem length, the number of growing leaves, including leaf width and length, as well as the number of flowers per bush (Uthairatanakit et al., 2007). Furthermore, the chitosan was proposed to enhance the growth and the number of PLBs of *Paphiopedilum sanderianum* (Rchb. f.) (Uthairatanakit et al., 2007).

It can also enhance the growth and development of *Phalaenopsis cornu-cervi* (Breda) Blume and Rchb.f. (Prasertsongskun and Chaipakdee, 2011), and supported seed germination and protocorm development of *Dendrobium* orchid (Kananont et al., 2010). Moreover, chitosan is usually used with other elicitors, such as jasmonic acid derivatives caused positive effects on shoot formation from PLB cultures of *Cymbidium insigne* (Shimasaki and Wang, 2009) and *Cymbidium kanran* (Shimasaki et al., 2009). Jin et al. (1999) confirmed that chitosan was the best biotic elicitor increasing the total production of anthraquinone colorants in Madder (*Rubia akane* Nakai). In addition, the supplementing of chitosan (15 mg/L) into 1/2 MS liquid medium promoted PLB growth of *Grammatophyllum speciosum* Blume with no changes in ploidy level of plantlets when tested by flow cytometry (Sopalun et al., 2010).

Hyaluronic acid (hyaluronan, HA) comprised of linear, unbranching, polyanionic disaccharide units consisting of glucuronic acid (GlcUA) an N-acetyl glucosamine (Glc-NAc). These linears were joined alternately by beta 1–3 and beta 1–4 glycosidic bonds (Figure 5) (Park et al., 2007). The molecular mass of HA varies between $2x10^5$ and $10xl0^6$ Da. The physiological properties are hardily influenced due to its polymeric and polyelectrolyte character. Moreover, the viscous nature of its solutions is also fine (Kogan et al., 2008) serving important biological functions in bacteria and higher animals including human. Nahar et al. (2011) reported that HA can be used as a plant growth regulator for orchid production or tissue culture. It increases the formation of PLBs, shoots and roots with very short duration of time in *Cymbidium*. Moreover, Kaewjampa et al. (2012) confirmed that HA has the ability to stimulate PLB proliferation of *Cymbidium in vitro* within short period.

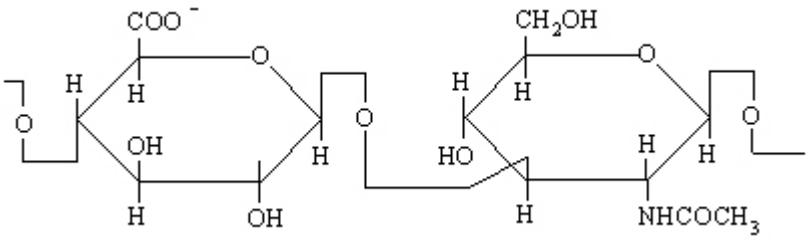

Figure 5. Structure of hyaluronic acid (HA) (Necas et al., 2008).

1.2.5 Leaf anatomical traits

As in previous era of the 19th century, the taxonomists were limited using the features of reproductive organs, this caused the floral characters were considered to be the most valuable characters to taxonomic relationships (Ogie-Odia et al., 2010). Thus entire non-reproductive organ, the leaf is the most broadly used in plant taxonomy (Ogie-Odia et al, 2010). Moreover, the leaf epidermis is the second most important character after cytology for solving taxonomic problems (Ogie-Odia et al., 2010). The use of leaf epidermal features in systematic has become popular and distinctive and has been used as a great tool to compare/determine the relationship with the epidermal features/characteristics among the taxa studies (Ogie-Odia et al, 2010)

The application of leaf anatomical traits in orchid studies were shown in the works of Mulgaonkar (2005) observing and comparison features of leaf epidermis in the stomata and their relation among six terricolous taxa, *Habernarin grandifloriformis* Blatt. and McCann., *H. rariflora* A. Rich., H. *gibsonii* Hook. F., *H. heyneana* Lindl., *Pecteilis susannae* (L.) Rafin., and *Spathoglottis plicata* Blume. Moreover, Guan et al. (2011) examined the leaf anatomical structure of *Paphiopedilum bellatulum, P. armenniacum, P. dianthum, Cypripedium flavum,* C. *lichiangense,* and *C. yunnanense*. It was found that *Paphiopedilum* have the leaf anatomical structure exhibited many xeromorphic feature related to demoting water loss and enhancing water-use capability. These conduce to growth and survival in karst habitats.

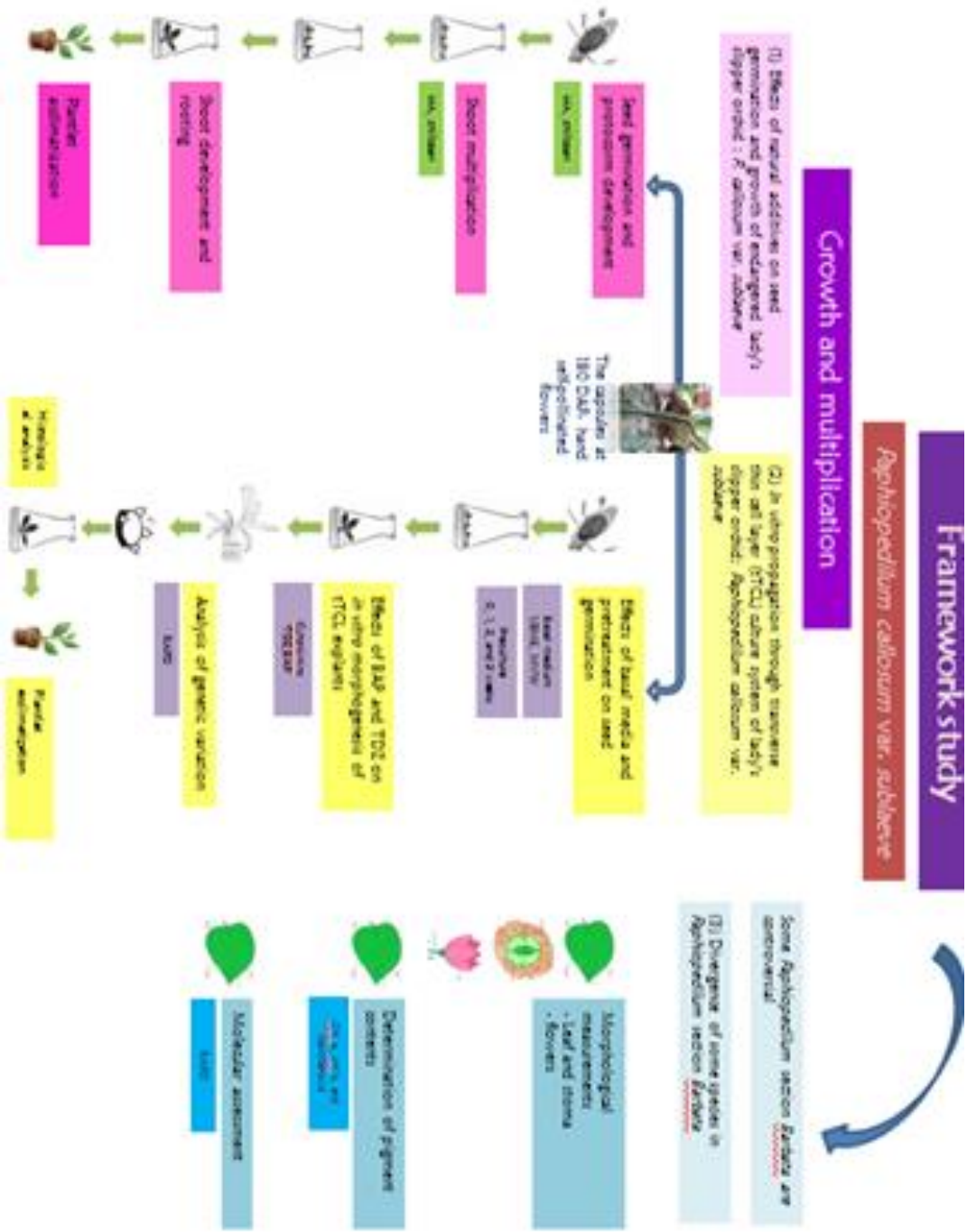

Figure 6. Study frame work

1.3 Objectives

1. To investigate the effects of chitosan and hyaluronic acid on seed development of *P. callosum* var. *sublaeve*.

2. To investigate the effect of cytokinins (TDZ and BAP) on PLB formation through culturing of thin cell layer explants.

3. To examine the genetic variations of *P. callosum* var. *sublaeve* between regenerated plants from tTCL technique and their mother plants by RAPD analysis.

4. To examine the relationships among *P. barbatum*, *P. callosum* var. *sublaeve*, *P. callosum* var. *callosum* and *P. callosum* var. *potentianum* by morphological characteristics and RAPD marker analysis.

CHAPTER 2

RESEARCH METHODOLOGY

2.1 Plant material

P. callosum var. *sublaeve* were maintained in the greenhouse of Department of Biology, Faculty of Science, Prince of Songkla University, Thailand. The capsules at 180 days after pollination (DAP) were collected from hand self-pollinated flowers. The seed viability was evaluated by 1% 2,3,5-Triphenyltetrazolium chloride (TTC) according to the method described by Vellupillai (1997). The viable seeds exhibiting dark red color were counted. Seed viability (SV) was reported as a percentage which was calculated as follows:

$$\% \text{ SV} = \frac{[\text{Red embryos}/(\text{numbers of red embryos + colorless embryos})] \times 100}{\text{Total number of embryos}}$$

2.2 Natural additives affecting on seed germination and protocorm development

2.2.1 Seed preculture

The capsules were surface-sterilized with 1.2% (v/v) sodium hypochlorite (NaOCl) containing 1-2 drops of Tween-20 for 20 min and rinse 2-3 times with sterile distilled water (DW). They were cut longitudinally and the seeds were scooped out and placed into a 125 ml Erlenmeyer flask containing 40 ml of sterile DW. For preculture, the seed suspension (5 ml) was pipetted into a 125 ml Erlenmeyer flask containing 20 ml of sterile DW supplemented with either HA (0, 0.01, 0.1, and 1.0 mg/L) or chitosan (0, 1, 5, and 10 mg/L). These cultures were maintained on a shaker at 50 rpm in the culture room (25±2^{o}C) under the dark condition for 2 weeks.

2.3 Seed germination and protocorm development

After preculture for 2 weeks, 1-ml seed suspension (approximately 294 seeds/ml) was placed into each 125 ml Erlenmeyer flask containing 20 ml of modified Vacin and Went (MVW) medium (Vacin and Went, 1949) supplemented with either HA (0, 0.01, 0.1, and 1.0 mg/L) or chitosan (0, 1, 5, and 10 mg/L). Each treatment was conducted with 15 replicates (culture flask). Visual observation was observed at 15-day intervals. The subculture was carried out in the same fresh medium every 2 months. The percentages of seed germination including germination index and protocorm formation were examined after 2 months of culture.

The seed germination percentage and the germination index were calculated by formula proposed in Pierik et al. (1988) as follows:

$$\% \text{ Seed germination} = \frac{[(B+C+D)]}{(A+B+C+D)} \times 100$$

$$\text{Germination index} = \frac{[10(1B+2C+3D)]}{(A+B+C+D)}$$

Where A is seeds containing an embryo but not germination (swollen), swollen seeds.

B is germinating, but not yet rupturing the seed coat.

C is seeds with embryos just rupturing the seed coat.

D is seeds with embryos completely out of the seed coat.

The germination parameters, for instance, germination percentage and germination index are measured. When germination does not occur at all, the germination index is 0. When all seeds germinate, they will reach the class value of 3 in which the germination rate of seed germination is 100% and the germination index is 30 (Pierik et al., 1988)

The percentage of seed/protocorms formation at each developmental stage was calculated by the number of seeds/protocorm in each stage dividing by the total number of cultured seeds in each flask.

2.4 Shoot multiplication

Three-month-old seedlings (2-3 cm in height) were used as materials for shoot multiplication. They were cultured on modified Murashige and Skoog (MMS) medium (Kaewubon et al., 2010) supplemented with 2 g/L peptone, 2 g/L activated charcoal and various concentrations of HA (0, 0.01, 0.1 and 1 mg/L) or chitosan (0, 1, 5 and 10 mg/L) for 2 months. All experiments consisted 9 culture flasks and each flask contained 1 seedling. Number of shoots and multiplication rate were examined after 2 months of culture.

2.5 Shoot development and rooting

Single shoot, 3-4 cm high derived from shoot multiplication was transferred to MMS medium (Kaewubon et al., 2010) for 2 months to induce root growth. These plantlets were then transferred to pots containing sphagnum moss and maintained in the shaded greenhouse. The survival rate of these plantlets was recorded after transplanting for one month.

2.6 Effects of basal media and pretreatment duration on seed germination

After sterilization of seeds, they were suspended in approximately 125 seeds/ml in a 125-ml culture flask containing 20 ml of DW for various preculture periods (0, 1, 2 or 3 weeks). The cultures were maintained on a shaker (50 rpm) in the culture room (25±2^{o}C) under the dark condition before being transferred to different basal media [1/2Murashige and Skoog (MS; Murashige and Skoog, 1962) or modified Vacin and Went (MVW; Vacin and Went, 1949) media]. All media were supplemented with 10 g/L sucrose, 1 mg/L chitosan, 1.0 g/L activated charcoal (AC) and solidified with 6.8 g/L agar in which all concentrations were optimized in initial trials. The media were adjusted to pH 5.2 and all cultures were incubated under a 16-h photoperiod at irradiance of 23 µmol/m^2/s provided by cool white fluorescent tubes (Philips). Only if the appearance of a swollen embryo with the ruptured testa would

be considered to have successfully germination. The experiment was performed with 3 replicates and repeated twice.

2.7 Effects of BAP and TDZ on *in vitro* morphogenesis from tTCL of shoot explant

Aseptic shoot (1-1.5 cm height) of 4-month-old seedling was cut transversely into 2 tTCL explants each with a thickness of 0.5-0.6 mm. These tTCL explants (2 slices/bottle) were inoculated on 10 ml of MVW medium supplemented with various concentrations of 6-Benzylaminopurine (BAP) (0, 1.0, 5.0 and 10.0 mg/L) and thidiazuron (TDZ) (0, 0.1, 0.5 and 1.0 mg/L) alone or in combination with 15 replications (bottle). All media containing 0.2% AC were solidified with a combination of 5.5 g/L agar and 1 g/L Phytagel. After culture for 2 weeks, all explants were transferred to AC-free MVW medium to promote morphogenesis of several organs. The percentage of browning explant, shoot and root formation and PLB formation were recorded after culture for 8 weeks.

2.8 Plantlet acclimatization

Regenerated shoots were transferred to MMS medium for plantlet growth (Kaewubon et al., 2010). The plantlets with 3-4 roots were then transplanted into a 3-inch pot containing sphagnum moss in a greenhouse and the survival rate was recorded at one month after transplanting.

2.9 Histological analysis

The seeds and *in vitro* morphogenesis responses were histologically confirmed. After culture for 8 weeks, PLBs, shoots, and roots derived from thin sections were collected and fixed in FAAII [formaldehyde: glacial acetic acid: 70% ethyl alcohol; 1:1:18 v/v/v] for 48 h. Fixed tissues were dehydrated in a tertiary-butyl-alcohol series, embedded in Histoplast PE and thin sectioned (6 µm thick) by a rotary microtome. Sections were stained with Delafield's hematoxylin and Safranin to

examine the general structure (Ruzin, 1999). Seed samples were stained with toluidine blue O (TBO) to detect lignin and some phenols (Feder and O'Brian, 1968).

2.10 Culture conditions

The pH of the media was adjusted to 5.2±0.1 with 1N NaOH or HCl prior to autoclaving at 121^{o}C for 20 min. All treatments/experiments were cultured at 25±2^{o}C under a 16-h photoperiod at irradiance of 23 µmol/m^{2}/s provided by cool white fluorescent tubes (Philips).

2.11 Statistical analysis

Each experiment was arranged in a completely randomized design (CRD). Fifteen replicates (for seed germination) and 9 replicates (for shoot multiplication) were performed for each treatment. Data were analyzed using SPSS 17.0 program for Windows (SPSS Inc. Chicago, IL, USA). Data of percentage of seedling in each developmental stage, percentage of seed germination, seed germination index, number of shoots and multiple shoot formation rates were subjected to analysis of variance (ANOVA) with mean separation ($P \leq 0.05$) by Duncan's multiple range tests. Data of percentage of protocorm formation were analyzed by using Kruskal-Wallis test.

For experiment of effects of basal media and pretreatment duration on seed germination, data were subjected to a two-way analysis of variance (ANOVA) followed by separating of mean using the Duncan's multiple range tests (DMRT) at $P<0.05$. The percentage of germinated seed was determined after culture for 30 days.

2.12 Divergence of some species in *Paphiopedilum* section *Barbata*

2.12.1 Plant materials collection

Five groups of *Paphiopedilum* plant samples (Table 1) in the section *Barbata* (*P. callosum* var. *callosum*, *P. callosum* var. *sublaeve*, *P. callosum* var. *potentianum* and *P. barbatum*) were collected from various sources based on their habitats. Some

plant parts were obtained from Mr. Teerapun Tothirakul at Management of Plant Research and Development of Doi Tung Development Project, Ms. Nopparat Thawinwathin at The Agricultural Development and Extension Center, Trang, and Mr. Suchart Wundee. These plants were acclimatized in greenhouse at the Department of Biology, Faculty of Science, Prince of Songkla University for 12 months before the beginning of experiment. The morphological data were measured based on Atwood (1984).

Table 1. Sources of plant samples.

Sample plants	A[1]	B[2]	C[3]	D[2]
Sources	Border of Malaysia	Border of Cambodia	Rattaphum district, Songkhla province	Border of Cambodia

Plant samples were received from Mr. Teerapun Tothirakul[1], Mr. Suchart Wundee[2] and Ms. Nopparat Thawinwathin[3].

A = Sample plants were collected from border of Malaysia

B = Sample plants were collected from border of Cambodia

C = Sample plants were collected from Rattaphum district, Songkhla province

D = Sample plants were collected from border of Cambodia

2.13 Morphological measurements and physiological assessment

2.13.1 Leaf and guard cell

The 2nd fully expanded leaf, not counting the small new leaf (< 5 cm long), was collected as sample for plant analysis since the 1st mature leaf is generally appeared at the 3rd-5th leaf from the top of the plant.

Lower epidermal of leaf samples was peeled and fixed in FAAII (formaldehyde: glacial acetic acid: 70% ethyl alcohol; 1:1:18 v/v/v) for 48 h, dehydrated in graded series of ethanol-TBA, embedded in paraffin wax, cut (6 µm thickness) by a rotary microtome (Shandon AS 325). The sections of leaf samples and

epidermal peels were double stained with Safranin and Fast green and Fast green staining, respectively (Johansen, 1940). Photographs were taken under a light microscope (Olympus, BX 51) and an in-built digital camera (Olympus, DP 71).

Digital images were processed using Digimizer version 4.6.1 image analysis software (MedCalc Software). The total mesophyll (TM), length of adaxial (Lad), width of adaxial (Wad), length of abaxial (Lab), width of abaxial (Wab), cuticle thickness of adaxial (CuTad), cuticle thickness of abaxial (CuTab), guard cell length (GL), and guard cell width (GW) were measured. The guard cell area (GA) was calculated according to James and Bell (2001). The stomatal density and the guard cell index (GI) of both adaxial and abaxial surfaces were calculated using the following formula with modification (Thakur and Patil, 2011).

$$GI = \frac{G}{E + G} \times 100$$

$$GA = 1/4 \times \pi \times SL \times SW$$

Where:

G = number of the guard cell per unit area

E = number of epidermal cells per same unit area

GL = guard cell length

GW = guard cell width

2.13.2 Flowers

The morphological data of flowers including size of dorsal sepal, petal, lip, staminode, synsepal and ovary was examined among A, B, C and D. Three fully developed flowers per species/variety were used as samples. These samples were photographed and processed using Digimizer version 4.6.1 image analysis software (MedCalc Software).

2.14 Determination of pigment contents

The second fully expanded leaves (n=3) were assembled as samples to determine the pigment contents. The leaf samples (0.1 g fresh weight) were extracted according to method of Mistra et al. (2010). These samples were pulverized in 4 ml of 80% (v/v) acetone by a clean mortar. The homogenized tissue was centrifuged at 10,000xg for 4 min to get the supernatant. The centrifugation was repeated three times in a series of 80% (v/v) acetone at 3, 2 and 1 ml, respectively. The final supernatant (10 ml in volume) was determined by measuring the absorbance at 480, 510, 645 and 663 nm. Carotenoid and total chlorophyll contents (chl a and chl b) were calculated in mg/g fresh weight using the following formulae (Misra et al., 2010).

$$\text{Total chlorophyll} = \frac{[20.2(A_{645}) + 8.02(A_{663})]\times V}{1000\times W}$$

$$\text{Chlorophyll a} = \frac{[12.7(A_{663}) + 2.63(A_{645})]\times V}{1000\times W}$$

$$\text{Chlorophyll b} = \frac{[22.9(A_{645}) - 4.68(A_{663})]\times V}{1000\times W}$$

$$\text{Carotenoid} = \frac{[7.6(A_{480}) - 2.63(A_{510})]\times V}{1000\times W}$$

Where:

A = optical density reading of the chlorophyll extract at specific indicated wavelength

V = final volume of the 80% acetone-chlorophyll extracted

W = fresh weight in grams of the tissue extracted

2.15 Analysis of genetic variation

2.15.1 Plant materials

A. Effects of BAP and TDZ on *in vitro* morphogenesis from tTCL of shoot explant

The mother (M) seedlings and their regenerated (R) plants were randomly collected to determine the genetic stability. Four-month-old seedlings which were cultured on 1/2MS medium containing 1 mg/L chitosan were used as mother plants. The R plants were collected from tTCL-derived plantlets which were cultured on MVW medium containing TDZ (0, 0.1, 0.5, and 1.0 mg/L) in combination with BAP (0, 1.0, 5.0 and 10.0 mg/L) for 12 weeks. The total genomic DNA was extracted from the young leaf samples (100 mg/sample) of M and R plants following the protocol described by Chung et al. (2006) with some adaptation. DNA concentration and purity were determined by spectrophotometer and the samples were diluted to a concentration of 20 ng/l.

B. Divergence of some species in *Paphiopedilum* section *Barbata* and DNA extraction

The second fully expanded leaf of each type of sample plants were used to be the samples for molecular analysis. *Paphiopedilum exul* and *Dendrobium crumenatum* orchids were used as the control groups.

The genomic DNA was isolated by modifying the method described by Chung et al. (2006). The quality and quantity of DNA were determined by spectrophotometer (Biodrop Duo Micro Volume Spectrophotometer).

2.15.2 RAPD amplification and electrophoresis

The amplification reaction was done in 24.5 µL which consisted of 2.5 µL 10x buffer (100 mM Tris-HCl, 500 mM KCl, 0.01% gelatin), 1 µL of template DNA, 2 µL dNTP mix (100 mM), 0.25 µL of 5 u/µL *Taq* DNA polymerase, 1.5 µL of

10 µM primer, and 17.25 µL deionized water. DNA amplification was carried out in a thermal cycler for an initial denaturation step of 3 min at 94^{o}C before beginning the cycling protocol. An amplification cycle consisted of 40 sec at 94^{o}C, 1 min at 37^{o}C and 1 min at 72^{o}C. A total of 40 cycles were performed. The cycling was terminated with a final extension at 72^{o}C for 10 min. After amplification, DNA fragments were separated by 1.5% agarose (molecular biology grade) gel electrophoresis at 100V in 0.5x Tris-acetate-EDTA (TAE) buffer (20 mM Tris, 10 mM acetate, 0.5 mM EDTA; pH8.0), stained with ethidium bromide (0.1µl/ml). The DNA bands were then photographed under ultraviolet light using a photo documentation system. A 100-base pair ladder was used to estimate the size of RAPD bands to nearest 50 base pairs.

Amplification product profiles were scored for the presence (1) or absence (0) of bands. Genetic similarity between pairs was calculated according to Jaccard's similarity coefficient, followed by cluster analysis by the SIMQUAL module (Anderson et al., 1993).

CHAPTER 3

EFFECTS OF NATURAL ADDITIVES ON ASYMBIOTIC SEED GERMINATION AND GROWTH OF ENDANGERED LADY'S SLIPPER ORCHID: *Paphiopedilum callosum* var. *sublaeve*

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3.1 Introduction

Many orchid populations are under extinction menace as a result of over-collection and habitat destruction (Zeng, 2012). *Paphiopedilum callosum* var. *sublaeve*, a lady's slipper orchid native to the southern Thailand, is protected under the appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES, 2013). This orchid species has sensationally beautiful flowers, marbled and evergreen foliage which make it delightful and distinctive (Ng and Saleh, 2011). *Paphiopedilum* orchid is generally propagated through the division of axillary bud of the mother plant which is a very time-consuming and its seed germination is also slow growth (Nhut et al., 2006; Long, 2010; Ng et al., 2010). Accordingly, technique of plant tissue culture is being used for large-scale plantlet production within a short period and has become an optimal solution for conservation of genus extinction (Nhut et al., 2006). Especially, the supplementation of natural substances to the medium can enhance *in vitro* seed germination, promote growth and prevent somaclonal variation (Pornpienpakdee et al., 2010). Chitosan and hyaluronic acid (HA) are polysaccharides which act as plant growth regulators that interest as safe materials for humans and the environment (Nge et al., 2006 and Nahar et al., 2012). These substances were added to germination media to improve seed germination and to retain somaclonal variation (Pornpienpakdee et al., 2010). Chitosan is a biodegradable polymer, which comprises of a copolymer of N-acetyl-D-glucosamine residues, linked by β-1,4 glycosidic bonds (Croisier and Jerome, 2013). The structure of chitosan relates to glycosaminoglycans (GAG) which is significant structural element of extracellular matrix of many tissues (Enrione et al., 2010). This chitosan was used to promote seed germination and protocorm development in many orchids such as *Dendrobium phalaenopsis* (Nge et al., 2006), *D. bigibbum* var. *compactum* and *D. formosum* (Kananont et al., 2010), *Grammatophyllum speciosum* (Sopalun et al., 2010) and *Cymbidium insigne* (Nahar et al., 2012). Hyaluronic acid (HA) is a natural polysaccharide which composed of alternating (1→4)-β linked D-glucoronic acid and (1→3)-β linked *N*-acetyl-D-glucosamine residues (Kogan et al, 2007). The application of HA in plant tissue culture has also been shown to enhance

the micropropagation in many orchid species, for instance, *Cymbidium dayanum* (Nahar et al., 2011), *C.* Waltz 'Idol' (Kaewjampa et al., 2012), and *Dendrobium kingianum* (Habiba et al., 2014). Therefore, present study aimed to examine the natural substances (HA and chitosan) affecting on seed germination, protocorm development and multiple shoot induction of *P. callosum* var. *sublaeve*.

3.2 Materials and methods

3.2.1 Plant material

P. callosum var. *sublaeve* (Figure 7A) were maintained in the greenhouse of Department of Biology, Faculty of Science, Prince of Songkla University, Thailand. The capsules at 180 days after pollination (DAP) were collected from hand self-pollinated flowers (Figure 7B). The seed viability was evaluated by 1% 2,3,5-Triphenyltetrazolium chloride (TTC) (Vellupillai, 1997). The viable seeds exhibiting dark red color (Figure 7C) were counted. Seed viability (SV) was reported as a percentage which was calculated as follows:

$$\% \text{ SV} = \frac{[\text{Red embryos}/(\text{numbers of red embryos + colorless embryos})]\times 100}{\text{Total number of embryos}}$$

3.2.2 Natural additives affecting on seed germination and protocorm development

3.2.2.1 Seed preculture

The capsules were surface-sterilized with 1.2% (v/v) sodium hypochlorite (NaOCl) containing 1-2 drops of Tween-20 for 20 min and rinse 2-3 times with sterile distilled water (DW). They were cut longitudinally and the seeds were scooped out and placed into a 125 ml Erlenmeyer flask containing 40 ml of sterile DW. For preculture, the seed suspension (5 ml) was pipetted into a 125 ml Erlenmeyer flask containing 20 ml of sterile DW supplemented with either HA (0, 0.01, 0.1, and 1.0 mg/L) or chitosan (0, 1, 5, and 10 mg/L). These cultures were maintained on a shaker at 50 rpm in the culture room (25±2^{o}C) under the dark condition for 2 weeks.

Figure 7. *Paphiopedilum callosum* var. *sublaeve* plant showing (A) single flower, (B) capsule (180 DAP) and (C) dark red embryo of viable seed stained with TTC.

3.2.1.2 Seed germination and protocorm development

After preculture for 2 weeks, 1-ml seed suspension (approximately 294 seeds/ml) was placed into each 125 ml Erlenmeyer flask containing 20 ml of modified Vacin and Went (MVW) medium (Vacin and Went, 1949) supplemented with either HA (0, 0.01, 0.1, and 1.0 mg/L) or chitosan (0, 1, 5, and 10 mg/L). Each treatment was conducted with 15 replicates (culture flask). Visual observation was observed at a 15-day interval. The subculture was carried out in the same fresh medium for every 2 months. The percentages of seed germination including germination index and protocorm formation were examined after 2 month of culture.

3.2.1.3 Evaluation of seed germination and growth

The seed germination percentage and the germination index were calculated by formulae proposed in Pierik et al. (1988) as follows:

$$\% \text{ Seed germination} = \frac{[100(B+C+D)]}{(A+B+C+D)}$$

$$\text{Germination index} = \frac{[10(1B+2C+3D)]}{(A+B+C+D)}$$

Where A is seeds containing an embryo but not germination (swollen), swollen seeds.

B is germinating, but not yet rupturing the seed coat.

C is seeds with embryos just rupturing the seed coat.

D is seeds with embryos completely out of the seed coat.

The percentage of seed/protocorms formation at each developmental stage was calculated by dividing the number of seed/protocorms in each stage by the total number of cultured seeds in each flask.

3.2.3 Shoot multiplication

Three-month-old seedlings (2-3 cm in height) were used as materials for shoot multiplication. They were cultured on modified Murashige and Skoog (MMS) medium (Kaewubon et al., 2010) added with 2 g/L peptone, 2 g/L activated charcoal and various concentrations of HA (0, 0.01, 0.1 and 1 mg/L) or chitosan (0, 1, 5 and 10 mg/L) for 2 months. All experiments consisted 9 culture flasks and each flask contained 1 seedling. Number of shoots and multiplication rate were examined after 2 months of culture.

3.2.4 Shoot development and rooting

Single shoot (3-4 cm. height derived from shoot multiplication) was transferred to MMS medium (Kaewubon et al., 2010) for 2 months to induce root growth. These plantlets were then transferred to pots containing sphagnum moss and maintained in the shaded greenhouse. The survival rate of these plantlets was recorded after transplanting for one month.

3.2.5 Culture conditions

The pH of the media was adjusted to 5.2±0.1 with 1N NaOH or HCl prior to autoclaving at 121^{o}C for 20 min. All treatments/experiments cultures were cultured at 25±2^{o}C under a 16-h photoperiod at irradiance of 23 µmol/m^{2}s provided by cool white fluorescent tubes (Philips).

3.2.6 Statistical analysis

Each experiment was arranged in a completely randomized design (CRD). Fifteen replicates (for seed germination) and 9 replicates (for shoot multiplication) were performed for each treatment. Data were analyzed using SPSS 17.0 program for Windows (SPSS Inc. Chicago, IL, USA). Data of percentage of seedling in each developmental stage, percentage of seed germination, seed germination index, number of shoots and multiple shoot formation rates were subjected to analysis of variance (ANOVA) with mean separation ($P \leq 0.05$) by Duncan's multiple range tests. Data of percentage of protocorm formation were analyzed by using Kruskal-Wallis test.

3.3 Results and discussion

3.3.1 Natural additives affecting on seed germination and protocorm formation

The viable seeds with dark red color due to the formation of red formazan were presented at 27.19%. Process of seed germination and protocorm development is presented in Table 2 and Figure 8. Seeds started to germinate after culture for 60 days. The highest seed germination percentage (8.34±1.01), germination index (0.16±0.02) and protocorm formation percentage (4.28±0.96) were observed on MVW medium added with 0.1 mg/L HA (Table 2, Figure 8a, 8b). This result conformed to Nahar et al. (2011) who reported that the application of 1 mg/L HA in MMS medium promoted the formation rate of protocorm like bodies (PLBs) and shoots of *Cymbidium dayanum*. In addition, 0.1 mg/L HA-supplemented MMS medium gave the highest number of PLBs (12.4) of *C*ymbidium Walts 'Idol' (Kaewjampa et al., 2012). Although HA, abiotic elicitor, was reported to enhance the

production of secondary metabolite in medicinal plant tissue culture (Zhou and Wu, 2006), it plays important roles in living organism including control of tissue hydration, water transport, tissue repair and various receptor-mediated functions in cell detachment (Kogan et al., 2008). Moreover, Lee and Spicer (2000) claimed that HA is essential for normal development in higher eukaryotes namely regulation of cell proliferation, migration and tissue architecture at multiple levels. However, visual observation of protocorm which cultured in MVW medium added with chitosan was green and more vigorous than those in HA. Unfortunately, it was possible that the HA application was not suitable for seed germination of *Paphiopedilum* species due to orchid seed coat presenting the impermeable testa which may act as a barrier to water and nutrients (Zhang et al., 2015) including HA permeation.

MVW medium supplemented with 1.0 mg/L chitosan was the most suitable medium for subsequent protocorm development of *P. callosum* var. *sublaeve*. This treatment gave seed germination percentage and protocorm formation at 4.89±0.54% and 2.47±0.46%, respectively. This treatment also provided the highest seed germination percentage (1.93±0.35) at stage C (seeds with embryos just rupturing the seed coat) and exhibited vigorous green protocorms (Figure 8C). In addition, Nge et al. (2006) and Nahar et al. (2011) revealed that chitosan presents a unique combination of properties and was widely available, inexpensive, environmentally friendly and as well as non-toxic to human. Chitosan may be involved in some signaling pathway related to auxin biosynthesis via a tryptophan-independent pathway which supported growth and development in plant (Uthairatanakij et al., 2007). It has been reported that chitosan may act as a plant growth stimulator in some plant species including orchids (Sopalun et al., 2010). Chitosan was effective for producing seed germination and protocorm formation in *Dendrobium phalaenopsis* (Nge et al., 2006) and *Grammatophyllum speciosum* (Sopalun et al., 2010). In the latter case, the application of 15 mg/L chitosan could enhance the PLBs growth rate. Therefore, chitosan might be suitable substrate for seed germination and protocorm formation of *P. callosum* due to the requirement for safety and reducing cost.

The process of seed germination of *P. callosum* var. *sublaeve* (Table 3, Figure 8) was divided into the following six categories according to developmental stages of embryos (Miyoshi and Mii, 1995).

Table 2. Effects of hyaluronic acid (HA) and chitosan on seed germination, germination index and protocorm formation of *Paphiopedilum callosum* var. *sublaeve*.

Natural additive	(mg/L)	Percentage of seedlings in each development stage (mean±SE) A	B	C	D	Total seed germination percentage	Seed germination index	%Protocorm formation*	visual observation of protocorms
Control	0	95.36±1.18^{a}	2.85±0.97^{ns}	0.71±0.46^{b}	0.36±0.36^{b}	4.65±1.18^{b}	0.06±0.02^{ab}	3.08±0.97^{ns}	Pale yellow
HA	0.01	95.15±1.57^{a}	3.59±1.21^{ns}	0.47±0.33^{b}	0.78±0.52^{b}	4.84±1.58^{b}	0.07±0.03^{ab}	1.51±0.66^{ns}	Pale and green
	0.1	91.67±1.01^{b}	3.75±0.77^{ns}	1.35±0.39^{ab}	3.23±0.64^{a}	8.34±1.01^{a}	0.16±0.02^{a}	4.28±0.96^{ns}	Pale and green
	1.0	96.25±0.45^{a}	2.23±0.46^{ns}	0.71±0.21^{b}	0.80±0.28^{b}	3.75±0.45^{b}	0.06±0.01^{b}	1.83±0.53^{ns}	Pale and green
Chitosan	1.0	95.11±0.54^{a}	2.16±0.56^{ns}	1.93±0.35^{a}	0.79±0.35^{b}	4.89±0.54^{b}	0.08±0.01^{ab}	2.47±0.46^{ns}	Green, ,healthy
	5.0	96.92±0.50^{a}	1.83±0.33^{ns}	0.77±0.27^{b}	0.48±0.22^{b}	3.34±0.47^{b}	0.05±0.01	1.23±0.29^{ns}	Green, Healthy
	10.0	94.90±0.88^{a}	2.79±0.67^{ns}	1.06±0.49^{ab}	1.25±0.44^{b}	5.10±0.89^{b}	0.08±0.02^{ab}	2.56±0.71^{ns}	Green, Healthy

Data were taken after culture on MVW medium supplemented with various additives for 3 months.

Means in column followed by the same letters were not significantly different at $P \leq 0.05$ as determined by DMRT.

*The Kruskal-Wallis test was used.

ns: non-significant.

A: swollen seeds, no embryo germination; B: swollen seeds, embryo germination but not yet rupturing the seed coat; C: seeds with embryos just rupturing the seed coat; and D: seeds with embryos completely out of the seed coat.

Table 3. Developmental stages of *P. callosum* var. *sublaeve* protocorms.

Stage	Days after culture (days)	Description
0, No germination	0-40	No growth of embryo occurs.
1, Pre-germination	40-45	Embryo swells to fill the seed coat.
2, Germination	40-50	Embryo emerges from the seed coat.
3, Protocorm	60-65	Embryo is completely discharged from the seed coat.
4, Rhizoid	60-75	Rhizoids are formed on the protocorm surface.
5, Shoot	70-85	Shoot is differentiated from the protocorm.

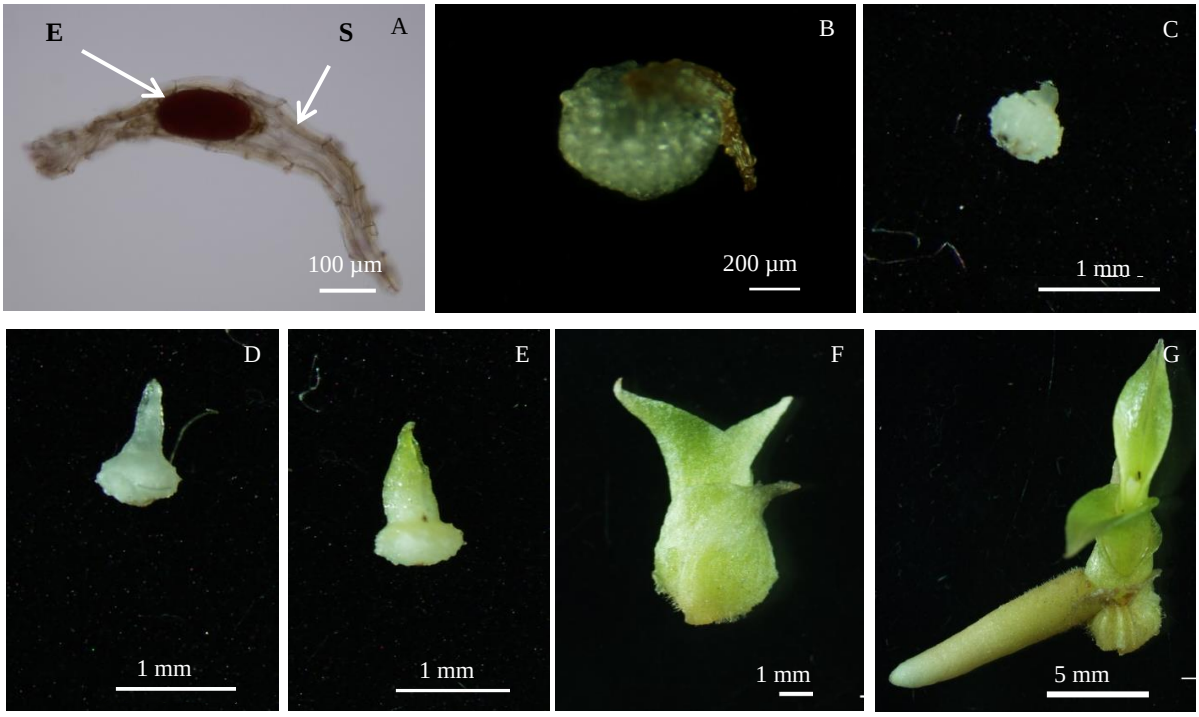

Figure 8. *In vitro* seed germination and seedling development of *P. callosum* var. *sublaeve* (A) stage 0; swelling and ungerminated seeds, (B) stage 1; testa ruptured, (C) stage 2; appearance of the shoot, (D) stage 3; appearance of the shoot and rhizoids, (E) stage 4; emergence and elongation of first leaf, (F) stage 5; presence of two or more leaves, (G) seedling with multiple leaves and roots.

3.3.2 Effects of natural additives on shoot multiplication

The effects of natural additives on number of shoots and shoot formation rate were shown in Table 4 and Figure 9. The highest number of shoot (3.22±0.36) and the maximum shoot formation rate (100%) were derived from the explants which were cultured on MMS medium containing 1.0 mg/L HA. In present experiment, HA at low concentration could induce shoot formation of *P. callosum*. This result was the same as Nahar et al. (2011) who reported that the optimum concentration for promoting the highest number of shoot (3.0±0.4) and shoot formation rate (9.3%) of *Cymbidium dayanum* tissue was 1.0 mg/L HA. Furthermore, Kaewjampa et al. (2012) reported that HA at 1.0 mg/L gave the highest number of shoots (2.7±0.5) in *Cymbidium* Waltz 'Idol'. Moreover, HA at the same concentration also gave the highest shoot formation rate (53.3% and 66.7%) in *Cymbidium kanran* Makino (Kamal et al., 2014) and *D. kingianum* (Habiba et al., 2014). However, Sultana et al. (2015) reported that 0.1 mg/L HA application was the optimal concentration for the highest shoot number in *Phalaenopsis* 'Fmk 02010'. Therefore, a function of HA was entirely different from orchid species to species (Sultana et al., 2015).

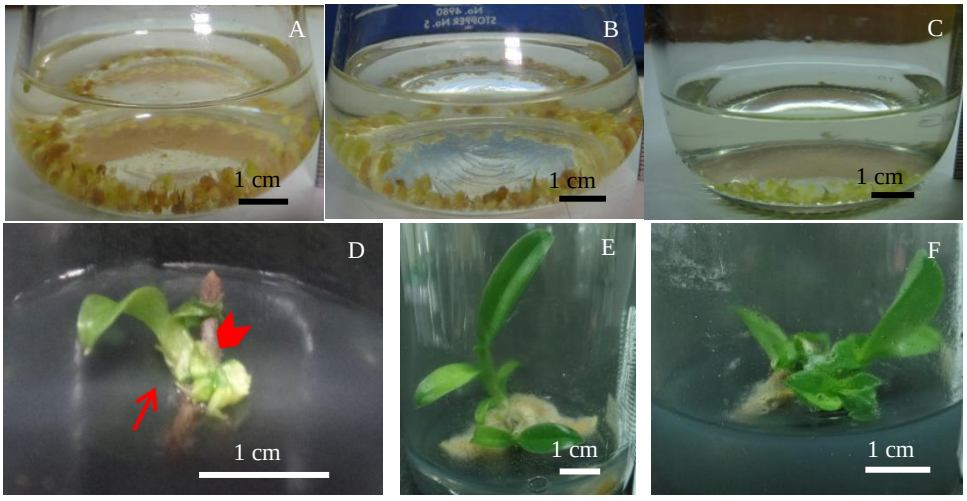

Figure 9. Protocorms development and shoot multiplication of *Paphiopedilum callosum* var. *sublaeve*. Sixty-day-old protocorms exhibiting (A) pale yellow protocorms were cultured in MVW medium (control), (B) pale and green protocorms were cultured in MVW medium containing HA (0.1 mg/L), (C) vigorous green

protocorms were cultured in MVW medium containing chitosan (1.0 mg/L.), (D) Seedling (arrow) presents a new shoot (arrow head) on MMS supplemented with 0.1 mg/L HA for 4 weeks. (E) Multiple shoots presenting vigorous growth on MMS supplemented with chitosan at 1.0 mg/L for 10 weeks. and (F) Multiple shoots exhibited more shoot number on MMS supplemented with HA at 1.0 mg/L for 10 weeks.

Table 4. Effects of hyaluronic acid (HA) and chitosan on number of shoots and multiple shoot formation rate of *P. callosum* var. *sublaeve*.

Natural additive (mg/L)		Number of shoots (shoots) (mean±SE)	Multiple shoot formation rate (%)
Control		1.00±0.37^{b}	55.56±17.57^{ab}
HA	0.01	1.33±0.76^{b}	44.44±17.57^{b}
	0.1	1.11±0.26^{b}	77.78±14.67^{ab}
	1.0	3.22±0.36^{a}	100.00±0.00^{a}
Chitosan	1.0	1.33±0.41^{b}	77.78±14.67^{ab}
	5.0	1.33±0.47^{b}	88.89±11.11^{ab}
	10.0	1.67±0.58^{b}	66.67±16.67^{ab}

Data were taken after culture on MMS medium supplemented with various additives for 2 months.

Means in column followed by the same letters were not significantly different at $P \leq 0.05$ as determined by DMRT.

3.3.3 Shoot development and rooting

Four-month-old plantlets (Figure 10A) which cultured on MMS medium supplemented with 50 g/L banana homogenate (Kaewubon et al., 2010) were transferred to sphagnum moss filled pots (Figure 10B). These plants presented at 90% survival rate after being transferred to the greenhouse for one month.

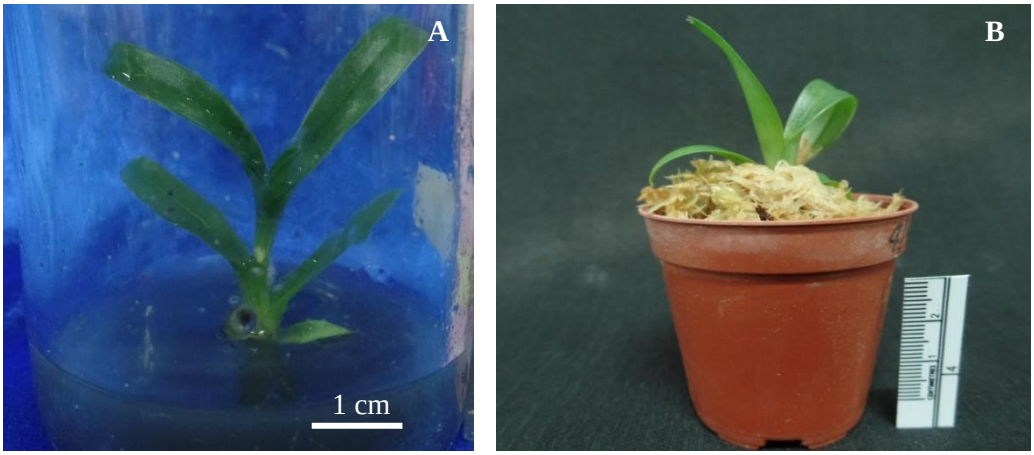

Figure 10. Multiple shoot-derived plantlet exhibits (A) vigorous shoot with root after culture on MMS medium supplemented with 50 g/L banana homogenate for 2 months (B) A representative plantlet after being transferred to the greenhouse for one month

3.4 Conclusion

In conclusion, the most appropriate procedure for seed germination and protocorm formation was performed by the pre-culturing in sterile DW added with 1.0 mg/L chitosan for the first 2 weeks in the dark, followed by the culture in MVW medium supplemented with 1.0 mg/L chitosan for further 3 months. This procedure provided healthy protocorms and seedlings. Moreover, MMS medium supplemented with 1.0 mg/L HA was suitable for shoot multiplication. Plantlets grew well with 90% survival rate in a shaded greenhouse. In the near future, the experiment to elucidate the effective protocol for seed germination and protocorm development of *P. callosum* var. *sublaeve* will be gained. Chitosan can be supplemented in culture medium to promote number of seed germination and shoot multiplication, followed by HA application to encourage quality of protocorms and seedlings.

CHAPTER 4

IN VITRO PROPAGATION THROUGH TRANSVERSE THIN CELL LAYER (tTCL) CULTURE SYSTEM OF LADY'S SLIPPER ORCHID : *Paphiopedilum callosum* var. *sublaeve*

This work was submitted to Songklanakarin Journal of Science and Technology (SJST) (in revision)

4.1. Introduction

Paphiopedilum, known as lady's slipper orchid, is a genus in the subfamily Cypripedioideae (Orchidaceae) (Cribb, 1998). Members of *Paphiopedilum*, listed in appendix I of CITES, are under extinction menace caused by over-collection and habitat destruction (Zeng et al, 2012). *Paphiopedilum callosum* var. *sublaeve*, a native species of southern Thailand, exhibits marvelous beautiful flowers, marbled and evergreen foliage (Figure 11A) and it is more important in preventing the extinction. However, the conventional propagation including axillary bud division should spend a period of time for producing a new shoot.

Thin cell layer (TCL) technique, based on the use of very small explants derived from a limited cell number of uniform tissue, is useful for reducing the time period, producing a high frequency of shoot regeneration and more competence than primary *in vitro* culture techniques (Zhao et al., 2007). This TCL culture system could be used for the large scale production required for plant conservation. For instance, PLBs of *Dendrobium malones* 'Victory' (Anjum et al., 2006) and *Xenikophyton smeeanum* (Reichb.f.) (Mulgund et al., 2011) were successfully induced from thin sections of leaf and shoot tips in a short period of time, respectively. Vyas et al. (2010) also revealed that the secondary PLBs were induced from tTCL of primary PLBs of *Cymbidium* Sleeping Nymph. However, somaclonal variation caused by growth regulators during seed germination, and development *in vitro* can arise and occur. Therefore, the effective molecular marker, such as RAPD marker has been introduced to investigate for the genetic stability. Consequently, this study was conducted to investigate *in vitro* PLB production of *P. callosum* var. *sublaeve* via tTCL technique and to determine genetic stability using RAPD marker.

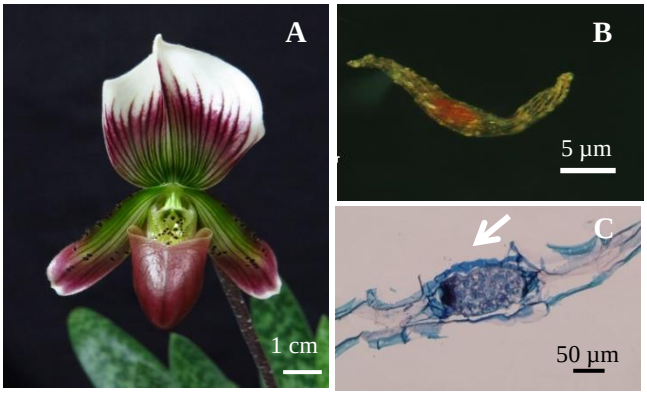

Figure 11. *Paphiopedilum callosum* var. *sublaeve* (A) Flower (B) Viable seed with dark red-stained embryo (TTC test) (C) Seed coat with greenish-blue (arrow, TBO staining).

4.2. Materials and methods

4.2.1 Plant material

The capsules at 180 days after pollination (DAP) were collected from hand self-pollinated flowers of *P. callosum* var. *sublaeve* maintained in the green-house of Department of Biology, Faculty of Science, Prince of Songkla University, Thailand. Seed viability was examined using 1% 2, 3, 5-Triphenyltetrazolium chloride (TTC) assay (Vujanovic et al., 2000).

4.2.2 Effects of basal media and pretreatment duration on seed germination.

The capsule was surface-sterilized with 1.2% sodium hypochlorite (NaOCl) for 20 min, rinsed with sterile distilled water (DW) for 2-3 times and cut longitudinally. Seeds were scooped out and placed into sterile DW. They were suspended in approximately 125 seeds/ml in a 125-ml culture flask containing 20 ml of DW for various preculture periods (0, 1, 2 or 3 weeks) The cultures were maintained on a shaker (50 rpm) in the culture room (25±2^{o}C) under the dark condition before being transferred to different basal media [1/2Murashige and Skoog (MS; Murashige and Skoog, 1962) or modified Vacin and Went (MVW; Vacin and

Went, 1949) media]. All media were supplemented with 10 g/L sucrose, 1 mg/L chitosan, 1.0 g/L activated charcoal (AC) and solidified with 6.8 g/L agar in which all concentrations were optimized in initial trials. The media were adjusted to pH 5.2 and all cultures were incubated under a 16-h photoperiod at irradiance of 23 µmol/m^2/s provided by cool white fluorescent tubes (Philips). Only if the appearance of a swollen embryo with the ruptured testa would be considered to have successfully germination. The experiment was performed with 3 replicates and repeated twice. Data were subjected to a two-way analysis of variance (ANOVA) followed by separating of mean using the Duncan's multiple range tests (DMRT) at $P<0.05$. The percentage of germinated seed was determined after culture for 30 days.

4.2.3 Effects of BAP and TDZ on *in vitro* morphogenesis from tTCL

Aseptic shoot (1-1.5 cm height) of 4-month-old seedling (Figure 12A) was cut transversely into 2 tTCL explants each with a thickness of 0.5-0.6 mm (Figure 12B). These tTCL explants (2 pieces/bottle) were inoculated on 10 ml of modified Vacin and Went (MVW) medium supplemented with various concentrations of 6-Benzylaminopurine (BAP) (0, 1.0, 5.0 and 10.0 mg/L) and thidiazuron (TDZ) (0, 0.1, 0.5 and 1.0 mg/L) alone or in combination with 15 replications (bottle). All media containing 0.2% AC were solidified with a combination of 5.5 g/L agar and 1 g/L Phytagel. After culture for 2 weeks, all explants were transferred to AC-free MVW medium to promote morphogenesis of several organs. The percentage of browning explant, shoot and root formation and PLB formation were recorded after culture for 8 weeks.

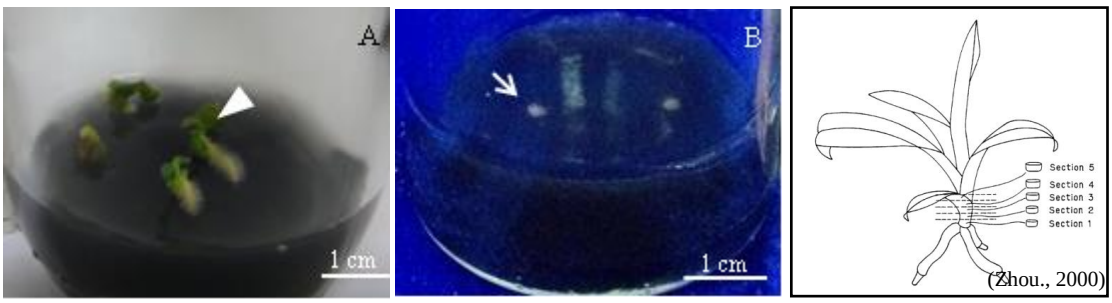

Figure 12. Explant sources for the tTCL culture system. (A) Four-month-old seedling on MVW showing shoot (arrow head) at size of 0.5-1.0 cm tall which was then cut

3.0 mm below the apex transversely into 2 pieces (B) The tTCL of 0.5 mm thickness (arrow) cultured on MVW medium added with 1.0 mg/L TDZ. and (C) Diagram presenting transversely thin cell layer (tTCL) technique.

4.2.4 Histological analysis

The seeds and *in vitro* morphogenesis responses were histologically confirmed. After culture for 8 weeks, PLBs, shoots, and roots derived from thin sections were collected and fixed in FAAII [formaldehyde: glacial acetic acid: 70% ethyl alcohol; 1:1:18 v/v/v] for 48 h. Fixed tissues were dehydrated in a tertiary-butyl-alcohol series, embedded in Histoplast PE and thin sectioned (6 µm thick) by a rotary microtome. Sections were stained with Delafield's hematoxylin and Safranin to examine the general structure (Ruzin, 1999). Seed samples were stained with toluidine blue O (TBO) to detect lignin and some phenols (Feder and O'Brian, 1968).

4.2.5 Plantlet acclimatization

Regenerated shoots were transferred to modified Murashige and Skoog (MMS) medium for plantlet growth (Kaewubon et al., 2010). The plantlets with 3-4 roots were then transplanted into a 3-inch pot containing sphagnum moss in a greenhouse and the survival rate was recorded at one month after transplanting.

4.2.6 Analysis of genetic variation

A. Plant materials and DNA isolation

The mother (M) seedlings and their regenerated (R) plants were randomly collected to determine the genetic stability. Four-month-old seedlings which were cultured on 1/2MS medium containing 1 mg/L chitosan were used as mother plants. The R plants were induced from tTCL explants which were cultured on MVW medium containing TDZ (0, 0.1, 0.5, and 1.0 mg/L) in combination with BAP (0, 1.0, 5.0 and 10.0 mg/L) for 12 weeks (Table 7). The total genomic DNA was extracted from the young leaf samples (100 mg/sample) of M and R plants following the protocol described by Chung et al. (2006) with some modification. DNA

concentration and purity were determined by spectrophotometer and the samples were diluted to a concentration of 20 ng/l.

B. DNA amplification

The amplification reaction was done in 24.5 µL which consisted of 2.5 µL 10x buffer (100 mM Tris-HCl, 500 mM KCl, 0.01% gelatin), 1 µL of template DNA, 2 µL dNTP mix (100 mM), 0.25 µL of 5 u/µL *Taq* DNA polymerase, 1.5 µL of 10 µM primer (Table 8), and 17.25 µL deionized water. DNA amplification was carried out in a thermal cycler for an initial denaturation step of 3 min at 94^{o}C before beginning the cycling protocol. An amplification cycle consisted of 40 sec at 94^{o}C, 1 min at 37^{o}C and 1 min at 72^{o}C. A total of 40 cycles were performed. The cycling was terminated with a final extension at 72^{o}C for 10 min. After amplification, DNA fragments were separated by 1.5% agarose (molecular biology grade) gel electrophoresis at 100V in 0.5x Tris-acetate-EDTA (TAE) buffer (20 mM Tris, 10 mM acetate, 0.5 mM EDTA; pH8.0), stained with ethidium bromide (0.1µl/ml). The DNA bands were then photographed under ultraviolet light using a photo documentation system. A 100-base pair ladder was used to estimate the size of RAPD bands to nearest 50 base pairs. In this study, 90 primers were taken for initial screening, as only 11 primers producing repeatable bands were analyzed.

Amplification product profiles were scored for the presence (1) or absence (0) of bands. Genetic similarity between pairs was calculated according to Jaccard's similarity coefficient, followed by cluster analysis by the SIMQUAL module (Anderson et al., 1993).

4.3. Results and Discussion

4.3.1 Effects of pretreatment duration and basal media on seed germination

The dark red-stained viable seeds (Figure 11B) were 28.0%. This present result exhibited seeds on 1/2 MS medium gave higher percentage of seed germination than that on VW medium. It was conflicted with Long et al. (2010) who reported that VW medium provided higher percentage of seed germination in four

Paphiopedilum species than that of 1/2 MS. Thus, the composition of macro- and micronutrients was one of factors required for growth and development. However, no significant difference were observed between1/2MS and VW media in seed germination of *Coelogyne nervosa* A. Rich (Abraham et al., 2012) and *P. wardii* Sumerh (Zeng et al., 2012).

Soaking seeds in water for 2 weeks, followed by a culture on 1/2MS gave vigorous, green seedlings with normal germination and the highest germination percentage (37.47±2.96%) which was significantly different from that on MVW (17.80±1.72%) (Table 5). In contrast, water-pretreated seeds for 0 and 1 week followed by culture on both 1/2MS and MVW exhibited slow germination (Table 6). The pale green seedlings were obtained from no water-pretreatment. Therefore, seed pretreatment with water for 2 weeks could enhance seed germination of this orchid.

There were interaction effects between medium type and water-pretreatment period on seed germination (Table 5). This study was similar to Godo et al. (2010) who reported that the water presoaking could enhance seed germination of *Calanthe tricarinata* Lindl. Waes and Debergh (1986) also reported that the pre-soaking of *Dactylorhiza maculata* seeds for 15 min in 5% $Ca(OCl)_2$ and 1% Tween-80 followed by 24 h in sterile DW was the optimal condition for its germination and the pretreatment period was a species-dependent. Many plant species needed the pretreatment step because a water soluble inhibitor could be leached and the seed coat became permeable to water and other nutrients. In this species, the seed coat were stained greenish blue with TBO (Figure 11C) indicating the presence of lignin and polyphenol in the cell wall. The hydrophobic characteristic of these substances was reported to be a crucial barrier to the uptake of water and nutrients (Lee et al., 2006). Consequently, the demolition of the barrier substances allowed more water and oxygen absorption to embryo. Therefore, changing the physical characteristics of the testa by shaking (Kauth et al., 2008) or soaking seeds in water (Linden, 1992) might improve seed germination due to making the testa tissue more permeable.

Table 5. Effects of pretreatment duration and basal medium type on seed germination of *P. callosum* var. *sublaeve.*

Basal medium	Percentage of seed germination (mean±SE)				
	Duration of pretreatment (weeks)				Avg basal medium
	0	1	2	3	
1/2 MS	16.86 ± 2.40[b]	17.33 ± 0.86[b]	37.47 ± 2.96[a]	19.67 ± 7.34[b]	22.83 ± 1.72
MVW	16.39 ± 1.68[b]	17.33 ± 1.13[b]	17.80 ± 1.72[b]	22.48 ± 8.88[b]	18.50 ± 1.72
Avg duration of pretreatment	16.62 ± 2.29	17.33 ± 2.29	27.63 ± 2.29	21.07 ±2.80	

Main factors	df	Mean square	F	*p*
Pretreatment duration (P)	3	304.546	4.834	*
Basal medium type (B)	1	200.335	3.180	ns
Interaction PxB	3	301.604	4.788	*
Error	36			
Total	44			

* = significant level at $\alpha = 0.05$, ns = not significant

The same letters in column are not significantly different at $P \leq 0.05$ as determined by DMRT.

Table 6. Effects of pretreatment duration and basal medium type on visual observation of seed germination and seedlings of *P. callosum* var. *sublaeve*.

Basal medium type	Duration time of pretreatment (weeks)	Visual observation	
		Germination	Seedling*
1/2MS	0	slow	pale green
	1	slow	green and healthy
	2	normal	green and vigorous
	3	normal	green and vigorous
MVW	0	slow	pale green
	1	slow	green and healthy
	2	normal	green and healthy
	3	normal	green and healthy

Data were taken after a culture for 60 days and seedling were cultured 120 days (*).

4.3.2 Effects of BAP and TDZ on morphogenesis of tTCL explants

A. PLB response

The highest percentage of PLB induction (46.67±6.67) and number of PLBs per explant (3.33±0.33) were obtained on MVW medium supplemented with 1.0 mg/L TDZ alone (Table 7). This result conformed to Niknejad et al. (2011) who reported that the highest percentage of PLB induction and number of PLB per explant of *Phalaenopsis gigantean* were gained from the using of 1.0 mg/L TDZ alone. In addition, basal segment explants of *Dendrobium* Sonia 'Earsakul' cultured on 1/2MS medium supplemented with 1.0 mg/L TDZ alone could be induced to form somatic embryos at 10.71% and at 0.5 PLB/explant (Juntada et al., 2015). The application of TDZ alone at higher concentration (3 mg/L) gave the highest number of PLBs per leaf explant in *Phaleanopsis bellina* (Rchb.f.) Christenson (Khoddamzadeh et al., 2011) and *Phaleanopsis* cv. 'Surabaya' (Balilashaki et al., 2015).

B. Shoot response

The highest percentage of shoot induction (40.00±5.16) and high number of shoots/explant (3.00±1.00) were observed in MVW medium containing 1.0 mg/L TDZ alone and 0.5 mg/L TDZ alone, respectively. Meanwhile, the same medium consisting of 5 mg/L BAP alone gave lower percentage of shoot induction (13.33±13.33) than those of 0.5-1.0 mg/L TDZ alone (Table 7). Srivastava et al. (2015) reported that application of 9.1 µM TDZ alone to Knudson C (KC) medium gave the highest number of shoot buds/shoot segment of *Aerides ringens* (Lindl.) Fischer. Besides, 1/2MS medium containing 1.0 mg/L TDZ alone was also suggested to provide higher number of shoot/explant than that of BA in *Dendrobium aqueum* Lindley (Parthibhan et al., 2015). Therefore, TDZ had a stronger effect than BAP on shoot induction of *P. callosum* var. *sublaeve*. This is possibly due to the ability of TDZ that is resistant to cytokinin oxidase (Huetteman and Preece, 1993) providing the internal suitable balance of cytokinin: auxin (Saxena et al., 1992) and enhancing the synthesis of adenine type cytokinins (Baghel and Bansal, 2015).

However, application of BAP alone was reported to provide the highest number of shoots/ explant of *Paphiopedilum callosum* (Wattanawikkit et al., 2011), *Aerides odorata* Lour (Devi et al., 2013) and *Vanda coerulea* (Jitsopakul et al., 2013).

The highest number of shoots/explant was obtained on MVW medium contained 0.1 mg/L TDZ in combination with 5.0 mg/L BAP but it was not significantly different with 0.5 mg/L TDZ alone. Whereas, the application of TDZ in combination with BAP was reported to give the maximum number of shoots/explant from *Rhynchostylis gigantean* (Le et al., 1999) and *Dendrobium chrysanthum* Wall. ex Lindl (Hajong et al., 2013). According to a model of cytokinin action in plant cell, the cytokinin-binding protein (CBP) has two different binding sites; one site binds adenine-type natural cytokinins and the other side binds phenylurea-type cytokinins (Nielsen et al., 1995; Guo et al., 2011). It was possible that shoot induction of *P. callosum* var. *sublaeve* was affected by both BA (binding to adenine-type CBC) and TDZ (binding to the phenylurea-type CBC). Moreover, TDZ (0.5 mg/L) combined with NAA (1.0 mg/L) could provide high number of micro-shoots/ seedling of hybrid orchid (*Aerides vandarum* Reichb.f x *Vanda stangeana* Reichb.f) (Kishor and Devi, 2009). Jitsopakul et al. (2013) also reported that MVW medium containing 2.0 mg/L TDZ in combination with 0.5 mg/L NAA provided high mean number of shoots/ explant in *Vanda coerulea*.

C. Root response

Root formation exhibited in almost all treatments, except the one containing 10.0 mg/L BAP alone could not induce root. This result was similar to Wattanawikkit et al. (2011) who revealed that 1/2MS medium supplemented with a high concentration of BAP at 100 µM could not induce root formation of *Paphiopedilum callosum*.

The highest percentage of root formation (30.00±12.38) and number of roots/ explant (1.33±0.33) were obtained on MVW medium added with 1.0 mg/L TDZ and MVW medium added with 0.1 mg/L TDZ combination with 5.0 mg/L BAP, respectively (Table 7). Thus, TDZ might provide the mobility of endogenous auxins (such as indol-3-acetic acid, IAA) or auxin-like bioregulators. The synergistic

effects of auxin and cytokinin combination may establish the inductive signal for root induction (Ahmadian et al., 2013) Therefore, TDZ at 1.0 mg/L in this study was able to enhance the percentage of root formation.

However, PGR-free medium gave the highest number of roots/explant (2.00±0.58). Majumder et al. (2010) reported that PGR-free KC medium could enhance the rooting ability of *Dendrobium farmeri* Paxt. within 3 weeks. This was because of the balance between endogenous auxin and cytokinin, key regulator of *in vitro* organogenesis (Nordstrom et al., 2004). Therefore, high endogenous auxin/ cytokinin ratio in explants cultured on PGR-free MVW medium could induce root formation.

D. Browning response and survival rate

Most explants gradually became brown after a culture for 3 weeks. Explants on MVW medium containing high BAP (10 mg/L) exhibited high browning (100%), low survival rate (10.00±6.83%), low PLB induction (6.67±4.21%) and low shoot formation (3.33±3.33%) (Table 7). This study conformed to Mondal et al. (2013) who revealed that BAP at high concentration inhibited PLB induction of *Doritis pulcherrima*. However, Jitsopakul et al. (2013) reported that shoot tips of *Vanda coerulea* cultured on MVW medium supplemented with 5.0 mg/L BAP provided high survival rate but gave rise to the low number of shoot and roots per explant. Besides, the application of BAP (10.0 mg/L) in combination with TDZ (0.1-1.0 mg/L) gave higher survival rate than that of 10 mg/L BAP alone.

Explants on MVW medium supplemented with TDZ at high concentration (1.0 mg/L) exhibited green, the lowest browning (76.67±12.02%) and the highest survival (70.00±4.47%). This result disagreed with Mulgund et al. (2011) who reported that high TDZ level leaded to browning, necrosis and eventually death of *Xenikophyton smeeanum* (Reichb.f). Therefore, the types with varying ratios of growth regulators may need adjustment depending on the plant species.

Table 7 Effects of BAP and TDZ on morphogenic responses of tTCL explants of *P. callosum* var. *sublaeve* cultured on MVW medium for 8 weeks.

Cytokinins (mg/L)		Percentage PLB induction (mean±SE)	Number of PLBs/explant (mean±SE)	Percentage root formation (mean±SE)	Number of roots/explant (mean±SE)	Percentage shoot formation (mean±SE)	Number of shoots/explant (mean±SE)	Browning percentage (mean±SE)	Survival Percentage (mean±SE)
TDZ	BAP								
0	0	3.33 ± 3.33^{b}	1.00 ± 0.57^{b}	3.33 ± 3.33^{b}	2.00 ± 0.58^{a}	13.33 ± 4.21^{b}	1.67 ± 0.33^{ab}	100.00 ± 0.00^{a}	20.00 ± 7.30^{b}
0	1.0	23.33 ±12.02^{b}	1.33 ± 0.33^{b}	6.67 ± 4.21^{b}	1.00 ± 0.58^{ab}	6.67 ± 6.67^{b}	2.00 ± 0.58^{ab}	96.67 ± 3.33^{a}	33.33 ±12.29^{b}
0	5.0	23.33 ±10.85^{b}	1.00 ± 0.57^{b}	13.33 ± 6.67^{ab}	0.67 ± 0.33^{ab}	13.33 ±13.33^{b}	1.00 ± 0.00^{b}	100.00 ± 0.00^{a}	40.00 ±13.66^{b}
0	10.0	6.67 ± 4.21^{b}	1.00 ± 0.57^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	3.33 ± 3.33^{b}	2.00 ± 0.58^{ab}	100.00 ± 0.00^{a}	10.00 ± 6.83^{b}
0.1	0	16.67 ± 9.54^{b}	1.00 ± 0.57^{b}	3.33 ± 3.33^{b}	1.00 ± 0.58^{ab}	3.33 ± 3.33^{b}	1.00 ± 0.58^{b}	100.00 ± 0.00^{a}	23.33 ± 9.54^{b}
0.5	0	16.67 ± 6.14^{b}	1.00 ± 0.58^{b}	13.33 ± 4.21^{ab}	0.67 ± 0.67^{ab}	16.67 ± 8.03^{b}	3.00 ± 1.00^{ab}	93.33 ± 4.21^{a}	40.00 ±10.33^{b}
1.0	0	46.67 ± 6.67^{a}	3.33 ± 0.33^{a}	30.00 ±12.38^{a}	1.00 ± 0.58^{ab}	40.00 ± 5.16^{a}	1.33 ± 0.33^{b}	76.67 ±12.02^{b}	70.00 ± 4.47^{a}
0.1	1.0	20.00 ± 7.30^{b}	0.33 ± 0.33^{b}	13.33 ± 6.67^{ab}	1.00 ± 0.58^{ab}	6.67 ± 6.67^{b}	1.00 ± 0.00^{b}	100.00 ± 0.00^{a}	30.00 ±11.25^{b}
0.1	5.0	6.67 ± 4.21^{b}	1.67 ± 0.88^{b}	10.00 ± 4.47^{ab}	1.33 ± 0.33^{ab}	13.33 ± 6.67^{b}	3.67 ± 1.45^{a}	100.00 ± 0.00^{a}	23.33 ± 9.54^{b}
0.1	10.0	20.00 ± 8.94^{b}	0.33 ± 0.33^{b}	10.00 ± 6.83^{ab}	1.00 ± 0.00^{ab}	3.33 ± 3.33^{b}	1.00 ± 0.58^{b}	93.33 ± 4.21^{a}	26.67 ± 8.43^{b}
0.5	1.0	10.00 ± 6.83^{b}	1.33 ± 0.67^{b}	10.00 ± 6.83^{ab}	0.67 ± 0.33^{ab}	16.67 ± 9.54^{b}	1.00 ± 0.58^{b}	96.67 ± 3.33^{a}	33.33 ± 9.89^{b}
0.5	5.0	6.67 ± 4.21^{b}	1.00 ± 0.58^{b}	10.00 ±10.00^{ab}	1.00 ± 0.58^{ab}	13.33 ± 9.89^{b}	1.00 ± 0.58^{b}	96.67 ± 3.33^{a}	26.67 ±11.15^{b}
0.5	10.0	16.67 ± 6.14^{b}	1.00 ± 0.58^{b}	10.00 ± 6.83^{ab}	0.33 ± 0.33^{b}	6.67 ± 4.21^{b}	1.00 ± 0.58^{b}	93.33 ± 4.21^{a}	30.00 ± 6.83^{b}
1.0	1.0	16.67 ± 6.14^{b}	1.00 ± 0.58^{b}	10.00 ± 4.47^{ab}	1.00 ± 0.58^{ab}	6.67 ± 6.67^{b}	1.00 ± 0.58^{b}	100.00 ± 0.00^{a}	23.33 ± 9.54^{b}
1.0	5.0	10.00 ± 6.83^{b}	0.33 ± 0.33^{b}	16.67 ± 6.14^{ab}	1.00 ± 0.58^{ab}	16.67 ± 9.54^{b}	1.00 ± 0.58^{b}	100.00 ± 0.00^{a}	36.67 ± 8.03^{b}
1.0	10.0	16.67 ± 6.14^{b}	1.33 ± 0.88^{b}	10.00 ± 4.47^{ab}	0.67 ± 0.33^{ab}	16.67 ± 8.03^{b}	1.33 ± 0.88^{b}	100.00 ± 0.00^{a}	36.67 ±14.06^{b}

Mean in each column followed by the same letters are not significantly different at P≤0.05 as determined by DMRT.

4.3.3 Histological assessment

The cytokinins affecting morphogenesis responses were histologically examined. The tTCL explants cultured on MVW medium containing 1 mg/L TDZ provided PLBs (arrow, Figure 13A), shoots (a dashed line with an arrow, Figure 13A) and root (arrow head, Figure 13A). Histological observation displayed the shoot with shoot apical meristem (SAM) and leaf primodium (LP) presenting cells with densely stained cytoplasm (Figure 13B). This zone had high meristematic activity involving formation of new meristematic cells. PLBs also exhibited the masses of small embryogenic cells containing dense cytoplasm and large nuclei (Figure 13C).

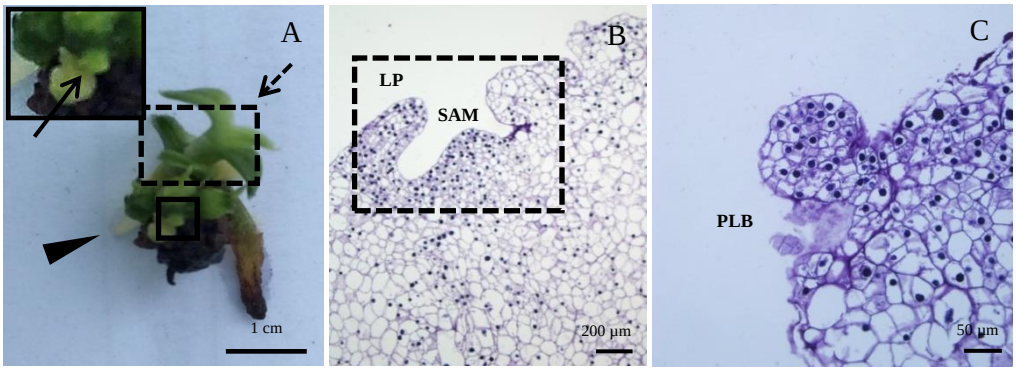

Figure 13. The transverse thin cell layer (tTCL) of shoot tips of *P. callosum* var.

sublaeve cultured on MVW medium supplemented with 1.0 mg/L TDZ exhibiting (A) shoot (a dashed line with an arrow), PLBs (arrow) and roots (arrow head). Longitudinal section of explant presenting (B) shoot and leaf primodium (C) PLB regenerated from tTCL. SAM, shoot apical meristem; LP, leaf primodium.

4.3.4 Plantlet acclimatization

Sixteen-week-old regenerated shoots on MVW medium supplemented with 1.0 mg/L TDZ (Figure 14A) were transferred to MMS medium. These plantlets displayed 3-4 roots (Figure 14B) after culture for 10 weeks and they were then transplanted into sphagnum moss with 80% survival rate in a greenhouse (Figure 14C).

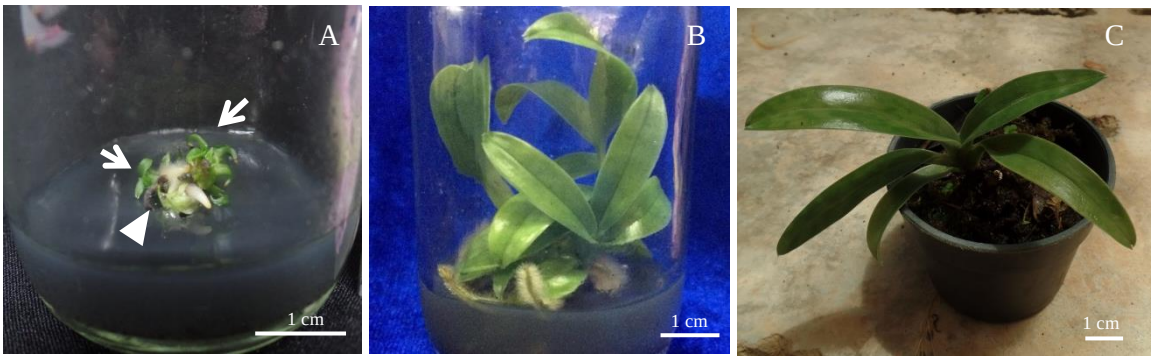

Figure 14. Growth promotion of *P. callosum* var. *sublaeve*.
(A) Clump of developing new shoots (arrows) originated from tTCL shoot piece (arrow head) cultured on MVW medium supplemented with 1 mg/L TDZ for 16 weeks (B) Regenerated plantlets after being transferred to growth-promoting medium for 10 weeks (C) Greenhouse-grown plants in pot containing sphagnum moss.

4.3.5 Analysis of genetic stability

Eight pairs of the regenerated plants and the mother plants were randomly chosen to examine the genetic stability. The eleven primers having 60-70% guanine-cytosine (GC) content and clear polymorphic DNA bands were successfully amplified (Table 8). These primers generated the fragments varied from 11 (OPU-13) to 18 (OPU-12) and the sizes were ranging from 100 bp (OPU-12) to 2,000 bp (OPA-04). A total of 159 bands were obtained with an average of 14.45 bands per primer

and 95% of these were polymorphism. Results indicated the percentage of GC was affected to amplification which conformed to the report of Gnat et al. (2015) who revealed that using primer having high GC% in RAPD technique supported to generate adequately high number of amplicons in amplification of *Astragalus glycyphyllos*. However, Padmalatha and Prasad (2008) claimed that percentage of GC was not affected to amplification of *Centella asiatica*. The RAPD profiles of 16 samples (8 pairs of M and their R plants) using OPU 12 were shown in Figure 15. Results revealed that the regenerated plants and the mother plants presenting identical RAPD profiles indicated the mother-regenerated plant pairs had a genetic stability.

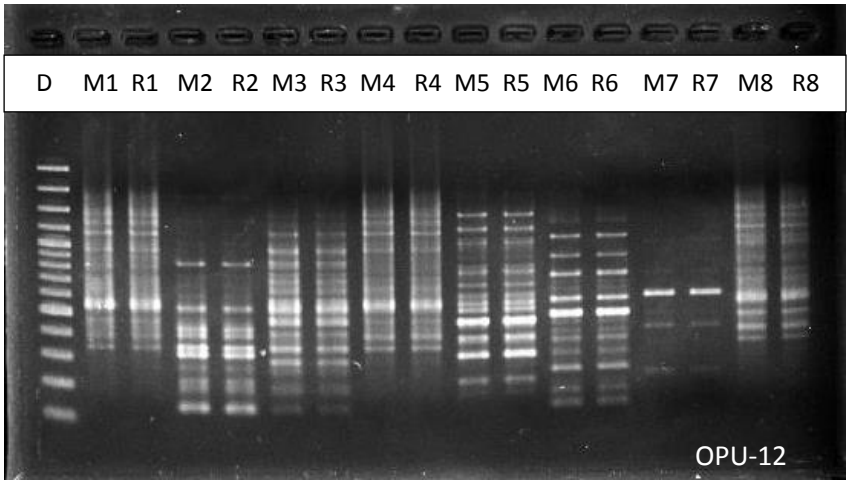

Figure 15. RAPD profiles of the mother seedlings (M) and their regenerated plants (R) generated by OPU-12. D: 100bp DNA ladder.

Table 8. Number and size ranges of the amplified RAPD bands of *P. callosum* var. *sublaeve* which were cultured on MVW containing different concentrations of TDZ and BAP for 8 weeks.

Primer	Sequence (5'-3')	Size ranges (bp)	GC%
OPA-04	AATCGGGCTG	180-2000	60
OPA-09	GGGTAACGCC	210-1500	70
OPU-12	TCACCAGCCA	100-1500	60
OPU-13	GGCTGGTTCC	210-1200	70
OPZ-3	CAGCACCGCA	200-1400	70
OPZ-11	CTCAGTCGCA	320-1800	60
OPA-A-11	ACCCGACCTG	200-1150	70
OPA-B-4	GGCACGCGTT	200-1800	70
OPA-B-8	GTTACGGACC	190-1100	60
OPA-D-15	TTTGCCCCGT	180-1300	60
UBC-719	GGTGGTTGGG	380-1600	70
Total			-
$\bar{x}$			-

4.4 Conclusions

The recent protocol for *in vitro* propagation of *P. callosum* var. *sublaeve* was found to cause no genetic variation. The water-pretreated seeds in dark for 2 weeks before being transferred were required for seed germination. The morphogenesis responses including PLBs, shoots and roots were successfully induced from tTCL explants of shoot tips cultured on MVW containing 1.0 mg/L TDZ. No genetic variation could be detected among the mother plants and their own regenerants as revealed by RAPD markers.

CHAPTER 5

DIVERGENCE OF SOME SPECIES IN *PAPHIOPEDILUM* SECTION *BARBATA*

5.1 Introduction

Paphiopedilum, the largest genus of slipper orchids comprising about 100 species (Guo et al., 2015), is severely controlled and monitored by CITES (Conventional on International Trade in Endangered Species of Wild Fauna and Flora) Appendix I (Pedersen et al, 2011). The genus *Paphiopedilum* was comprised of 3 subgenera *Parvisepalum*, *Brachypetalum* and *Paphiopedilum*. In the latter case, subgenus *Paphiopedilum* was composed of 5 sections, namely, *Coryopedilum, Pardalopetalum, Cochlopetalum*, *Paphiopedilum* and *Barbata* (Cribb, 1998). However, some plants in section *Barbata* such as *P. callosum* (including 3 varieties) and *P. barbatum* were still controversial and required the supporting data to clarify their characteristics.

Paphiopedilum callosum, native plant of Southeast Asia, especially in Thailand, Cambodia, Laos and Vietnam, is one of species in section *Barbata*. This species can be separated into 3 varieties: *sublaeve*, *potentianum* and *callosum* (Pedersen et al, 2011). Few differences among these varieties, for instance, the occurrence of warts on the upper margin petals of *P. callosum* var. *sublaeve* and var. *callosum*, but absence in var. *potentianum*, were revealed (Cribb, 1998). Moreover, dorsal sepals of var. *callosum* are wider than those of var. *sublaeve* and the sigmoid-shaped petals in var. *callosum* are predominately showed more than in var. *sublaeve*. Cribb (1998) reported that var. *sublaeve* provided some floral characteristics which were between *P. callosum* and *P. barbatum*. *Paphiopedilum barbatum* widely distributed throughout peninsular of Malaysia, Penang Island off the Northwest coast and below the Thailand border, was found to differ from *P. callosum*. Interestingly, these two species were also proposed to be conspecific according to the variation of their habitats that were actually continuous between the extremes of northern Thailand for *P. callosum* and southern Malaysian for *P. barbatum* (Cribb, 1998).

Cox et al. (1998) reported that orchids in section *Barbata* have large genomes, widely ranged and highly chromosome number (2n=28-42). Moreover, the slipper orchids in section *Barbata* were rapidly adapted to environment including the

forest floors (Atwood, 1984; Cox et al., 1998). These led to confusion of the species identification having resembled characteristics.

Szlachetko et al. (2013) also suggested that the morphological, phytogeographic, cytological and molecular analyses could be used for orchid classification. The morphological characteristics including traits of anthers (Dressler and Dodson, 1960), stamen number, and 3-chambered ovary (Atwood, 1984) had only infrequently been couched in terms of explicitly phylogenetic studies (Freudenstein and Rasmussen, 1999).Various DNA markers have been recently applied in many orchid phylogenetic studies, such as random amplified polymorphic DNA (RAPD) marker in *Neotinea ustulata* (Harastova-Sobotkova et al., 2005), Inter-simple sequence repeat (ISSR) marker in *Cymbidium sinense* (Lu et al., 2013) and nuclear ribosome internal transcribed spacer (ITS) in *Angraecum*-alliance (Szlachetko et al., 2013).

Since slipper orchids are difficult to identify without their flowers, the original methods of classification namely; the morphological and physiological systems, should be supported by molecular technique (Choi, 2006; Trung et al., 2013). Molecular data are likely to play a very important role in determining the genetic relationship among plants and providing the novel genetic classification that often coincides with primary taxonomy (Jobst et al., 1998).

Leaf trait is one of morphological characteristics reflecting adaptation of plants to environment (Dunbar-Co et al., 2009). The genetic markers have furnished a helpful equipment to reinforce or negate the unclear classification (Harastova-Sobotkova et al., 2005). The RAPD technique is a fingerprinting using short, random and oligonucleotide primers to search the variation in the whole genomic DNA (William et al., 1990). RAPD marker was used to classify the members of Orchidaceae (Lim et al., 1999), to study relationship and distinguish taxa up to the specific level in *Phalaenopsis* and *Paraphalaenopsis* (Goh et al., 2005) and investigate the genetic variation in 30 orchids (Miano, 2015). Both of the morphological features and molecular analysis (PCR-RFLP) were used to confirm the relationship of new hybrid orchid (*Ophrys lutea* x *O. tarentina*) (Pellegrino et al., 2008). In addition, Tang et al. (2015) also proposed the new framework on the

complex phylogenetic relationships between *Amitostigma* and other genera by morphological and molecular analyses (using nrITS sequences).

This present study aimed to examine the relationship among orchid members in section *Barbata* including 3 varieties of *P. callosum* (*P. callosum* var. *sublaeve, P. callosum* var. *callosum* and *P. callosum* var. *potentianum*) and *P. barbatum*. by both morphological characteristics and molecular (RAPD marker) analysis.

5.2 Materials and methods

5.2.1. Plant materials

Four groups of *Paphiopedilum* plant samples (Table 9) in the section *Barbata* (*P. callosum* var. *callosum, P. callosum* var. *sublaeve*, *P. callosum* var. *potentianum* and *P. barbatum*) were collected from various locations based on their habitats. Some plant parts of them were obtained from Mr. Teerapun Tothirakul at Management of Plant Research and Development of Doi Tung Development Project, Ms. Nopparat Thawinwathin at The Agricultural Development and Extension Center, Trang, and Mr. Suchart Wundee. These plants were maintained grown in greenhouse at the Department of Biology, Faculty of Science, Prince of Songkla University for 12 months before starting of experiment. The morphological data were measured based on Atwood (1984).

Table 9. Sources of plant samples.

Sample plants	A[1]	B[2]	C[3]	D[2]
Sources	Border of Malaysia	Border of Cambodia	Rattaphum District, Songkhla Province	Border of Cambodia

Plant samples were received from Mr. Teerapun Tothirakul[1], Mr. Suchart Wundee[2] and Ms. Nopparat Thawinwathin[3].

Where A = Sample plants were collected from border of Malaysia

B = Sample plants were collected from border of Cambodia

C = Sample plants were collected from Rattaphum district, Songkhla province

D = Sample plants were collected from border of Cambodia

5.2.2 Morphological measurements and physiological assessment

A. Leaf and guard cell

The 2nd fully expanded leaf, not counting the small new leaf (< 5 cm long), was collected as sample for plant analysis since the 1st mature leaf is generally appeared at the 3rd-5th leaf from the top of the plant.

Leaf samples and lower epidermal peels of leaf blade were fixed in FAAII (formaldehyde: glacial acetic acid: 70% ethyl alcohol; 1:1:18 v/v/v) for 48 h, dehydrated in graded series of ethanol-TBA, embedded in paraffin wax, thin sectioned (6 µm thickness) by a rotary microtome (Shandon AS 325). The sections of leaf samples and epidermal peels were then stained with double staining (Safranin and Fast green) and fast green staining, respectively (Johansen, 1940). Photographs were taken under a light microscope (Olympus, BX 51) and an in-built digital camera (Olympus, DP 71).

Digital images were processed using Digimizer version 4.6.1 image analysis software (MedCalc Software). The total mesophyll (TM), long of adaxial (Lad), wide of adaxial (Wad), long of abaxial (Lab), wide of abaxial (Wab), cuticle thickness of adaxial (CuTad), cuticle thickness of abaxial (CuTab), guard cell length (GL), and guard cell width (GW) were measured. The guard cell area (GA) was calculated followed by James and Bell (2001). The guard cell density and the guard cell index (GI) of both adaxial and abaxial surfaces were calculated using the following formula with modification (Thakur and Patil, 2011).

$$GI = \frac{G}{E + G} \times 100$$

$$GA = 1/4 \times \pi \times GL \times GW$$

Where:

G = number of the guard cell per unit area

E = number of epidermal cells per same unit area

GL = guard cell length

GW = guard cell width

B. Flowers

The morphological data of flowers including size of dorsal sepal, petal, lip, staminode, synsepal and ovary was examined among A, B, C and D. Three fully developed flowers per species/variety were used as samples. These samples were photographed and processed using Digimizer version 4.6.1 image analysis software (MedCalc Software).

5.2.3 Determination of pigment contents

The second fully expanded leaves (n=3) were assembled as samples to determine the pigment contents. The leaf samples (0.1 g fresh weight) were extracted according to method of Mistra et al. (2010). These samples were pulverized in 4 ml of 80% (v/v) acetone by a clean mortar. The homogenized tissue was centrifuged at 10,000xg for 4 min to get the supernatant. The centrifugation was repeated three times in a series of 80% (v/v) acetone at 3, 2 and 1 ml, respectively. The final volume of supernatant at 10 ml was determined by measuring the absorbance at 480, 510, 645 and 663 nm. Carotenoid and total chlorophyll contents (chl a and chl b) were calculated in mg/g fresh weight using the following formulae (Misra et al., 2010).

$$\text{Total chlorophyll} = \frac{[20.2(A_{645}) + 8.02(A_{663})]\times V}{1000\times W}$$

$$\text{Chlorophyll a} = \frac{[12.7(A_{663}) + 2.63(A_{645})]\times V}{1000\times W}$$

$$\text{Chlorophyll b} = \frac{[22.9(A_{645}) - 4.68(A_{663})]\times V}{1000\times W}$$

$$\text{Carotenoid} = \frac{[7.6(A_{480}) - 2.63(A_{510})]\times V}{1000\times W}$$

Where:

A = optical density reading of the chlorophyll extract at specific indicated wavelength

V = final volume of the 80% acetone-chlorophyll extracted

W = fresh weight in grams of the tissue extracted

5.2.4 Molecular assessment

A. Plant materials for DNA extraction

The second fully expanded leaf of each type of sample plants were used to be the samples for molecular analysis. *Paphiopedilum exul* and *Dendrobium crumenatum* orchids were used as the control groups.

The genomic DNA was isolated by modifying the method described by Chung et al. (2006). The quality and quantity of DNA were determined by spectrophotometer (Biodrop Duo Micro Volume Spectrophotometer).

B. RAPD amplification and electrophoresis

The PCR mixture was RAPD amplified by using amplification condition explained by Chung et al. (2006). The PCR reaction containing 1 µL of template DNA, 2.5 µL of 10x buffer (100 mM Tris-HCl, 500 mM KCl, 0.01% gelatin), 2 µL dNTP mix (100 mM), 0.25 µL of 5 u/µL *Taq* DNA polymerase, 1.5 µL of 10 µM primer, and 17.25 µL deionized water, was carried out in 24.5 µL total volumes. The reaction programs for RAPD were implemented in the thermal cycler at 94^{o}C for 3 min in an initial denaturation step. An amplification cycles comprised of 40 sec at 94^{o}C, 1 min at 37^{o}C and 1 min at 72^{o}C. A total of 40 cycles were operated

and the termination of the cycling was final extended at 72^{o}C for 10 min. Subsequently, the DNA fragments were isolated by 1.5% agarose gel electrophoresis at 100V in 0.5x Tris-acetate-EDTA (TAE) buffer (20 mM Tris, 10mM acetate, 0.5 mM EDTA; pH 8.0), stained with 0.1µl/ml ethidium bromide. The bands of DNA were photographed under ultraviolet light utilizing a photo documentation system (Gel Doc^{TM} EZ Gel Documentation System, Bio-RAD).

C. RAPD data analysis

The product profiles of amplification were scored for the presence (1) or absence (0) of bands. Genetic similarity based on Jaccard's coefficient was calculated using the SIMQUAL module (Anderson et al., 1993) and arranged into a similarity matrix. Dendrogram was created utilizing a NTSYS version 2.10m software package by following the UPGMA selection of the SAHN module.

5.3 Results

5.3.1 Leaf morphology and internal structure

Leaf and stoma traits were compared among four plant samples, namely, A, B, C and D. It was found that B provided the longest and widest of adaxial epidermal cells. In general, the adaxial epidermal cells of *Paphiopedilum* leaves are usually larger than abaxial cells (Guan et al., 2011) which coincided with our results (Table 10, Figure 16). This sample also gave the maximum of abaxial epidermal cells at 66.82 ± 2.06 µm long and 88.83 ± 6.34 µm wide (Table 10).

Cuticles were existed on both sides of the lamina. In this study, sample D provided the maximum of the cuticle thickness of adaxial layer but not significantly different from sample A. Meanwhile, the cuticle thickness of adaxial layer was not significantly different among plant samples. This is due to these samples were raised in the same place, caused these plants adapted to the same environment. The cuticle of plants prevented water losses through transpiration, excessive transpiration, permitted

gas exchange. Moreover, cuticles were physical impediment for resisting the pests and pathogens (Yeats and Rose, 2013; Yang et al., 2016).

Guan et al. (2011) reported that the guard cells of *Paphiopedilum* were only appeared on the abaxial cell layer which resembled with our results (Figure 16 and 18). The guard cell index (GI), guard cell density (GD), guard cell length (GL), guard cell width (GW-two guard cells of the closed stoma) and stomata area (SA) were proposed in Table 10 and Figure 17. The highest SI and SL among plant samples were found in sample A which was in agreement with dendrogram (Figure 22) showing the separation into the subcluster.

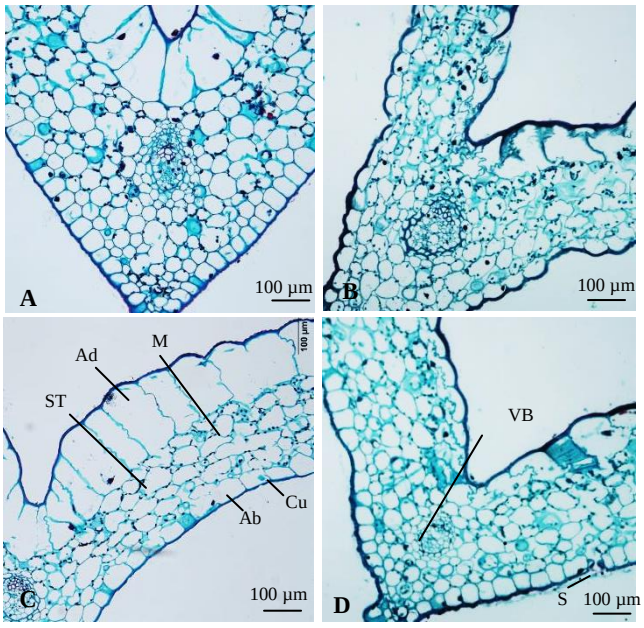

Figure 16. Transverse sections of leaf samples of (A) sample A, (B) sample B, (C) sample C and (D) sample D.

Cu, cuticle; Ad, adaxial epidermis; PT, palisade tissue; ST, spongy tissue; M, mesophyll cells; VB, vascular bundle; Ab, abaxial epidermis; S, stoma

Table 10. Leaf and stomatal traits, leaf pigment contents of *Paphiopedilum* samples.

Parameters	Species/varieties			
	A	B	C	D
LL	15.08±1.13[a]	10.89±0.67[b]	15.83±1.65[a]	13.61±1.18[ab]
WL	3.29±0.26[ab]	2.72±0.14[bc]	3.64±0.37[a]	2.37±0.17[c]
LL : WL	4.69±0.71[a]	4.01±0.21[a]	4.51±0.86[a]	5.73±0.09[a]
SI (%)	37.22 ± 2.02[a]	15.32 ± 1.12[c]	15.44 ± 1.38[c]	32.85 ± 1.64[b]
SD (mm^{-2})	13.47 ± 0.89[a]	6.40 ± 0.49[b]	6.40 ± 0.62[b]	14.13 ± 0.89[a]
SL(µm)	67.28 ±1.46[a]	62.48 ± 1.05[b]	62.67 ± 1.12[b]	57.38 ± 0.80[c]
SW (µm)	45.05 ± 1.00[b]	50.74 ± 1.39[a]	44.45 ± 0.46[b]	41.66 ± 0.75[c]
SA (μm^2)	2391.88 ± 90.26[a]	2490.02 ± 77.03[a]	2189.27 ± 45.76[b]	1879.85 ± 46.46[c]
MT (µm)	240.08 ± 12.57[a]	241.09 ± 6.77[a]	203.06 ± 4.72[b]	232.14 ± 12.72[a]
Lad (µm)	105.05 ± 6.93[c]	281.86 ± 11.64[a]	195.37 ± 6.18[b]	49.22 ± 6.65[d]
Wad (µm)	94.09 ± 6.06[b]	154.03 ± 4.17[a]	96.81 ± 6.04[b]	69.30 ± 6.91[c]
Lab (µm)	51.35 ± 3.48[b]	66.82 ± 2.06[a]	50.15 ± 1.41[b]	74.61 ± 8.09[a]
Wab (µm)	59.56 ± 2.30[b]	88.83 ± 6.34[a]	61.31 ± 2.67[b]	62.47 ± 5.89[b]
CuTab (µm)	3.69 ± 0.37[a]	2.19 ± 0.08[a]	2.58 ± 0.16[a]	4.21 ± 0.42[a]
CuTad (µm)	3.29 ± 0.24[a]	3.32 ± 0.16[b]	3.33 ± 0.17[b]	3.96 ± 0.45[a]
Tchl (mg g^{-1} fw)	1.32 ± 0.18[a]	1.35 ± 0.23[a]	0.94 ± 0.07[a]	1.14 ± 0.14[a]
chl a (mg g^{-1} fw)	1.12 ± 0.16[a]	1.11 ± 0.18[a]	0.80 ± 0.06[a]	0.94 ± 0.11[a]
chl b (mg g^{-1} fw)	0.38 ± 0.05[a]	0.43 ± 0.08[a]	0.27 ± 0.02[a]	0.36 ± 0.04[a]
carotenoid (mg g^{-1}fw)	0.45 ± 0.05[a]	0.39 ± 0.07[ab]	0.27 ± 0.02[b]	0.41 ± 0.04[ab]
chl a: chl b	2.99 ± 0.09[a]	2.70 ± 0.11[b]	3.07 ± 0.13[a]	2.62 ± 0.01[b]

Different letters in the same row indicating statistically significant difference $P < 0.05$ (ANOVA)

LL, length of leaf; WL, width of leaf; SI, stomatal index; SD, stomatal density; SL, length of stomata; SW, width of stomata; SA, stomata area ; MT, mesophyll thickness; Lad, length of adaxial epidermal cell; Wad, width of adaxial epidermal cell;

Lab, length of abaxial epidermal cell; Wab, width of abaxial epidermal cell; CuTad, cuticle thickness of adaxial surface; CuTab, cuticle thickness of abaxial surface; Total chl, total chlorophyll content; chl a, chlorophyll a content; chl b, chlorophyll b content, chl a: chl b, chlorophyll a : chlorophyll b

The elliptical stomata were lightly sunken to the epidermal cells (Figure 18) and the guard cell walls were abundantly covered with cuticle. The outer ledges covered the stomatal pore, lead to a large antechamber above the stoma (Figure 18). Jeffree et al. (1971) reported that the antechamber is cavity impeding the evaporation of water and the transfer of CO_2 pathway. Therefore, the crucial function of the sunken stoma and the antechamber is limitation the stomatal opening and exclusion water transpiration. Yang et al. (2016) also revealed that the stomatal density of orchidaceae including *Paphiopedilum* was relatively low, compared with other angiosperm leaves. The report was coincided with our study providing the stomatal density in sample D (14.13 ± 0.89 mm^{-2}) was lower than previous report indicating the low values in *P. armeniacum* (29.14±1.99 mm^{-2}.) (Guan et al., 2011).These leaf traits in *Paphiopedilum* species could assist their adaptation to arid environment (Guan et al., 2011) and to water deficits (Yang et al., 2016). The natural dwelling of this plant species was customarily appeared by low soil water content and periodic water insufficiency (Guan et al., 2011).

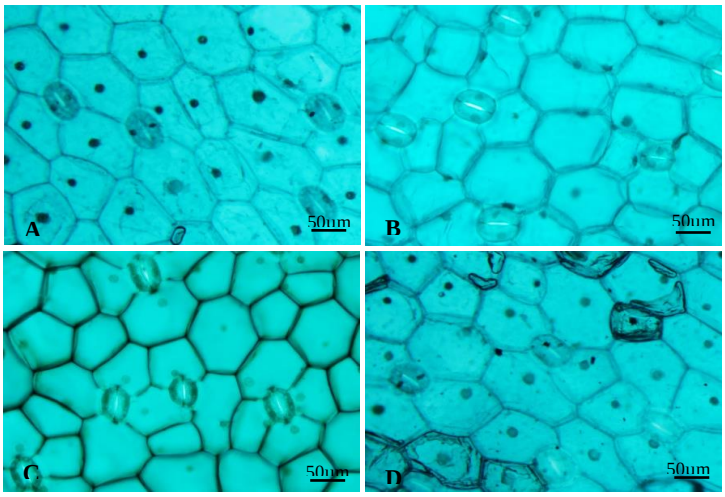

Figure 17. Abaxial epidermal leaf peels of *Paphiopedilum* species (A) sample A, (B) sample B, (C) sample C and (D) sample D.

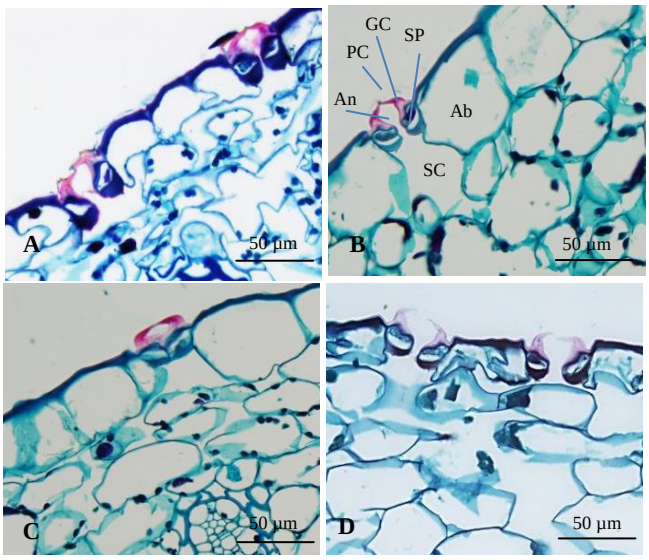

Figure 18. Transverse sections of leaves of *Paphiopedilum* species showing sunken stomata of (A) sample A, (B) sample B, (C) sample C and (D) sample D.

GC, guard cell; Ab, abaxial epidermis; PC, prominent outer stomatal ledge ; SP, stomatal pore; An, antechamber; SC, substomatal cavity.

The important pigments which are actually associated with the physiological performance are chlorophylls (chlorophyll a, chlorophyll b). The contents of chlorophyll (chl) in leaves are also associated with plant stress and senescence (Sims and Gamon, 2002; Steele et al., 2008). The contents of total chl, chl a, chl b and carotenoids among plant samples were shown in Table 10. It was found that the contents of total chl, chl a, chl b, and carotenoid among plant samples were not significantly different, this may be due to these plants were acclimatized in the same place. The highest ratio of chl a to chl b was observed in sample C, but not significantly differed with sample A.

The leaves of sample B and sample D have more mottled dark green than those of sample A and sample C (Figure 19). This observation supported the presumption of Cribb (1988) who reported that *Paphiopedilum* from Border of Malaysia (sample A) is extremely associated with *Paphiopedilum* from Rattaphum District, Songkhla Province (sample C). Furthermore, the determination of the leaf sizes among plant samples found that sample C furnished the highest of leaf size (long and wide) but not significantly different from sample A (Table 10). Cribb (1998)

identified the leaf characteristics of *Paphiopedilum* in section *Barbata* as tessellate leaf which corresponded to this present study. However, the appearance of blotch patterns among sample plants was not different (Atwood, 1984). Tessellated leaf has a crucial role as camouflage in defenses the anti-herbivore of plants growing in sun-flecked light conditions. However, there is not distinctly demonstrated that this is adaptation of *Paphiopedilum* (Givnish, 1990).

Table 11. Determination of various parameters in flower of *Paphiopedilum* samples.

Parameters		Sample plants			
mean ± SE		A	B	C	D
Flower across (cm)		12.34±1.44[a]	10.76±0.80[ab]	9.67±0.55[ab]	8.01±0.03[b]
	L (cm)	4.37±0.84[a]	4.94±0.09[a]	4.89±0.18[a]	4.60±0.44[a]
Dorsal sepal	W (cm)	4.55±0.35[a]	5.17±0.10[a]	4.62±0.13[a]	2.90±0.07[b]
	L : W	0.95±0.14[b]	0.96±0.03[b]	1.06±0.02[b]	1.58±0.11[a]
Petal	L (cm)	5.24±0.42[a]	5.63±0.39[a]	4.60±0.36[a]	4.94±0.22[a]
	W (cm)	1.53±0.19[a]	1.06±0.06[a]	1.34±0.06[ab]	1.05±0.02[b]
	L : W	3.47±0.33[b]	5.34±0.45[a]	3.45±0.29[b]	4.69±0.29[a]
Lip	L (cm)	3.24±0.30[ab]	4.36±0.72[a]	3.65±0.54[ab]	2.67±0.05[b]
	W (cm)	3.02±0.52[a]	2.36±0.22[ab]	2.18±0.20[ab]	1.86±0.04[b]
	L : W	1.10±0.12[a]	1.90±0.39[a]	1.72±0.33[a]	1.43±0.01[a]
Staminode	L (cm)	0.85±0.13[a]	0.97±0.11[a]	0.62±0.16[a]	0.60±0.06[a]
	W (cm)	1.37±0.45[a]	0.90±0.22[a]	1.03±0.05[a]	0.92±0.01[a]
Synsepal	L (cm)	2.84±0.32[a]	3.05±0.01[a]	3.05±0.26[a]	3.44±0.25[a]
	W (cm)	0.98±0.04[a]	0.86±0.15[a]	1.51±0.36[a]	1.04±0.01[a]
Ovary	L (cm)	6.04±0.68[a]	3.44±0.11[b]	2.75±0.25[b]	2.79±0.01[b]
	W (cm)	0.53±0.02[ab]	0.62±0.09[a]	0.51±0.02[ab]	0.38±0.01[b]

Different letters in the same row indicating statistically significant difference $P < 0.05$ (ANOVA), L, length; W, width

The morphological traits of flowers among four samples were exhibited in Table 11. It was found that sample A provided the highest flower cross, lip width, and ovary long. The flower across of sample B and C were not significantly different, while sample C gave the least value. This result agreed with the dendrogram which separated plants into three groups (Figure 22).

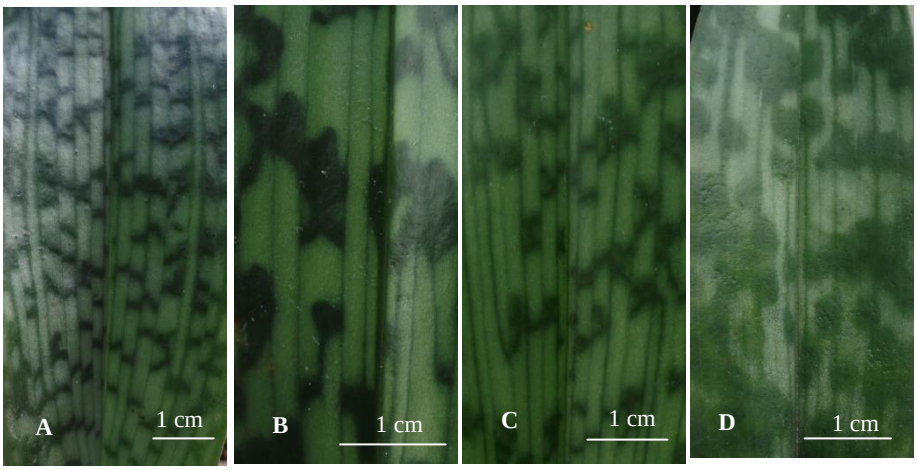

Figure 19. Mottled leaf features of *Paphiopedilum* species (A) sample A, (B) sample B, (C) sample C and (D) sample D.

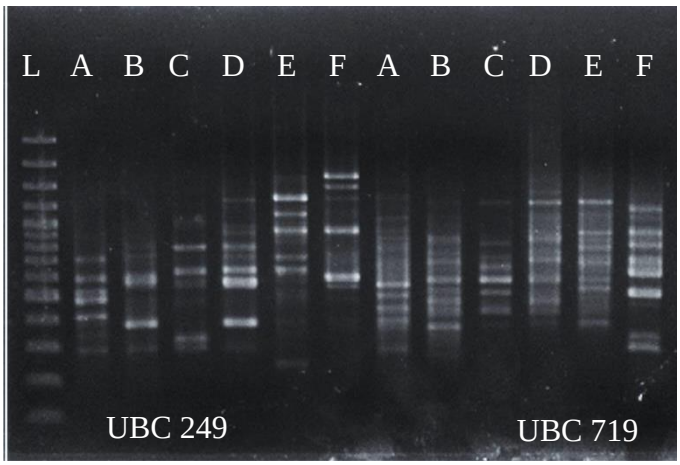

Figure 20. RAPD profiles of the plant samples by UBC-249, UBC-719. A; sample A, B; sample B, C; sample C, D; sample D, E; *Paphiopedilum exul* (control group), F; *Dendrobium crumenatum* (control group). L: 100 bp DNA ladder.

Table 12. List of primers, their sequences and GC contents of the amplified fragments generated by 12 RAPD primers.

Primer	Sequence (5´- 3´)	GC%
OPA-4	AATCGGGCTG	60
OPA-9	GGGTAACGCC	70
OPU-12	TCACCAGCCA	60
OPU-13	GGCTGGTTCC	70
OPZ-03	CAGCACCGCA	70
OPZ-11	CTCAGTCGCA	60
OPA-B-04	GGCACGCGTT	70
OPA-B-08	GTTACGGACC	60
OPA-D-04	GTAGGCCTCA	60
OPA-D-15	TTTGCCCCGT	60
UBC-249	GCATCTACCG	60
UBC-719	CCCACCCACA	70

The estimation size of bands used 100-base pair ladder (Thermo Fisher Scientific Inc.) to nearest 50 base pairs. Total of 90 primers were screened. However, only 12 primers and their guanine-cytosine (GC) contents (Table 12) provided reproducible bands. These primers produced total 181 bands, in which being 177 (97.79%) bands were polymorphic. The bands were characterized based on size and range from approximately 0.12-3.00 kb. (Figure 20). For identifying relationship among plant samples, UPGMA was utilized to generate a dendrogram based on Jaccard's genetic distance (Figure 22) and pairwise Jaccard's coefficients of similarity for all accessions are shown in Table 11. Values of genetic distance based on RAPD

(Table 13) ranged from 0.746 (sample B and sample C) to 0.541 (sample A and sample D), indicating that plant sample B and C were the greatest similar and plant sample A and D were the most different. The flower characteristics of plant samples were exhibited in Figure 21. These molecular results were conformed to the morphological data shown in the Table 11. The flower characteristics, such as lip, dorsal sepal, synsepal, petal, staminode, and ovary are the structures carrying taxonomically considerable information (Atwood, 1984).

The molecular analysis and the morphological sorting were exhibited in Figure 22 and Table 14. The lip color, the color and angle with horizontal of dorsal sepal separated plant samples into two groups. Meanwhile, the length of synsepal, the color of petal, the angle and wart of petal, the color of staminode and ovary separated plant samples into three groups.

The lips of sample A, B, and C are maroon color, while sample D is maroon and green at lower. Moreover, the dorsal sepal of sample A, B and C has white color, striped purple and lined green at base, though sample D has white lined with green.

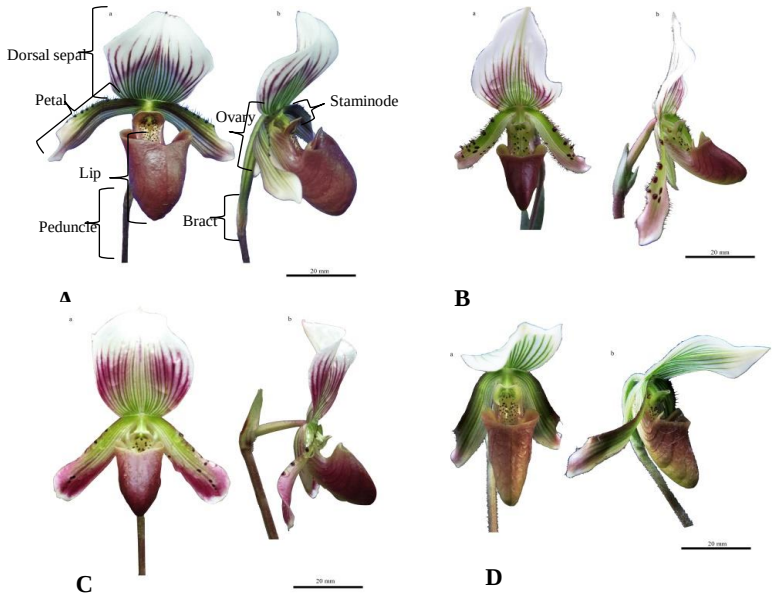

Figure 21. Flowers of *Paphiopedilum* species (A) sample A, (B) sample B, (C) sample C and (D) sample D.

In addition, the length of synsepal of sample A (2.84 cm) is differed from sample B and C (3.05 cm) and D (3.44 cm). The petal traits of sample A is green at upper and striped with purple at middle, white color at lower and lined with purple and light purple at tip. Sample B and C have light green color at upper associated with warts and lined with green, white color at lower and lined with green, purple color at tip, the petal is sigmoidal. At the same time, sample D has light green color at upper, lined green and white color lined green and purple at tip. The angle with horizontal of the petal of sample A (ca. 30.79^{o}) is separated from B and C (ca. 40.67^{o} and 46.00^{o}). The petal of D presents the angle with horizontal at ca. 62.56^{o}. Moreover, the warts at petal separated samples into three groups, 1) sample A is rarely warts at upper surface, 2) sample B has warts at upper, middle and lower surfaces of petal, while sample C has not wart at lower surface 3) Sample D has not wart at the petal both lower and upper.

The staminode color is also crucial trait separating the plant samples. Sample A has purple and green color at the middle and smeared with dark, sample B and C have white color smeared with green at the middle and sample D has light green color smeared with green.

The ovary color of sample A differs from sample B, C and D. Sample A has light green color at the middle of ovary and smeared with dark purple. Sample B and C have green color and striped with dark green, while the ovary of sample D has green color.

Table 13. Percentage of similarity matrix among plant samples based on similarity of Jaccard's estimation.

	A	B	C	D
A	100			
B	72.9	100		
C	64.1	74.6	100	
D	54.1	64.6	61.3	100

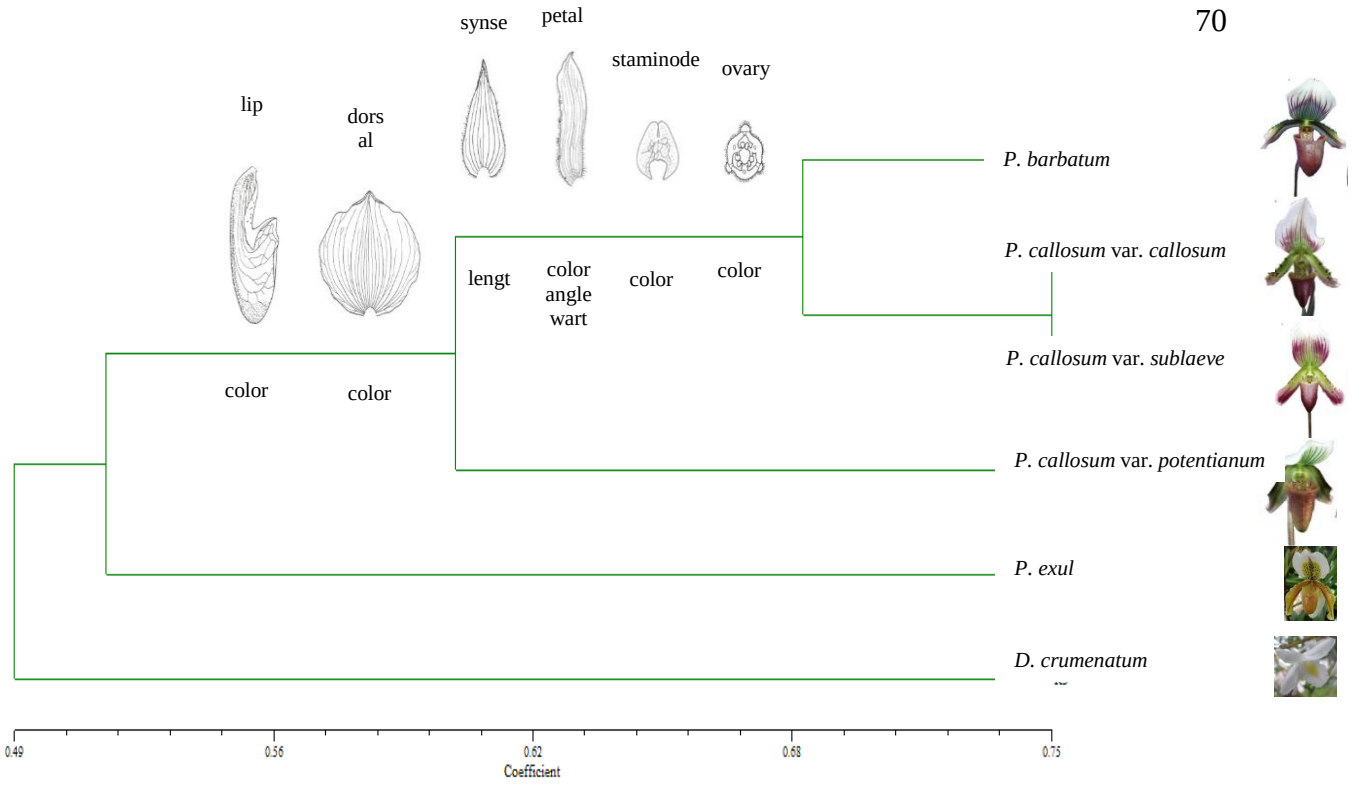

Figure 22. Dendrogram based on a combination of morphological traits and molecular RAPD markers.

The plant samples were identified by using the morphological characteristics based on Atwood (1984). The characteristics of plant samples A, B, C and D were collected from different places, sample A, B, C, and D was probably *P. barbatum*, *P. callosum* var. *callosum*, *P. callosum* var. *sublaeve* and *P. callosum* var. *potentianum* , respectively (Table 11).

Table 14. The main important characters of plant samples.

Traits/Plant samples	A	B	C	D
Leaf				
Number	5	3	4-5	3
Length (cm)	15.08	10.89	15.83	13.61
Width (cm)	3.29	2.72	3.64	2.37
Ciliate	ciliate at base	ciliate at base	ciliate at base	ciliate at base
Tessellate	tessellated pale and dark green above, pale green below	mottled pale and dark green above, pale green below	tessellated pale and dark green above, pale green below	mottled pale and dark green above, pale green below
Shape	oblong-elliptic	oblong-elliptic	oblong-elliptic	oblong-elliptic
Apices	acute	acute	acute	acute
Surfaces	wax	wax	wax	wax
Texture	leathery	leathery	leathery	leathery
Vein	abaxial keels on the mid vein	abaxial keels on the mid vein	abaxial keels on the mid vein	abaxial keels on the mid vein
Inflorescence				
Number	1	1	1	1
Peduncle color	dark purple	dark purple	dark purple	green and dark brown
Length (cm)	28.84	26.38	20.83	14.96
Bracts				
Shape	ovate	ovate	ovate	ovate
Apices	acute	acute	acute	acute
Length (cm)	1.027	1.7	2.77	1.703
Width (cm)	0.423	0.574	0.942	0.536
Color	dark green	green	green	green
Flower				
Flower across (cm)	12.34	10.76	9.67	8.01
Dorsal sepals				
Color	white with striped purple and lined green at base	white with striped purple and lined green at base	white with striped purple and lined green at base	white lined green

Table 14. The main important characters of plant samples (continued).

Traits/Plant samples	A	B	C	D
Shape	ovate	ovate	ovate	ovate
Apices	apiculate	apiculate	apiculate	apiculate
Length (cm)	4.37	4.94	4.89	4.6
Width (cm)	4.55	5.17	4.62	2.9
Angle with horizontal	ca. 73.24^{o}	ca. 81.714 ^{o}	ca. 90.469 ^{o}	ca. 17.314 ^{o}
Ciliate	pubescent on outer surface	pubescent on outer surface	pubescent on outer surface	pubescent on outer surface
Synsepal				
Color	lined with green and lightly flushed with purple at base	white at base lined with purple and light green at tip lined with green	white striped green and purple	light green lined green
Shape	ovate	ovate	ovate	ovate
Apices	acute	acute	acute	acute
Length (cm)	2.84	3.05	3.05	3.44
Width (cm)	0.98	0.86	1.51	1.04
Petal				
Color	white with purple upper, striped with veined and dark warts on upper margin	light green at upper associated with warts lined with green, white at lower lined with green, purple at tip,	light green at upper associated with warts, white in the middle, purple at tip striped with green and purple,	light green at upper lined green and white lined green and purple at tip
Shape	lanceolate	lanceolate, sigmoid	Lanceolate, sigmoid less than sample B	landceolate,
Angle with horizontal	ca. 30.79 ^{o}	ca. 40.67 ^{o}	ca. 46.00 ^{o}	ca. 62.56 ^{o}
Length (cm)	5.24	5.63	4.6	4.94
Width (cm)	1.53	1.06	1.34	1.05
L/W	3.47	5.34	3.45	4.69
Ciliate	ciliate about half of upper and lower, base of petal	ciliate about third quarter of upper and lower, base of petal	ciliate about half of upper and lower of petal	rarely ciliate
Upper ciliate long (cm)	0.104	0.104	0.2729	0.163

Table 14. The main important characters of plant samples (continued).

Traits/Plant samples	**A**	**B**	**C**	**D**
Lower ciliate long (cm)	0.10	0.08	0.21	0.14
Warty	rarely at upper petal	wart upper, lower and middle of petal	wart upper and middle of petal	none
Lip				
Color	maroon, flushed with dark maroon inside	maroon, flushed with dark maroon inside	maroon, flushed with dark maroon inside	maroon and green at lower
Warty	warty at incurve side-lobes	warty at incurve side-lobes	warty at incurve side-lobes	warty at incurve side-lobes
Ciliate	sparsely at tip margin	sparsely at tip margin	sparsely at tip margin	sparsely at tip margin
Length (cm)	3.24	4.36	3.65	2.67
Width (cm)	3.02	2.36	2.18	1.86
Shape	auricles present near apex	auricles present near apex	auricles present near apex	auricles present near apex
Angle with horizontal	ca. 56.76 ^{o}	ca. 34.96 ^{o}	ca. 53.12 ^{o}	ca. 65.06 ^{o}
Staminode				
Color	purple, green at the middle and smeared with dark purple	white smeared with green at the middle	white striped with green and purple	light green smeared green
Shape	lunate	lunate	lunate	lunate
Length (cm)	0.85	0.97	0.62	0.60
Width (cm)	1.37	0.90	1.03	0.92
Pattern	Apical lateral teeth falcate 3 teeth	Apical lateral teeth falcate 3 teeth	Apical lateral teeth falcate 3 teeth	Apical lateral teeth falcate 3 teeth
Ovary				
color	light green shaded with purple	light green and purple striped with green	light green and purple striped with green	green
Length (cm)	6.04	3.44	2.75	2.79
Width (cm)	1.53	0.62	0.51	0.38
Presumption plant	*P. barbatum*	*P.callosum* var. *callosum*	*P. callosum* var. *sublaeve*	*P. callosum* var. *potentianum*

5.4 Conclusions

The morphological identification and molecular analysis in this study were clearly known some relationship among plant samples in section *Barbata* having controversial. It is concluded that 1) samples were collected from Border of Malaysia and Cambodia was probably *P. barbatum*, *P.callosum* var. *callosum*, and *P.callosum* var. *potentianum*, respectively. Meanwhile, *P. callosum* var. *sublaeve* was probably from Rattaphum District, Songkhla Province in Thailand. 2) *P. callosum* var. *sublaeve* were the most similar with *P. callosum* var. *callosum*, and *P. barbatum* were the most different from *P. callosum* var. *potentianum*.

CHAPTER 6

CONCLUSIONS

The well-established protocol for seed germination and protocorm formation of *P. callosum* var. *sublaeve* was performed by the pre-culturing in sterile DW added with 1.0 mg/L chitosan for the first 2 weeks in the dark condition followed by the culture in MVW medium supplemented with 1.0 mg/L chitosan for the further 3 months. Moreover, MMS medium supplemented with HA (1.0 mg/L) was appropriate for shoot multiplication. Plantlets grew well with 90% survival rate in a shaded greenhouse. HA can be incorporated in culture medium to promote number of seed germination (quantity) and shoot multiplication followed by chitosan application to encourage quality protocorms and seedlings.

There were no genetic variation among the mother plants of *P. callosum* var. *sublaeve* and their own regenerated plants cultured by the *in vitro* propagation through transverse thin cell layer (tTCL) culture system. Seed germination required the water-pretreated seeds in dark for 2 weeks before being transferred, and the morphogenesis responses including PLBs, shoots and roots were successfully induced from tTCL explants of shoot tips cultured on MVW containing 1.0 mg/L TDZ.

The relationship of this species with other closed relative plants revealed that *P. callosum* var. *sublaeve* was morphologically and phylogenetically closer to *P. callosum* var. *callosum* than other candidate species.

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APPENDICES

APPENDIX A

Vacin and Went (VW) medium

Components	Quantity
Macroelements	
Potassium nitrate, KNO_3	525 mg
Potassium phosphate, KH_2PO_4	250 mg
Magnesium sulfate, $MgSO_4.7H_2O$	250 mg
Ammonium sulfate, $(NH_4)_2SO_4$	500 mg
Tricalcium phosphate, $Ca_3(PO_4)_2$	200 mg
Microelements	
Manganese sulfate, $MnSO_4.H_2O$	6.8 mg
Iron	
Ferrous sulfate, $FeSO_4.7H_2O$	27.8 mg
Disodium ethylene diaminetetraacetate, Na_2EDTA	37.3 mg
Sugar	
Sucrose	20 g

(Source: Vacin and Went, 1949)

Murashige and Skoog (MS) medium

Components		Quantity/liter	
Macroelements			
Ammonium nitrate	NH_4NO_3	165	mg
Potassium nitrate	KNO_3	190	mg
Calcium chloride	$CaCl_2 \cdot H_2O$	44	mg
Magnesium sulfate	$MgSO_4 \cdot 7H_2O$	37	mg
Potassium phosphate	KH_2PO_4	17	mg
Microelements			
Boric acid	H_3BO_3	6.2	mg
Manganese sulfate	$MnSO_4 \cdot 4H_2O$	22.3	mg
Zinc sulfate	$ZnSO_4 \cdot H_2O$	8.6	mg
Potassium iodide	KI	0.083	mg
Sodium molybdate	$NaMO_4 \cdot 2H_2O$	0.25	mg
Copper (II) sulfate	$CuSO_4 \cdot 5H_2O$	0.025	mg
Cobalt (II) chloride	$CoCl_2 \cdot 6H_2O$	0.025	mg
Iron			
Iron (II) sulfate	$FeSO_4 \cdot 7H_2O$	27.8	mg
Disodium ethylene diaminetetraacetate	$Na_2EDTA \cdot 2H_2O$	37.3	mg
Vitamin			
Nicotinic acid		0.5	mg

Murashige and Skoog (MS) medium (cont.)

Components	Quantity/liter	
Pyridoxine·HCl	0.5	mg
Thiamine·HCl	0.1	mg
Myo-inositol	10	mg
Others		
Glycine (Amino acid)	2	mg
Sucrose (Carbon source)	20	g
Agar (solidifying agent)	6.8	g

(Source: Murashige and Skoog, 1962)

APPENDIX B

Components of FAA II solution

Formaldehyde	5 % (v/v)
Glacial acetic acid	5 % (v/v)
70 % alcohol	90 % (v/v)

Dehydration solution

No.	Total alcohol (%)	Composition (mL)				
		Ethanol				
		TBA	95 % alcohol	100 % alcohol	Water	Other
1	5	-	5	-	95	
2	10	-	10	-	90	
3	20	-	20	-	80	
4	30	-	30	-	70	
5	50	10	40	-	50	
6	70	20	50	-	30	-
7	85	35	50	-	15	-
8	95	55	40	-	5	-
9	100	75	-	25	-	-
10	-	100	-	-	-	Eosin
11	-	100	-	-	-	-
12*	-	100	-	-	-	Paraffin oil 100

TBA: tertiary butyl alcohol

* Equal of TBA:paraffin oil = 1:1

Deparaffinization process

1.	xylene substitute I	3	min
2.	xylene substitute II	3	min
3.	absolute alcohol : xylene substitute	3	min
4.	absolute alcohol I	2	min
5.	absolute alcohol II	2	min
6.	95 % alcohol I	2	min
7.	95 % alcohol II	2	min
8.	70 % alcohol I	2	min
9.	70 % alcohol II	2	min
10.	50 % alcohol I (optional)	2	min
11.	50 % alcohol II (optional)	2	min

Delafied's hematoxylin and safranin staining

Reagents:

1) Delafied's hematoxylin

Hematoxylin	8	g
95 % ethanol	250	mL
Ammonium alum	16	g
Distilled water	800	mL
Glycerin	400	mL
Potassium permanganate	0.4	g

2) Safranin O

Safranin O	2.5	g
95 % ethanol	10	mL
Distilled water	90	mL

3) 0.1 % lithium carbonate

Lithium carbonate	0.1	g
Distilled water	100	mL

4) Acidulated water

Concentrated HCl	1-2	drops
Distilled water	100	mL

Staining procedure:

1.	Deparaffinization and bring to distilled water		
2.	Stain in Delafied's hematoxylin	25	min
3.	Rinse in tap water	2	min
4.	Destain in acidulate water	6	dips
5.	Rinse with tap water	1-2	dips
6.	Place in 0.1 % lithium carbonate	2	min
7.	Rinse in tap water	1-2	dips
8.	Counterstain with safranin	3	min
9.	Wash in tap water	3-4	dips
10.	Treat with acidulate water	1-2	dips
11.	Immerse in 0.1 % lithium carbonate	2-3	sec
12.	Dehydrate through graded alcohol		
13.	Clear in Xylene		
14.	Mount in Hi-mo		

Result:

Nucleus	Blue
Cytoplasm	Light pink

1 % toluidine blue O staining

Reagents:

1 % toluidine blue O

Toluidine blue O (TBO)	1	g
Sodium borate	1	g
Distilled water	100	mL

Staining procedure:

1.	Deparaffinization and bring to distilled water		
2.	Stain in 1 % TBO	1-2	min
3.	Rinse with distilled water		
4.	Dehydrate quickly through two changes of 95 % and 100 % alcohol	10	dips each
5.	Clear in xylene		

6. Mount in Hi-mo

Result:

Nucleus	Blue
Phenolic compound	Green

Periodic acid-Schiff's reaction

Reagents:

1) 1 % periodic acid

Periodic acid	1	g
Distilled	100	mL

2) Schiff's reagent

3) Harris's hemaoxylin

Hematoxylin	5	g
Absoloute alcohol	50	mL
Aluminum Ammonium Sulfate	100	g
Distilled water	1000	mL
Mercuric oxide	2.5	g

4) 1 % acid alcohol

70 % Ethanol	99	mL
Concentrated HCl	1	mL

Staining procedure:

1.	Deparaffinization and bring to distilled water		
2.	Place section in 1 % periodic acid	15	min
3.	Wash in tab water	3	min
4.	Wash in distilled water	1	min
5.	Treat in Schiff's reagent	15	min
6.	Wash in tab water	10	min
7.	Counterstain in Harris's hematoxylin	10	min
8.	Wash in distilled water	5	min
9.	Differentiate in 1 % acid alcohol	1-2	dips
10.	Wash in tap water	1-2	min
11.	Dehydrate through graded alcohol		

12. Clear in Xylene
13. Mount in Hi-mo

Result:

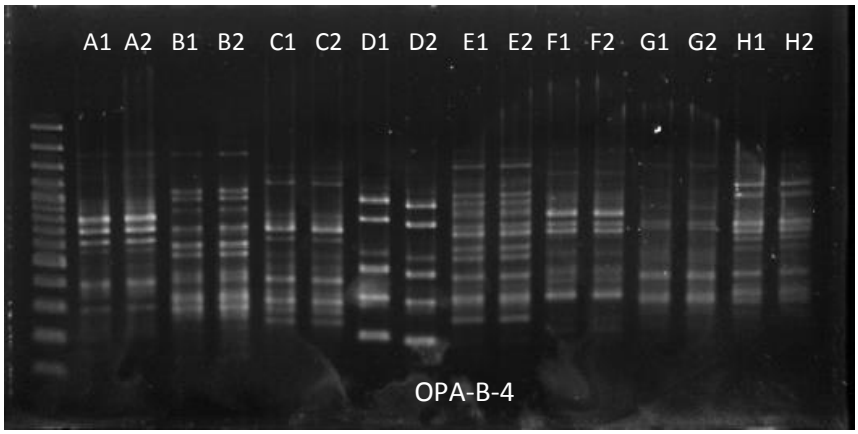
Insoluble carbohydrate	Magenta
Nucleus	Blue

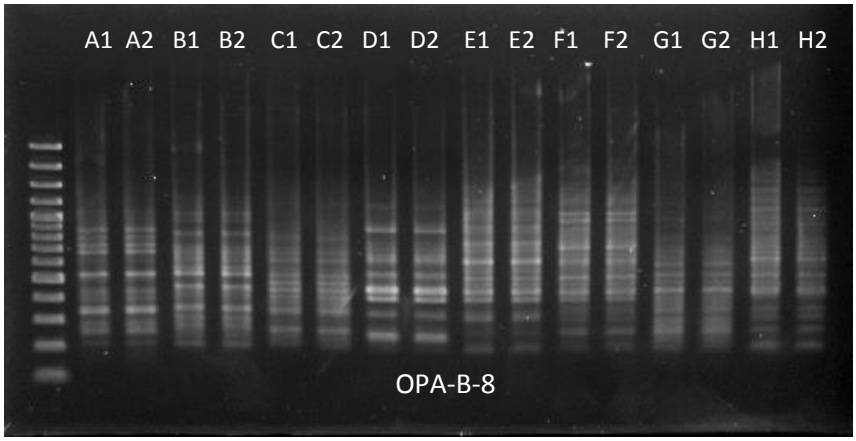
Solution for determination of chlorophyll and carotenoid content

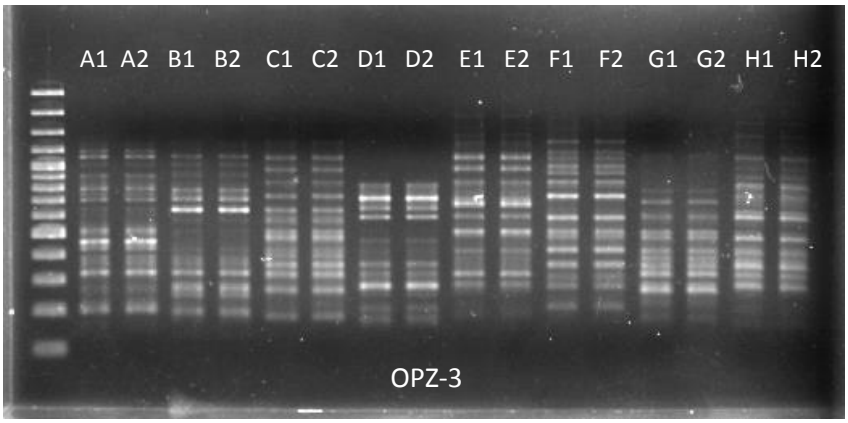
Acetone	80 % (v/v)

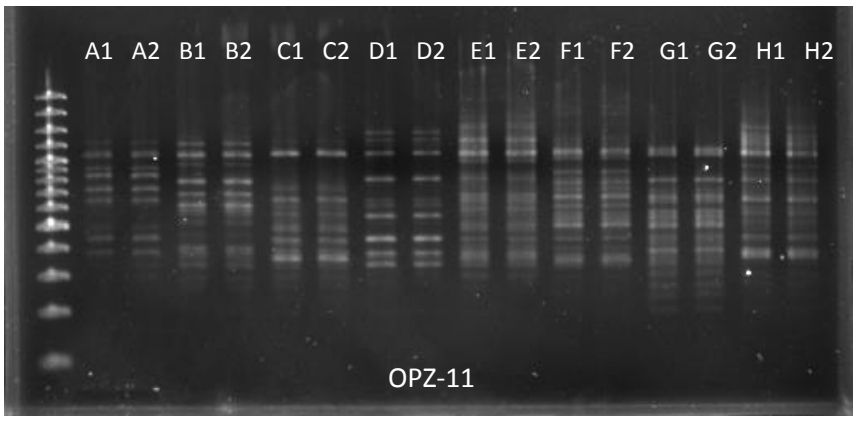
APPENDIX C

Gel Electrophoresis of PCR products (for CHAPTER 4)





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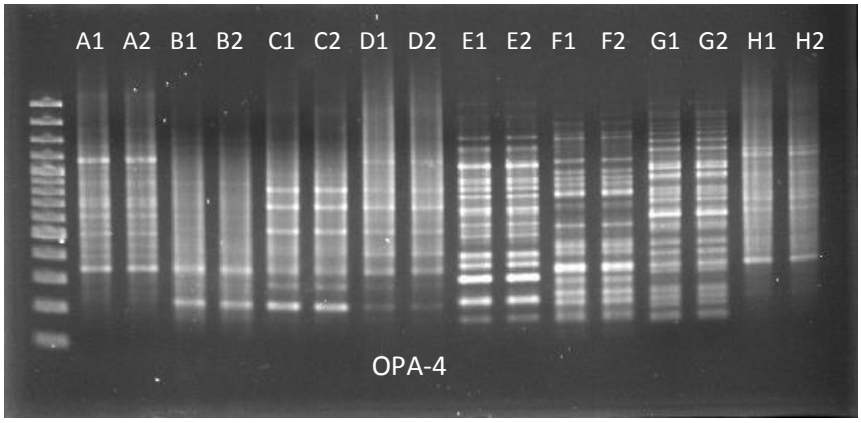
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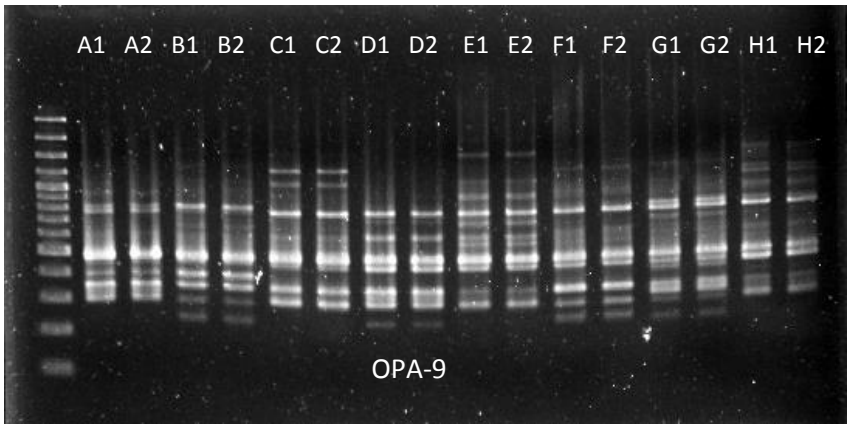
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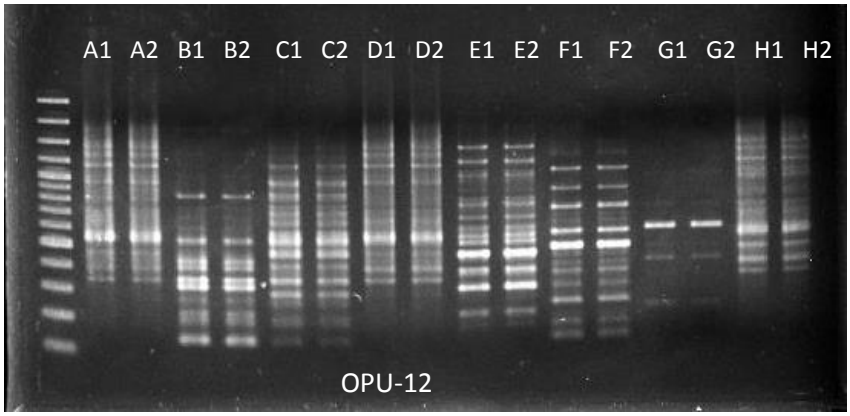
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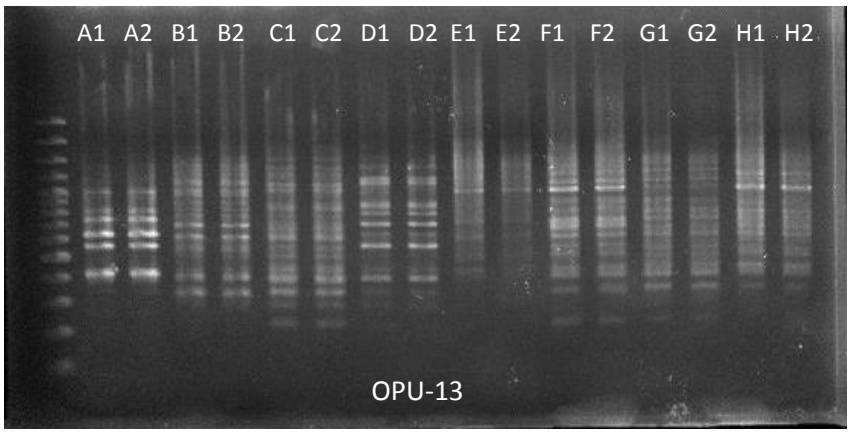
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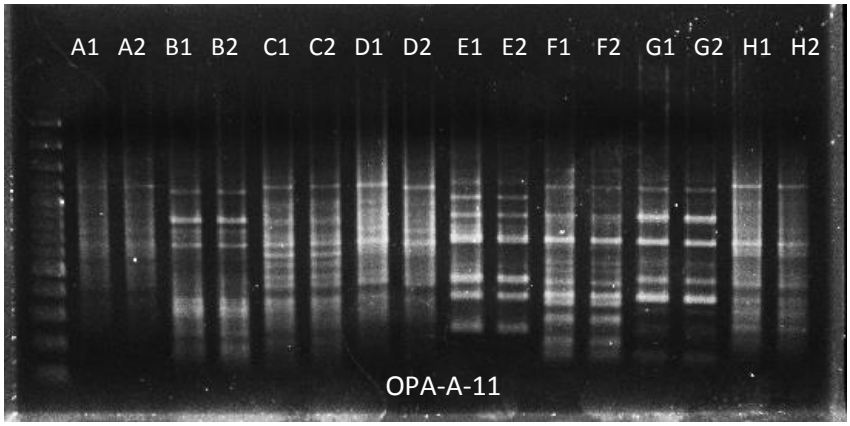
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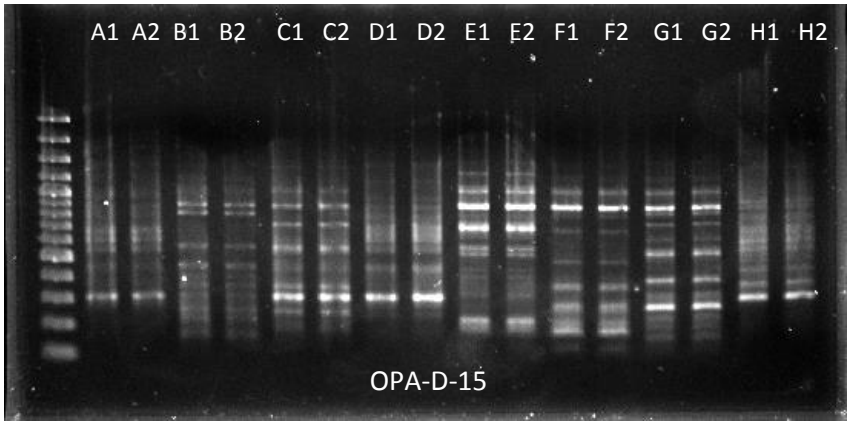
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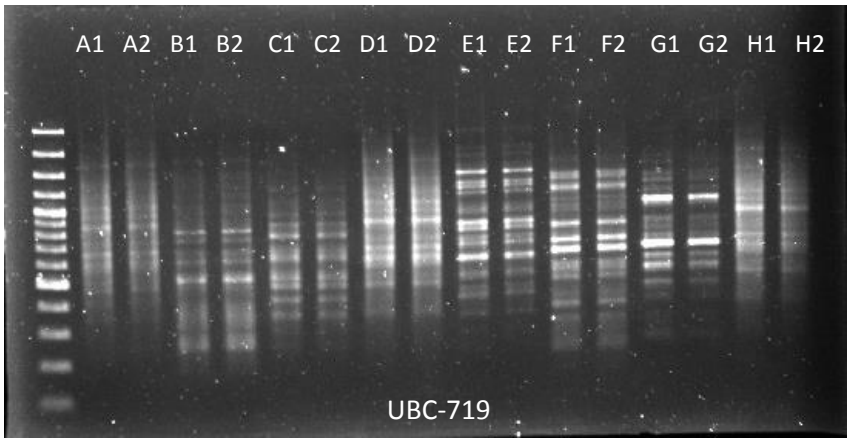
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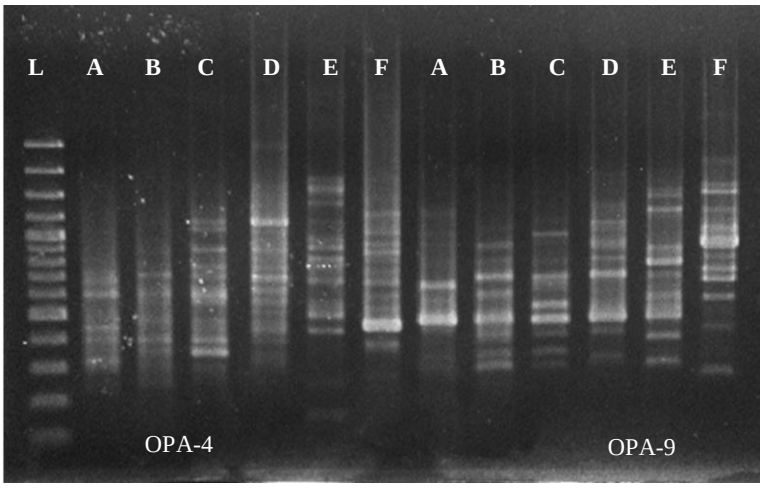
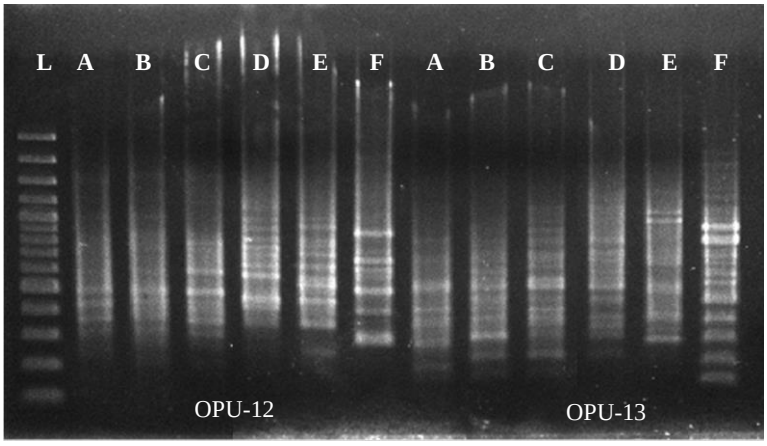
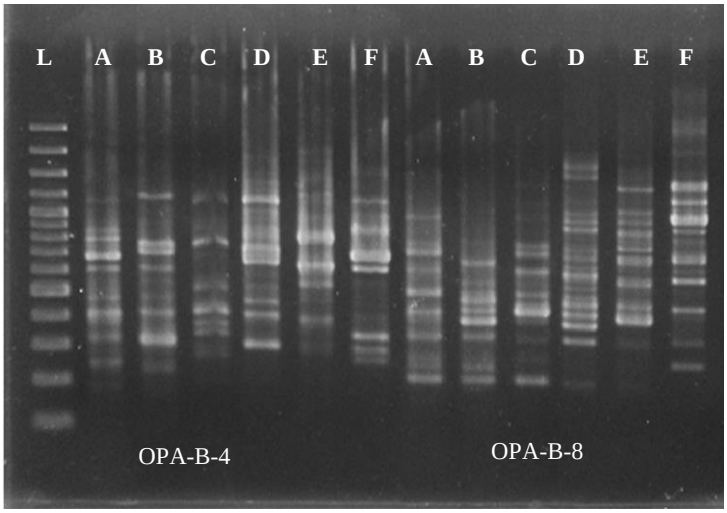
Gel Electrophoresis of PCR products (for CHAPTER 5)



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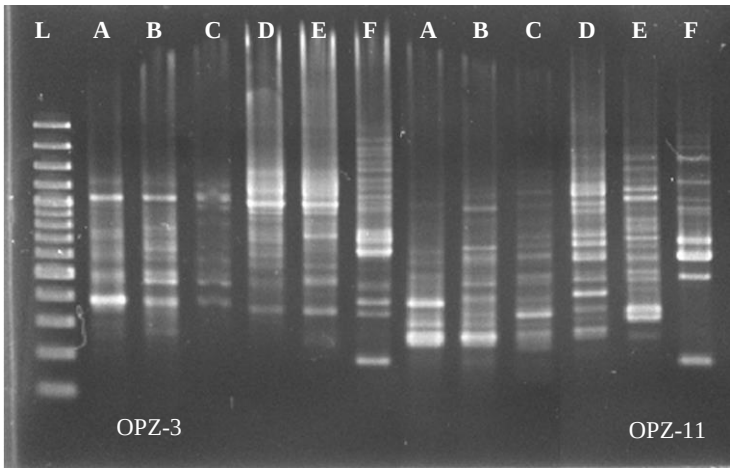
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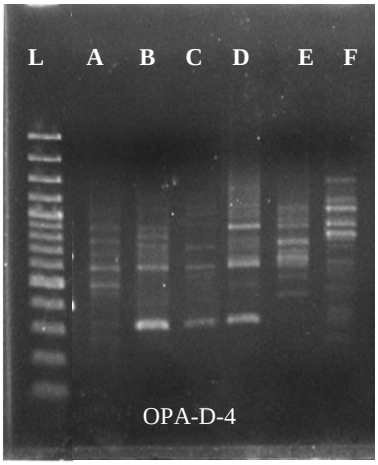
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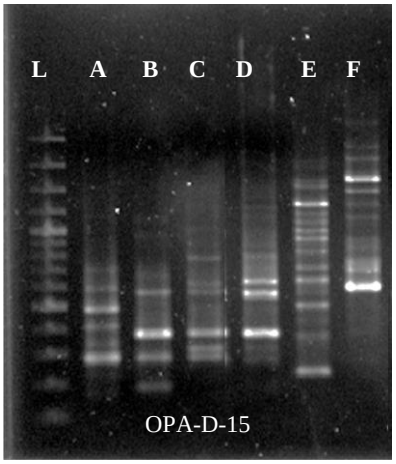
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VITAE

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Educational Attainment

Degree	Name of Institution	Year of Graduation
B.Sc. (Biotechnology)	Prince of Songkla University	1999
M.Sc. (Biotechnology)	Prince of Songkla University	2004

Scholarship Awards during Enrolment

1) Princess of Naradhiwas University's Academic Development Scholarship.
2) PhD Thesis Research Scholarship, Graduated School, Prince of Songkla University.

List of Publications and Proceedings

Paper I: Wattanapan, N., Nualsri, J., and Meesawat, U. 2016. Effects of natural additives on asymbiotic seed germination and growth of endangered lady's slipper orchid: *Paphiopedilum callosum* var. *sublaeve*. Princess of Naradhiwas University Journal. 8(3): 109–120.

Paper II: Wattanapan, N., Nualsri, J., and Meesawat, U. 2016. *In vitro* propagation through transverse thin cell layer (tTCL) culture system of lady's slipper orchid : *Paphiopedilum callosum* var. *sublaeve.* Songklanakarin Journal of Science and Technology (under revision).