CHARACTERISTICS, GROWTH AND DEVELOPMENT CYCLE, AND CROSSABILITY OF *Eulophia macrobulbon* (Par. & Rchb. f.) Hook. f. AND *E. spectabilis* (Dennst.) Suresh.

PONGNATEE PINTAJAM

DOCTOR OF PHILOSOPHY
IN HORTICULTURE

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GRADUATE SCHOOL
CHIANG MAI UNIVERSITY
APRIL 2019

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A THESIS SUBMITTED TO CHIANG MAI UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN HORTICULTURE

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Examination Committee

..................................Chairman
(Assoc. Prof. Dr. Theeranuch Jaroenkit)

..................................Member
(Assoc. Prof. Dr. Nuttha Potapohn)

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..................................Co-advisor
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Dissertation Title Characteristics, Growth and Development Cycle, and Crossability of *Eulophia macrobulbon* (Par. & Rchb. f.) Hook. f. and *E. spectabilis* (Dennst.) Suresh.

Author Mr. Pongnatee Pintajam

Degree Doctor of Philosophy (Horticulture)

Advisory Committee

Assoc. Prof. Dr. Nuttha	Potapohn	Advisor
Prof. Dr. Soraya	Ruamrungsri	Co-advisor
Asst. Prof. Dr. Chantalak	Tiyayon	Co-advisor

ABSTRACT

Characteristics, growth and development, and crossability of *Eulophia macrobulbon* (Par. & Rchb. f.) Hook. f. and *E. spectabilis* (Dennst.) Suresh. were studied in order to provide basic information for future breeding program. These researches are divided into 3 parts. First part involved in morphology, growth cycle, anatomy of flower bud during development, cytological study, and genetic relationship. Second part was crossability of two *Eulophia* and the last part was physiological study on growth and development after storing rhizome at low temperature.

Characteristics of the *E. macrobulbon* and *E. spectabilis* under cultivation condition were recorded. The results showed that several parts of morphological character of both terrestrial orchids were similar, i.e. root, rhizome, leaf, fruit, and seed, except inflorescence and flower which were different, i.e. flower shape, size, color, and number of flowers.

Growth cycles of both terrestrial orchids were deciduous type and undergone the dormant period throughout dry season. *E. macrobulbon* started vegetative shoot at early February. After that inflorescence developed from base of vegetative shoot and appeared above ground before leaves, during March to April. The aerial part of this

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plant began to die back and stayed dormant from November to January of the next year. While *E. spectabilis* started vegetative growth by shoot emerged in mid-March. Inflorescence developed at the same time as leaves. Mature inflorescence appeared between April to late May. Dormancy began in November and ended in March of the next year.

Histological flower bud development of *E. macrobulbon* and *E. spectabilis* was investigated by paraffin embedding technique. The result showed that flower bud of *E. macrobulbon* developed from the lateral of young bud on the second week from bud break. After that, it grew and developed to form floral primordia at the fourth week from bud break. Flower bud of *E. spectabilis* developed at the first week from bud break and develop to form floral primordia at the forth week from bud break.

Root tips of each species were employed for chromosome number study using Feulgen's squash method. Suitable procedure for chromosome counting was conducted. It was found that good sampling time for *E. macrobulbon* was at 9.00 A.M. whereas *E. spectabilis* was 10.00 A.M. The suitable pre-treatment duration with 0.002 M 8-hydroxyquinoline for *E. macrobulbon* and *E. spectabilis* was 24 hours. Chromosome number of *E. macrobulbon* and *E. spectabilis* was 2n=48 and 2n=52, respectively.

Genetic relationship of genus *Eulophia* was analyzed by RAPD technique. The resulted showed that OPA4, OPF1, and OPF13 primers could distinguish this genus into two groups corresponding to morphological characteristics.

Part 2 involved intraspecific, interspecific, and intergeneric hybridization between *E. macrobulbon*, *E. spectabilis*, and some terrestrial orchids that were related with *Eulophia.* Hand pollination was conducted. These studies revealed that selfed pollination of *E. macrobulbon* and *E. spectabilis* was great successful whereas interspecific hybridization between of *E. macrobulbon* and *E. spectabilis* was possible when *E. spectabilis* was used as the female parent. The intergeneric hybridization of *Eulophia* with *Geodorum attenuatum*, and *Spathoglottis affinis* was successful when using *G. attenuatum* as female parent.

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Part 3 was physiological study by storing rhizome of *E. macrobulbon* and *E. spectabilis* at low temperature, 15°C, for 0, 30, 60, and 90 days. The result showed that storing rhizome at 15°C for 60 and 90 days could delay growth and flowering of *E. spectabilis*. However, flower quality was very poor. On the other hand, low temperature could delay germination of *E. macrobulbon* but there was no flower in all treatments.

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หัวข้อดุษฎีนิพนธ์ ลักษณะ วงจรการเจริญเติบโต และความสามารถในการผสมข้ามของว่านอึ่งและว่านหัวครู

ผู้เขียน นายพงษ์นที ปินตาแจ่ม

ปริญญา วิทยาศาสตรดุษฎีบัณฑิต (พืชสวน)

คณะกรรมการที่ปรึกษา

รองศาสตราจารย์ ดร.ณัฐา โพธาภรณ์	อาจารย์ที่ปรึกษาหลัก
ศาสตราจารย์ ดร. โสระยา ร่วมรังษี	อาจารย์ที่ปรึกษาร่วม
ผู้ช่วยศาสตราจารย์ ดร. ฉันทลักษณ์ ติยายน	อาจารย์ที่ปรึกษาร่วม

บทคัดย่อ

การศึกษาลักษณะ วงจรการเจริญเติบโตและความสามารถในการผสมข้ามของว่านอึ่ง [*Eulophia macrobulbon* (Par. & Rchb. f.) Hook. f.] และว่านหัวครู [*E. spectabilis* (Dennst.) Suresh.] มีวัตถุประสงค์เพื่อใช้เป็นข้อมูลพื้นฐานในการพัฒนาเป็นไม้ดอกเศรษฐกิจในอนาคต การศึกษาครั้งนี้แบ่งการทดลองออกเป็น 3 ส่วน ส่วนที่ 1 ประกอบด้วยการศึกษาลักษณะทางสัณฐานวิทยา วงจรการเจริญเติบโต ลักษณะทางกายวิภาคของตาดอกระหว่างการพัฒนา การศึกษาเซลล์วิทยา และความสัมพันธ์ทางพันธุกรรม ส่วนที่ 2 เป็นการศึกษาความสามารถในการผสมข้ามของกล้วยไม้สกุลยูโลเฟียทั้งสองชนิด และส่วนสุดท้ายเป็นการศึกษาทางสรีรวิทยา โดยเก็บรักษาหัวกล้วยไม้ดินทั้งสองชนิดที่อุณหภูมิต่ำ พร้อมทั้งศึกษาการเจริญเติบโตหลังจากทำการเก็บรักษา

จากการศึกษาลักษณะของว่านอึ่งและว่านหัวครูในสภาพปลูกเลี้ยง พบว่า ลักษณะทางสัณฐานวิทยาส่วนต่างๆ ของกล้วยไม้ดินทั้งสองชนิดมีความคล้ายคลึงกัน เช่น ลักษณะของราก ลำต้นใต้ดิน ใบ ฝัก และเมล็ด เป็นต้น ยกเว้น ลักษณะของช่อดอกและดอกที่มีความแตกต่างกันทั้งด้านรูปร่าง ขนาด สี และจำนวนดอกต่อช่อ

วงจรการเจริญเติบโตของกล้วยไม้ดินทั้งสองชนิดมีลักษณะการเจริญเติบโตของต้นและดอกสลับกับการพักตัว โดยว่านอึ่งเริ่มแทงหน่อใบจากตาในช่วงต้นเดือนกุมภาพันธ์ ต่อมาช่อดอกได้มีการพัฒนาจากโคนของหน่อใบจนกระทั่งโผล่พ้นดินก่อนใบ การเจริญเติบโตของช่อดอกอยู่ในช่วงเดือน

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มีนาคมถึงเดือนเมษายน ตามด้วยการเจริญเติบโตทางต้น และเข้าสู่ระยะพักตัวระหว่างเดือนพฤศจิกายนถึงเดือนมกราคมของปีถัดไป ส่วนว่านหัวครูเริ่มแทงหน่อใบประมาณกลางเดือนมีนาคม โดยมีการแทงช่อดอกในช่วงเวลาไล่เลี่ยกันกับใบ ช่อดอกเจริญเติบโตเต็มที่ช่วงเดือนเมษายนถึงปลายเดือนพฤษภาคม หลังจากนั้นจะเข้าสู่ระยะพักตัวระหว่างเดือนพฤศจิกายนถึงเดือนมีนาคมของปีถัดไป

การศึกษาเนื้อเยื่อวิทยาของการพัฒนาตาดอกของว่านอึ่งและว่านหัวครู โดยใช้วิธีการฝังตัวอย่างเนื้อเยื่อในพาราฟิน พบว่า ตาดอกของว่านอึ่งมีการสร้างบริเวณด้านข้างของหน่อในสัปดาห์ที่ 2 หลังจากตาเริ่มพัฒนาและมีการพัฒนาจนเกิดจุดกำเนิดตาดอกในสัปดาห์ที่ 4 หลังจากตาเริ่มพัฒนา ในขณะที่ตาดอกของว่านหัวครูเริ่มมีการสร้างตั้งแต่สัปดาห์ที่ 1 หลังจากตาเริ่มพัฒนาและมีการพัฒนาจนเกิดจุดกำเนิดตาดอกในสัปดาห์ที่ 4 หลังจากตาเริ่มพัฒนา

การศึกษาโครโมโซมจากเนื้อเยื่อปลายราก โดยใช้วิธี Feulgen's squash พบว่า วิธีการที่เหมาะที่สุดสำหรับว่านอึ่ง คือ การเก็บตัวอย่างปลายรากเวลา 9.00 น. และหยุดวงชีพของเซลล์ด้วย 0.002 M 8-hydroxyquinoline เป็นเวลา 24 ชั่วโมง ส่วนวิธีการที่เหมาะสมที่สุดสำหรับว่านหัวครู คือ การเก็บตัวอย่างปลายรากเวลา 10.00 น. และหยุดวงชีพของเซลล์เป็นเวลา 24 ชั่วโมง เช่นกัน และจำนวนโครโมโซมของว่านอึ่งและว่านหัวครู คือ 2n=48 และ 2n=52 ตามลำดับ

จากการวิเคราะห์ความสัมพันธ์ทางพันธุกรรมด้วยเทคนิค RAPD พบว่า สามารถใช้ไพรเมอร์ OPA4, OPF1 และ OPF13 ในการจัดจำแนกกล้วยไม้สกุลยูโลเฟียได้เป็น 2 กลุ่ม ซึ่งสอดคล้องกับลักษณะทางสัณฐานวิทยาของกล้วยไม้แต่ละชนิดในกลุ่มนี้

ในส่วนที่ 2 ซึ่งประกอบด้วยการศึกษาความสามารถในการผสมข้ามภายในชนิดเดียวกัน การผสมข้ามชนิด และการผสมข้ามสกุลของว่านอึ่งและว่านหัวครูและกล้วยไม้ดินบางชนิดที่มีความใกล้เคียงกับกล้วยไม้กลุ่มยูโลเฟีย พบว่า ว่านอึ่งและว่านหัวครูสามารถผสมตัวเองได้ดี ในขณะที่การผสมข้ามชนิดระหว่างว่านอึ่งและว่านหัวครูสามารถเกิดขึ้นได้ถ้าใช้ว่านหัวครูเป็นต้นแม่พันธุ์ ส่วนการผสมข้ามสกุลระหว่างกล้วยไม้ดินยูโลเฟียทั้งสองชนิดกับว่านจูงนางและเอื้องดินใบหมาก พบว่าสามารถสำเร็จได้หากใช้ว่านจูงนางเป็นแม่พันธุ์

ส่วนที่ 3 การศึกษาทางสรีรวิทยาโดยการเก็บรักษาหัวพันธุ์ว่านอึ่งและว่านหัวครูที่อุณหภูมิ 15°C เป็นเวลา 0, 30, 60 และ 90 วัน ผลการทดลองแสดงให้เห็นว่า การเก็บรักษาหัวพันธุ์ที่ 15°C เป็นเวลา 60 และ90 วัน สามารถชะลอการเจริญเติบโตและการออกดอกของว่านหัวครูได้ แต่มีผลกระทบทำให้คุณภาพดอกลดลง ในขณะที่การเก็บรักษาที่อุณหภูมิต่ำสามารถชะลอการเจริญเติบโตของว่านอึ่งได้ แต่ไม่พบการออกดอกในทุกกรรมวิธี

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STATEMENT OF ORIGINALITY

1. *Eulophia macrobulbon* (Par. & Pchb. f.) Hook. f. and *E. spectabilis* (Dennst.) Suresh. are terrestrial orchid which have potential to be utilized as ornamental plant as well as cut flower. Thus, understanding basic information i.e. morphology, growth cycle, flower bud development, cytogenetics, and genetic relationship, will be useful for future development.

2. Orchid is one of the family that can be hybridized with the species and genus, therefore, to achieve new variety, crossability of genus *Eulophia*; intraspecific, interspecific, and intergeneric hybridization, have been conducted to improve novelty terrestrial orchid from native orchid of Thailand.

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ข้อความแห่งการริเริ่ม

1. ว่านอึ่งและว่านหัวครูเป็นกล้วยไม้ดินกลุ่มหนึ่งที่มีศักยภาพที่จะพัฒนาเพื่อใช้เป็นไม้ประดับและไม้ตัดดอก ดังนั้น การศึกษาและเข้าใจข้อมูลพื้นฐาน เช่น ข้อมูลทางสัณฐานวิทยา วงจรชีวิต การพัฒนาตาดอก เซลล์วิทยา และความสัมพันธ์ทางพันธุกรรม จะเป็นประโยชน์ต่อการพัฒนาสายพันธุ์กล้วยไม้ดินเหล่านี้ต่อไป

2. กล้วยไม้เป็นไม้ดอกกลุ่มหนึ่งที่สามารถผสมข้ามได้อย่างกว้างขวางเพื่อพัฒนาสายพันธุ์ใหม่ ทั้งการผสมข้ามชนิดและการผสมข้ามสกุล เพราะฉะนั้น การศึกษาความสามารถในการผสมภายในชนิดเดียวกัน การผสมข้ามชนิด และการผสมข้ามสกุลจึงมีความจำเป็น เพื่อสร้างกล้วยไม้ดินสายพันธุ์ใหม่จากกล้วยไม้พื้นเมืองที่พบในประเทศไทย

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CHAPTER 1

INTRODUCTION

Orchid is monocotyledon plant which belongs to family Orchidaceace. It is one of the largest family that has number of plants approximately 17,000-35,000 species (Dressler, 1998). Plant in this family has a long history of evolution, then, each species can adapt to environment surround them, for example, aquatic orchid (*Epipactis flavas*) is orchid that can grow in water without rotting (Supanatananont, 2012), epiphytic orchids that have root system adhesion to tree (Sagrik, 1973), and terrestrial orchids which evolve stem and root to be storage organ. Furthermore, flower of orchid adapts to have colorful flower to attract pollinators. In history of human, orchids have been improved for commercial use in so many ways, instance of medicinal plant, seasoning, and ornamental plant. Majority of commercial orchid are epiphytic orchid. Whereas, terrestrial orchid is not very well known although there are many species of terrestrial orchid that have potential to be used as commercial orchids in Thailand, such as *Calanthe*, *Cymbidium*, *Eulophia*, *Geodorum*, *Paphiopedilum*, and *Spathoglottis* (Thaitong, 2002)

Eulophia belongs to subfamily Epidendrodiae and tribe Cymbidiae. Underground stem of orchid in this genus is corm. Inflorescence is raceme and usually arises from the base of pseudobulb. Peduncle is erect and approximately 30-40 cm long. There are many numbers of flowers with various colors (Bose and Bhattacharjee, 1980). Besides, the good characteristics, *Eulophia* is sturdy and strong. It is easy to transport. Thus *Eulophia* is suitable to improve to be used as cut flower and ornamental plant, especially *Eulophia macrobulbon* (Par & Pchb. f.) Hook. f. and *Eulophia spectabilis* (Dennst.) Suresh. which can be found all around in Thailand and they are very easy to cultivate.

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However, little information on *Eulophia* is available. Studies on life cycle, growth and development, crossability and certain characteristics will enhance utilization of orchids in this genus. In addition, this information will be useful for novelty improvement of native orchid.

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CHAPTER 2

LITERATURE REVIEW

Orchid is a monocotyledonous plant belonging to the family Orchidaceae. It is a perennial plant and there are many species, approximately 25,000 species. They can be found around all regions in the world and have different growth pattern depending on environment, for example epiphytic orchid, lithophytic orchid, aquatic orchid, saprophytic orchid, and terrestrial orchid (Sitthersutjathan, 2006; Thaitong, 2000).

Terrestrial orchid is an orchid that grows and develops on ground. It is deciduous plant that has bulb or rhizome for storage nutrient. Their growth pattern composes of vegetative growth, reproductive growth, and dormant period. There are many genera of terrestrial orchid in Thailand, such as *Calanthe*, *Eulophia*, *Geodorum*, *Paphiopedilum*, and *Spathoglottis* (Thaitong, 2000).

2.1 *Eulophia* R. Br. ex Lindl.

Eulophia is a terrestrial orchid which belongs to tribe *Cymbidiae* and subtribe *Cyrtopodiinae* (Pridgeon, 1992). The name of *Eulophia* derives from word "Eu" that means "well" and word "lophos" that means "plume". Therefore, *Eulophia* means plant that has big crest on lip of flower (Sitthersutjathan, 2006; Pridgeon, 1992). It has sympodial growth pattern. Tuberous stem is big and function as organ. This organ forms corm to partly aerial pseudobulbs which are mostly positioned on an elongate rhizome (Linder and Kurzweil, 1999). Those subterranean tubers have been maintained for a few seasons but are easily lost unless great care is paid to the watering regime (Pridgeon, 1992). Each node of corm has white tissue. There are many leaves per plant, which are long. Pseudostem is a compactly fold leaf base (Sitthersutjathan, 2006). Inflorescence usually arises from side or base of tuberous stem. It is single or branched depended on species. Flower usually resupinate at anthesis. (Bose and Bhattacharjee, 1980; Linder and Kurzweil, 1999). Flower has various colors (Pridgeon, 1992). Sepal and petal are

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free, spreading, and almost equal. Lip is erect. Spur and column are short. There are two pollinia (Bose and Bhattacharjee, 1980).

There are approximately 200 species of *Eulophia* all around the world (Seidenfaden, 1983) with its centre of diversity is in Africa (Beaman *et al*., 1997). Generally, *Eulophia* distribute in a wide variety of habitats, from swamps to forest and sandy beaches to mountain grassland or semi-desert (Pridgeon, 1992). In Thailand, only 12 species have been found (Seidenfaden, 1983) that could be divided into two groups; the first group having distinct sidelobes and the second without sidelobes on lip (Seidenfaden and Smitinand, 1961).

Cultivation of *Eulophia* can be done using mixture of leaf soil, and sand, with some nodules of charcoal (Williams, 1894). Temperature which is suitable for growth and development of this genus, is depended on habitat. Normally, *Eulophia* can grow well at 15.5°C to 29.7°C in summer and 15.5°C in winter (Bose and Bhattacharjee, 1980).

2.2 Botanical aspect of *Eulophia macrobulbon* Par. & Rchb. f. and *E. spectabilis* (Dennst.) Suresh.

2.2.1 *E. macrobulbon* Par. & Rchb. f.

E. macrobulbon is terrestrial orchid with large subterraneous tubers (Bose and Bhattacharjee, 1980). Underground stem form is corm, dark purple in color. Leaf is oblanceolate, approximately 10 × 35 cm, and red-purple. Leaf apex is acute. Inflorescence is raceme. Flowering period is about March to April (Sitthersutjathan, 2006). Generally, flowers appear before vegetative growth (Seidenfaden and Smitinand, 1961). Inflorescence has a lot of flowers, 1 cm in diameter, purple with white lip. Sepal is oblong-elliptic. Petal is elliptic (Sitthersutjathan, 2006). Both of them are red (Onetanaphut, 1997). Lip is triangular obtuse with 2 distinct keels at terminal. Terminal lip is white. Spur is short about 3-5 mm, long and narrow (Seidenfaden and Smitinand, 1961; Onetanaphut, 1997). This terrestrial orchid can be found in dry dipterocarp forest at elevation 750 to 1,100 m above sea level (Sitthersutjathan, 2006).

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2.2.2 *E. spectabilis* (Dennst.) Suresh.

E. spectabilis is terrestrial orchid that has round subterraneous tubers (Thaitong, 2000). Underground stem form is corm, 6 × 30 cm (Sitthersutjathan, 2006), enclosed by several lanceolate sheaths (Wood, 2011). Leaf shape is lanceolate, size is approximately 3-4 cm × 20-50 cm. Leaf base folds to form pseudostem (Thaitong, 2000). Inflorescence is a raceme, erect or little bend, 1 m long, approximately 12-20 flowers (Sitthersutjathan, 2006). Peduncle is 30-70 cm long, fleshy, bearing several 2-6 cm long ovate-elliptic, acute to acuminate sterile sheath (Wood, 2011). Flower size is about 2 cm and has various colors, white, yellow-white, green, and purple (Sitthersutjathan, 2006; Thaitong, 2000). Sepal and petal are oblanceolate. Lip, 3 lobed, is ovate-oblong and strongly curved at right angles to spur. Side lobe is obscure and rounded while mid-lobe is obtuse, margin crenulate and nerves minutely papillose (Wood, 2011). Spur is large and short (Sitthersutjathan, 2006). Pedicel with ovary is slender, 2 to 2.5 cm long (Wood, 2011). Flowering period is April to June (Thaitong, 2000).

E. spectabilis can grow on open grassy area, lowland dipterocarp forest, rubber plantations, in light shade or full sunlight at 900 m elevation (Wood, 2011).

Generally, this terrestrial orchid distributes from India, Sri Lanka, and the Himalayan region to China and Indochina through Malaysia and Indonesia, the Philippines, New Guinea east to Fiji, and Tonga (Wood, 2011).

Botanical study of *E. macrobulbon* and *E. spectabilis* is less available, only some terrestrial orchids which related to both *Eulophia* have been reported. Prarasi (2006) studied characterization of *E. graminea* Lindl. The study revealed root of *E. graminea* was fibrous type. Underground stem was corm which grows and developed on ground surface. Leaf was simple type. Leaf shape was linear, acuminate acute apex, attenuate base, and entire margin. Inflorescence was raceme and branch. Flower was bilaterally symmetry. It composed of three sepals, two petals and a lip. Sepals were brown-green, linear, acuminate acute apex and attenuate base. Petals were linear with acuminate acute apex. Lip was red-brown with pink appendage on upper lip. It has two pollinia. Fruit was septicidal capsule and oblong.

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In year 2007, Chanaken reported on morphology of *E. andamanensis* Rchb.f. It was found that this terrestrial orchid has fibrous root. Underground stems grow on ground surface. The new bulb generated from bud of old bulb. Leaf was simple, linear, entire margin, acuminate acute apex and attenuate base. Inflorescence was raceme and erect. Flower was complete flower, bilaterally symmetry. It composed of three sepals, two petals and a lip. Sepals were linear, acuminate acute apex and entire margin. Petals were linear, acuminate acute apex, and green. Lip was white with brown-red network. Pollen was two. Fruit was septicidal capsule and oblong (Chanaken, 2007).

There are reports on studies of another terrestrial orchids. Research was conducted at the Huai Hong Khrai Royal Development Study Center (Ruchivanichkul, 2006). It was found that morphology of *G. recurvum* (Roxb.) Alston and *G. siamense* Rolfe ex Downie was similar, they had fibrous root system and cormous stem type. Leaves were obovate-lanceolate or lanceolate with attenuate bases and acute tip. Inflorescences were raceme with drooping rachis. Flower was bisexual, bilateral symmetry, composed of three sepals, two petals and a lip. The flower of *G. recurvum* was white in color with yellow-patched pointed-tip and densed network of purplish-red veins. In the other hand, *G. siamense* was light green in color. The lip was light green with brown streaks with yellow lip-tip.

Thongsan (2008) studied about characterization of *Geodorum* spp. collected from Khun Mae Kwuang National Reserved Forest. It showed that morphological of seven *Geodorum* was the same, fibrous root system and cormous type stem. The leaves were alternate phyllotaxis, obovate and oblanceolate with acute apex and attenuate base. Flower was perfect flower and bilateral symmetry, each having three sepals, two petals and a lip. The color was usually white with freckles of different colors and pattern.

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2.3 Growth and development cycle of *Eulophia* R. Br. ex Lindl.

Eulophia is terrestrial orchid that has underground stem**.** Growth pattern of these orchids is of deciduous type. They undergone the dormant period throughout dry season by leaving aerial part to die and growing again in rainy season (Thorut, 1994).

Correll (1950) reported that the subterraneous tuber is sympodial growth pattern that can be horizontal or ascending, for example in *Isotria*, *Spiranthes*, *Cleistes*, and

Pogonia. Normally, tissue of terrestrial orchid constitutes connect between the old and young tubers and the base of the leafy shoot (Correll, 1950).

Study of growth and development cycle of *Eulophia* and terrestrial orchid which closed related to *Eulophia*, have been reported. Prarasi (2006) studied annual growth cycle of *E. graminea* Lindl. at Huai Hong Khrai Royal Development Study Center. The plant performed had the growth period alternating with dormancy. The cycle started with inflorescence emergence at the first week of January followed by the growth of leaves at the second week of May. The plant dormant after new pseudobulb formation was completed, between November to late December. In the following year, there was a report on growth cycle of *E. andamanensis* Rchb. f. was conducted by Chanaken (2007). Annual growth cycle of this terrestrial orchid started with vegetative growth in January, while reproductive growth emerged between February to March. Leaf and pseudobulb continued to grow and develop until December.

Ruchivanichkul *et al.* (2008) studied annual growth cycle of *Geodorum recurvum* (Roxb.) Alston. It showed that the terrestrial orchid was of deciduous type which stayed dormant from November to February of the next year. The plant emerged a vegetative shoot again from the base of the corms at March, following by inflorescence emerging. Flowering period is April to mid-May. Aerial part continued to grow until October and die back again in November. In the same year, Ruchivanichkul (2008) studied growth cycle and development of *G. siamense* Rolfe ex Downie. It revealed that growth cycle of this orchid was the same pattern as *G. recurvum*, but occurred in different period. The vegetative shoot of *G. siamense* emerged at March, the floral shoot appeared at mid-April. The aerial part continued to grow until September and go to dormant between October to February.

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2.4 Floral bud development

Flower development of some plant responds to change of environment. Different kinds of plants respond in different ways. The flowering involves the response of the genes and their products to the environment (Salisbury, 1963).

Most investigation concluded that at the time of flowering, the vegetative shoot undergoes various physiological and histological changes and transforms into a reproductive shoot (Fahn, 1977).

Normally, floral process consists of 4 stages. The first is maturation stage. Plant which ready for flowering has to be at suitable age, nutrient storage, and environment. This stage is depended on species, for example, maturation stage of pea is 5 weeks, pineapple is more than 8 months and tree group, which vegetative growth alternating reproductive growth takes a time (Techapinyawat, 2005).

The second is induction stage. This stage is the first step of flowering. Metabolism of plant changes because of factor such as light, temperature, and plant age. Floral hormones are activated and moved to meristematic tissue for development to floral bud. Reproductive meristem possesses a structure fundamentally different from the vegetative meristem. A meristematic mantle is present in the apices of reproductive axes, but it is not formed from a transformation of the tunica-corpus layer like vegetative apex. The two superficial layers of the mantle give rise to the floral parts and contribute cells to a parenchymatous core (Grégoire,1938).

Philipson (1947, 1949) reported that basic function of the vegetative apex is to promote longitudinal growth of the axis, while that of the reproductive apex is to produce a meristematic envelope with a large surface area from which the parts of a flower or flower develop. Inside this meristematic envelope there is a rib meristem consisting of relatively large, vacuolated cells.

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The first change of reproductive meristem process is the increase of mitotic activity on the boundary between the central mother cell zone and the rib meristem zone. The cells of central mother cell zone become smaller and richer in protoplasm. In this way all the cells above the rib meristem are added to the tunica, the cells of which are more or less isodiametric and are relatively small. Following these change, mitotic activity and growth ceases, or almost so, in the cell of the rib meristem and of the pith below it. Thus, in the apex a parenchymatous pith surrounded by meristematic cell develops. Only the flower parts or the bract, the axillary branches of the inflorescence, and the flower themselves develop from these meristematic cell depending on the

species (Fahn, 1977). The third stage is initiation of floral primordia. Tissues grow and develop to floral bud (Techapinyawat, 2005). The fourth stage is floral development or organogenesis. Floral primordia develop floral parts; calyx, corolla, androecium, and gynoecium (Techapinyawat, 2005). After that, each of floral part grows to mature stage until anthesis.

Beyer (1942) distinguished stage of flower initiation and development of flower bulb. Stage I is vegetative stage which has leaf formation without flower initiation and floral organ formation. Stage II indicates that floral formation begins. In flower bulb, meristem tissue changes to dome forming. On the next stage, floral bud will continually develop to have the first floral primordium. This stage can be indicated with Pr, Primordium. Normally, Pr stage will occur in flower bulb with multiple flowers such as *Hyacinthus* and *Lillium.* After that, floral primordium develops to have floral organ which can be classified from flower basin order. First is outer perianthe can be designated with P1 and the inner perianthe can be designated with P2. For the male formation, the outer stamen and inner stamen formation are indicated by A1; Androecium, and A2, respectively. Finally, G; Gynoecium, indicated that the female organ formation. Order of floral organ development is different depend on plant species. In some flower bulb, there are different organ formations, for example Bract; Br, in *Lillium*, Bracteola; Bo, in *Lillium*, and Paracorolla; Po, in *Narcissus*.

Microtechnique is a technique to study on anatomical structure of plant. It usually requires some preparation of the plant sample to facilitate observation, especially; the more complex and massive tissues are usually sliced into very thin slices with freehand or with microtome. Samples are sufficiently rigid to be cut with embedded in a supporting matrix before sectioning. After that, the sections are stained and mounted to make temporary or permanent slides. The method used for preparation of a given subject depends on the character of the plant sample, including slides, equipment, reagent, and time (Sass, 1971).

Sass (1971) distinguished steps of microtechnique for botany as follows;

- Collect and subdivide plant sample; the preservation of structural details of cells and tissues is influenced by the condition of the plant at the collecting time and by the subsequent preparation for killing and fixing.

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- Killing and fixing plant tissues; it is one of the most important steps in the processing of tissues for botanical microtechnique. The killing protoplasm by killing agent must maintain or fix the structure and render the mass of plant sample firm enough to withstand the necessary handling. One of the most useful types of killing and fixing agent is Formaldehyde-Acetic acid-Alcohol agent (FAA). This fluid is stable, has good hardening action, and could store plant sample for years. They are suitable for large or impervious organs such as woody branch, tough herbaceous stem, and old root.
- Dehydration for embedding; objective of this step is to remove water from the fixed and hardened tissues. Dehydration has some washing action, and makes the plant sample firm and hard. This step consists of treating the plant sample with a series of solutions containing increasing concentrations of the dehydrating agent and decreasing concentration of water. Tertiary butyl alcohol (TBA) is the most favorite dehydrating reagent (Johansen, 1940). They have greatly extended the range of usefulness of the paraffin method by making it possible to cut plants sample that are rendered hard and brittle by ethyl or propyl alcohol or acetone.
- Infiltration and embedding in paraffin wax; the paraffin matrix in embedded tissues serves to support the tissues against the impact of the knife and to hold the parts in proper relation to each other after the sections have been cut. Infiltration consists of gradually increasing the concentration of paraffin in the solvent containing the tissues and decreasing the concentration of solvent.
- Microtome sectioning of material in paraffin; plant sample embedded in paraffin is cut with a rotary microtome, in which the knife is stationary and the piece of tissue is moved up and down past the cutting edge. Successive sample slices remain attached to each other, forming a ribbon of paraffin.
- Staining paraffin sections; the objective of this step is dye to external or internal structure for easy investigation. There are many dyes for histological study depending on the purpose structure, for example, safranin O and fast green.

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In terrestrial orchid, Prarasi (2006) studied young inflorescence of *Eulophia graminea* Lindl. It showed that 6 florets appeared on 0.17 cm young inflorescence and they had complete structure at this stage.

Chanaken (2007) studied histology on flower bud development of *Eulophia andamanensis* Rchb. f. It was found that there are 3 shoots on early bud. The middle shoot is vegetative shoot, which is dome and has leaf primordia. The floral primordia on floral shoot appeared at week 3 and had fully developed in week 5. In the following year, histological study on *Phaius tankervilleae* (Banks ex I' Heriter) Blume flower bud development was conducted. It was found that the inner tissue of bud at 1st week comprised a shoot apical meristem and a developed bract. In the 4th week more bract primordia were found. The flower primordia were initiated in the 5th week. After that, flower primordia developed into sepals, lip, and petals, in the 10th week column and in the 12th week rostellum (Chanaken, 2008).

Thahanthai (2010) studied floral development of terrestrial orchid, *Liparis paradoxa* (Lindl.) Rchb. f., *L. regnieri* Finet, *L. siamensis* Rolfe ex Downie, *L. sutepensis* Rolfe ex Downie and *Malaxis latifolia* J. E. The results showed that floral development of all species was the same. Inflorescence of each species was initiated from the apical meristem changing from vegetative to reproductive. Floral whorl developed inwardly from the outermost one. Developmental stages of inflorescence could be described as I, II, Br, Ca, Co, A, G. Timing of each stage until blooming periods depended on species.

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2.5 Cytogenetics

Cytogenetics combines two words, "cytology" and "genetic". "Cytology" means study of chromosomes and other cell components and "genetics" means study of inheritance. Therefore, content of this study includes chromosome handlings, function of chromosome, chromosome movement, behavior chromosome, number and structure of chromosomes and expression of genes (Singh, 1993).

Cytogenetics has assumed great significance in orchid breeding, for example chromosome number or chromosome structure can be indicated to relationship of parent plants (Soontornchainaksaeng, 2005) and terminology diploids, triploids, tetraploids,

pentaploids, hexaploids, and polyploids, are in common usage among orchids breeders. With wide species crosses, the hybrids are generally infertile due to poor pairing of the parental chromosomes at meiosis. If the chromosome number of such hybrids is doubled, the tetraploids or amphidiploids may be expected to be fully fertile, since they will have two sets of chromosome, or genomes, each of the parental species (Kamemoto and Sagarik, 1975).

Variation in chromosome number appears among the orchid species of Thailand. Some species, for example *Vanda denisoniana*, *Aerides odorata*, and *Doritis pulcherrima*, have both diploid and tetraploid varieties occurring in the wilds. These diploid and tetraploid varieties differ markedly in their breeding behavior. A tetraploid variety is expected to exhibit a strong influence on the progeny due to transmission of two sets of chromosomes. Besides, some orchid can be identified with cytogenetic technique, for example in genus *Paphiopedilum*, *P. callosum*, and *P. barbatum* can be readily separated from other species on the basis of chromosome number. Accordingly, chromosome numbers are important in distinguishing species or in discussions of breeding (Kamemoto and Sagarik, 1975).

Chromosome number and chromosome structure can be studied from various meristematic tissues at metaphase stage at which chromosomes have become more contracted in cell cycle (Chaiyasut, 1989; Singh, 1993; Stebbins, 1971). Normally, the tissues that can be taken to somatic chromosome study are root tip, the calyx base, young bud, the tip of young leaf or endosperm inside the seeds (Apisitwanich and Masuthon, 2000; Chaiyasut, 1989; Jones and Luchsinger, 1979; Pignone *et al*., 1994; Roy *et al*, 2010; Withner, 1974).

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Among chromosomal techniques, Feulgen's squash method is the most popular method to study chromosome number that gives flattened chromosome aligning at the same level (Campiranon, 2003; Dyer, 1979; Krasaechai, 1996). Basic principles of this method consist of pretreatment, fixative, maceration, and staining specimens.

- Pre-treatment is a step to stop spindle fiber production during cell division. Inhibiting spindle fiber production makes chromosome spread around cell. There are pre-treatment reagents to use, for example, colchicine, p-dichlorobenzene, 8-hydroxyquinoline, and naphthalene derivatives.

- Fixative is a procedure to inhibit DNA degradation metabolism to maintain chromosome of living cell. Acetic-alcohol fixative; 1:3, is a common reagent to use. The effect of acid is attributed to their breaking some of the cross-linkages between protein molecules and making available a number of lypophil radicles which associate water molecules (Woodroffe, 1941).
- Acid macerating method is a method which uses high concentration acid at suitable time and temperature to separate overlap cell to single cell. Hydrochloric acid 1M at $60^{o}C$ is a suitable reagent.
- Staining, proper staining reagent is used to dye chromosome for easy investigation. There are many dyes for chromosome depending on the purpose of the study, for example, Lacto-propionic orcein, carbo-fuchsin.

Modification of chemical and time of each step depended on species and objective of the experiment. In terrestrial orchid, Félix and Guerra (2000) studied cytogenetic and cytotaxonomy of some Brazillian species of Cymbidoid orchid having 44 species belong to 20 genera. In this study, they reported that chromosome number of *Eulophia* is 2n=32 to 2n=66.

Prarasi (2006) studied chromosome number of *E. graminea* Lindl. Sampling of root-tip should be taken at 11.00 A.M., pre-treatment with para-dichlorobenzene, fixed and then stained with carbol-fuchsin for 1 hour. It revealed that chromosome number of this species was 2n=56.

Bunnag and Theerakulpisut (2007) studied cytogenetics of some orchid species in plant genetic conservation at Khok Phu Ta Ka, Amphoe Phu Wiang, Khon Kaen. It was found that *Eulophia andamanensis* Rchb. f., *Geodorum citrinum* Jack, *Habenaria dentata* (Sw.) Schltr., and *Pecteilis susannae* L. Raf. have chromosome number 2n=42, 40, 38, and 42, respectively.

Sukkasem (2007) studied characterization, growth and development of *Calanthe cardioglossa* Schltr. It was reported that this terrestrial orchid should be prepared by sampling at 8.00 A.M., pre-treatment in para-dichlorobenzene for 36 hours and staining in carbol-fuchsin for 30 minutes. Chromosome number of the plant was 2n=44.

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In year 2008, Ruchivanichkul reported that root-tip of *Geodorum recurvum* (Roxb.) Alston and *G. siamense* Rolfe ex Downie should be collected at 11.00 A.M., pre-treatment in para-dichlorobenzene for 3 and 2 hours, respectively. The suitable staining duration in carbol-fuchsin for both terrestrial orchids was 6 and 12 hours, respectively. Chromosome number of *G. recurvum* was 2n=128 and *G. siamense* was 2n=54.

In the same year, Saetung (2008) investigated chromosome number of bamboo orchid, *Arundiana graminifolia* (D.Don) Hochr. It was found that sampling root tip at 8.00-10.00 A.M., pre-treatment root tip in para-dichlorobenzene for 3 hours, and staining with carbol-fuchsin for 30 minutes were the best method for chromosome counting for this orchid. Chromosome number of *A. graminifolia* was 2n=2x=40.

Thainuruk (2008) investigated chromosome number of *Nervilia aragoana* Gaud from 3 different locations. It showed that the best treatment of root-tip sampling was 11.00 A.M., pre-treatment in para-dichlorobenzene for 1 hour, and staining sample in carbol-fuchsin at least 30 minutes. Chromosome number of all orchid samples was the same number, 2n=72.

Boonaree (2010) studied chromosome number of 8 *Spathoglottis* species. It was found that chromosome number of *S. hardingiana*, *S. kimballiana*, and *S. vanoverburgii* was 2n=38 whereas chromosome number of *S. affinis*, *S. plicata*, and *S. petri* was 2n=40. Chromosome number of hybrids, hybrid of *S. vanoverburgii* × *S. affinis* was 2n=4x=80 and hybrid of [*S. vanocverburgii* × *S. affinis*] × *S. plicata* was 2n=3x=60.

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2.6 Molecular markers for genetic analysis

One of objectives of molecular marker is for genetic diversity identification of organism, between and within species, or between and within population, or between individuals.

DNA molecular marker is an efficient technique because of its property. DNA molecule is more stable than protein, it can be maintained at cold temperature for a long period. DNA can be found at every cell therefore they can be detected at all tissues, at any time and environment.

Random amplified polymorphic DNA (RAPD) is a DNA fingerprinting technique depending on Polymerase chain reaction (PCR) technique. This technique used a single short primer, 10-12 nucleotides long, to amplify random base of genome (William *et al*., 1990). After amplification, the amplified DNA patterns can be determined by agarose gel electrophoresis. This technique is suitable to use on unknown base sequence information of targets because of arbitrary primer. Besides, it can display on classification and genetic relationship between species. DNA fingerprint of different species should be different and of closed species should be similar or closed (Newton and Graham, 1994)

DNA fingerprint is useful for plant breeding program. Each position of DNA relates to phenotype and inherits to progeny. Therefore, this technique is used for selection of parent to be used for breeding and to confirm correct hybrid. Besides, the knowledge of genome relationship is often very useful in interspecific hybridization and intergeneric hybridization (Kamemoto and Sagarik, 1975).

Genetic relationship study of *Eulophia* is less available, only some terrestrial orchids are revealed. Choi *et al*. (2006) reported that RAPD could determine the intraspecific and interspecific relationship of oriental *Cymbidium* species. The *Cymbidium* could be divided into 2 clusters based upon ecological traits. One trait was temperate zone that included 3 species; *C. aloifolium*, *C. insigne*, and *C. lowianum*. Another trait was subtropical *Cymbidium* that comprised 12 species; *C. sinense*, *C. faberi*, *C. kanran*, *C. formosanum*, *C. forrestii*, *C. goeringii*, *C. lancifolium*, and *C. aspidistrifolium*.

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In the same year, Chung *et al.* determined genetic diversity and relationship between *Paphiopedilum* and *Phragmipedilum* species and cultivars using RAPD. They found that these orchids could be separated into 2 major subgroups. The first subgroup included all *Paphiopedilum* species and 8 *Phragmipedilum* species. The second subgroup comprised *Phrag. longiflorum*, *Phragmipedium* hybrids i.e. ‘Belle Hogue Point’, ‘Bakara LeAnn’, ‘Mem Dick Clement’, ‘Don Wimber’, and ‘Hanne Popow’ (Chung *et al.*, 2006)

In year 2014, Sinumporn studied genetic relationship of *Habenaria* and *Pecteilis* using RAPD technique. The result showed that dendrogram derived from 15 primer combination could distinguish flower color of *Habenaria* and *Pecteilis*. They could separate white flower from colored flower, white color group included *H. lindleyana*, *H. myriotricha*, *P. hawkesiana*, and *P. susanae* and a colored flower group was *H. rhodocheila* and *H. xanthocheila*. However, their result could not separate a group of *Habenaria* from *Pecteilis* (Sinumporn, 2014).

2.7 Crossabilities of *Eulophia* R. Br. ex Lindl.

In the nature, the pattern of selfing pollination within flower, autogamy, can be classified depending on occurring of anther and stigmas.

- Anther and stigma occur in same time, it can be called 'dichogamy'.
- Anther and stigma occur in the same location, it can be called 'herkogamy'.

Pattern of pollination which occurs on different flower pollination, allogamy, can be classified depending on species of parent

- If selfing within same flower species, it can be called 'Geitonogamy' in nature, 'Intraspecific hybridization' in breeding.
- If crossing between different species, it can be called 'Xenogamy' in nature, 'Interspecific hybridization' in breeding (Richards, 1997).

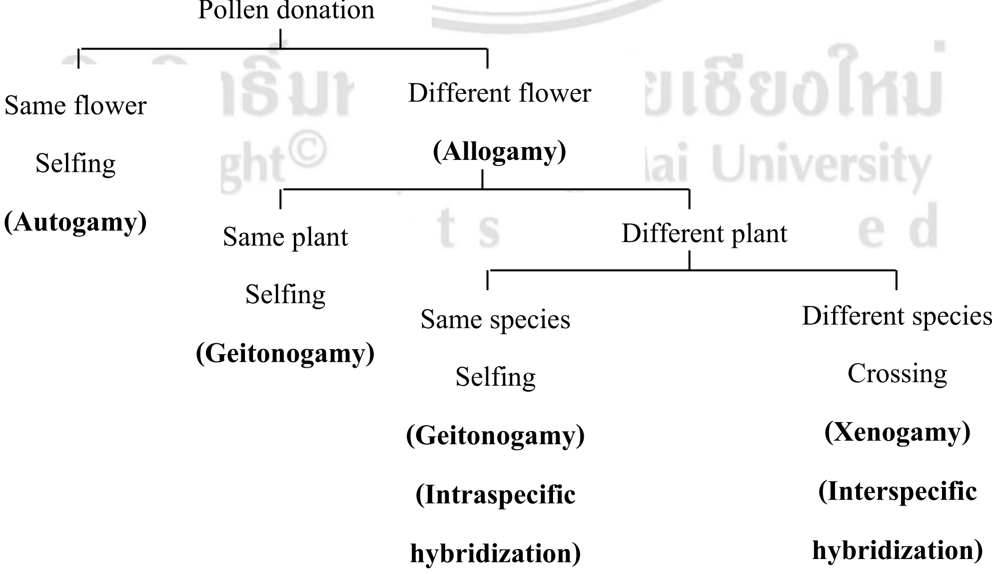

Figure 2.1 Patterns of pollen transfer within and between flowers and plants

All orchids are relatively specialized flowers. The invention of the column has encouraged the evolution of the pollinium. Numbers of ovules occur in an ovary is roughly equal as pollen nuclei occur in a pollinium. That makes fruit of orchid has very numbers of seeds after fertilization and seed is very small because of constraints of space (Richards, 1997).

The column of orchid requires very accurate patterns of behavior by the pollinator because target areas; pollinia and stigma basin, are very small. Thus, orchids tend to have very complex and pollinator-specific flowers to facilities their pollination, for example *Ophrys* can be cross-pollinated by male *Hymenoptera* which pseudo copulate with the flowers. The wasp; *Vespa* spp., visitors are attracted to the colored of *Epipactic palustris*, *E. helleborine*, *E. purpurata*, *E. atrorubens*, *E. microphylla*, and *E. gigantean* (Richards, 1997).

Self-incompatibility defined as the inability of a fertile hermaphorodite seed plant to produce zygotes after pollination (Nettancourt, 1977). This mechanism may be result from the failure of pollen grains to adhere to the stigma, or germinate on the stigma, or the failure of pollen tubes to penetrate the stigma and grow down the style (Richards, 1997)

Interspecific; crosses between species within the same genus, and intergeneric hybridization; crosses between different genus, are important method to increase the genetic diversity of species for breeding program. When sources of variation for character of interest, e.g. floral color or floral form, cannot be found within wild species, it needs to be looking from related species or genera and examine the possibility to hybridization from them into the one of interest (Brown *et al*., 2014).

Successful interspecific or intergeneric hybridization should be considered when (Brown *et al*., 2014):

- The interest expression of a character is not available within the gene pool of adapted genotype, or their unadapted counterparts from the same species.
- Acceptable expression for this character has been shown within a related species of genera.

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- It is possible to hybridization from the related species into the cultivated species.

In order to make a successful hybrid between two different species, there are number of factors need to be considered to ensure successful gene combination (Brown *et al*., 2014).

The first stage, the male and female gametes from different genotype must be fertilization and form a zygote. Failure at this stage can result from (Brown *et al*., 2014):

- Inability of pollen grain to germinate on the receptive stigma of the female parent. It can result from proteins on the pistil that interacts unfavorably with proteins in the pollen.
- Failure of pollen tubes to develop and grow down through style, or non-attraction of the pollen tube towards the ovary.
- Inability of male gametes that reach the embryo sac to actually fuse with egg cell.
- Inability of the nuclei from pollen and egg to fuse.

Sometimes success in interspecific hybridization may be undirectional, for example style length is longer than pollen tube that means pollen tube cannot reach to the ovary, and these can be overcome by the reciprocal cross. Therefore, successful hybrids might be possible from the crossing A × B, but difficult or successful when crossing as B × A (Brown *et al*., 2014).

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After fertilization, failure of seeds to develop and maturity can result from embryo or endosperm abortion. Besides, failure embryo of fruit development is another factor makes to mature seeds fail (Brown *et al*., 2014).

Maintenance of successful fruit and flower after fertilization is a factor of a dependency on having a sufficient number of developing embryos. In some interspecific or intergeneric hybrids, the number of fertilized ovule is too low. That might be due to have not enough growth regulators to stimulate seed development, for example auxin

and gibberellic acid, to make fruit development to mature. Thus, application external growth regulator to enhance fruit retention is requested (Brown *et al*., 2014).

Crossability study of *Eulophia* is less available, only some terrestrial orchids have been reported. Wongnan *et al.* (2010) studied intersectional crossability of some *Cymbidium* species; *Iridorchis*, *Cymbidium*, *Jensoa* and two hybrids. Intraspecific and interspecific of all *Cymbidium* were done as well as reciprocal cross. The results revealed that the best section of *Cymbidium* that could be crossed was *Jensoa*, at 100%, whereas incompatibilities were found in crosses between *Cymbidium* × *Iridorchis* and *Cymbidium* × hybrid.

In the following year, Kaeochumchuen (2011) studied crossability between *Spathoglottis hybrid* 'Julalux' and yellow hybrid. The results showed that selfing of *Spathoglottis hybrid* 'Julalux' and yellow hybrid were 54.00% and 71.43%, respectively. Whereas, interspecific hybridization of *Spathoglottis hybrid* 'Julalux' × yellow hybrid and reciprocal cross was 69.23% and 50.00%, respectively.

Kawchadee *et al.* (2012) studied interspecific and intergeneric crossability of some *Habenaria* and *Pecteilis*. It was found that fruit set of selfing were very successful whereas *H. erichmichaelii* pink, *H. rhodocheila*, *H. xanthocheila* were good as female plant for interspecific and intergeneric hybridization.

Tongkham *et al.* (2015) studied intersubgeneric crossability of some *Paphiopedilum* species, *Brachypetalum*, *Cochlopetalum*, *Paphiopedilum*, *Parvisepalum*, *Polyantha*, and *Sigmatopetalum*. Eighteen species were selected to study of each subgenus. Intersubgeneric hybridization of all those *Paphiopedilum* was done as well as reciprocal crosses. Moreover, *Brachypetalum*, *Polyantha*, and *Sigmatopetalum* could be crossed with all subgenus of *Paphiopedilum*. Whereas, intersubgeneric crossability of *Parvisepalum* with other subgenus was great successful, expect cross with *Cochlopetalum*.

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2.8 Physiological studies

Dormancy is a stage of a healthy bulb characterized by little or no external growth of the sprout or roots (Hertogh and Nard, 1993). Lang (1987) reported that dormancy contains a meristem with subdivision of:

- Endodormancy: dormancy response to an environmental or endogenous signal perceived by the organ itself.
- Paradormancy: dormancy response to a biochemical signal from another organ.
- Ecodormancy: dormancy result from one or more unsuitable environmental factors.

Generally, dormancy; in terms of horticultural, means no appearance of shoot or root. This occurrence might be due to unsuitable environment. However, internal examination reveals that some activities, development of leaf primordia, inflorescence formation occur during dormant period. Plant with storage organs are "dormant", exhibit no above-ground growth, during unfavorable period, and starts to grow again when conditions suitable. Generally, leaves are developed during suitable environment for growth whereas flowers appear when available insects are present for pollination (Rees, 2009).

Understanding of plant physiology, especially on flower bulbs, can assist flower production in controlling growth and development at desirable period (Rees, 2009). Several environmental factors can affect bulb growth and development, especially temperature. Hartsema (1961) reports that temperature is the major external factor to control growth, development, and flowering ornamental bulbs. In the nature, periodicity, growth and development of bulb are mainly affected by a seasonal thermoperiodicity. That constitutes the basis of the techniques used for the control of growth and flowering

The optimum temperature on flowering bulbs is depended on species. For tulip, the range is 17-20°C as the optimum range as indicated by earliness of initiation. Hyacinth and lily have higher optimum; 20-25°C, than tulip and narcissus, and iris and allium at 9-13°C (Rees, 2009).

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On all plant, temperature is important for respiration and energy supplying process of unplanted flower bulb. Low temperature reduces respiration rate and carbohydrate metabolism rate within unplanted flower bulb. Beside, increasing of respiration following time has direct influence on decreasing of soluble solid, pH and increasing of acididity, flavonoids and total phenolic compound content in unplant. That has relation on process and rate of the morphological development (Hertogh and Nard, 1993).

About effect of storage period on *Eulophia* at low temperature is less available, only some terrestrial orchid and flower bulb have been reported. Wongin (2010) studied effect of temperature and bulb storage duration on growth and flowering of *Hippeastrum* hybrid cv. Susan. Bulbs were selected and kept at 10°C before use after that they were kept at different temperature, 10 and 15°C for 4, 12 and 20 weeks. The result showed that storing bulbs at 10 and 15°C for 4-12 weeks, increased leaf length, leaf width, inflorescence stalk circumference, inflorescence stalk length, diameter of flower and bulb quality were better than other treatment.

Kitidee *et al.* (2015) studied effect of temperature and bulb storage duration on growth and flowering of *Habenaria lindleyana* Steud. Bulbs were stored at 10°C, 15°C and room temperature, about 25-30°C, and storage duration for 90, 180, 270 and 360 days. The results showed that storage temperature at 10 and 15°C for 180 days tended to delay germination and flowering of *H. lindleyana*. Later, in 2018, Kitidee *et al.* reported effect of temperature and bulb storage duration on growth and flowering of *Habenaria erichmichaelii* and *Pecteilis sagarilkii*. Bulbs were storage at 10°C, 15°C and room temperature, about 25-30°C, and storage duration for 90, 180, 270 and 360 days. It was found that the storage temperature at 10 and 15°C could extent flowering of both terrestrial for 360 days. Flowers of *H. erichmichaelii* and *P. sagarikii* gave the good quality, were stored at 10°C for 180 days (Kitidee *et al.*, 2018).

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CHAPTER 3

MATERIALS AND METHODS

The research concerned several aspects of studies involving 2 species of terrestrial orchid; i.e. *Eulophia macrobulbon* (Par. & Rchb. f.) Hook. f. and *E. spectabilis* (Dennst.) Suresh. The studies were divided into 3 parts, 1) characteristic studies, 2) crossability studies, and 3) physiological experiment to delay flowering of these orchids. The materials and methods of individual study can be described as follows:

3.1 Characterizations and growth pattern

This part is composed of 5 studies of *Eulophia* characterization; i.e. morphological studies, growth cycle, anatomical of floral bud, cytological studies, and genetic relationship. Materials and methods used in each experiment were described as follows:

3.1.1 Morphological characterization

Morphology of the plant parts, i.e. root, stem, leaf, inflorescence, flower, fruit, and seed of *E. macrobulbon* and *E. spectabilis* were individually studied at their mature stage.

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3.1.1.1 Materials

3.1.1.1.1 Plant materials

Five bulbs of *E. macrobulbon* and *E. spectabilis* were selected. The plants of both terrestrial orchids were obtained from the orchid nursery, Department of Plant and Soil Sciences, Mae Hia Agriculture Center for Research, Demonstration and Training, Faculty of Agriculture, Muang district, Chiang Mai.

3.1.1.1.2 Scientific instruments

3.1.1.1.2.1 vernier caliper

3.1.1.1.2.2 miscellaneous tool: forceps, needles, paintbrushes, and label

3.1.1.1.2.3 microscopic slides and cover slips

3.1.1.1.2.4 compound microscope; OLYMPUS CX31, Japan, with microscopic camera set; OLYMPUS CX21, Japan

3.1.1.1.2.5 The Royal Horticultural Society's color chart; PO Box 313, London

3.1.1.1.2.6 digital camera; Panasonic DMC-FS3, China

3.1.1.1.2.7 recording tool: notebook, pencil, eraser, and ruler

3.1.1.2 Methods

Investigation of the plant parts; i.e. root, stem, leaf, inflorescence, flower, fruit, and seed of both *Eulophia* was carried out. Morphology, number, and size of each organ were recorded at their mature stage, categorized as listed below, and digital photography of individual plant parts were taken.

3.1.1.2.1 Root

Record was made on shape and color of root. Diameter and length of root were also measured.

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3.1.1.2.2 Stem

Shape and color of *E. macrobulbon* and *E. spectabilis* bulb were observed. Number of bulbs per plant, number of nodes, and diameter of each tuberous stem were also recorded.

3.1.1.2.3 Leaf

Leaf phyllotaxy was identified. Characteristics of leaf blade, leaf apex, leaf base, leaf margin, and leaf color were described. Measurement was done on width, length, thickness, leaf number, and amount of veins appeared on leaf.

3.1.1.2.4 Inflorescence

Inflorescence type, shape, and color of peduncle were examined. Length and width of inflorescence were recorded as well as length and diameter of peduncle.

3.1.1.2.5 Flower

Floral symmetry, shape, and color of flower, floral whorl arrangement and component of individuals were evaluated. Width and length of flower, sepal, petal, labellum (lip), column, pollen, anther cap, and pedicel were measured.

3.1.1.2.6 Fruit

Type, shape, and color of fruits were examined. The width and length were measured.

3.1.1.2.7 Seed

Investigation of pollen morphology was conducted under compound microscope. Shape and size of pollen were recorded.

3.1.2 Growth cycle

Character and numerical data about growth at vegetative, reproductive, and dormancy stages of *E. macrobulbon* and *E. spectabilis* were recorded. Measurement was made on individual part every month and digital photography was taken to evaluate their growth pattern.

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3.1.3 Anatomical study of flower bud development

Anatomical structure of flower bud development of *E. macrobulbon* and *E. spectabilis* was investigated from longitudinal sections. These sections were permanently prepared using paraffin embedding method followed by Johansen (1940).

3.1.3.1 Materials

3.1.3.1.1 Plant materials

Buds of *E. macrobulbon* and *E. spectabilis* were studied in this experiment. The samples were taken from plants at different stages of development, 1-4 weeks from bud break of both species.

3.1.3.1.2 Chemical reagents for permanent slide preparation

3.1.3.1.2.1 formalin-acetic acid-alcohol mixture (FAA) for killing and fixing

3.1.3.1.2.2 95% ethyl alcohol, absolute ethyl alcohol, tertiary butyl alcohol (TBA) paraffin oil, and distilled water for dehydration (Sass, 1971)

3.1.3.1.2.3 paraffin; Paraplast Plus®

3.1.3.1.2.4 albumin adhesive

3.1.3.1.2.5 xylene, ether, clove oil, safanin O, and fast green for staining

3.1.3.1.2.6 canada balsam; Merck

3.1.3.1.3 Scientific apparatus

3.1.3.1.3.1 compound microscope; OLYMPUS CX31, Japan, with microscopic camera set; OLYMPUS CX21, Japan

3.1.3.1.3.2 microscopic slides and cover slip

3.1.3.1.3.3 vacuum with desiccators

3.1.3.1.3.4 glass bottles and vials

3.1.3.1.3.5 heat block and thermometer

3.1.3.1.3.6 paraffin embedding oven; 56°C

3.1.3.1.3.7 card paper for paraffin blocks

3.1.3.1.3.8 wooden block; 2×2×2 cm^3 and 2×3×2 cm^3, saturated with paraffin

3.1.3.1.3.9 rotary microtome; MODEL YIDI 1508, with microtome knife

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3.1.3.1.3.10 slide drying bench; Thermo Scientific MH6616, Britian

3.1.3.1.3.11 staining jars

3.1.3.1.3.12 miscellaneous tool: alcohol flame, slide boxes, droppers, forceps, labels, needles, paintbrushes, pencils, and scalpel handles with blades.

3.1.3.2 Methods

Chemical reagents and components of chemical solutions in this technique have been shown in Appendix A. The preparation procedures of permanent slides for histological studies of bud were described as follow:

3.1.3.2.1 Killing and Fixing

Tissues of bud were sampled, killed and fixed in formalin-acetic acid-alcohol mixture (FAA). Then, vacuum suction samples were done and kept in FAA at least 24 hours at room temperature. After that, fixed samples were dehydrated with a series of alcohol mixture as dehydration procedure.

3.1.3.2.2 Dehydration

Fixed samples were immersed sequentially in a series of dehydration having the percentages of alcohol mixtures being 50%, 70%, 85%, 95%, and 100%, each for 24 hours at room temperature (Sass, 1971). When the whole process was completed, dehydrated tissues were ready to paraffin infiltration.

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3.1.3.2.3 Paraffin infiltration

Paraffin infiltration started from soaking dehydrated samples into pure tertiary butyl alcohol (TBA) for 24 hours at room temperature, 3 times, before being infiltrated in a mixture of TBA and paraffin oil; 1:1, for 24 hours at room temperature. After that, samples were moved to pure paraffin oil for 24 hour at room temperature. While paraffin chip; Paraplast plus®, were melt in beaker kept in hot air oven at 56°C, then the sample in pure paraffin oil were moved to melted paraffin. They were left for a complete infiltration in the oven, at least for a month, before taken out for embedding**.**

3.1.3.2.4 Paraffin embedding

Melt paraffin; Paraplast plus®, was prepared and kept in the hot air oven to be used for embedding. After that, it was poured into paper boats to make a paraffin block to use for embedding sample. Orienting of the samples when embedding in paraffin was done. Air bubbles in the paraffin were eliminated with a heated needle. The embedded paraffin blocks were left hardened before taken to section.

3.1.3.2.5 Sectioning

Sectioning was made by rotary microtome. The paraffin block containing embedding sample was cut into cube or rectangular shape depending on shape and size of sample. After that, the paraffin block was attached on wooden blocks saturated with paraffin. The thickness of the paraffin ribbons was adjusted to 16 micrometers. Selected ribbon of tissue section was pasted with albumin adhesive on microscopic slides. The ribbons were left to dry before moving for staining.

3.1.3.2.6 Staining

Completely dried ribbons mounted on the slides were stained with safranin O and fast green. They were first immersed in xylene until all paraffin dissolved away, approximately 3-5 minutes. Then, the slides were hydrated in a series of ethanol and its mixtures starting from absolute ethanol: xylene; 1:1 and absolute alcohol: ether; 1:1, before passing through diluted ethanol of 95%, 70%, 50%, and 30%, respectively, each for 5 minutes. After that, the slides were placed in staining jars with safranin O for 3 days before going through dehydration process. This process started with distilled water, 3 times, and slides were moved to series of ethanol from 30%, 50%, 70%, and 95%, respectively, each for 5 minutes. Then, samples were stained with fast green about 1 minute and wash sample with clove oil at least 5 minutes. The slides were consequently immersed in absolute ethanol, 1:1 mixture of xylene and ethanol, and finished in 100% xylene, each for 5 minutes. After the whole process was done the slides were ready for permanent mounting.

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3.1.3.2.7 Mounting

Stained sample slides were mounted with cover slips using Canada balsam as an adhesive. They were left to dry before microscopic investigation.

3.1.3.2.8 Microscopic investigation

Permanent slides of the tissue samples were investigated under, OLYMPUS CX31, compound light microscope. Photomicrographing; OLYMPUS CX21, was conducted for histological studies.

3.1.4 Chromosome investigation

In this experiment, investigation of root-tip chromosomes of *E. macrobulbon* and *E. spectabilis* was conducted as indicate by Feulgen's squash method modified by Vitayasak (1996) and varying tissue sampling treatment and pre-treatment to obtain the most suitable method being a protocol and applicable for both terrestrial orchids.

3.1.4.1 Materials

3.1.4.1.1 Plant materials

The growing root of *E. macrobulbon* and *E. spectabilis* which were translucent white with the tips opaque cream to white were sampled by length of 0.5-1.0 cm.

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3.1.4.1.2 Chemical agents

3.1.4.1.2.1 8-hydroxyquinoline for pre-treatment

3.1.4.1.2.2 glacial acetic acid and 95% ethyl alcohol for fixation

3.1.4.1.2.3 hydrochloric acid for maceration

3.1.4.1.2.4 carbol-fuchsin for staining

3.1.4.1.3 Scientific apparatus

3.1.4.1.3.1 vials and glass

3.1.4.1.3.2 heat block and thermometer

3.1.4.1.3.3 microscopic slides and cover slips

3.1.4.1.3.4 compound microscope; OLYMPUS CX31, Japan, with microscopic camera set; OLYMPUS CX21, Japan

3.1.4.1.3.5 miscellaneous; needle, forceps, scissors, and label

3.1.4.2 Methods

Chemical reagent and preparing method of solution used in this investigation appear in Appendix B. The procedure of chromosome study is as follow:

3.1.4.2.1 Root-tip sampling

Root-tip of *E. macrobulbon* and *E. spectabilis* were sampled at different time; 8:00, 9:00, 10:00, and 11:00 A.M. Then, the samples were washed by shaking in distilled water before moved to vials, waiting to be pre-treated.

3.1.4.2.2 Pre-treatment of root samples

Root-tip of *E. macrobulbon* and *E. spectabilis* were collected at suitable sampling time which could yield chromosome of both terrestrial orchids at metaphase stage. The root-tip samples were moved to 0.002 M 8-hydroxyquinoline for 12, 24, and 36 hours at 10ºC before fixing.

3.1.4.2.3 Fixation

Pre-treatment root-tip was fixed in solution of 1:3 acetic acid and 95% ethyl alcohol for 7 minutes to prevent distortion of chromosome.

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3.1.4.2.4 Maceration

Fixed samples were macerated in hydrolytic solution of 1 N hydrochloric acid in warm bath at 60ºC for 10 minutes. After that, the root-tip was moved for staining.

3.1.4.2.5 Staining

Macerated root-tips were stained in carbol-fuchsin solution for 10 minutes. Then, stained root-tips were squashed.

3.1.4.2.6 Squashing

Stained samples were individually placed on microscopic slide with a drop of carbol-fuchsin. The root caps were removed before the roots were squashed, crushed, and spread on microscopic slide to prevent overlap cell and the tissue was covered by cover slips. Slides were sealed with nail polisher and ready for investigation

3.1.4.2.7 Microscopic investigation

The slides were examined under compound microscope for chromosome counting. Chromosome number from cell which intact and well scattered chromosome at least 10 sample of each species were recorded and taken photograph.

3.1.5 Genetic relationship

The objective of this experiment was to study genetic relationship of genus *Eulophia* using randomly amplified polymorphic DNA (RAPD) to provide basic information for breeding program.

3.1.5.1 Materials

3.1.5.1.1 Plant materials

Young leaves of 5 species; *Eulophia macrobulbon* (Par. & Rchb. f.) Hook. f., *E. spectabilis* (Dennst.) Suresh., *E. andamanensis* Rchb. f., *E. graminea* Lindl., and *E. flava* (Lindl.) Hook. f., total of 12 samples, were collected (Figure 3.1).

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3.1.5.1.2 Chemical agent

3.1.5.1.2.1 Cetyltrimethyl ammonium bromide (CTAB) Sodium chloride (NaCl), Tris-HCl, Ethylene diamine tetra-acetic acid (EDTA), Polyvinyl pyrrolidone 40, Proteinase K, Chloroform, Isoamyl alcohol, Isopropanol, Ammonium acetate, Ethyl alcohol, RNase A, and Liquid nitrogen for extraction.

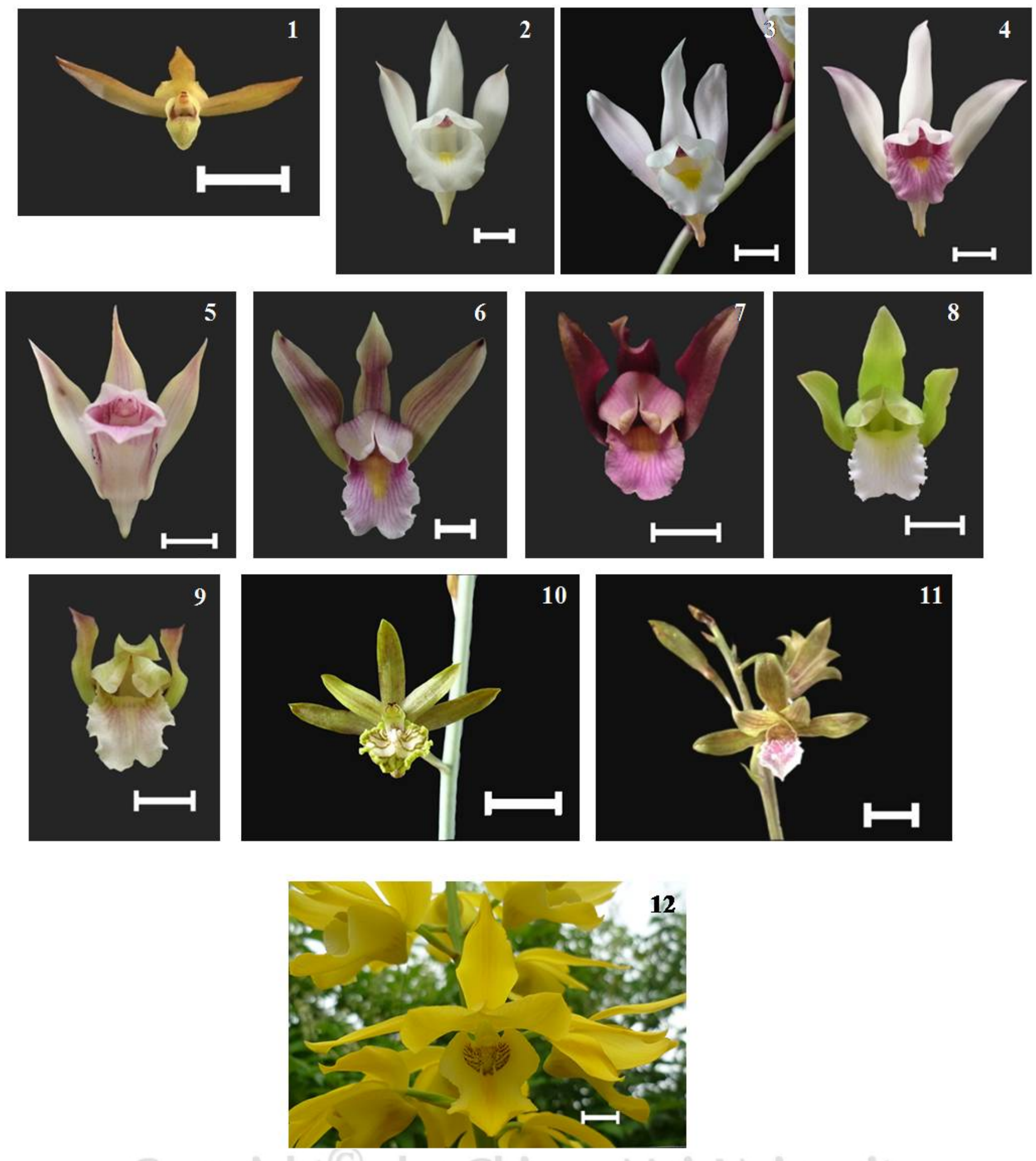

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Figure 3.1 *Eulophia* used in genetic relationship analysis: 1) *E. macrobulbon*, 2) *E. spectabilis*; white flower, white lip with yellow stripe, 3) *E. spectabilis*; pink-white flower, white lip with yellow stripe, 4) *E. spectabilis*; pink-white flower, pink lip with yellow stripe, 5) *E. spectabilis*; pink flower, pink lip with yellow stripe, 6) *E. spectabilis*; green-pink flower, pink lip with yellow stripe, 7) *E. spectabilis*; dark pink to red flower, pink lip, 8) *E. spectabilis*; yellow-green flower, white lip, 9) *E. spectabilis*; pink-green flower, pink lip, 10) *E. andamanensis*, 11) *E. graminea*, 12) *E. flava* (scale bar = 1 cm)

3.1.5.1.2.2 PCR reaction buffer, Magnesium chloride ($MgCl_2$), Deoxyribonnucleoside triphosphate; dNTP mix, 5u *Taq* DNA polymerase, and Primer for DNA amplification

3.1.5.1.2.3 Agarose, 100 to 3,000 bp DNA marker, Ethidium bromide, and 6x loading dye for gel electrophoresis

3.1.5.1.3 Scientific apparatus

3.1.5.1.3.1 autoclave

3.1.5.1.3.2 automatic pipette P2, P20, P100, P200 and P1000; Gilson Medical Electronics S.A., France,and pipette tip

3.1.5.1.3.3 -20ºC freezer

3.1.5.1.3.4 high speed microcentrifuge

3.1.5.1.3.5 microwave oven

3.1.5.1.3.6 mortar

3.1.5.1.3.7 vertex mixer

3.1.5.1.3.8 pH meter

3.1.5.1.3.9 electrophoresis apparatus; BIO-RAD, USA

3.1.5.1.3.10 gel documentation; BIO-RAD, USA

3.1.5.1.3.11 thermal cycler; Perkin Elmer Gene Amp PCR System 2400, Perkin-Elmer Cetus Co., Norwalk, Connecticut, USA

3.1.5.1.3.12 nanodrop 2000; Thermo Scientific, USA

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3.1.5.2 Methods

Chemical reagents and components of chemical solution in this experiment are shown in Appendix C. The genetic relationship studies are conducted as follows:

3.1.5.2.1 DNA extraction

Genomic DNA of each *Eulophia* was extracted from leaves using modified cetytrimethylammonium bromide (CTAB) method (Taywiya, 2010). Fresh leaf tissue; 100 mg, was placed in a mortar and ground to a powder in liquid nitrogen, 1 ml extract buffer; 2% CTAB, 5 M NaCl, 0.5 M EDTA, 1 M Tris-HCl pH 8.0, and 1% polyvinylpyrrolidone, was added and homogenized. Proteinase K, 1 mg/µl, volume 10 µl was added in a mixture and treated at 60°C for 30 minutes, mixed every 10 minutes, then, extracted once with 500 µl chloroform-isoamyl alcohol; 24:1 v/v, 500 µl to obtain a clear supernatant. Supernatant containing the plant genomic DNA was transferred to a fresh tube after centrifugation at 10,000 rpm for 10 minutes at 4°C. The DNA was precipitated from the supernatant by adding one volume of cold isopropanol, then, and incubated at 4°C overnight, and then centrifuged at 10,000 rpm for 10 minutes at 4°C. After that, DNA pellet was rinsed with cold buffer; 10 mM ammonium acetate, 75% ethanol, and cold 75% ethanol and centrifuged at 10,000 rpm for 5 minutes. The DNA was then air dried. The dried DNA was resuspended in 40 µl of TE buffer; 10 mMTris-HCl pH 8 and 1 mM EDTA, and 10 unit of RNase A was added into the mixture, then, put into incubator at 37°C for 30 minutes. Concentration of DNA was determined by measuring the absorbance at 260 nm. The DNA quality was assessed by running on a 1.2% agarose gel stained with ethidium bromide.

3.1.5.2.2 RAPD amplification

RAPD amplification was performed in a total volume of 20 µl that contained; 10 ng template DNA, 100 µM dNTP mix, 100 ng OPA or OPF primer, 1x PCR buffer, 1.5 mM $MgCl_2$, 0.8 unit *Taq* DNA polymerase and sterilized water. Amplification was performed in a thermal cycler; Perkin Elmer Gene Amp PCR System 2400, Perkin-Elmer Cetus Co', Norwalk, Connecticut, USA. The PCR program was conducted as indicated by Sinumporn (2014). Denaturation was performed at 94°C for 3 minutes before beginning the cycling protocol. An amplification cycle consisted of 30 second at 94°C, 1 minute at 43.6^{o}C, and 1 minute at 72°C. A total of 40 cycles were performed. The cycling was terminated with final extension at 72°C for 8 min. Amplified products were examined on a 1.8 % agarose gel stained with ethidium bromide.

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3.1.5.2.3 **Data analysis**

RAPD bands were recorded as present; 1 or absent; 0 and assembled into data matrix, genetic distances were calculated using UPGMA, unweighted pair group method with arithmetic averages, for clustering and drawing a dendrogram.

3.2 Crossability studies

Intraspecific, interspecific, and intergeneric crossabilities of *E. macrobulbon* and *E. spectabilis* were studies in order to provide basic information for future breeding program. Each of experiment consists of hybridization, seed viability, and seed germination studies.

3.2.1 Materials

3.2.1.1 Plant materials

E. macrobulbon (Par. & Rchb. f.) Hook. f. and *E. spectabilis* (Dennst.) Suresh., *Geodorum attenuatum* Griff, and *Spathoglottis affinis* de Vries were cultivated at orchid nursery, Mae Hia Agricultural Center for Research, Demonstration and Training, Muang district, Chiang Mai University, Chiang Mai province.

3.2.1.2 Chemical agents

3.2.1.2.1 60% lacto-propionic orcein

3.2.1.2.2 20% Clorox

3.2.1.2.3 sterile distilled water

3.2.1.2.4 Vacin and Went (VW, 1949) medium

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3.2.1.3 Scientific instruments

3.2.1.3.1 orchid breeding tools; toothpick and plastic label

3.2.1.3.2 digital camera; Panasonic DMC-FS3, China

3.2.1.3.3 recording tool: notebook, pencil, eraser, and ruler

3.2.1.3.4 microscopic slides and cover slips

3.2.1.3.5 compound microscope; OLYMPUS CX31, Japan, with microscopic camera set; OLYMPUS CX21, Japan

3.2.1.3.6 laminar air flow

3.2.1.3.7 autoclave

3.2.1.3.8 12 oz jar

3.2.1.3.9 miscellaneous; needle, forceps, scissors, scalpel handles with blades, and alcohol frame

3.2.2 Methods

3.2.2.1 Hybridization studies

3.2.2.1.1 Intraspecific hybridization

E. macrobulbon and *E. spectabilis* were employed for selfing. Hybridization was done during 8:00 A.M. to 10:00 A.M. Anther cap was removed, by using clean toothpick to touch pollinia which were at the top of column of male parent flower. After that, pollinia were carried and pressed into the stigma which was under the male part of female parent flower which was removed its pollinia. Each of terrestrial orchids of hybridization was done at least 5 flowers. Percentage of fruit set was recorded by measuring from ration of number of hybridized flower and number of seed pots.

3.2.2.1.2 Interspecific hybridization

Hybridization between *E. macrobulbon* and *E. spectabilis* was done as well as reciprocal crosses as described in 3.2.2.1.1

3.2.2.1.3 Intergeneric hybridization

E. macrobulbon and *E. spectabilis* were crossed with other terrestrial orchids which are in the same subfamily, for example *G. attenuatum* and *S. affinis* and reciprocal crosses were also made as described in 3.2.2.1.1

3.2.2.2 Seed viability

Seed viability was done followed by modified Wongnan and Potapohn's methods (2010). Seeds were stained with 60% lacto-propionic orcein for 10 minutes and seeds were examined under compound microscope. Viable seeds showed white embryo inside whereas non-viable seed showed empty embryo or atrophy. Seed

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viability of each hybrid was done using 3 fruits and 5 samples per fruit. Percentage of seed viability was recorded by counting number of viable seeds per total seed counted on microscopic slides and then calculated viable seed percentage.

3.2.2.3 Seed germination

Mature fruit, *E. macrobulbon*, *E. spectabilis*, and *G. attenuatum* were approximately 5 months, *S. affinis* was approximately 1 month, were steriled with 20% Clorox for 20 minutes. After that, fruit were rinsed with steriled distilled water 3 times in laminar air flow cabinet. Sterilized fruits were dissected longitudinally with a sterile surgical blade. Seeds were scooped out and sown on Vacin and Went (VW, 1949) medium. Seeds were spread on surface VW medium in 12 oz jar. Jars were closed and kept at 25°C in dark condition. Three capsules were employed in each species and 3 jars were used per capsules. Percentage of seed germination was recorded after 180 days from seed sown; Germination ratio was calculated by total area of media, which seeds were sown; as 100%, area of seed germination was measured and then compared to total area, germinated seed in all area was 100%, no germinated seed was 0% and in between was calculated according to germinated seeds area.

3.3 Physiological studies

This part composed of 2 studies, effect of storage period of *E. macrobulbon* and *E. spectabilis* at low temperature on flowering and total non-structural carbohydrate (TNC) analysis of both terrestrial orchids in various treatments.

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3.3.1 Effect of storage period on *E. macrobulbon* and *E. spectabilis* at low temperature

3.3.1.1 Materials

3.3.1.1.1 Plant materials

Sixteen bulbs, same size and weight, of *E. macrobulbon* and *E. spectabilis* were selected. Both *Eulophia* were grown at the orchid nursery, Department of Plant and Soil Sciences, Mae Hia Agriculture Center for Research, Demonstration, and Training, Faculty of Agriculture, Chiang Mai University, Muang district, Chiang Mai.

3.3.1.1.2 Scientific instruments

3.3.1.1.2.1 cold storage

3.3.1.1.2.2 basket

3.3.1.1.2.3 vernier caliper

3.3.1.1.2.4 digital camera; Panasonic DMC-FS3, China

3.3.1.1.2.5 recording tool: notebook, pencil, eraser, and ruler

3.3.1.2 Methods

Bulb of *E. macrobulbon* and *E. spectabilis* were stored at 15ºC for 0; room temperature, 30, 60, and 90 days. After treatment, 4 bulbs of each species were grown in eight-inch plastic pod using sand and coconut duct ratio 1:1. Completely Randomized Design (CRD) was used in this experiment. Day of germination, plant height, percentages of flowering, peduncle length, and flower size were recorded.

3.3.1.2.1 Day of germination

Numbers of day of bud that emerge from bulb in each treatment were recorded.

3.3.1.2.2 Plant height

Lengths of pseudostem from base of pseudostem to apex were measured at mature stage.

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3.3.1.2.3 Percentage of flowering

Percentages of flowering were calculated from ration of number plant that gave flower per plant in each treatment.

3.3.1.2.4 Peduncle length

Lengths of peduncle were recorded from base of peduncle to apex.

3.3.1.2.5 **Flower size**

Mature flower size were recorded. The length of the flower was measured from left to right petals.

3.3.2 Total non-structural carbohydrate

3.3.2.1 Materials

3.3.2.1.1 Plant materials

Twenty-seven bulbs of *E. macrobulbon* and *E. spectabilis* were selected. Both *Eulophia* were grown at the orchid nursery, Department of Plant and Soil Sciences, Mae Hia Agriculture Center for Research, Demonstration and Training, Faculty of Agriculture, Chiang Mai University, Muang district, Chiang Mai.

3.3.2.1.2 Chemical agent

3.3.2.1.2.1 Anhydrous sodium carbonate (Na_2CO_3), Sodium potassium titrate ($NaKC_4H_4O_6$), Sodium bicarbonate ($NaHCO_3$), Anhydrous Sodium Sulfate ($NaSO_4$), Copper Sulfate ($CuSo_4.5H_20$), and Sulfuric acid (H_2SO_4) for preparing Nelson's alkaline copper reagent.

3.3.2.1.2.2 Ammoniummolybdate [$(NH_4)_6Mo_7O_24.4H_2O$], Sodium dehydrogenarsenate ($Na_2HArSO_4.7H_2O$), and Sulfuric acid (H_2SO_4) for preparing arsenomolybolic acid reagent.

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3.3.2.1.2.3 Sulfuric acid (H_2SO_4) and Sodium hydroxide (NaOH) for pH adjustment.

3.3.2.1.3 Scientific apparatus

3.3.2.1.3.1 water bath; DAIKI SCIENTIFIC Dk-WB002, Korea

3.3.2.1.3.2 pH meter; Sartorious PB11,Germany

3.3.2.1.3.3 volumetric flask100 ml

3.3.2.1.3.4 Whatman #5 paper

3.3.2.1.3.5 pipette

3.3.2.1.3.6 vertex mixer; Scientific Industries GENIE 2, USA

3.3.2.1.3.7 spectrophotometer; Thermo Scientific GRNESYS 10SVIS, Germany

3.3.2.2 Methods

Chemical reagents and component of chemical solution in this experiment are shown in Appendix E. TNC analysis was done as follow:

3.3.2.2.1 TNC extraction

The first bulb meant the new bulb which developed in this year, the second bulb meant the bulb from the last year, and the third bulb meant the oldest bulb from the last second year (Figure 3.2) derived from each treatment were sliced and dried at 60ºC for 72 hours. Dried samples; 0.2 g, were grounded to powder. H_2SO_4, 0.2 N; 40 ml, was added in sample and incubated at 100ºC for 60 minutes. The mixture was adjusted pH to 7.0 and sterilized water was added to final volume at 100 ml. Then, the mixture was infiltrated with whatman #5 paper before analyze TNC.

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Figure 3.2 *Eulophia macrobulbon* and *E. spectabilis* bulb for TNC extraction

1) the first bulb; the new bulb

2) the second bulb; the old bulb from the last year

3) the third bulb; the oldest bulb

3.3.2.2.2 TNC analysis

Standard curve was done using D-glucose concentration 0, 0.02, 0.06, 0.08, 0.10, 0.12, 0.14, 0.16, 0.18, and 0.20 mg/ml.

Extraction sample; 1 ml, was added with 1 ml Nelson's alkaline copper reagent and incubated in boiled water for 20 minutes. After that, 1 ml Arsenomolybolic acid reagent was added in mixture and shake to be homogenized. The mixtures were adjusted volume to 10 ml. Finally, concentrations of D-glucose of each treatment were determined by measuring the absorbance at 540 nm and compared with standard curve.

Total nonstructural carbohydrate was calculated from volume of D-glucose per sample dried weights.

$$\text{TNC} = \frac{\text{(mg) glocose equaivalent } \times \text{volume make}}{\text{Wt. of sample } \times \text{volume take}}$$

volume make = final volume after adjust pH to 7

volume take = volume of solution that measure the absorbance

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CHAPTER 4

RESULTS

Results of this research are described in 3 parts according to specific objectives as designated earlier. Part 1; characterization and growth pattern of *Eulophia macrobulbon* (Par. & Rchb. f.) Hook. f. and *E. spectabilis* (Dennst.) Suresh. This part included the studies involving morphology, growth cycle, anatomy of flower bud, cytological study, and genetic relationship between both terrestrial orchids and other species of *Eulophia*, Part 2; intraspecific, interspecific crossabilities of two *Eulophia*, and intergeneric crossability between *E. macrobulbon*, *E. spectabilis*, *Geodorum attenuatum* Griff. and *Spathoglottis affinis* de Vries. The final part was physiological study; rhizome of *E. macrobulbon* and *E. spectabilis* were kept at low temperature, growth and development after storing were observed.

4.1 Characterizations and growth pattern

4.1.1 Morphological characterization

Morphology of the plant parts, i.e. root, stem, leaf, inflorescence, and fruit of 2 species were individually studied and taken a picture at their mature stage. Descriptive morphological characteristics of the plant species were recorded.

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4.1.1.1 *Eulophia macrobulbon* (Par. & Rchb. f.) Hook. f.

4.1.1.1.1 Root

Observations revealed that the roots of this plant were fibrous type that germinated from base of tuberous stem and spread around tuberous stem. They were thick, fleshy, unbranched, and white (N155A) when they were still young but became grayed-orange (164B) when they were older (Figure 4.1). There were root hairs on surface of root, and root tips were entire and green-white (157A). A single

Figure 4.1 Root of *Eulophia macrobulbon* (Par. & Rchb. f.) Hook. f.
(scale bar = 1 cm)

plant had 9-17 roots. Diameter and length of these roots were 0.39±0.03 cm and 7.70±4.08 cm, respectively.

4.1.1.1.2 Stem

Tuberous stem of *E. macrobulbon* was rhizome type which grew on ground surface level. It was roundish. It had approximately 5-8 nodes per tuberous stem which were found on every base of internodes. Normally, tuberous stem of this terrestrial orchid consisted of 2-3 rhizomes, attached together. Diameter of each tuberous stem was 7.03±0.96 cm. Young tuberous stem was smooth and grayed-purple (183A) when tuberous got older, the surface shrank to casing wrinkled and the surface became darker in color (Figure 4.2).

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Figure 4.2 Stem of *Eulophia macrobulbon* (Par. & Rchb. f.) Hook. f.
(scale bar = 1 cm)

4.1.1.1.3 Leaf

A plant, developed from a vegetative bud, there were 2-3 leaves, oblanceolate, entire-margined. Leaf apex was acute while leaf base was narrowly cuneate. Leaf venation was parallel. There were 5-11 veins in each leaf. Leaf base was compactly fold and form pseudostem (Figure 4.3). Width, length, and thickness of leaf were 7.65±1.83 cm, 29.78±13.32 cm, and 0.01±0.00 cm, respectively. The color of the leaf was yellow-green (148A). Leaf arrangement was alternate.

Figure 4.3 Leaf of *Eulophia macrobulbon* (Par. & Rchb. f.) Hook. f. (scale bar = 1 cm)

4.1.1.1.4 Inflorescence

Inflorescence was raceme, emerging from lateral bud of the new developed shoot. Usually, 1-2 inflorescences were found on each stem and appeared before leaf. The length including peduncle was 20.07±9.28 cm. Peduncle was straight, smooth, approximately 0.76±0.13 cm in diameter and there were 1-2 nodes on each peduncle. It was yellow-green (145D) in color. Flower blooms started from peduncle base and there were approximately 42.12±24.54 flowers per inflorescence (Figure 4.4).

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Figure 4.4 Inflorescence of *Eulophia macrobulbon* (Par. & Rchb. f.) Hook. f. (scale bar = 1 cm)

4.1.1.1.5 Flower

Flower was perfect flower with bilateral symmetry. Mature flower was 2.45±0.22 cm in length and 1.16±0.21 cm in width. Flower had 6 perianths; composed of 2 whorls. The outer whorl had 3 sepals. The inner whorl had 2 petals and 1 modified petal; labellum. Each of them was free, spreading, and almost equal in size. Sepal consisted of a dorsal sepal and 2 lateral sepals. A dorsal sepal was at back of column. It was lanceolate, acute apex, entire margin, brown (200A, 200B) in color, 0.36±0.05 cm in width, and 1.31±0.13 cm in length. The shape of lateral sepal was lanceolate, acute apex, and entire margin. It was brown (200A, 200B) in color. Width and length of this organ was 0.32±0.04 cm and 1.53±0.11 cm, respectively. Petal constructed of 2 lateral petals and a labellum. Lateral petal was elliptic, acute apex, entire margin, and purple (N77A) in color. Width and length of lateral petal was 0.41±0.06 cm and 1.12±0.10 cm, respectively. Labellum was narrowly oblong, yellow-green (150C) in color, and bigger than other perianth. Lateral lobes were short. End lobe was curved downward, spreading, and frilly margin. There were greyed-purple spot (187A) at labellum base and yellow ridges on upper surface. This perianth was 0.37±0.05 cm in width and 1.22±0.10 cm in length. Column was yellow-green (145B)

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in color and small approximately 0.66±0.05 cm Anther cap (Operculum) was bulging, grayed-purple (187B), 0.09±0.02 cm in width and 0.15±0.03 cm in length. Pollen composed of 2 pollinia that were round, yellow (7A), and had viscidium. Stigma was 1.29±0.01 cm. Pedicel was approximately 1.54±0.23 cm in length, taper, had ridges on surface, and purple (N77B) (Figure 4.5 and 4.6).

Figure 4.5 Flower of *Eulophia macrobulbon* (Par. & Rchb. f.) Hook. f. (scale bar = 1 cm)

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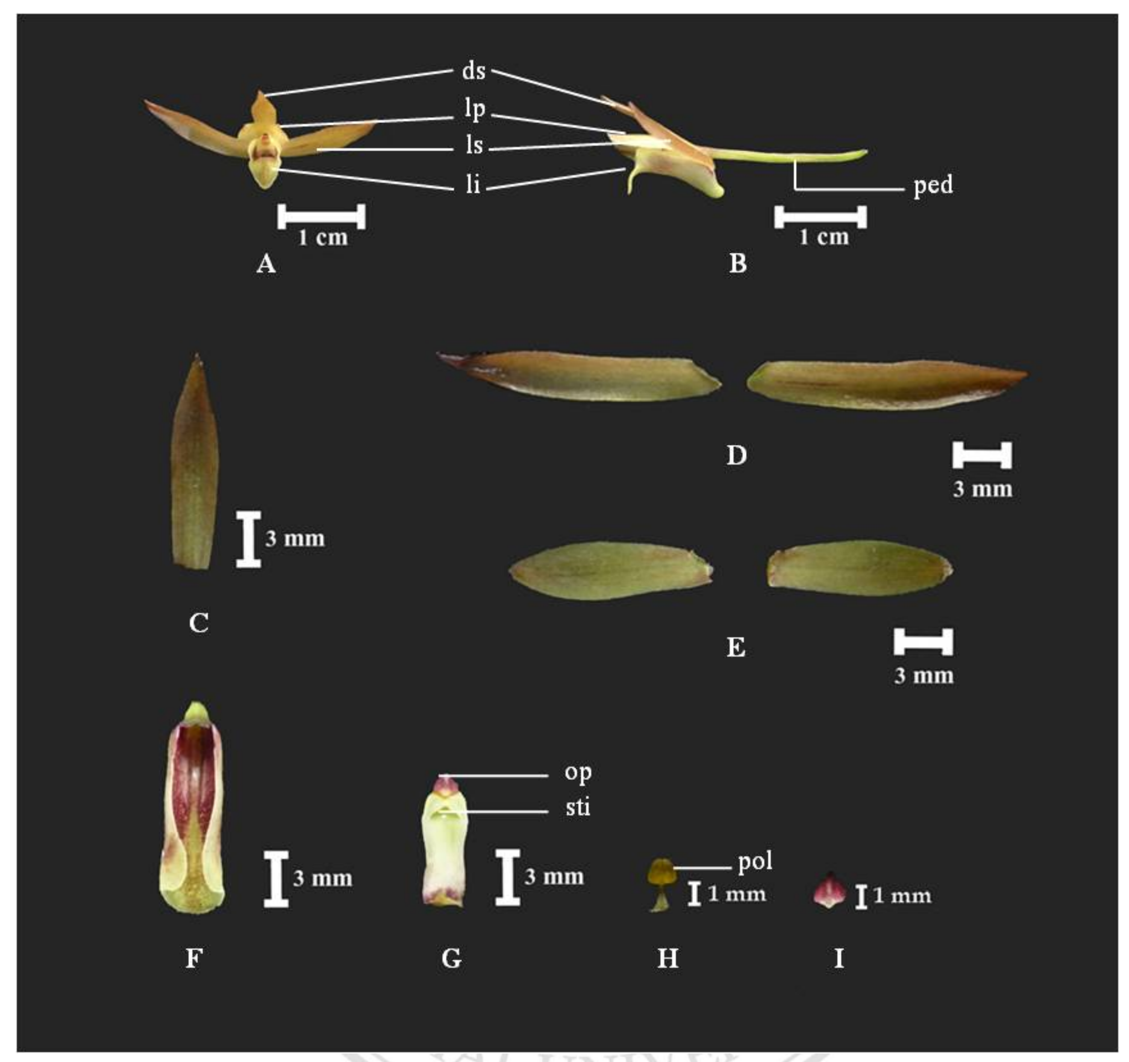

Figure 4.6 Flower structure of *Eulophia macrobulbon* (Par. & Rchb. f.) Hook. f.; A) flower (front side), B) flower (lateral side), C) dorsal sepal, D) lateral sepal, E) lateral petal, F) labellum, G) column, H) pollinia, I) operculum ds = dorsal sepal, lp = lateral petal, ls = lateral sepal, li = labellum, ped = pedicel, op = operculum, sti = stigma, pol = pollinia

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4.1.1.1.6 Fruit

Fruit was septicidal capsule which was narrowly elliptic to elliptic, 1.75±0.25 cm in width and 5.00±0.25 cm in length. The color of young fruit was green (143A). It turned to yellow at mature stage (Figure 4.7).

Figure 4.7 Fruit of *Eulophia macrobulbon* (Par. & Rchb. f.) Hook. f. (scale bar = 1 cm)

4.1.1.1.7 Seed

Seeds were very small, approximately 1.08±0.17 mm long under microscope. They were long tapering end, rough net surface, and transparent. There was a embryo inside (Figure 4.8).

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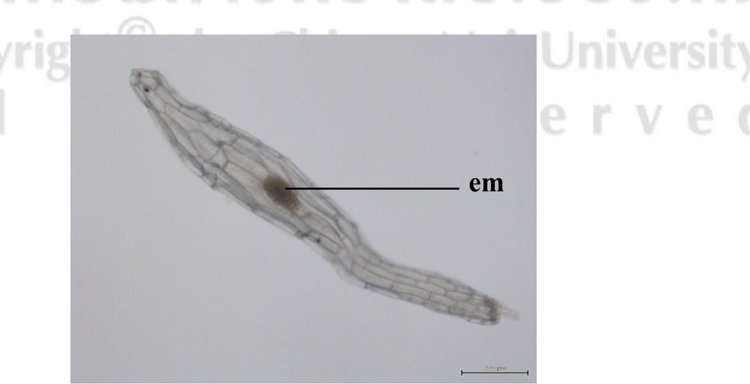

Figure 4.8 Seed of *Eulophia macrobulbon* (Par. & Rchb. f.) Hook. f. (scale bar = 200 µm) em = embryo

4.1.1.2 *Eulophia spectabilis* (Dennst.) Suresh.

4.1.1.2.1 Root

Roots of *E. spectabilis* were fibrous type which germinated from base of tuberous stem and spread around tuberous stem. They were thick, fleshy, unbranched, and white (155A) when they were still young and became grayed-orange (164B) when they were older. There were 7-10 roots per plant. Root was 0.40±0.10 cm in diameter and 9.07±3.37 cm in length (Figure 4.9).

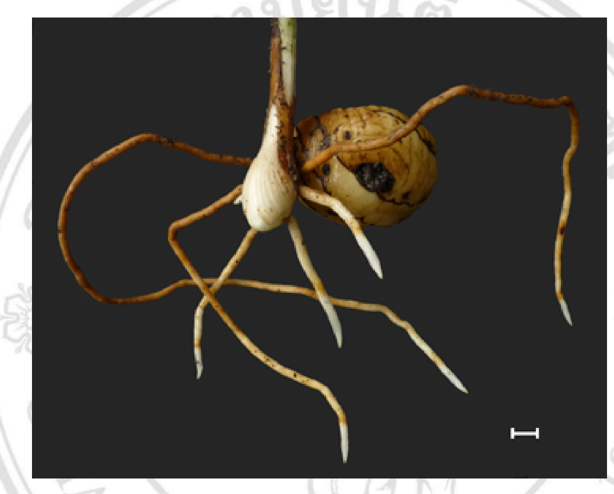

Figure 4.9 Root of *Eulophia spectabilis* (Dennst.) Suresh. (scale bar = 1 cm)

4.1.1.2.2 Stem

Stem was rhizome type that could be found on ground surface level. It was roundish to obtuse, entire surface and green (143A) when they were young and surface was wrinkled and darker color when they became older. It had 7-11 nodes, and there was bud on every base of internode. Diameter of stem was 5.89±0.83 cm (Figure 4.10).

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Figure 4.10 Stem of *Eulophia spectabilis* (Dennst.) Suresh. (scale bar = 1 cm)

4.1.1.2.3 Leaf

Leaf was of simple type with alternate phyllotaxy, 2-4 leaves per plant. Leaf blade, green (143A) in color, was lanceolate. The width × length of leaf were 2.41±0.99 × 29.00±13.44 cm. Leaf apex was narrowly acute. Leaf base was narrowly cuneate. Leaf margin was entire. Leaf venation was parallel, 8-10 veins per leaf. Leaf base was compactly fold and form pseudostem (Figure 4.11).

Figure 4.11 Leaf of *Eulophia spectabilis* (Dennst.) Suresh. (scale bar = 5 cm)

4.1.1.2.4 Inflorescence

Inflorescence of this orchid was raceme that emerged from lateral bud of new developed shoot. It could be found 1-2 inflorescences per plant and normally show as the same time as leaf. Peduncle was straight, entire, waxy, green (143C), and there are 2-3 nodes per peduncle. Measurement of peduncle revealed that 0.60±0.30 cm in diameter, 39.90±6.51 cm in length, 8.00±3.00 flowers were found in an inflorescence. These flowers bloomed acropetally (Figure 4.12).

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Figure 4.12 Inflorescence of *Eulophia spectabilis* (Dennst.) Suresh. (scale bar = 1 cm)

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4.1.1.2.5 Flower

Flower was perfect flower with bilateral symmetry. Mature flower was 2.55±1.15 cm in length and 2.60±0.91 cm in width. Flower of this terrestrial orchid had various color; white flower and white lip with yellow stripe, pink-white flower and white lip with yellow stripe, pink-white flower and pink lip with yellow stripe, pink flower and pink lip with yellow stripe, green-pink flower and pink lip with yellow stripe, dark pink to red flower and pink lip, yellow-green flower and white lip, pink-green flower and pink lip. Flower had 6 perianths; compose of 2 whorls. The outer

whorl had 3 sepals. The inner whorl had 2 petals and 1 modified petal; labellum. Each of them was free, spreading, and almost equal. The outer whorl consisted of a dorsal sepal and 2 lateral sepals. A dorsal sepal was behind column. Shape of this perianth was lanceolate, acute apex, entire margin, and a sight twisted. Width and length of dorsal sepal were 0.80±0.25 cm and 3.00±0.90 cm, respectively. A lateral sepal was also lanceolate, acute apex, entire margin and a sight twisted. It was 0.70±0.21 cm in width and 2.93±0.83 cm in length. The inner whorl constructed of 2 lateral petals and labellum. Lateral petals were ovate, rounded apex, and entire margin. The size of lateral petals was 0.80±0.20 cm in width and 2.40±0.35 cm in length. Labellum that was larger than other perianth and narrowly oblong. Lateral lobe was short. End lobe was curved downward spread and frilly margin. There were many ridges and hair on upper surface. The size of lip was 1.15±0.30 cm in width and 2.35±0.35 cm in length. Column was taper and approximately 1.10±0.20 cm long. Pollen consisted of 2 pollinia, that were taper and the size was 0.40±0.12 cm. Pistil which was under pollen, was small basin, approximately 3.54±0.49 mm. Pedicel approximately 3.02±0.75 cm in length, taper, and had ridges on surface (Figure 4.13-4.20).

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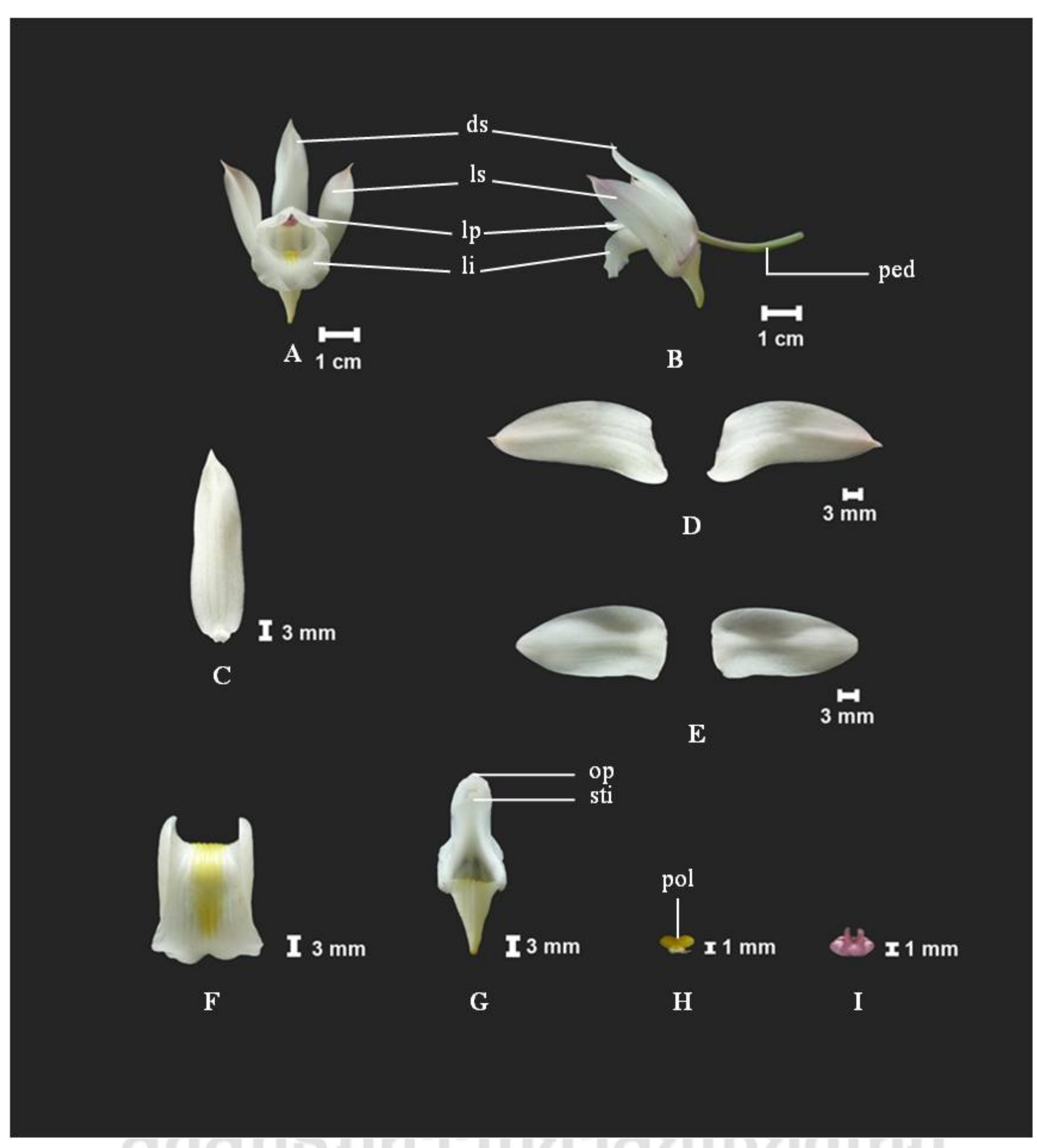

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Figure 4.13 Flower structure of *Eulophia spectabilis* (Dennst.) Suresh.; white flower and white lip with yellow stripe
A) flower (front side), B) flower (lateral side), C) dorsal sepal, D) lateral sepal, E) lateral petal, F) labellum, G) column, H) pollinia, I) operculum
ds = dorsal sepal, lp = lateral petal, ls = lateral sepal, li = labellum, ped = pedicel, op = operculum, sti = stigma, pol = pollinia

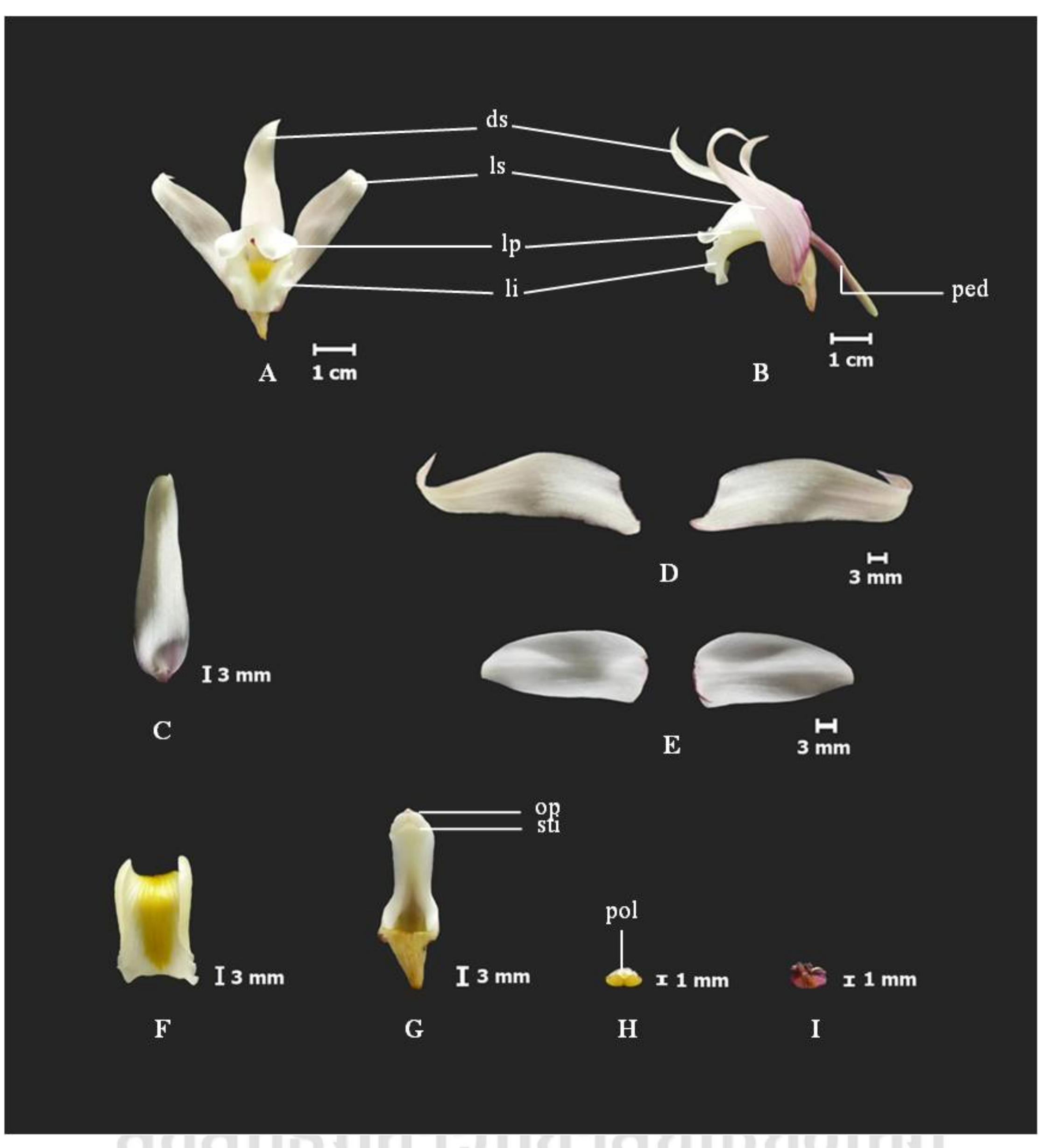

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Figure 4.14 Flower structure of *Eulophia spectabilis* (Dennst.) Suresh.; pink-white flower and white lip with yellow stripe

A) flower (front side), B) flower (lateral side), C) dorsal sepal, D) lateral sepal, E) lateral petal, F) labellum, G) column, H) pollinia, I) operculum

ds = dorsal sepal, lp = lateral petal, ls = lateral sepal, li = labellum, ped = pedicel, op = operculum, sti = stigma, pol = pollinia

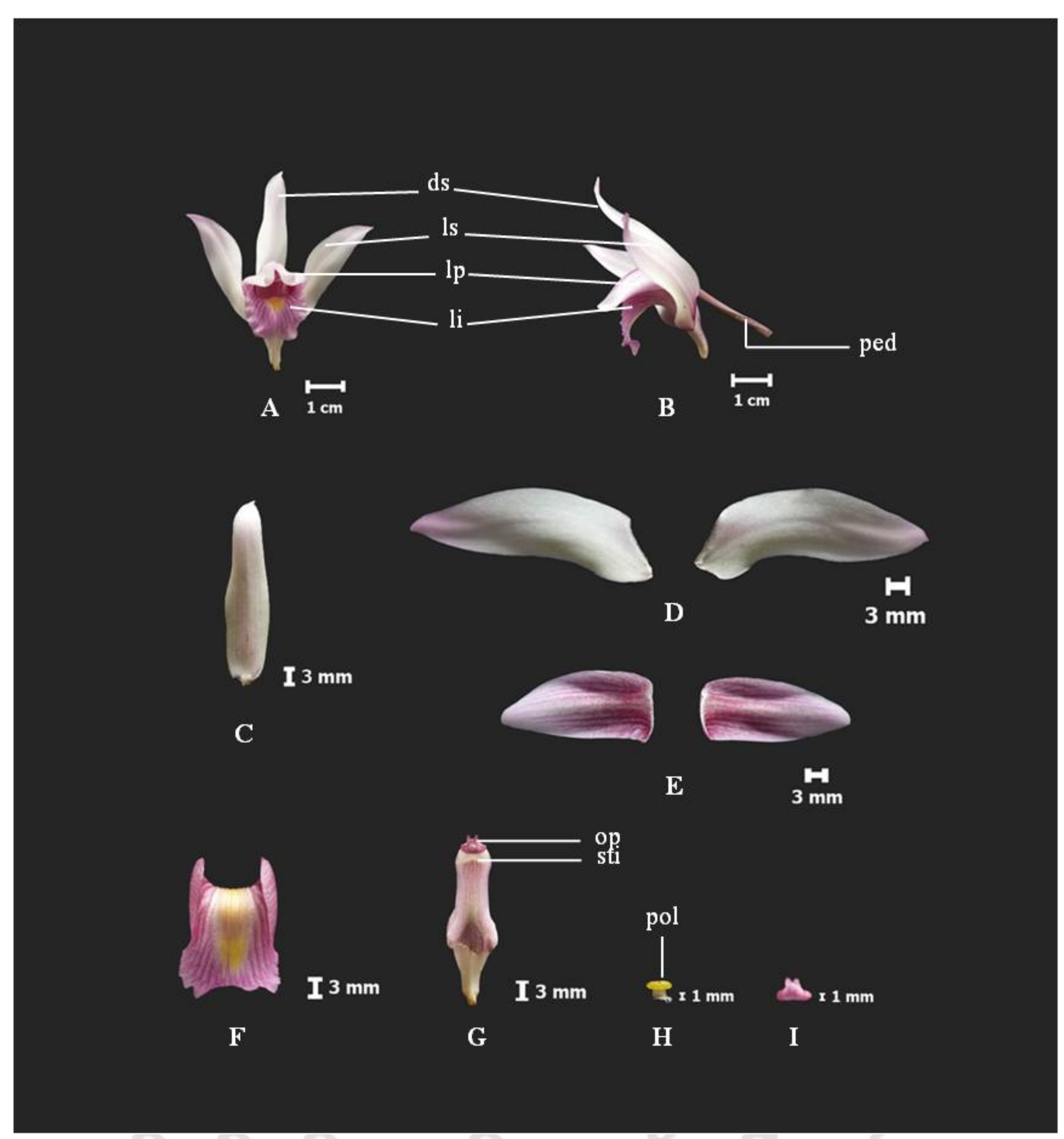

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Figure 4.15 Flower structure of *Eulophia spectabilis* (Dennst.) Suresh.; pink-white flower and pink lip with yellow stripe

A) flower (front side), B) flower (lateral side), C) dorsal sepal, D) lateral sepal, E) lateral petal, F) labellum, G) column, H) pollinia, I) operculum

ds = dorsal sepal, lp = lateral petal, ls = lateral sepal, li = labellum, ped = pedicel, op = operculum, sti = stigma, pol = pollinia

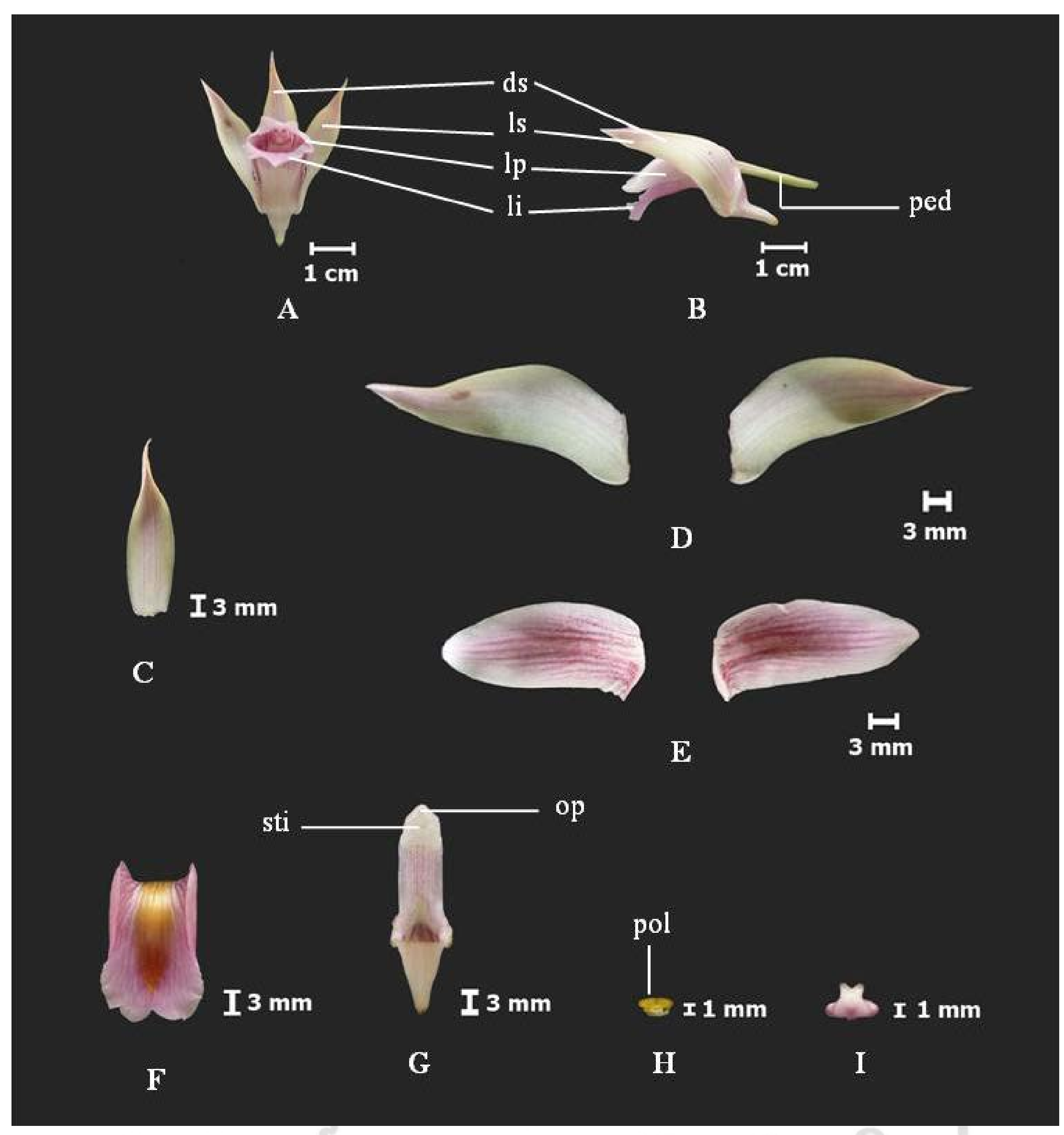

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Figure 4.16 Flower structure of *Eulophia spectabilis* (Dennst.) Suresh.; pink flower and pink lip with yellow stripe

A) flower (front side), B) flower (lateral side), C) dorsal sepal, D) lateral sepal, E) lateral petal, F) labellum, G) column, H) pollinia, I) operculum

ds = dorsal sepal, lp = lateral petal, ls = lateral sepal, li = labellum, ped = pedicel, op = operculum, sti = stigma, pol = pollinia

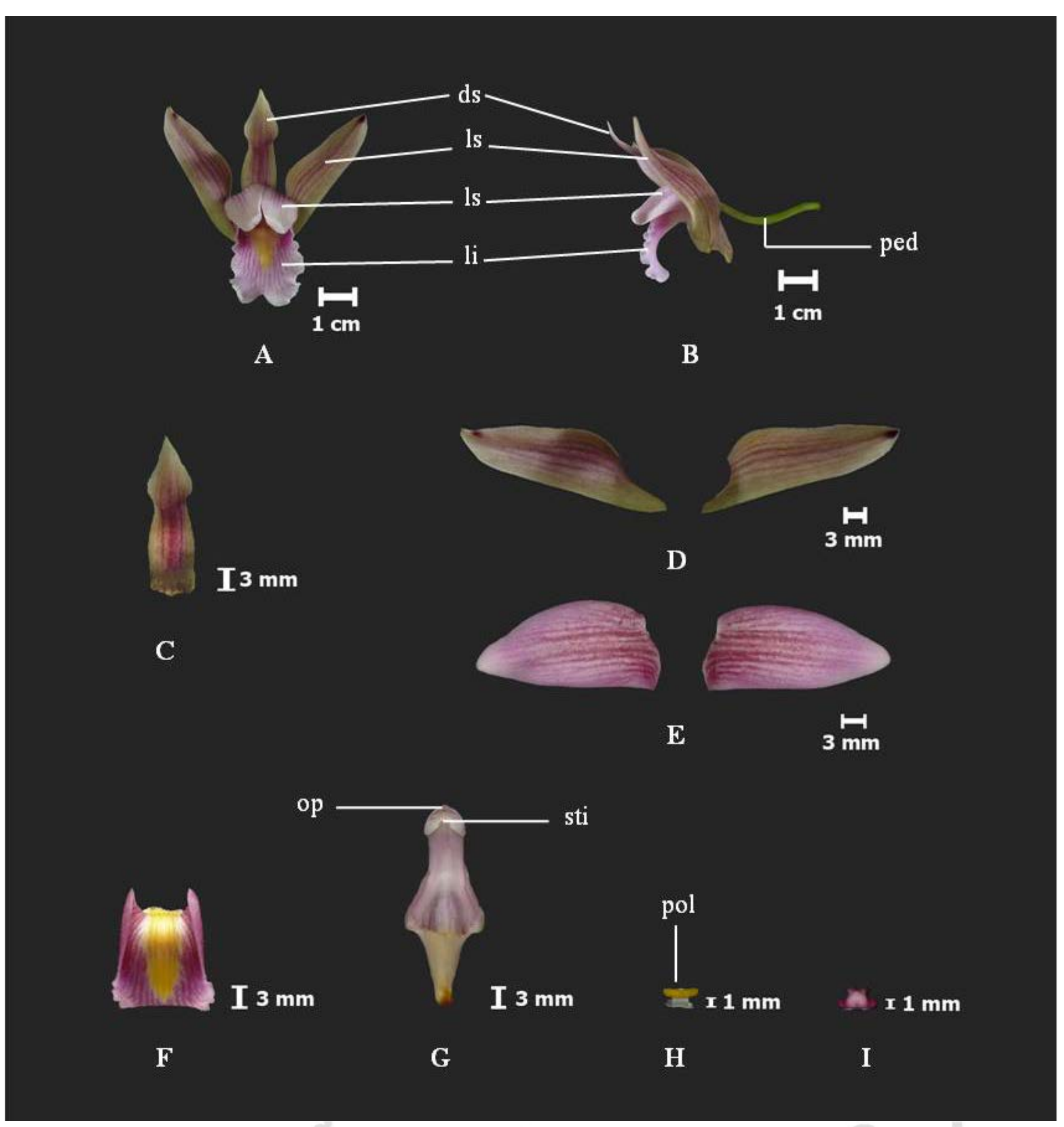

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Figure 4.17 Flower structure of *Eulophia spectabilis* (Dennst.) Suresh.; green-pink flower and pink lip with yellow stripe
A) flower (front side), B) flower (lateral side), C) dorsal sepal, D) lateral sepal, E) lateral petal, F) labellum, G) column, H) pollinia, I) operculum
ds = dorsal sepal, lp = lateral petal, ls = lateral sepal, li = labellum, ped = pedicel, op = operculum, sti = stigma, pol = pollinia

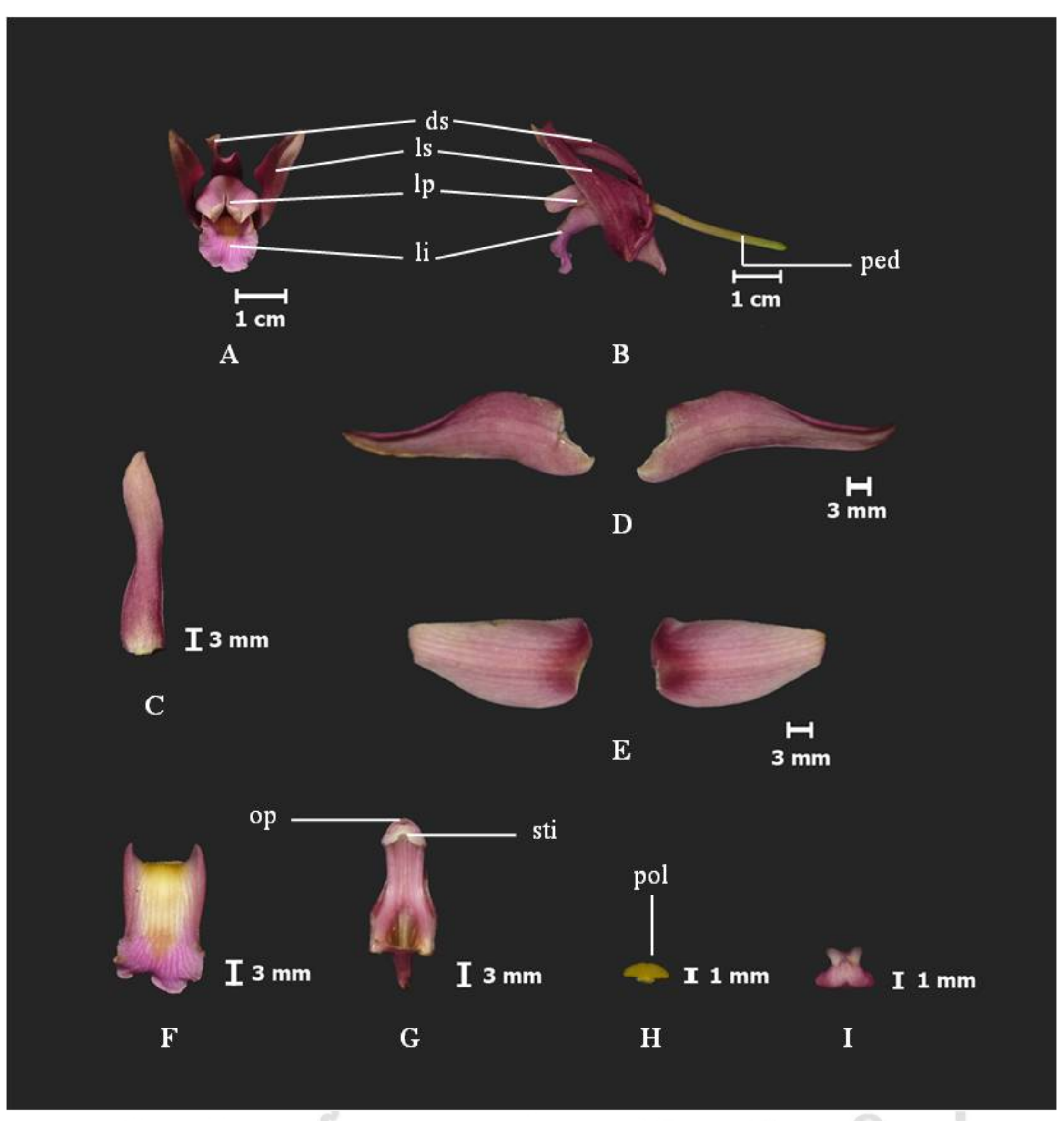

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Figure 4.18 Flower structure of *Eulophia spectabilis* (Dennst.) Suresh.; dark pink to red flower and pink lip

A) flower (front side), B) flower (lateral side), C) dorsal sepal, D) lateral sepal, E) lateral petal, F) labellum, G) column, H) pollinia, I) operculum

ds = dorsal sepal, lp = lateral petal, ls = lateral sepal, li = labellum, ped = pedicel, op = operculum, sti = stigma, pol = pollinia

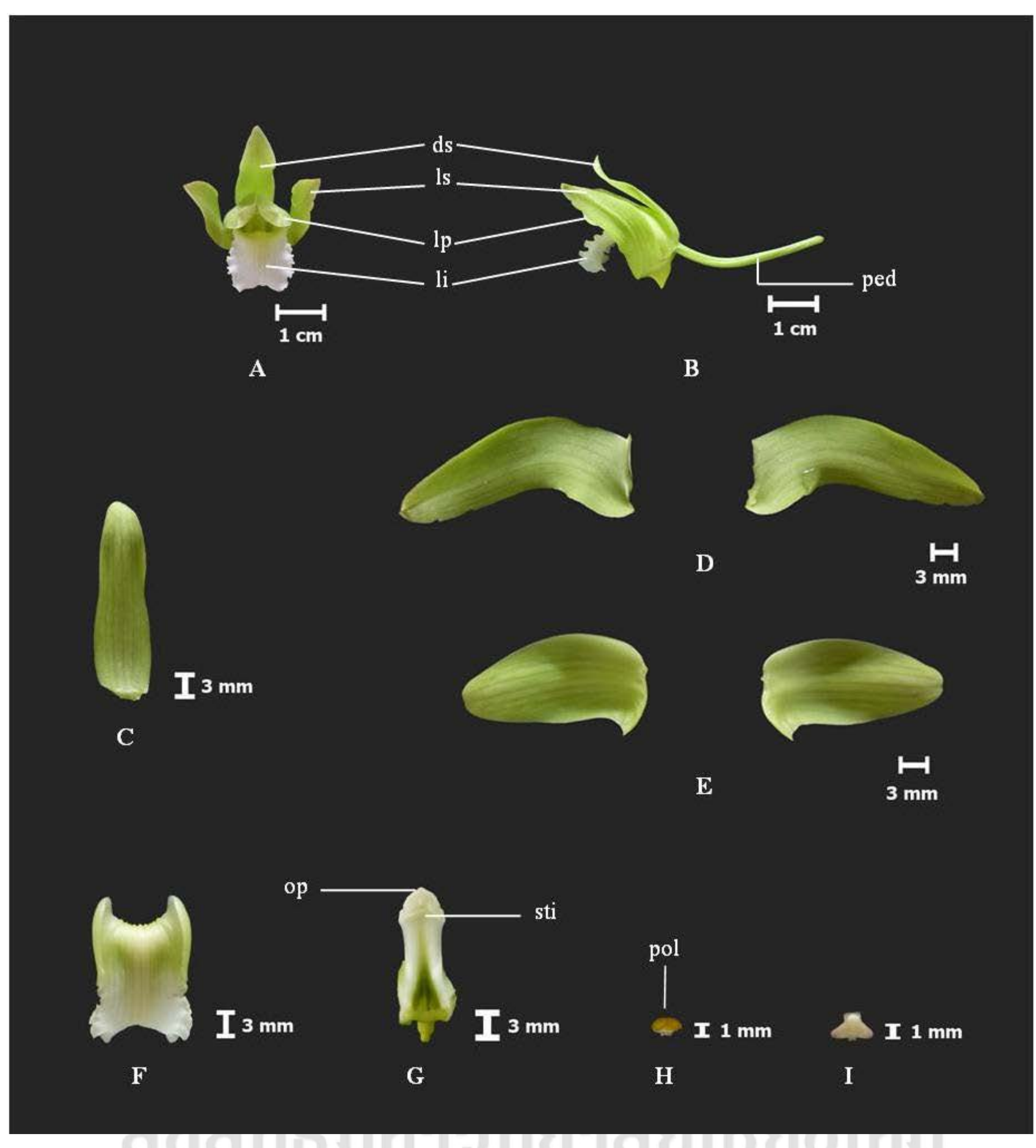

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Figure 4.19 Flower structure of *Eulophia spectabilis* (Dennst.) Suresh.; yellow-green flower and white lip

A) flower (front side), B) flower (lateral side), C) dorsal sepal, D) lateral sepal, E) lateral petal, F) labellum, G) column, H) pollinia, I) operculum

ds = dorsal sepal, lp = lateral petal, ls = lateral sepal, li = labellum, ped = pedicel, op = operculum, sti = stigma, pol = pollinia

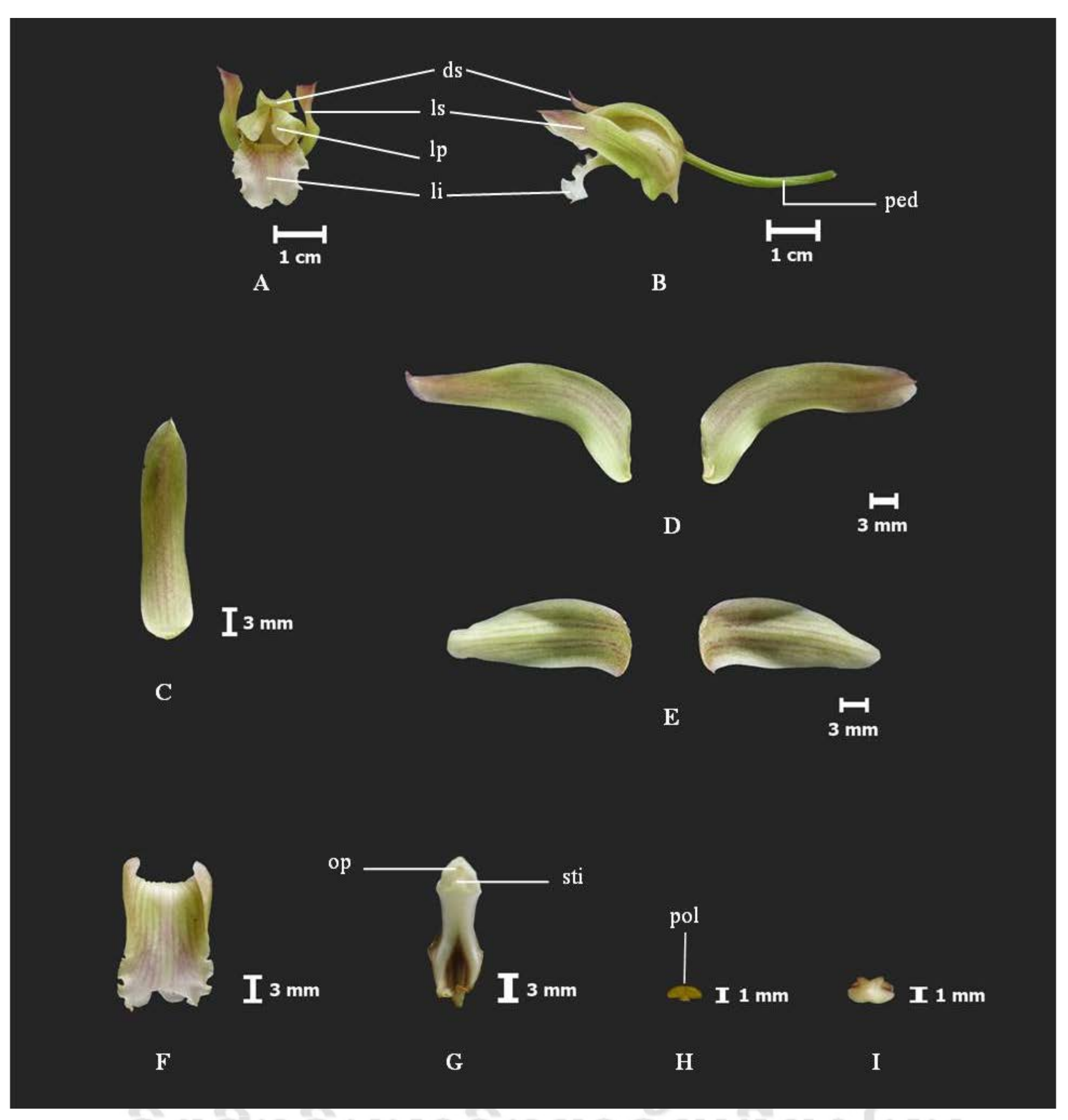

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Figure 4.20 Flower structure of *Eulophia spectabilis* (Dennst.) Suresh.; pink-green flower and pink lip

A) flower (front side), B) flower (lateral side), C) dorsal sepal, D) lateral sepal, E) lateral petal, F) labellum, G) column, H) pollinia, I) operculum

ds = dorsal sepal, lp = lateral petal, ls = lateral sepal, li = labellum, ped = pedicel, op = operculum, sti = stigma, pol = pollinia

4.1.1.2.6 Fruit

Fruit was of septicidal capsule type. It was narrowly elliptic to elliptic. The size of fruit was 2.20±0.25 cm in width and 7.20±0.80 cm in length. The color of young fruit was green (143A) and become yellow when older (Figure 4.21).

Figure 4.21 Fruit of *Eulophia spectabilis* (Dennst.) Suresh. (scale bar = 1 cm)

4.1.1.2.7 Seed

Seeds were very small approximately 1.35±0.05 mm. Seed was long, taper, transparent, and had rough net surface when examine by microscope. There was embryo inside (Figure 4.22).

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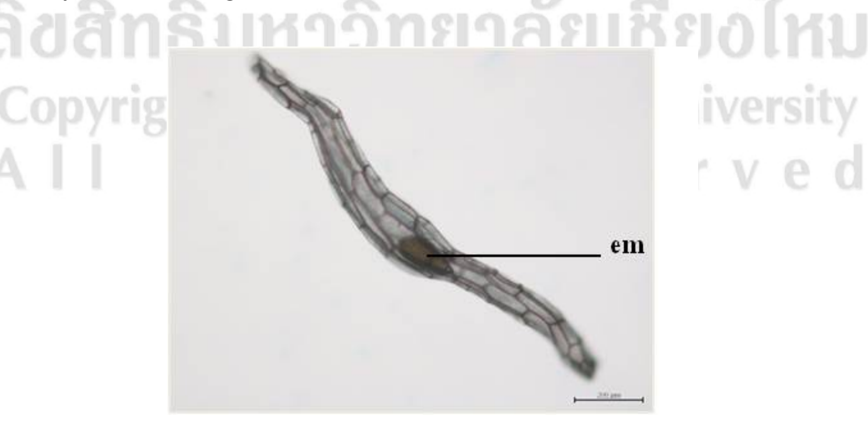

Figure 4.22 Seed of *Eulophia spectabilis* (Dennst.) Suresh.
(scale bar = 200 μm) em = embryo

4.1.2 Growth cycle

Growth and development of plants grown in pots were observed their growth patterns from dormancy stage until the next cycle of dormancy.

4.1.2.1 *E. macrobulbon*

Growth cycle of *E. macrobulbon* was of deciduous type and undergone the dormant period throughout dry season. It started vegetative shoot from bud that was at base of underground stem at early-February. After that, inflorescence emerged from base of vegetative shoot and appeared above ground before leaves during mid-February to mid-March. It continued to elongate and enlarge until most of the florets basipetally reached their anthesis in mid-March to the first week of April. In nature, fruit of this terrestrial orchid could be set approximately the third week of March. Mature fruit could be found and split to spread seeds in February of the next year.

During inflorescence was mature; April, vegetative shoot rapidly developed until it developed in full length by early-May. After that, underground stem of *E. macrobulbon* enlarge size. Base of vegetative shoot size increased and transform to new rhizome that was roundish. There were apparently nodes. Increasing size of this plant would be stop in October. Finally, the aerial part of this terrestrial orchid began to die back and stay dormant from November to January of the following year. Old rhizome was dry, hard, and still attached to new rhizome.

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A diagram concluding the stage of growth of the plant at various time of the year is shown in Figure 4.23. Illustration of plant parts at different stages of growth is presented in Figure 4.24.

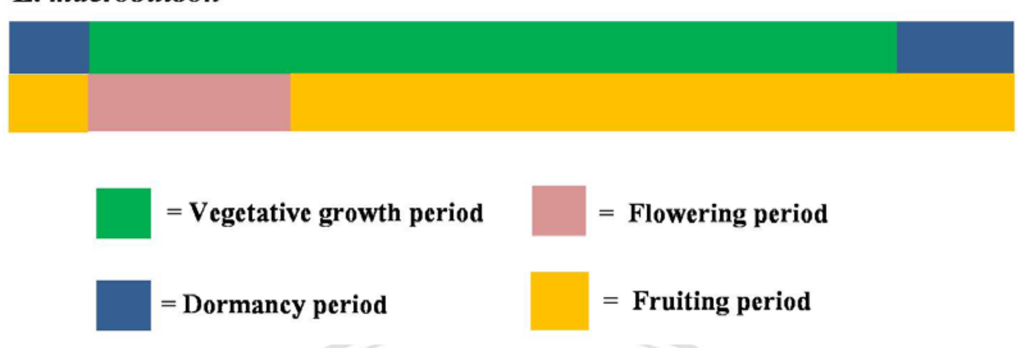

Figure 4.23 Diagram of life cycle of *Eulophia macrobulbon* (Par. & Rchb. f.) Hook. f.

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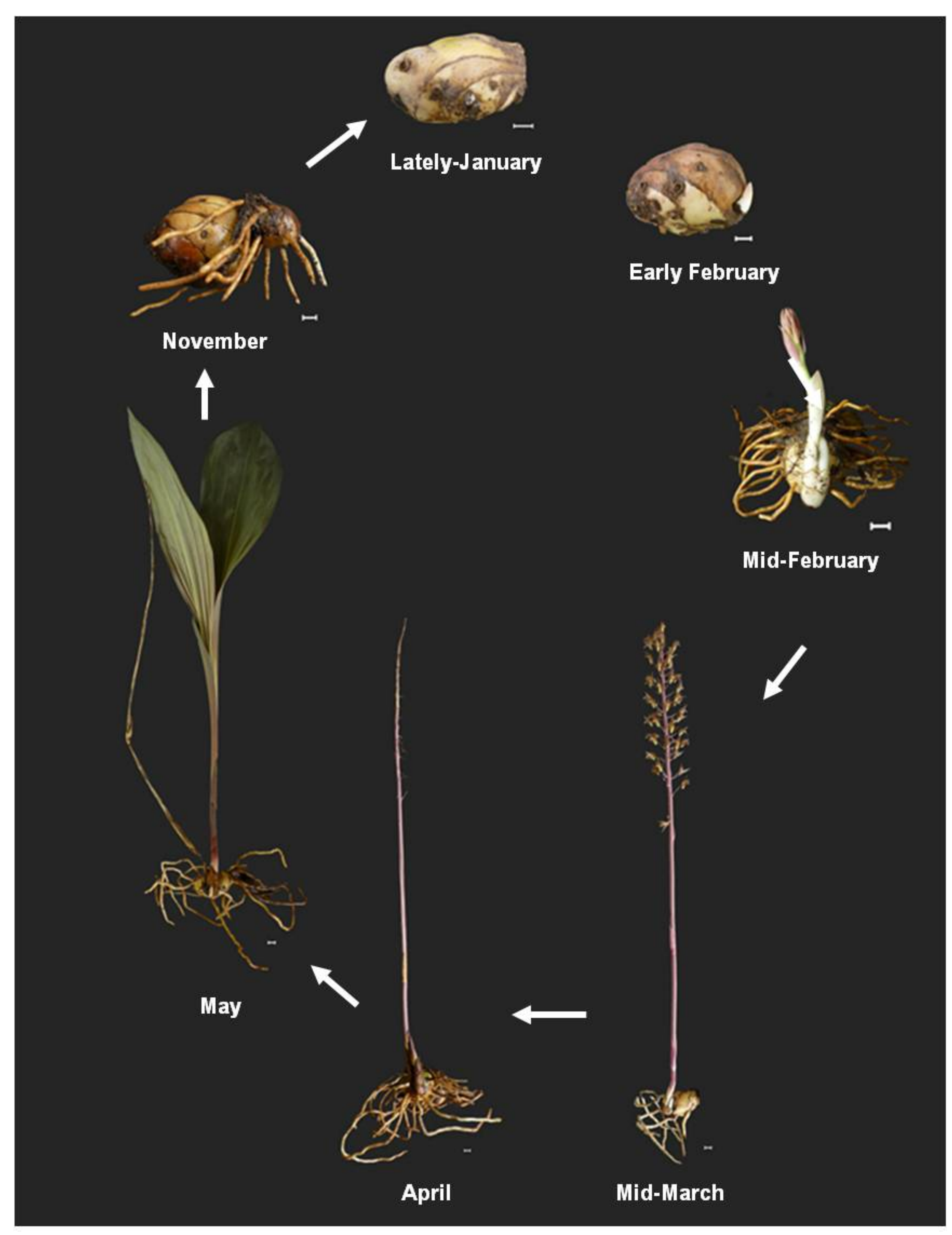

Figure 4.24 Annual growth and development of *Eulophia macrobulbon* (Par. & Rchb. f.) Hook. f. (scale bar = 1 cm)

4.1.2.2 *E. spectabilis*

Growth cycle of *E. spectabilis* was deciduous type. Vegetative shoot and reproductive shoot emerged from bud which was at base of rhizome. This terrestrial orchid, leaves emerged and developed above ground before inflorescence. After that, inflorescence rapidly developed until mature as same as mature leaves; April to late May. Flower blooms from the base of inflorescence toward apex. Fruit could occur in nature. It settled from the second week of May and dispersed seeds in March of the next year. When leaves were mature, base of vegetative shoot would expand and change to new rhizome which was roundish. There were apparently nodes. Size expansion of this terrestrial orchid would stop in October. Dormancy began in November and ended in March of the next year. Old rhizome was dry, hard, and sticks with new rhizome.

A diagram concluding the stage of growth of the plant at various time of the year is shown in Figure 4.25 Illustration of plant part at different stages of growth is presented in Figure 4.26

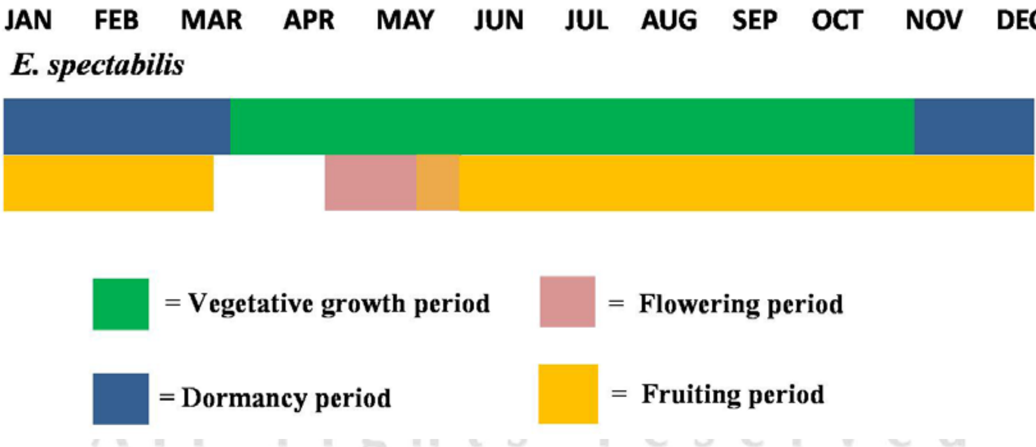

Figure 4.25 Diagram of life cycle of *Eulophia spectabilis* (Dennst.) Suresh.

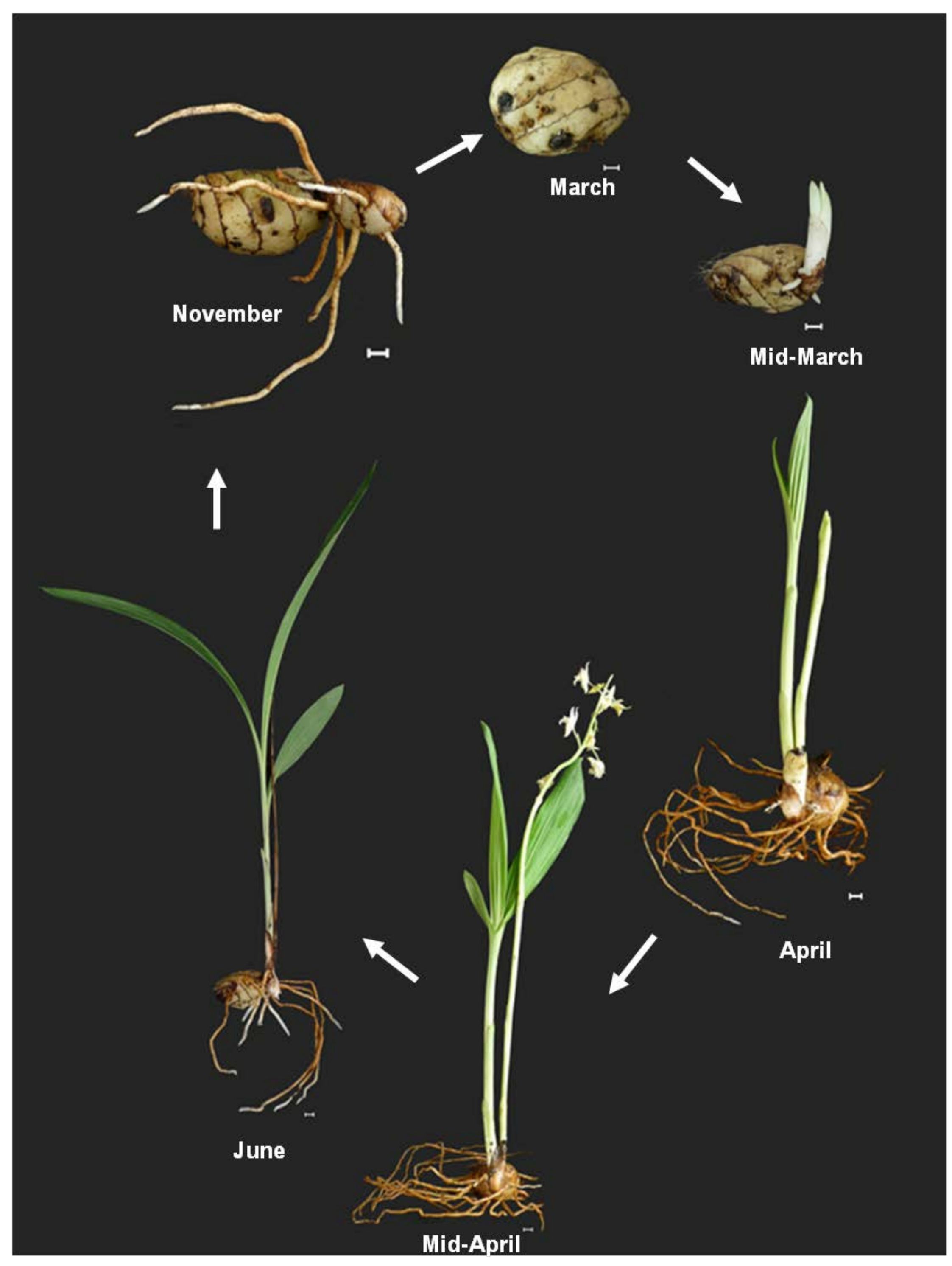

Figure 4.26 Annual growth and development of *Eulophia spectabilis* (Dennst.) Suresh. (scale bar = 1 cm)

4.1.3 Anatomical study of flower bud development

Anatomical characteristics of *E. macrobulbon* and *E. spectabilis* were investigated from longitudinal section of flower bud. Permanent slides of those sections were prepared via paraffin embedding technique modified by Johansen (1940). The samples of both species were taken at different plant growth periods; 1-4 week from bud break.

4.1.3.1 *E. macrobulbon*

Anatomical investigation of flower bud development on *E. macrobulbon* showed that there were 2 types of bud in shoot; vegetative bud and floral bud. Growth and development of flower bud are reported as follow;

Week 1; Shoot of *E. macrobulbon* started to emerge from underground stem at early-February. Size of bud at this stage was approximately 0.32±0.02 cm. Longitudinal cut showed that there was a bud appeared in the shoot, at the middle of the bud. It was a small dome. Amount of cells were divided. This week, only “Apical meristem” was formed. There was “Leaf primordia” on lateral side. It would develop further into leaf; vegetative bud (Figure 4.27).

Week 2; Shoot size at this time was approximately 0.35±0.03 cm. During this period, there were two buds; one was in the middle and the second bud; the first lateral bud, was at the base of axillary leaf (Figure 4.28). The first bud developed more than the first week and leaves were form in an alternative direction, left and right.

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Week 3; Shoot size was approximately 0.65±0.15 cm. Two to four buds were found in this week. The middle bud; first bud-vegetative bud, still grew to have many leaves. The first lateral bud was larger than the second one and had bract on apex bud. The second lateral bud was formed in axillary leaf of opposite of the first lateral bud (Figure 4.29).

Week 4; At this week, shoot size was approximately 0.70-1.20 cm. Three to five shoots which composed of a middle bud; vegetative bud, and the lateral bud; 2-6 buds, appeared at this week. The middle bud continued to grow. There

were many leaves and started to fold. The first lateral bud elongated and had many bracts that covered apex bud and floral primordia were found at this stage. This bud would develop further to be inflorescence. They were at the base of axillary leaf which was outer area where in the opposite of the previous lateral bud (Figure 4.30).

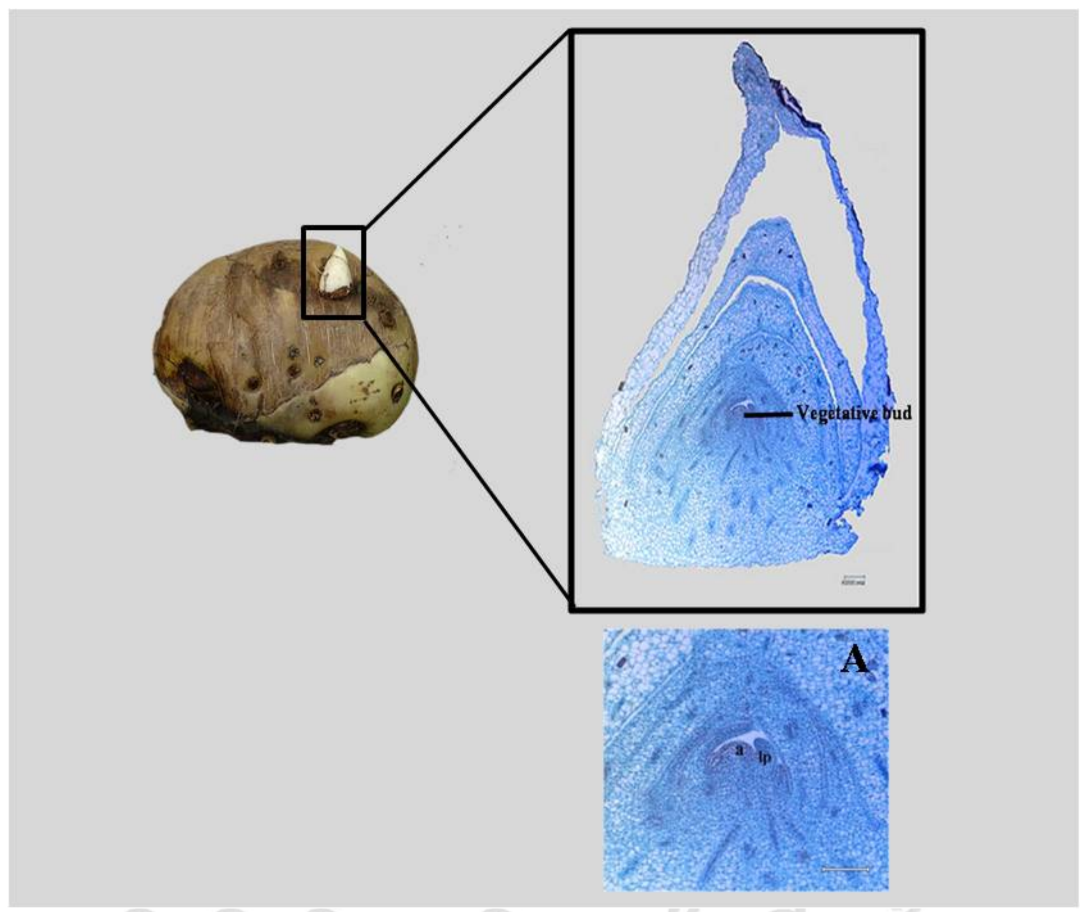

Figure 4.27 Longitudinal section of *Eulophia macrobulbon* (Par. & Pchb. f.) Hook. f. bud at week 1; A) vegetative bud (scale bar = 200 µm)
a = apical meristem, lp = leaf primordia

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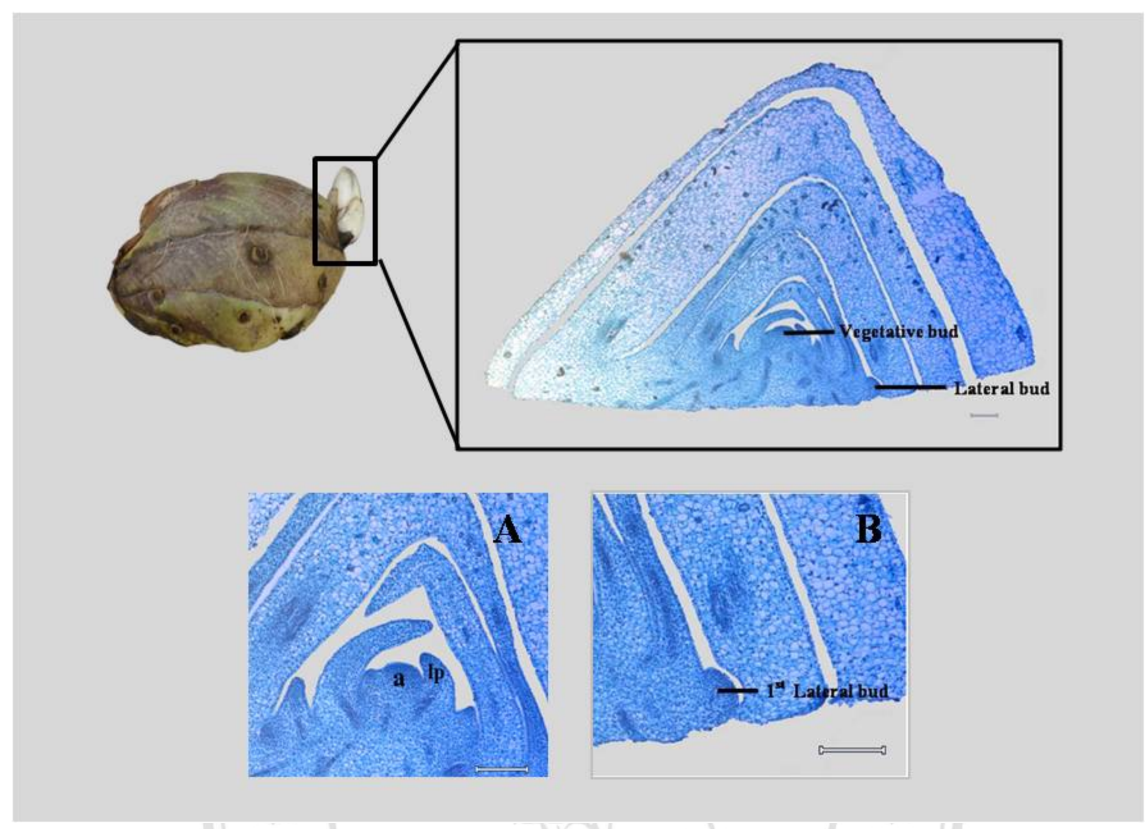

Figure 4.28 Longitudinal section of *Eulophia macrobulbon* (Par. & Pchb. f.) Hook. f. bud at week 2; A) vegetative bud, B) 1st lateral bud (scale bar = 200 μm) a = apical meristem, lp = leaf primordia

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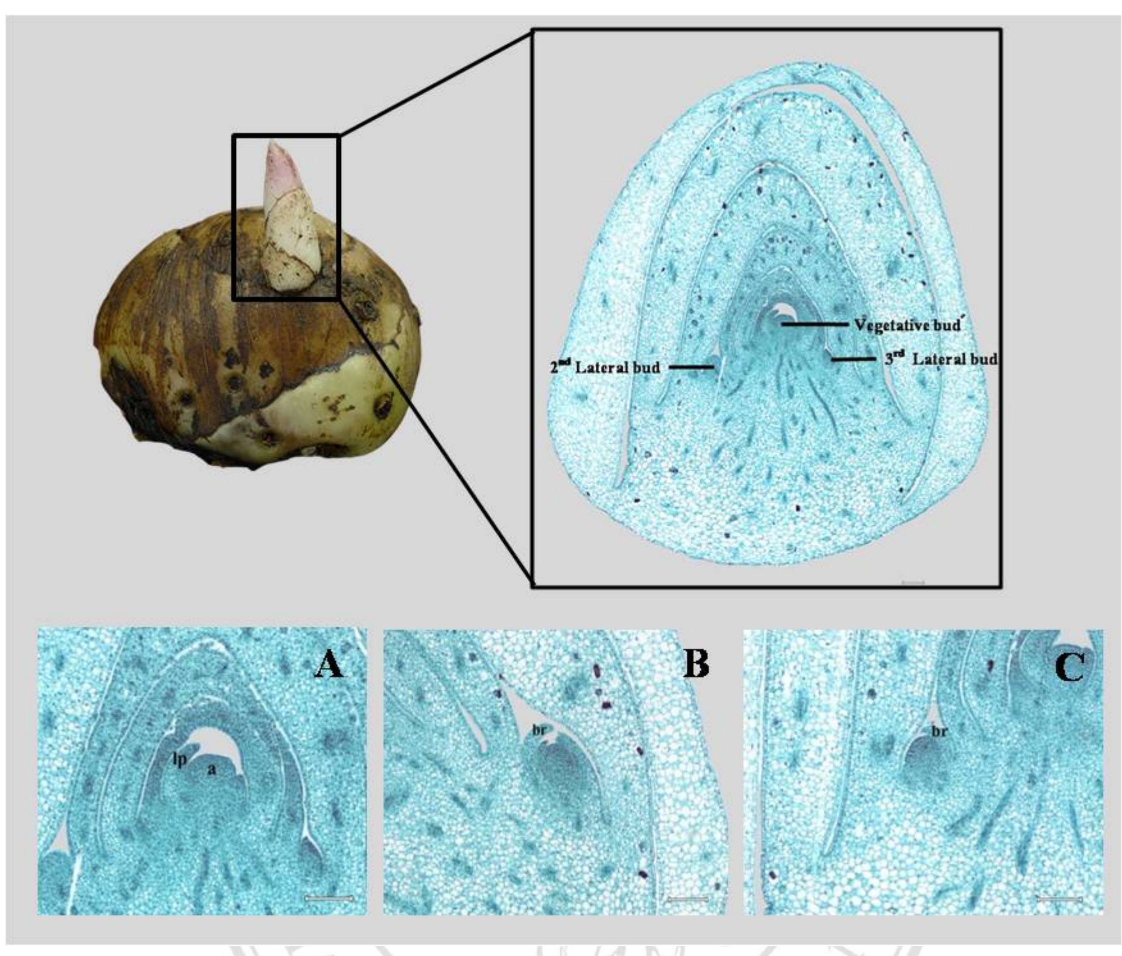

Figure 4.29 Longitudinal section of *Eulophia macrobulbon* (Par. & Pchb. f.) Hook. f. bud at week 3; A) vegetative bud, B) 1st lateral bud, C) 2nd lateral bud (scale bar = 200 µm)

a = apical meristem, lp = leaf primordia, br = bract

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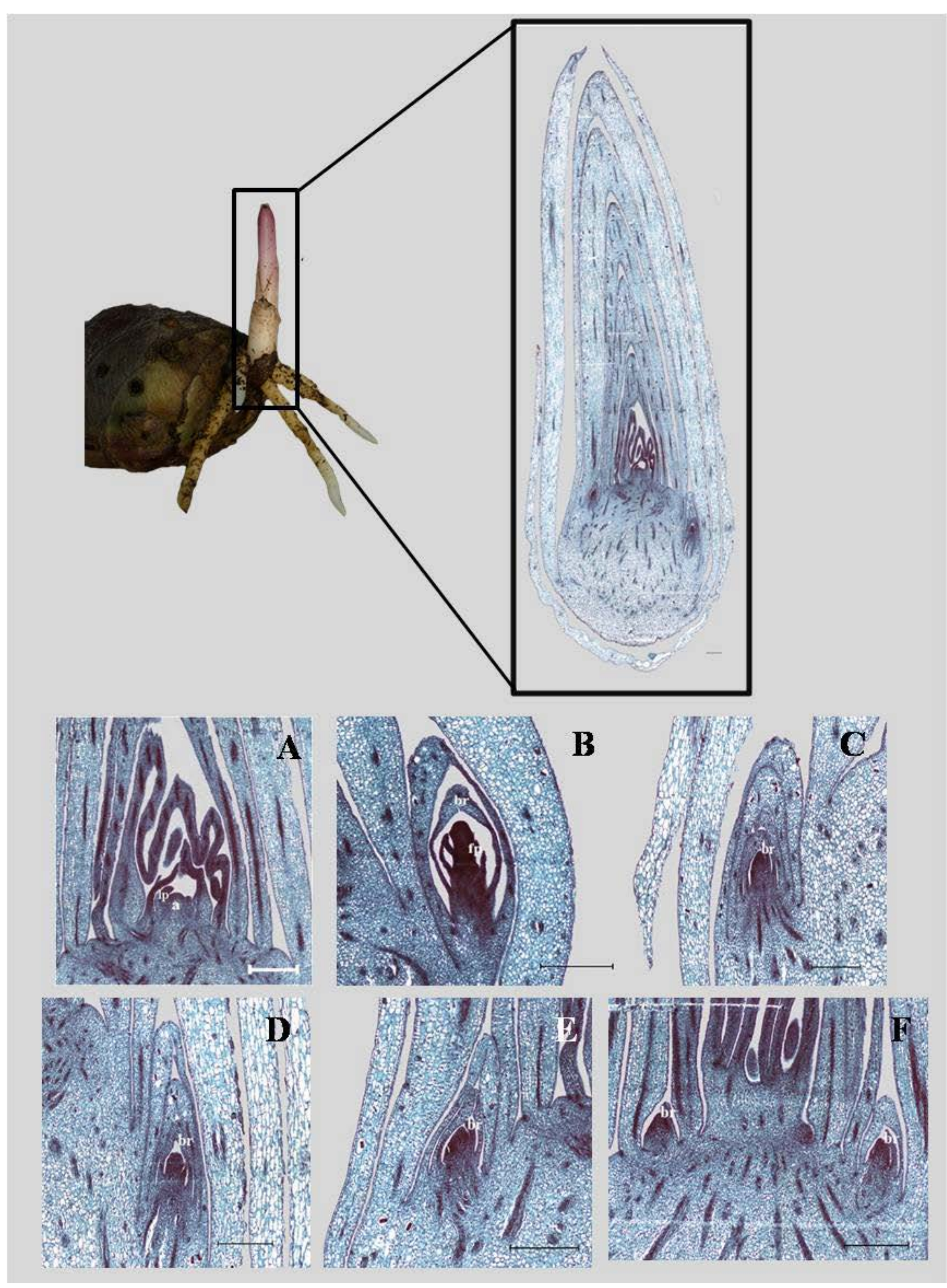

Figure 4.30 Longitudinal section of *Eulophia macrobulbon* (Par. & Pchb. f.) Hook. f. bud at week 4; A) vegetative bud , B) 1st lateral bud, C) 2nd lateral bud, D) 3rd lateral bud, E) 4th lateral bud, F) 5th and 6th lateral bud (scale bar = 500 µm)

a = apical meristem, lp = leaf primordia, br = bract, fb = floral primordia

4.1.3.2 *E. spectabilis*

Histological study on flower bud development of *E. spectabilis* was conducted. It showed that 2 types of bud could be found in shoot; vegetative bud and floral bud. Growth and development of flower bud of this terrestrial orchid are reported as follow;

Week 1; Shoot of *E. spectabilis* emerged from underground stem in March. Shoot size was approximately 0.45±0.05 cm. Longitudinal cut showed that there were 2 buds in the shoot. At this time, characteristics of the first bud which was at the middle of the shoot was a small dome. There were many dividing cells. Apical meristem and leaf primordia, where on lateral side, were formed. It would develop further to form leaf; vegetative bud. The second bud; the first lateral bud, was at base of axillary leaf (Figure 4.31).

Week 2-3; Shoot size was approximately 0.50±0.20 cm. During this period, there were 2-5 buds. They consisted of the middle bud; vegetative bud, and lateral buds; 1-3 buds. The middle bud developed more than the first week and leaf developed in an alternative way, left and right. The first lateral bud was larger than the first week and had bract on apex bud. The other lateral buds were at the base of axillary leaf which was in the opposite of outer area of the previous lateral bud (Figure 4.32-4.33).

Week 4; At this week, size of shoot was approximately 1.15±0.35 cm. 3-6 buds, which composed of the middle bud and 2-5 lateral buds, appeared at this week. A middle bud continued to grown. There are many leaves and started to fold. The first lateral bud elongated and had many bracts that covered apex bud. Floral primordia were found at this stage. This bud would develop further to be inflorescence. The other lateral buds were at the base of axillary leaf which was in the opposite of outer area of the previous lateral bud (Figure 4.34).

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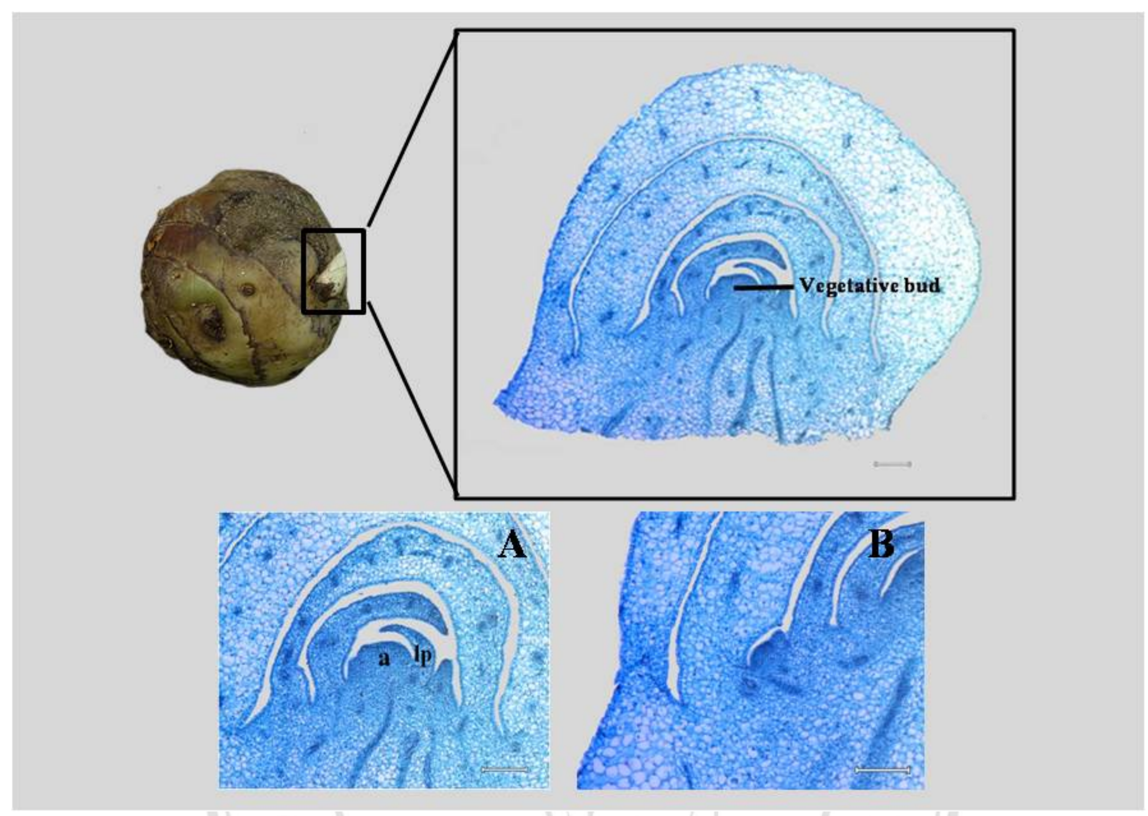

Figure 4.31 Longitudinal section of *Eulophia spectabilis* (Dennst.) Suresh. bud at week 1; A) vegetative bud, B) 1st lateral bud (scale bar = 200 µm) a = apical meristem, lp = leaf primordia

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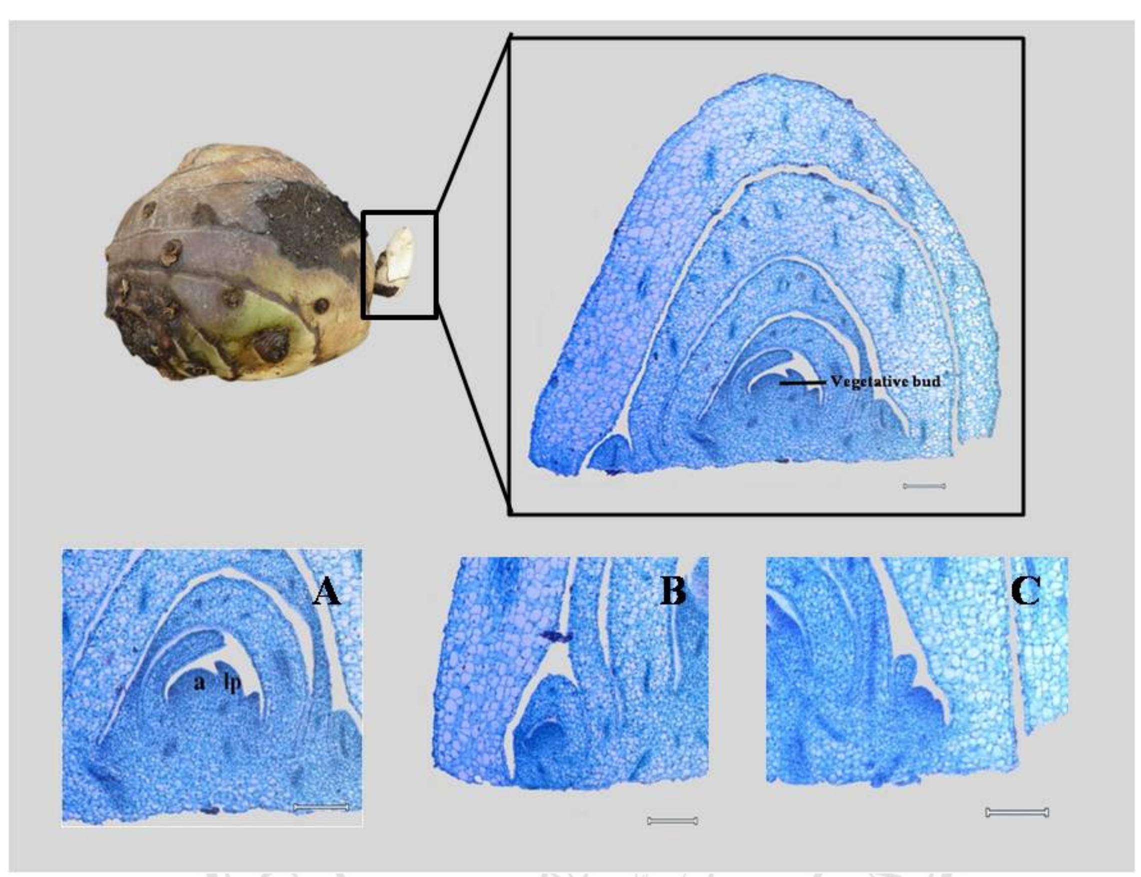

Figure 4.32 Longitudinal section of *Eulophia spectabilis* (Dennst.) Suresh. bud at week 2; A) vegetative bud, B) 1st lateral bud, C) 2nd lateral bud (scale bar = 200 μm)

a = apical meristem, lp = leaf primordia, br = bract

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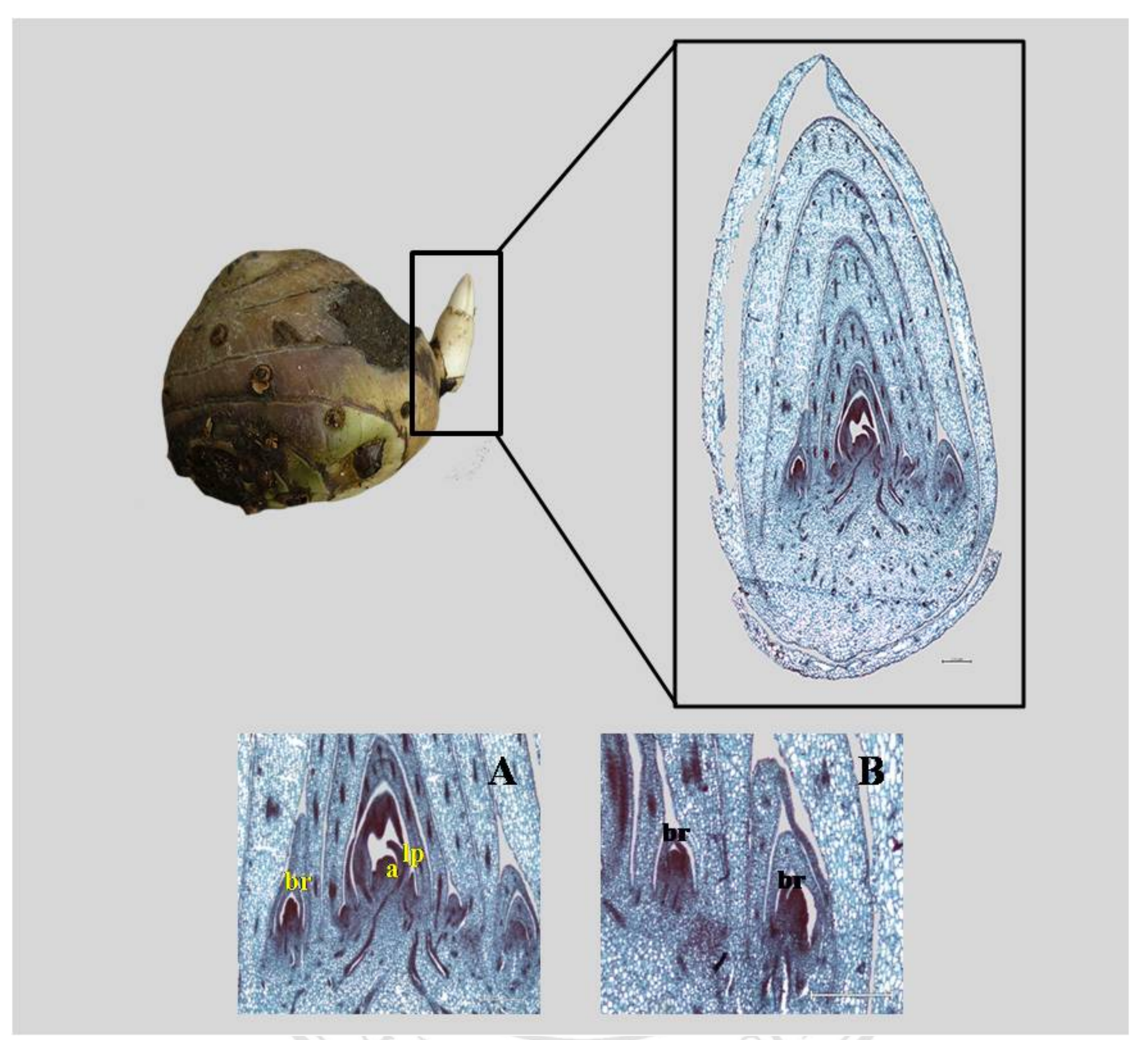

Figure 4.33 Longitudinal section of *Eulophia spectabilis* (Dennst.) Suresh. bud at week 3; A) vegetative bud and 2nd Lateral bud, B) 1st and 3rd lateral bud (scale bar = 200 µm)

a = apical meristem, lp = leaf primordia, br = bract

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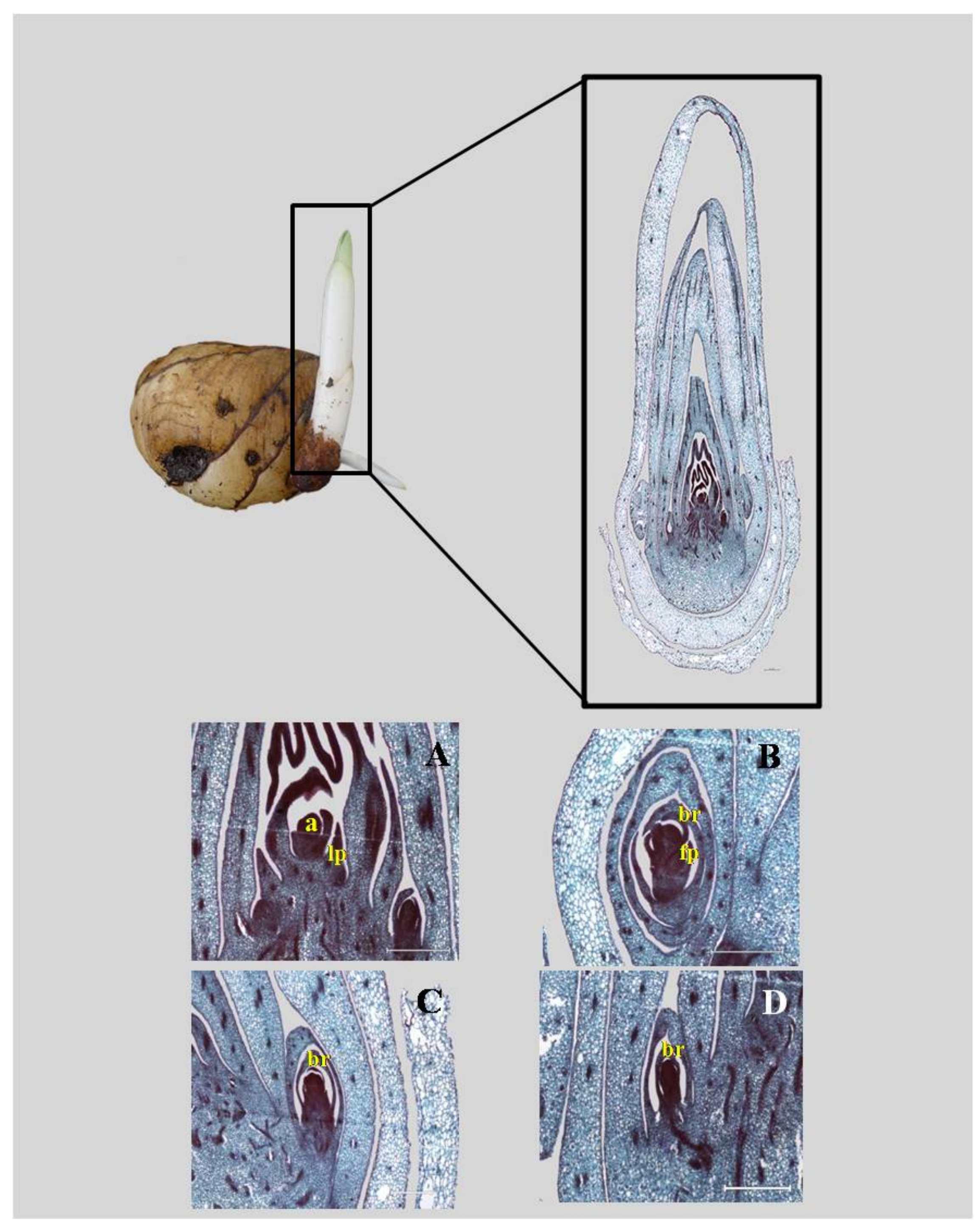

Figure 4.34 Longitudinal section of *Eulophia spectabilis* (Dennst.) Suresh. bud at week 4; A) vegetative bud, B) 1st lateral bud C) 2nd lateral bud, D) 3rd Lateral bud (scale bar = 500 µm)

a = apical meristem, lp = leaf primordia, br = bract, fp = floral primorda

4.1.4 Chromosome investigation

Objective of this study was to develop suitable procedures for chromosome investigation of *E. macrobulbon* and *E. spectabilis*. Experiments were conducted on root-tip tissue preparing for chromosome counting.

Tissue preparation technique was conducted as indicated by Feulgen's squash method modified by Vitayasak (1996). These cytological studies composed of 2 experiments. The first experiment was tissue sampling to find suitable sampling time which could yield plant chromosome at metaphase stage. Another experiment was to find the best pre-treatment duration to make chromosome contracted and scattered well.

4.1.4.1 *E. macrobulbon*

4.1.4.1.1 Sampling

Root-tip of *E. macrobulbon* were sampled at different time; 8.00, 9.00, 10.00, and 11.00 A.M. The root-tips in each treatment were fixed, hydrolyzed, and stained following the regular Feulgen's squash method before being investigated under compound microscope

The result of this experiment revealed that the best sampling time was at 9.00 A.M. since the cell of the root tissue were mostly in metaphase stage of mitosis. The tissues sampled at 8.00 A.M. obtained cell at prophase stage while those taken at 10.00 A.M. and 11 A.M. were in telophase stage (Figure 4.35).

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4.1.4.1.2 Pre-treatment

Root-tip samples taken at 9.00 A.M. were pre-treated in 0.002 M 8-hydroxyquinoline solution for 12, 24, and 36 hours. After that, samples were fixed, hydrolyzed, stained, and examined under compound microscope. It was found that 24 hours was the best pre-treatment duration to make chromosome of *E. macrobulbon* contracted and scattered (Figure 4.36).

Chromosome counting under compound microscope showed that chromosome number of *E. macrobulbon* was 2n=48.

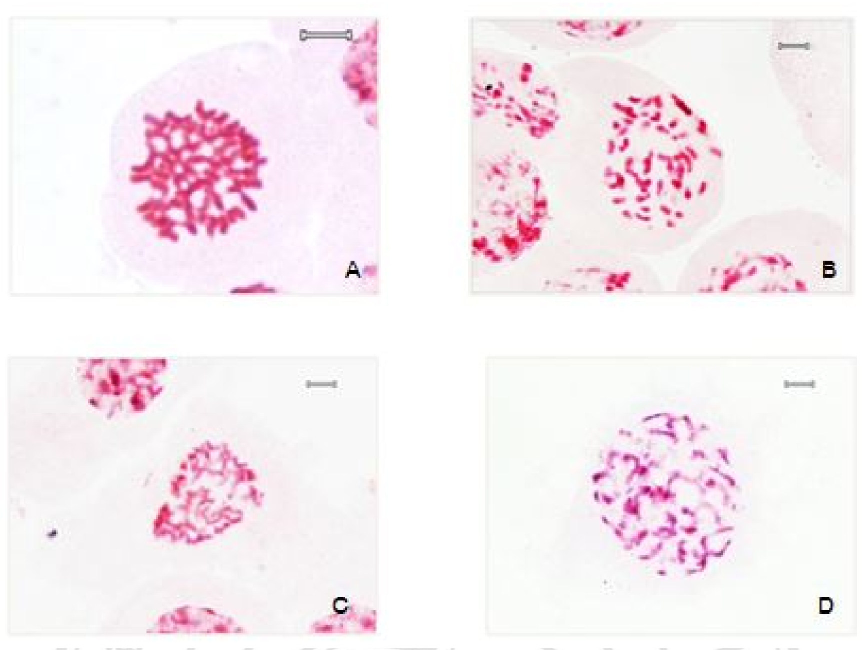

Figure 4.35 Somatic chromosomes in *Eulophia macrobulbon* root-tip cells collected at different times; A) 8:00 A.M., B) 9:00 A.M., C) 10:00 A.M., D) 11:00 A.M. (scale bar = 5 µm)

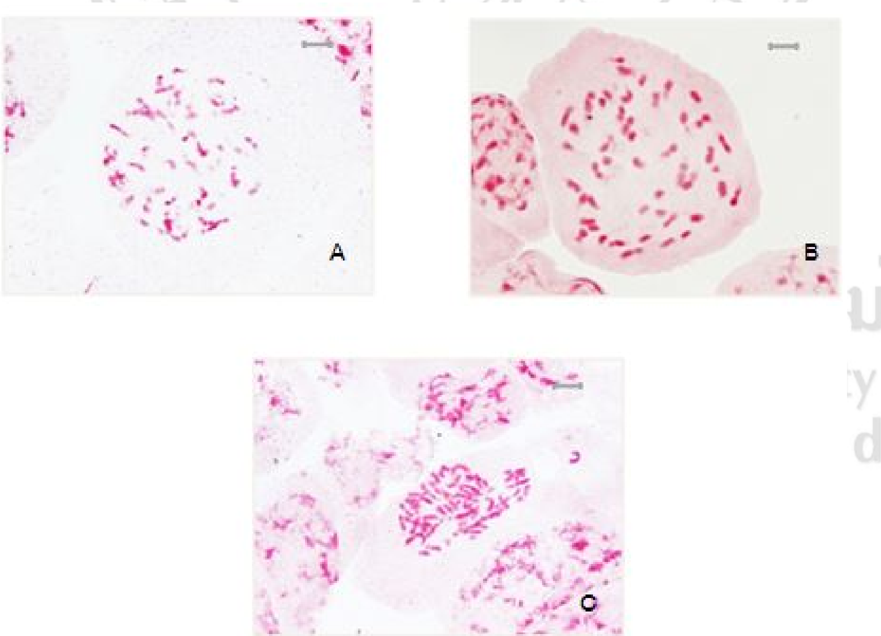

Figure 4.36 Somatic chromosomes in *Eulophia macrobulbon* root-tip cells soaked in pre-treatment solution at different duration; A) 12 hours, B) 24 hours, C) 48 hours (scale bar = 5 µm)

4.1.4.2 *E. spectabilis*

4.1.4.1.1 Sampling

Root tip of *E. spectabilis* were sampled at different time; 8.00, 9.00, 10.00, and 11.00 A.M. The root-tips in each time were fixed, hydrolyzed, and stained as indicated by regular Feulgen's squash method before being investigated under compound microscope**.**

Cell from root-tip sampled at 10.00 A.M. could yield cell at metaphase stage more than cell collected at other time. Prophase cell was found in sampled at 8.00 and 9.00 A.M., while anaphase cells occurred at 11.00 A.M. (Figure 4.37).

4.1.4.1.2 Pre-treatment

Root-tip samples taken at 10.00 A.M. were pre-treated in 0.002 M 8-hydroxyquinoline solution for 12, 24, and 36 hours. The result showed that 24 hours was the best duration to make chromosome of this terrestrial orchid contracted and spreaded (Figure 4.38).

Chromosome counting under compound microscope showed that chromosome number of *E. spectabilis* was 2n=52.

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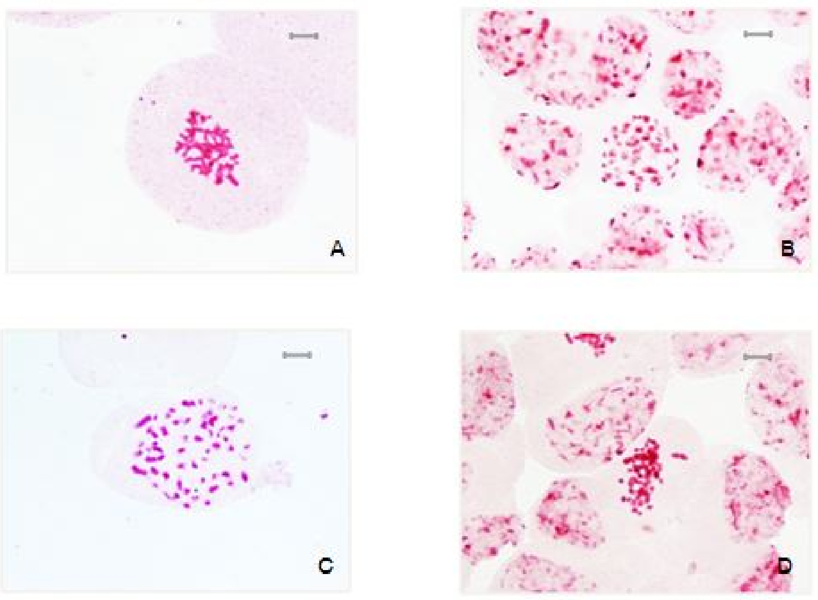

Figure 4.37 Somatic chromosomes in *Eulophia spectabilis* root-tip cells collected at different times; A) 8:00 A.M., B) 9:00 A.M., C) 10:00 A.M., D) 11:00 A.M. (scale bar = 5 µm)

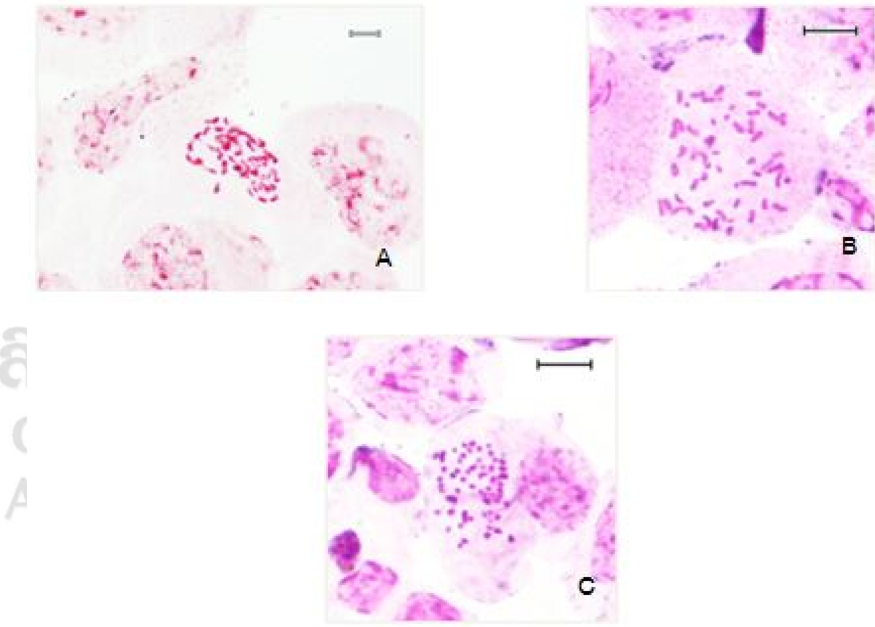

Figure 4.38 Somatic chromosomes in *Eulophia spectabilis* root-tip cells soaked in pre-treatment solution at different duration; A) 12 hours, B) 24 hours, C) 48 hours (scale bar = 5 µm)

4.1.5 Genetic relationship

Analysis of genetic relationship of genus *Eulophia* by RAPD technique was conducted. The objective of this experiment was to distinguish genetic diversity and relationship of *Eulophia* to provide basic information for breeding program.

This experiment was divided into 3 parts, DNA extraction, RAPD analysis, and analysis of genetic relationship.

4.1.5.1 DNA extraction

Leaf DNAs of plant material were extracted by CTAB method modified by Taywiya (2010). DNA qualities were checked by electrophoresis in 1.5% agarose gel. The electrophoresis result showed single DNA bands from each CTAB-extracted sample (Figure 4.39). DNA contents were quantified by nanodrop spectrophotometer 2000 (Thermo Scientific, USA) at 260 and 280 nanometer. $A_{260/280}$ ratios of twelve independently isolated samples extracted by CTAB were 1.53 to 1.87 (Table 4.1)

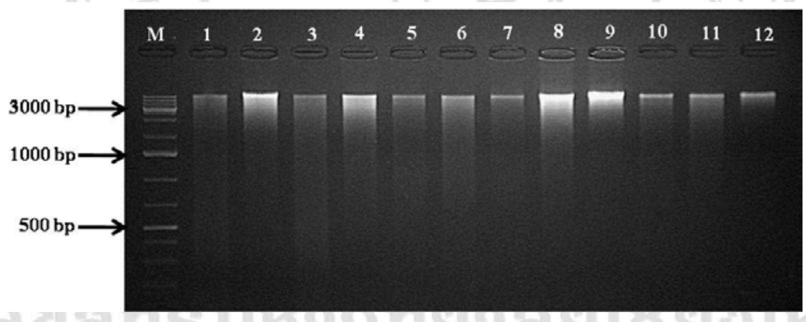

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Figure 4.39 Gel electrophoresis of *Eulophia* DNA extracted by CTAB buffer; M) 1 kb bps plus DNA ladder, 1) *E. macrobulbon*, 2) *E. spectabilis*; white, white lip with yellow stripe, 3) *E. spectabilis*; pink-white, white lip with yellow stripe, 4) *E. spectabilis*; pink-white, pink lip with yellow stripe, 5) *E. spectabilis*; pink, pink lip with yellow stripe, 6) *E. spectabilis* (green-pink, pink lip with yellow stripe), 7) *E. spectabilis*; dark pink to red, pink lip, 8) *E. spectabilis*; yellow-green, white lip, 9) *E. spectabilis*; pink-green, pink lip, 10) *E. andamanensis*, 11) *E. graminea*, 12) *E. flava*

Table 4.1 DNA concentration and absorbance ratio ($A_{260/280}$) of DNA extracted by CTAB buffers

Number	Name	DNA concentration (ng/µl)	$A_{260/280}$
1	*E. macrobulbon*	361.9	1.69
2	*E. spectabilis*; white, white lip with yellow stripe	681.8	1.58
3	*E. spectabilis*; pink-white, white lip with yellow stripe	321.0	1.69
4	*E. spectabilis*; pink-white, pink lip with yellow stripe	230.9	1.71
5	*E. spectabilis*; pink, pink lip with yellow stripe	508.7	1.59
6	*E. spectabilis*; green-pink, pink lip with yellow stripe	245.8	1.65
7	*E. spectabilis*; dark pink to red, pink lip	513.4	1.53
8	*E. spectabilis*; yellow-green, white lip	1218.4	1.83
9	*E. spectabilis*; pink-green, pink lip	1399.4	1.87
10	*E. andamanensis*	705.8	1.83
11	*E. graminea*	1097.2	1.86
12	*E. flava*	1293.9	1.74

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4.1.5.2 RAPD analysis

Genetic relationships of twelve terrestrial orchids were investigated. Thirty three decamer primers; OPA1-20, OPF1-3, OPF6-9, OPF11-13, OPF16, and OPF18-19 (Appendix D) were screened. The results showed that 21 primers; OPA2, OPA3, OPA4, OPA5, OPA6, OPA7, OPA8, OPA9, OPA10, OPA11, OPA12, OPA16, OPA18, OPA20, OPF1, OPF2, OPF3, OPF6, OPF9, OPF12, and OPF13, could reveal in *Eulophia* (Appendix E). There were 710 polymorphic bands (Table 4.2-4.3). Banding patterns from these 21 primers were recorded and analyzed by UPGMA for identifying genetic relationship.

Table 4.2 PCR amplification by 14 OPA primers

Number	Primer	Size of DNA bands (bps)	Total no. of bands	No. of polymorphic bands	% polymorphism
1	OPA2	2181-313	56	53	94.64
2	OPA3	1732-510	58	58	100.00
3	OPA4	1249-393	43	39	90.70
4	OPA5	1774-553	25	23	92.00
5	OPA6	2891-415	38	33	86.84
6	OPA7	1650-590	33	33	100.00
7	OPA8	982-643	18	17	94.44
8	OPA9	1871-1712	16	9	56.25
9	OPA10	1849-443	48	41	85.42
10	OPA11	1341-507	29	26	89.66
11	OPA12	1500-500	19	18	94.73
12	OPA16	1696-450	32	30	93.75
13	OPA18	1650-300	38	34	89.47
14	OPA20	1717-468	71	70	98.59
	Total		524	484	
	Average		37.43	34.57	92.37

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Table 4.3 PCR amplification by 7 OPF primers

Number	Primer	Size of DNA bands (bps)	Total no. of bands	No. of polymorphic bands	% polymorphism
1	OPF1	1292-428	46	45	97.82
2	OPF2	1046-383	18	12	66.67
3	OPF3	1732-748	13	11	84.61
4	OPF6	1458-447	29	26	89.65
5	OPF9	2000-500	40	37	92.50
6	OPF12	1539-346	57	56	98.24
7	OPF13	2139-268	61	39	63.93
	Total		264	226	
	Average		33.00	28.25	85.60

4.1.5.3 Analysis of genetic relationship

Genetic relationships among 12 samples of 5 species from *Eulophia* genera were estimated by NTSYSpc version 2.01cc program and mode dendrogram by using unweighted pair groups with arthmetric mean (UPGMA).

Combination of 3 primers, OPA4, OPF1, and OPF13, could separate *Eulophia* into 2 major groups at 47% similarity. These primers could cluster all *E. spectabilis*, *E. macrobulbon*, and *E. flava*. Another group comprised *E. andamanensis* and *E. graminea* (Figure 4.40).

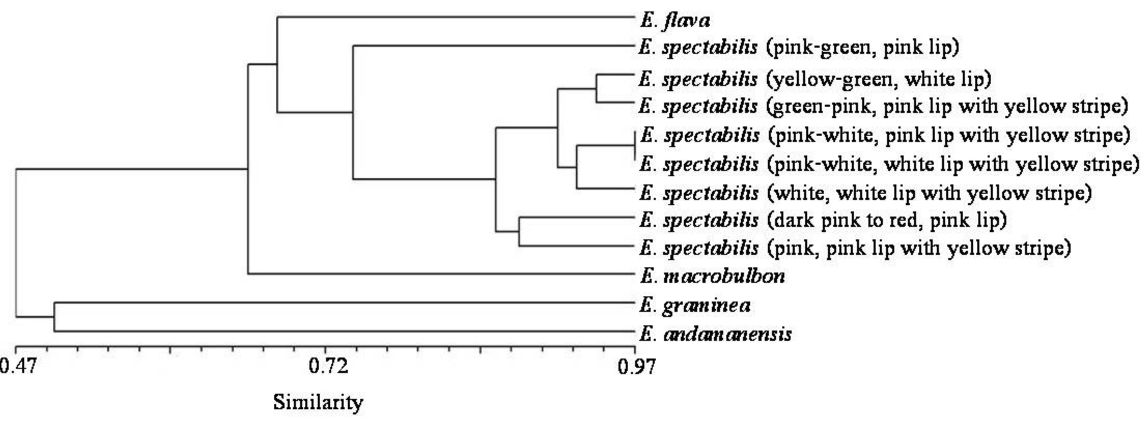

Figure 4.40 Dendrogram of *Eulophia* based on three primers

4.2 Crossabilities studies

Intraspecific, interspecific, and intergeneric hybridization between *E. macrobulbon*, *E. spectabilis*, and some terrestrial orchids that were related with both *Eulophia* were conducted. This study was done in order to provide basic information for future breeding program.

Each crossability hybridization study was tested for 3 steps. The first step was percentage of fruit set. The second step was seed viability study using modified Wongnan's method (2010).The final step was study of seed germination by using tissue culture technique on Vacin and Went (1949) medium (VW).

4.2.1 Intraspecific hybridization

Percentages of fruit set from selfing of *E. macrobulbon* and *E. spectabilis* were 86.67% and 92.30%, respectively (Table 4.4). Flower of *E. macrobulbon* and *E. spectabilis* withered at 1 month after pollination. These seed pods started to enlarge at 2 months and fruit mature appeared at 5 months after pollination (Figure 4.41 and 4.44). Seed viability of both terrestrial orchids was 87.22% and 97.68%, respectively (Table 4.4). Viable seed of self *E. macrobulbon* and *E. spectabilis* could be distinguished from non viable seed; viable seed showed very clear stained cell whereas non viable seed had no stained seed (Figure 4.42 and 4.45). Seeds that were cultivated *in vitro* could germinate at 83.33% and 95.50%, respectively (Table 4.4). Seed of *E. macrobulbon* and *E. spectabilis* could germinate within 2 months and develop to protocorms at 3 months. Seedlings of both terrestrial orchid appeared at 5 months (Figure 4.43 and 4.46).

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Table 4.4 Percentage of fruit set, seed viability, and seed germination from *Eulophia* hybridization

Female plant	Male plant	Number of hybridized flowers	Number of seed pods	Fruit set (%)	Seed viability (%)	Seed germination (%)
Intraspecific hybridization						
E. macrobulbon	*E. macrobulbon*	15	13	86.67	87.22 ± 6.00	83.33 ± 15.27
E. spectabilis	*E. spectabilis*	13	12	92.30	97.68 ± 7.10	95.50 ± 7.07

Figure 4.41 Seed pod of selfed *Eulophia macrobulbon* at different times; A) 1 month, B) 2 months, C) 3 months, D) 4 months, E) 5 months after pollination (scale bar = 1 cm)

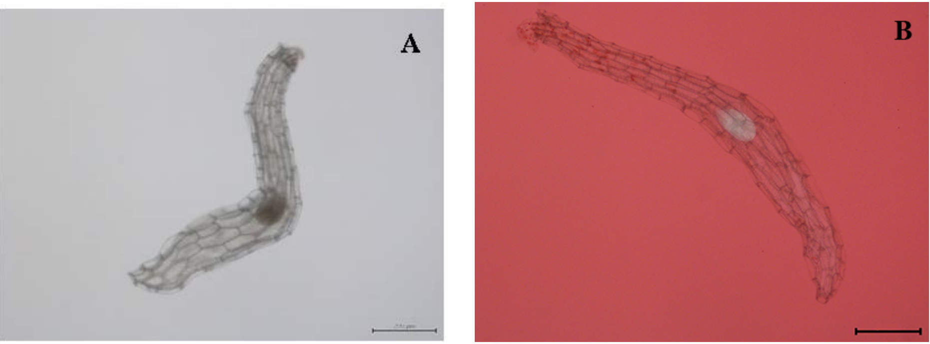

Figure 4.42 Seed of selfed *Eulophia macrobulbon* from 5 months pod; A) seed under microscope, B) stained seed with lacto-propionic orcein under microscope (scale bar = 200 µm)

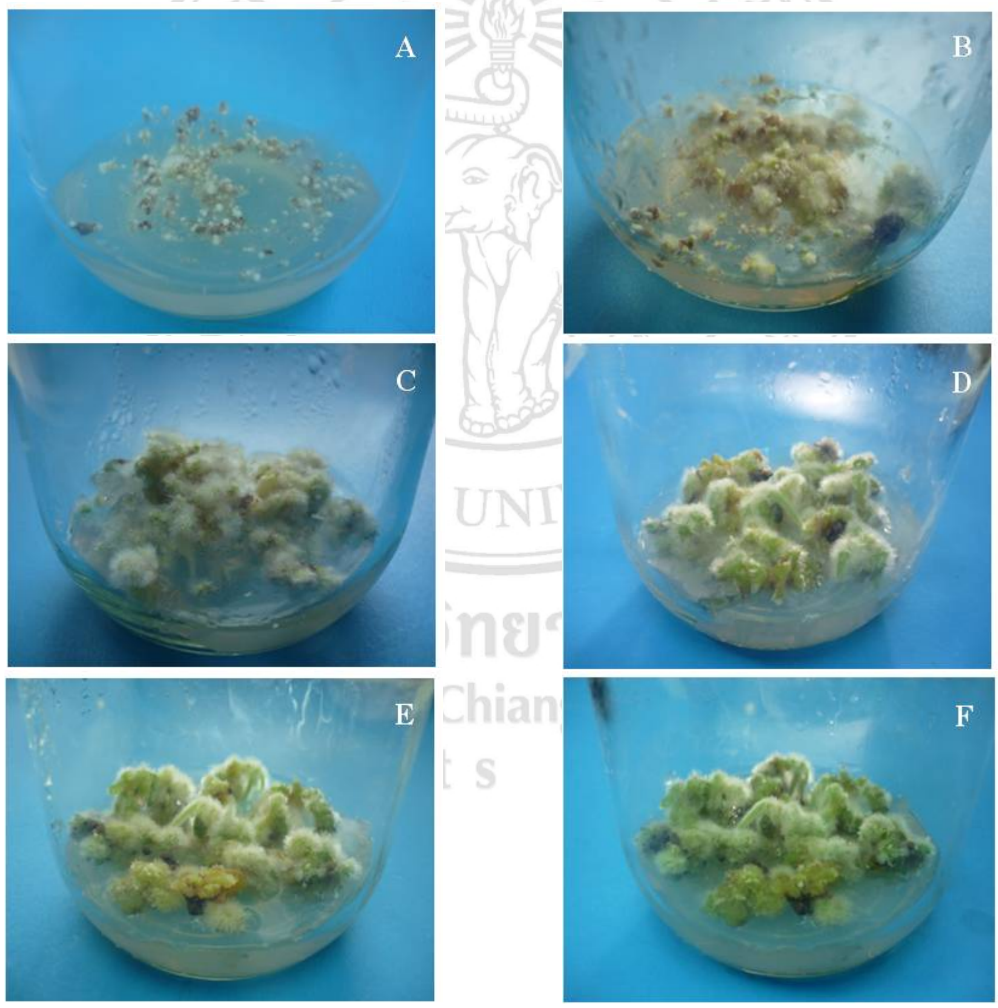

Figure 4.43 Seed germination of selfed *Eulophia macrobulbon* at different times; A) 1 month, B) 2 months, C) 3 months, D) 4 months, E) 5 months, F) 6 months after sowing

Figure 4.44 Seed pod of selfed *Eulophia spectabilis* at different times; A) 1 month, B) 2 months, C) 3 months, D) 4 months, E) 5 months after pollination (scale bar = 1 cm)

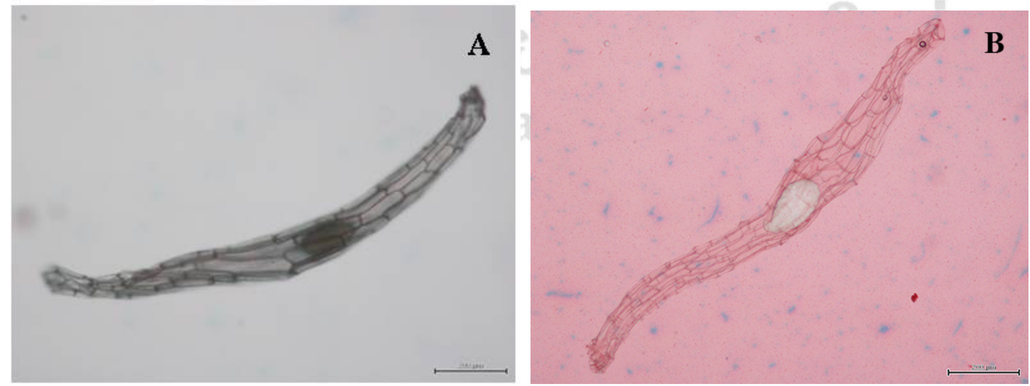

Figure 4.45 Seed of selfed *Eulophia spectabilis* from 5 months pod; A) seed under microscope, B) stained seed with lacto-propionic orcein under microscope (scale bar = 200 µm)

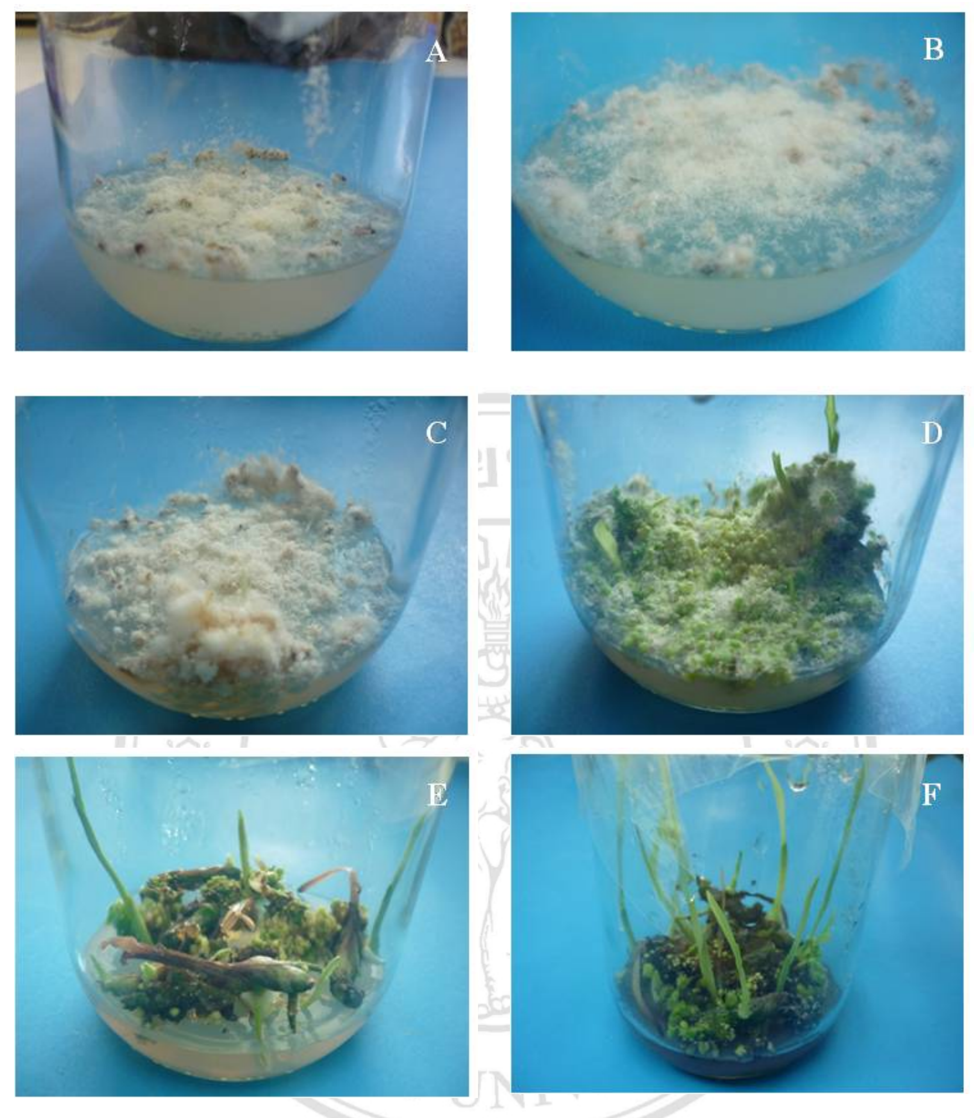

Figure 4.46 Seed germination of selfed *Eulophia spectabilis* at different times; A) 1 month, B) 2 months, C) 3 months, D) 4 months, E) 5 months, F) 6 months after sowing

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4.2.2 Interspecific hybridization

Interspecific hybridization between *E. macrobulbon* and *E. spectabilis* did not occur when using *E. macrobulbon* as female parent. When *E. spectabilis* was used as female parent, 100% fruit set was found (Table 4.5). Seed pod of *E. spectabilis* × *E. macrobulbon* enlarged at 2 months after pollination. The mature fruit could be harvested at 5 months after pollination (Figure 4.47). Seed viability and seed germination of *E. spectabilis* × *E. macrobulbon* were 98.13% and 86.25%, respectively (Table 4.5). Viable seed of this hybrid could be distinguished from non viable seed; viable seed showed very clear stained cell whereas non viable seed had no stained seed (Figure 4.48). Seed of *E. spectabilis* × *E. macrobulbon* started to geminate at 3 months and developed to young plant at 4 months (Figure 4.49).

Table 4.5 Percentage of fruit set, seed viability, and seed germination from interspecific *Eulophia* hybridization

Female plant	**Male plant**	**Number of hybridized flowers**	**Number of seed pods**	**Fruit set (%)**	**Seed viability (%)**	**Seed germination (%)**
Interspecific hybridization						
E. macrobulbon	*E. spectabilis*	20	0	0	-[1]	-[1]
E. spectabilis	*E. macrobulbon*	13	13	100	98.13 ± 1.77	86.25 ± 13.78

[1] Data unavailable due to no fruit set

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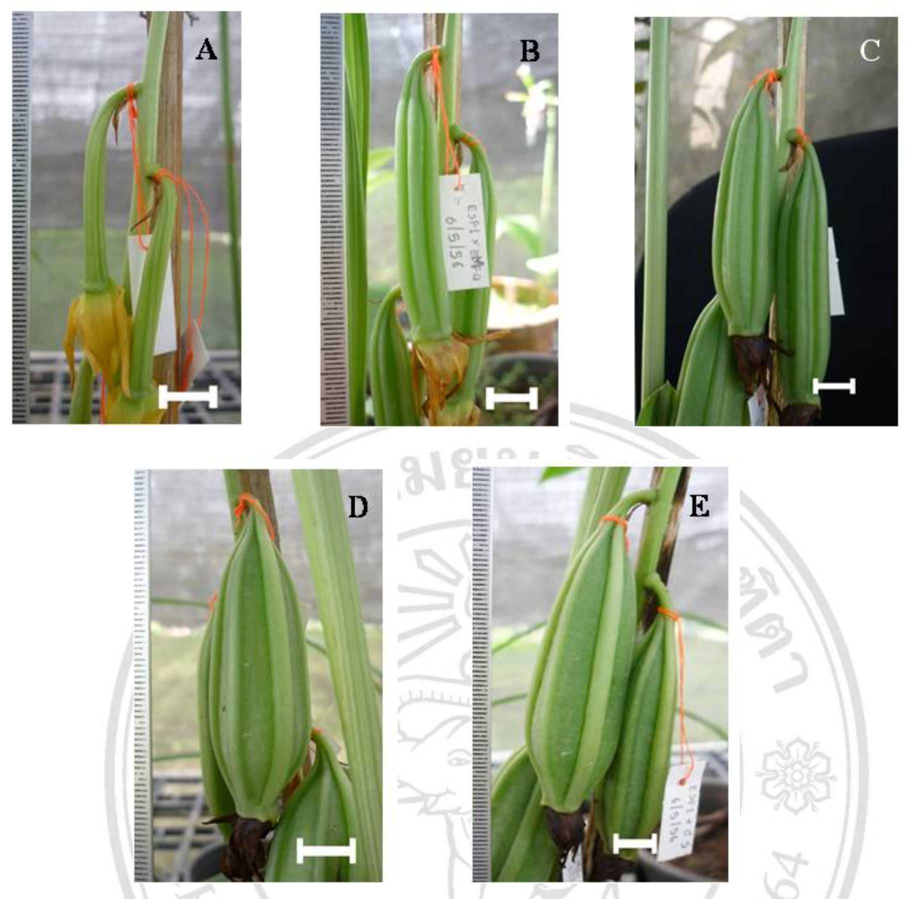

Figure 4.47 Seed pod of *Eulophia spectabilis* × *Eulophia macrobulbon* at different times; A) 1 month, B) 2 months, C) 3 months, D) 4 months, E) 5 months after pollination (scale bar = 1 cm)

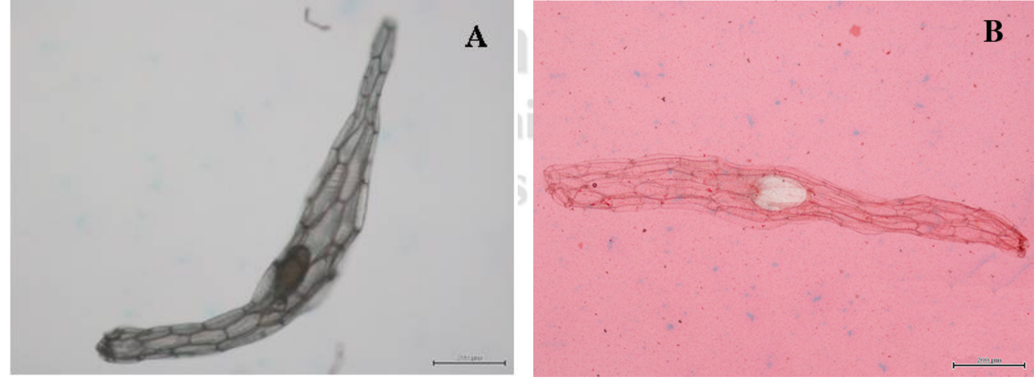

Figure 4.48 Seed of *Eulophia spectabilis* × *Eulophia macrobulbon* from 5 months pod; A) seed under microscope, B) stained seed with lacto-propionic orcein under microscope (scale bar = 200 µm)

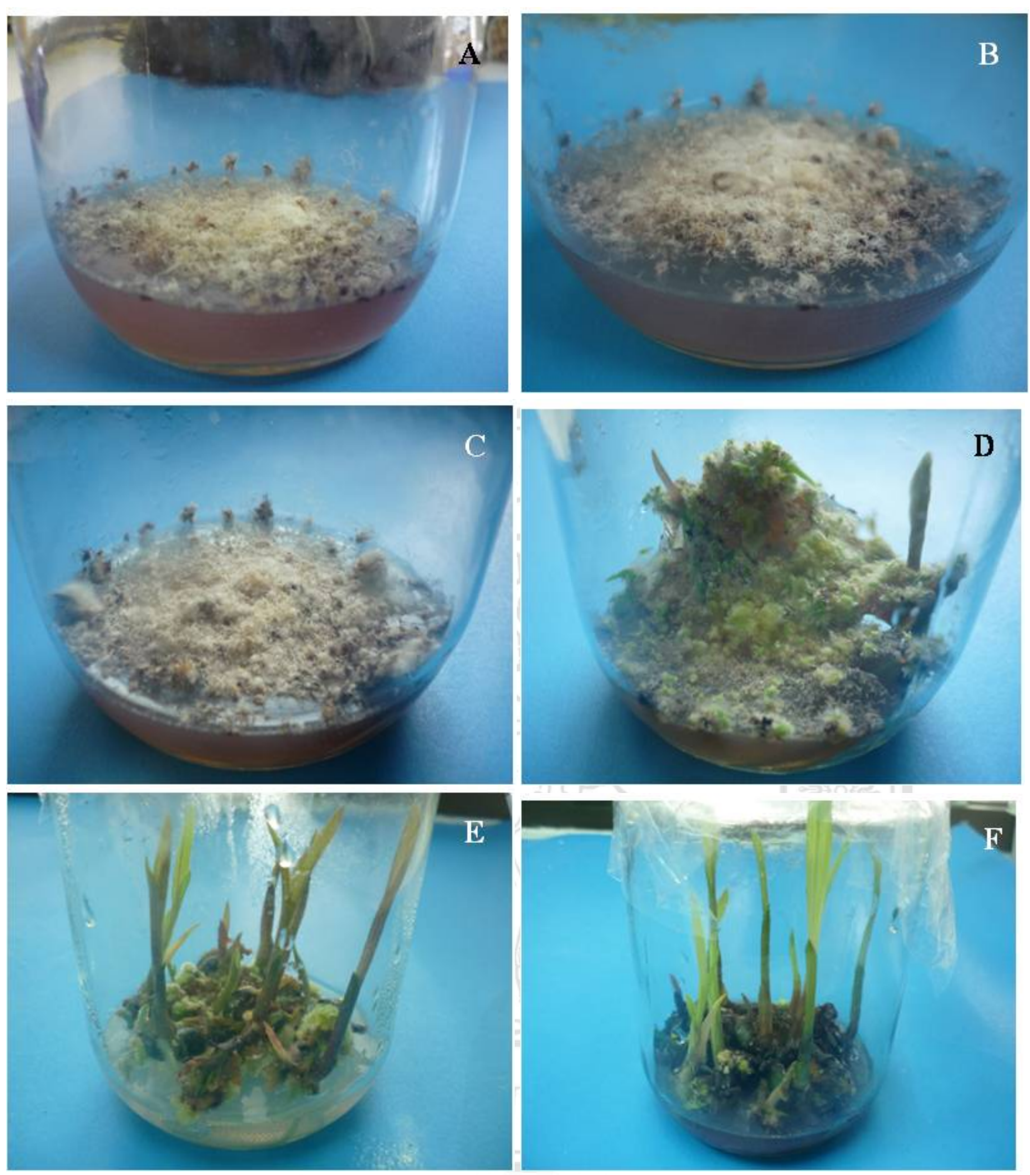

Figure 4.49 Micropropagation of *Eulophia spectabilis* × *Eulophia macrobulbon* at different times: A) 1 month, B) 2 months, C) 3 months, D) 4 months, E) 5 months, F) 6 months after sowing

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4.2.3 Intergeneric hybridization

Study of intergeneric crossability between *Eulophia* and terrestrial orchids which are in the same subfamily *Geodorum* and *Spathoglottis* was conducted. It was found that percentage of fruit set, seed viability, and seed germination of *G. attenuatum* × *E. macrobulbon* were 92.31%, 87.29%, and 57.50%, respectively (Table 4.6). The seed pod of this hybrid started to enlarge at 2 months and developed to a mature fruit at 5 months after pollination (Figure 4.50). Viable seed of *G. attenuatum* × *E. macrobulbon* could be distinguished from non viable seed; viable seed showed very clear stained cell whereas non viable seed had no stained seed (Figure 4.51). Seed of this hybrid germinated on VW medium at 4 months and developed to seedling at 6 months (Figure 4.52). Whereas, hybridization of *E. macrobulbon* × *G. attenuatum*, percentage of fruit set was 21.43%. Seed pods of this hybrid were small. Growth and development of this cross was slow and viable seed was no found inside the capsule (Figure 4.53 and 4.54). Besides, seed could not geminate (Figure 4.55). On the other hand, fruit set of hybridization between *G. attenuatum* × *E. spectabilis* could yield fruit set at 85.71%, seed viability was 54.64%. The mature seed pod could be harvested at 5 months after pollination (Figure 4.56). Viable seed of this hybrid showed very clear stained cell (Figure 4.57). Seed germination was 24.00%, seeds germinate at 2 months, seedlings appeared at 6 months (Figure 4.58), and when reciprocal crosses were done, 100% fruit set was found. The fruit of this hybrid developed to mature fruit at 5 months (Figure 4.59). Seed viability and seed germination of *E. spectabilis* × *G. attenuatum* were 76.59% and 16.17%, respectively (Table 4.6). Viable seed of this hybrid had stained cell (Figure 4.60). Seed started to germinate on VW medium at 2 months. Seedling of this hybrid appeared at 6 months (Figure 4.61).

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Fruit set of *S. affinis* × *E. macrobulbon* was only 34.48%. The mature seed pod could be harvested at 1 month (Figure 4.62). None of the seed was viable (Figure 4.63) and could not germinate on VW medium (Figure 4.64) and the reciprocal cross was also failure, whereas, hybridization between *E. spectabilis* and *S. affinis* was not successful (Table 4.6).

Table 4.6 Percentage of fruit set, seed viability, and seed germination from intergeneric *Eulophia* hybridization

Female plant	Male plant	Number of hybridized flowers	Number of seed pods	Fruit set (%)	Seed viability (%)	Seed germination (%)
Intergeneric hybridization						
G. attenuatum	*E. macrobulbon*	13	12	92.31	87.29 ± 7.85	57.50 ± 10.82
E. macrobulbon	*G. attenuatum*	14	3	21.43	0	0
G. attenuatum	*E. spectabilis*	14	12	85.71	54.64 ± 2.73	24.00 ± 5.45
E. spectabilis	*G. attenuatum*	11	11	100	76.59 ± 17.88	16.67 ± 5.77
S. affinis	*E. macrobulbon*	29	10	34.48	0	0
E. macrobulbon	*S. affinis*	12	0	0	na	na
S. affinis	*E. spectabilis*	26	0	0	na	na
E. spectabilis	*S. affinis*	29	0	0	na	na

na= data not applicable due to no fruit set

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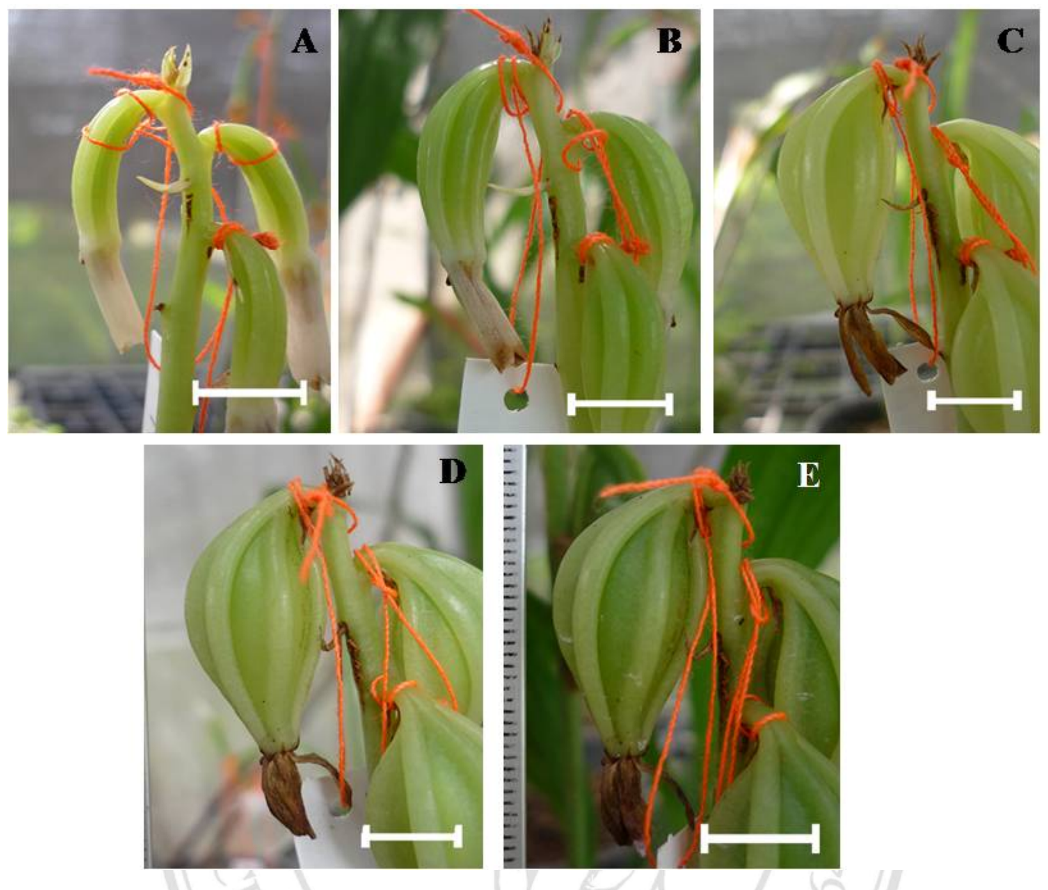

Figure 4.50 Seed pod of *Geodorum attenuatum* × *Eulophia macrobulbon* at different times; A) 1 month, B) 2 months, C) 3 months, D) 4 months, E) 5 months after pollination (scale bar = 1 cm)

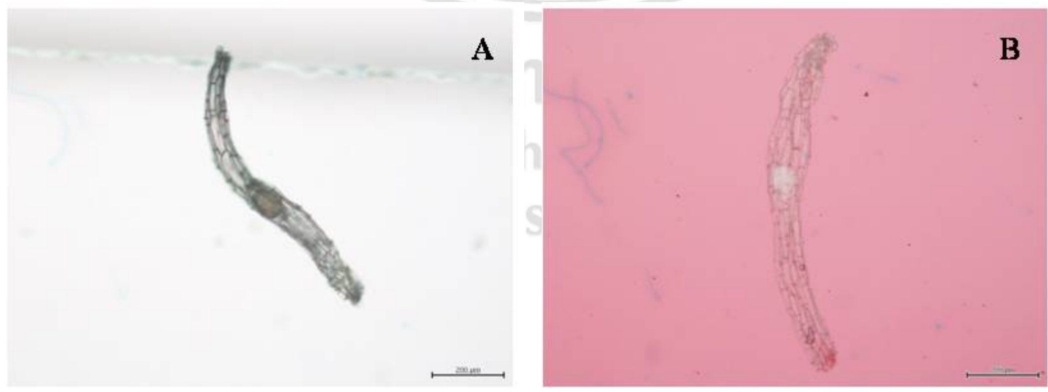

Figure 4.51 Seed of *Geodorum attenuatum* × *Eulophia macrobulbon* from 5 months pod; A) seed under microscope, B) stained seed with lacto-propionic orcein under microscope (scale bar = 200 µm)

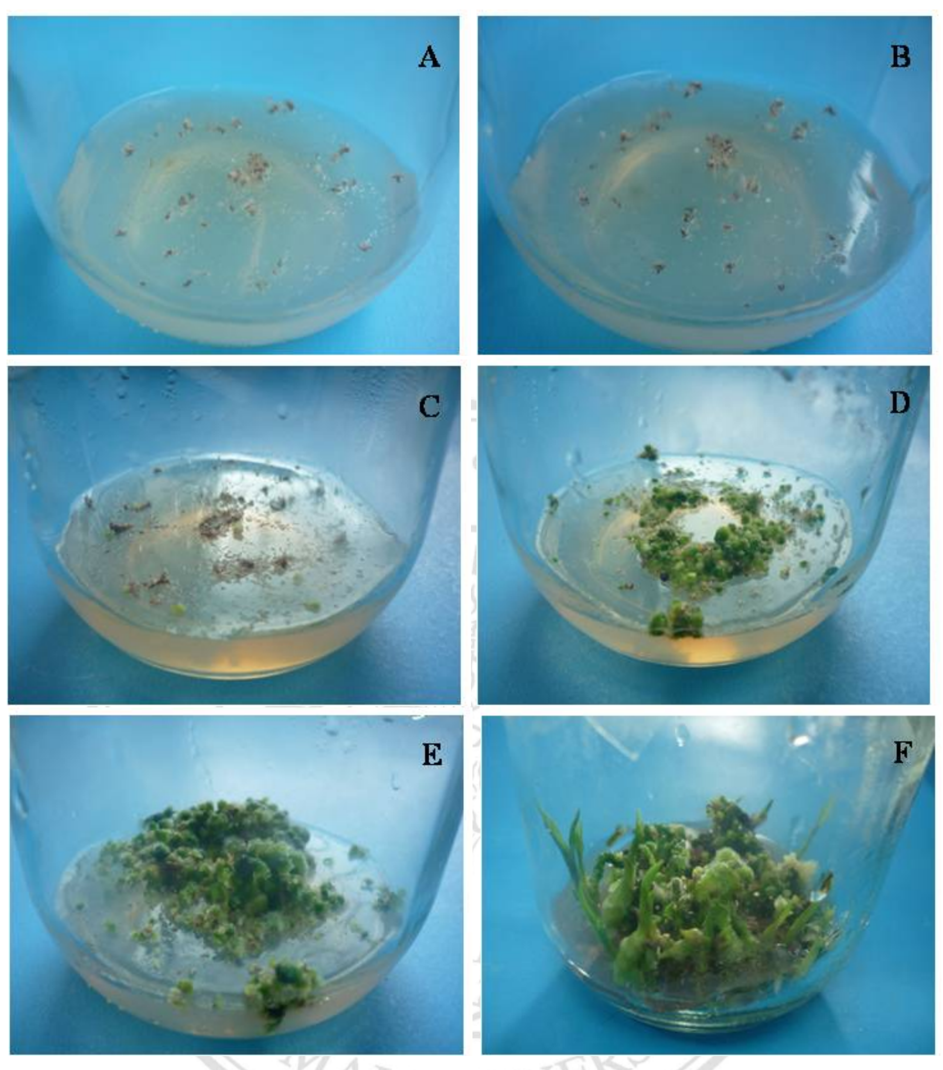

Figure 4.52 Micropropagation of *Geodorum attenuatum* × *Eulophia macrobulbon* at different times; A) 1 month, B) 2 months, C) 3 months, D) 4 months, E) 5 months, F) 6 months after sowing

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Figure 4.53 Seed pod of *Eulophia macrobulbon* × *Geodorum attenuatum* at different times; A) 1 month, B) 2 months, C) 3 months, D) 4 months, E) 5 months after pollination (scale bar = 1 cm)

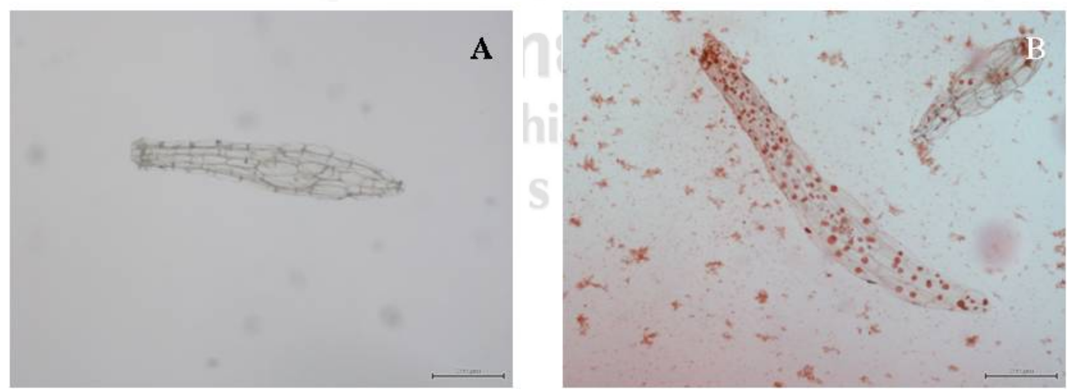

Figure 4.54 Seed of *Eulophia macrobulbon* × *Geodorum attenuatum* from 5 months pod; A) seed under microscope, B) stained seed with lacto-propionic orcein under microscope (scale bar = 200 µm)

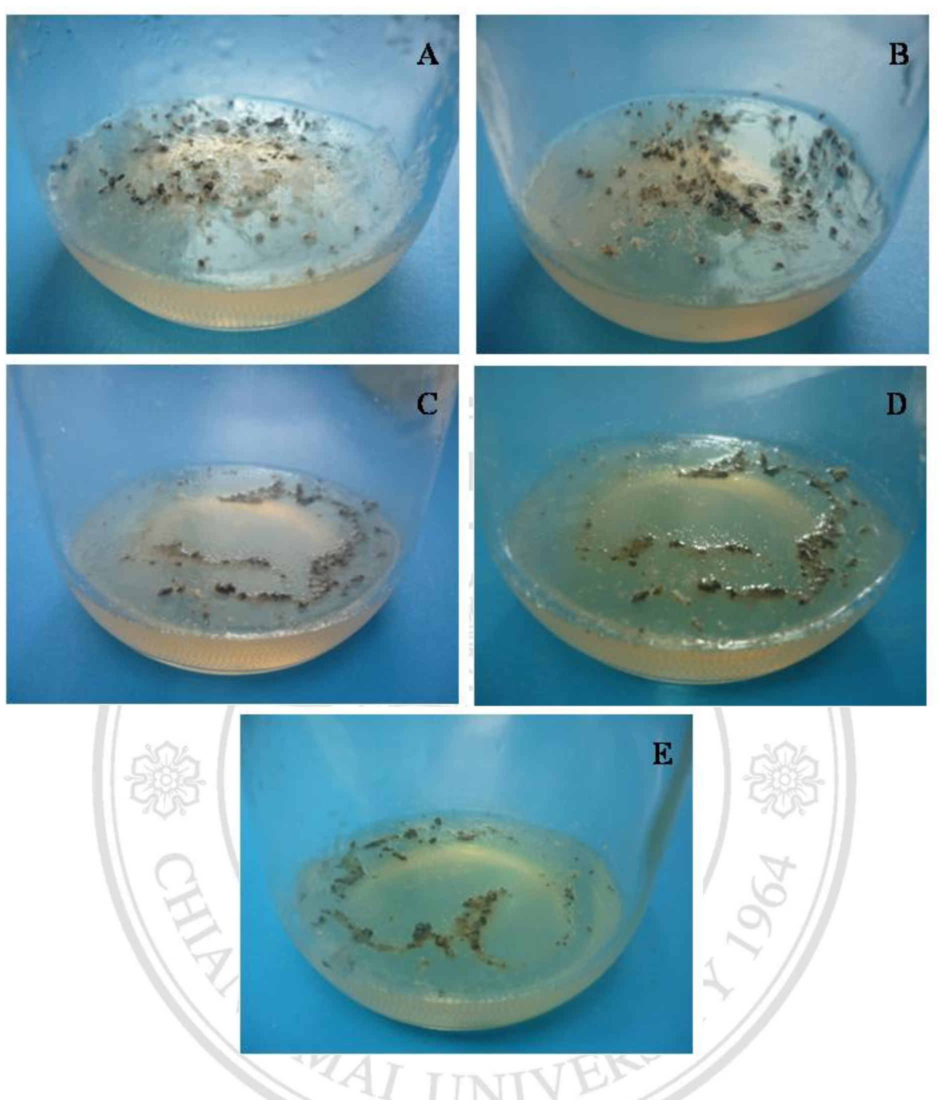

Figure 4.55 Micropropagation of *Eulophia macrobulbon* × *Geodorum attenuatum* at different times; A) 1 month, B) 2 months, C) 3 months, D) 4 months, E) 5 months, F) 6 months after sowing

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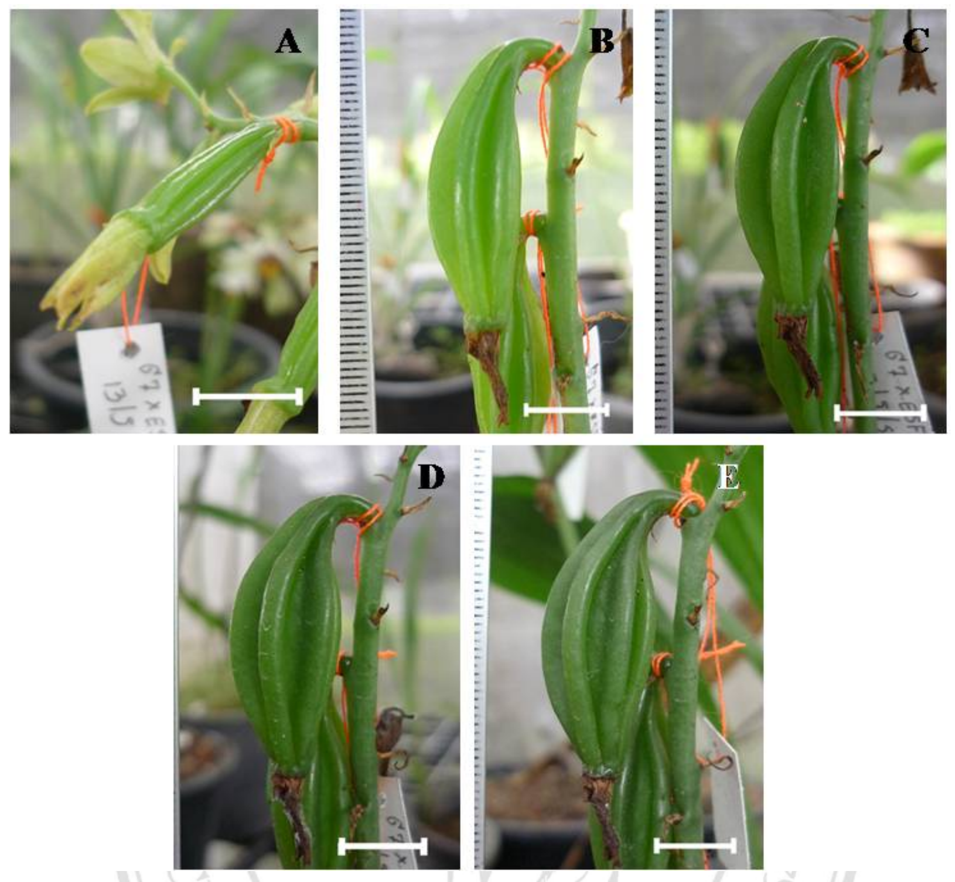

Figure 4.56 Seed pod of *Geodorum attenuatum* × *Eulophia spectabilis* at different times; A) 1 month, B) 2 months, C) 3 months, D) 4 months, E) 5 months after pollination (scale bar = 1 cm)

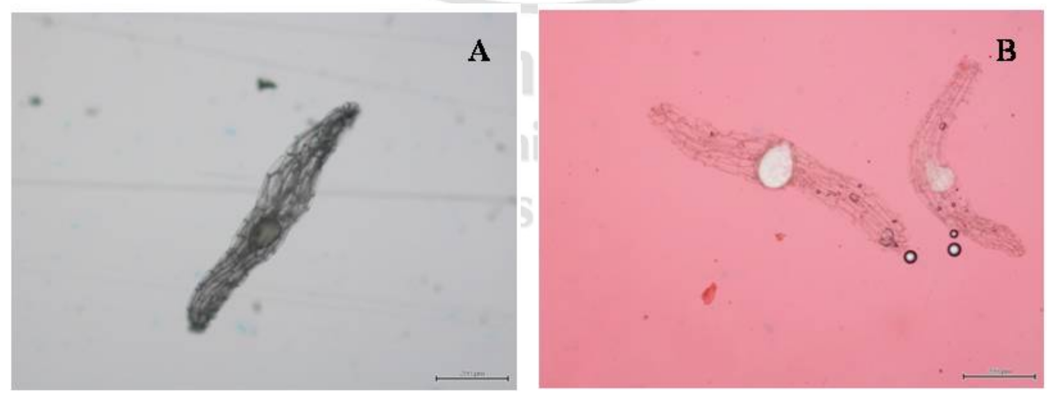

Figure 4.57 Seed of *Geodorum attenuatum* × *Eulophia spectabilis* from 5 months pod; A) seed under microscope, B) stained seed with lacto-propionic orcein under microscope (scale bar = 200 µm)

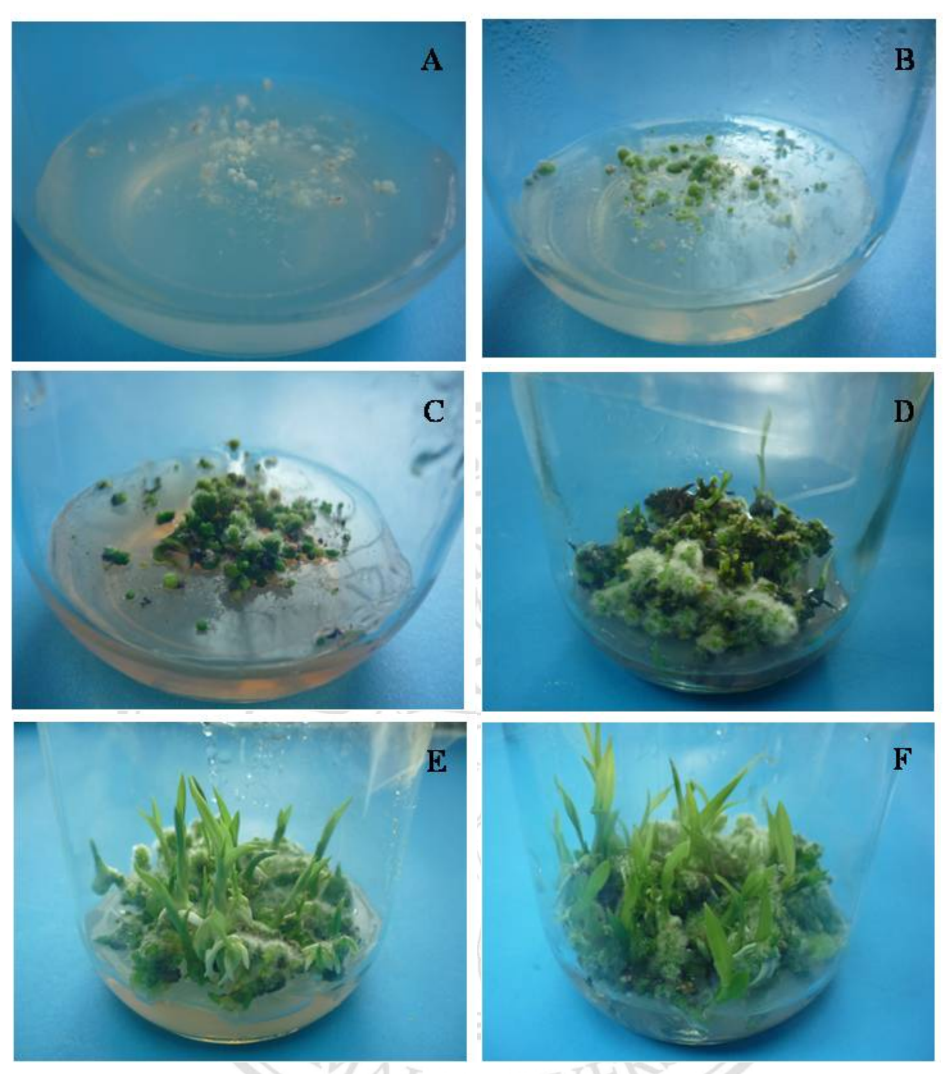

Figure 4.58 Micropropagation of *Geodorum attenuatum* × *Eulophia spectabilis* at different times; A) 1 month, B) 2 months, C) 3 months, D) 4 months, E) 5 months, F) 6 months after sowing

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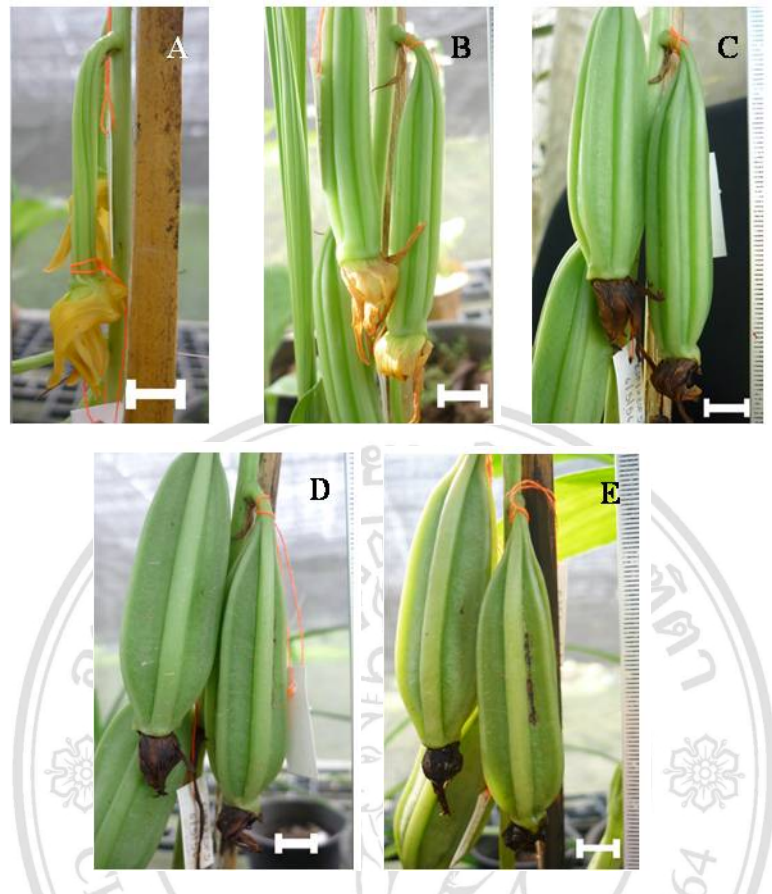

Figure 4.59 Seed pod of *Eulophia spectabilis* × *Geodorum attenuatum* at different times; A) 1 month, B) 2 months, C) 3 months, D) 4 months, E) 5 months after pollination (scale bar = 1 cm)

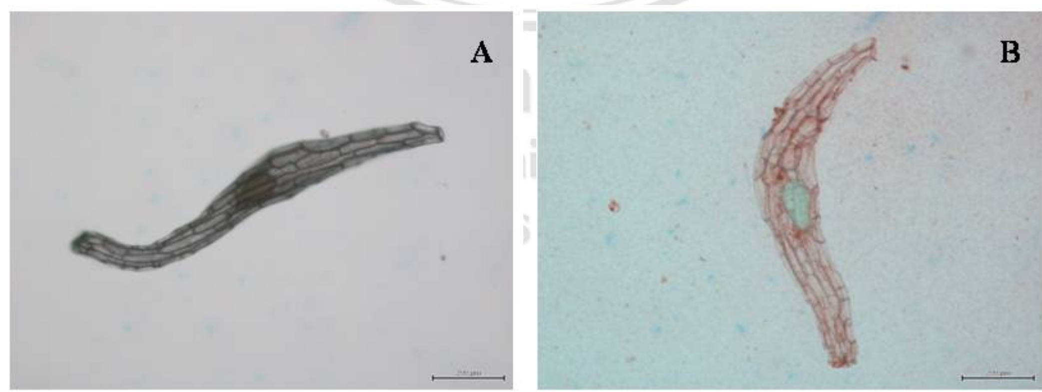

Figure 4.60 Seed of *Eulophia spectabilis* × *Geodorum attenuatum*in from 5 months pod; A) seed under microscope B) stained seed with lacto-propionic orcein under microscope (scale bar = 200 µm)

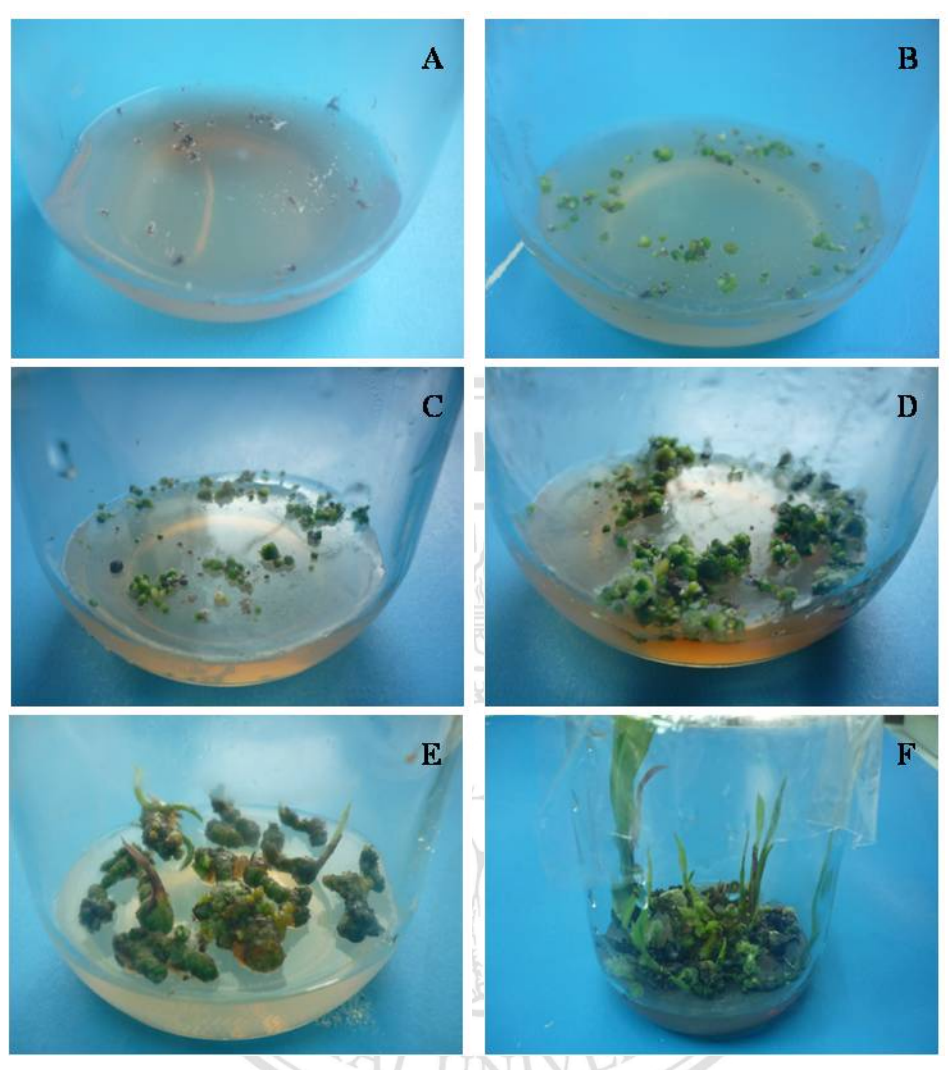

Figure 4.61 Micropropagation of *Eulophia spectabilis* × *Geodorum attenuatum* at different times; A) 1 month, B) 2 months, C) 3 months, D) 4 months, E) 5 months, F) 6 months after sowing

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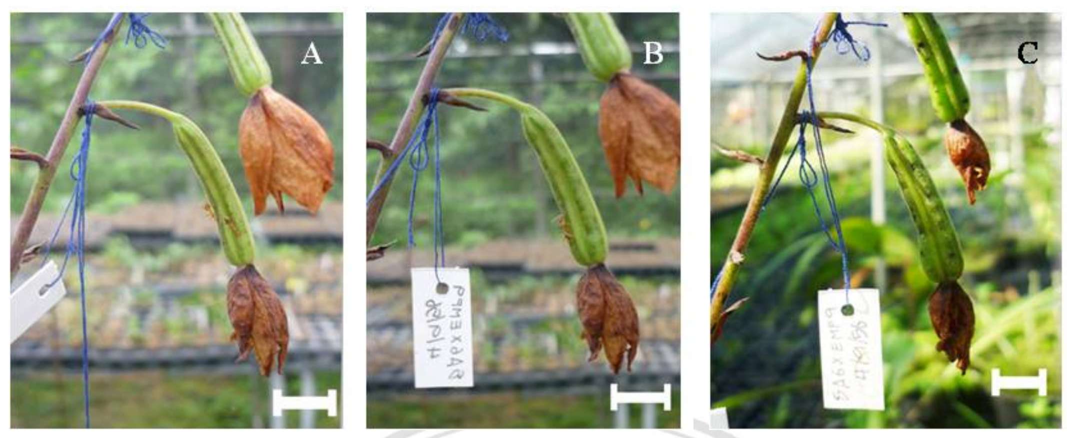

Figure 4.62 Seed pod of *Spathoglottis affinis* × *Eulophia macrobulbon* at different times; A) 5 days, B) 15 days, C) 30 days after pollination (scale bar = 1 cm)

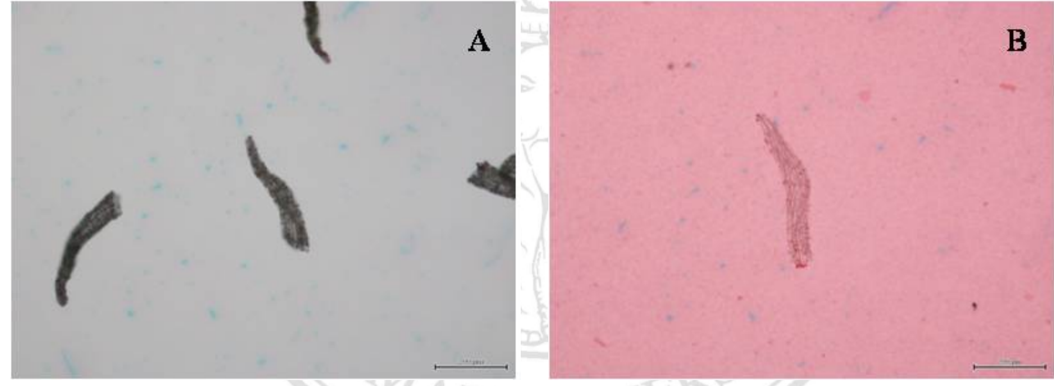

Figure 4.63 Seed of *Spathoglottis affinis* × *Eulophia macrobulbon* from 1 month pod; A) seed under microscope B) stained seed with lacto-propionic orcein under microscope (scale bar = 200 µm)

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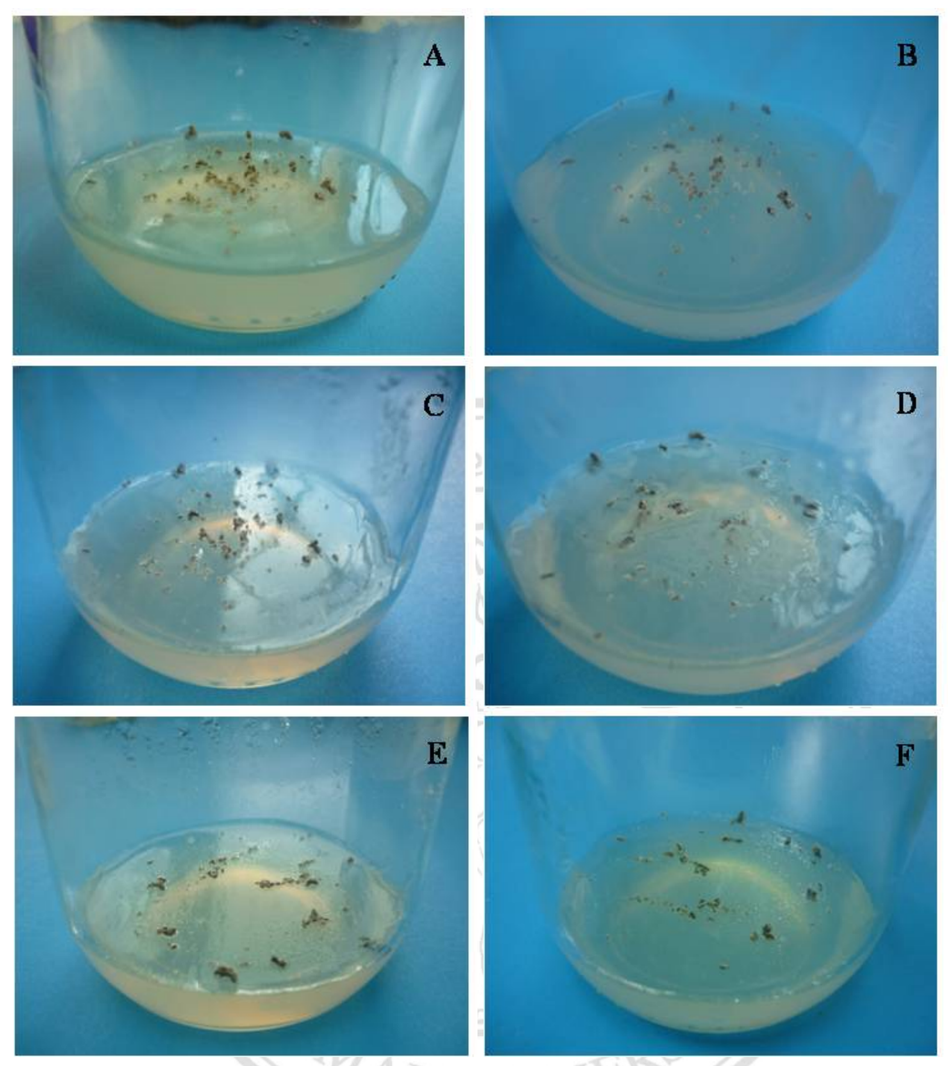

Figure 4.64 Micropropagation of *Spathoglottis affinis* × *Eulophia macrobulbon* at different times; A) 1 month, B) 2 months, C) 3 months, D) 4 months, E) 5 months, F) 6 months after sowing

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4.3 Physiological studies

Rhizome of *E. macrobulbon* and *E. spectabilis* were kept at low temperature for different period, 0, 30, 60, and 90 days. After rhizome of each specie was stored at each treatment, they were planted in 8-inch pot. Growth and development was recorded as well as TNC analysis.

4.3.1 Effect of storage period on *E. macrobulbon* and *E. spectabilis* at low temperature

4.3.1.1 Days of germination

Rhizome of *E. macrobulbon* and *E. spectabilis* was stored at 15°C for 0; room temperature, 30, 60, and 90 days. It was found that germination of *E. macrobulbon* which was stored at 15°C for 60 and 90 days was slower than controlled plant, 148 and 149.25 days, respectively. Whereas, storing rhizome at 15°C for 30 day was no different with control treatment (Table 4.7).

Day of germination of *E. spectabilis* which was stored at 15°C for 90 days could delay than other treatment; 174 days. While, day of germination of *E. spectabilis* which was stored at 15°C for 0, 30, and 60 days, were 136, 162 and 163 days, respectively (Table 4.8).

4.3.1.2 Plant height

Plant height of *E. macrobulbon* which stored at 15°C for 90 days was significantly shorter than controlled plant; 29.20 cm, while, plant height of *E. macrobulbon* which stored at 15°Cfor 0, 30, and 60 days was 46.67, 61.23, and 50.36 cm, respectively (Table 4.7).

Plant height of *E. spectabilis* was decreasing over period of storage at low temperature. Plant height of *E. spectabilis* that stored at 15°C for 0, 30, 60, and 90 days, were 87.70, 43.77, 40.67, and 18.22 cm, respectively (Table 4.8).

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4.3.1.3 Flowering

The low temperature of all storage period had no effect on flowering of *E. macrobulbon*, whereas, flowering percentage of *E. spectabilis* that stored at 15°C for 0, 60, and 90 days was 50, 25, and 50, respectively. The low temperature caused peduncle length significantly shorter than controlled plant. Peduncle of *E. spectabilis* which stored at 15°C for 60 and 90 day were 10.76 and 13.00 cm, respectively, whereas peduncle of controlled plant was 23.16 cm. Flower size was not different from controlled plant. Flower diameter of *E. spectabilis* that stored at 15°C for 0, 60, and 90 days, were 1.86, 1.83, and 1.80 cm, respectively (Table 4.8).

Table 4.7 Growth of *E. macrobulbon* stored at 15°C for different period

Period of storage at 15°C	Day of germination (days)	Plant height (cm)	Flower percentage (%)
0 days	134.25a	46.67a	0
30 days	132.00a	61.23b	0
60 days	148.00b	50.36a	0
90 days	149.25b	29.20c	0
$Duncan_{0.05}$	10.71	13.29	-

Mean within column followed by different letter are significantly at P<0.05.

Table 4.8 Growth of *E. spectabilis* stored at 15°C for different period

Period of storage at 15°C	Day of germination (days)	Plant height (cm)	Flower percentage (%)	Peduncle length (cm)	Flower size (cm)
0 days	136.00a	87.70a	50	23.16a	1.86
30 days	162.00b	43.77b	0	-[1/]	-[1/]
60 days	163.00b	40.67b	25	10.76b	1.83
90 days	174.00c	18.22c	50	13.00b	1.80
$Duncan_{0.05}$	14.65	25.69	-	6.03	NS.[2/]

Mean within column followed by different letter are significantly at P<0.05.

[1/]no flowering and [2/]not significantly different.

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4.3.2 Total non-structural carbohydrate analysis.

TNC analysis of *E. macrobulbon* and *E. spectabilis* rhizome stored at 15°C for different period was conducted on different rhizome age; first rhizome means new rhizome that grows and develops at present year, second rhizome means old rhizome from last year and third rhizome means rhizome from year before last year.

The result showed that temperature at 15°C had no effect on TNC of all rhizomes of *E. macrobulbon* when compare with control (Table 4.9).

Table 4.9 Content of total nonstructural carbohydrate (TNC) in storage rhizome of *E. macrobulbon* under different storage period

Rhizome	Treatment	TNC (mg ml^{-1} of D-glucose/dry weight sample)			
		0 day	30 days	60 days	90 days
1st	Control	398.74±143.88	458.24±51.91	424.21±12.60	424.06±20.36
	15°C		495.80±54.74	399.53±7.24	483.36±39.10
2nd	Control	241.07±20.81	237.15±30.01	227.35±11.76	225.06±24.07
	15°C		225.81±12.44	231.18±13.38	260.83±35.84
3rd	Control	270.75±165.55	229.49±23.75	151.83±63.17	172.67±44.39
	15°C		204.21±27.63	171.38±47.49	230.53±20.17

The result showed that 15°C had no effected on TNC of the whole bulb of *E. spectabilis* when compare with control (Table 4.10).

Table 4.10 Content of total nonstructural carbohydrate (TNC) in storage rhizome of *E. spectabilis* under different storage period

Rhizome	Treatment	TNC (mg ml^{-1} of D-glucose/dry weight sample)			
		0 day	30 days	60 days	90 days
1st	Control	406.25±38.80	498.28±40.76	562.36±26.20	492.91±26.99
	15°C		453.01±13.58	516.69±31.89	518.08±26.42
2nd	Control	527.84±64.49	545.52±18.20	621.26±29.99	550.62±47.79
	15°C		579.27±44.57	600.07±58.21	541.46±26.78
3rd	Control	450.82±175.72	442.26±122.15	454.70±111.20	301.67±134.75
	15°C		521.26±60.61	358.68±91.60	304.16±140.05

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CHAPTER 5

DISCUSSION

Studies of characteristics, growth and development cycle, and crossability of *Eulophia macrobulbon* (Par & Pchb. f.) Hook. f. and *E. spectabilis* (Dennst.) Suresh. were conducted. Both terrestrial orchids have potential to be used as cut flower and ornamental plant. They produce good flower size, shape, and number of flowers.

5.1 Characterizations and growth pattern

5.1.1 Morphological characterization

Morphological characterization of vegetative and reproductive growth of *E. macrobulbon* and *E. spectabilis* is compared as follows (Table 5.1).

Table 5.1 Morphological characterization of *Eulophia macrobulbon* and *E. spectabilis*

Characterization	*E. macrobulbon*	*E. spectabilis*
1. root		
• Morphology	Fibrous root, thick, fleshy, and not branched.	Fibrous root, thick, fleshy, and not branched.
• Color	White color when young and become strong yellowish brown when older.	White color when young and become strong yellowish brown when older.
2. Stem		
• Morphology	Rhizome type, roundish, grows on ground surface level.	Rhizome type, roundish, grows on ground surface level.
• Color	Gray-purple color when it is young.	Green color when it is young.
• Size	Diameter is 5.5-7.0 cm when mature.	Diameter is 5.5-7.0 cm when mature.

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Table 5.1 Morphological characterization of *Eulophia macrobulbon* and *E. spectabilis* (continued)

Characterization	*E. macrobulbon*	*E. spectabilis*
3. Leaves		
• Morphology	Simple leaf, oblanceoloate shape alternate arrangement, parallel venation, 5-11 veins, leaf base is compactly fold forming pseudostem.	Simple leaf, lanceoloate shape alternate arrangement, parallel venation, 5-11 veins, leaf base is compactly fold forming pseudostem.
• Leaf number	2-4 leaves.	2-4 leaves.
• Color	Yellow-green, 148A.	Green, 143A.
4. Inflorescence		
• Morphology	Raceme type, straight peduncle, acropetally blooming, and usually appears before leaf.	Raceme type, straight peduncle, acropetally blooming, and usually appears at the same time as leaf.
• Number of inflorescence	1-2 inflorescences.	1-2 inflorescences.
Flower		
• Morphology	Bisexual flower, bilateral symmetry, and has 6 perianths. A dorsal sepal and lateral sepal are lanceolate shape. Lateral petal is elliptic, labellum is narrowly oblong and larger than other perianths, 2 pollinia.	Bisexual flower, bilateral symmetry, small and has 6 perianths. A dorsal sepal and lateral sepal are lanceolate shape. Lateral petal is ovate, labellum is narrowly oblong and larger than other perianths, 2 pollinia.
• Flower size	Approximately 2.2-2.6 cm.	Approximately 1.5-3.0 cm .
• Color	Flower is brown with white pink lip.	There are various colors, white flower and white lip with yellow stripe, pink-white flower and white lip with yellow stripe, pink-white flower and pink lip with yellow stripe, pink flower and pink lip with yellow stripe, green-pink flower and pink lip with yellow stripe, dark pink to red flower and pink lip, yellow-green flower and white lip, pink-green flower and pink lip.
• Number of flowers	20-60 flowers.	5-10 flowers.

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Table 5.1 Morphological characterization of *Eulophia macrobulbon* and *E. spectabilis* (continued)

Characterization	*E. macrobulbon*	*E. spectabilis*
6. Fruit		
• Morphology	Septicidal capsule type, narrowly elliptic to elliptic.	Septicidal capsule type, narrowly elliptic to elliptic.
• Color	Green color when young and becomes yellow when old.	Green color when young and becomes yellow when old.
7. Seed		
• Morphology	Very small, looks like yellow dust, long taper, transparent, and has rough net surface when examine under microscope. There is embryo inside.	Very small, looks like yellow dust, long taper, transparent, and has rough net surface when examine under microscope. There is embryo inside.
• Seed size	Approximately 0.9-1.4 mm long	Approximately 0.9-1.4 mm long

Morphological characterization of stem, leaves, flower were similar to description by Bose and Bhattacharjee (1980), Seidenfaden (1983), Sittheesutjathan (2006), Sittheesutjathan and Kritsanachandee (2007) and Thaitong (2000)

Generally, terrestrial orchid has a simple root system each plant develops only few roots that are usually thick, fleshy and unbranched root (Fuchs and Ziegenspeck, 1925). These are general characteristics of root system of plant symbiosis with mycorrhiza (Brundrett and Kendrick, 1988).

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Underground stem of *E. macrobulbon* and *E. spectabilis* was hard, roundish, apparent node and it had horizontal growing pattern. Besides, shoot and root arise at only a side. Therefore, they could be identified as “rhizome” (Hertogh and Nard, 1993)

Many species of terrestrial orchid have rhizome which later on swell to form a underground stem, for example *Arethusa*, *Calopogon*, *Calypso*, *Tipularia*. The stem serve mainly as storage organs for nutrient, which is drawn for shoot initiation and seeds set (Zimmerman and Whigham, 1992).

About reproductive growth, flower shape of each species of orchid has specific morphological character to attract specific pollinator for pollination (Richards, 1999).

The fruit of plant in Orchidaceae family is generally dehiscent that usually occurs after pollination, when mature, it ruptures along the midline of each carpel and between the carpel but remain joined at the apex (Cribb, 1999).

Orchids can produce large amounts of seeds, and each capsule can contain up to four million seeds (Arditti and Ghani, 2000). The seeds of terrestrial orchids measure from 0.07 to 0.40 mm across and from 0.11 to 1.97 mm in length, including the testa (Harvais, 1974; Arditti *et al*., 1979). Besides, most orchid species have small seeds with undifferentiated embryos and mass of undifferentiated cell, which can be compared to the globular stage of the embryo of dicotyledon (Ariditti, 1992). Hirt (1906) reported that embryo of orchid seed covers with inflated air-filled testa that has long tapering ends. That is characteristics of wind dispersal seed.

5.1.2 Growth cycle

Study of growth and development cycle of *E. macrobulbon* and *E. spectabilis* were conducted. It was found that growth and development cycle of both terrestrial orchid was resemble, deciduous type, and undergone the dormant period throughout dry season.

Growth cycle of *E. macrobulbon* started vegetative shoot from bud that was at base of underground stem, after that inflorescence emerged from the base of vegetative shoot and appeared above ground before leaves. After leaves are in full length, underground stem stored nutrition. That made base of vegetative shoot increased and transformed to new rhizome. Finally, the aerial part began to die and stay dormant.

For *E. spectabilis*, vegetative shoot and reproductive shoot emerged from bud that was at base of rhizome. Inflorescence of this orchid developed at the same time as vegetative organ; leaves and pseudostem. When vegetative organ was mature, base of vegetative shoot would be a store for nutrition and expand and change to new rhizome. After that, dormancy of this terrestrial orchid began in dry season.

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Vegetative development of both *Eulophia* started from vegetative shoot emerged from old rhizome. After that, roots developed from base of vegetative shoot and followed by development of aerial shoot form to leaves. The root appeared before any leaves because transpiration from leaves induced uptake of water (Rasmussen, 1995). After that, base of bud was swollen to form a rhizome for storing nutrient, in particular carbohydrates and water (Zimmerman and Whigham, 1992).

In this genus, storage organ had annual increment in size and number. The first corm usually formed at the base of the first leafy shoot (Rasmussen, 1995).

Terrestrial orchid in *Eulophia* genus can be found in deciduous forest which there is dry season and rainy season. That makes these terrestrial orchids adapt to dormancy with storage organ or rhizome in dry season and grow again in rainy season (Thorut, 1994). That coincides with the results of this experiment. Vegetative and reproductive growth of *E. macrobulbon* depends on temperature, it starts to germinate when temperature increases whereas growth of *E. spectabilis* depends on rainfall, it starts to germinate at the beginning of rainy season. The dormancy of both terrestrial orchids occurred at low rainfall and temperature (Figure 5.1).

5.1.3 Anatomical study of flower bud development

Histological study on flower bud development of *E. macrobulbon* and *E. spectabilis* was conducted from longitudinal section at different period of flower bud. It was found that flower bud development of both plants were similar, but occurred at different time. Both terrestrial orchids had 2 types of shoot in bud; vegetative shoot and floral shoot.

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Bud of *E. macrobulbon* consisted of apical meristem which was located in the middle of the bud, and leaf primordia on lateral side at the first week after bud break. There was a shoot that was at axillary leaf on the following week and developed to form floral shoot that showed floral primordia at the fourth week after bud break (Figure 5.2).

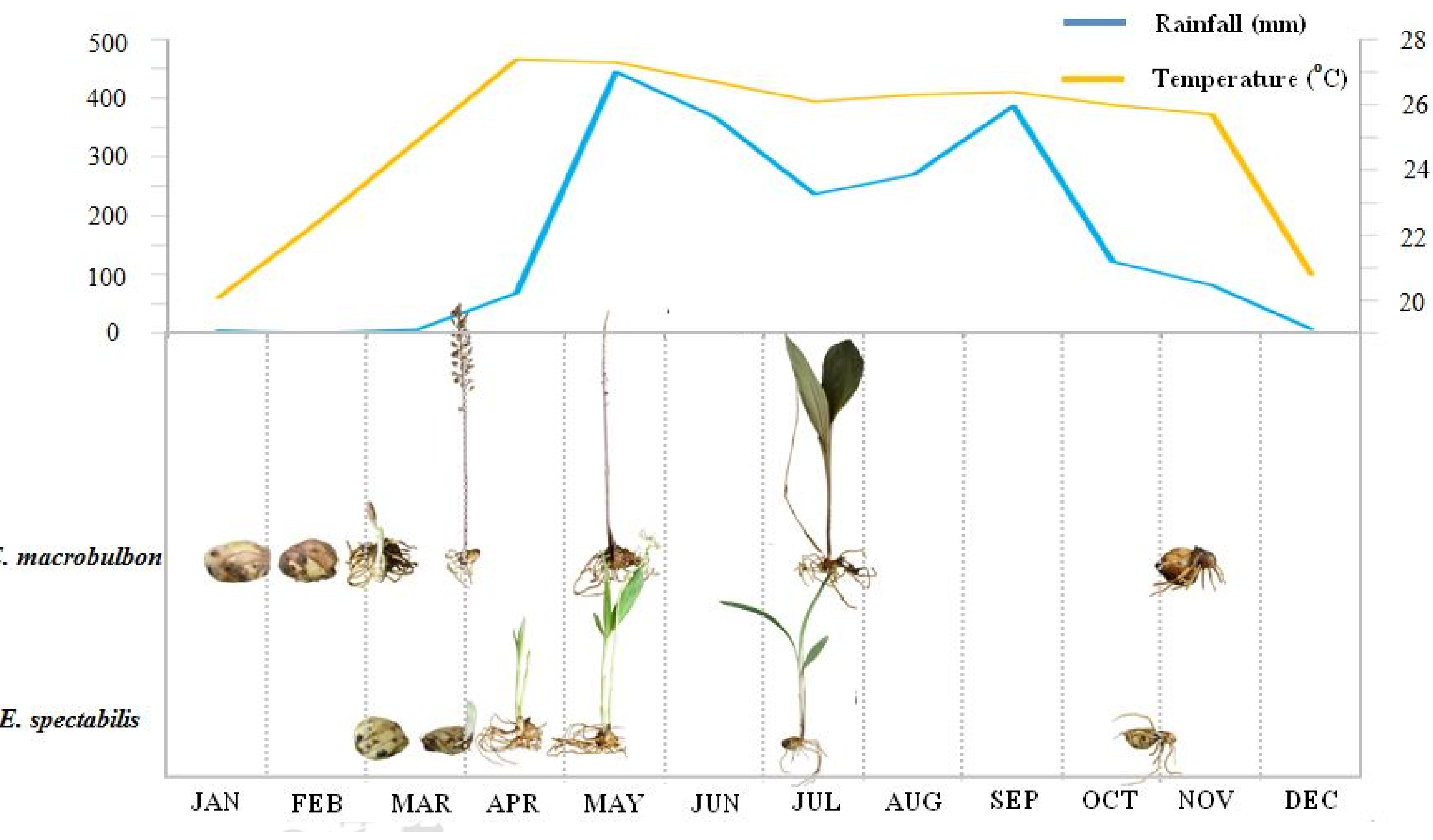

Figure 5.1 Growth cycle of *Eulophia macrobulbon* and *E. spectabilis* comparison along with rainfall and temperature data

In *E. spectabilis*, there were apical meristem, leaf primordia, and shoot that would develop to form floral shoot similar to *E. macrobulbon*, but all of these occurred at the first week after bud break (Figure 5.3).

The early development of flower bud of both terrestrial orchids was similar as report by Fahn (1977). He reported that the first change of flower bud showed by increasing of mitotic activity on the boundary between the central zone and the rib meristem zone. Thus, their cells are small and high in protoplasm. This phenomenon occurs in flower bud of *E. macrobulbon* at early February and *E. spectabilis* at early March. After division of meristematic cell, development of other organs begins. This stage can be found in flower bud of *E. macrobulbon* at mid February and *E. spectabilis* at mid March.

Anatomy characteristics of flower bud development of *E. macrobulbon* and *E. spectabilis* was the same as *E. andamanensis* Rchb. f. (Chanaken, 2007), *E. graminea* Lindl. (Prarasi, 2006), Geodorum *recurvum* (Roxb.) Alston and *G. siamense* Rolfe ex Downie (Sota, 2007), these orchids are in the same tribe.

According to study of growth cycle, the results indicated that inflorescence of *E. macrobulbon* and *E. spectabilis* emerge from base of vegetative shoot, and could be found approximately 1-2 inflorescences. Anatomy study confirmed that inflorescence of both terrestrial orchids developed from the first lateral bud at axillary leaf, and emerge from base of vegetative shoot.

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5.1.4 Chromosome investigation

Investigation of root-tip chromosome of *E. macrobulbon* and *E. spectabilis* was made in order to develop suitable method for chromosome counting of these terrestrial orchids (Table 5.2).

The results of this study coincided with study of Felix and Guerra (2000), which reported that *Eulophia* in Brazil had chromosome number between 2n=33 to 2n=56.

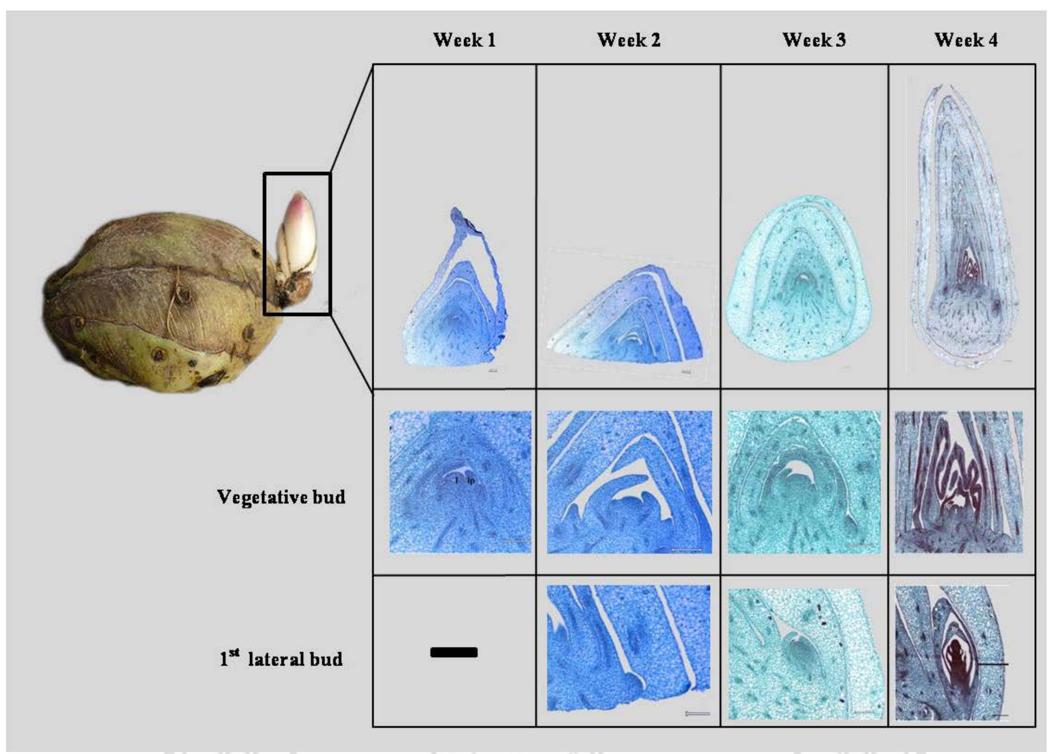

Figure 5.2 Longitudinal section of bud development of *Eulophia macrobulbon* (Par. & Pchb. f.) Hook. f.

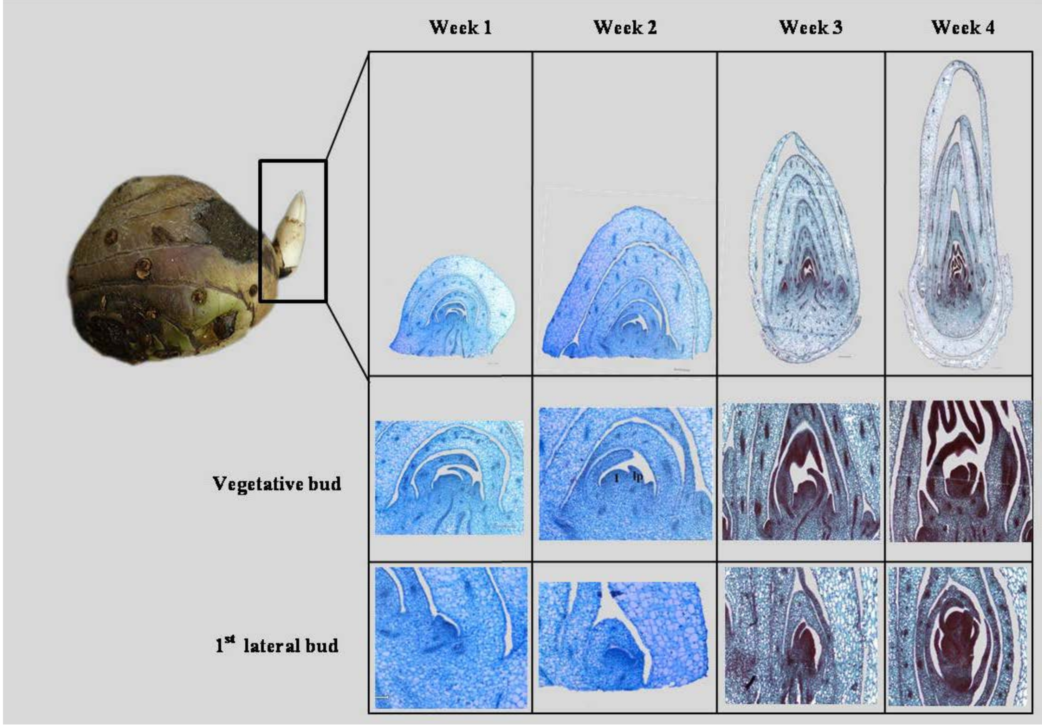

Figure 5.3 Longitudinal section of bud development of *Eulophia spectabilis* (Dennst.) Suresh.

Table 5.2 Chromosome investigation procedure of *Eulophia macrobulbon* and *E. spectabilis*

Treatment	*E. macrobulbon*	*E. spectabilis*
1. Sampling time	9:00 A.M.	10:00 A.M.
2. Pre-treatment period	24 hours	24 hours
3. Chromosome number	2n=48	2n=52

In Thailand, there are some reports about chromosome investigation of *Eulophia*. Prarasi (2006) reported that chromosome number of *E. graminea* that found at Huai Hong Khrai Royal Development Study Center was 2n=56. The following year, Chanaken (2007) studied cytogenetics of *E. andamanensis* and chromosome number of this terrestrial orchid was 2n=42.

5.1.5 Genetic relationship

Analysis of genetic relationship of genus *Eulophia* by RAPD technique was done in order to distinguish genetic diversity and relationship of terrestrial orchid in *Eulophia* genus.

Primer screening of *Eulophia* were conducted in order to select the primer to amplify polymorphic band in all terrestrial orchid in *Eulophia*. Thirty three primers of OPA and OPF primer sets, 20 primers of OPA and 13 primers of OPF, were used. It was found that twenty-one primers could reveal polymorphism band in sample.

The result from cluster analysis of genetic relationship by UPGMA and dendrogram showed that single primer cannot distinguish *Eulophia* in correspondence with their morphological characteristics (Appendix E).

However, *Eulophia* sample could be separated into two groups using combination of OPA4, OPF1, and OPF13 primers. In the first cluster, *E. andamanensis* and *E. graminea* were related, genetic similarity is 0.529. Both terrestrial orchids could be grouped according to vegetative organ morphology. Their stem is rhizome type that grows above ground level. Leaf is linear and lanceolate. The difference of *E. andamanensis* and *E. graminea* is flower. Flower of *E. andamanensis*, sepals and petals

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are pale green in color. Lip is green at the base and white at the centre with maroon vein along lip length, whereas flower of *E. graminea* is greenish underneath with dark purple color.

The second cluster consisted of all color *E. spectabilis*, *E. macrobulbon*, and *E. flava*. Shape of their tuberous stem is rhizome, they all have rhizome at underground level. Inflorescence is raceme and usually arises from upper node of tuberous stem.

At 0.647 similarity, *E. macrobulbon* separated from *E. spectabilis* and *E. flava* according to flower morphology; Flower of *E. macrobulbon* is small, approximately 1 cm, purple-red with white lip and there are many numbers of flowers per inflorescence while *E. spectabilis* and *E. flava* have few flowers.

The difference that separates *E. flava* from *E. spectabilis* is flower character; *E. flava* being a large flower, lemon yellow, and fragrant whereas flower of *E. spectabilis* is smaller than *E. flava*, various colors, white, pink, red, green, and having no fragrance. Genetic similarity between *E. flava* and *E. spectabilis* is about 0.588-0.733.

E. spectabilis is terrestrial orchid with various flower colors depend on ecological habit. Flower size is approximately 3 cm. The result of dendrogram from cluster analysis showed that all color *E. spectabilis* were related. Genetic similarity is about 0.735-0.941. White flower with pink and yellow mid white lip of *E. spectabilis* is closely related to white flower with pink and stripe pink lip *E. spectabilis*.

RAPD technique could be employed to distinguish species in other orchids, for example, *Cymbidium* (Choi *et al*. 2006) *Paphiopedilum* and *Phragmipedilum* (Chung *et al.* 2006), and *Habenaria* and *Pecteilis* (Sinumporn, 2014).

5.2 Crossabilities studies

Crossability of intraspecific, interspecific, and intergeneric hybridization between *E. macrobulbon*, *E. spectabilis*, and some terrestrial orchids which are closely related with both *Eulophia* were performed.

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5.2.1 Intraspecific hybridization

According to experiment 2.1, crossability of intraspecific hybridization, selfing of *E. macrobulbon* and *E. spectabilis* was very successful. Percentages of fruit set, seed viability, and percentages of germination of both terrestrial orchid were high. That means both terrestrial orchids were self-compatible plants. This result was similar to other terrestrial orchid, for example, *Habenaria erichmichaelii* Hance, *H. rhodocheila* Hance, *H. xanthochelia* Ridl. (Kawchadee *et al*., 2012), *Cymbidium insigne*, *C. lowianum*, *C. sinense*, and *C. tracyanum* (Wongnan, 2010). Seed viability from selfed of these orchid species were almost 100%.

In nature, self-compatibility orchids can be classified into 2 types.

- Autogamy; selfed within same flower, can occur in some terrestrial orchid species which produce numerous small fruit for example *Corallorhiza odontorhiza* and *Lister corduts*.
- Allogamy; selfed between flowers, flower in this types has barrier to prevent pollination within same flower, for example ecological or structure barriers, in particular the rostellum tissue. However, pollination possibly occurs with the availability of pollinator (Dafni and Bernhardt, 1990).

In addition, there are self-incompatibility orchid in nature. Approximately 10% of orchid species are self-incompatible. This mechanism is useful for the formation and maintenance of the high diversity and adaptation of orchid (Fujii *et. al*., 2016) for example, *Dendrobium* species, self-incompatible mechanism of this genus is controlled by gene that control growth of the pollen.

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5.2.2 Interspecific hybridization

Interspecific hybridization between *E. macrobulbon* and *E. spectabilis* revealed that hybridization of two species could be done when *E. spectabilis*, that flower size was larger than *E. macrobulbon*, was used as female parent. The result was similar to hybridization of *Ascocentrum ampullaceum* (Roxb.) Schltr. var. *auranticum*, *Vanda* genus in the present, and *Vanda coerulea* Griff. Crosses between the two species could only be made when *V. coerulea* was used as female parent. Flower size of *V.*

coerulea is approximately 10.0 cm whereas *A. ampullaceum* (Roxb.) Schltr. var. *auranticum* is approximately 1.5-2.5 cm (Rajkumar *et al.*, 2005). On terrestrial orchid, Tongkham *et al.* (2015) reported hybridization of *Paphiopedilum jackii* × *P. villosum* were successful when *P. villosum* was used as male parent. Flower size of *P. jackii* (approximately 7.00 cm) was smaller than *P. villosum* (approximately 9.00 cm).

5.2.3 Intergeneric hybridization

Study of intergeneric crossability between *Eulophia* and some terrestrial orchids which are in the same subfamily; *Geodorum* and *Spathoglottis*, was conducted. It was found that hybridization between *Geodorum attenuatum* and both *Eulophia* was successful when using *G. attenuatum* as female parent.

Fruit set of cross between *S. affinis* and *E. macrobulbon* was found. However, when seed pod was harvest, there was no viable seed. Therefore, fruit development is not a good indicator of successful breeding. Even though fruit set occurs, no seed viability can be found, especially fruit set of intergeneric hybridization. Fruit can develop due to the stimulation of auxin from pollen when pollination occurs (Techapinyawat, 2005). There is evidence that pollen extract could stimulate orchid's ovary to develop without fertilization (Arditti and Knauft, 1969)

5.2.4 Factor involved in interspecific or intergeneric hybridization of *E. macrobulbon* and *E. spectabilis*

Interspecific and intergeneric hybridization are important method to develop new species when sources of variation for interested character cannot be found within species, but can be found in closely related species. However, there are many factors to make a successful hybrid involving two different species especially in orchid that could be crossed between many species and genus.

According to Rakpaibulsombat (1997), there are five main reasons that cause orchid unsuccessful hybridization.

5.2.4.1 Sterility of one of the parent

5.2.4.2 Very different chromosome number of the parents

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5.2.4.3 Environmental should be suitable for growth and development of female plant. For example, if the female parent is a temperate plant, it should be grow at a low enough temperature to enable interspecific or intergeneric hybridization.

5.2.4.4 Genetic relationship; hybridization of interspecific or intergeneric would be successful if the parents are closely related.

5.2.4.5 An unsuitable parent plant. If flower size of parent plant is very different, the style of female parent is not suitable for pollen tube of male parent to reach the ovary.

For the first reason, the results in experiment 2.1, crossability of intraspecific hybridization, showed that *E. macrobulbon* and *E. spectabilis* were self-fertile because they can set seed after selfing. For second reason, result of experiment 1.4; chromosome number of both terrestrial orchids was not too far different. Chromosome number of *E. macrobulbon* and *E. spectabilis* were 2n=48 and 2n=52, respectively. Interestingly, seed pod was successful when *E. spectabilis* that greater chromosome number than *E. macrobulbon was* used as female. This was similar to the study of crossability of four varieties *Phalaenopsis equestris*, including two white and two pink forms. The results showed that failure of seed production was found when the tetraploid plants were used as pollen parents to cross with the diploid varieties (Tang and Chen, 2007). Therefore, in order to enhance the breeding efficiency, a breeder has to use the greater chromosome species/varieties as female parent.

For third reason, environment that is suitable for female parent, both *E. macrobulbon* and *E. spectabilis* are tropical plant that can found at 250 to 2,100 m above sea level. That means hybridization could be successfully when use both terrestrial orchid as female plant in general area of Thailand.

For fourth reason, in interspecific hybridization the results of experiment 1.5, genetic relationship by RAPD technique, showed that molecular technique could group plant species of *Eulophia*; *E. macrobulbon*, *E. spectabilis* and *E. flava*. These results could confirm genetic distance of *E. macrobulbon* and *E. spectabilis* that are closely related, genetic similarity is 0.618-0.765. Therefore, hybridization between both *Eulophia* was great potential to success. There were evidence about relationship between genetic distance and crossability of terrestrial orchids. In experiment of

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Kawchadee *et al.* (2012), interspecific hybridization between *Habenaria rhodocheila* × *H. xanthocheila* and reciprocal cross were more successful than hybridization between *H. lindleyana* × *H. rhodocheila* and *H. lindleyana* × *H. xanthocheila*. According to Sinumporn (2014), reported that *H. lindleyana* could be separate from group of *H. rhodocheila* and *H. xanthocheila* by RAPD technique.

For the fifth reason, flower size of *E. macrobulbon* was much smaller than that of *E spectabilis.* The ovary tube of *E. macrobulbon* is too narrow for penetration by pollen tube of *E. spectabilis*. This may be the reason that make hybridization of *E. macrobulbon* × *E. spectabilis* failure.

In intergeneric hybridization between *E. macrobulbon* or *E. spectabilis* and some terrestrial orchids which are in the same subfamily. *Geodorum* could cross with *Eulophia* better than *Spathoglottis* that might be due to different chromosome numbers; *G. attenuatum* is 2n=52 (Thongsan, 2008) whereas *S. affinis* is 2n=40 (Boonaree, 2010). Besides, *Geodoeum,* Subfamily: Epidendroideae, Tribe: Cymbidiaeae, is closely related to *Eulophia* than *Spathoglottis*, Subfamily: Epidendrodieae, Tribe: Collabieae.

5.3 Physiological studies

Rhizome of *E. macrobulbon* and *E. spectabilis* were stored at 15°C for 30, 60, and 90 days in order to find the suitable period of keeping rhizome for extending flowering period. The results revealed that low temperature could delay the day of germination of *E. macrobulbon* and *E. spectabilis*, but it had effect on decreasing plant size, when period of stored rhizome at low temperature was increased. In addition, it was found that *E. macrobulbon* could not give flower in any low temperature storage treatment whereas *E. spectabilis* could give flower.

The reasons that cause germination of underground stem to be slower than control, might be temperature. Hartsema (1961) reports that temperature is major external factor controlling growth, development, and flowering of ornamental bulbs. For temperate flower bulb, low temperature is a factor that breaks dormancy for example, in *Arisaema sikokianum* (Fukai *et al.,* 2006), Lilly (Langens-Gerrits, 2003) and *Zephyra elegans* (Yaὶ ῆ ez *et al.,*2004).

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For *Eulophia* which is tropical orchid, heat accumulation might be a factor that breaks dormancy. Storing rhizome of *E. macrobulbon* and *E. spectabilis* at low temperature might demolish heat accumulation inside. Therefore, when rhizomes were planted, they need to accumulate heat before germination. The results coincided with Rodnoi (2013) reported that dormancy break of *Geodorum*; terrestrial orchid which is in the same tribe as *Eulophia*, should be related with both moisture and temperature. Underground stem must accumulate enough heat to break dormancy and require moisture for new shoot emergence.

Quality of plant size and flower are lower when period of stored rhizome is increased, that might be due to that low temperature might have changed and canceled endogenous hormones, poor quality of flower and inflorescence was found. However, the results form total non-structural carbohydrate (TNC) analysis of this study which indicated that TNC of treated rhizome was not different from controlled plant.

The result is similar to experiment of many flower bulbs, for example, Wongin *et al.* (2010) studied effect of bulb storage duration on growth and flowering of *Hippeastrum* hybrid cv. Susan. Bulbs were selected and kept in cold room at 10°C for 8 weeks before use for experiment. Then, bulbs were divided into six groups and each group was kept continuously in cold room at 10 and 15°C for 4, 12, and 20 weeks. The results showed that the bulbs stored at 10 and 15°C for 20 weeks had smaller leaf length, leaf width, peduncle diameter, peduncle circumference, peduncle length, diameter of flower, and bulb quality than controlled bulb and bulbs stored at low temperature for 4 and 12 weeks. Later, Phrompanya *et al.* (2014) studied effect of bulb storage temperature on growth and flowering of *Eucomis*. Bulbs were selected and stored at room temperature, 5±2 and 10±2°C for 2 and 4 months. The result showed that bulbs stored at low temperatures for 4 months had less plant height and lower flower qualities than those stored at low temperature for 2 months.

Kitidee *et al.* (2015) studied effect of temperature and bulb storage duration on growth and flowering of *Habenaria lindleyana* Steud. Tubers were stored at 10°C, 15°C and room temperature; 25-30°C, and stored duration of 90, 180, 270 and 360 days. The results showed that storage temperature at 10 and 15°C for 180 tend to delay germination and flowering. In addition, Kitidee *et al.* (2018) studied effect of

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temperature and bulb storage duration on growth and flowering of *Habenaria erichimichelii* and *Pecteilis sagarilkii*. Bulbs were stored at 10ºC, 15ºC and room temperature; 25-30ºC, and storage duration of 90, 180, 270 and 360 days. It was found that storing bulb at 10 and 15ºC could extend flowering of both orchid for 360 days. Treatment that gave good quality flower of *H. erichmichelii* and *P. sagarikii*, was 10ºC for 180 days.

Besides, germination of both terrestrial orchids was delayed might be due to, temperature, rainfall, and light intensity were not suitable for fully growth and development (Appendix F). Quality of plant and flower were lower than control. The results coincide with *Curcuma alismatifolia* Gagnep. Payakaihapon *et al.* (2006) studied effect of long day on off-season flowering of *C. alismatifolia* Gagnep. Bulbs of *C. alismatifolia* were selected and kept at 15±2ºC and planting on off-season, August and October. After that, plants were grown under natural condition and long-day condition, supplying 2 hours of incandescent light at night on different day of growth, 20, 30, 40 and 50 days after planting. It was found that long-day condition could enhance plant growth and flowering. Plant height and quality of flower of plant in long-day treatment were significantly greater than plant grown under natural condition. The results of this experiment indicated that environment had effect on off-season growth and development of stored bulb.

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CHAPTER 6

CONCLUSIONS

6.1 Characterizations and growth pattern

Eulophia macrobulbon: Root is fibrous type. Stem is a rhizome. Leaf is simple type, alternate arrangement, oblanceolate shape, and leaf base is compactly fold forming pseudobulb. Inflorescence is raceme and usually shows before leaf. Peduncle is straight. Flower is bisexual with bilateral symmetry. Flower is small; 2.2-2.6 cm, that has 6 perianth. There are a dorsal sepal, 2 lateral sepals, 2 lateral petals and a labellum. Flower color for overview is brown. Fruit is septicidal capsule type. Seeds are very small. It is long taper, transparent, and has rough net surface

Growth cycle of *E. macrobulbon* is deciduous type and undergoes the dormant period throughout dry season. It starts vegetative shoot in early February, after that inflorescence develops from the base of vegetative shoot and appears above ground before leaves, during March to April. The aerial parts of this plant begin to die back and stay dormant from November to January of the consecutive year.

Flower bud of *E. macrobulbon* forms from the lateral bud which is bases of axillary leaf at the second week after bud break. After that, it grows and develops to form floral primordia at the fourth week from bud break.

Chromosome number of *E. macrobulbon* is 2n=48, sample should be collected at 9.00 A.M. and pre-treatment with 0.002 M 8-hydroxyquinoline for 24 hours.

Eulophia spectabilis: Root is fibrous root. Tuberous stem is rhizome. Leaf is simple type, alternate arrangement, lanceolate shape and leaf base is compactly fold forming pseudobulb. Inflorescence is raceme and usually shows at the same time as leaf. Peduncle is straight. Flower is bisexual with bilateral symmetry. Flower is

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approximately 1.5-3.0 cm that has 6 perianths. There are a dorsal sepal, 2 lateral sepals, 2 lateral petals and a labellum. Flower has various colors; white flower and white lip with yellow stripe, pink-white flower and white lip with yellow stripe, pink-white flower and pink lip with yellow stripe, pink flower and pink lip with yellow stripe, green-pink flower and pink lip with yellow stripe, dark pink to red flower and pink lip, yellow-green flower and white lip, pink-green flower and pink lip. Fruit is septicidal capsule type. Seeds are very small. It is long taper, transparent and has rough net surface.

Growth cycle of *E. spectabilis* is deciduous type and undergoes the dormant period throughout dry season. *E. spectabilis* starts vegetative growth by shoot emerge in mid-March, inflorescence develops at the same time. Mature inflorescence appears between Aprils to late May. Dormancy begins in November and ends in March of the next year.

Flower bud of *E. spectabilis* develops from the lateral bud at the base of axillary leaf at the first week from bud break. After that, it grows and develops to form floral primordia at the fourth week from bud break.

Chromosome number of *E. spectabilis* is 2n=52, sample should be collected at 10.00 A.M. and pre-treatment with 0.002 M 8-hydroxyquinoline for 24 hours.

RAPD technique provides information on the relationship of species which belong to *Eulophia*. Three primers; OPA4, OPF1, and OPF13, could distinguish some of *Eulophia* in this study into two groups corresponding to morphological characteristics.

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6.2 Crossabilities studies

Intraspecific hybridization of *E. macrobulbon* and *E. spectabilis* is great success whereas interspecific hybridization between of *E. macrobulbon* and *E. spectabilis* is possible only when *E. spectabilis* is used as the female parent. The intergeneric hybridization of both *Eulophia* and *G. attenuatum*, and *S. affine* could occur when use *G. attenuatum* as female parent.

6.3 Physiological studies

Rhizome of *E. spectabilis* could be stored at 15ºC for 90 days but low temperature had effect on plant height and flower quality. As of *E. macrobulbon*, low temperature could delay germination of *E. macrobulbon* but there was no flower in all treatments.

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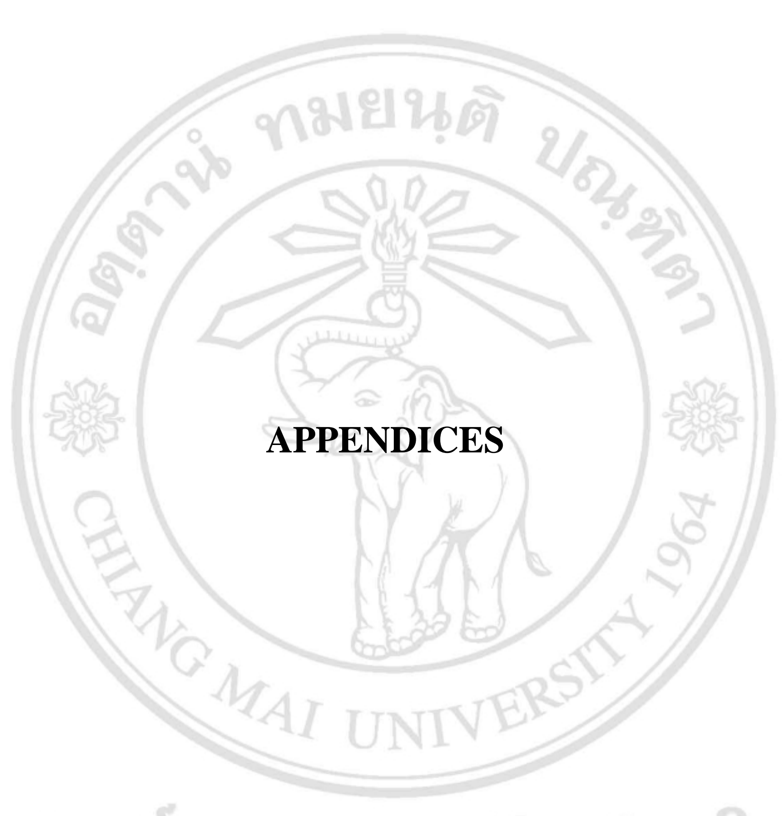

APPENDICES

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APPENDIX A

Chemical reagent preparation of microtechnique

Anatomical structure microtechnique by paraffin embedding method (Johansen, 1940 and Sass, 1966)

1. **Killing and fixation solution**

FAA or Formalin-Acetic acid-Alcohol solution contains

95% ethyl alcohol	50	ml
glacial acetic acid	5	ml
formalin	10	ml
distilled water	35	ml

2. **Dehydration solution**

Solvent proportion	Concentration of alcohol				
	50%	70%	85%	95%	100%
95% ethyl alcohol	40	50	50	45	-
absolute alcohol	-	-	-	-	25
tertiary butyl alcohol	10	20	35	55	75
distilled water	50	30	15	-	-

3. **Adhesive solution**

Stock solution:	albumin	1	ml
	distilled water	49	ml

when use, dilute 1 ml of the stock solution with distilled water to 50 ml

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4. Stain

Stain	Chemicals	
Safranin	safranin O ($C_{20}H_{19}N_4Cl$)	4 ml
	metyl cellosolve	200 ml
	95% ethyl alcohol	100 ml
	sodium acetate	4 g
	formalin	8 ml
	distilled water	100 ml
Fast Green	fast green FCF ($C_{37}H_{34}N_2O_{10}S_3Na_2$)	0.15 g
	methyl cellosolve	100 ml
	absolute ethyl alcohol	100 ml
	clove oil	100 ml

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APPENDIX B

Reagent preparation of chromosome investigation

Root-tip chromosome investigation via Feulgen' s squash method (Vitayasak, 1996)

1. **Pre-treatment agent**

    0.002 8-hydroxyquinoline solution contain

8-hydroxyquinoline	0.29	g
distilled water	1,000	ml

2. **Fixative agent**

    Acetic-alcohol fixative solution contains glacial acetic acid and absolute ethanol in ratio 1:3 (should be prepared this solution before used).

3. **Acid macerating fluid agent**

    1 M hydrochloric acid prepare from 37% hydrochloric acid 12.076 ml mixed with distilled water 1,000 ml.

4. **Stain**

    Preparation of carbol-fuchsin staining solution was conducted based on Chen' recipe (1992) as follow:

    Fluid A: dissolve 3 g of basic fuchsin in 10 ml of 70% ethanol then add 90 ml of 5% phenol

    Fluid B: mix 6 ml of glacial acetic acid and 6 ml of 37% formaldehyde into 55 ml of fluid A

    The fluid B, 5-10 ml, was mixed with 95 ml of 45% acetic acid and 1.8 g sorbital then stirred with magnetic stirrer for several minutes.

    Fresh carbol-fuchsin stain fluid was ready after 30-45 days of incubation.

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APPENDIX C

Chemical reagent preparation for RAPD

CTAB buffer

5M NaCl	28 ml
1M Tris-HCl pH 8	10 ml
0.5 mM EDTA pH 8	4 ml
CTAB	2 g
PVPP	1 g

Add H_2O to100 ml

Wash buffer

Ammonium acetate	10 mM
Ethanol	75 %

TE buffer

2M Tris-HCl pH 8	500 µl
0.5 mM EDTA pH 8	100 µl

Added ddH_2O to 500 ml

0.5M EDTA pH 7.5 and 8

EDTA ($Na_2 \cdot 2H_2O$)	186.1 g

Added ddH_2O to 1,000 ml

Adjust pH as needed

1.5% Agarose gel

Agarose	1.5 g
1X TBE	1,000 ml

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APPENDIX D

Nucleotide sequence of RAPD primer

Primer		Primer	
Primer	Sequences 5’-3’	Primer	Sequences 5’-3’
OPA-01	5’-CAGGCCCTTC-3’	OPA-11	5’-CAATCGCCGT-3’
OPA-02	5’-TGCCGAGCTG-3’	OPA-12	5’-TCGGCGATAG-3’
OPA-03	5’-AGTCAGCCAC-3’	OPA-13	5’-CAGCACCCAC-3’
OPA-04	5’-AATCGGGCTG-3’	OPA-14	5’-TCTGTGCTGG-3’
OPA-05	5’-AGGGGTCTTG-3’	OPA-15	5’-TTCCGAACCC-3’
OPA-06	5’-GGTCCCTGAC-3’	OPA-16	5’-AGCCAG CGA A-3’
OPA-07	5’-GAAACGGGTG-3’	OPA-17	5’-GAC CGC TTG T-3’
OPA-08	5’-GTGACGTAGG-3’	OPA-18	5’-AGGTGACCGT-3’
OPA-09	5’-GGGTAACGCC-3’	OPA-19	5’-CAAACGTCGG-3’
OPA-10	5’-GTGATCGCAG-3’	OPA-20	5’-GTTGCGATCC-3’
OPF-01	5’-ACGGATCCTG-3’	OPF-11	5’-TTGGTACCCC-3’
OPF-02	5’-GAG GAT CCC T-3’	OPF-12	5’-ACGGTACCAG-3’
OPF-03	5’-CCT GAT CAC C -3’	OPF-13	5’-GGCTGCAGAA-3’
OPF-04	5’-GGT GAT CAG G-3’	OPF-14	5’-TGC TGC AGG T-3’
OPF-05	5’-CCG AAT TCC C-3’	OPF-15	5’-CCA GTA CTC C-3’
OPF-06	5’-GGGAATTCG G-3’	OPF-16	5’-GGAGTACTGG-3’
OPF-07	5’-CCGATATCCC-3’	OPF-17	5’-AAC CCG GGA A-3’
OPF-08	5’-GGGATATCGG-3’	OPF-18	5’-TTC CCG GGT T-3’
OPF-09	5’-CCAAGCTTCC-3’	OPF-19	5’-CCT CTA GAC C-3’
OPF-10	5’-GGA AGC TTG G-3’	OPF-20	5’-GGT CTA GAG G-3’

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APPENDIX E

RAPD profile of *Eulophia*

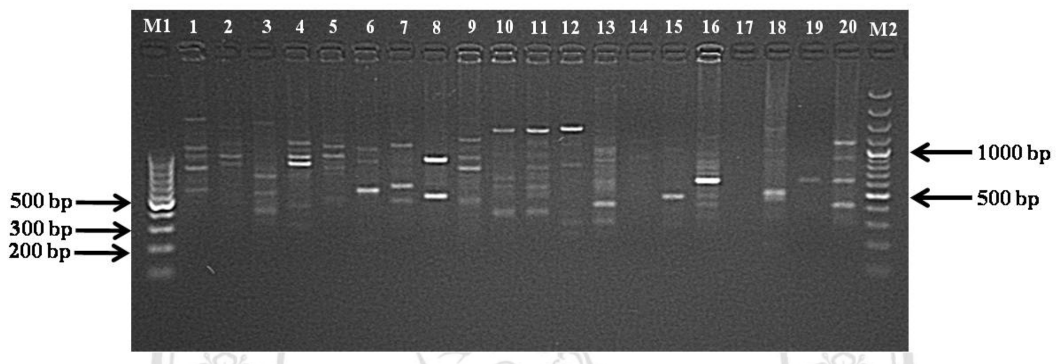

Figure 1 Primer screening of *Eulophia* by OPA primer set; M1) 100 bps DNA ladder,1) OPA1, 2) OPA2, 3) OPA3, 4) OPA4, 5) OPA5, 6) OPA6, 7) OPA7, 8) OPA8, 9) OPA9, 10) OPA10, 11) OPA11, 12) OPA12, 13) OPA13, 14) OPA14, 15) OPA15, 16) OPA16, 17) OPA17, 18) OPA18, 19) OPA19, 20) OPA20, M2) 100 bps plus DNA ladder

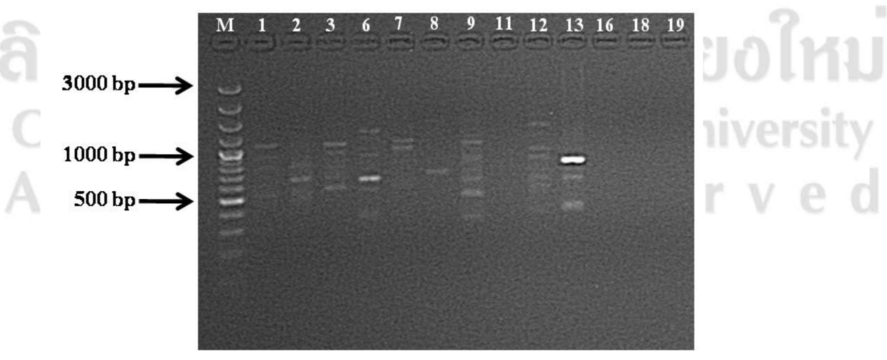

Figure 2 Primer screening of *Eulophia* by OPF primer set; M) 100 bps plus DNA ladder, 1) OPF1, 2) OPF2, 3) OPF3, 6) OPF6, 7) OPF7, 8) OPF8, 9) OPF9, 11) OPF11, 12) OPF12, 13) OPF13, 16) OPA16, 18) OPF18, 19) OPF19

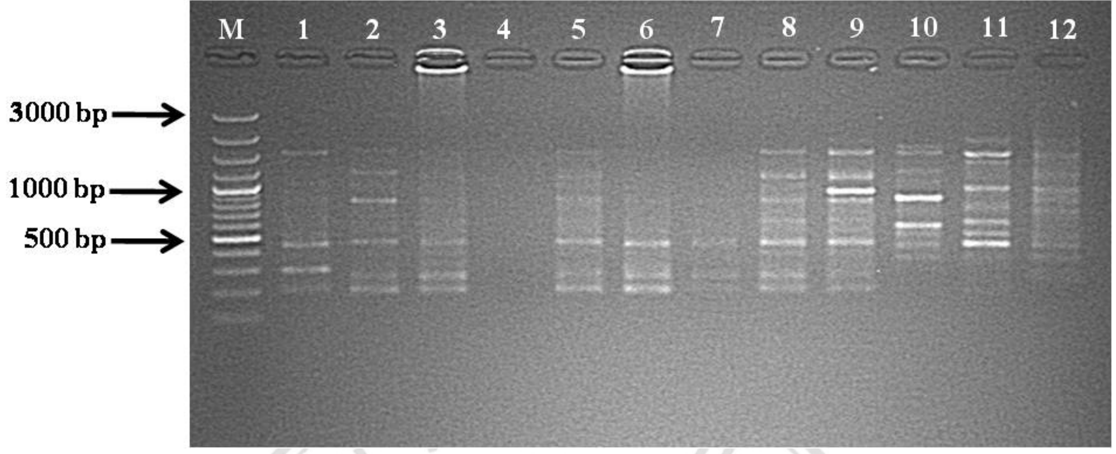

Figure 3 RAPD profile of *Eulophia* generated by OPA2 primer; M) 100 bps plus DNA ladder, 1) *E. macrobulbon*, 2) *E. spectabilis*; white, white lip with yellow stripe, 3) *E. spectabilis*; pink-white, white lip with yellow stripe, 4) *E. spectabilis*; pink-white, pink lip with yellow stripe, 5) *E. spectabilis*; pink, pink lip with yellow stripe, 6) *E. spectabilis*; green-pink, pink lip with yellow stripe, 7) *E. spectabilis*; dark pink to red, pink lip, 8) *E. spectabilis*; yellow-green, white lip, 9) *E. spectabilis*; pink-green, pink lip, 10) *E. andamanensis*, 11) *E. graminea*, 12) *E. flava*

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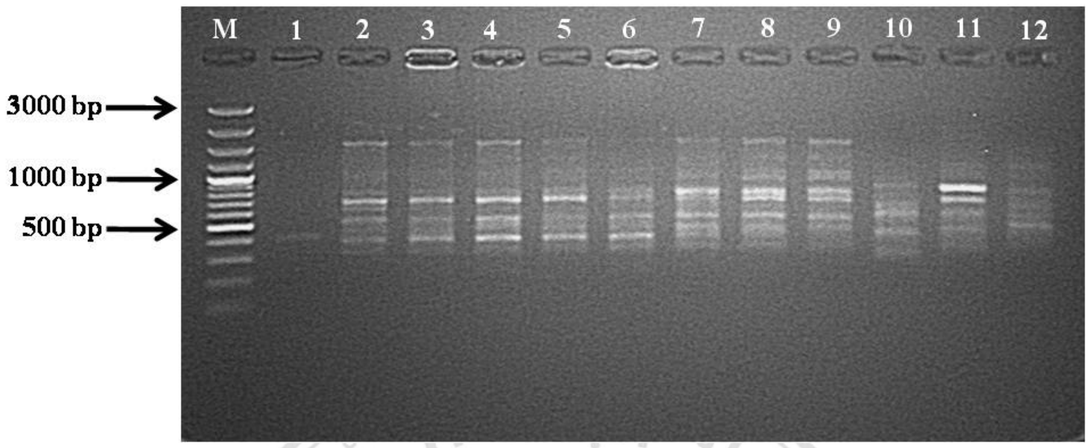

Figure 4 RAPD profile of *Eulophia* generated by OPA3 primer; M) 100 bps plus DNA ladder, 1) *E. macrobulbon*, 2) *E. spectabilis*; white, white lip with yellow stripe, 3) *E. spectabilis*; pink-white, white lip with yellow stripe, 4) *E. spectabilis*; pink-white, pink lip with yellow stripe, 5) *E. spectabilis*; pink, pink lip with yellow stripe, 6) *E. spectabilis*; green-pink, pink lip with yellow stripe, 7) *E. spectabilis*; dark pink to red, pink lip, 8) *E. spectabilis*; yellow-green, white lip, 9) *E. spectabilis*; pink-green, pink lip, 10) *E. andamanensis*, 11) *E. graminea*, 12) *E. flava*

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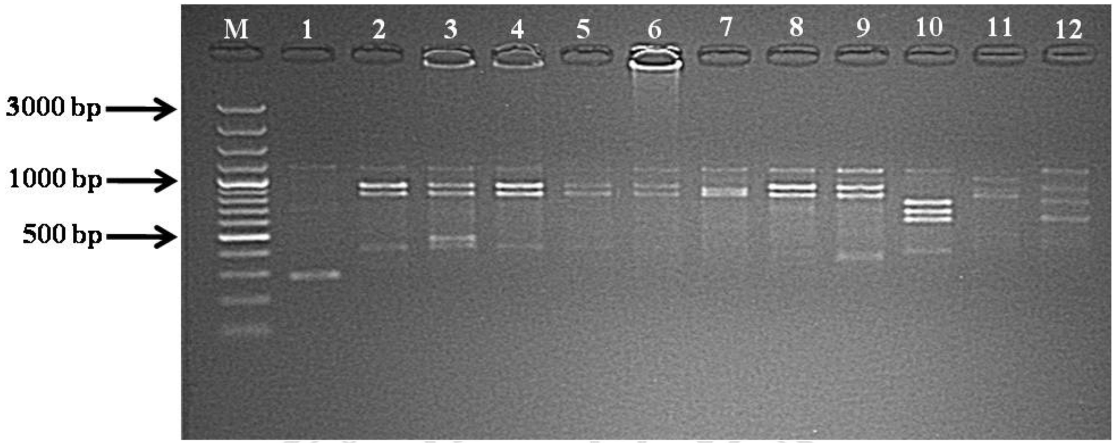

Figure 5 RAPD profile of *Eulophia* generated by OPA4 primer; M) 100 bps plus DNA ladder, 1) *E. macrobulbon*, 2) *E. spectabilis*; white, white lip with yellow stripe, 3) *E. spectabilis*; pink-white, white lip with yellow stripe, 4) *E. spectabilis*; pink-white, pink lip with yellow stripe, 5) *E. spectabilis*; pink, pink lip with yellow stripe, 6) *E. spectabilis*; green-pink, pink lip with yellow stripe, 7) *E. spectabilis*; dark pink to red, pink lip, 8) *E. spectabilis*; yellow-green, white lip, 9) *E. spectabilis*; pink-green, pink lip, 10) *E. andamanensis*, 11) *E. graminea*, 12) *E. flava*

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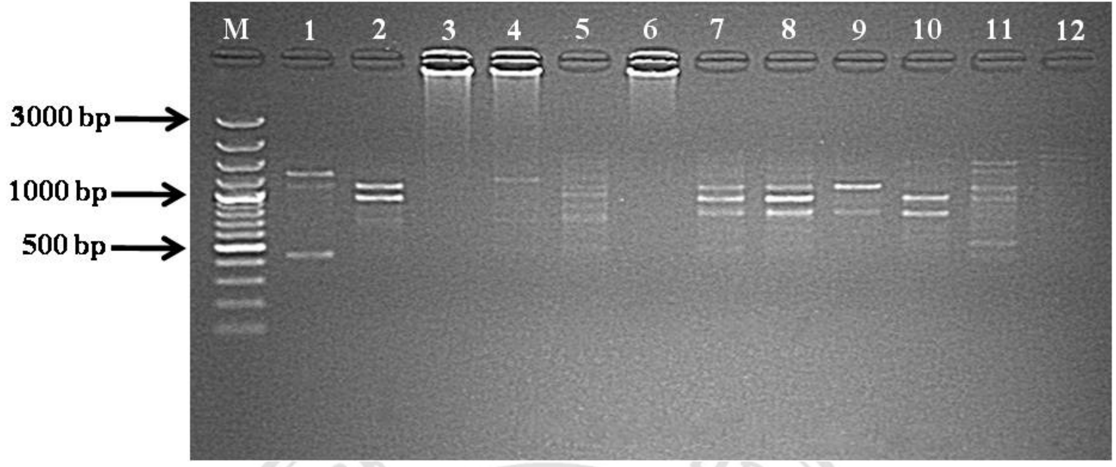

Figure 6 RAPD profile of *Eulophia* generated by OPA5 primer; M) 100 bps plus DNA ladder, 1) *E. macrobulbon*, 2) *E. spectabilis*; white, white lip with yellow stripe, 3) *E. spectabilis*; pink-white, white lip with yellow stripe, 4) *E. spectabilis*; pink-white, pink lip with yellow stripe, 5) *E. spectabilis*; pink, pink lip with yellow stripe, 6) *E. spectabilis*; green-pink, pink lip with yellow stripe, 7) *E. spectabilis*; dark pink to red, pink lip, 8) *E. spectabilis*; yellow-green, white lip, 9) *E. spectabilis*; pink-green, pink lip, 10) *E. andamanensis*, 11) *E. graminea*, 12) *E. flava*

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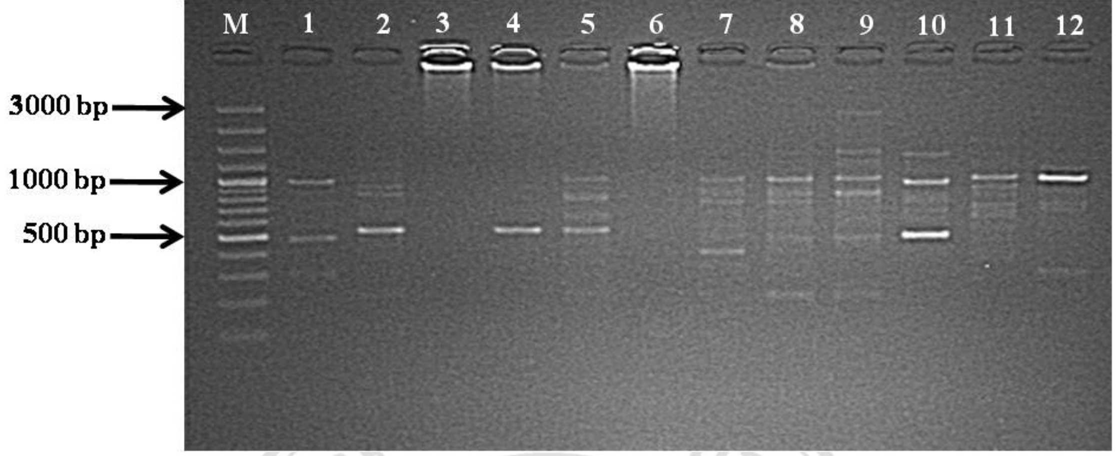

Figure 7 RAPD profile of *Eulophia* generated by OPA6 primer; M) 100 bps plus DNA ladder, 1) *E. macrobulbon*, 2) *E. spectabilis*; white, white lip with yellow stripe, 3) *E. spectabilis*; pink-white, white lip with yellow stripe, 4) *E. spectabilis*; pink-white, pink lip with yellow stripe, 5) *E. spectabilis*; pink, pink lip with yellow stripe, 6) *E. spectabilis*; green-pink, pink lip with yellow stripe, 7) *E. spectabilis*; dark pink to red, pink lip, 8) *E. spectabilis*; yellow-green, white lip, 9) *E. spectabilis*; pink-green, pink lip, 10) *E. andamanensis*, 11) *E. graminea*, 12) *E. flava*

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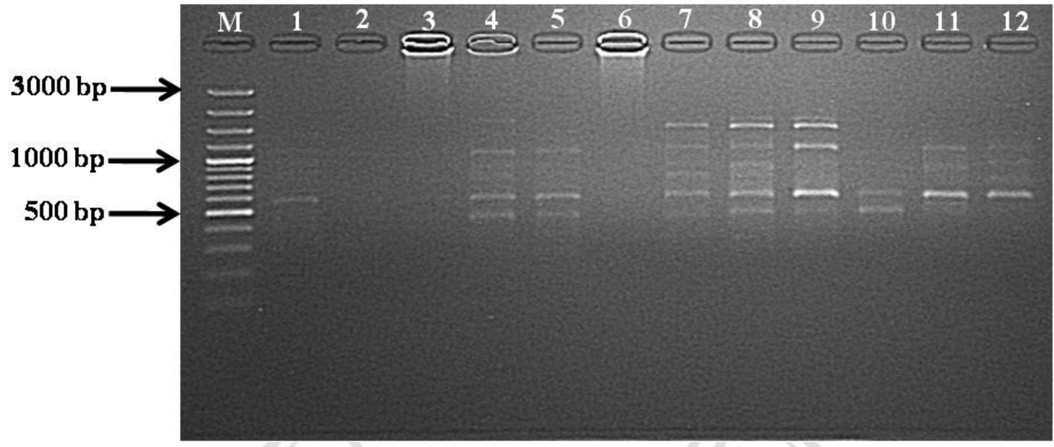

Figure 8 RAPD profile of *Eulophia* generated by OPA7 primer; M) 100 bps plus DNA ladder, 1) *E. macrobulbon*, 2) *E. spectabilis*; white, white lip with yellow stripe, 3) *E. spectabilis*; pink-white, white lip with yellow stripe, 4) *E. spectabilis*; pink-white, pink lip with yellow stripe, 5) *E. spectabilis*; pink, pink lip with yellow stripe, 6) *E. spectabilis*; green-pink, pink lip with yellow stripe, 7) *E. spectabilis*; dark pink to red, pink lip, 8) *E. spectabilis*; yellow-green, white lip, 9) *E. spectabilis*; pink-green, pink lip, 10) *E. andamanensis*, 11) *E. graminea*, 12) *E. flava*

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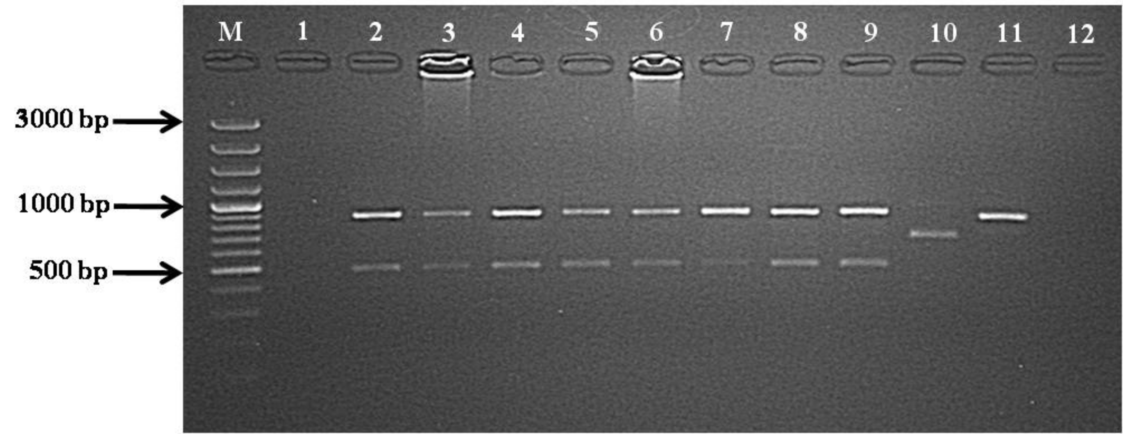

Figure 9 RAPD profile of *Eulophia* generated by OPA8 primer; M) 100 bps plus DNA ladder, 1) *E. macrobulbon*, 2) *E. spectabilis*; white, white lip with yellow stripe, 3) *E. spectabilis*; pink-white, white lip with yellow stripe, 4) *E. spectabilis*; pink-white, pink lip with yellow stripe, 5) *E. spectabilis*; pink, pink lip with yellow stripe, 6) *E. spectabilis*; green-pink, pink lip with yellow stripe, 7) *E. spectabilis*; dark pink to red, pink lip, 8) *E. spectabilis*; yellow-green, white lip, 9) *E. spectabilis*; pink-green, pink lip, 10) *E. andamanensis*, 11) *E. graminea*, 12) *E. flava*

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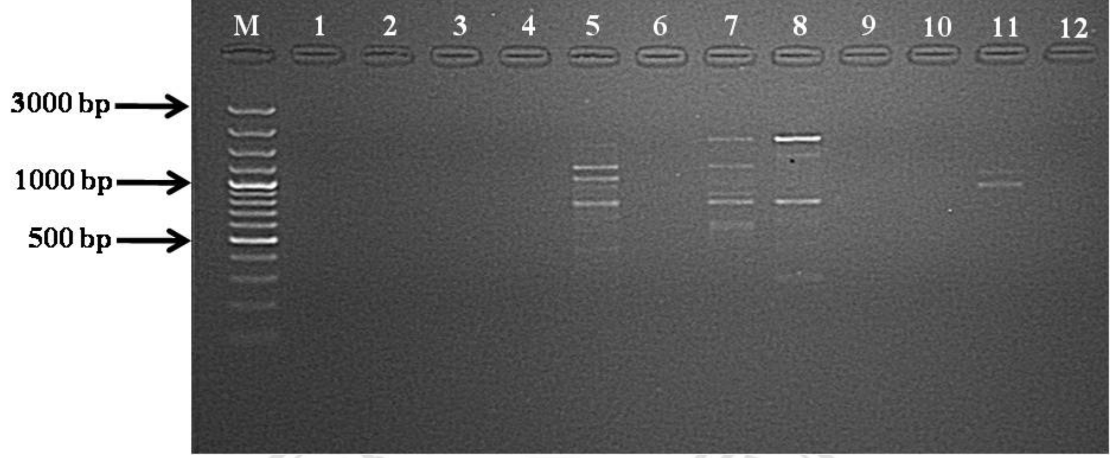

Figure 10 RAPD profile of *Eulophia* generated by OPA9 primer; M) 100 bps plus DNA ladder, 1) *E. macrobulbon*, 2) *E. spectabilis*; white, white lip with yellow stripe, 3) *E. spectabilis*; pink-white, white lip with yellow stripe, 4) *E. spectabilis*; pink-white, pink lip with yellow stripe, 5) *E. spectabilis*; pink, pink lip with yellow stripe, 6) *E. spectabilis*; green-pink, pink lip with yellow stripe, 7) *E. spectabilis*; dark pink to red, pink lip, 8) *E. spectabilis*; yellow-green, white lip, 9) *E. spectabilis*; pink-green, pink lip, 10) *E. andamanensis*, 11) *E. graminea*, 12) *E. flava*

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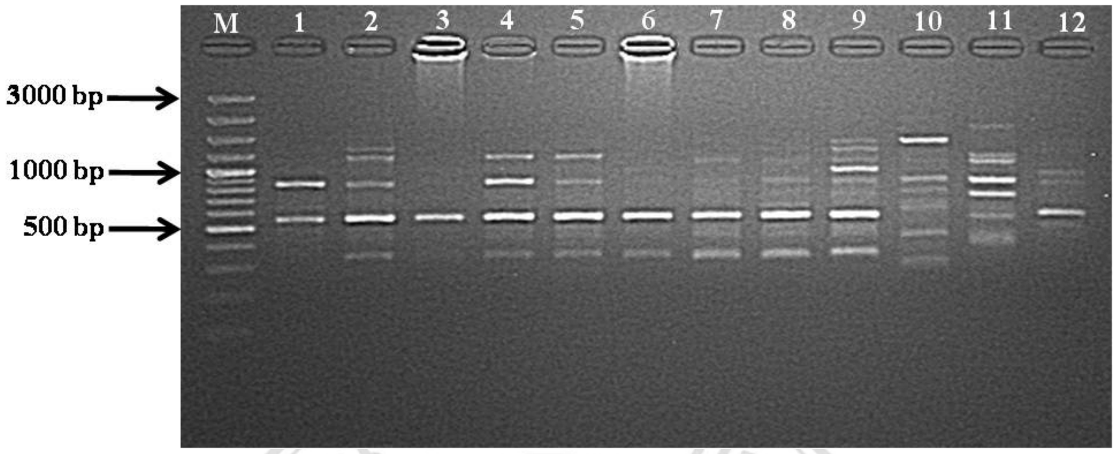

Figure 11 RAPD profile of *Eulophia* generated by OPA10 primer; M) 100 bps plus DNA ladder, 1) *E. macrobulbon*, 2) *E. spectabilis*; white, white lip with yellow stripe, 3) *E. spectabilis*; pink-white, white lip with yellow stripe, 4) *E. spectabilis*; pink-white, pink lip with yellow stripe, 5) *E. spectabilis*; pink, pink lip with yellow stripe, 6) *E. spectabilis*; green-pink, pink lip with yellow stripe, 7) *E. spectabilis*; dark pink to red, pink lip, 8) *E. spectabilis*; yellow-green, white lip, 9) *E. spectabilis*; pink-green, pink lip, 10) *E. andamanensis*, 11) *E. graminea*, 12) *E. flava*

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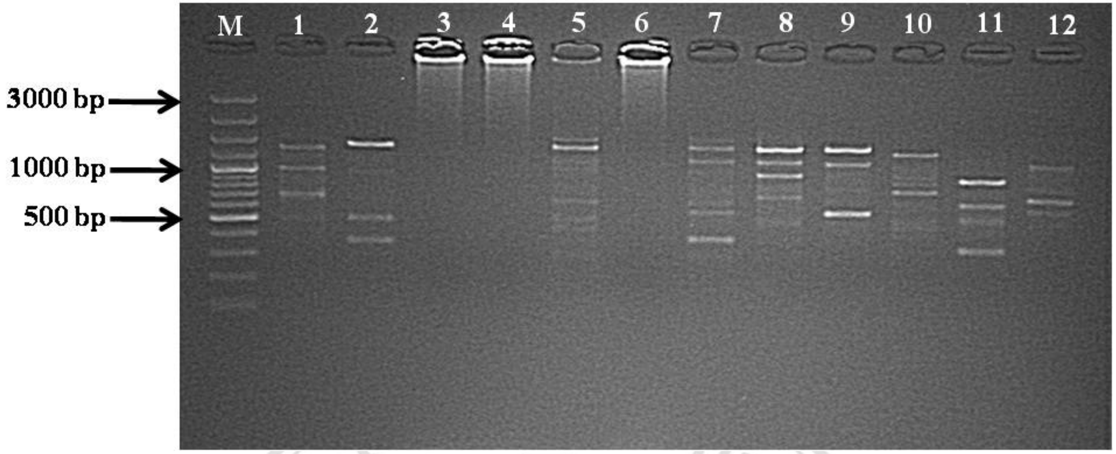

Figure 12 RAPD profile of *Eulophia* generated by OPA11 primer; M) 100 bps plus DNA ladder, 1) *E. macrobulbon*, 2) *E. spectabilis*; white, white lip with yellow stripe, 3) *E. spectabilis*; pink-white, white lip with yellow stripe, 4) *E. spectabilis*; pink-white, pink lip with yellow stripe, 5) *E. spectabilis*; pink, pink lip with yellow stripe, 6) *E. spectabilis*; green-pink, pink lip with yellow stripe, 7) *E. spectabilis*; dark pink to red, pink lip, 8) *E. spectabilis*; yellow-green, white lip, 9) *E. spectabilis*; pink-green, pink lip, 10) *E. andamanensis*, 11) *E. graminea*, 12) *E. flava*

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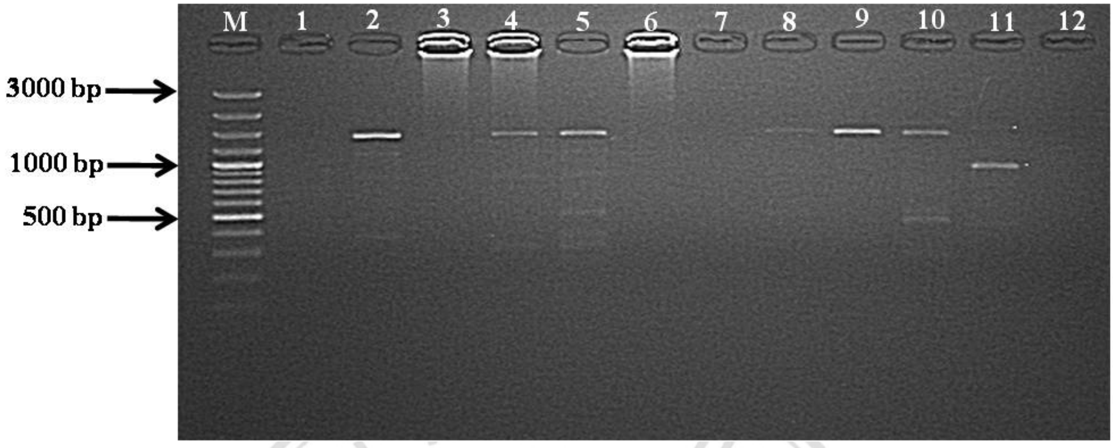

Figure 13 RAPD profile of *Eulophia* generated by OPA12 primer; M) 100 bps plus DNA ladder, 1) *E. macrobulbon*, 2) *E. spectabilis*; white, white lip with yellow stripe, 3) *E. spectabilis*; pink-white, white lip with yellow stripe, 4) *E. spectabilis*; pink-white, pink lip with yellow stripe, 5) *E. spectabilis*; pink, pink lip with yellow stripe, 6) *E. spectabilis*; green-pink, pink lip with yellow stripe, 7) *E. spectabilis*; dark pink to red, pink lip, 8) *E. spectabilis*; yellow-green, white lip, 9) *E. spectabilis*; pink-green, pink lip, 10) *E. andamanensis*, 11) *E. graminea*, 12) *E. flava*

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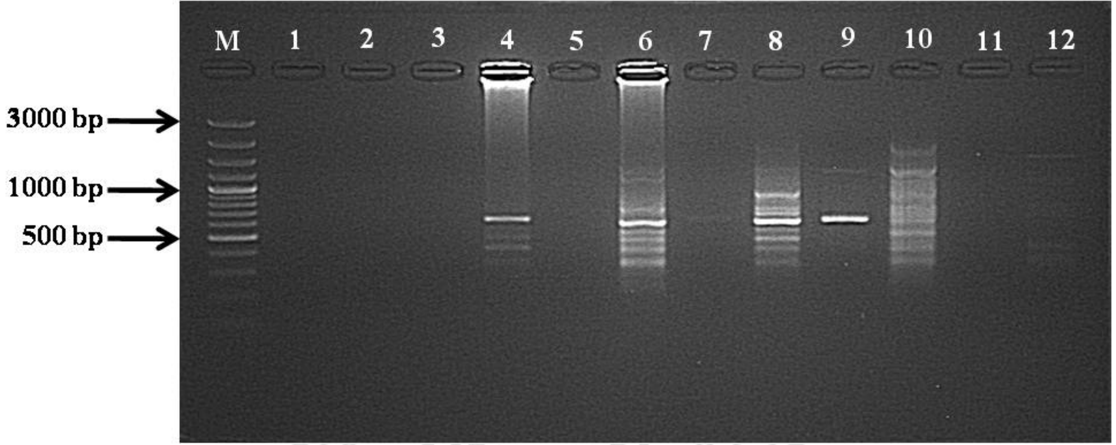

Figure 14 RAPD profile of *Eulophia* generated by OPA16 primer; M) 100 bps plus DNA ladder, 1) *E. macrobulbon*, 2) *E. spectabilis*; white, white lip with yellow stripe, 3) *E. spectabilis*; pink-white, white lip with yellow stripe, 4) *E. spectabilis*; pink-white, pink lip with yellow stripe, 5) *E. spectabilis*; pink, pink lip with yellow stripe, 6) *E. spectabilis*; green-pink, pink lip with yellow stripe, 7) *E. spectabilis*; dark pink to red, pink lip, 8) *E. spectabilis*; yellow-green, white lip, 9) *E. spectabilis*; pink-green, pink lip, 10) *E. andamanensis*, 11) *E. graminea*, 12) *E. flava*

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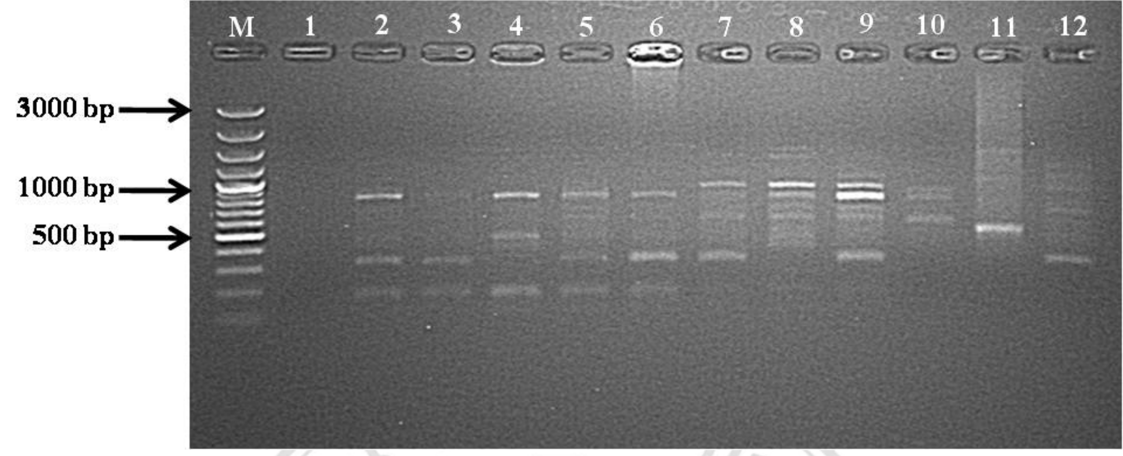

Figure 15 RAPD profile of *Eulophia* generated by OPA18 primer; M) 100 bps plus DNA ladder, 1) *E. macrobulbon*, 2) *E. spectabilis*; white, white lip with yellow stripe, 3) *E. spectabilis*; pink-white, white lip with yellow stripe, 4) *E. spectabilis*; pink-white, pink lip with yellow stripe, 5) *E. spectabilis*; pink, pink lip with yellow stripe, 6) *E. spectabilis*; green-pink, pink lip with yellow stripe, 7) *E. spectabilis*; dark pink to red, pink lip, 8) *E. spectabilis*; yellow-green, white lip, 9) *E. spectabilis*; pink-green, pink lip, 10) *E. andamanensis*, 11) *E. graminea*, 12) *E. flava*

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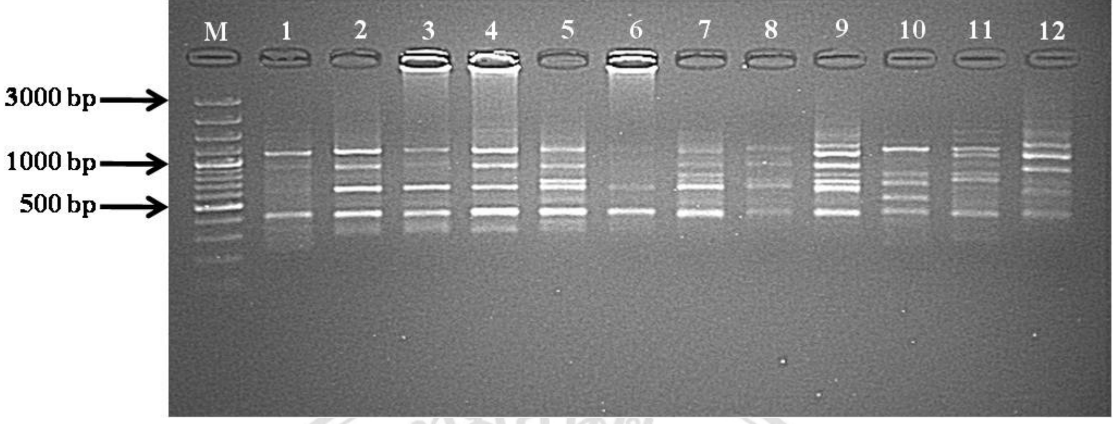

Figure 16 RAPD profile of *Eulophia* generated by OPA20 ; M) 100 bps plus DNA ladder, 1) *E. macrobulbon*, 2) *E. spectabilis*; white, white lip with yellow stripe, 3) *E. spectabilis*; pink-white, white lip with yellow stripe, 4) *E. spectabilis*; pink-white, pink lip with yellow stripe, 5) *E. spectabilis*; pink, pink lip with yellow stripe, 6) *E. spectabilis*; green-pink, pink lip with yellow stripe, 7) *E. spectabilis*; dark pink to red, pink lip, 8) *E. spectabilis*; yellow-green, white lip, 9) *E. spectabilis*; pink-green, pink lip, 10) *E. andamanensis*, 11) *E. graminea*, 12) *E. flava*

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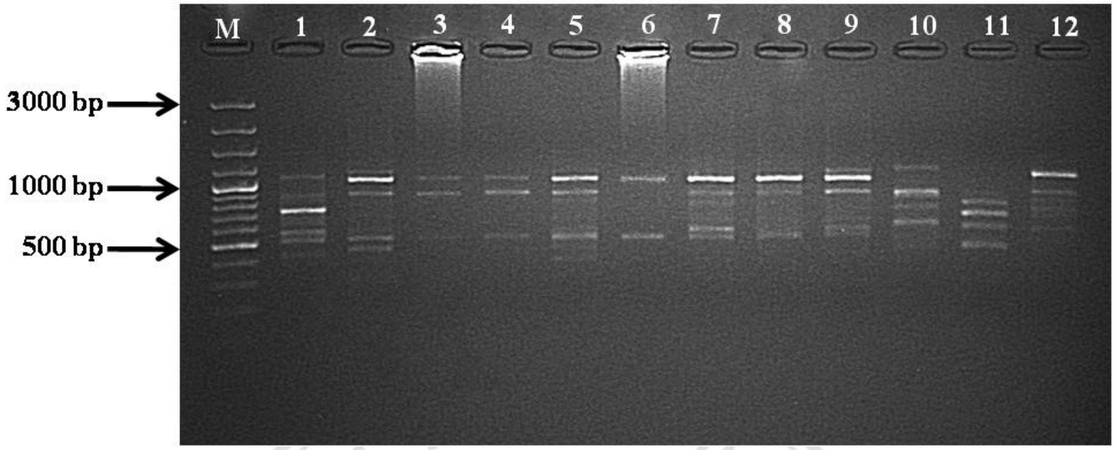

Figure 17 RAPD profile of *Eulophia* generated by OPF1 primer; M) 100 bps plus DNA ladder, 1) *E. macrobulbon*, 2) *E. spectabilis*; white, white lip with yellow stripe, 3) *E. spectabilis*; pink-white, white lip with yellow stripe, 4) *E. spectabilis*; pink-white, pink lip with yellow stripe, 5) *E. spectabilis*; pink, pink lip with yellow stripe, 6) *E. spectabilis*; green-pink, pink lip with yellow stripe, 7) *E. spectabilis*; dark pink to red, pink lip, 8) *E. spectabilis*; yellow-green, white lip, 9) *E. spectabilis*; pink-green, pink lip, 10) *E. andamanensis*, 11) *E. graminea*, 12) *E. flava*

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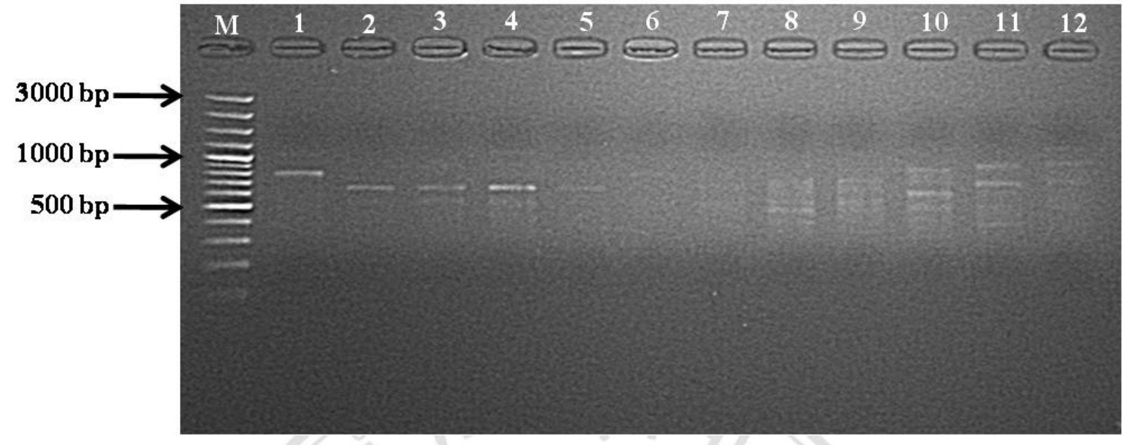

Figure 18 RAPD profile of *Eulophia* generated by OPF2 primer; M) 100 bps plus DNA ladder, 1) *E. macrobulbon*, 2) *E. spectabilis*; white, white lip with yellow stripe, 3) *E. spectabilis*; pink-white, white lip with yellow stripe, 4) *E. spectabilis*; pink-white, pink lip with yellow stripe, 5) *E. spectabilis*; pink, pink lip with yellow stripe, 6) *E. spectabilis*; green-pink, pink lip with yellow stripe, 7) *E. spectabilis*; dark pink to red, pink lip, 8) *E. spectabilis*; yellow-green, white lip, 9) *E. spectabilis*; pink-green, pink lip, 10) *E. andamanensis*, 11) *E. graminea*, 12) *E. flava*

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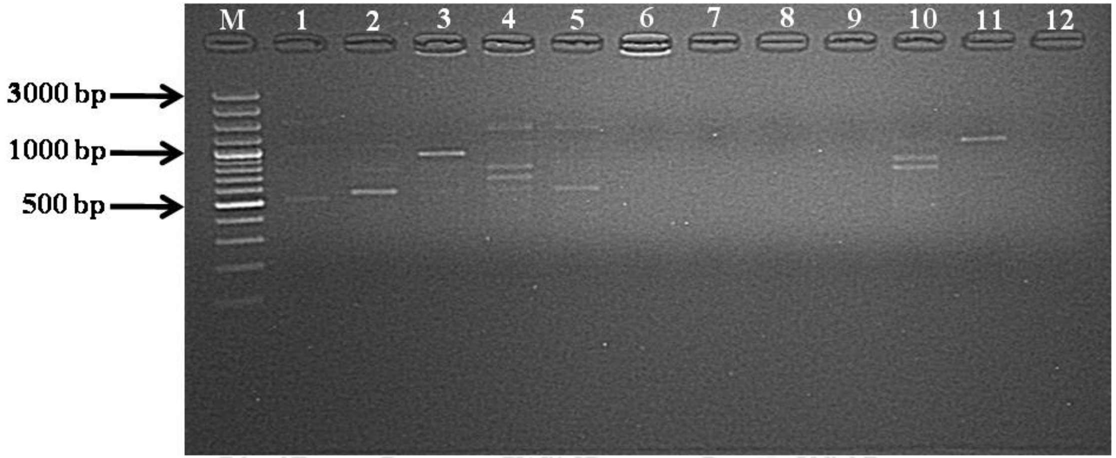

Figure 19 RAPD profile of *Eulophia* generated by OPF3 primer; M) 100 bps plus DNA ladder, 1) *E. macrobulbon*, 2) *E. spectabilis*; white, white lip with yellow stripe, 3) *E. spectabilis*; pink-white, white lip with yellow stripe, 4) *E. spectabilis*; pink-white, pink lip with yellow stripe, 5) *E. spectabilis*; pink, pink lip with yellow stripe, 6) *E. spectabilis*; green-pink, pink lip with yellow stripe, 7) *E. spectabilis*; dark pink to red, pink lip, 8) *E. spectabilis*; yellow-green, white lip, 9) *E. spectabilis*; pink-green, pink lip, 10) *E. andamanensis*, 11) *E. graminea*, 12) *E. flava*

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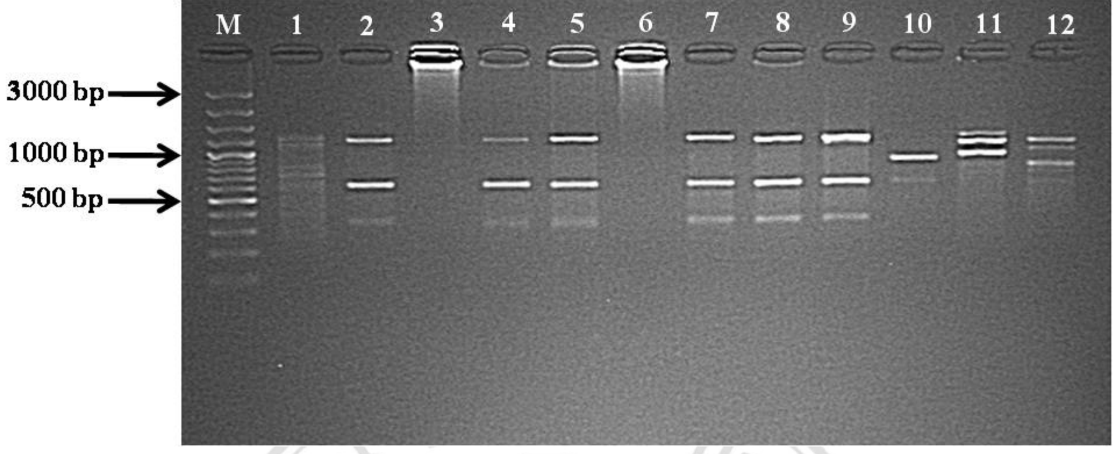

Figure 20 RAPD profile of *Eulophia* generated by OPF6 primer; M) 100 bps plus DNA ladder, 1) *E. macrobulbon*, 2) *E. spectabilis*; white, white lip with yellow stripe, 3) *E. spectabilis*; pink-white, white lip with yellow stripe, 4) *E. spectabilis*; pink-white, pink lip with yellow stripe, 5) *E. spectabilis*; pink, pink lip with yellow stripe, 6) *E. spectabilis*; green-pink, pink lip with yellow stripe, 7) *E. spectabilis*; dark pink to red, pink lip, 8) *E. spectabilis*; yellow-green, white lip, 9) *E. spectabilis*; pink-green, pink lip, 10) *E. andamanensis*, 11) *E. graminea*, 12) *E. flava*

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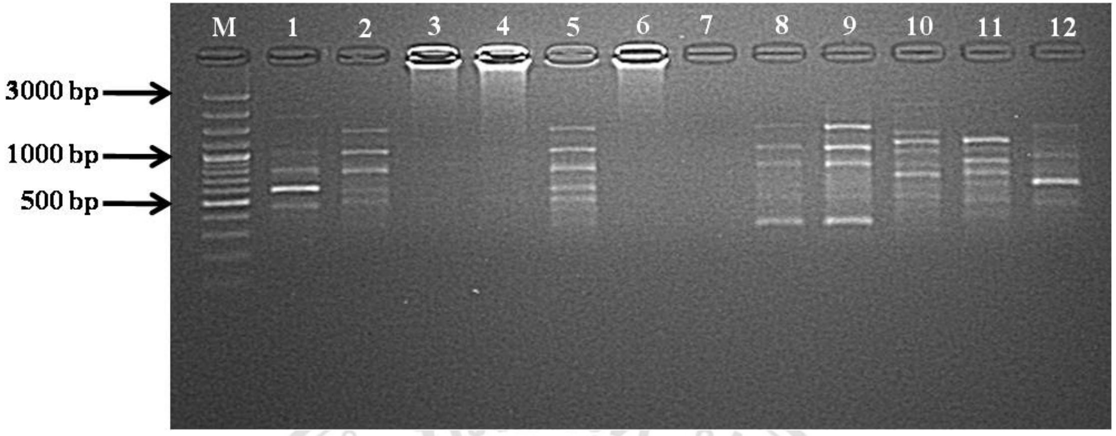

Figure 21 RAPD profile of *Eulophia* generated by OPF9 primer; M) 100 bps plus DNA ladder, 1) *E. macrobulbon*, 2) *E. spectabilis*; white, white lip with yellow stripe, 3) *E. spectabilis*; pink-white, white lip with yellow stripe, 4) *E. spectabilis*; pink-white, pink lip with yellow stripe, 5) *E. spectabilis*; pink, pink lip with yellow stripe, 6) *E. spectabilis*; green-pink, pink lip with yellow stripe, 7) *E. spectabilis*; dark pink to red, pink lip, 8) *E. spectabilis*; yellow-green, white lip, 9) *E. spectabilis*; pink-green, pink lip, 10) *E. andamanensis*, 11) *E. graminea*, 12) *E. flava*

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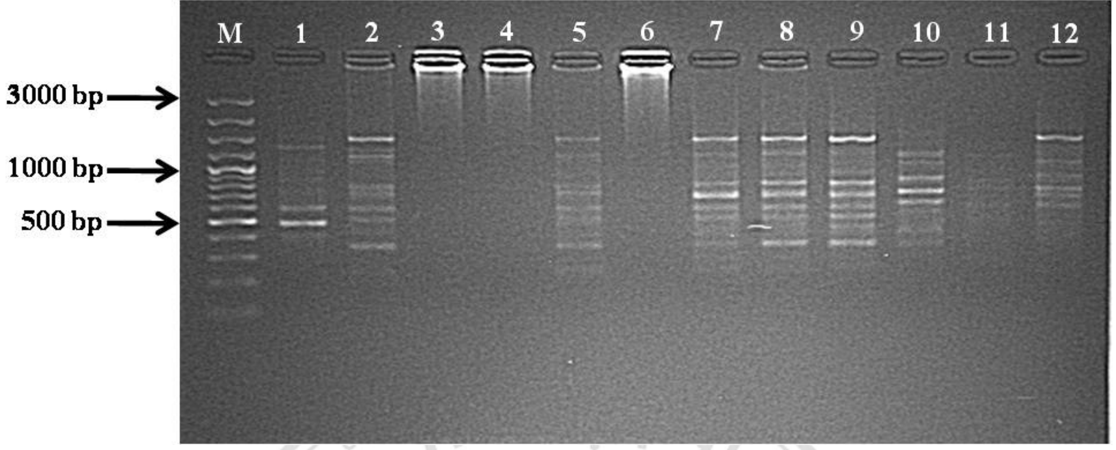

Figure 22 RAPD profile of *Eulophia* generated by OPF12 primer; M) 100 bps plus DNA ladder, 1) *E. macrobulbon*, 2) *E. spectabilis*; white, white lip with yellow stripe, 3) *E. spectabilis*; pink-white, white lip with yellow stripe, 4) *E. spectabilis*; pink-white, pink lip with yellow stripe, 5) *E. spectabilis*; pink, pink lip with yellow stripe, 6) *E. spectabilis*; green-pink, pink lip with yellow stripe, 7) *E. spectabilis*; dark pink to red, pink lip, 8) *E. spectabilis*; yellow-green, white lip, 9) *E. spectabilis*; pink-green, pink lip, 10) *E. andamanensis*, 11) *E. graminea*, 12) *E. flava*

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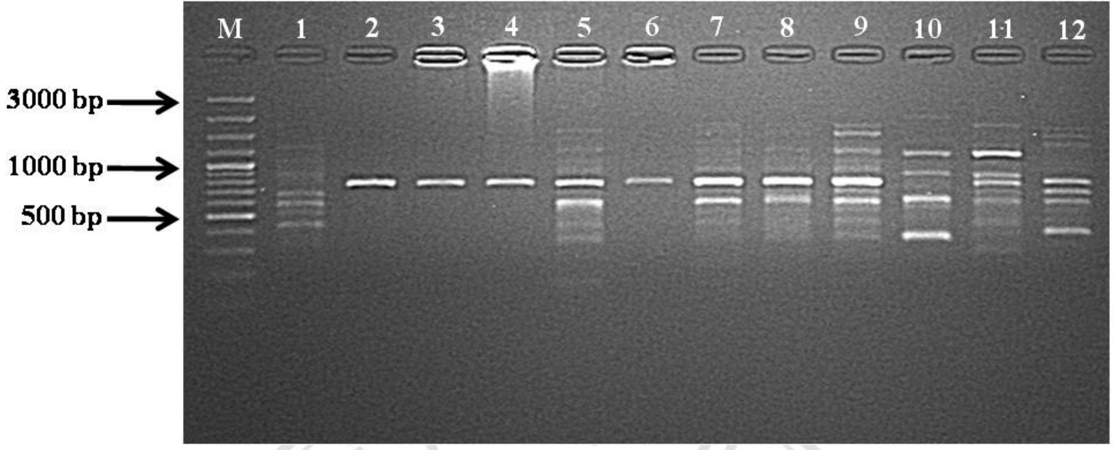

Figure 23 RAPD profile of *Eulophia* generated by OPF13 primer; M) 100 bps plus DNA ladder, 1) *E. macrobulbon*, 2) *E. spectabilis*; white, white lip with yellow stripe, 3) *E. spectabilis*; pink-white, white lip with yellow stripe, 4) *E. spectabilis*; pink-white, pink lip with yellow stripe, 5) *E. spectabilis*; pink, pink lip with yellow stripe, 6) *E. spectabilis*; green-pink, pink lip with yellow stripe, 7) *E. spectabilis*; dark pink to red, pink lip, 8) *E. spectabilis*; yellow-green, white lip, 9) *E. spectabilis*; pink-green, pink lip, 10) *E. andamanensis*, 11) *E. graminea*, 12) *E. flava*

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OPA 4 primers could separate *E. andamanensis* from other *Eulophia* at 45% similarity. At 61% similarity, OPA 4 primer could separate *E. macrobulbon* from other *Eulophia* (Figure 24).

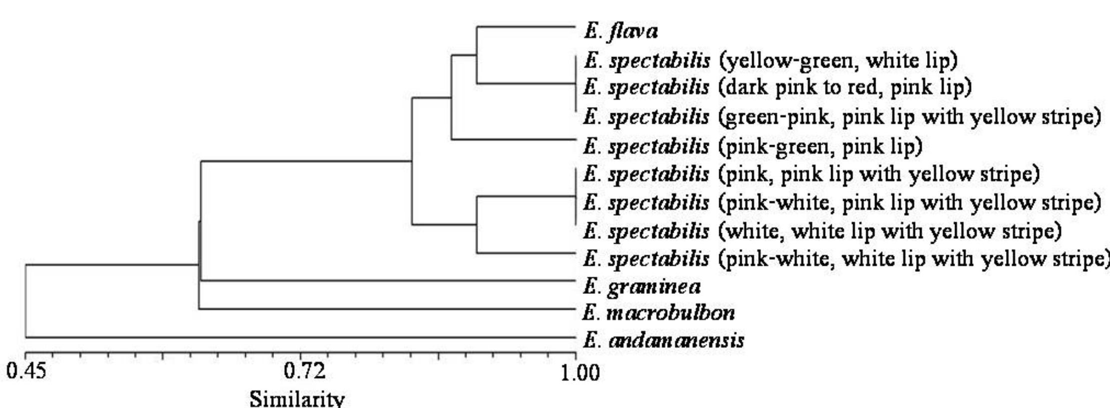

Figure 24 Dendrogram of *Eulophia* based on OPA4 primer

OPF1 primer could separate terrestrial orchid into 2 groups at 33% similarity. The first group consisted of *E. graminea* and *E.* andamanensis. Another group consisted of *E. spectabilis*, *E. flava* and *E. macrobulbon*. The primer could separate subgroup *E. spectabilis*; pink-green, pink lip, and *E. Spectabilis*; white, white lip with yellow stripe, from other *E. spectabilis* at 70% similarity (Figure 25).

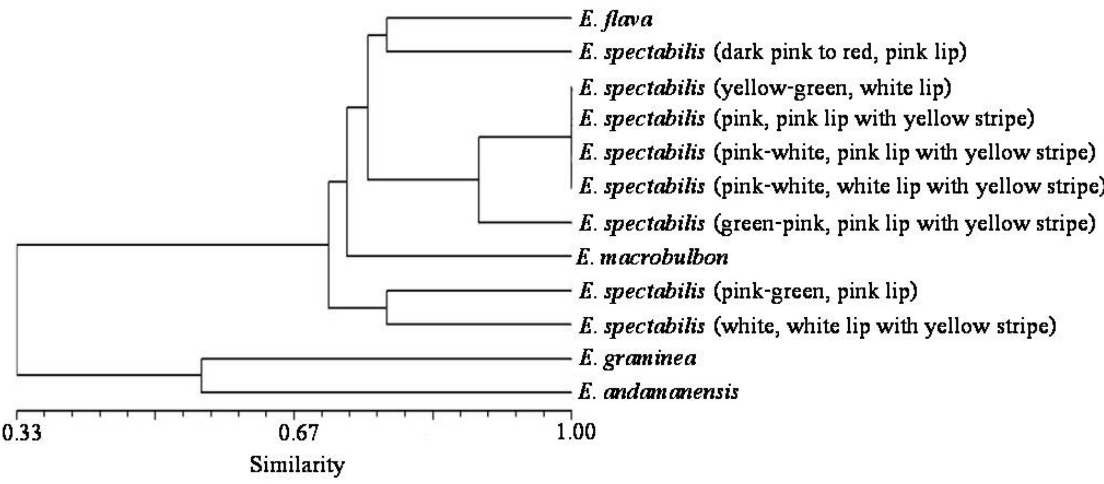

Figure 25 Dendrogram of *Eulophia* based on OPF1 primer

OPF13 primer could separate *E. flava* from other *Eulophia* at 50% similarity. At 54% similarity, OPF13 could separate *E. andamanensis* from *E. macrobulbon*, *E. graminea*, and all *E. spectabilis* (Figure 26).

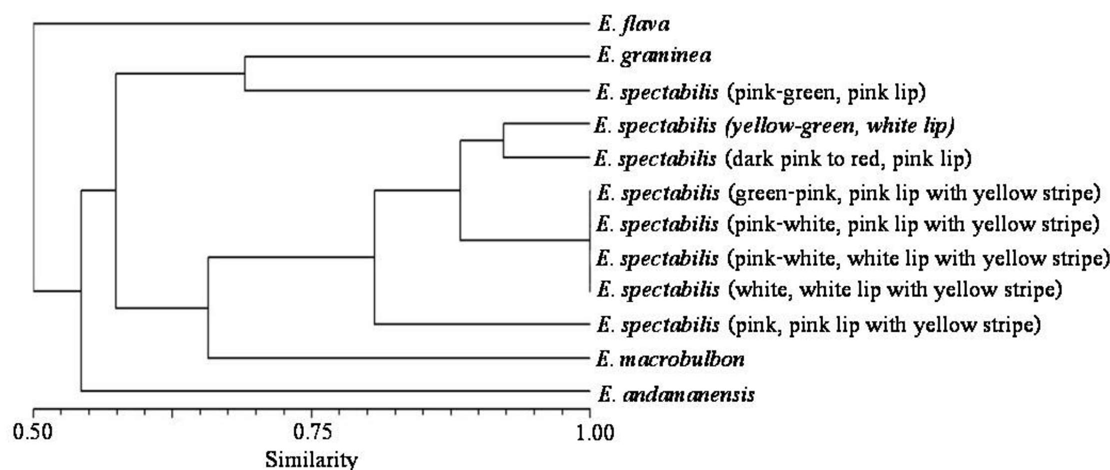

Figure 26 Dendrogram of *Eulophia* based on OPF13 primer

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Appendix F

Atmosphere data between physiological studies

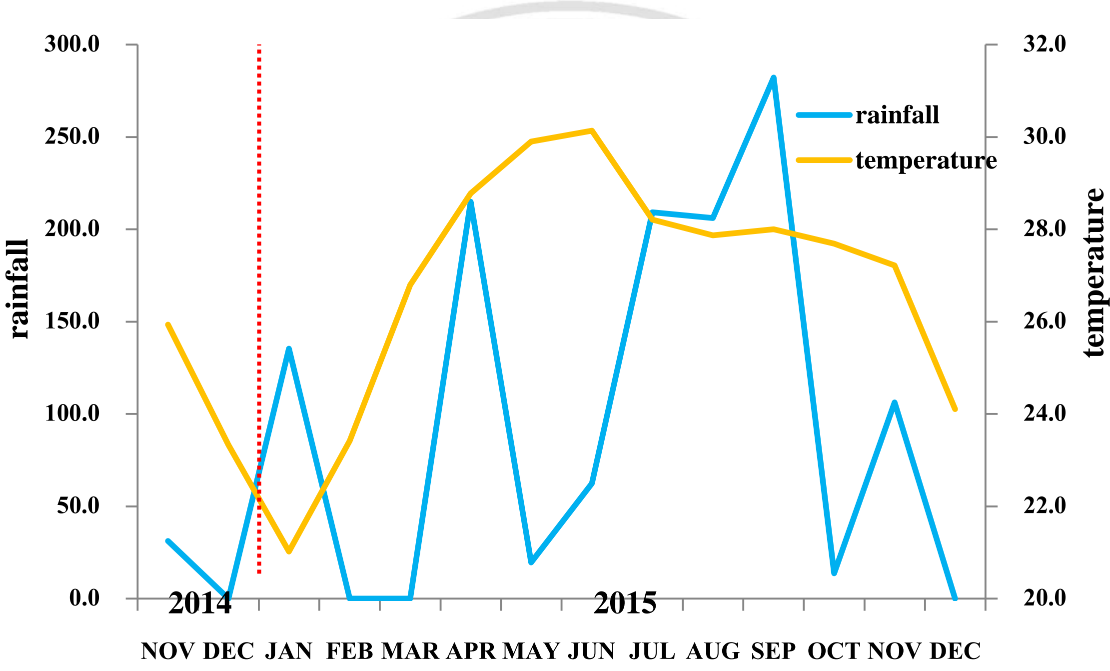

Figure 27 Rainfall and temperature data between physiological studies

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Appendix G

Chemical reagent preparation for total non-structural carbohydrate analysis

1. Nelson's alkaline copper reagent

Preparation of Nelson's alkaline copper reagent solution was conducted as follow:

- Reagent A

Anhydrous sodium carbonate (Na_2CO_3)	25 g
Sodium potassium titrate	25 g
Sodium bicarbonate ($NaHCO_3$)	20 g
Anhydrous Sodium sulfate (Na_2SO_4)	200 g

add H_2O to1000 ml

- Reagent B

Copper sulfate ($CuSO_4 \cdot 5H_2O$)	15 g

mix with H_2O 100 ml and add sulfuric acid 2 drops

- Mix Reagent A 20 ml with reagent B 0.8 ml
- Nelson's alkaline copper reagent was ready to used immediately

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2. Arsenomolybolic acid reagent

Preparation of Arsenomolybolic acid reagent solution was conducted as follow:

- Reagent A

Ammoniummolybdate [$(NH_4)_6Mo_7O_{24} \cdot 4H20$] 25 g were solute in water 450 ml, then add sulfuric acid 21 ml

- Reagent B

Solute Sodium dehydrogenarseate ($Na_2HAsO_4 \cdot 7H_2O$) in water 25 ml

- Mix all reagents A and B.
- Arsenomolybolic acid reagent was ready after 2 days of incubation.

CURRICULUM VITAE

Author's Name	Mr. Pongnatee Pintajam
Date of Birth	January 24, 1989
Place of Birth	Lamphun Province, Thailand
Education	2007-2011 B.S. (Science), Chiang Mai University, Thailand
Publications	Pintajam, P. and N. Potapohn. 2015. Effect of Cold Storage Period on Flower Delaying of *Eulophia macrobulbon* (Par. & Pchb. f.) Hook. f. and *E. spectabilis* (Dennst.) Suresh. King Mongkut's Agricultural Journal. 33: 242-245. Pintajam, P., C. Tiyayon and N. Potapohn. 2016. Interspecific and Intergeneric Crossabilities of *Eulophia macrobulbon* (Par. & Pchb. f.) Hook. f. and *E. spectabilis* (Dennst.) Suresh. Journal of Agriculture. 32(3): 299-308 Pintajam, P., W. Bundithya and N. Potapohn. 2018. Intraspecific and Interspecific Crossability of Some *Eulophia* Species. Maejo International Journal of Science and Technology. 12(3): 241-250

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