# *In Vitro* Flowering of Dwarf *Dendrobium*

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

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**ชื่อวิทยานิพนธ์** การออกดอกในหลอดทดลองของกล้วยไม้หวายแคระ
**ผู้เขียน** นางสาวปานจันทน์ สุจริตธุรการ
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## บทคัดย่อ

การชักนำโพรโทคอร์มไลค์บอดี้จากโพรโทคอร์มของกล้วยไม้หวายแคระ (*Dendrobium* Queen Pink x *Dendrobium* Phakbung) ที่มีประสิทธิภาพ เพื่อพัฒนาการขยายพันธุ์ในปริมาณมาก ได้ประสบความสำเร็จ พบว่าความเข้มข้นที่แตกต่างกันของ $N^6$-benzyladenine (BA) และ thidiazuron (TDZ) มีผลต่อการชักนำโพรโทคอร์มไลค์บอดี้ โดย TDZ มีประสิทธิภาพสูงกว่า BA ในอาหารเหลวสูตร Murashige and Skoog (MS) ดัดแปลงที่เติม TDZ ความเข้มข้น 18 ไมโครโมลาร์ สามารถชักนำโพรโทคอร์มไลค์บอดี้สูงสุด คือ 86.4 เปอร์เซ็นต์และจำนวนโพรโทคอร์มไลค์บอดี้ต่อโพรโทคอร์มสูงสุด คือ 3.6 หลังจากเพาะเลี้ยง 9 สัปดาห์ โพรโทคอร์มไลค์บอดี้ที่ชักนำ ได้พัฒนาขึ้นจากผิวของโพรโทคอร์มโดย ไม่ผ่านแคลลัส เมื่อย้ายโพรโทคอร์มไลค์บอดี้ไปเลี้ยงบนอาหารสูตร MS ที่ปราศจากสารควบคุมการเจริญเติบโต พบว่าโพรโทคอร์มไลค์บอดี้สามารถพัฒนาเป็นยอดและรากภายใน 6 สัปดาห์ และพัฒนาเป็นต้นพืชที่ปกติหลังจากการย้ายปลูกไปไว้ที่โรงเรือน 12 สัปดาห์

ผลของ BA paclobutrazol (PBZ) น้ำตาลซูโครส ช่วงแสง ความต้องการ ธาตุอาหาร (ความเข้มข้นต่ำของไนโตรเจนและความเข้มข้นสูงของฟอสฟอรัส หรือ NP treatment) และอาหาร 2 ชั้น ต่อการชักนำการออกดอกในหลอดทดลองของกล้วยไม้หวายแคระ พบว่า อาหารสูตร MS ที่มี BA ความเข้มข้น 22 ไมโครโมลาร์ น้ำตาลซูโครสความเข้ มข้น 30 กรัมต่อลิตร ที่ช่วงแสง 12 ชั่วโมงต่อวัน ให้เปอร์เซ็นต์การเกิดตาดอก 57.14 และจำนวนช่อดอกเฉลี่ยสูงสุด 1.14 ช่อดอกต่อชิ้นส่วนพืช หลังจากการเพาะเลี้ยง 4 เดือน อย่างไรก็ตาม ตาดอกเหล่านี้ไม่บานและเหี่ยวแห้งในเวลาต่อมา สำหรับความเข้มข้นของ PBZ 0.085 ไมโครโมลาร์ ภายใต้ช่วงแสง 16 ชั่วโมงต่อวัน ส่งเสริมการชักนำตาดอก ผลิตตาดอกได้มากที่สุด 20 เปอร์เซ็นต์ และจำนวนช่อดอกสูงสุด 0.4 ช่อดอกต่อชิ้นส่วนพืช หลังจากการเพาะเลี้ยง 4 เดือน ตาดอกเหล่านี้สามารถบานเป็นดอกภายใน 2-3 เดือน ดอกที่ชักนำได้เป็นดอกปกติตามสัณฐานวิทย า ประกอบด้วยกลีบเลี้ยง 3 กลีบ กลีบดอก 2 กลีบ และกลีบปาก 1 กลีบ ก้อนเรณูและอวัยวะเพศเมีย การใช้ NP treatment ร่วมกับ BA ความเข้มข้น 22 ไมโครโมลาร์ มีผลต่อการชักนำตา ดอกจากการทดลอง พบว่า อาหาร ที่มีความเข้มข้น ของไนโตรเจนรวม 8.6 มิลลิโมลาร์และความเข้มข้นของฟอสฟอรัสรวม 6.25 มิลลิโมลาร์ ($N_7P_5$) ที่ช่วงแสง 16 ชั่วโมงต่อวัน ทำให้เกิดตา

ดอก 30 เปอร์เซ็นต์ และจำนวนช่อดอกสูงสุด 1.4 ช่อดอกต่อชิ้นส่วน หลังจากการเพาะเลี้ยง 4 เดือน อย่างไรก็ตาม ตาดอกที่เกิดขึ้นเหล่านี้ไม่สามารถพัฒนาไปเป็นดอกและบานในเวลาต่อมา การทดลองในอาหาร 2 ชั้นที่มี BA ความเข้มข้น 22 ไมโครโมลาร์ ภายใต้ช่วงแสง 16 ชั่วโมงต่อวัน พบว่ามีประสิทธิภาพสูงที่สุดในการชักนำตาดอก สามารถชักนำ จำนวนช่อดอก 3.4 ช่อดอกต่อชิ้นส่วนพืช และการเกิดตาดอก 54.55 เปอร์เซ็นต์ หลังจากการเพาะเลี้ยง 3 เดือน ตาดอกเหล่านี้บานเป็นดอกภายใน 2-3 เดือน หลังจากตาดอกเกิดขึ้น ดอกที่ชักนำได้ประกอบด้วยกลีบเลี้ยง 3 กลีบ กลีบดอก 2 กลีบ และกลีบปาก 1 กลีบ ก้อนเรณูและอวัยวะเพศเมีย จากการทดลองพบว่า อาหาร 2 ชั้นที่มีน้ำมะพร้าวเพียงอย่างเดียวไม่สามารถชักนำการออกดอกได้

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## ABSTRACT

An efficient protocorm-like body (PLB) induction from protocorms of dwarf *Dendrobium* (*Dendrobium* Queen Pink x *Dendrobium* Phakbung) was accomplished in order to develop mass-scale propagation. The effect of various concentrations of $N^6$-benzyladenine (BA) and thidiazuron (TDZ) on the *in vitro* induction of PLBs was studied. TDZ was found to be a more effective inducer of PLBs and their proliferation than BA. The highest percentage for the induction of PLBs (86.4 %) and the highest number of PLBs (3.6) per protocorm were observed after 9 weeks of culture in modified Murashige and Skoog (MS) liquid medium supplemented with 18 μM TDZ. PLBs developed directly from the surface of protocorms without the intermediate formation of callus. Subsequently, PLBs were transferred to the basal MS medium without plant growth regulators (PGRs) upon which they developed into shoots and roots in 6 weeks. They grew well and developed into normal plantlets after 12 weeks of transplantation.

The effect of BA, paclobutrazol (PBZ) , sucrose, photoperiod, nutrient requirements (low concentration of nitrogen (N) and high concentration of phosphorus (P) or NP treatment), and the two-layered medium on *in vitro* flowering induction of dwarf *Dendrobium* were examined. The results showed that MS medium containing 22 μM BA and 30 g/l sucrose at 12-h photoperiod gave the percentage of floral buds (57.14 %) and the best average of 1.14 inflorescences per explant was produced after 4 months of culture. However, these floral buds did not proceed to open and wilt later. Only concentration of PBZ at 0.085 μM under 16-h photoperiod promoted floral buds induction that showed maximum percentage (20 %) of cultures producing floral buds and number of inflorescences (0.4) per explant after 4 months of culture. These floral buds proceeded to bloom within 2-3 months. The flowers were normal in morphology comprised of three sepals, two petals and one lip or labellum,

pollinia and female organ. The NP treatment combined with 22 μM BA affected the induction of floral buds. The NP medium containing 8.6 mM total N and 6.25 mM total P ($N_7P_5$ medium) at 16-h photoperiod gave floral buds formation (30 %) and the highest number of inflorescences (1.4) per explant after 4 months of culture. Nevertheless, these formed floral buds could not further develop to flowers and proceed to open. The best efficiency of plantlets producing floral buds was found in two-layered medium containing 22 μM BA under 16-h photoperiod that exhibited 54.55 % floral buds formation and the best response for number of inflorescences (3.4) per explant after 3 months of culture. These floral buds bloomed within 2-3 months after floral buds had produced. The flowers composed of three sepals, two petals and one lip or labellum, pollinia and female organ. In two-layered medium added CW singly did not induce floral bud formation.

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Panjan Sujjaritthurakarn

# CONTENTS

**CONTENTS (Continued)**

## CONTENTS (Continued)

# LIST OF TABLES

# LIST OF FIGURES

# LIST OF ABBREVIATIONS AND SYMBOLS

| | | |
|---|---|---|
| ANOVA | = | Analysis of variance |
| ASEAN | = | Association of the South East Asian Nations |
| BA | = | Benzyladenine |
| C | = | Carbohydrates |
| CW | = | Coconut water |
| DN | = | Day-neutral |
| DZ | = | Dihydrozeatin |
| DZR | = | Dihydrozeatin riboside |
| DZ9G | = | Dihydrozeatin-9-glucoside |
| DZ5P | = | Dihydrozeatin riboside 50-monophosphate |
| $GA_3$ | = | Gibberellic acid |
| h | = | hour |
| HPLC | = | High performance liquid chromatography |
| iP | = | $N^6$-($\Delta^2$-isopentenyl)-adenine |
| iPA | = | $N^6$-($\Delta^2$-isopentenyl)-adenosine |
| iPAMP | = | $N^6$-($\Delta^2$-isopentenyl)-adenoside-5'-monophosphate |
| iP9G | = | $N^6$-($\Delta^2$-isopentenyl)-adenine-9-glucoside |
| iP | = | $N^6$-($\Delta^2$-isopentenyl)-adenine |
| KC | = | Knudson C medium |
| KN | = | Kinetin |
| kPa | = | kilopascals |
| l | = | lip |
| LD | = | Long day |
| MS | = | Murashige and Skoog medium |
| N | = | Nitrogen |
| $NH_4^+$ | = | Ammonium ion |
| $NO_3^-$ | = | Nitrate ion |
| NP | = | Low N and high P concentrations |
| P | = | Phosphorous |
| p | = | petals |

| | | |
|---|---|---|
| PBZ | = | Paclobutrazol |
| PGRs | = | Plant growth regulators |
| PLBs | = | Protocorm like bodies |
| s | = | sepals |
| SAM | = | Shoot apical meristem |
| SD | = | Short day |
| TDZ | = | Thidiazuron |
| US | = | United States |
| VW | = | Vacin and Went medium |
| Z | = | Zeatin |
| ZR | = | Zeatin riboside |
| Z9G | = | Zeatin-9-glucoside |
| Z5P | = | Zeatin riboside 5’-monophosphate |

# CHAPTER 1
# INTRODUCTION

## 1.1 Introduction

Orchids (Family: Orchidaceae), one of the largest, most diverse, and most important categories of botanically and commercially significant flowering plant families with 20,000-30,000 species (Cronquist, 1981; Dressler, 1981), are popular horticultural and ornamental plants mostly not only because of their exquisite flowers but also for their long shelf life. Moreover, orchids attract almost every kind of individual including professional breeders, amateurs and normal collectors (Nambiar *et al*., 2012) because they are prized for their unique and beautiful shaped flowers with fascinated colors and intriguing long lasting flowers. The floral characteristics of orchids cover an exceptionally wide range of different shape, form, size, and coloration, persistence of blooms up to perfection and their ability to travel long distances uplifted them to one of the top ten cut flowers in the international market. As a result, they are exceedingly valued in floriculture trade as species or hybrids for potted plants or cut flowers (Martin and Madassery, 2006; Thomas and Michael, 2007). Today orchid growing is more than just an industry; it is an international business (Griesbach, 2002). The increase in popularity of orchids in Asia, Europe and the United States has led to continued increase in worldwide orchid production (Winkelmann *et al*., 2006). Also, with increasing demand for orchid cut flowers and potted plants, the need to generate new commercial cultivars is constantly expanding. The last decades witnessed release of several new cultivars and observed an increased demand for orchid cut flowers. Orchids occupy 8% share of the global floricultural trade (Martin and Madassery, 2006) and has the potential to alter the economic landscape of a country. At present orchids are a million dollar industry in several countries like Thailand, Australia, Singapore, Malaysia and several others (Chugh *et al*., 2009). Orchids are marketed both as cut flowers and potted plants. The largest

exporters of potted orchids are Taiwan, Thailand, United Kingdom, Italy, Japan, New Zealand and Brazil, respectively while the largest importer of potted orchids is the United States. Today orchids, such as *Cymbidium*, *Dendrobium*, *Oncidium* and *Phalaenopsis* are marketed globally and the orchid industry has contributed substantially to the economy of many ASEAN (Association of the South East Asian Nations) countries (Hew, 1994; Laws, 1995). Orchids have become the most valuable potted flowering crop in the United States with a reported wholesale value of $160 million in 2009, up 26 percent from the previous year. About 19.5 million potted orchids were sold in 2009 with a mean wholesale price of $8.20 per pot (U.S. Department of Agriculture, 2010). Thailand, the world's sixth largest exporter of cut flowers, earns 30 million US$ a year from orchid exports (Reddy, 2008). In 2010, Thai orchid export value was 86.3 million US$ accounting for 76% of all Thai floricultural products combined. Export of orchid flowers was 72.9 million US$ and 13.4 million US$ for orchid plants. Major markets were Japan, United States, China, Italy and the Netherlands (Lekawatana, 2012).

Several categories of orchids have been successful for use as blooming potted plant. *Dendrobium* orchid is one such category, with increasing popularity in the international floricultural scene. It is a highly evolved, diversified group and the second-largest orchid genus comprising of more than 1,000 natural species (Puchooa, 2004; Xu *et al*., 2006). While historically a cut flower commodity, *Dendrobiums* are increasingly being used as potted plants for interiorscaping of hotel and restaurants. For example, the use for decoration is in resort destinations or also increasingly visible in offices and home. Moreover, they are made excellent gifts for all occasions (Kuehnle, 2006). In several countries, such as Thailand where it is currently the world largest exporter of *Dendrobium* orchid (Lekawatana, 2012), China, Taiwan, The Phillipines, United States, Japan and Germany, potted *Dendrobium* orchids are produced in a mass scale. Furthermore, they are grown primarily as ornamentals and is valued as cut flowers because of various elegant colors of floriferous flowering, their high number of flowers per inflorescence and recurrent flowering (Martin and Madassery, 2006; Nambiar *et al*., 2012). These hybrids have the great variety of gorgeous flower sprays (Puchooa, 2004), their capability in blooming continuously and a prolonged post-harvest life relative to other orchid hybrids. Therefore, they

occupy a foremost position in floriculture trade, especially in ornamental cut flower industry (Kuehnle, 2006). Generally, *Dendrobiums* are reproduced asexually by division of offshoots, but the multiplication rate is very low with only 2-4 plants per year (Martin and Madassery, 2006; Nasiruddin *et al.*, 2003). In addition, vegetative propagation does not provide sufficient clones within short timeframe. Sexual propagation to produce complete plants through orchid seeds is difficult because their seeds are minute and have no endosperm. Consequently, they need symbiotic fungi in order to germinate (Anjum *et al.*, 2006; Thomas and Michael, 2007).

Both neither the processes of symbiotic germination nor the chronology of seedling development have been reliably established and the identity of the fungal partner(s) remains unclear (McKendrick *et al*., 2002). McKendrick *et al*. (2002) studied the results of an analysis of the symbiotic germination and development of *Neottia nidus-avis* (L) Rich, orchidaceous myco-heterotrophic plants (MHPs), and they found that identification of fungal symbionts was demonstrated in seed packets in which germination took place there was frequently observed a rhizomorph-forming fungus that varied from black-brown to bright orange-brown and was closely associated with the germinating seeds (Figure 1a, b, f). The involvement of this fungus in seed germination was unproven. Microscopic examination of seedlings showed that the fungus routinely infecting seeds and often found on the testa of uninfected seeds in which germination had reached the imbibition stage (1a) was fine (typically 1.25 µm wide), hyaline, with infrequent septa and no clamp connections. The hyphal walls of the fine fungus varied from being hyaline to having a brick-red pigmentation. The fungus formed simple multistranded rhizomorphs in which the hyphae occasionally carried clear stellate crystals (possibly calcium oxalate) on their surfaces. A fungus with somewhat coarser hyaline hyphae (typically 2.5 µm wide) was also occasionally observed infecting the plants and in close association with the seed and seed coats (McKendrick *et al*., 2002).

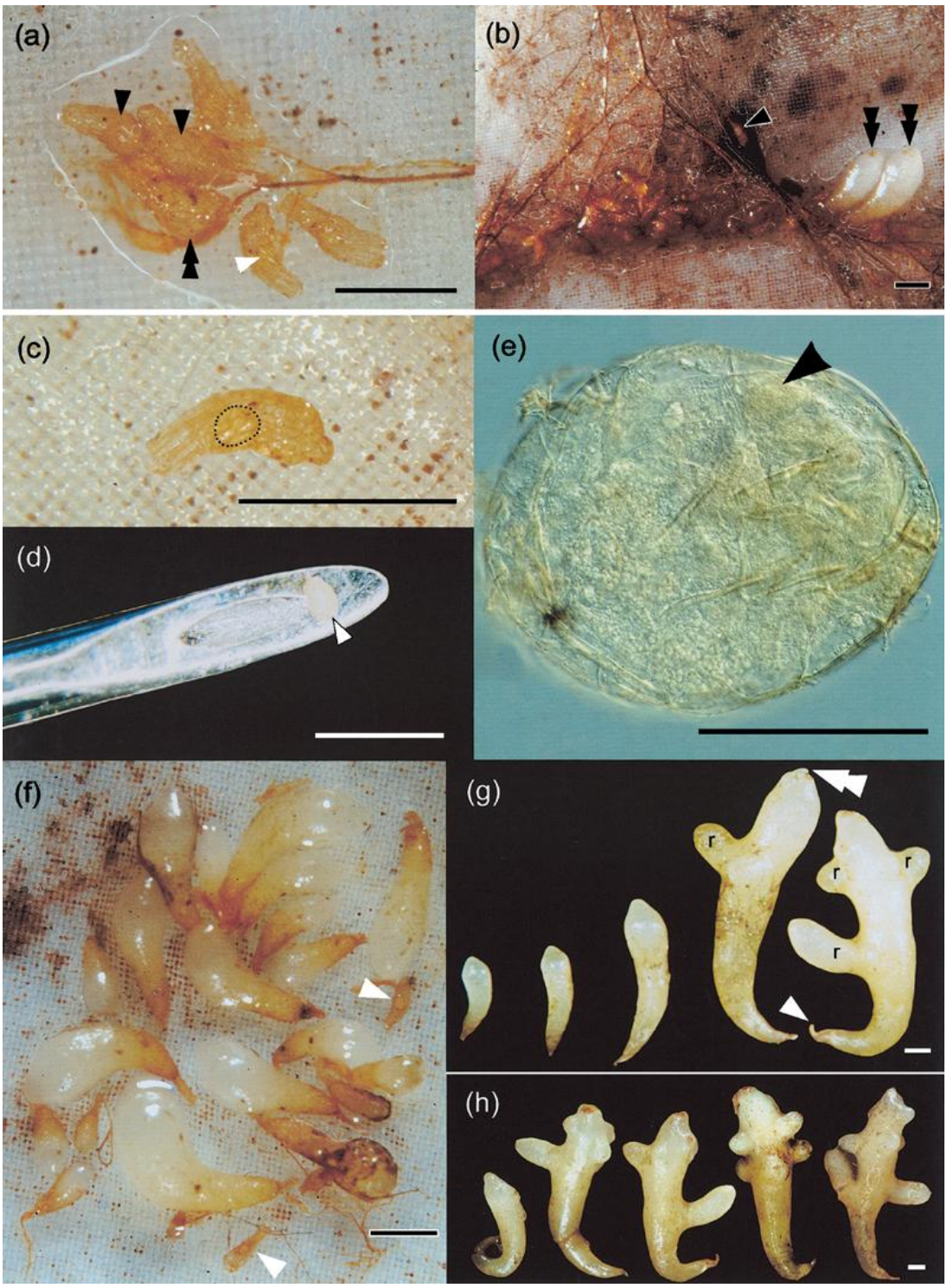


Figure 1 Stages in development of *Neottia nidus-avis*.

(a) Seeds in various stages of germination accompanied by orange-brown rhizomorph-forming fungus: Stage 0, ungerminated seed (white arrow); Stage 1, swollen embryo colonized by fungus (single black arrow); Stage 2,

protocorm with ruptured testa (double black arrow). Bar = 1 mm. (b) A mesh packet with some seedlings at a more advanced stage of development and accompanied by a brown rhizomorph-forming fungus: Stage 1, swollen embryo colonized by fungus (single black arrow); Stage 2, protocorms that have fully ruptured their testa and have developed a well-defined apical meristem (double black arrows). Bar = 1 mm. (c) Detail of ungerminated seed showing the unexpanded embryo (circled by dotted black line) fully enclosed in the testa. Bar = 1 mm. (d) Germinated seed (Stage 1a, expanded seedling before fungal infection) on a dissecting needle following removal of the testa. Bar = 1 mm. (e) Detail of a germinated seedling (Stage 1c) with testa removed. Fungal pelotons have formed, one of which is indicated (black arrow). Bar = 0.25 mm. (f) Seedlings in various stages of development in a single packet. Development ranges from ungerminated seed (Stage 0, white arrows) to large Stage 2 seedlings. Note accompanying brown rhizomorph-forming fungus. Bar = 1 mm. (g) Progressive development of seedlings in a single packet ranging from Stage 2 (left side, unbranched seedlings) to Stage 3 seedlings with many rootlets (r) forming. The translucence of the apical meristem of the stage 2 seedlings results from the cells lacking both pelotons and starch accumulation in this region. Note the development of a shoot bud with a rudimentary scale-leaf on the largest seedling (double arrow). The testa of the original seeds are visible (single arrow shows one example). Bar = 1 mm. (h) Representative examples of seedlings at Stage 3, showing early development of the characteristic short rootlets that grow at right angles to the main axis and form the ‘bird’s nest’ appearance from which the plant derives its name. Bar = 1 mm.

The light microscope observations suggested that germination (Stage 1a, increased translucence of seed, attributed to mobilization of stored carbon compounds) was initiated before fungal penetration. In many of these cases the distinctive fine and hyaline fungus with simple septa was seen on the testa of the seeds. This initial stage of germination occurred only in the plots in which seeds subsequently became infected. Since the plots containing adults appeared otherwise

identical to those from which they were absent, it appears that the breaking of seed dormancy requires the fungal partner. The failure of the light microscope studies to detect fungal infection of seeds at this stage leads to the hypothesis that germination may be initiated by a chemical signal from the fungus. The hypothesized mechanism, which requires further investigation, would enable *Neottia nidus-avis* seeds to remain dormant for long periods of time before germination is initiated by its essential fungal partner(s) growing close to the seed (McKendrick *et al*., 2002). In another fully myco-heterotrophic orchid, *Corallorhiza trifida*, it was found that breaking of seed dormancy, again recognized by increased translucence of seeds, occurred only after infection of the suspensor region by a specific fungal partner (McKendrick *et al*., 2000). Growth in length and breadth of seedlings of *Corallorhiza trifida* then swiftly followed even before fungal pelotons developed in the adjacent cells. In *Neottia nidus-avis* the breaking of dormancy (Stage 1a) and the initial infection before peloton formation (Stage 1b) led initially to very modest increases in the seed dimensions. The main growth of *Neottia nidus-avis* occurred only following peloton formation. Previous studies on another terrestrial orchid by Yoder *et al*. (2000) have shown that infection of seeds and the development of pelotons results in greatly increased water content, and this, together with expansion of cell volumes and vacuolation (Rasmussen, 1990), may account for much of the rapid increase in seedling volumes once fungal infection is established. The fungus-dependent initiation of germination seen in *Corallorhiza trifida* and *Neottia nidus-avis* contrasts with the situation described in some common initially myco-heterotrophic orchids such as *Dactylorhiza majalis* (Rchb. F.) in which the initial stages of germination can occur in the absence of symbiotic fungi, even to the point of extensive cell division and the formation of rhizoids (Rasmussen, 1995). These differences may reflect adaptations to the contrasted patterns of specificity and distribution of the fungi colonising these orchids. *Neottia nidus-avis* shows a remarkable ability to control the growth of its fungal symbiont. Infection commences with penetration of the cells at the base (micropylar end) of the embryo by which it was attached to the parent plant. From here it spreads into adjacent cells in which the first pelotons are formed. It is not known whether the attachment cells provide a chemical attractant to the fungus or if the walls of these cells are the most easily penetrated by fungi. Infection later spreads towards the

chalazal end of the embryo but does not enter the meristem, which continues to develop, eventually forming a shoot primordium (Figure 1g, h). In the larger seedlings there are distinct regions of peloton-containing cells extending from the base of the seedling to a region below the apical meristem (McKendrick *et al*., 2002). McKendrick *et al*. (2002) hypothesize that the *Sebacina dimitica*-like fungus infecting *Neottia nidus-avis* at Galley Down may obtain its carbon primarily through mycorrhizal associations with *Fagus sylvatica* L. (the only autotrophic plant consistently present in the vicinity of *Neottia nidus-avis* at the site.

However, seed are usually germinated *in vitro* on a simple medium containing minerals and sugars (Knudson, 1946). Besides the seedling progenies are heterozygous, and do not warrant true-to-type plants of hybrid cultivars. Conventional *Dendrobium* hybrid breeding could take up to 5 years (Fadelah, 2006) that is time consuming. Orchid propagation involves pollination, seedpod maturation, protocorm development, *in vitro* growth of seedlings and subsequent *in vivo* establishment of seedlings. This entire cycle could be between 3 and 5 years depending on the genotypes involved (Kamemoto *et al*., 1999). Thus, to keep in pace with the increasing demand, methods for rapid *in vitro* propagation of orchids and several *in vitro* cultural protocols have been developed in this genus (Arditti and Ernst, 1993; Chugh *et al.*, 2009). Micropropagation of orchids has for many years played an important role as a means for rapid propagation of commercially valuable cultivars progressed well. Obviously, the development of protocols for rapid and large scale clonal multiplication of highly priced cultivars of *Dendrobium* has considerable commercial value (Martin and Madassery, 2006).

Dwarf *Dendrobium* (*Dendrobium* Queen Pink x *Dendrobium* Phakbung), one of the tropical *Dendrobium* hybrids, is a sympodial epiphytic orchid. It is a species with small pseudobulbs and white flowers with purple lip (Figure 2). Also, its flowers have longevity and blooms many times a year. It is generally used as a flowering-potted for ornamental plants. Nowadays more and more, it has potential commercial value and is used as gifts, souvenirs, or decorations at official or social functions. However, in many commercially important *Dendrobium* hybrids have juvenile periods range at least 2–5 years from seedlings to reach maturity and flowering stage (Fadelah, 2006; Hee *et al*., 2007; Sim *et al*., 2007) that delay in

flowering. Then it is a major problem in the propagation and breeding of them (Wang *et al*., 2009). Orchid breeders usually take a few years to grow the thousands of seedlings from each seedpod to maturity before flower quality can be evaluated (Sim *et al*., 2007). Juvenility (vegetative growth) refers to the early phase of plant growth during which flowering does not occur normally under natural conditions (Hew and Yong, 1997). The period of vegetative growth from seed to flowering varies with species and hybrids (Sim *et al*., 2007). Therefore, in order to reduce the long juvenile phase, it is important to study *in vitro* flowering of *Dendrobium*s. *In vitro* systems are used to study the physiological and molecular basis of the orchid flowering process, to hasten flowering in slow-maturing orchids and to improve the control of forcing and flower-timing (Mudalige and Kuehnle, 2004). Protocols to induce early *in vitro* flowering have been developed in several *Dendrobium* orchids (Ferreira *et al*., 2006; Sim *et al*., 2007; Wang *et al*., 1997) and could shorten the time required for flowering, which could be used to get an early indication of floral characteristics (Hee *et al*., 2007). Furthermore, shortening of juvenile phase can provide a model system for studying flowering initiation and development that might facilitate the elucidation of the flowering mechanism. The advance of an early *in vitro* flowering method will have significant impact on the orchid industry, so there is a need to develop a method to speed up the flowering process of *Dendrobium* in order to be competitive in the ever growing orchid industry (Nambiar *et al*., 2012). In addition, such miniaturized orchid plantlets with flowers have potential commercial value as gifts or decoration.

Figure 2 Flowers of Dwarf *Dendrobium* (*Dendrobium* Queen Pink x *Dendrobium* Phakbung)

## 1.2 Literature review

### 1.2.1 Protocormlike body (PLB) induction

The production of orchid seedlings from seeds involves three successive phases: germination, formation of protocorms, and seedling development (Mitra *et al.*, 1976). The development of protocorms from germinated seed and the subsequent induction of PLBs, from different tissues as explants has become a reliable method for breeding orchids. Propagation by formation of PLBs is a preferred option because of the large number of PLBs that can be obtained within a short period of time. The need for mass propagation has led to the development of *in vitro* methods

such as the propagation of large-scale PLBs using shoot tips (Malabadi *et al.*, 2005; Sheela *et al.*, 2004), leaf segments (Martin and Madassery, 2006; Park *et al.*, 2002a), protocorms (Sheelavanthmath *et al.*, 2005; Teng *et al.*, 2004), flower stalks (Chen and Chang, 2000; Chen *et al.*, 2002), stem segments (Luo *et al.*, 2008) and root tips (Manners *et al.*, 2010; Park *et al.*, 2003). PLBs can proliferate rapidly and can readily regenerate into complete plantlets, so they are also the most general target tissue for genetic transformation studies in orchids (Liau *et al.*, 2003; Sreeramanan *et al.*, 2008). Moreover, PLBs are well-differentiated tissues that are sometimes regarded as orchid embryos that can develop two distinct bipolar structures, namely, the shoot and root meristem. Thus, these structures are able to convert to plantlets easily when grown on plant growth regulator-free medium (Ng and Saleh, 2011). The induction and proliferation of PLBs from various explants in orchids was investigated by the role of plant growth regulators (PGRs), for example, thidiazuron (TDZ: N-phenyl-N'-1,2,3-thidiazol-5-ylurea) and benzyladenine (Martin and Madassery, 2006; Mohanty *et al*., 2012; Roy *et al.*, 2007). However, along with changing of plant materials, response of PGRs is greatly changed. Thus, functions of the exogenous PGRs are quite different from orchid species to species (Naing *et al*., 2011).

TDZ, a phenylurea derivative and a non-purine cytokinin compound, is not catabolized via cytokinin oxidase (Hare and Van, 1994; Kishor and Devi, 2009). Cytokinin oxidase have been believed to be a copper-containing amine oxidase (Burch and Horgan, 1989; Hare and Van, 1994) and is the key enzyme of cytokinin degradation in plants resulting in formation of adenine-type compounds and corresponding isopentenyl aldehyde (Galuszka *et al*., 2001). It catalyzes the oxidation of cytokinins bearing unsaturated isoprenoid side chains, using molecular oxygen as the oxidant. Cytokinin oxidase acts by removing the $N^6$-substituted isoprene chain of cytokinins or their ribonucleosides to produce active cytokinins consist of an adenine (ade) and the corresponding aldehyde (Armstrong, 1994; Hare and Van, 1994). Previously, TDZ was reported to be effective in the regeneration of a number of orchid species, such as *Doritaenopsis* (Ernst, 1994), *Phalaenopsis* (Chen and Piluek, 1995), *Cymbidium* (Chang and Chang, 1998; Nayak *et al.*, 1997), *Oncidium* (Chen *et al.*, 1999; Chen and Chang, 2001), and *Dendrobium* (Roy *et al.*, 2007).

BA ($N^6$-benzyladenine) is an adenine-type cytokinin. The beneficial effect of BA has also been described for the regeneration of several orchids, including the genera *Oncidium* (Kalimuthu *et al.*, 2007), *Geodorum* (Sheelavantmath *et al.*, 2000), *Vanda* (Decruse *et al.*, 2003), *Dendrobium* (Martin and Madassery, 2006; Nayak *et al.*, 2002), *Vanilla* (Geetha and Shetty, 2000), and *Cymbidium* (Paek and Yeung, 1991; Taixeira da Silva and Tanaka, 2006).

### 1.2.2 Phenomenon of flowering

Angiosperms pass through three phases in their life cycle: juvenile (non-competent) e.g. germination, vegetative adult (competent but not induced), i.e. vegetative growth, and reproductive (competent and induced), such as flowering and fertilization (Poethig, 1990; Simpson *et al.*, 1999). The duration of each phase varies from a very short period to a very long one that can last for many years (Scorza, 1982). The switch from juvenility to vegetative adult phase is controlled by endogenous systems (Simpson *et al.*, 1999). Physiologically, the change from juvenile to adult plant is expressed as a change from non-competency to competency (Poethig, 1990). Competent meristems are responsive to environmental or autonomous signals that eventually lead to flower formation.

Flowering is considered as a complex mechanism, is meaningful in developmental and physiological science and is sensitive to the environment. (Nandagopal, 2006; Nujeen and Te-chato, 2007; Vu *et al*., 2006) which is determined by complex interactions between both external cues like light (photoperiodism and intensity), temperature (vernalization and thermoperiodism) and nutrient availability and internal factors that have been identified using extensive molecular genetic analyses (Isabel and Caroline, 2006; Wu *et al*., 2008). These different internal factors as well as environmental factors are known to participate in the various steps that lead to flower formation (Tanimoto *et al*., 1985). The reproductive stage, a unique phenological phenomenon, is one of the crucial events and is vital for the completion of the life cycle in angiosperm plants and seed production (Ziv and Naor, 2006). The timing of the transition from vegetative growth to flowering is of paramount importance in agriculture, horticulture, and plant breeding (Corbesier and Coupland, 2006) because flowering is the first step of sexual reproduction (Bernier *et al*., 1993).

Flower formation involves the switch from vegetative stage to reproductive stage of growth and it is known to be under the control of a so-called “switch-on” mechanism. Basically, this mechanism is governed by ensembles of flowering time and meristem identity genes with a complex hierarchy (Nilsson and Weigel, 1997; Simon *et al*., 1996). This process involves the transition of vegetative shoot apex to form either an inflorescence or a floral meristem, and followed by initiation and subsequent maturation of the floral organs (Sim *et al*., 2007) and is expressed by changes in the morphology and developmental physiology of the bud meristem (Simpson *et al*., 1999). The nature of the signal that induces flowering, however, remains unclear (Corbesier and Coupland, 2006). Notwithstanding the considerable intensive efforts have been expanded on the elucidation of flower initiation spanning several decades, knowledge obtained in this aspect so far is still very limited (Bernier, 1988; Bernier *et al*., 1993; Macha kova and Krekule, 2002). A restriction of flowering is juvenility. Many perennial plants must pass through a juvenile phase of vegetative development before they reach the adult phase and are able to flower (Hopkins, 1999). For instance, the large family, the Orchidaceae, usually have long juvenile periods - up to 13 years (Arditti, 1992), delayed transition to reproductive development and prolonged breeding programs**.** The shortening of juvenility, especially in commercial varieties and hybrids, is biotechnological interest. In addition, the entire flowering mechanism has not been completely understood because the flowering physiology is difficult to study using traditional methods (Zhang, 2007). Therefore, plant tissue culture techniques for flowering can be an important tool to achieve these goals because these systems could micropropagate vegetative shoots, then reduce juvenility, induce and develop flowering and their organs. They have been studied and manipulated for a large number of plants *in vitro.* The advances in cell and tissue culture techniques provide an excellent system for studying the physiological and molecular biology aspects of plant science, including flowering**.** Moreover, the advantage of tissue culture techniques is their ability to control environmental conditions as well as media components like nutrients, vitamins, promoting and retarding PGRs. Media components, growth conditions, and the source of explants affect the morphological response (Ziv and Naor, 2006).

### 1.2.3 *In vitro* flowering

*In vitro* flowering is known as flowering occurs in tissue cultures under *in vitro* conditions (Franklin *et al*., 2011) that can be manipulated and provide an alternative controlled system necessary to study flower induction, as well as inflorescence and flower morphogenesis (Ziv and Naor, 2006), and is considered a convenient means to study the switch-on flowering mechanism. It can be used to study either specific aspects of flowering or the whole mechanism of the reproductive process (Dewir *et al*., 2007) such as floral initiation, floral organ development, and floral senescence (Goh, 1992). Generally, flowering occurs more quickly under *in vitro* condition than the field condition in *Pisum sativum* L. (Franklin *et al*., 2000), *Dendrobium* Chao Praya Smile (Hee *et al*., 2007), *Vigna mungo* L. (Ignacimuthu *et al*., 1997), *Bambusa arundinacea* Willd and *Dendrocalamus brandisii* Kurz. (Nadgauda *et al*., 1990), *Arabidopsis thaliana* (L.) Heynh**.** (Ochatt and Sangwan 2008) and *Nicotiana plumbaginifolia* Viv. (Trinh *et al*., 1987), which would be extremely useful for studies related to flowering of plants that have a long juvenile period, such as orchids under natural conditions (Hee *et al*., 2007; Nadgauda *et al*., 1990). The ability of inducing orchids to flower *in vitro* greatly reduced the time required (from years to months) for reaching the maturity stage necessary for flowering. For examples, *in vitro* flowering of *Oncidium varicosum* Mericlones was observed after 8-9 months (Kerbauy, 1984), the *in vitro* flower of *Dendrobium candidum* Wall ex Lindl. was induced within 3-6 months (Wang *et al*., 1993), and *Cymbidium niveo-marginatum* Mak *in vitro* flowers were observed within 3 months (Kostenyuk *et al*., 1999).

Then, establishing a reliable procedure for precocious flowering would decrease the breeding period and the costs of the commercial production of plants. It also allows researchers to produce flowers year-round (Lin *et al*., 2003). However, flowers formed *in vitro* appear to be extremely tiny (miniature) which is a limitation of the pot plant industry but rather useful in the *in vitro* bouquet industry (Sudhakaran *et al*., 2006). Preceding reports indicated that reasons for studying flower formation *in vitro* can be summarized. First of all, to provide an ideal experimental system for studying the mechanism of phase transition to reproductive growth, floral induction and development as these experiments can be controlled and repeated (Silva and Tan

Nhut, 2003; Sudhakaran *et al*., 2006; Taylor and Staden, 2006). Secondly, to apply as a tool to accelerate breeding programs of distant varieties by synchronization of flowering (Zhang, 2007), or can be adjusted to commercial production of specific compounds from floral organs (Ziv and Naor, 2006). Thirdly, to provide important method for the study of molecular and genetic mechanisms of flower induction and for assisting breeding programs (Kostenyuk *et al*., 1999). Finally, to provide an early, synchronized and profuse flowering pot plants for commercial market through integration of *in vitro* flowering as a part of the large-scale micropropagation process (Dewir *et al*., 2006). *In vitro* techniques have opened new avenues of research into flowering, enabling the researchers to vary single parameters to measure the effects of each on flowering (Dewir *et al*., 2007).

Previously, incidences of *in vitro* flowering have been reported in many plant species, for example, *Bambusa edulis* (Odashima) Keng (Lin *et al*., 2003; 2004; 2005a), *Celosia argentea* var. *plumosa* 'Fresh Look Red' (Bodhipadma *et al*., 2011), *Cucumis sativus* L. (Kielkowska and Havey, 2011), *Dendrocalamus latiflorus* Munro. (Lin *et al*., 2007), *Dioscorea zingiberensis* C. H. Wright (Huang *et al*., 2009), *Fagopyrum esculentum* L. (Kachonpadungkitti *et al*., 2001), *Gypsophila paniculata* L. (Kanchanapoom *et al*., 2011), *Kniphofia leucocephala* Baijnath. (Taylor *et al*., 2005), *Panax ginseng* C.A. Meyer (Lin *et al*., 2005b), *Perilla frutescens* (L.) Britton (Zhang, 2007), *Phoenix dactylifera* L. (Masmoudi-Allouche *et al*., 2010), *Pisum sativum* L. (Franklin *et al*., 2000), *Rosa hybrida* L. (Vu *et al*., 2006; Wang *et al*., 2002; Kanchanapoom *et al*., 2009; 2010), *Sinningia speciosa* Hiern (Zhang *et al*., 2012), *Spathiphyllum* (Dewir *et al*., 2007), *Vitex negundo* L. (Gantait *et al*., 2012). Investigations of *in vitro* flower induction have been described in several orchid species and their hybrids, for instance, *Cymbidium ensifolium* (L.) Swartz var. *misericors* (Hayata) T. P. Lin (Chang and Chang, 2003), *Dendrobium friedericksianum* Rchb.f (Te-chato *et al*., 2009; Prasertsongsakul, 2007), *Dendrobium* Madame Thong-In (Sim *et al*., 2007; 2008), *Dendrobium nobile* Lindl. (Wang *et al*., 2009), *Doriella* (Duan and Yazawa, 1994a), *Oncidium varicosum* (Kerbauy, 1984), *Phalaenopsis* Cygnus 'Silky Moon' (Rojanawong *et al*., 2006), *Phalaenopsis* Pink Leopard 'Petra' (Duan and Yazawa, 1995). The developmental pattern and ability of explants to form flowers *in vitro* depend on numerous factors, such as PGRs (Campos

and Kerbauy, 2004; Hee *et al*., 2007), sucrose (Rojanawong *et al.*, 2006; Singh *et al*., 2006), photoperiod (Kachonpadungkitti *et al*., 2001; Vaz *et al*., 2004) and nutrient requirements (e.g. the concentration of nitrogen (N) and phosphorus (P) in the medium) (Kostenyuk *et al*., 1999; Tee *et al*., 2008) and any changes in one of their components might change the morphogenetic expression. These environmental cues can promote the synthesis of a floral stimulus that is transported through phloem to the shoot apical meristem (SAM) and subsequently induces flower initiation (Bernier and Perilleux, 2005).

Translocation via the phloem provides the most important long-distance transport pathway of the plant. Whilst the xylem tubes transport mainly mineral-containing water from the roots to the shoots, the phloem is responsible for the translocation of organic compounds from the sites of synthesis to the developing and non-photosynthetic tissues. The sieve elements (SEs)-companion cells (CCs) complex is the functional entity responsible for the longdistance phloem translocation not only of photoassimilates, amino acids, organic acids, secondary metabolites, ions, peptides and phytohormones, but also a large range of macromolecules, including proteins, small RNAs and microRNAs (miRs) (Dinant *et al*., 2010; Turgeon and Wolf, 2009). The transport of molecules from the CCs or adjacent parenchyma cells to the SEs takes place either through the apoplasm, based on a series of carriers and pumps, present on the plasma membrane of SEs and CCs, or through the pore-plasmodesma units at the CC-SE interface (Dinant and Lemoine, 2010). Most macromolecules present in the SEs are synthesized in the CCs (Turgeon and Wolf, 2009). The entry of macromolecules into the SE takes place via the plasmodesmata, which can be either selective or passive via diffusion in a size-dependent manner. The loading of metabolites can follow either symplasmic or apoplasmic routes. Interestingly, it has been proposed (Van Bel *et al*., 2011) that the role of the phloem, including production, release and distribution of signalling molecules, may also encompass modulation and amplification of signals along the long-distance transport conduit. The driving force for long-distance transport in the SE makes use of a turgor gradient due to variations in photosynthate accumulation along the conduit that create a hydraulic pressure gradient (Knoblauch and Peters, 2010). As long distance floral signal transport is now accepted as more complex than the movement of a single type

of signal molecule (Bernier and Perilleux, 2005), the endogenous compounds translocated within the phloem, along with their involvement in sink-strength regulation may be involved in regulation of the juvenile-to-adult and vegetative-to-reproductive phase transitions.

Cells at the SAM summit serve as stem cells that split continuously to displace daughter cells to the surrounding peripheral region, where they are incorporated into differentiating leaf or flower primordia. A balance between creation of new meristematic cells by division and departure of cells from the meristem by differentiation is required to maintain a functional SAM. (Weidong *et al*., 2000) Flower is also a kind of lateral organs, which is differentiated from the shoot apical. The development of flower includes a series of progressive processes (MA, 1994; Meyerowitz *et al*., 1991). The first step is flowering determination (or floral induction), in which the vegetative meristem (VM) can be affected by the environmental factors (e.g. photoperiod and low temperature) and endogenous signals. Once the signals come to SAM, the SAM switches to the inflorescence meristem (IM), and then forms the floral meristem (FM) (Datta and Das, 2002). The second step of flowering (flower evocation), there are not any visible changes in morphology, but the function of the SAM has been changed fundamentally. The last step of flowering is the formation and development of floral organs (floral organogenesis). The cells in the IM will differentiate into different flower organs. (Weidong *et al*., 2000). The floral organization in each fold or whorl is determined by a unique combination of three organ identity genes or ABC-class genes (ABC model) (Datta and Das, 2002). ABC-class genes act lonely or together to give rise to sepals (A), petals (A+B), stamens (B+C), and carpels (C) (Meesawat, 2005). The basic structure of a complete flower of angiosperm consists of four different floral organs, in particular, sepals (whorl 1), petals (whorl 2), stamens (whorl 3) and pistils (whorl 4) are initiated sequentially in the flanks of the FM to form the whorl of calyx, corolla, androecium and gynocium, respectively (Scott, 1993).

In these processes, the flowering determination is the base of others, and directly controls the flowering time. Three major theories attempt to explain the complex phenomena, the physiological and biochemical control of the transition to flowering.

1. The florigen/antflorigen concept (Evans, 1971; Lang, 1952). It suggests that flowering is induced by some substances; for example, floral promoter and inhibitor are each a simple, specific, and universal hormone (Lang, 1984) produced in leaf which could be transmissible from the "donor" to the "recipients". But the florigen has not been isolated and identified yet so far.

2. The nutrient diversion hypothesis. According to this model, it is proposed that the inductive treatments result in an increase in the amount of assimilates moving to the apical meristem, which in turn induces flowering (Bernier, 1988), whatever the nature of the involved environmental factors, is a means of modifying the source/sink relationships within the plant in such a way that the shoot apex receives a better supply of assimilates than under noninductive conditions. (Sachs and Hackett, 1983)

3. The multifactorial control model. It is proposed that a number of promoters and inhibitors, including phytohormones and assimilates, are involved in controlling the developmental transition. According to this model, flowering can only occur when the repressive and inductive factors are present at the apex in the appropriate concentrations and at the suitable time. The multifactorial control model has been supported by the successful cloning of a series of genes identified to promote flowering (Koornneef et al., 1998; Weller et al., 1997)

In spite of the generalization for various components, functions and activities, specific requirements must be studied separately for each given species or even cultivar (Rastogi and Sawheny, 1989).

#### 1.2.3.1 Plant growth regulators

The function of PGRs in regenerating flowers and floral organ development *in vitro* is discussed extensively in prior reviews (He and Loh, 2002; Nair *et al*., 2007; Naor *et al*., 2004; Taixeira da Silva, 2003). Indeed, different PGRs requirements have been associated with flowerings which are dependent on the physiological stage of the explant, particularly its endogenous hormone levels (Tanimoto *et al*., 1985). Preceding procedure showed correlations between PGRs concentration and morphological changes in relation to flowering (Corbesier *et al*.,

2003; Vu *et al*., 2006), and it seems that changes in PGRs ratios are necessary for the development of different floral whorls or organs. When an explant is a floral bud or organ it is clear that PGRs can affect organ morphogenesis and development of various flower organs. However, if the explant is a vegetative bud or tissue, PGRs can affect the flower induction phase, the floral organ development, or both. (Ziv and Naor, 2006). In orchids, control by PGRs of flowering has attracted attention because of the need in commercial orchid production to resolve problems of flowering periodicity (Hew and Clifford, 1993), so they play an important role as a mean to produce *in vitro* flowering. Several *in vitro* systems to stimulate flowering have been established together with types and levels of PGRs to enhance the efficiency of flower induction, but they were species-dependent (Huang *et al*., 2009). In this study, PGRs that affect *in vitro* flowering of dwarf *Dendrobium* are BA and paclobutrazol (PBZ).

BA have been implicated in many plant processes, including cell division (Zhang *et al*., 2005), organ formation and regeneration (Al-Ramamneh *et al*., 2006), axillary bud growth, lateral branching growth, apical dominance and floral development (Taylor *et al*., 2005). Cytokinins are believed to be the root-to-shoot signal that is responsible for the vegetative to floral transition (Bernier *et al*., 1993), and have been associated with floral induction and development, both qualitative and quantitative (Bernier *et al*., 1993; Dewitte *et al*., 1999; Havelange *et al*., 2000). Thus, they are considered as a critical physiological signal in triggering the process of flowering (Bernier *et al*., 1993; Bonhomme *et al*., 2000; Lejeunne *et al*., 1994).

The effects of BA on *in vitro* flowering were observed in many orchids. For example, *Dendrobium* Madame Thong-In (Sim *et al*., 2007) and *Dendrobium* Chao Praya Smile (Hee *et al*., 2007) protocorms were cultured on modified Knudson C medium (KC) (Knudson, 1946) two-layered (liquid over Gelrite-solidified) medium containing 22.2 µM BA and 11.1 µM BA respectively that was found to enhance inflorescence initiation and flower bud formation in 5-6 months from germination. In *Dendrobium friedericksianum* Rchb.f, plantlets were implanted on VW (Vacin and Went, 1949) medium supplemented with 10 mg/l BA, and were induced to flowering after 80 days of culture (Prasertsongsakul, 2007). Moreover, adventitious shoots of *Phalaenopsis* Pink Leopard ‘Petra’ were cultured on VW

medium containing 44 µM BA, and formed floral buds after 120 days of culture (Duan and Yazawa, 1995).

PBZ [(2RS, 3RS)-1-(4-chlorophenyl)-4, 4-dimethyl-2-(1H-1, 2, 4 triazol-1-yl) pentan-3-ol] is a plant growth retardant that has recently been applied in plant tissue culture with various effects. Generally, it reduces elongation of the internodes of higher plants *in vitro*. Similar results are also observed in reduction in leaf size, dark green color of leaves and thickening of root (Graebe, 1987). There have been few reports on the effect of growth retardants on plantlets grown *in vitro*, such as an inhibitory effect on shoot growth (Ozmen *et al*., 2003; Ziauka and Kuusiene, 2010). Furthermore, it has also been shown to enhance *in vitro* flowering (Pileuk *et al*., 1986; Wang *et al*., 2006; Zheng and Pang, 2006).

The induction of vegetative shoot apical meristem to inflorescence meristem was studied when *Dendrobium friedericksianum* Rchb.f shoots were cultured on MS (Murashige and Skoog, 1962) supplemented with 0.05 mg/l PBZ, and promoted floral bud induction after 3 months of culture (Te-chato *et al*., 2009). Seedlings of *Dendrobium nobile* Lindl. produced floral buds precociously on a defined basal medium (½ MS) containing 0.5 mg/l PBZ within 4 months of culture (Wang *et al*., 2009). In *Saposhnikovia divaricata* (Turcz.) Schischk., PBZ was also shown to form *in vitro* flowering which embryogenic calluses were cultured on MS medium supplemented with 1.02 µM PBZ , and time to flowering was 40 days (Qiao *et al*., 2009).

#### 1.2.3.2 Sucrose

Basically, sucrose is known to be the carbon source of necessary choice for *in vitro* flowering (Rastogi and Sawhney, 1986; Scorza, 1982). Previous studies showed that flower development could be modulated by sugar, as exogenous sugars delay senescence in many species of cut flowers (Van Doorn, 2004). High and low concentrations of sucrose in the growth medium have been described to affect floral transition, with seemingly opposite effects (Zhang *et al*., 2012). In *Spathiphyllum cannifolium* cv. Sunny Sails, sucrose concentration at 3 or 6 % induced inflorescence development in 83-85 % of the plantlets, respectively. High sucrose

concentrations at 9 and 12 % reduced the inflorescence development to 0-10 % (Dewir *et al*., 2007). Saccharides (sucrose, glucose, maltose, lactose, raffinose) are supplemented in culture media for reliable induction and development of flowers in different species, sucrose being the most commonly used (Sudhakaran *et al*., 2006; Taylor and van Staden 2006).

In some other reports, the role of sucrose on *in vitro* flowering is also studied. For instance, regenerated *Gypsophila paniculata* L. shoots could be induced to flower efficiently when cultured on MS medium containing 13.3 µM BA and 50 mg/l sucrose after 8 weeks of culture (Kanchanapoom *et al*., 2011). *Phalaenopsis* Cygnus 'Silky Moon' seedlings were precultured for 4 months on modified VW medium containing 30 g/l sucrose, then transferred onto flower bud initiation medium supplemented with 66.6 µM BA and flower buds were induced after culturing for 40-120 days (Rojanawong *et al.*, 2006). Shoots of *Rosa hybrida* (hybrid tea) cv. 'First Prize' were induced to flower *in vitro* on MS medium containing 45 g/l sucrose without PGRs after 3 months of culture (Vu *et al*., 2006). Sepals of *Sinningia speciosa* were cultured on a modified MS medium supplemented with 0.1 mg/l BAP and 20 g/l sucrose that floral buds were formed directly from explants without prior formation of shoots and leaves (Zhang *et al*., 2012).

#### 1.2.3.3 Photoperiod

The duration of daylight changes with a predictable pattern along the year, providing a reliable environmental signal for the varying seasons. The ability to sense and respond to changes in day length is known as photoperiodism. Photoperiodic flowering, which refers to a plant's ability to promote flowering by measuring the changes in day length, is regulated by the internal circadian clock and external signals (Hayama and Coupland, 2004; Thomas and Vince-Prue, 1997; Yanovsky and Kay, 2003). In many plants, the time of flowering is strongly influenced by photoperiod, which synchronizes the floral transition with the favorable season of the year, so it is important in commercial production to know how the flower initiation and development are affected by photoperiod (Islam *et al*., 2005). Garner and Allard (1920) showed the use of controlled photoperiodic conditions for

the first time that day length can determine the time of flowering, being the first photoperiodism phenomenon documented. Plants have been classified differently into three photoperiodic groups according to their flowering response to day length. In long day (LD) plants flowering is promoted by daily periods of light longer than a critical day length; whereas, plants that accelerate flowering in response to day length below a critical threshold are called short day (SD) plants. Day-neutral (DN) plants flower at the same time irrespectively of the photoperiodic conditions (Jarillo *et al*., 2008). The photoperiodic effects on orchid flowerings have been reported and the number of species or hybrids examined, such as *Cattleya, Cymbidium, Dendrobium, Paphiopedilum* and *Phalaenopsis*. Mostly, orchids which respond to SD or LD photoperiods are temperate in origin. Many tropical species flower at various times throughout the year. They are DN plants (Goh, 1992).

Over the years, physiological and biochemical studies have led to three models for explaining the regulation of flowering time. The first is the concept of a universal flowering hormone-like substance, which was first postulated by Mikhail Chailakhyan (Chailakhyan, 1936a, b, 1937). The florigen (from Latin, flora, “flower”; and Greek, “genesis”) concept was based on the transmissibility of floral inductive signals across grafts between reproductive donor stems and juvenile recipients in *Nicotiana tabacum* plants. It was proposed that florigen was synthesized in leaves under inductive daylength conditions and transported to stimulate the initiation of flowering in the SAM via the phloem. The detection of a graft transmissible floral antagonist also led to the theory of a competing "antflorigen" (Thomas and Vince-Prue 1997). Many years of research were spent researching for the florigen and antflorigen molecules in the phloem exudates of several plant species, but their molecular character has remained elusive until some recent advances. The difficulty to dichotomize the hypothetical floral hormone-like substance from phloem-transported assimilates reformed to a second model, the nutrient diversion hypothesis (Thomas and Vince-Prue 1997). This model suggested that floral-promotive conditions result in an increase in the amount of photosynthates translocating to the SAM, which in turn promotes floral induction. The hypothesis that photosynthate translocation is uniquely important in the promotion of floral initiation was displaced by the multifactorial control hypothesis (Bernier and Perilleux 2005). This model

proposed that everal inducers and repressors, including plant hormones and photosynthates, systematize the vegetative-to-reproductive phase transition. According to the multifactorial control hypothesis, floral initiation can only be triggered when the limiting determinants are present at the SAM, at the right dose and time. Furthermore, this model attempted to systematize the diversity of floral responses by suggesting that different genetic, biochemical and physiological factors could be limiting for floral induction in different genotypes and/or under diverse abiotic and biotic conditions (Matsoukas *et al*., 2012)

The florigen should have the following traits. First of all, it is produced in leaves and transmitted to the SAM, where the florigen triggers flowering. Secondly, the florigen can transmit in all directions and different distances. Thirdly, the substance is the same as or similar to each other in structure in different species. Finally**,** the florigen is produced under special conditions, so it is different from other metabolized substances (Bernier *et al*., 1993).

The effects of photoperiod on *in vitro* flowering in embryogenic cultures of *Citrus nobilis* Lour ×*Citrus deliciosa* Tenora was observed that 12-h photoperiod was most effective for flowering on MS medium containing 2 mg/l KN with 40 g/l sucrose after 4 weeks of culture (Singh *et al*., 2006). Both long day (16-h photoperiod) and short day (8-h photoperiod) conditions induced flowers of *Fagopyrum esculentum* L. from node segments when cultures were maintained on ½ MS medium containing 0.1 µM kinetin after 12 weeks of culture (Kachonpadungkitti *et al*., 2001). In *Psygmorchis pusilla*, a positive relationship was observed between long days and floral spike formation, significantly increasing floral spike number under longer day. Both 12 and 16-h photoperiods resulted in perfectly opened flowers. The flower buds that had been kept in darkness did not open. However, plant incubation under 20-h photoperiod or longer days negatively affected floral bud development, inhibiting anthesis and reducing flower longevity (Vaz *et al*., 2004). Length of photoperiod significantly influenced *in vitro* flowering of *Vitex negundo* L. When the micro-plants were cultured on MS medium pH 5.7 at 25 ± 1ºC temperature, 7 mg/l $GA_3$ and among the other photoperiodic conditions (from 8/16 h to 20/4 h). The 12/12 h photoperiod gave the highest frequency of flowering, all the resultant flowers were normal, and showed flowering within 18 days (Gantait *et al*., 2012).

#### 1.2.3.4 Nutrients

While the PGRs, sucrose and photoperiod signaling pathways have been subjected to extensive analysis, little is known about how nutrients regulate floral induction. Tissue culture media contain mineral and sugar sources in addition to PGRs and other organic constituents. It was found that *in vitro* flowering was also influenced by the levels and ratios of the two major components, carbohydrates (C) and minerals. High concentrations of N in media promoted vegetative growth but inhibited flowering, which competed more efficiently for C from the medium (Dielen *et al*., 2001). According to the floral nutrient diversion hypothesis, C and N are known as C/N ratios increase in buds during flower induction (Sachs, 1977). Thus, reducing nitrogen level enhanced *in vitro* flowering in orchids, such as *Cymbidium niveo-marginatum* Mak (Kostenyuk *et al*., 1999), *Dendrobium* Sonia 17 (Tee *et al*., 2008), *Doritis* (Duan and Yazawa, 1994a), *Phalaenopsis* Cygnus 'Silky Moon' (Rojanawong *et al.*, 2006), *Phalaenopsis* Pink Leopard 'Petra' (Duan and Yazawa, 1995). Besides, the effect of high P contents in the medium was found to stimulate *in vitro* flowering in *Cymbidium niveo-marginatum* Mak (Kostenyuk *et al*., 1999), *Dendrobium* Sonia 17 (Tee *et al*., 2008), *Doriella*, *Phalaenopsis* and *Dendrobium* (Duan and Yazawa, 1994b).

Induction of early *in vitro* flowering of *Cymbidium niveo-marginatum* Mak was investigated when shoots in the presence of BA in the MS medium which was reduced to 1/20 of N and increased to 5 of P within 90 days (Kostenyuk *et al*., 1999). Flowering buds of *xDoriella* Tiny were observed on low N media supplemented with 14.4 μM BA after 80 days. The optimal N concentration was 6-9 mM and a high ratio of $NH_4^+$/ $NO_3^-$ was beneficial (Duan and Yazawa, 1994a). When the high phosphorous was used in concentration corresponding to 1.25X of the full strength MS medium and the low nitrogen content was reduced to 0.25X, the high P/low N medium was effective to induce inflorescences of *Dendrobium* Sonia 17 plantlets after 3 months (Tee *et al*., 2008). In *Phalaenopsis* Pink Leopard 'Petra', the lowest tested concentration of N (4.5 mM) in VW medium was the most effective for formation of floral buds after 4 months of culture (Duan and Yazawa, 1995). Moreover, P supply provided favorable conditions for *in vitro* flowering of

*Cymbidium ensifoium* (L.) Swartz var. *misericors* (Hayata) T. P. Lin (Chang and Chang, 2003).

## 1.3 Objectives of the research

1.3.1 To study the effects of PGRs on the induction and proliferation of PLBs from protocorms of dwarf *Dendrobium*.

1.3.2. To study the effects of PGRs, sucrose, photoperiod, nutrients and a two-layered (liquid over agar-solidified) medium on *in vitro* flowering of dwarf *Dendrobium*

# CHAPTER 2
# MATERIALS AND METHODS

## 2.1 Investigation of PLB induction and proliferation of dwarf *Dendrobium*

### 2.1.1 Plant materials

Healthy plants of dwarf *Dendrobium* (*Dendrobium* Queen Pink x *Dendrobium* Phakbung) were planted in pots and grown under the greenhouse at Department of Biology, Prince of Songkla University, Hat Yai, Songkhla, Thailand. Flowers were hand pollinated and a pair of bipartite pollinia was picked and deposited on the stigma of a flower during pollination. The pollinated plants were maintained in the greenhouse. The 3-month-old mature and well-developed seedpods were collected for seed germination experiments.

### 2.1.2 Culture medium and conditions

MS medium containing 3 % (w/v) sucrose, 8.2 g/l agar, and 15 % (v/v) coconut water (CW) was used throughout the experiments as the basal medium. The pH of the medium was adjusted to 5.5 prior to autoclaving at 121ºC at 1 kPa for 20 min. All cultures were incubated at 25±1°C under a 16/8 (day/night) photoperiod provided with white fluorescent tubes at an intensity of 1,960 lux.

### 2.1.3 Seed culture

Seeds of mature seedpods were cultured immediately after collection. Seed pods were surface disinfected by immersion in 95 % ethanol and flamed, then they were dissected longitudinally and the seeds were cultured on MS medium.

### 2.1.4 Influence of TDZ and BA on the induction and proliferation of PLBs

The 2-month-old green protocorms were then used for induction of PLBs by transferring to 40 ml of the MS liquid medium supplemented with different concentrations of TDZ (4.5, 9, 13.5, 18 or 22.5 μM) and BA (4.4, 8.8, 13.2, 17.6 or 22 μM) in 100 ml Erlenmeyer flasks on a rotary shaker at 120 rpm for proliferation and multiplication.

### 2.1.5 Plantlet regeneration and transplantation

PLBs were transferred to the MS medium for plantlet regeneration. Plantlets with three or four well-developed roots were taken out from the culture medium and washed thoroughly under running tap water to remove agar. They were acclimatized in pots containing wetted coconut husk. After hardening, the transplanted plantlets were grown in the greenhouse.

### 2.1.6 Experimental design and data analysis

Five protocorms were implanted per flask and subcultures of these protocorms were carried out every 3 weeks. Experiments were carried out in a completely randomized design and repeated twice with each treatment having 25 replicates. After 9 weeks of culture, explants were evaluated in terms of the percentage of PLBs formed and the number of PLBs per explant. The data were analyzed statistically using one-way analysis of variance (ANOVA). The mean values were compared using Duncan's multiple range test at $P \leq 0.05$.

## 2.2 Investigation of *in vitro* flowering of dwarf *Dendrobium*

### 2.2.1 Plant materials

Three or four leaf stage plantlets without roots of dwarf *Dendrobium* were maintained on MS basal medium, and were used as source of explants for this experiment.

### 2.2.2 Culture media and conditions

MS medium containing 3 % (w/v) sucrose and 8.2 g/l agar was used throughout the experiments as the basal medium. The pH of the medium was adjusted to 5.5 prior to autoclaving at 121°C at 1 kPa for 20 min. All cultures were incubated at 25±1°C under white fluorescent tubes at an intensity of 1,960 lux. To examine the effect of photoperiod, three light/dark cycles i.e. 8/16, 12/12 and 16/8 were used in monitoring flowering *in vitro*. The explants were carried out in glass-capped culture jars (8 oz capacity) each containing 25 ml of medium.

### 2.2.3 Influence of BA and sucrose on *in vitro* flowering

To test the effect of BA and sucrose on *in vitro* flowering, the explants were aseptically cultured on MS medium containing 0, 4.4, 8.8, 13.2, 17.6 or 22 μM BA in combination with different sucrose concentrations at 0, 30, 60 or 90 mg/l.

### 2.2.4 Influence of PBZ on *in vitro* flowering

To investigate the effect of PBZ on *in vitro* flowering, the explants were cultured on MS medium supplemented with 0, 0.017, 0.034, 0.051, 0.068, 0.085, 0.170, 0.255 or 0.340 μM PBZ.

### 2.2.5 Influence of low nitrogen (N) and high phosphorous (P) concentration in medium (NP medium) on *in vitro* flowering

The changing total N concentration was reduced to 7X of the full strength MS medium (8.6 mM N) and total P concentration was increased to 3X of the full strength MS medium (3.75 mM P) and supplemented with 22 μM BA. Thus, the culture system was coded as $N_7P_3$ accordingly (Table 1).

Table 1 NP medium on *in vitro* flowering

| Media | N (mM) | P (mM) | BA (μM) |
|---|---|---|---|
| Control (MS) | 60.01 | 1.25 | 0 |
| $N_7P_3$ | 8.6 | 3.75 | 0 |
| $N_7P_5$ | 8.6 | 6.25 | 0 |
| $N_{20}P_3$ | 3 | 3.75 | 0 |
| $N_{20}P_5$ | 3 | 6.25 | 0 |
| $N_{25}P_3$ | 2.4 | 3.75 | 0 |
| $N_{25}P_5$ | 2.4 | 6.25 | 0 |
| $N_7P_3$ | 8.6 | 3.75 | 22 |
| $N_7P_5$ | 8.6 | 6.25 | 22 |
| $N_{20}P_3$ | 3 | 3.75 | 22 |
| $N_{20}P_5$ | 3 | 6.25 | 22 |
| $N_{25}P_3$ | 2.4 | 3.75 | 22 |
| $N_{25}P_5$ | 2.4 | 6.25 | 22 |

### 2.2.6 Influence of a two-layered medium on *in vitro* flowering

Explants were cultured on a two-layered medium that consisted of 50 ml of 8.2 g/L agar solidified MS medium containing 22 μM BA and 15 % (v/v) CW (BACW medium), or without 15 % (v/v) CW (BA medium) overlaid with 20 ml of liquid medium of the same composition. Thus, the culture system was coded accordingly (Table 2).

Table 2 Two-layered MS medium on *in vitro* flowering

| Media | Liquid layer | | Agar-solidified layer | |
|---|---|---|---|---|
| | BA (μM) | CW (%) | BA (μM) | CW (%) |
| Control | 0 | 0 | 0 | 0 |
| CW | 0 | 15 | 0 | 15 |
| BA | 22 | 0 | 22 | 0 |
| BA+CW | 22 | 15 | 22 | 15 |

### 2.2.7 Experimental design and data analysis

One explant was planted per jar and experiments were carried out in a completely randomized design and repeated twice with each treatment having 20 replicates. After culture for 4 months, explants were evaluated in terms of the percentage of floral buds formed and the number of floral buds per explant. The data were analyzed statistically using one-way analysis of variance (ANOVA). The mean values were compared using Duncan's multiple range test at $P \leq 0.0$.

# CHAPTER 3
# RESULTS

## 3.1 Investigation of PLB induction and plant regeneration of dwarf *Dendrobium*

### 3.1.1 Effects of TDZ and BA on the induction and proliferation of PLBs

Seed germination occurred after 2 weeks of culture. The germinating seeds increased in size, became swollen, and later turned green. Seeds grew into protocorms after 2 months (Figure 3A). Subsequently, the protocorms developed into PLBs after 6 weeks of culture on the PLB induction media. Formation of PLBs occurred directly on the surface of protocorms in the growth regulator medium. The efficiency of protocorms producing PLBs was dependent on the types and concentrations of cytokinins in the medium. The highest percentage of forming PLBs (86.4 %) and the highest number of PLBs per explant (3.6) were found in modified MS liquid medium supplemented with 18 μM TDZ after 9 weeks of culture (Figure 3B, Table 3). Of the various concentrations of BA tested, the best response was recorded for the medium containing 4.4 μM BA, when 56 % produced PLBs with an average of 0.9 PLBs per explant. TDZ was more effective than BA in inducing PLBs. Direct formation of PLBs was observed from protocorms without the intermediate formation of callus.

### 3.1.2 Plantlet regeneration and transplantation

PLBs were transferred to the basal MS medium upon which they developed into shoots and roots in 6 weeks (Figure 3C). The regenerated plantlets were then potted to wetted coconut husk and kept in greenhouse (Figure 3D). They grew well and developed into normal plants after 12 weeks of transplantation.

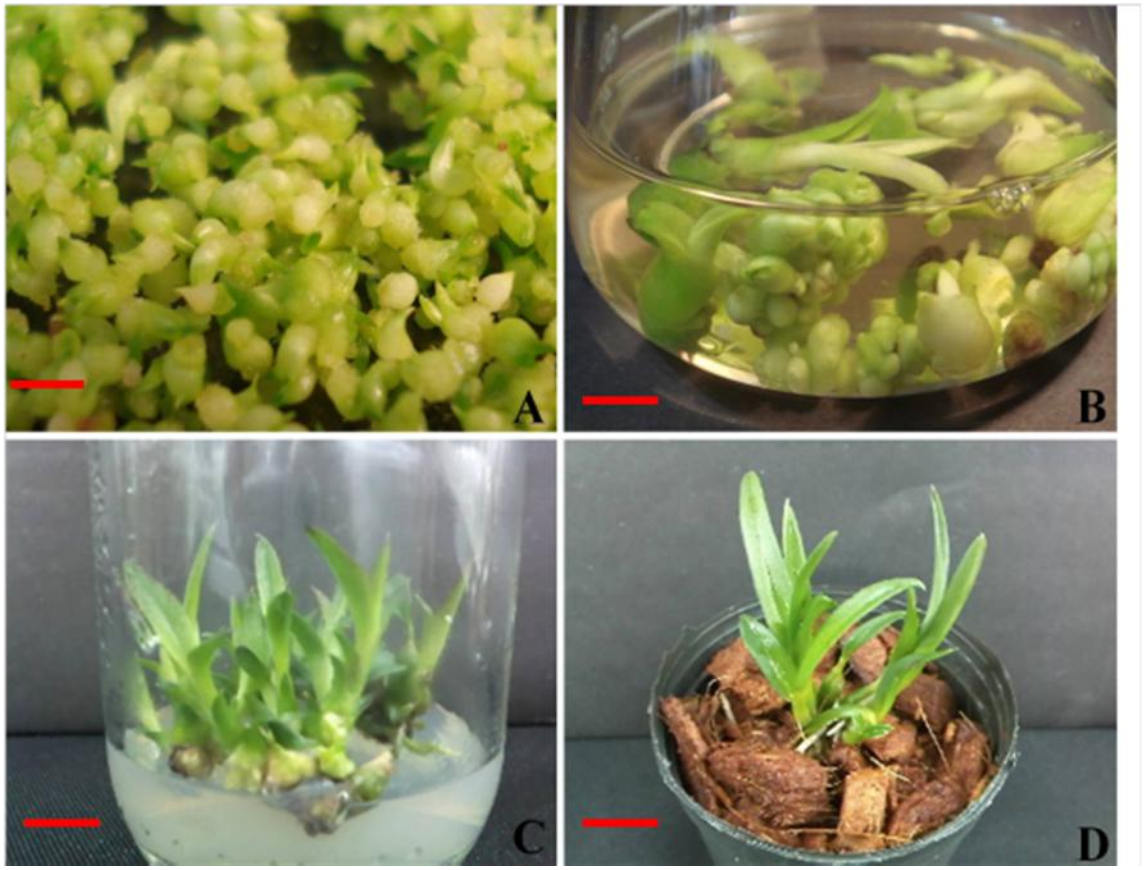


Figure 3 PLB induction and plant regeneration of dwarf *Dendrobium*

(A) 2-month-old green protocorms Bar = 1 cm. (B) PLBs development from protocorms after 9 weeks of culture in MS liquid medium with 18 μM TDZ Bar = 2 cm. (C) Plantlets developed on MS medium Bar = 1 cm.

(D) Acclimatized plants Bar = 3 cm.

Table 3 Effects of TDZ and BA on the induction of PLBs from protocorms of dwarf *Dendrobium* after 9 weeks of culture

| Cytokinin concentration (μM) | PLB formation (%) (Mean ± SE)* | Number of PLBs per explant (Mean ± SE)* |
|---|---|---|
| Control | 14.4± 5.2$^{e}$ | 0.2± 0.1$^{c}$ |
| TDZ | | |
| 4.5 | 43.2 ± 8.1$^{bcd}$ | 0.6 ± 0.2$^{bc}$ |
| 9.0 | 30.4 ± 7.3$^{de}$ | 0.3 ± 0.1$^{c}$ |
| 13.5 | 32.0 ± 6.8$^{cde}$ | 0.4 ± 0.1$^{bc}$ |
| 18 | 86.4 ± 6.1$^{a}$ | 3.6 ± 0.5$^{a}$ |
| 22.5 | 25.6 ± 8.0$^{de}$ | 1.5 ± 0.8$^{b}$ |
| BA | | |
| 4.4 | 56.0 ± 8.5$^{b}$ | 0.9 ± 0.2$^{bc}$ |
| 8.8 | 41.6 ± 8.6$^{bcd}$ | 0.8 ± 0.3$^{bc}$ |
| 13.2 | 36.0 ± 8.5$^{bcde}$ | 0.5 ± 0.1$^{bc}$ |
| 17.6 | 54.4 ± 8.8$^{bc}$ | 0.9 ± 0.2$^{bc}$ |
| 22 | 25.6 ± 6.2$^{de}$ | 0.3 ± 0.1$^{c}$ |

*Means within a column followed by the same letter are not significantly different by Duncan's multiple range test ($P \leq 0.05$).

## 3.2 Investigation of *in vitro* flowering of dwarf *Dendrobium*

### 3.2.1 Effects of combination of BA and sucrose on *in vitro* flowering

Different concentrations of BA, sucrose, and duration of photoperiods were tested to find out their optimum level for *in vitro* flowering of dwarf *Dendrobium.* Results obtained revealed that 22 μM BA and 30 g/l sucrose at 16-h photoperiod gave the highest percentage of floral buds (64.29 %) although the number of flowers (0.79) per explant was produced after 4 months of culture (Figure 4, Table 4). Whereas 57.14 % plantlets formed inflorescences with the best average of 1.14 flowers per explant in the medium containing 22 μM BA and 30 g/l sucrose at 12-h photoperiod (Figure 5, Table 4). However, these floral buds did not proceed to open and withered soon after they appeared. Plantlets grown in the absence of BA or sucrose added singly or in combination on basal medium containing 4.4 or 8.8 μM BA supplemented with all concentrations of sucrose did not produce floral buds at three photoperiods tested.

Table 4 Effects of different combinations of BA, sucrose, and photoperiod on *in vitro* flowering of dwarf *Dendrobium* after 4 months of culture

| BA (μM) | Sucrose (g/l) | Photoperiod (light/dark) | Floral buds formation (%) | Number of inflorescences per explant (Mean±SE)* |
|---|---|---|---|---|
| 0 | 0 | 8/16 | 0 | 0±0 [c] |
| | | 12/12 | 0 | 0±0 [c] |
| | | 16/8 | 0 | 0±0 [c] |
| 0 | 30 | 8/16 | 0 | 0±0 [c] |
| | | 12/12 | 0 | 0±0 [c] |
| | | 16/8 | 0 | 0±0 [c] |
| 0 | 60 | 8/16 | 0 | 0±0 [c] |
| | | 12/12 | 0 | 0±0 [c] |
| | | 16/8 | 0 | 0±0 [c] |
| 0 | 90 | 8/16 | 0 | 0±0 [c] |
| | | 12/12 | 0 | 0±0 [c] |
| | | 16/8 | 0 | 0±0 [c] |
| 4.4 | 30 | 8/16 | 0 | 0±0 [c] |
| | | 12/12 | 0 | 0±0 [c] |
| | | 16/8 | 0 | 0±0 [c] |
| 4.4 | 60 | 8/16 | 0 | 0±0 [c] |
| | | 12/12 | 0 | 0±0 [c] |
| | | 16/8 | 0 | 0±0 [c] |
| 4.4 | 90 | 8/16 | 0 | 0±0 [c] |
| | | 12/12 | 0 | 0±0 [c] |
| | | 16/8 | 0 | 0±0 [c] |
| 8.8 | 30 | 8/16 | 0 | 0±0 [c] |
| | | 12/12 | 0 | 0±0 [c] |
| | | 16/8 | 0 | 0±0 [c] |

| | | | | |
|---|---|---|---|---|
| 8.8 | 60 | 8/16 | 0 | 0±0 [c] |
| | | 12/12 | 0 | 0±0 [c] |
| | | 16/8 | 0 | 0±0 [c] |
| 8.8 | 90 | 8/16 | 0 | 0±0 [c] |
| | | 12/12 | 0 | 0±0 [c] |
| | | 16/8 | 0 | 0±0 [c] |
| 13.2 | 30 | 8/16 | 0 | 0±0 [c] |
| | | 12/12 | 0 | 0±0 [c] |
| | | 16/8 | 0 | 0±0 [c] |
| 13.2 | 60 | 8/16 | 0 | 0±0 [c] |
| | | 12/12 | 0 | 0±0 [c] |
| | | 16/8 | 0 | 0±0 [c] |
| 13.2 | 90 | 8/16 | 14.29 | 0.14±0.14 [bc] |
| | | 12/12 | 0 | 0±0 [c] |
| | | 16/8 | 0 | 0±0 [c] |
| 17.6 | 30 | 8/16 | 0 | 0±0 [c] |
| | | 12/12 | 0 | 0±0 [c] |
| | | 16/8 | 0 | 0±0 [c] |
| 17.6 | 60 | 8/16 | 28.57 | 0.57±0.43 [abc] |
| | | 12/12 | 42.86 | 0.43±0.20 [bc] |
| | | 16/8 | 28.57 | 0.29±0.18 [bc] |
| 17.6 | 90 | 8/16 | 28.57 | 0.29±0.18 [bc] |
| | | 12/12 | 14.29 | 0.29±0.29 [bc] |
| | | 16/8 | 28.57 | 0.57±0.43 [abc] |
| 22 | 30 | 8/16 | 0 | 0±0 [c] |
| | | 12/12 | 57.14 | 1.14±0.55 [a] |
| | | 16/8 | 64.29 | 0.79±0.15 [ab] |
| 22 | 60 | 8/16 | 42.86 | 1.14±0.40 [a] |
| | | 12/12 | 57.14 | 0.71±0.29 [abc] |
| | | 16/8 | 50 | 0.29±0.18 [bc] |

| | | | | |
|---|---|---|---|---|
| 22 | 90 | 8/16 | 14.29 | $0.57\pm0.57^{abc}$ |
| | | 12/12 | 0 | $0\pm0^{c}$ |
| | | 16/8 | 0 | $0\pm0^{c}$ |

*Means within a column followed by the same letter are not significantly different by Duncan's multiple range test ($P\leq0.05$).

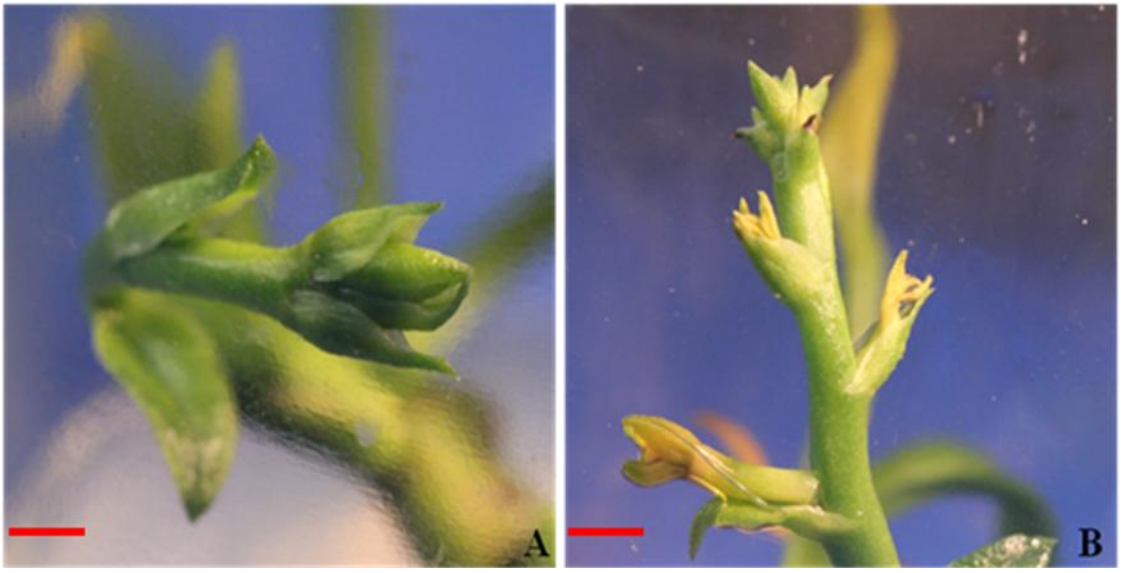


Figure 4 Plantlets with inflorescence stalks cultured on MS medium containing 22 μM BA under 16-h photoperiod after 4 months of culture

(A) Young inflorescence emerged from explants Bar = 1 cm. (B) Several flowers in one inflorescence Bar = 1 cm.

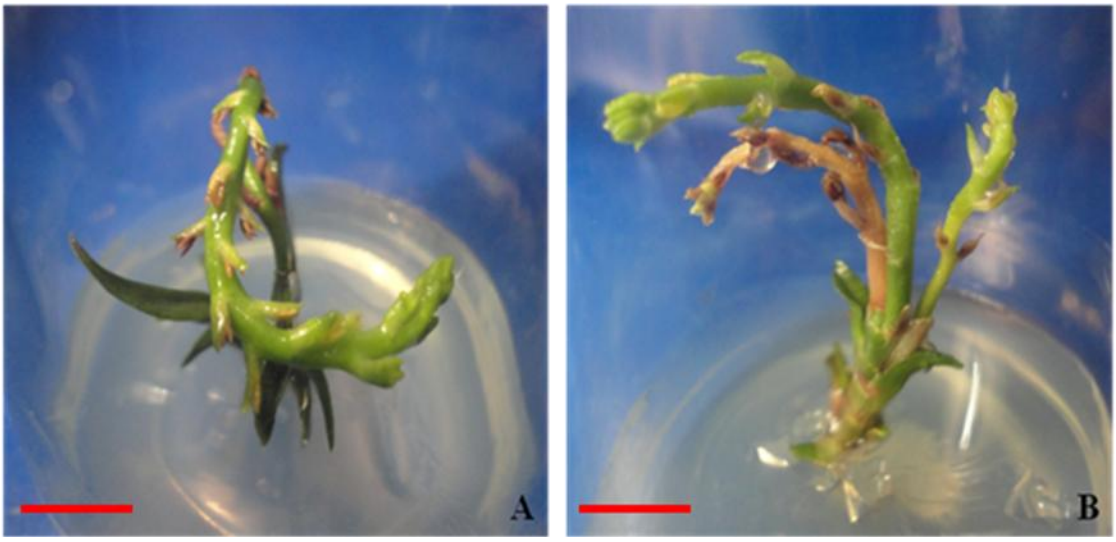


Figure 5 Plantlets with inflorescence stalks cultured on MS medium containing 22 μM BA under 12-h photoperiod after 4 months of culture
(A) One inflorescence per explant Bar = 1 cm. (B) Several inflorescences per explant Bar = 1 cm.

### 3.2.2 Effect of PBZ on *in vitro* flowering

Only 0.085 μM PBZ under 16-h photoperiod promoted the most effective floral bud induction of dwarf *Dendrobium* that showed maximum percentage (20 %) of cultures producing floral buds and number of flowers (0.4) per explant after 4 months of culture (Table 5). These floral buds proceeded to open flowers within 2-3 weeks. The flowers were normal in morphology comprised of three sepals, two petals and one lip or labellum, pollinia and female organ (Figure 6). However, such flowers were undersized with a diameter of 1 cm compared to their *in vivo* counterparts, and there was difference in their coloration that was green.

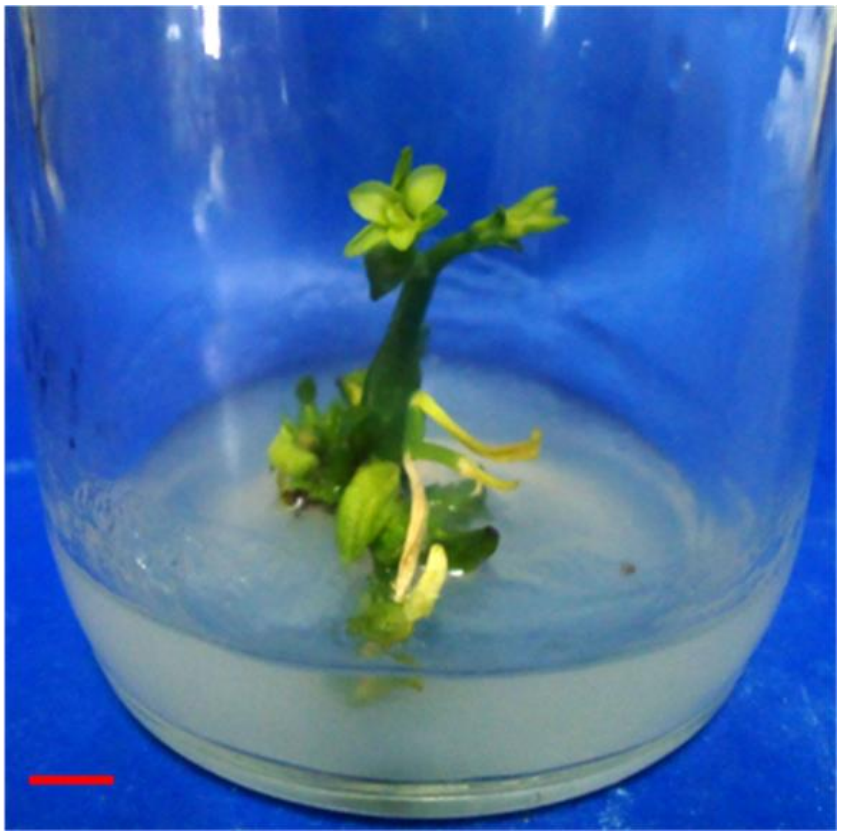

Figure 6 *In vitro* flowering of dwarf *Dendrobium* on MS medium containing 0.085µM PBZ with two normal flowers blooming after 2-3 weeks of culture Bar = 1cm.

Table 5 Effects of PBZ and photoperiod on *in vitro* flowering of dwarf *Dendrobium* after 4 months of culture

| PBZ (μM) | Photoperiod (light/dark) | Floral buds formation (%) | Number of inflorescences per explant (Mean±SE)* |
|---|---|---|---|
| 0 | 8/16 | 0 | 0±0 [b] |
| | 12/12 | 0 | 0±0 [b] |
| | 16/8 | 0 | 0±0 [b] |
| 0.017 | 8/16 | 0 | 0±0 [b] |
| | 12/12 | 0 | 0±0 [b] |
| | 16/8 | 0 | 0±0 [b] |
| 0.034 | 8/16 | 0 | 0±0 [b] |
| | 12/12 | 0 | 0±0 [b] |
| | 16/8 | 0 | 0±0 [b] |
| 0.051 | 8/16 | 0 | 0±0 [b] |
| | 12/12 | 0 | 0±0 [b] |
| | 16/8 | 0 | 0±0 [b] |
| 0.068 | 8/16 | 0 | 0±0 [b] |
| | 12/12 | 0 | 0±0 [b] |
| | 16/8 | 0 | 0±0 [b] |
| 0.085 | 8/16 | 0 | 0±0 [b] |
| | 12/12 | 0 | 0±0 [b] |
| | 16/8 | 20 | 0.4±0.4 [a] |
| 0.255 | 8/16 | 0 | 0±0 [b] |
| | 12/12 | 0 | 0±0 [b] |
| | 16/8 | 0 | 0±0 [b] |
| 0.340 | 8/16 | 0 | 0±0 [b] |
| | 12/12 | 0 | 0±0 [b] |
| | 16/8 | 0 | 0±0 [b] |

*Means within a column followed by the same letter are not significantly different by Duncan's multiple range test ($P \leq 0.05$).

### 3.2.3 Effects of low nitrogen (N) and high phosphorous (P) concentrations on *in vitro* flowering

*In vitro* flowering of dwarf *Dendrobium* was unable to induce in the control and modified medium with low N and high P contents (NP treatment) without BA during the whole experimental period (4 months). Both treatments did not affect *in vitro* flowering formation. The NP treatment combined with BA affected the induction of floral buds (10-44.44 %) after 4 months of culture (Table 6). $N_7P_5$ medium (8.6 mM N and 6.25 mM P) at 16-h photoperiod gave the highest number of flowers (1.4) per explant while floral buds formation (30 %) (Figure 7A) was low compared to $N_{25}P_5$ medium (2.4 mM N and 6.25 mM P) at 16-h photoperiod resulted in maximum floral buds (44.44 %) even though the number of flowers (0.56) per explant (Figure 7B). The floral buds formed in both media could not develop to flowers and proceeded to open further. Within 2-3 weeks, all of these floral buds would wilt and turn brown.

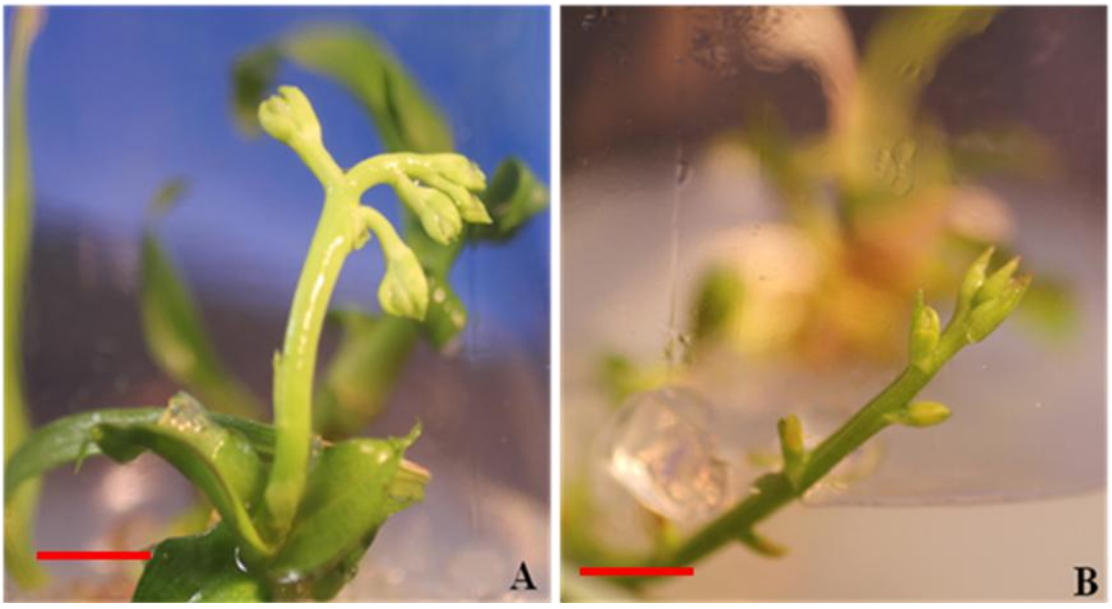


Figure 7 A plantlet with an inflorescence stalk cultured on NP treatment containing 22 µM BA under 16-h photoperiod after 4 months of culture
(A) $N_7P_5$ medium Bar = 1 cm. (B) $N_{25}P_5$ medium Bar = 1 cm.

Table 6 Effects of total nitrogen and phosphorus concentration and photoperiod on *in vitro* flowering of dwarf *Dendrobium* after 4 months of culture

| Media | N (mM) | P (mM) | BA (μM) | Photoperiod (light/dark) | Floral buds formation (%) | Number ofinflorescences per explant (Mean±SE)* |
|---|---|---|---|---|---|---|
| Control (MS) | 60.01 | 1.25 | 0 | 8/16 | 0 | 0±0 $^{c}$ |
| | | | | 12/12 | 0 | 0±0 $^{c}$ |
| | | | | 16/8 | 0 | 0±0 $^{c}$ |
| $N_7P_3$ | 8.6 | 3.75 | 0 | 8/16 | 0 | 0±0 $^{c}$ |
| | | | | 12/12 | 0 | 0±0 $^{c}$ |
| | | | | 16/8 | 0 | 0±0 $^{c}$ |
| $N_7P_5$ | 8.6 | 6.25 | 0 | 8/16 | 0 | 0±0 $^{c}$ |
| | | | | 12/12 | 0 | 0±0 $^{c}$ |
| | | | | 16/8 | 0 | 0±0 $^{c}$ |
| $N_{20}P_3$ | 3 | 3.75 | 0 | 8/16 | 0 | 0±0 $^{c}$ |
| | | | | 12/12 | 0 | 0±0 $^{c}$ |
| | | | | 16/8 | 0 | 0±0 $^{c}$ |
| $N_{20}P_5$ | 3 | 6.25 | 0 | 8/16 | 0 | 0±0 $^{c}$ |
| | | | | 12/12 | 0 | 0±0 $^{c}$ |
| | | | | 16/8 | 0 | 0±0 $^{c}$ |
| $N_{25}P_3$ | 2.4 | 3.75 | 0 | 8/16 | 0 | 0±0 $^{c}$ |
| | | | | 12/12 | 0 | 0±0 $^{c}$ |
| | | | | 16/8 | 0 | 0±0 $^{c}$ |
| $N_{25}P_5$ | 2.4 | 6.25 | 0 | 8/16 | 0 | 0±0 $^{c}$ |
| | | | | 12/12 | 0 | 0±0 $^{c}$ |
| | | | | 16/8 | 0 | 0±0 $^{c}$ |

| | | | | | | |
|---|---|---|---|---|---|---|
| $N_7P_3$ | 8.6 | 3.75 | 22 | 8/16 | 0 | 0±0 [c] |
| | | | | 12/12 | 20 | 0.2±0.2 [c] |
| | | | | 16/8 | 20 | 0.1±0.1 [c] |
| $N_7P_5$ | 8.6 | 6.25 | 22 | 8/16 | 0 | 0±0 [c] |
| | | | | 12/12 | 0 | 0±0 [c] |
| | | | | 16/8 | 30 | 1.4±0.88 [a] |
| $N_{20}P_3$ | 3 | 3.75 | 22 | 8/16 | 0 | 0±0 [c] |
| | | | | 12/12 | 0 | 0±0 [c] |
| | | | | 16/8 | 40 | 1.2±0.53 [ab] |
| $N_{20}P_5$ | 3 | 6.25 | 22 | 8/16 | 0 | 0±0 [c] |
| | | | | 12/12 | 20 | 0.4±0.4 [c] |
| | | | | 16/8 | 40 | 0.5±0.22 [bc] |
| $N_{25}P_3$ | 2.4 | 3.75 | 22 | 8/16 | 20 | 0.2±0.2 [c] |
| | | | | 12/12 | 0 | 0±0 [c] |
| | | | | 16/8 | 10 | 0.2±0.2 [c] |
| $N_{25}P_5$ | 2.4 | 6.25 | 22 | 8/16 | 0 | 0±0 [c] |
| | | | | 12/12 | 20 | 0.2±0.2 [c] |
| | | | | 16/8 | 44.44 | 0.56±0.24 [bc] |

*Means within a column followed by the same letter are not significantly different by Duncan's multiple range test ($P \leq 0.05$).

### 3.2.4 Effect of a two-layered MS medium on *in vitro* flowering

In the control or CW added singly in two-layered medium without BA did not promote any flowering formation. The efficiency of plantlets producing floral buds was found in two-layered MS medium containing 22 μM BA under 16-h photoperiod that exhibited the best response for number of flowers (3.4) per explant but resulted in 54.55 % floral bud formation after 3 months of culture (Figure 8, Table 7). 3 % out of these induce floral buds from plantlets and bloomed within 2-3 months after floral buds had produced, while the remaining floral buds withered or stopped development. Despite the small size of *in vitro* flowers as compared to the field flowers, their development was normal. The flowers were yellow in color with a diameter of 1.3 cm and composed of three sepals, two petals and one lip or labellum, pollinia and female organ (Figure 9). Although the highest floral bud induction (80 %) was shown in medium supplemented with 22 μM BA under 12-h photoperiod, the number of flowers (1.8) per explant was reduced (Figure 10) and these floral buds did not proceed to open and withered soon after they appeared.

Table 7 Effects of two-layered MS medium combined with BA, CW and photoperiod on *in vitro* flowering of dwarf *Dendrobium* after 3 months of culture

| Media | Photoperiod (light/dark) | Floral buds formation (%) | Number ofinflorescences per explant (Mean±SE)* |
|---|---|---|---|
| Control | 8/16 | 0 | 0±0 [c] |
| | 12/12 | 0 | 0±0 [c] |
| | 16/8 | 0 | 0±0 [c] |
| CW | 8/16 | 0 | 0±0 [c] |
| | 12/12 | 0 | 0±0 [c] |
| | 16/8 | 0 | 0±0 [c] |
| BA | 8/16 | 80 | 1.6±0.5 [b] |
| | 12/12 | 80 | 1.8±0.6 [b] |
| | 16/8 | 54.55 | 3.4±0.5 [a] |
| BA+CW | 8/16 | 40 | 0.6±0.4 [c] |
| | 12/12 | 80 | 1.4±0.5 [b] |
| | 16/8 | 30 | 0.4±0.2 [c] |

*Means within a column followed by the same letter are not significantly different by Duncan's multiple range test ($P \leq 0.05$).


Figure 8 Plantlets with inflorescence stalks in two-layered medium containing 22 μM BA under 16-h photoperiod after 3 months of culture

(A) One inflorescence per shoot with 5 florets Bar = 1.5 cm. (B) Several florets per inflorescence Bar = 1.5 cm. (C) Two inflorescences with high rate of floral bud induction Bar = 2 cm. (D) Floral buds withered or stopped development Bar = 2 cm.

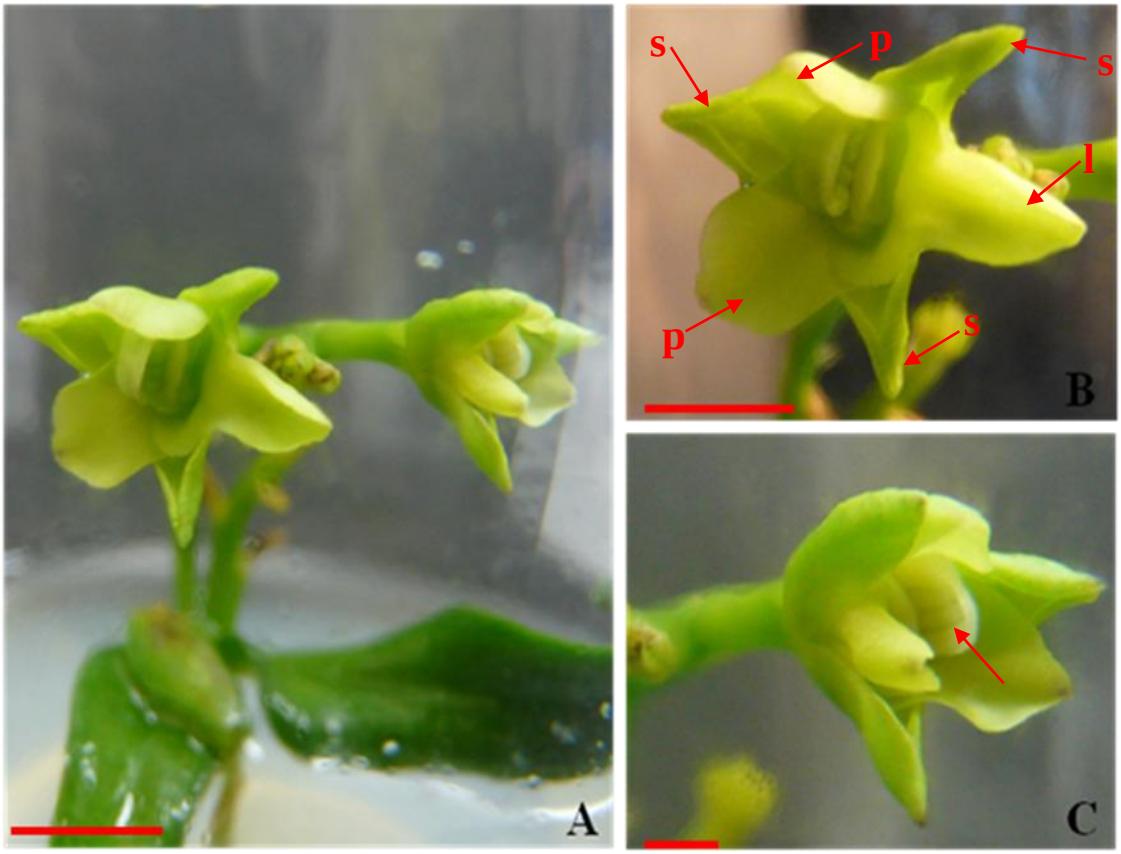


Figure 9 *In vitro* flowering of dwarf *Dendrobium* in two-layered medium supplemented with 22 μM BA under 16-h photoperiod after 3 months of culture

(A) An inflorescence with two opened flowers Bar = 10 mm.

(B) The complete flowers composed of three sepals (s), two petals (p) and one lip (l) (arrows) Bar = 5 mm. (C) Pollinia (arrow) Bar = 1 cm.

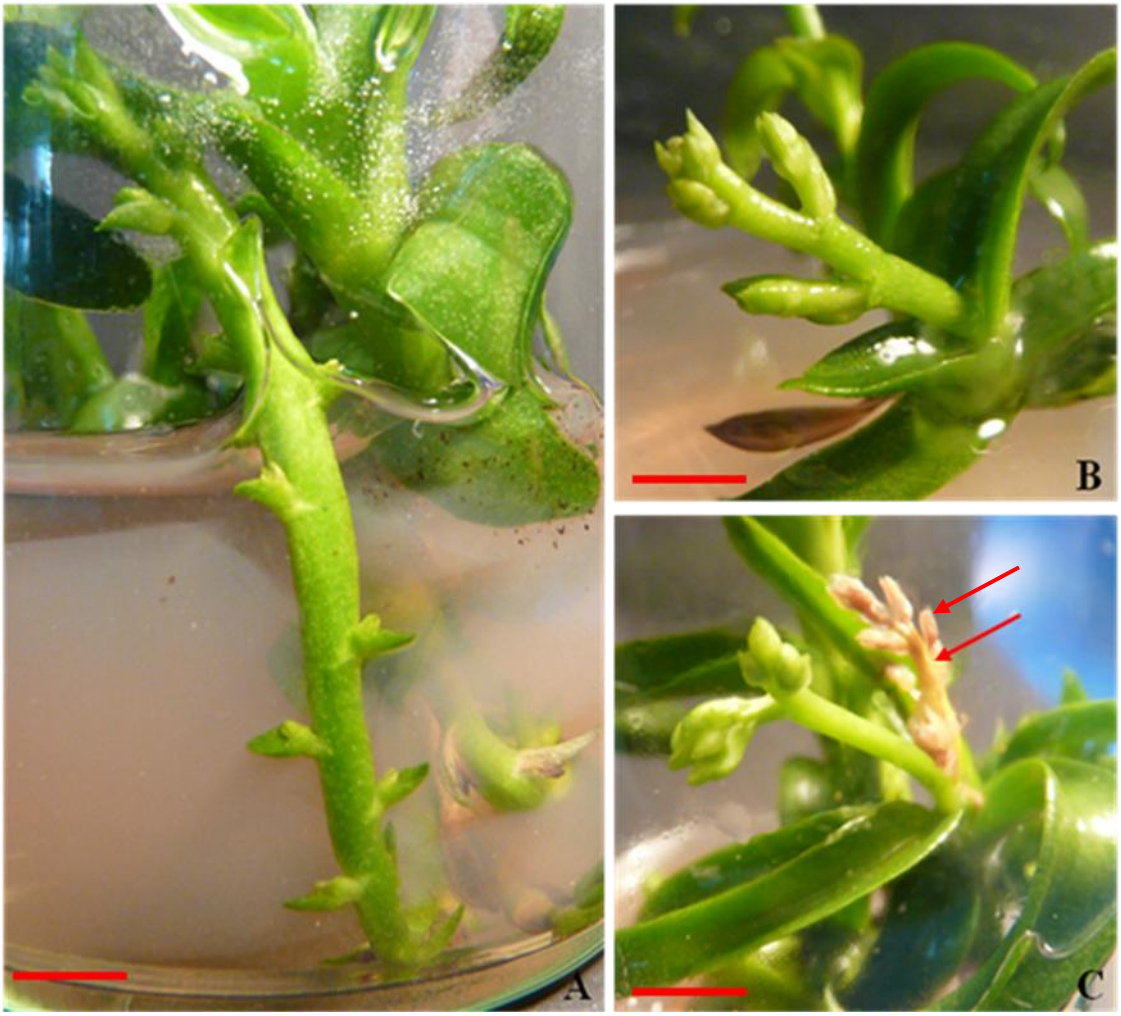


Figure 10 Plantlets produced inflorescence stalks in two-layered medium supplemented with 22 μM BA under 12-h photoperiod after 3 months of culture

(A) High frequency of floral buds per inflorescence Bar = 1.5 cm. (B) Four florets per inflorescence Bar = 2 cm. (C) The withered inflorescence (arrow) Bar = 2 cm.

# CHAPTER 4
# DISCUSSION

## 4.1 PLB induction and plant regeneration of dwarf *Dendrobium*

The types and concentrations of PGRs play an important role in *in vitro* propagation of many orchid species (Arditti and Ernst, 1993). Differences in the induction rate for PLBs were observed between the treatments of TDZ and BA. Comparatively, TDZ gave a superior response to BA for inducing PLBs in dwarf *Dendrobium*. TDZ has been previously used successfully to induce PLBs and the subsequent proliferation in *Dendrobium* 'Chiengmai Pink' (Chung *et al.*, 2005; 2009), *Dendrobium chrysotoxum* Lindl. (Roy *et al.*, 2007) and *Vanda coerulea* Griff. Ex Linda (Malabadi *et al.*, 2004). In this study, TDZ at a particular concentration strongly stimulated the formation of PLBs. In a similar way, TDZ was found to be suitable for production of PLBs from thin leaf sections of a *Doritaenopsis* hybrid (Park *et al.*, 2002b). This study also clearly shows that TDZ used alone was more effective than BA in PLBs induction and proliferation. This result was also in agreement with the observations in *Epidendrum radicans* (Chen *et al.*, 2002), *Doritaenopsis* (Park *et al.*, 2003), and *Phalaenopsis* (Kuo *et al.*, 2005).

TDZ is believed to be the best synthetic cytokinin present for the regeneration of numerous plant species. TDZ has gained a considerable attention during past decades due to its efficient role in plant cell and tissue culture. Wide array of physiological responses were observed in response to TDZ application in different plant species. Other reports showed that TDZ may modify endogenous plant growth regulators, either directly or indirectly and produce reactions in cell/tissue, necessary for its division/regeneration. Other possibilities include modification in cell membrane, energy levels, nutrient absorption, transport and assimilation, etc. (Guo *et al*., 2011).

Originally, TDZ was classified as a type of cytokinin that induces many responses that were similar to the responses induced by natural cytokinins. It was proved that TDZ, unlike traditional phytohormones, individual fulfilled the requirements of various regenerative responses of many different plant species. Recently, the morpho-regulatory potential of TDZ has led to its application in plant tissue culture for the development of feasible morphogenetic systems. TDZ emerged as an effective bioregulant in cell and tissue cultures in wide array of plant species (Hosseini-Nasr and Rashid, 2000; Li *et al*., 2000; Matand and Prakash, 2007; Svetla *et al*., 2003).

TDZ application revealed great deal about Skoog and Miller (1957) postulate about morphogenesis. However, a short exposure to TDZ effectively induces a range of different morphogenetic responses. Pretreatment with TDZ can predispose a tissue to accept other inductive stimuli. Alternately, exposure to TDZ can commit a tissue to regenerative route that is expressed even after the inductive stimulus is removed. Moreover, TDZ trigger a basic survival mechanism in plant tissues that includes asexual reproduction for species survival.

To summarize the biochemical and biophysical responses of plant cell to TDZ and to discern the mechanism(s) involved in the induction of morphogenic potential in plants.

**Metamorphosis of TDZ**

Exploitation of TDZ in plant cell culture systems for induction of adventitious shoot regeneration produced a considerable interest in understanding the plant morphogenesis and different physiological parameters. The complex nature of the biochemical and morphological responses that have been reported for plant tissues exposed to TDZ has provided some indication of the cascade of physiological reactions within the plant tissues Mok and Mok (1985) found that TDZ did not metabolize in callus tissue of *Phaseolus lunatus* within the first 48 h of culture, following which the primary metabolites of TDZ were glucoside residue. In study with *Pelargonium* X *hortorum Bailey*, there was a limited oxidation of the TDZ molecule within the etiolated hypocotyl explants as evidenced by the limited evolution of $14CO_2$ (Murch and Saxena, 2001a). Few reports have shown that TDZ

molecule remains largely intact within the plant tissues (Murch and Saxena, 2001b). TDZ molecule may exist in several forms within the plant tissue. Free TDZ molecules would be recovered in the ethanol soluble fractions, while TDZ molecules that had become associated with a protein or cell wall components which were sequestered would have been recovered in the ethanol insoluble fraction (Atkins and Canvin, 1971). These data may open many possibilities for the mode of TDZ action within the plant tissues including the induction of a metabolite stress response and the modulation of protein mediated responses.

**TDZ liaised responses**

It was firstly used as a defoliant for cotton. Premature activation of the abscission zone affects water potential or chlorophyll content in some members of Malvaceae family (Grossmann, 1991). Although, TDZ protected chlorophyll degradation in detached leaves of geranium (Murthy *et al*., 1998; Visser *et al*., 1995). To encourage the growth of radish cotyledons and persuade adventitious shoots on tobacco leaf, TDZ was observed effective (Thomas and Katterman, 1986). Numerous plant species were induced to viable regeneration via TDZ application (Cocu *et al*., 2004; Faisal *et al*., 2005; Malik and Saxena, 1992).

Some examples of the diversity of physiological effects mediated by TDZ include efficient seed germination, expedited bud break, induction and stimulation of sprouting, cotyledonary growth and development, formation of trichomes and stomata appearance on floral parts and cluster and berry weight of grapes (Babiker *et al*., 1992; Baskakov *et al*., 1981; Lin *et al*., 1994). More recently, the morpho-regulatory potential of the chemical has led to its application in plant cell, tissue and organ culture for the betterment of regeneration protocols. It was also reported that TDZ-induced better response than BA in shoot regeneration in peanut (Gairi and Rashid, 2004; Victor *et al*., 1999). Since 2000, a considerable increase in the number of reports involving TDZ in the induction of regeneration responses has been recorded. TDZ had been reported for the production of economically important secondary metabolites in some plant species (Nabila *et al*., 2003). The exogenous application of TDZ affects concentration of endogenous plant growth regulators in some members of dicots. TDZ affects pathways of purines and cytokinin metabolisms

(Capelle *et al*., 1983; Laloue and Fox, 1989; Mok *et al*., 1982). However, TDZ is involved in modification of cell membranes, energy levels, nutrient uptake and nutrient assimilation. In depth study of the physiological responses of plant cells to TDZ will lead to an insight of the process of morphogenesis.

**Range of TDZ mediated morphogenesis**

Plant cells have the potential to reproduce intact plant via organogenesis or embryogenesis. Plant growth regulators play a role of backbone in process potentials, dedifferentiation and redifferentiation. These regenerative processes in cell and tissue cultures may be provoked by TDZ alone and in collaboration with other plant growth regulators. An array of complex physiological mechanisms like, functions of an intact molecule in both alone and in engaged system are involved in TDZ-treated somatic embryogenesis and also, TDZ-treated tissues maintain and enhance the accumulation and transport of auxin. All these results suggest that TDZ has a keen role in the induction of stimulation of plant growth regulator processes and physiological maintenance of plant tissues during culture process. The ability of the explant tissue to survive the applied stresses of the culture process seems to be an integral part of the morphogenic phenomena and some studies provide indication of the factors involved in the regulation of plant regeneration (Murch and Saxena, 2001a).

The developmental pathway representing somatic embryogenesis in competent cells was stimulated by low fraction of TDZ (Jones *et al*., 2007); however, functions of TDZ are quite different from plant species to species. Sophisticated combinations of exogenous and endogenous plant growth substances regulate a process called somatic embryogenesis (Komamine *et al*., 1992; Skoog and Miller, 1957). *In vitro* alteration of auxin to cytokinin ratio induces somatic embryogenesis from somatic cells, whereas TDZ alone has the potential to induce somatic embryogenesis in several species (Murthy *et al*., 1998). It has been postulated that the concentration of endogenous phytohormones affect TDZ-induced morphogenesis. Murch *et al*. (1999) verified that TDZ-stimulated propagation causes accretion of abscisic acid, proline and particular ions (Tang and Newton, 2005a). Also, the TDZ mediated alteration in the cytokinin biosynthetic pathway might be responsible for the

depletion of the endogenous 2iP pool and the elevated concentrations of the other purine metabolites (Victor *et al*., 1999; Zhang *et al*., 2005).

**Mechanism for TDZ action**

Involvement of phytohormones

TDZ is a powerful regulator of *in vitro* plant regeneration and subsequent growth (Murthy *et al*., 1998). Recent studies showed that TDZ is frequently associated with the metabolism of PGRs. Actually; TDZ was categorized as cytokinin due to induction of natural cytokinins like responses. Later, an increase in endogenous auxin, ethylene and ABA is recorded in response to TDZ-treatment (Murthy *et al*., 1995; Murthy *et al*., 1998; Yip and Yang, 1986).

It is reported that TDZ's action in development is much closed to cytokinin metabolism in plant cell. Casanoval *et al*. (2004) determined the results of TDZ on endogenous plant growth regulators in organogenesis as low TDZ levels (0.0 to 0.005 µmol/l) induce ZR, while high concentrations (0.5 µmol/l) are associated with isopentenyl adenine (ip) that results in rapid cell division and the stimulation of shoot organogenesis I, that is, petals and maximum carnation cultivar leaves (*Dianthus* spp.). It is elucidated that phenylurea compounds can modify preexisting cytokinins which results in the transformation of tissues independent of cytokinins. Many reports showed that TDZ replaces purine based cytokinins in in vitro and this is confirmed via purine metabolism inhibitors like, diaminopurine (DAP) halted TDZ stimulated somatic embryogenesis in geranium and peanut (Murthy *et al*., 1998).

Purines biosynthesis and storage is enhanced by TDZ. This may possibly occur via different strategies like, over synthesis, low catabolism rate or providing active cytokinin molecules stored in in-active forms (Capelle *et al*., 1983; Casanova *et al*., 2004; Ferreira *et al*., 2006; Jones *et al*., 2007; Murch *et al*., 2001; Murthy *et al*., 1998; Victor *et al*., 1999; Zhang *et al*., 2005). Besides, Laloue and Fox (1989) reported that phenylurea can prevent the breakdown of purines by inhibiting cytokinin oxidase.

The structure of TDZ is totally different from naturally occurring purine-based cytokinins. However, certain findings have shown that normally, pure purine based cytokinin have not induced few morphogenic cascades like somatic

embryogenesis (Victor *et al*., 1999). The mechanism in morphogenesis induced by zeatin riboside (ZR) and TDZ was studied by Anna *et al*. (2006). When the phytohormone supply is preceded by 3-day preculture, an alteration in response appears which is in favor of TDZ induced callus, while ZR activity is strongly inhibited. Therefore, it is assured that TDZ possess additional properties which are apparent via altered mechanisms. The two clusters of growth regulators, purine based cytokinin and phenylurea induce biotic response recommended a common location in the presence of competitive inhibitors (Hutchinson *et al*., 1996; Victor *et al*., 1999). Even though TDZ have proved to be the best option for adventitious shoot organogenesis from carnation petals (Nakano *et al*., 1994), but still, it is a mystery that whether phenylurea derivatives effect endogenous cytokinin metabolism directly or indirectly.

Involvement of enzymes

TDZ treatment resulted in accumulation of phenols, catalase (CAT) and peroxidase (POD) (Wang *et al*., 1991a). Todor and Iordanka (1995) investigated that TDZ enhances POD activity. Tang and Newton (2005) determined TDZ influences on antioxidant enzymes performance during morphogenesis of Eastern white pine. They showed that during culture, at the beginning, POD activity decreased and increased at later stages of shoot bud formation and CAT activity linearly decreased throughout the complete culture duration. All these findings showed that TDZ-induced direct adventitious shooting in Eastern white pine resulted in decreased activities of POD and CAT. POD is a multifunctional enzyme. As said by Edreva (1988) and Mamaghani *et al*. (2010) that various active forms of peroxidase involved in growth regulation, development and organogenesis. TDZ promotes the activities of POD, which may be one reason for shoot regeneration or embryo formation. It is remarkable that cytokinin oxidase may be involved in TDZ induced physiological responses. It has been reported that TDZ hampers the action of cytokynin oxidase possibly via a composite, freely occurring uncompetitive mechanism (Hare *et al*., 1994). Apart from antioxidant enzymes, it was reported that TDZ application causes alterations in nitrate reductase, ATP, ribulose diphosphate carboxylase oxidase and pentose phosphate enzymes (Kulaeva *et al*., 1982; Mok *et al*., 1987). So, it can be determined that morphological changes appeared in TDZ-induced tissues and organs

might be the consequence of alterations in enzyme kinetics. Wang *et al*. (1991b) reported that many of the TDZ-stimulated enzymes were associated with cell walls, membranes and membrane fluidity was modified.

Involvement of ions

TDZ treatment promoted accumulation of mineral ions which induced process of regeneration. Somatic embryogenesis was induced by incorporation of zinc, copper or sodium into the culture medium of carrots. Thus, it was inferred that application of TDZ enhanced accumulation of minerals or other metabolites and predisposes the explant to stress. In recent years, calcium signal response to TDZ has been made a point of doing. Calcium, a ubiquitous second messenger, plays the role of a facilitator of stimulus response pairing in the regulation of miscellaneous cellular activities (Allen and Schroeder, 2001). A calcium ion prickle produces when the calcium ion level in plants is getting to its peak due to maximum calcium ion influx and swift to basal level via calcium ion efflux, various stimuli responsible are: light, gravity, physio-chemical and biological stresses and hormones.

Response of plant cells to different hormones is due to external calcium concentration or rise in the level of this cation in the tissue (Trewavas, 1999). Thus, TDZ was classified as plant growth regulator. The mechanism of TDZ in the response of plant cells, especially in the plant tissue and organ culture was conjectured in relation with the balance of calcium concentration in plant tissue. This is substantiated by the effectiveness of TDZ at concentration of externally supplied calcium which is insufficient for shoot formation.

**Special apprehensions**

Amino acids

Zhang *et al*. (2006) reported that the transcription levels persuaded by TDZ were observed to vary all over TDZ treatment. TDZ persuaded the transcription of stress related genes, comprising proline dehydrogenase in both the treatments that is, early (day 2) and late stage (day 28), which are indicative of amino acid responses from the TDZ-treated callus.

Effects of concentration

TDZ induced shoot organogenesis of African violet explants, whereas at higher doses (5 to 10 µM) somatic embryos were formed. TDZ is a urea based cytokinin and therefore, is non-degradable by cytokinin oxidase enzymes in plant tissues. This quality causes TDZ to be persistent in tissues, hence, transforming them from cytokinin dependence to cytokinin autonomy. Makara *et al*. (2010) reported that TDZ had a carry-over effect that enabled shoots to continue proliferation on a hormone free medium as the culture cycles increased and that this effect was significantly ($P < 0.05$) higher than that of BAP. Various plants may need altered incubation periods for differentiation and morphogenesis. This is probably due to the capacity of TDZ in stimulating endogenous cytokinin biosynthesis or in altering cytokinin metabolism (Sankhla *et al*., 1994; Zhou *et al*., 1994).

Involvement of plant growth regulators

Although, TDZ alone is highly effective in inducing plant cell redifferentiation such as White Sim petals or other cultivars and species (Frey and Janick, 1991, Ricci *et al*., 2001). Both BA and TDZ can bind to a receptor, a cytokinin-binding protein (CBP) which has two binding sites. One site binds adenine-type cytokinins naturally, while the other is able to bind phenylurea-type cytokinins. The functions of combined BA and TDZ enhance the shoot morphogenesis possibly due to their active binding to both CBP sites. Similar results have been reported by Tegeder *et al*. (1995).

Morel (1960) was the first to report the shoot tip culture in orchids for mass propagation. Normally, plant regeneration and mass propagation from various types of explants have been achieved by four protocols. The first is adventitious bud induction through direct organogenesis. The second is protocorm induction which eventually develops into plantlets. The third is PLB regeneration through direct embryogenesis. The fourth is the transverse thin cell layer culture. According to these protocols, bud formation via organogenesis and direct PLB formation through embryogenesis are used as the cultural conditions from the explants (Zhao *et al*., 2008).

Regeneration of PLBs is comparable to the somatic embryogenesis pathway in orchids (Morel, 1974). Formation of PLBs can be classified into two types. The first is the direct formation of PLBs from protocorms, shoot tips, root tips, and stem segments through direct embryogenesis (Luo *et al.*, 2008; Mayer *et al.*, 2010; Naing *et al.*, 2011). The second is indirect formation of PLBs from callus (Hong *et al.*, 2008; Huang and Chung, 2010; Ng and Saleh, 2011). In the present experiment, PLBs developed directly from the protocorms without callus formation. Similar result has been demonstrated for *Aerides crispum* and histological observations reveled that the PLBs have originated from the subepidermal layer of the protocorm sections (Sheelavanthmath *et al.*, 2005). Direct somatic embryogenesis on leaf explants of *Oncidium* 'Gower Ramsey' were generally formed from the epidermal layers or sub-epidermal layers of the explants (Chen *et al*., 1999). Histology of direct somatic embryogenesis from leaf explants of *Phalaenopsis amabilis* were originated from the epidermal layer of a leaf explants (Chen and Chang, 2006). However, in this study, PLBs were not investigated in histological observations.

Lee and Phillips (1988) attributed this point as being of major importance because plants produced by direct regeneration will exhibit greater genetic stability than those produced by callus. Furthermore, regenerated plantlets, produced through the direct formation of PLB, produced fewer variants. Moreover, tissue cultures of orchids have not been focused on callus because of their slower growth rate and increased necrosis during culture (Zhao *et al.*, 2008). In the present study, the proficiency for inducing PLBs from protocorms is in agreement with the finding of Sheelavanthmath *et al.* (2005) who illustrated that juvenile explants like protocorms and young leaves were important for the efficient induction of PLBs and the subsequent regeneration of plants in *Aerides crispum*.

## 4.2 Factors affecting *in vitro* flowering of dwarf *Dendrobium*

### 4.2.1 Effects of plant growth regulators on *in vitro* flowering

A number of the inductive effect of BA on *in vitro* flowering reports have been shown and described in orchids (Duan and Yazawa, 1995; Hee *et al*., 2007; Kostenyuk *et al*., 1999; Rojanawong *et al.*, 2006; Sim *et al*., 2007; Tee *et al*., 2008). Cytokinins are important signals in flowering (Bernie *et al*., 1993; Bonhomme *et al*., 2000; Lejeunne *et al*., 1994) and also a common requirement for *in vitro* flowering (Bernier, 1988; Nitsch and Nitsch, 1967; Peeters *et al*., 1991). The role of cytokinins in floral evocation might be in controlling early mitotic activity, precocious initiation of axillary meristems, and increased rate of appendage production by the meristems (Scorza and Janick, 1980). Moreover, cytokinins possibly regulated floral development through genes controlling shoot apical meristem activity (Lindsay *et al*., 2006). Also, it was cited as a probable component of a multi-factored flowering stimulus (Bernier, 1988). Bernier *et al*. (1993) found cytokinins as constituents of the floral stimulus transported in phloem to the apex in response to a photoperiodic treatment inducing flowering. In this study, BA was necessary for transition of vegetative shoot apical meristem to inflorescence meristem. This was demonstrated in the absence of BA did not produce floral buds. Similarly results were observed on induction of flowering of *Doriella* Tiny (*Doritis pulcherrima* × *Kingiella philippinensis*) explants *in vitro* where floral buds were only initiated in the presence of BA (Duan and Yazawa, 1994a). In dwarf *Dendrobium*, BA alone in the medium was effective for floral induction. A similar way, MS medium with BA induced floral bud formation for *Dendrobium candidum* Wall ex Lindl. (Wang *et al*., 1997). However, BA combined with NP treatment promoted the number of flowers per explant that was increased compared to BA alone in the medium. This result agrees with the preceding findings in *Dendrobium* Sonia 17 (Tee *et al*., 2008) and *Phalaenopsis* Pink Leopard 'Petra' (Duan and Yazawa, 1995). In addition, Kostenyuk *et al*. (1999) have applied BA in combination with NP and root-excised treatment to some other orchid species, for instance, *Cymbidium goeringii*, *Cymbidium. kanran*, and *Dendrobium phalaenopsis* hybrid. The induction of flowering was achieved in all

these orchids (including dwarf *Dendrobium* in our study). This implies possible similarity in the mechanism controlling transition to flowering in the orchid species tested. However, PGRs requirements of plants for *in vitro* flowering are variable. These data indicated that BA can induce floral buds on explants, and it is essential in activating the switch-on mechanism for flower induction under the present experimental condition.

PBZ is one of the plant growth regulators which is frequently used wide in agriculture both foliage and flowering plant production (Te-chato *et al*., 2009). The main uses of PBZ for this purpose were reported in field production of fruit crops, e.g. apple tree (cv. Red Delicious), sweet cherry (*Prunus avium* L.), sour cherry (*Prunus cerasus*) (Meilan, 1997) and mango (*Mangifera indica* L.) (Rahim *et al*., 2011). The inhibition of vegetative growth and inducing early flowering in most woody angiosperms and ornamental potted plants; for example, *Dendrobium* Hepa (Pileuk *et al*., 1986) was apparent. Moreover, PBZ could induce flower bud initiation in *Eucalyptus globulus* Labill. and *Eucalyptus nitens* (Dean & Maid.) ex Maid (Griffin *et al*., 1993; Hasan and Reid, 1995) when it was applied as a collar drench, foliar spray, or trunk injection. For floral bud induction *in vitro* was also shown to form *in vitro* flowering in some orchids, such as *Cymbidium goeringii* (Reichenbach f.) Reichenbach f. and *Cymbidium hybridium* L. (Zheng and Pang, 2006), *Dendrobium moniliforme* (L.) Sw. (Wang *et al.,* 2006) and *Dendrobium nobile* Lindl. (Wang *et al*., 2009). A similar result was shown in our study on dwarf *Dendrobium* that PBZ promoted floral bud formation.

*In vitro* flowering in almost all orchid species was performed by manipulation the culture media with BA (Hee *et al*., 2007; Sim *et al*., 2007; Tee *et al*., 2008). In the present experiment also succeeded in floral bud induction with BA that has a stronger inductive effect than PBZ on the *in vitro* flowering of dwarf *Dendrobium*. This result is also in agreement with the observations in *Dendrobium friedericksianum* Rchb.f (Nujeen, 2007) and *Cymbidium niveo-marginatum* Mak (Kostenyuk *et al*., 1999). In contrast, treatment with PBZ produced normal *in vitro* flowering in dwarf *Dendrobium*. Te-chato *et al*. (2009) suggested that PBZ might block the activity of some cytokinins which induce abnormality of flowering *in vitro* and have previously reported that replacing BA with PBZ at low concentration (0.05

mg/l) promoted normal flowering; nevertheless, the function of PBZ on normal flowering development was not clearly understood. Similarly, a result was shown that PBZ totally blocked the inductive effects of cytokinin when it was applied with BA (Kostenyuk *et al*., 1999).

### 4.2.2 Effect of sucrose on *in vitro* flowering

Sucrose is indispensable for *in vitro* flowering which is generally known as carbon sources in culture media for the vegetative growth, reliable floral induction and development of flowers. Addition of sucrose to the medium is necessary for induction of floral stimulus (Singh *et al*., 2006). Jumin and Nito (1996) defined that addition of 3-7 % sucrose to the medium may be necessary for floral induction of *Fortunella hindsii*. In the present investigation, the effect of different concentrations of sucrose on flower induction was studied. The frequency and efficiency of flowering were higher in the presence of 30 g/l sucrose and this result coincided with earlier reports in *Perilla frutescens* L. (Zhang, 2007), *Pisum sativum* L. (Franklin *et al*., 2000) and *Spathiphyllum cannifolium* cv. Sunny Sails (Dewir *et al*., 2007). This indicates that sucrose, which is the main carbohydrate transported in flower organs, was required at specific levels for flower induction and maturity in this morphogenic system (Zhang *et al*., 2012). Corbesier *et al*. (1998) illustrated that sucrose levels have been postulated to play an important role in determining floral initiation time, and flower-inducing treatments may also result in an increased transport of carbohydrates from leaves to SAM. This increase in sucrose flow to SAM prior to flowering suggests that sucrose levels may have a role to play in regulating floral induction, and that their movement to the SAM is not simply a response to increasing metabolic activity (Bernier *et al*., 1993). Furthermore, Havelange *et al*. (2000) revealed that sucrose and cytokinins interact with each other for floral induction in *Sinapis alba* L. by moving between shoot and root. In our system, BA and sucrose together could induce flowering indicated that dwarf *Dendrobium* is not cultivar-dependent. However, they were only a factor needed in floral bud induction or initial development but other factors are required to help them develop fully in later stages of *in vitro* floral morphogenesis.

### 4.2.3 Effect of photoperiod on *in vitro* flowering

The most predictable factor in many plant species to time their reproduction (flowering) is the length of the light periods or day length (Bernier and Perilleux, 2005) and dark periods to which the plant is exposed, i.e. it is photoperiod dependent (Krekule *et al*., 1989). In the field, it is known that the photoperiod is important for flower induction (photoperiodically sensitive plants) (Zeevaart, 1983). This experiment also explored the mode of *in vitro* flower induction control in relation to the photoperiodic requirements of dwarf *Dendrobium*. The photoperiodic requirements for *in vivo* dwarf *Dendrobium* flowering are not yet fully known. Nevertheless, under *in vitro* conditions, flower induction was obtained by varying in three tested photoperiod exposures photoperiod exposure (8/16 h, 12/12 h and 16/8 h day/night). These data provide additional evidence that flowering occurred in all of photoperiods which are DN conditions. Similarly, *in vivo* dwarf *Dendrobium* flowers many times a year and is one of the tropical *Dendrobium* hybrids, so it is DN plant. Consequently, these observations provide further evidence that it is able to flower, whatever the photoperiod, as long as sufficient photosynthetic activity is available. A similar situation was demonstrated in dwarf *Dendrobium* (*Dendrobium* Thai Siri X *Dendrobium* Thai Compactum) when *in vitro* flowering induction formed in SD (8-h light photoperiod) conditions but it is not SD plant because its flowers were also produced in LD (24-h light photoperiod) conditions (Teerawatsakul, 2003). As shown in this work that non specific photoperiod can induce *in vitro* flowering in dwarf *Dendrobium*. Similarly, the results were demonstrated that photoperiod affects the induction of flowers in *Citrus nobilis* Lour X *Citrus deliciosa* Tenora (Singh *et al*., 2006), *Phoenix dactylifera* L. (Masmoudi-Allouche *et al*., 2010), *Psygmorchis pusilla* Dodson and Dressler (Vaz *et al*., 2004) and *Vitex negundo* L. (Gantait *et al*., 2012).

### 4.2.4 Effects of nutrients on *in vitro* flowering

Generally, the nutritional status of plants greatly affects the sensitivity of the major flowering mechanism to both suppressive and inductive stimuli (Hirst and Ferree, 1995; Kerbauy, 1984; Sachs and Hackett, 1983; Yoneda, 1989), although the interactions between nutrition and flowering mechanism are still unclear. N is the

most important inorganic nutrient for plant growth (Marschner, 1995; Miller *et al*., 2007). The major source of N is usually nitrate, with ammonium and amino acids also sometimes making a contribution (Miller *et al*., 2007). The N supply affects all levels of plant function, from metabolism through to allocation and development (Crawford, 1995; Hirel *et al*., 2007; Lea and Azevedo, 2006; Marschner, 1995; Stitt and Krapp, 1999; Zhang *et al*., 2007). At a cellular level, N regulates nitrate and ammonium uptake and reduction, N and carbon metabolism, secondary metabolism and cellular growth (Gutierrez *et al*., 2007; Scheible *et al*., 1997a, 2000, 2004; Vidal and Gutierrez, 2008; Wang *et al*., 2000, 2003). N regulates developmental processes like germination (Alboresi *et al*., 2005), shoot-root allocation (Scheible *et al*., 1997b; Stitt and Krapp, 1999), lateral root growth (Tian *et al*., 2008; Zhang and Ford, 1998; Zhang *et al*., 1999, 2007), the timing of flowering (Bernier *et al*., 1993; Dickens and Van Staden, 1988; Klebs, 1913) and senescence (Vanacker *et al*., 2006; Wang *et al*., 2000).

Studies with genotypes exhibiting low nitrate reductase (NR) activity have shown that some of the responses to N are triggered by nitrate. NR-deficient genotypes accumulate high levels of nitrate but contain low levels of amino acids and other N-containing metabolites, low protein and have low rates of growth (Scheible *et al*., 1997a, b; Wang *et al*., 2004). Nitrate induces genes required for the uptake and reduction of nitrate, ammonium assimilation, the oxidative pentose pathway, and glycolysis and organic acid metabolism (Crawford, 1995; Gutierrez *et al*., 2007; Scheible *et al*., 1997a, 2000; Wang *et al*., 2004), and represses phenylpropanoid metabolism (Fritz *et al*., 2006). Nitrate regulates also shoot-root allocation (Scheible *et al*., 1997b), root architecture (Vidal *et al*., 2010), and triggers a local stimulation of lateral root growth (Tian *et al*., 2008; Zhang and Forde, 1998; Zhang *et al*., 1999). The details of the signalling pathway still need to be elucidated. High-affinity nitrate transporters might play a role in sensing nitrate (Little *et al*., 2005; Remans *et al*., 2006). Nitrate-dependent induction of IPT3 in the roots leads to increased synthesis and export of cytokinins to the shoot (Sakakibara *et al*., 1998; Takei *et al*., 2004). In Arabidopsis plants induced to flower by exposure to a single 22-h-long day, cytokinins increases correlate with the successive steps of the floral transition (Corbesier *et al*., 2003). Transcript profiling has identified many transcription factors,

protein kinases and protein phosphatases that are rapidly induced or repressed by nitrate (Gutierrez *et al*., 2007; Scheible *et al*., 2004; Wang *et al*., 2003).

Other responses require metabolisation of nitrate, and are presumably triggered by metabolites lying further downstream in N metabolism, by accompanying changes in other pathways, or by changes in cellular activities that occur when the N supply increases. For example, downstream signalling regulates large sets of genes involved in cellular growth, including genes encoding components of the protein synthesis apparatus (Gutierrez *et al*., 2007; Scheible *et al*., 2004; Wang *et al*., 2004). There are probably multiple mechanisms for sensing changes that occur when nitrate is assimilated. In bacteria and fungi the PII protein acts as a sensor for 2-oxoglutarate and glutamate, two metabolites at the interface between N and carbon metabolism. Plants contain putative homologues to the PII protein (Hsieh *et al*., 1998; Smith *et al*., 2003), which have been shown to bind 2-oxoglutarate (Kamberov *et al*., 1995; Moorhead and Smith, 2003; Smith *et al*., 2003). Plants also contain a large family of glutamate receptor-like (GLR) genes (Filleur *et al*., 2005; Forde and Lea, 2007; Kang and Turano, 2003).

N deficiency often induces early flowering (Bernier *et al*., 1993; Dickens and van Staden, 1988; Klebs, 1913). Marin *et al*. (2011) who investigated how nitrate regulates flowering, by exploiting genetic tools available in *Arabidopsis* and they reported that flowering was accelerated when plants grown on low nitrate compared to the cultures grown on high nitrate. Several studies have shown that the formation of floral buds is affected by the nitrogen content of the medium (Duan and Yazawa, 1994a; 1995; Tanaka, 1986; Wada and Totsuka, 1982). Duan and Yazawa (1994b) studied the *in vitro* flowering in *Doriella*, *Phalaenopsis* and *Dendrobium*, and they found that high N decreased or discouraged the floral buds formation. In addition, low N content in the culture medium improved the induction of floral buds. Different concentrations of N and P in the medium affected the induction of *in vitro* flowering for orchids (Duan and Yazawa, 1994ab; Kostenyuk *et al*., 1999; Tee *et al*., 2008). In the present experiment, very low N and high P supply alone did not induce floral bud in dwarf *Dendrobium*, but it could flower efficiently when cultured on MS medium containing BA with the NP treatment. This effect coincided with earlier reports of *Cymbidium niveo-marginatum* Mak that very low N and high P supply

alone did not stimulate reproductive development; nevertheless, the low nitrogen supply clearly provided favorable conditions for the inductive action of other promotive agents, such as BA and root excision (Kostenyuk *et al*., 1999). In *Doriella* Tiny and *Phalaenopsis* Pink Leopard 'Petra', plants were induced to flower by cytokinin and low nitrogen supply (Duan and Yazawa, 1994a; 1995). In dwarf *Dendrobium*, the maximum number of flowers per explant (1.2-1.4) was found on modified MS medium with low N (3-8.6 mM) and high P (3.75-6.25 mM) content. Similar results were shown when almost 90 % of plants flowering in *Cymbidium niveo-marginatum* Mak were induced on NP treatment with low N (3 mM) and high P (6.25 mM) concentration (Kostenyuk *et al*., 1999). Furthermore, the effects of N/P ratio on *in vitro* flowering induction was studied in *Dendrobium* Sonia 17, 52 % plantlets cultured on the medium containing BA with low N and high P content formed inflorescence while only 20 % plantlets formed inflorescences in the half-strength MS medium containing BA without any modification of the N/P ratio after four months (Tee *et al*., 2008). Kostenyuk *et al*., (1999) indicated that the *Cymbidium* plantlets cultured in the medium containing BA with low N and high P content achieved higher number of *in vitro* flowers compared to the cultures that were grown in the half-strength MS medium containing BA without modified N/P ratio. These effects are in line with our finding revealed that MS medium supplement with BA and modified NP treatment gave more number of flowers per explant than the medium containing BA alone. Moreover, increasing the concentration of ammonium nitrate in the medium tends to reduce the *in vitro* flowering response. This investigation was demonstrated in *Pisum sativum* L. (Franklin *et al*., 2000) and *Perilla frutescens* (Zhang, 2007) which suggest that ammonium nitrate plays a positive role in inducing *in vitro* flowers at lower concentrations and inhibits flowering at higher concentrations. Vu *et al*. (2006) attributed that an efficient induction of *in vitro* flowering in response to the dilution of the inorganic and organic salts, which especially related to reduction in nitrogen, specifically $NH_4^+$ ions. However, the physiological role of ammonium nitrate on *in vitro* flowering is not clear.

### 4.2.5 Effect of two-layered medium on *in vitro* flowering

In dwarf *Dendrobium*, plantlets incubated in MS agar-solidified medium supplemented with either 15 % CW or both 15 % CW and various concentration of BA did not produce normal flowers even after 4 months in culture but with the same nutrient composition in a two-layered medium flower buds were initiated after 3 months of culture. A similar effect was observed in *Dendrobium* Madame Thong-In when protocorms transferred to two-layered medium containing BA of 22.2 $\mu$M with or without 15 % CW which produced inflorescence stalks within 9 week; however, they could not form inflorescence stalk even after 18 weeks in Gelrite-solidified medium with the same nutrient composition (Sim *et al*., 2007). Sim *et al*. (2007) suggested that besides the composition of nutrients and culture conditions, physical state of the medium also played an important role for flower buds induction or transition of vegetative meristems to inflorescence meristems. Kerbauy (1984) also revealed that medium composition and environmental conditions seemed to accelerate the growth rate of the pseudobulbs and thus shortened the vegetative period in *Oncidium varicosum*. Furthermore, flowers of pepper (*Capsicum fruitescens* L.) were only initiated in liquid medium and not in agar medium, so the factors responsible for flowering attributed the physical culture environment and not the nutrient medium because shoot tips were grown on the same MS medium without growth regulators for the culture systems used (Tisserat and Galletta, 1995). Capability of nutrient uptake by plants in Gelrite-solidified media which there are impurities from gelling agent may affect flower initiation in Gelrite-solidified media compared to liquid medium. Nonetheless, liquid layer alone did not provide the necessary support and did not encourage normal flower development as orchid is not an aquatic plant. Consequently, the two-layered culture system was adopted in dwarf *Dendrobium* because this strategy was reported supplies the better alternative for growth, and especially promoted normal flower development in orchids (Sim *et al*., 2007; Hee *et al*., 2007). In this experiment, the two-layered medium and added BA rapidly enhanced precocious *in vitro* flowering and complete flowers of dwarf *Dendrobium*. A similar situation was shown in *Dendrobium* Chao Praya Smile that the two-layer culture system containing BA at 11.1 $\mu$M induced the highest percentage of flowering in plantlets within 6 months from germination (Hee *et al*.,

2007), and *Dendrobium* Madame Thong-In that the two-layered medium strategy allowed normal development of flower buds to maturity, and addition of BA in the upper liquid layer not only increase flower bud formation but also normal development of flowers (Sim *et al*., 2007). Nowadays, there were a few reports on *in vitro* flowering study using two-layered medium. On the other hand, the two-layered method had been used with success in protoplast cultures of *Petunia* (Power *et al*., 1976), *Linum usitatissimum* (Barakat and Cocking, 1983), *Lithospermum erythrorhizon* (Maeda *et al*., 1983). Also, it was found that two-layered method enhanced somatic embryo formation as compared to Gelrite-solidified and liquid media in *Citrus mitis* (Sim *et al*., 1988). These data was indicated that besides the PGRs, sucrose, photoperiod and nutrients in the culture medium, physical state of the medium, for instant, agar-solidified or the two layer medium also played an important role in *in vitro* flowering of dwarf *Dendrobium*.

### 4.2.6 Effect of coconut water on *in vitro* flowering

Coconut water (CW) contains sugars, vitamins, amino acids and PGRs (Raghavan, 1966; Tulecke *et al*., 1961). In the present experiment, CW alone in the medium was not effective for floral induction, but CW combined with BA increased precocious inflorescence stalks formation and produced floral bud induction. This result agreed with the findings of Sim *et al*. (2007) who reported that in the presence of CW and BA enhanced earlier formation of inflorescence stalks and promoted flower bud induction of *Dendrobium* Madame Thong-In. These data showed the complexity of flowering process as CW alone is not enough for successful flowering.

### 4.2.7 Effect of root excision on *in vitro* flowering

The flower-promoting effect of root pruning is well known in some woody plants (Fossard, 1972; Meilan, 1997). Although cytokinin synthesis occurs in the root of higher plants (Davies, 1995), a specific signal in tobacco roots was also reported to prevent flowering (McDaniel, 1996). This observation was indicated in *Cymbidium niveo-marginatum* Mak that the abolishment of anti-floral signals by root

excision may be the reason which explants were found slight induction of orchid flowering *in vitro*, even when BA was not employed (Kostenyuk *et al*., 1999). In the present study, plantlets without roots were used as explants to induce floral buds. Similar to *Cymbidium niveo-marginatum* Mak, the inclusion of root-excised combined with all cultures significantly promoted the transition to flowering (Kostenyuk *et al*., 1999).

### 4.2.8 Effect of flower colors in *in vitro* flowering

Flower color is due to three different pigments - chlorophyll, flavonoids, and carotenoids. Chlorophyll is located in small "packets" called chloroplasts found throughout the petal and sepal cells. This pigment is responsible for green color and is fat or lipid soluble. The carotenoids are also found within small "packets" in the cells. The "packets" containing carotenoids are called chromoplasts. These pigments are responsible for yellow and orange colors and are also lipid soluble. The flavonoids, unlike the other two pigments, are located within the cellular vacuole which occupies most of the cell volume. Anthocyanins are responsible for red and blue color and water soluble (Griesbach, 1992b).

Each pigment is the result of a different sequence or pathway of biochemical reactions. The production of each pigment is independent of the other two. Thus, a block in the flavonoid pathway has no effect on the carotenoid and chlorophyll sequences. For example, in white flowered forms of many of the red spotted rhododendron, the flavonoids which are usually present in the spots are lacking due to an absence of a critical enzyme in the flavonoid biosynthesis pathway. The carotenoids, however, are unaffected. Therefore, the spots are yellow on a white background (Griesbach, 1992b).

Flower color is the result of mixing the three pigments (flavonoids, chlorophyll and carotenoids) in different proportions. For example, a flower of 'Vulcan' appears red because of the presence of red flavonoids and the absence of both chlorophyll and carotenoids. On the other hand, the flowers of *Rhododendron japonicum* appear orange because of the presence of red flavonoids combined with orange carotenoids. Similarly, *Rhododendron sanguineum* flowers appear brown

because of the presence of red flavonoids combined with green chloroplasts. By mixing and matching the three pigments, an endless array of different colors can be created (Griesbach, 1992b).

Very little is known about the biochemistry of carotenoids and chlorophyll as related to flower color. However, a lot of information is known about flavonoid biochemistry and flower color. The flavonoids can be subdivided into several groups - anthocyanins, flavonols, aurones, chalkones and gossypetins (Griesbach, 1992b).

Light and temperature can also dramatically affect flower color. High light intensity during flower development can also lead to more vibrant coloration. At high light intensity, photosynthesis is occurring at a very rapid rate which leads to the production of increased amounts of sugar. At cool temperatures the plant's growth is slowed down, limiting the amount of sugar needed for respiration. Cool temperatures and high light intensity thus allow the plant to accumulate a reserve of sugar. Sugar molecules are bound to anthocyanin molecules and have the effect of stabilizing color. In addition, at high light intensities, increased anthocyanin production occurs. Anthocyanins help protect the cell from harmful effect of increased irradiation. All these factors coupled together lead to an increase in anthocyanin under cool temperatures and high light intensity. High light intensity and high temperatures can cause the anthocyanins to break down and lead to fading. In order to retain the vibrant color, the flowers, after opening, could be placed in a low light intensity, cool environment to preventfading (Griesbach, 1992b).

Besides environmentally induced fading or intensity differences, there are genes which control the amount of anthocyanins produced. These genes can either increase the amount of pigment per cell or increase the number of cells producing pigment. When comparing plants for differences in color intensity, one must be careful to separate differences due to genetics from differences due to culture or environment (Griesbach, 1992b).

Under low light cut flowers often fade in color. Low lightintensities are also known to reduce flowercolorin plants (Larson, 1992) as was described in *Petunia hybrida* (Weiss and Halevy, 1991), Rosa *hybrida* (Mortensen and Moe, 1995) and *Chrysanthemum* and *Poinsettia* (Nell *et al*., 1990). In lisianthus (*Eustoma*

*grandiflorum* GRISE.), flowers that opened under low light were paler than normal. A 25% decrease in lightintensity was related to a 30% reduction in the concentration of anthocyanin and a 40% reduction in colorintensity (Griesbach, 1992a).

A requirement for carbohydrates was demonstrated in cut flowers of *Petunia hybrida* (Weiss and Halevy, 1991) and *Antirrhinum majus* linn. (Sang *et al*., 1991), by exogenously supplying sucrose to promote anthocyanin production. Kawabata *et al*. (1995) reported that the concentration of anthocyanin increased as the light intensity increased from 1000 to 15,000 lux.

*In vivo* flower colors of dwarf *Dendrobium* is white on petals and sepals with purple lip but *in vitro* flowers are green and yellow. In this study, the light intensity might affect the changing of flower colors. Plantlets with flowers were transferred to the greenhouse; however, to further study the effect of light intensity on *in vitro* flower color.

# CHAPTER 5
# CONCLUSIONS

## 5.1 PLB induction and plant regeneration of dwarf *Dendrobium*

The efficient induction of PLBs and their proliferation from protocorms of dwarf *Dendrobium* was achieved for large-scale propagation. It was found that 18 μM TDZ on MS medium was the optimum concentration for inducing PLBs (86 %), and PLBs developed directly from protocorms without the intermediate formation of callus after 9 weeks of culture. Then PLBs were transferred to the basal MS medium without PGRs upon which they developed into shoots and roots in 6 weeks.

## 5.2 *In vitro* flowering of dwarf *Dendrobium*

Precocious *in vitro* flowering of dwarf *Dendrobium* was accomplished when plantlets without roots were cultured on inductive medium containing 22 μM BA and 30 g/l sucrose under 12-h photoperiod that promoted number of inflorescences per explant (1.14), but they could not develop to flowers. However, both the same nutrient composition in a two-layered medium and substitution BA with PBZ on agar-solidified medium under 16-h photoperiod produced normal flowers per explant (3.4 and 0.4 respectively). The flowers were yellow and green in color with a diameter of 1-1.3 cm and composed of three sepals, two petals and one lip, male and female organ. In addition, the low N (8.6 mM) and high P (6.25 mM) concentration in $N_7P_5$ medium under 16-h photoperiod had a positive effect on floral bud induction that formed number of inflorescences per explant (1.4) although they did not proceed to open. All experiments investigated, the morphology of plantlets

bearing floral buds could not form when BA was excluded. In general, flower morphogenesis is known to be controlled by hormonal, environmental, and nutritional factors. This protocol revealed that besides the PGRs and nutrients in the culture medium, physical state of the medium; for example, two-layered or agar-solidified medium, also played an important role in *in vitro* flowering

Seeds were used as plant material to initiate in this culture. The process of seed germination to flowering *in vitro* took about 8-9 months, but it takes about 30 months in the greenhouse for dwarf *Dendrobium*. For this reason, the juvenile period shortened the time required for normal evaluation (at least 2 years), reduces the labor costs and optimizes the space required for normal orchid breeding. This avenue will be considerably beneficial for orchid breeders and assistant for the orchid cut flower industry. Furthermore, our results might contribute to the clarification of the physiological process of floral formation that would be useful to researchers who study orchid flower regulation and development.

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# APPENDICES

# APPENDIX 1

## Composition of Murashige and Skoog (MS) medium (1962)

| | 1 liter |
|---|---|
| **Macroelements** | |
| Ammonium nitrate, $NH_4 NO_3$ | 1,650 mg |
| Potassium nitrate, $KNO_3$ | 1,900 mg |
| Calcium chloride, $CaCl_2.2H_2O$ | 440 mg |
| Potassium dihydrogen phosphate, $KH_2PO_4$ | 170 mg |
| Magnesium sulfate, $MgSO_4.7H_2O$ | 370 mg |
| **Chelated iron** | |
| Disodium ethylene diaminetetraacetate, $Na_2$-EDTA | 37.3 mg |
| Ferrous sulfate, $FeSO_4.7H_2O$ | 27.8 mg |
| **Microelements** | |
| Boric acid, $H_3BO_3$ | 6.2 mg |
| Manganese sulfate, $MnSO_4.4H_2O$ | 16.9 mg |
| Zinc sulfate, $ZnSO_4.7H_2O$ | 6.14 mg |
| Potassium iodide, KI | 0.83 mg |
| Sodium molybdate, $Na_2MoO_4.2H_2O$ | 0.25 mg |
| Copper sulfate, $CuSO_4.5H_2O$ | 0.025 mg |
| Cobalt chloride, $CoCl_2.6H_2O$ | 0.025 mg |
| **Growth factor** | |
| Myo-inositol | 100 mg |

**Organic addenda**

| | |
|---|---|
| Glycine | 2 mg |
| Coconut water | 150 ml |

**Vitamins**

| | |
|---|---|
| Thiamine HCl | 0.1 mg |
| Nicotinic acid | 0.5 mg |
| Pyridoxin HCl | 0.5 mg |

**Sugar**

| | |
|---|---|
| Sucrose | 30,000 mg |

# APPENDIX 2

## Composition of coconut water (CW)

| Chemical constituents | Mature Coconut Water (%) | Tender Coconut Water (%) |
|---|---|---|
| Total solids | 5.4 | 6.5 |
| Reducing sugars | 0.2 | 4.4 |
| Minerals | 0.5 | 0.6 |
| Protein | 0.1 | 0.01 |
| Fat | 0.1 | 0.01 |
| Acidity (mg) | 60.0 | 120.0 |
| pH | 5.2 | 4.5 |
| Potassium (mg) | 247.0 | 290.0 |
| Sodium (mg)% | 48.0 | 42.0 |
| Calcium (mg)% | 40.0 | 44.0 |
| Magnesium (mg) | 15.0 | 10.0 |
| Phosphorous (mg) | 6.3 | 9.2 |
| Iron (mg) | 79.0 | 106.0 |
| Copper (mg) | 26.0 | 26.0 |

Source: Krishnankutty, 1987

**(**http://www.navimpex.com/fic_bdd/produits_pdf_fr_fichier/12500044750_Tender_Coconut_Water.pdf**)**

| Amino Acid | (% of total protein) |
|---|---|
| Alanine | 2.41 |
| Arginine | 10.75 |
| Aspartic acid | 3.60 |
| Cystine | 0.97 - 1.17 |
| Glutamic acid | 9.76 - 14.5 |
| Histidine | 1.95 - 2.05 |
| Leucine | 1.95 - 4.18 |
| Lysine | 1.95 - 4.57 |
| Proline | 1.21 - 4.12 |
| Phenylalanine | 1.23 |
| Serine | 0.59 - 0.91 |
| Tyrosine | 2.83 - 3.00 |

Source: Pradera *et al*., 1942 (http://www.navimpex.com/fic_bdd/produits_pdf_fr_fichier/12500044750_Tender_Coconut_Water.pdf)

| Vitamins of B Group | µg/ml |
|---|---|
| Nicotinic acid | 0.64 |
| Pantothenic acid | 0.52 |
| Biotin | 0.02 |
| Riboflavin | < 0.01 |
| Folic acid | 0.003 |
| Thiamine | Trace |
| Pyridoxine | Trace |

Source: The Wealth of India, 1950 (http://www.navimpex.com/fic_bdd/produits_pdf_fr_fichier/12500044750_Tender_Coconut_Water.pdf)

**Cytokinins (pmol/ml) in autoclaved and unautoclavedcoconut water**

| Cytokinin (pmol $ml^{-1}$ ± SE) | Unautoclaved coconut water | Autoclaved coconut water |
|---|---|---|
| Z | 1.8 ± 0.3 | 2.7 ± 0.0 |
| Z5P | 1.3 ± 0.1 | 2.6 ± 0.4 |
| Z9G | 1.3 ± 0.1 | 1.5 ± 0.0 |
| ZR | 136.3 ± 4.7 | 142.8 ± 3.4 |
| DZ | 0.8 ± 0.2 | 0.5 ± 0.1 |
| DZ5P | 0.5 ± 0.1 | 0.6 ± 0.1 |
| DZ9G | 1.1 ± 0.1 | 1.2 ± 0.1 |
| DZR | 1.7 ± 0.0 | 2.1 ± 0.4 |
| iP | 3.4 ± 0.6 | 2.1 ± 0.3 |
| iPAMP | 0.2 ± 0.1 | 0.3 ± 0.2 |
| iP9G | 1.6 ± 0.2 | 1.2 ± 0.1 |
| iPA | 0.1 ± 0.1 | 0.1 ± 0.1 |

Source: Sim *et al*., 2008

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**List of Publication and Proceedings**

1. Sujjaritthurakarn, P. and Kanchanapoom, K. 2011. Efficient direct protocorm-like bodies induction of dwarf *Dendrobium* using thidiazuron. *Notulae Scientia Biologicae*. 3(4): 88-92.
2. Sujjaritthurakarn, P. and Kanchanapoom, K. 2010. Protocorm-like bodies induction from protocorms of dwarf *Dendrobium*. The 7th IMT-GT UNINET and The 3rd Joint International PSU-UNS Conferences on Bioscience for the future 2010, 7-8 October 2010, 60th Anniversary of His Majesty the King's Accession to the Throne International Convention Center, Prince of Songkla University, Hat Yai, Songkhla, Thailand.
3. Sujjaritthurakarn, P. and Kanchanapoom, K. 2012. *In vitro* flowering of dwarf *Dendrobium*. The International Symposium on Orchids and Ornamental Plants on the International Horticultural Exposition: Royal Flora Ratchaphruek 2011, 9-12 January 2012, Imperial Mae Ping, Chiang Mai, Thailand.