# Micropropagation of *Phalaenopsis cornu-cervi* (Breda) Blume & Rchb. f. and Conservation *In Vitro*

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**ชื่อวิทยานิพนธ์** การขยายพันธุ์กล้วยไม้เขากวางอ่อนและการอนุรักษ์พันธุกรรมในหลอดทดลอง

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## บทคัดย่อ

เพาะเมล็ดกล้วยไม้เขากวางอ่อน [*Phalaenopsis cornu-cervi* (Breda) Blume & Rchb. f.] อายุ 6 เดือนหลังการผสมเกสรบนอาหารสูตร Murashige and Skoog (MS, 1962) ที่เติมน้ำมะพร้าว 15 เปอร์เซ็นต์ ในสภาวะปลอดเชื้อ หลังจาก 2 เดือนของการเพาะเลี้ยง เมล็ดกล้วยไม้งอกเป็นโปรโตคอร์มและใช้เป็นชิ้นส่วนเริ่มต้นในการชักนำและเพิ่มปริมาณ protocorm-like bodies (PLB) ในอาหารเหลวสูตรต่างๆ คือ MS Vancin and Went (VW, 1949) และสูตร New Dogashima (ND, 1993) ก่อนการเพาะเลี้ยงเตรียมโปรโตคอร์มด้วยวิธีต่างๆ พบว่า อาหารเหลวทุกสูตรที่เติมหรือไม่เติมน้ำมะพร้าว 15 เปอร์เซ็นต์ ไม่สามารถชักนำหรือส่งเสริมการเพิ่มปริมาณ PLB ได้ เนื่องจากโปรโตคอร์มปล่อยสารประกอบฟีโนลิคออกมา การตัดโปรโตคอร์มออกเป็นสองส่วนตามยาวแล้วเพาะเลี้ยงในอาหารเหลวสูตร VW ที่เติมกรดแอสคอร์บิคเข้มข้น 150 มิลลิกรัมต่อลิตรเป็นเวลา 50 วันส่งเสริมให้มีการเพิ่มปริมาณของ PLB สูงสุด 21 PLB ต่อชิ้นส่วน การสร้างบาดแผลโดยใช้ใบมีดกรีดรอบๆ โปรโตคอร์มแล้วเพาะเลี้ยงบนอาหารสูตร MS ที่ลดความเข้มข้นขององค์ประกอบลงครึ่งหนึ่ง (½ MS) เติม NAA เข้มข้น 0.1 มิลลิกรัมต่อลิตรและ TDZ เข้มข้น 0.1 มิลลิกรัมต่อลิตร เป็นเวลา 6 สัปดาห์ ส่งเสริมการสร้าง PLB ขนาดเล็ก ขนาดกลาง และขนาดใหญ่ ได้ 13.9 10.7 และ 11 PLB ต่อชิ้นส่วน ตามลำดับ เมื่อวางเลี้ยง PLB เหล่านี้บนอาหารสูตร ND เติมผงถ่าน 0.2 เปอร์เซ็นต์และน้ำตาลซูโครส 4 เปอร์เซ็นต์ เป็นเวลา 5 เดือน ส่งเสริมการเจริญทางลำต้นดีที่สุด และมีอัตรารอดชีวิตหลังย้ายปลูก 100 เปอร์เซ็นต์ ไม่พบการเปลี่ยนแปลงทางสัณฐานวิทยาและการเปลี่ยนแปลงชุดของโครโมโซมเมื่อตรวจสอบด้วยเครื่องโฟลไซโตมิเตอร์

การการเก็บรักษาโปรโตคอร์มที่มีอายุต่างกันในไนโตรเจนเหลวด้วยวิธีการต่างๆ การเตรียมโปรโตคอร์มโดยวิธี encapsulation-dehydration ให้ความมีชีวิตและการงอกหลังเพาะเลี้ยงบนอาหารชักนำการงอกสูงกว่าวิธี encapsulation-vitrification อย่างไรก็ตาม เมื่อแช่แข็งโปรโตคอร์มในไนโตรเจนเหลวเป็นเวลา 1 วันไม่สามารถส่งเสริมให้โปรโตคอร์มมีชีวิตรอดได้ แม้ว่ามีการปรับปรุงวิธี encapsulation-dehydration ร่วมกับ cold-hardening หรือร่วมกับการแช่ใน

สารละลายน้ำตาลซูโครสที่เวลาต่างๆ กัน ก็ยังไม่ประสบความสำเร็จในการเก็บรักษาในไนโตรเจนเหลว ดังนั้นการเก็บรักษา PLB ของกล้วยไม้เขากวางอ่อนในรูปของเมล็ดเทียม (หุ้ม PLB ด้วยโซเดียมแอลจิเนตเข้มข้น 3 เปอร์เซ็นต์และแคลเซียมคลอไรด์เข้มข้น 100 มิลลิโมลาร์ ละลายในอาหารเหลวสูตร ND ที่เติมน้ำตาลซูโครส 2 เปอร์เซ็นต์) และเก็บรักษาที่อุณหภูมิ 25±1 องศาเซลเซียสในหลอดทดลองเป็นเวลา 30 วัน เป็นวิธีการที่เหมาะสมที่สุดสำหรับการเก็บรักษาพันธุกรรมกล้วยไม้เขากวางอ่อน เมื่อต้องการชักนำการงอกก็นำเมล็ดเทียมมาวางเลี้ยงบนอาหารสูตร ND ที่เติมน้ำตาลซูโครส 4 เปอร์เซ็นต์ร่วมกับผงถ่าน 0.2 เปอร์เซ็นต์ให้อัตราการงอก 100 เปอร์เซ็นต์

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# ABSTRACT

Seeds of *Phalaenopsis cornu-cervi* (Breda) Blume & Rchb. f. were collected 6 months after pollination. All seeds were aseptically germinated *in vitro* on Murashige and Skoog (MS, 1962) medium supplemented with 15% coconut water (CW). After 2 months of culture, the seeds germinated into protocorms and used as initial explants for induction and proliferation of protocorm-like bodies (PLB) in various kinds of culture media [MS, Vancin and Went (VW, 1949) medium, New Dogashima (ND, 1993) medium] using different treatments. The results showed that all culture media with or without 15% CW could not induce PLB owing to the exudates (phenolic compounds) released from cultured protocorms. Protocorms cut longitudinally into half (bisected protocorms) and cultured in VW liquid medium supplemented with 150 mg/l ascorbic acid gave the best proliferation of PLB at 21 PLB/explant after being cultured for 50 days. Wounded protocorms obtained by tapping into the wound around the protocorms with a sharp razor blade and cultured on half strength Murashige and Skoog (½ MS) medium supplemented with 0.1 mg/l NAA and 0.1 mg/l TDZ for 6 weeks gave the highest percentage of PLB formation and each explant produced small, medium and large sizes of PLB at 13.9, 10.7 and 11 PLB per explant, respectively. Upon transferring these PLB to ND medium supplemented with 0.2% (w/v) activated charcoal (AC) and 4% (w/v) sucrose for 5 months the best results in vegetative growth and survival rate were obtained at 100%. Morphological characteristics of those plantlets were normal and ploidy level was the same as controlled plantlets as revealed by flow cytometry.

The protocorms at different ages were also preserved in different methods, in liquid nitrogen (LN). Preparation of protocorms by encapsulation-dehydration gave the higher survival rate and germination percentage of protocorms

than those obtained by encapsulation-vitrification. However, survival rate of the protocorms was not obtained after cryopreservation in LN for one day. Even though those preparations were modified by cold-hardening or sucrose pretreatment at various periods, the survival rate of encapsulated PLB after cryopreservation in LN was not improved. Therefore, encapsulation of the PLB using 3% sodium alginate and 100 mM calcium chloride dissolved in ND liquid medium supplemented 2% (w/v) sucrose and storage at 25±1°C *in vitro* for 30 days was optimum for germplasm storage of *Phalaenopsis cornu-cervi* (Breda) Blume & Rchb. f. Germination and proliferation of encapsulated PLB could be performed effectively at 100% on ND medium supplemented with 4% (w/v) sucrose and 0.2% (w/v) activated charcoal (AC).

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# LIST OF ABBREVIATIONS AND SYMBOLS

| | | |
|---|---|---|
| AC | = | activated charcoal |
| ANOVA | = | analysis of variance |
| API | = | air-driven periodic immersion |
| BA | = | 6-benzyladenine |
| BAP | = | benzylaminopurine |
| BE | = | banana extract |
| $CaCl_2$ | = | calcium chloride |
| cm | = | centimeter |
| CRD | = | completely randomized design |
| CW | = | coconut water |
| 2, 4-D | = | 2, 4-dichlorophenoxyacetic acid |
| DMRT | = | Duncan's multiple range test |
| DMSO | = | dimethyl sulfoxide |
| e.g. | = | example gratia (Latin), for example |
| et al. | = | et alli (Latin), and others |
| FCM | = | flow cytometry |
| g | = | gram |
| g/l | = | gram per liter |
| GI | = | Growth Index |
| IAA | = | indole acetic acid |
| ISSR | = | inter simple sequence repeats |
| KC | = | Knudson C |
| LN | = | liquid nitrogen |
| MAP | = | months after pollination |
| ml | = | milliliter |
| MS | = | Murashige and Skoog medium |
| mm | = | millimeter |
| mM | = | millimolar |
| mg/l | = | milligram per liter |

# LIST OF ABBREVIATIONS AND SYMBOLS (Continued)

| | | |
|---|---|---|
| M | = | Molar |
| NAA | = | α-naphthalene acetic acid |
| ND | = | New Dogashima medium |
| nm | = | nanometer |
| PGRs | = | plant growth regulators |
| PI | = | propidium iodide |
| PLB | = | protocorm-like bodies |
| POD | = | peroxidase |
| PPFD | = | photosynthetic photon flux density |
| PPO | = | polyphenoloxidase |
| PVP | = | polyvinylpyrolidone |
| PVS | = | plant vitrification solution |
| PVS2 | = | plant vitrification solution formula 2 |
| RAPD | = | random amplified polymorphic DNA |
| rpm | = | round per minute |
| SE | = | standard error |
| TDZ | = | thidiazuron |
| VW | = | Vacin and Went medium |
| v/v | = | volume per volume |
| w/v | = | weight per volume |
| µl | = | microliter |
| µm | = | micrometer |
| µmol $m^{-2}$ $s^{-1}$ | = | micromole per square meter per second |
| °C | = | degree Celsius |
| % | = | percentage |

# CHAPTER 1

# INTRODUCTION

## 1.1 Introduction about orchids

Orchids are prized ornamentals that have flourished to be the second most popular cut flowers, as well as potted floriculture crop, with wholesale prices estimated at $126 million (USDA, 2008). In Thailand, orchids are very important commercial plants and produce an annual income for Thailand of 2 billion Baht (Rojanawong *et al*., 2006). *Phalaenopsis* (Orchidaceae), commonly known as moth orchids, their potted plants and cut flower production have high economic value in international flower markets. (Gow *et al*., 2009).

Sexual propagation to produce complete plants through orchid seeds is difficult because their seeds are tiny, no endosperm and in nature they must be symbiotic with some kinds of fungi in order to germinate (Anjum *et al*., 2006; Thomas and Michael, 2007).

Orchid seeds are unusual in several respects. At maturity, they possess rudimentary embryos that have developed only as far as the globular stage. Because they lack a storage organ (such as an endosperm or large cotyledon), they have little nutrient reserve in comparison with more well studied agricultural species (Lee *et al*., 2008). Their rudimentary nature is partly responsible for their requirements for long times and fungal symbionts for germination. The fungal symbioses that orchid seeds require to germinate in nature are difficult to establish artificially, and orchid growing has flourished only since Knudson (1946) demonstrated that orchid seeds could germinate and develop into seedlings with high frequency when they were aseptically cultured on nutrient media. Since then, *in vitro* seed germination protocols have been established for many orchid species (Arditti, 1977). Tissue culture has for many years played an important role as a mean to propagate orchids and several *in vitro* cultural protocols have been developed in genus *Phalaenopsis* (Gow *et al*., 2009).

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 seeds and the subsequent

induction of protocorm-like bodies (PLB), from different tissues as explants has become a reliable method for breeding orchids. Propagation by formation of PLB is a preferred option because of the large number of PLB that can be obtained within a short period of time.

Protocols for the mass propagation and development of *in vitro* methods have been investigated in many explants of orchids through PLB formation, such as the propagation of large-scale PLB using: shoot tips (Roy and Banerjee, 2003; Malabadi *et al*., 2005; Sheela *et al*., 2004), leaf segments (Martin and Madassery, 2006), 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). PLB 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, PLB are well-differentiated tissues that are sometimes regarded as orchid embryos that can develop two distinct bipolar structures, namely the shoot and root meristems. Thus, these structures are able to convert into plantlets easily when grown on plant growth regulator-free medium (Ng and Saleh, 2011).

### 1.2 Micropropagation of *Phalaenopsis*

*Phalaenopsis*, commonly known as moth orchids, have long arching sprays. This genus distributed throughout Southeast Asia with a few species extending from Taiwan, Sikkhim to Australia and the Pacific (Teob, 1989). Most *Phalaenopsis* grow on trees as epiphytes, but a few attach themselves to the surface of rocks as lithophytes. In Thailand, the genus *Phalaenopsis* comprises of 2-3 epiphytic orchid species, such as *Phalaenopsis cornu-cervi* (Breda) Blume & Rchb. f. and *Phalaenopsis decumbens* Holtt. This genus is a monopodial epiphytic orchid which is difficult to propagate vegetatively (Kosir *et al*., 2004) and naturally slowly growth.

*Phalaenopsis*, a member in the family Orchidaceae, is a popular genus in horticultural and ornamental plants because of the successful production of beautiful cultivars derived from interspecific, as well as intergeneric hybridization with *Doritis pulcherrima*, achieved by conventional cross breeding during the last few decades (Shrestha *et al*., 2007). It is a very important commercial plant for both flowering-potted

plants and cut flower, which widely propagate by tissue culture technique (Tokuhara and Mii, 1993). This technique can be used not only for rapid and large-scale propagation of the species but also for *ex situ* conservation. Tissue culture has been used widely for mass propagation of superior varieties of *Phalaenopsis* (Tokuhara and Mii, 1993). Many protocols have been developed for large-scale propagations of a number of orchid species (including *Cymbidium*, *Vanda*, *Phaphiopedilum* and *Phalaenopsis*) through *in vitro* culture of various plant parts (Arditti and Ernst, 1993).

Protocols for *Phalaenopsis in vitro* micropropagation utilize flower stalk buds (Arditti, 1977; Tanaka and Sakanishi, 1978; Tokuhara and Mii, 1993; Tokuhara and Mii, 2001; Kosir *et al*., 2004) entire shoots, shoot tips, stem nodes (Griesbach, 1983), leaf tissues/segment (Tanaka and Sakanishi, 1980; Park *et al*., 2002b; Gow *et al*., 2008, 2009) or root tips culture (Tanaka *et al*., 1976; Park *et al*., 2003) were reported. Unfortunately, these methods are very difficult and inefficient. Propagation through protocorms derived from seedling proliferation has been studied by Yam *et al*. (1991) and Chen *et al*. (2000).

The conventional *in vitro* culture protocols had been developed in genus *Phalaenopsis* and usually for the propagating purpose via protocorm-like body formation, shoot multiplication and callus culture (Tanaka *et al*., 1975; Arditti and Ernst, 1993; Tokuhara and Mii, 1993; Ernst, 1994; Chen and Piluek, 1995; Duan *et al*., 1996; Ishii *et al*., 1998; Islam and Ichihashi, 1999; Chen *et al*., 2000; Young *et al*., 2000; Tokuhara and Mii, 2001; Park *et al*., 2002a). Recently, regeneration systems through direct somatic embryogenesis had been developed using leaf explants (Kuo *et al*., 2005; Chen and Chang, 2006; Chen and Chang, 2001). In addition, the requirements of growth regulators for inducing direct embryo formation were established (Gow *et al*., 2008). However, further investigations on culture condition, medium composition and physiological status are needed to optimize the protocol for practical use in regenerating transgenic plants or mass propagation in this orchid.

The protocorm is a structure unique to orchids, including *Phalaenopsis*. It is the earliest structure formed during embryo development during orchid seed germination (Ishii *et al*., 1998). Proliferation of protocorms and protocorm-like bodies (PLB) is often the only means of increasing the number of orchids, which produce few seeds or may not germinate well. In an effort to increase and/or accelerate proliferation,

propagators have resorted to media which contain very high hormone levels (Arditti, 1977). Vajrabhaya (1997) reported that some media containing hormones may accelerate the rate of increase, but they can also bring about undesirable mutations. These undesirable side effects may be reduced or eliminated through the use of media, which induce proliferation of protocorms or PLB but contain low concentrations of hormones (Gu *et al*., 1987) or by using hormone-free media supplemented with complex additives. Based on research by previous investigators we determined the optimal conditions required for *Phlaenopsis in vitro* culture and micropropagation. Thus, it is found that medium choice, concentrations of mineral nutrients (Hinnen *et al*., 1989; Tokuhara and Mii, 1993; Kosir *et al*., 2004), addition of complex additives, such as banana homogenate (Yam *et al*., 1991) and coconut water (Yam *et al*., 1991; Ishii *et al*., 1998), explant source (Ishii *et al*., 1998), maturity (Chen *et al*., 2000), and size (Park *et al*., 2002b) profoundly affect the success culture procedures.

### 1.3 *Phalaenopsis cornu-cervi* (Breda) Blume & Rchb. f.

*Phalaenopsis cornu-cervi* (Breda) Blume & Rchb. f. (2n=2x=38) is a monopodial orchid (Figure 1) which is difficult to propagate vegetatively. Mass propagation of this species is limited. Tissue culture techniques using shoot tips and axillary buds as explants are used widely in commercial orchid micropropagation. However, *Phalaenopsis cornu-cervi* (Breda) Blume & Rchb. f. has short stems. *In vitro* culture using shoot tips may lead to the loss of the mother plant. Many authors have developed large-scale propagations through various explants of orchid species, such as shoot tips (Martin and Madassery, 2006), leaf segments (Park *et al*., 2002b; Teng *et al*., 2004; Sheelavanthmath *et al*., 2005) and root tips (Park *et al*., 2003). Unfortunately, these methods are very difficult to propagate *Phalaenopsis*. Another problem encountered long time required for growth and multiplication of PLB during *in vitro* culture of *Phalaenopsis*.

Tissue culture method acts as the powerful approach to propagate the number of plants. Micropropagation was different among plant species, types of explants and culture media. The source of carbon in culture medium is a very important component for proliferation of protocorm-like bodies (PLB) in many orchids (Sopalun *et al*., 2010). Commonly used of carbon source is sucrose, glucose or fructose. Reports

from several laboratories emphasize the importance of the source and concentration of sugars on the promotion of *in vitro* orchid seed germination, as well as plant growth (Ernest, 1967). However, browning of the PLB of *P. cornu-cervi* (Breda) Blume & Rchb. f. is a problem during PLB development. Browning is the result from the accumulation of phenolic compounds that causes loss of growth capacity and tissue death during culture. To solve these problems, activated charcoal (AC) is widely used in culture medium. AC has a very fine network of pores with large inner surface area on which many substances can be adsorbed. AC is often used in tissue culture to improve cell growth and development (Pan and van Staden, 1998). It plays a critical role in orchid seed germination of *Cypripedium flavum* (Yan *et al*., 2006). The addition of AC to both liquid and semi-solid media is a recognized practice and its influence in growth and development may be attributed mainly to the adsorption of inhibitory compounds in the culture medium and substancially decreasing the toxic metabolites, phenolic exudation and brown exudates accumulation (Fridborg *et al*., 1978).

Thus, in this study, we describe an efficient induction of PLB from starting explants of *P. cornu-cervi* (Breda) Blume & Rchb.f. and reduce the time required for the growth and multiplication of PLB in order to mass propagation of this species and aimed to find out appropriate culture media for multiplication of plantlets from asymbiotic culture of seeds of *P. cornu-cervi* (Breda) Blume & Rchb. f.

**1.4 Effects of natural additives on orchid tissue culture**

A large number of complex additives like peptone, carrot juice, tomato juice, beef extract, potato extract, coconut water (CW) and banana extract (BE) are commonly added to culture media for orchid tissue culture. Beneficial effects of those organic additives added to medium on seedling growth have been reported in many orchid species like *Aranda* Deborah (Goh and Wong, 1990), *V. coerulea* (Seeni and Latha, 2000), *V. spathulata* (Decruse *et al*., 2003), *Dendrobium tosaense* (Lo *et al*., 2004), *Paphiopedilum* orchid (Ng and Saleh, 2011), *Renanthera* Tom Thumb ‘Qilin’ (Wu *et al*., 2012).

As early as 1954, Steward and Simmonds reported that substances that stimulate cell divisions in carrot cells are presented in the formative layers of banana fruit, and that alcoholic extracts of receptive ovaries were about 38% as effective as

whole coconut milk. Banana pulp is a rich source of natural cytokinins which inhibit culture initiation but promotes differentiation and growth of shoots at later stages (Withner, 1974; Arditti and Ernst, 1993). Substances of natural cytokinins, as well as auxins and gibberellins (Khalifah, 1966a, b) have been found in banana fruits. Promotory effect of BE on increase in number and growth of seedling roots was observed in *Cattleya aurantiaca* (Arditti, 1968) and *D. tosaense* (Lo *et al*., 2004) seedling growth. Vyas *et al*. (2009) have reported significantly increase in more number of roots per shoot, as well as length of roots of *Dendrobium lituiflorum* Lindl. on KC medium supplemented with BE.

Coconut water is a complex additive which contains many nutritional and/or hormonal substances (Dix and Van Staden, 1982). It has a marked growth promoting effect on a variety of plant tissues. It can incorporate in culture media with no loss of activity as a result of autoclaving or by exposure to ordinary laboratory conditions for limited periods of time (Mauney *et al*., 1952). It is commonly added to culture media to stimulate callus or protocorm formation and the range usually used is 10-25% (v/v). The promotory effect with regard to morphogenesis is related to its growth regulator content specially cytokinins (Letham, 1974; Van Staden and Drewes, 1975). Goh and Wong (1990) achieved PLB formation on inflorescence tip explants of the monopodial orchid hybrid *Aranda* 'Deborah' using CW and BAP supplemented KC medium. Lakshmanan *et al*. (1995) reported the formation of PLB from thin sections of shoot tips of *Aranda* Deborah on treatment with CW (5-25%, v/v). CW also increased the rate of survival of thin section explants. The attempt to replace CW with BAP and sugar by the same workers altered the course of development from PLB formation to callus production which implies that CW plays a regulatory role in PLB differentiation.

### 1.5 Automation of orchid mass propagation

One of the recent advances in the field of plant tissue culture is the culture of cells, somatic embryos, organogenetic propagules like bulblets, corms, microtubers or shoots in liquid suspension in bioreactors. The use of bioreactors for micropropagation helps in scaling-up of production and decreases the cost of production (Park *et al*., 2000). Aitken-Christie *et al*. (1995) suggested the possibility of mass production of protocorms in bioreactors.

An immensely useful technique for rapid mass production of orchid PLB from leaf segments was reported by Park *et al*. (2000). They established a protocol for multiplication of *Phalaenopsis* hybrid PLB formed on leaf segments in a bioreactor and further conversion of the PLB into plantlets. About 18,000 PLB were harvested from 20 g of inoculum in 2-litre Hyponex medium in a temporary immersion culture with attached charcoal filter after eight weeks of inoculation. This protocol can be used for other orchids also with little modification thereby reducing the space, labour and cost of mass propagation of orchids. Mass proliferation of PLB of *Doritaenopsis* induced from lateral buds on flower stalks in liquid medium in an air-driven periodic immersion (API) bioreactor was reported by Liu *et al*. (2001). Wu *et al*. (2007) formulated a simple protocol for *in vitro* mass propagation of *Anoectochilus formosanus* using an automated low cost bioreactor system by a two-step culture. Shoot tips were cultured in 3-litre balloon type bioreactor (BTBB) with an aeration volume of 0.006 vvm and then transferred to 3-litre BTBB containing a 0.75-litre Hyponex medium with 2 g/l peptone and 0.5 g/l activated charcoal for shoot elongation and rooting. Mass production of orchid plantlets using bioreactors is very beneficial for orchids like *Anoectochilus* which have secondary metabolites with medicinal properties and commonly collected from the wild by both common man and pharmaceutical companies.

**1.6 Problems in orchid micropropagation**

Though orchid micropropagation has shown spectacular development in the recent years, the wide spread use of micropropagation is believed to be still handicapped due to some major problems that have been highlighted below.

A problem usually encountered when dealing with explants isolated from mature plants is the release of exudates into the medium. Orchid cells in tissue culture exude a large quantity of phenolic compounds that become toxic to the cells when oxidized. Quick transfer of the explants to fresh media is often recommended to avoid possible inhibitory effects of exudates (Compton and Preece, 1986). Addition of activated charcoal and ascorbic acid to the medium can overcome the inhibitory effects of phenolic compounds released into the medium and have been generally used in culture media of some orchids, e.g. *R. imschootiana* (Seeni and Latha, 1992), *V. coerulea* (Seeni and Latha, 2000). Activated charcoal seems to adsorb the toxic

substances that may form in culture medium as a result of autoclaving or be released by the explants. It may also stimulate rooting by absorbing the toxins and excluding light from the medium (Paek and Murthy, 1977). Eymar *et al*. (2000) reported that the addition of AC in culture medium increased and maintained pH levels during culturing, increased the nitrogen uptake and improved growth and visual aspects of the explants and reduced the inhibitory effect of exogenous cytokinin on root growth. However, AC is likely to interfere with other additives as well. Ascorbic acid (vitamin C) has been also reported to inhibit or prevent the exudation of phenols as well (Arditti and Ernst, 1993).

It was found that TDZ (the adenine-type cytokinins like BAP) is more effective than other cytokinins in inducing shoot bud differentiation from various explants (Ernst, 1994; Nayak *et al*., 1997a, b). However, the drawback of using TDZ in regeneration studies includes difficulty in elongation and rooting of regenerated shoots. This may be due to the high cytokinin activity and persistence of TDZ in the tissue compared to other adenine-type cytokinins (Huetteman and Preece, 1993). Nayak *et al*. (1997a, b) overcame the problem of shoot elongation in *Acampe praemorsa* (Roxb.) Blatter and McCann., *Cymbidium aloifolium* (L.) Sw., *Dendrobium aphyllum* (Roxb.) Fisch. and *Dendrobium moschatum* (Buch.-Ham.) by incorporating an auxin (NAA) at lower concentrations along with TDZ or by transferring the shoot clumps to a medium containing different phytohormones (BAP and NAA).

Transplantation stage continues to be a major bottleneck in the micropropagation of many orchids. A substantial number of micropropagated plants do not survive after transferring from *in vitro* conditions to greenhouse or field environment. The greenhouse and field have substantially lower relative humidity, higher light level that is stressful to micropropagated plants compared to *in vitro* conditions. The benefit of any micropropagation system can, however, only is fully realized by the successful transfer of plantlets from tissue culture vessels to the ambient conditions found *ex vitro* (Hazarika, 2003). Acclimatization of most micropropagated plants can be hastened by *in vitro* hardening of plantlets or after transplantation under decreasing the transpiration rate by applying anti-transpirants including ABA or by increasing photosynthetic rate and elevated $CO_2$ concentration (Pospisilova *et al*., 1999). Development of photosynthetic capacity is very important for *in vitro* plantlets

and was shown to be improved by altering their environmental growth conditions, such as increasing light intensity, humidity, air temperature or $CO_2$ concentration in *Doritaenopsis* (Jeon *et al*., 2005). They reported that leaves of high light grown plants showed higher increase in wax formation than that on low light intensity. A good growing culture medium has some properties, such as maximum water holding capacity, porosity and drainage which is essential for proper growth and development of *in vitro*-raised seedlings of orchids. The survival percentage and growth performance of the seedlings in potting substrate consisting of brick : charcoal at the ratio of 2 : 1 mulched with moss (*Sphagnum* sp.) were found to be higher (80% survival) than that grown in potting substrate consisting of brick : charcoal : tree fern in the ratio 2 : 1 :1. Mulching of the planting medium with moss increases the water retaining capacity of the medium (Kishor *et al*., 2006). Franco *et al*. (2007) evaluated the effect of ten substrates, some organics (pine bark, coconut fibre and wood shavings), some inerts (polystyrene foam), vegetable coal and their combinations on morphometric and phenotypic traits in the hardening phase of *in vitro* grown plantlets of *Cattleya trianae*. Coconut fiber alone or mix in equal parts with pine bark and coal was the most efficient substrate with the highest survival rates. Hardened plants displayed characteristics like vigour, waxy texture and dark green leaves and velamen formation in the roots.

Long-term benefits of micropropagation, lies in the production of clonally uniform plants and hence somaclonal variation has to be discarded or minimized (Vijay Rani and Raina, 2000). High concentrations of plant growth regulators and long periods of culture are thought to be the main causes of variation in plants cultured *in vitro* (George and Sherrington, 1984). Chen *et al*. (1998) have reported considerable somaclonal variations in flower morphology, including colour and shape, occur in *Phalaenopsis* True Lady ‘‘B79-19’’ regenerants derived from tissue culture using molecular marker, random amplified polymorphic DNA (RAPD). In addition, biochemical traits, such as isozymes can help in the identification of somaclonal variations as a complement to monitoring morphological traits. Although the exact cause of mutations occurring in tissue cultured plants is not clearly known, the available evidence indicates that the use of pre-existing meristems (apical or axillary) as explant tissues, which minimize the requirement of growth regulators to induce growth

and development, may help to maintain clonal stability of plants derived *in vitro* to a great extent (Prakash *et al*., 1996).

### 1.7 Cryopreservation of orchids

Due to difficult to propagate vegetatively, mass propagation of *Phalaenopsis cornu-cervi* (Breda) Blume & Rchb. f. is limited. At present, *in vivo* field preservation is a major drain on time, manpower and space besides considerable risk of loss due to disease and environmental stress. Conservation of its germplasm through tissue culture requires frequent maintenance. Regular subcultures also increase the risk of tissue loss due to adverse culture conditions and somaclonal variation (Khoddamzadeh *et al*., 2010; Watt *et al*., 2009; Goncalves *et al*., 2010). To alleviate problems associated with culture maintenance, cryopreservation technique has been developed for the long-term conservation of several valuable germplasms. Cryopreservation offers long-term storage capability, high genetic stability along with minimal storage space, safety, repeatability and low maintenance requirements of germplasm (Engelmann, 1997; Pacheco *et al*., 2009; Hazubska-Przbyl *et al*., 2010).

Various approaches of cryopreservation, such as vitrification, encapsulation-dehydration, encapsulation-vitrification and air-drying have been developed and used with varying degrees of success to preserve diverse species of plants (Xue *et al*., 2008; Hazubska-Przybyl *et al*., 2010; Hua and Rong, 2010; Peng-Fei *et al*., 2012; Mohanty *et al*., 2012). Among of these cryopreservation procedures, the encapsulation-dehydration technique is easy to handle, and avoids the use of an expensive programmable freezer and the toxic effect of cryoprotectants, such as plant vitrification solution (PVS) (Tsai *et al*., 2009). Cryopreservation of seeds, shoot tips, protocorms and protocorm-like bodies (PLB) of many orchids has been successfully attempted for short and long-term conservation. However, vitrification and air-drying methods have resulted in both low and slow rates of regrowth of plantlets in case of orchids (Bian *et al*., 2002); whereas, encapsulation-dehydration and encapsulation-vitrification are comparatively more appropriate methods for orchid cryopreservation with higher success rate (Yin and Hong, 2009; Subramaniam *et al*., 2011). Encapsulated or artificial seeds have been reported as having advantages over non-encapsulated explants (Das *et al*., 2011) hence they have wider applications for germplasm storage in

cryopreservation studies. Though encapsulation-vitrification and encapsulation-dehydration are the most widely applicable methods of germplasm storage (Hirai and Sakai, 1999), Khoddamzadeh *et al*., (2011) reported that these methods can be used mainly for cryopreservation of shoot-tips and only few PLB (small vegetative parts of orchids that develop into whole plants). Limited number of studies has been reported on cryopreservation of protocorms and PLB of *Dendrobium* (Chen *et al*., 2001; Lurswijidjarus and Thammasiri, 2004; Pornchuti and Thammasiri, 2008; Yin and Hong, 2009; Anthony *et al*., 2010; Pouzi *et al*., 2011; Subramaniam *et al*., 2011). There are no reports on the cryopreservation of protocorms or PLB of *Phalaenopsis cornu-cervi* (Breda) Blume & Rchb. f. using encapsulation-dehydration and encapsulation-vitrification method.

**1.8 Artificial seed production**

Establishment of gene banks for *ex situ* conservation of plant germplasm in the form of field gene bank, seed gene banks, *in vitro* collection, and cryogenically preserved tissues is a common practice (Withers, 1983; Rao, 2004; Borner, 2006). Alginate encapsulation provides a viable approach for *in vitro* germplasm conservation as it combines the advantages of clonal multiplication with those of seed propagation and storage (Standardi and Piccioni, 1998; Ara *et al*., 2000).

Synthetic seed production technology via alginate encapsulation is presently considered as an efficient choice for both propagation and short-term to mid-term storage, in a number of commercially important orchids (Corrie and Tandon, 1993; Saiprasad and Polisetty, 2003; Mohanraj *et al*., 2009). This system provides a low-cost, high-volume propagation (Saiprasad and Polisetty, 2003). Other advantages of synthetic seeds include: easy handling all through storage and transportation, storage potential without any loss in viability and also upholding of clonal property of the regenerating plantlets (Ghosh and Sen, 1994; Germana *et al*., 2011). Notably, alginate coat of encapsulated explants shield plant tissues from physical and environmental injury, reduces dehydration, and offers mechanical pressure to grip the explants inside gel matrix during storage (Ara *et al*., 2000). The use of PLB for synthetic seeds development in orchids has been proven to be the most efficient (Saiprasad and Polisetty, 2003) over other organs due to its superior regenerative character. In this

perspective, the intervention of synthetic seed technology could be handy for the exchange of germplasm of this elite hybrid between laboratories and flowering-potted plant industries because of the miniature bead size and due to easy handling of these structures. Throughout the past years, substantial efforts have been made for propagation and storage of PLB via encapsulation for a number of commercial orchid hybrids, such as *Dendrobium* 'Sonia', *Oncidium* 'Gower Ramsay' (Saiprasad and Polisetty, 2003) and orchid species *Cymbidium giganteum* Wall. (Corrie and Tandon, 1993), *Cattleya leopoldii* (Saiprasad and Polisetty, 2003), *Coelogyne breviscapa* Lindl. (Mohanraj *et al*., 2009), *Aranda* Wan Chark Kuan 'Blue' x *Vanda coerulea* Grifft. ex. Lindl. (Gantait *et al*., 2012).

In view of the importance of *Phalaenopsis cornu-cervi* (Breda) Blume & Rchb. f. and the advantages of the synthetic seed technology, the present research was intended to develop a competent protocol for synthetic seed production in *Phalaenopsis cornu-cervi* (Breda) Blume & Rchb. f., storage and consequent plant regeneration in order to facilitate distribution of propagation material, as well as, exchange of germplasm material. Consequently, the influence of different storage temperature and duration on germination and conversion of the capsules was assessed.

### 1.9 Research objectives

1) To develop protocols for *P. cornu-cervi* (Breda) Blume & Rchb. f. micro-propagation *in vitro*.
2) To study the techniques of cryopreservation methods on survival rate of cryopreserved protocorm and PLB.
3) To study the effects of different storage temperatures and time on germination of encapsulated PLB of *P. cornu-cervi* (Breda) Blume & Rchb. f.

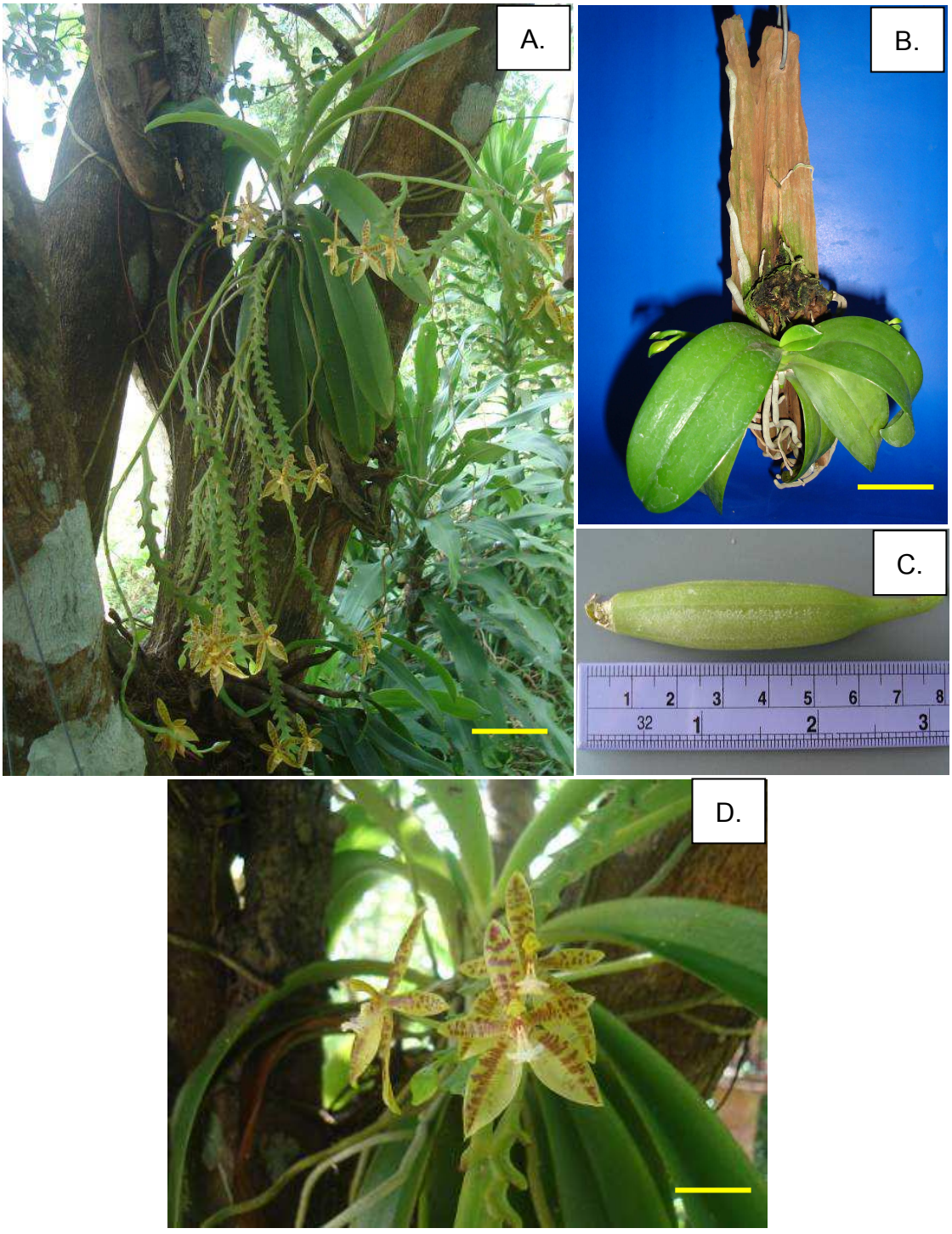



Figure 1. Characteristics of *Phalaenopsis cornu-cervi* (Breda) Blume & Rchb. f. (Bar=1 cm)

A, B: Five and one-year-old plant of *P. cornu-cervi* (Breda) Blume & Rchb. f., respectively.

C: An immature pod of *P. cornu-cervi* (Breda) Blume & Rchb. f. (6 MAP).

D: Flowers of *P. cornu-cervi* (Breda) Blume & Rchb. f.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1 Micropropagation of *Phalaenopsis cornu-cervi* (Breda) Blume & Rchb. f.

### 2.1.1 Seed germination, protocorm formation and plantlet development.

Immature pods of *P. cornu-cervi* (Breda) Blume & Rchb. f. were collected at 6 months after pollination (MAP). Each pod was cleaned by washing with running tap water for a few minutes, subsequently soaking in 95% ethanol and flaming for a few seconds. The pods were longitudinally cut into half on a sterile Petri dish. The seeds were aseptically sown on Murashige and Skoog (MS, 1962) medium supplemented with 15% (v/v) coconut water (CW) in order to induce protocorms from seed germination. The cultures were incubated for 3 months at 25±1ºC under a 16 hour photoperiod with light supplied by cool-white fluorescent lamps at an intensity of 60 µmol $m^{-2}$ $s^{-1}$ photosynthetic photon flux density (PPFD). After 3 months of culture, small plantlets were subcultured to MS medium supplemented with 15% (v/v) coconut water (CW) and 0.2% (w/v) activated charcoal (AC). Developments of plantlets from seeds were investigated.

### 2.1.2 Study on the effects of culture media on growth and PLB induction from protocorms *in vitro.*

Protocorms derived from seeds germinated for 49 days in experiment 2.1.1 were cultured in three culture media; MS liquid medium, Vacin and Went (VW, 1949) liquid medium and New Dogashima (ND, 1993) liquid medium (Tokuhara and Mii, 1993) either with or without 15% (v/v) coconut water (CW). The pH of MS, VW and ND liquid medium were adjusted to 5.7, 5.0 and 5.2, respectively with 1 N KOH or 1 N HCl prior to autoclaving for 15 minutes at 121°C. The cultures were incubated at 25±1ºC under 60 µmol $m^{-2}$ $s^{-1}$ for 16 hour photoperiod. After 2 months of culture, growth and development of PLB in all culture media were investigated and statistically compared among culture media.

2.1.3 Study on the effects of ascorbic acid and culture media on PLB induction from bisected and protocorms *in vitro.*

Bisected protocorms and protocorms at Growth Index3 (GI3) (Arditti and Heaky, 1979) were cultured in ND or VW liquid medium supplemented with ascorbic acid at various concentrations. All culture media supplemented with 15% (v/v) coconut water (CW). The pH of both culture media was adjusted to 5.2 and 5.0, respectively with 1 N KOH or 1 N HCl prior to autoclaving for 15 minutes at 121°C. The cultures were incubated at 25±1ºC under 60 µmol $m^{-2}$ $s^{-1}$ for 16 hour photoperiod. After 3 weeks of culture, percentage of PLB formation, number of PLB per explant and fresh weight (g) of PLB in all culture media were investigated and statistically compared.

2.1.4 Study on the effects of starting explants and PGRs on PLB formation.

Immature pods of *P. cornu-cervi* (Breda) Blume & Rchb. f. were collected at 6 MAP and aseptically grown in *in vitro* conditions in the same method as described in experiment 2.1.1. After 2 to 3 months of culture, germinated protocorms at GI3 (Arditti and Heaky, 1979) and cluster of two-leaf seedlings were obtained. Four starting explants; protocorms, bisected protocorms which were longitudinal cut into half, wounded protocorms which were prepared by hand strafing of the protocorms with a sharp razor blade, and leaf segments were used as starting explants for proliferation of PLB.

The basal medium used in this study was half-strength MS (½ MS) medium supplemented with 2% (w/v) sucrose and different concentrations of α-naphthalene acetic acid (NAA) (0, 0.1 and 1.0 mg/l), thidiazuron (TDZ) (0, 0.1, 1.0 and 3.0 mg/l) and benzyladenine (BA) (0.1, 1.0, 3.0 and 10.0 mg/l) and 15% (w/v) coconut water (CW). These PGRs were used alone or in combination and added prior to autoclaving. The culture media were solidified with 0.75% (w/v) agar-agar (commercial grade). The pH of the media was adjusted to 5.7 with 1 N KOH or 1 N HCl prior to autoclaving for 15 minutes at 121°C. All types of explants were placed on the surfaces of ½ MS medium and maintained at 25±1 °C under a 16 hour photoperiod with light supplied by cool-white fluorescent lamps at an intensity of 10 µmol $m^{-2}$ $s^{-1}$ PPFD.

The percentage of PLB formation and the mean number of PLB per explant were scored and compared statistically after 45 days of culture.

2.1.5 Study on the effects of culture media, concentrations of sucrose and activated charcoal on plantlet growth.

Six-month-old green pods obtained from self-pollination were collected from 5-year-old plants of *P. cornu-cervi* (Breda) Blume & Rchb. f. The seeds were aseptically sown on MS medium with 3% (w/v) sucrose and 0.75% (w/v) agar-agar (commercial grade) in bottles, each containing 25 ml of culture medium. This medium was supplemented with 15% (v/v) coconut water (CW) to induce protocorms. All cultures were maintained at 25±1°C under a 16 hour photoperiod with cool-white fluorescent lamps at an intensity of 10 µmol $m^{-2}$ $s^{-1}$ PPFD. After 2 to 3 months of culture, these seeds germinated into protocorms at GI3 (Arditti and Heaky, 1979) (about 5 mm-long). These wounded protocorm segments which were prepared by hand strafing of the protocorms with a sharp razor blade were cultured on ½ MS medium supplemented with 0.1 mg/l NAA and 0.1 mg/l TDZ for induction of PLB. The PLB were excised and transferred to ND medium or MS medium supplemented with 0, 2, 3 or 4% (w/v) sucrose with or without 0.2% (w/v) activated charcoal (AC) for plantlet regeneration. All culture media were supplemented with 15% (v/v) coconut water. The culture medium was solidified with 0.70% (w/v) agar-agar (commercial grade). The pH of the ND and MS medium were adjusted to 5.2 and 5.6, respectively with 1 N KOH or 1 N HCl prior to autoclaving for 15 minutes at 121°C. All types of explants were placed on the surfaces of these media and the cultures were maintained at 25±1°C under a 16 hour photoperiod of cool-white fluorescent lamps at an intensity of 10 µmol $m^{-2}$ $s^{-1}$ PPFD.

The survival percentage [ (number of survival plantlets/number of total PLB inoculated) x 100], fresh weight, plantlet height, number of leaves per plantlet, leaf length, leaf width, number of roots per plantlet and root length were recorded and statistically compared after 5 months of culture using completely randomized design (CRD). Analysis of variance and Duncan' s multiple range test were used for comparison among treatment means. At least twenty cultures were raised for each treatment and all experiments were repeated two times.

After 6 months of culture, the plantlets with 2 cm in height consisted of 5-6 leaves and 4-6 roots were removed from bottles. Plantlets were rinsed thoroughly with tap water to remove residual nutrients and agar from the plant tissue. The plantlets were then transplanted to pots. The seedlings were grown in the greenhouse with about 60% shading and 80% relative humidity. The young plants were sprayed with water twice a day. The survival rate was recorded after 2 months of culture in the greenhouse.

2.1.6 Study on ploidy instability analysis using flow cytometry (FCM).

To analyze their ploidy stability of complete plantlets of *P. cornu-cervi* (Breda) Blume & Rchb. f. derived from wounded protocorms which were cultured on half-strength MS medium (Murashige and Skoog, 1962) supplemented with different concentrations of PGRs. Approximately 0.5 $cm^2$ of four months young leaves of plantlets from control plants or treated plants were cut and placed in a 90-mm Petri dish. These leaves pieces were soaked with 1 ml of an extraction buffer (50 mol/L Tris-HCl, 0.5% polyvinylpyrrolidone, 0.01% Triton-X, 0.63% sodium sulfite, pH 7.5) for 5 minutes and chopped with a sharp razor blade. After mixing leaf tissue and buffer together it was filtered through 50-µm nylon mesh. The nuclear suspension was stained with 100 µl of 0.1% propidium iodide (PI) solution and incubated for at least 5 minutes at room temperature. The fluorescent intensities of each sample were measured by an EPICS XL, equipped with a 488 nm argon laser with a long path filter (Beckman Coulter, Tokyo, Japan) according to Ishigaki *et al*. (2009).

2.1.7 Plant regeneration and transfer to the greenhouse.

After 6 months of culture, the plantlets with 2 cm in height consisted of 5-6 leaves and 4-6 roots were removed from bottles. Plantlets were rinsed thoroughly with tap water to remove residual nutrients and agar from the plant tissue. The plantlets were then transplanted to pots. The seedlings were grown in the greenhouse with about 60% shading and 80% relative humidity. The young plants were sprayed with water twice a day. The survival rate was recorded after 2 months of culture in the greenhouse.

2.1.8 Experimental Design and Data Analysis.

Experiments were performed in a complete randomized design (CRD). The data were analyzed by ANOVA using SPSS version 11.5 and the mean values were separated using Duncan's multiple range test (DMRT) at a 5% probability level.

## 2.2 Cryopreservation of *Phalaenopsis cornu-cervi* (Breda) Blume & Rchb. f.

2.2.1 Study on the effects of cryopreservation methods and ages of protocorm on survival rate of cryopreserved protocorms.

2.2.1.1 Plant materials.

Six-month-old green pods obtained from self-pollination were collected from 5-year-old plants of *P. cornu-cervi* (Breda) Blume & Rchb. f. Each pod was cleaned by washing with running tap water for a few minutes, subsequently soaked in 95% ethanol and flamed. The pods were longitudinally cut into half on a sterile Petri-dish and the seeds were aseptically sown on the surface of MS (Murashige and Skoog, 1962) medium supplemented with 3% (w/v) sucrose, 15% (v/v) coconut water (CW) and 0.75% (w/v) agar-agar (commercial grade) in bottles, each containing 25 ml of the medium. All cultures were maintained at 25±1°C under a 16 hour photoperiod with light supplied by cool-white fluorescent lamps at an intensity of 10 µmol $m^{-2}$ $s^{-1}$ photosynthetic photon flux density (PPFD). After 49 days and 2 months of culture, the seeds germinated into protocorms. These protocorms were used for cryopreservation.

2.2.1.2 Cryopreservation methods.

1) Directly plunged into liquid nitrogen (LN).

Protocorms derived from 49 days (1-2 mm protocorms size) and 2 months of culture (3-4 mm protocorms size) were placed in a 2.0 ml cryotube, then directly plunged into LN for 1 day in the dark. The cryotubes were taken out of LN tank and rapidly warmed in a water bath at 38±2°C for 2 minutes. Cryopreserved protocorms were cultured on regrowth medium which was ND medium with 4.0% (w/v) sucrose, 0.7% (w/v) agar, 15% (v/v) coconut water (CW) and 0.2% (w/v) activated charcoal (AC) and kept in the dark for 1 week. Cultures were again transferred to fresh regrowth medium and maintained at 25±1°C under a 16 hour

photoperiod with light supplied by cool-white fluorescent lamps at an intensity of 10 µmol $m^{-2}$ $s^{-1}$ PPFD. For control, non-frozen protocorms were cultured on regrowth medium for plantlet regeneration.

2) Encapsulation-vitrification method.

Protocorms derived from 49 days (1-2 mm protocorms size) and 2 months of culture (3-4 mm protocorms size) were selected and encapsulated in an alginate matrix composed of 3% (w/v) Na-alginate plus 0.4 M sucrose in ND liquid medium, adjusted to pH 5.2. Drops of this solution were dispensed with a pipette into 100 mM $CaCl_2$ solution supplemented with 0.4 M sucrose, and kept for 30 minutes at 25±1°C. Encapsulated protocorms (about 6-7 mm in diameter) were then removed from the solution and washed in sterile distilled water for 3 times. Encapsulated protocorms were then precultured in ND liquid medium supplemented with 0.3 M sucrose for 3 days on a rotary shaker at 110 rpm (25±1°C). Encapsulated protocorms were rapidly surface-dried by plating them on a sterilized filter paper and loaded in a loading solution (2.0 M glycerol and 0.4 M sucrose) for 60 minutes at 25±1°C, then dehydrated with PVS2 solution [30% (w/v) glycerol, 15% (w/v) ethylene glycol, and 15% (w/v) dimethyl sulfoxide (DMSO) and 0.4 M sucrose in ND liquid medium] at either 25±1°C or 0°C for 60 minutes.

10 beads of encapsulated protocorms were placed in a 2.0 ml cryotube, then directly plunged into LN for 1 day in the dark. The cryotubes were taken out of LN tank and rapidly warmed in a water bath at 38±2°C for 2 minutes. After thawing, the PVS2 was replaced with 0.5 ml of 1.2 M sucrose in ND solution (unloading solution) and kept at 25±1°C for 20 minutes prior to culture on regrowth medium and kept in the dark for 1 week. The cultures were then transferred to fresh regrowth medium and maintained at 25±1°C under a 16 hour photoperiod with light supplied by cool-white fluorescent lamps at an intensity of 10 µmol $m^{-2}$ $s^{-1}$ PPFD. For control, encapsulated protocorms were dehydrated with PVS2 at either 25±1°C or 0°C for 60 minutes, and then cultured directly on regrowth medium for plantlet regeneration.

3) Encapsulation-dehydration method.

Protocorms derived from 49 days (1-2 mm protocorms size) and 2 months of culture (3-4 mm protocorms size) were selected and encapsulated in an alginate matrix composed of 3% (w/v) Na-alginate plus 0.4 M sucrose in ND

liquid medium, adjusted to pH 5.2. Drops of this solution were dispensed with a pipette into 100 mM $CaCl_2$ solution supplemented with 0.4 M sucrose, and kept for 30 minutes at 25±1°C. Encapsulated protocorms (about 6-7 mm in diameter) were then removed from the solution and washed in sterile distilled water for 3 times. Beads were then precultured in ND liquid medium supplemented with 0.5 M sucrose for 2 days on a rotary shaker at 110 rpm (25±1°C). Subsequently, encapsulated protocorms were placed on a sterile filter paper laid in open Petri-dishes (9 cm in diameter) and exposed to sterile air-flow at 80 ft/min from the laminar air-flow cabinet at 25±1°C for 5 hours. After dehydration, 10 dried beads were placed in a 2.0 ml cryotube, then directly plunged into LN for 1 day in the dark. The cryotubes were taken out of LN tank and rapidly thawed in a water bath at 38±2°C for 2 minutes. Cryopreserved protocorms were cultured on regrowth medium and kept in the dark for 1 week. The cultures were then transferred to fresh regrowth medium and maintained at 25±1°C under a 16 hour photoperiod with light supplied by cool-white fluorescent lamps at an intensity of 10 µmol $m^{-2}$ $s^{-1}$ PPFD. For control, dehydrated beads by the above procedures were cultured directly on regrowth medium for plantlet regeneration.

2.2.1.3 Regrowth rate.

After 2 months of culture, regrowth rate was calculated on the basis of protocorms forming plantlets from total number of encapsulated protocorms.

2.2.1.4 Morphological study.

The plantlets with well-developed shoots and roots were selected and rinsed thoroughly with tap water to remove residual nutrients and agar from the plant tissue. The plantlets were transferred to plastic basket containing foams for 1 month and then transplanted to pots containing sphagnum moss. The plantlets were grown in the greenhouse, with about 60% shading and 80% relative humidity. The young plants were sprayed with water twice a day. After 0, 3 and 6 months of culture in the greenhouse, the survival rate, plantlet height, root length, number of roots and leaves/plantlet were recorded.

2.2.1.5 Ploidy stability analysis using flow cytometry.

To analyze their ploidy stability, 6-month-old plantlets developed from non-cryopreserved and cryopreserved protocorms were collected and subjected to

FCM analysis according to the method described by Ishigaki *et al*. (2009). Nuclei were analyzed in the same way as described in 2.1.6.

2.2.2 Study on the effects of dehydration time on survival rate of cryopreserved encapsulated PLB.

2.2.2.1 Plant materials.

PLB derived from one protocorm developed from one seed on ½ MS medium supplemented with 0.1 mg/l NAA in combination with 0.1 mg/l TDZ and 3% sucrose, pH at 5.7 were used as plant material. Cultures were maintained at 25±1°C under a 16 hour photoperiod with light supplied by cool-white fluorescent lamps at an intensity of 10 µmol $m^{-2}$ $s^{-1}$ PPFD for 2 months. PLB reaching diameter of 0.5 cm was used for the following experiments.

2.2.2.2 Encapsulation-dehydration method.

PLB were selected and encapsulated in an alginate matrix composed of 3% (w/v) Na-alginate plus 0.4 M sucrose in ND liquid medium, adjusted to pH 5.2. Drops of this solution were dispensed with a pipette into 100 mM $CaCl_2$ solution supplemented with 0.4 M sucrose, and kept for 30 minutes at 25±1°C. Encapsulated PLB (about 6-7 mm in diameter) were then removed from the solution and washed in sterile distilled water for 3 times and then were blotted dried with sterile filter paper. For osmotic desiccation, encapsulated PLB were placed on a sterile filter paper laid in open Petri-dishes (9 cm in diameter) and then exposed to sterile air-flow at 80 ft/min from the laminar air-flow cabinet at 25±1°C for 0-7 hours. After dehydration, 10 dried beads were placed in a 2.0 ml cryotube, then directly plunged into LN for 1 day in the dark. The cryotubes were taken out of LN tank and rapidly warmed in a water bath at 38±2°C for 2 minutes. Cryopreserved PLB were cultured on regrowth medium and kept in the dark for 1 week. The cultures were then transferred to fresh regrowth medium and maintained at 25±1°C under a 16 hour photoperiod with light supplied by cool-white fluorescent lamps at an intensity of 10 µmol $m^{-2}$ $s^{-1}$ PPFD. For control, dehydrated beads by the above procedures were cultured directly on regrowth medium for plantlet regeneration.

2.2.2.3 Water content determination.

Water content was determined for encapsulated PLB following dehydration in the laminar air-flow cabinet for 0-7 hours. Dry weight of the beads was measured after drying in the oven at 60°C for 1 day. The percentage of water content was calculated on the basis of the fresh weight.

2.2.2.4 Regrowth rate.

After 2 months of culture, regrowth rate was calculated on the basis of PLB forming plantlets from encapsulated PLB.

2.2.2.5 Morphological study.

The plantlets with well-developed shoots and roots were selected and rinsed thoroughly with tap water to remove residual nutrients and agar from the plant tissue. The plantlets were transferred to plastic basket containing foams for 1 month and then transplanted to pots containing sphagnum moss. The plantlets were grown in the greenhouse, with about 60% shading and 80% relative humidity. The young plants were sprayed with water twice a day. After 0, 3 and 6 months of culture in the greenhouse, the survival rate, plantlet height, root length, number of roots and leaves /plantlet were recorded.

2.2.2.6 Ploidy stability analysis using flow cytometry.

To analyze their ploidy stability, 6-month-old plantlets developed from non-cryopreserved and cryopreserved PLB were collected and subjected to FCM analysis according to the method described by Ishigaki *et al*. (2009). Nuclei were analyzed in the same way as described in 2.1.6

2.2.3 Study on the effects of cold-hardening and dehydration time on survival rate of cryopreserved encapsulated PLB.

2.2.3.1 Plant materials.

PLB derived from one protocorm germinated from one seed on ½ MS medium supplemented with 0.1 mg/l NAA in combination with 0.1 mg/l TDZ and 3% (w/v) sucrose, adjusted to pH 5.7 were used. The cultures were maintained at 25±1°C under a 16 hour photoperiod with light supplied by cool-white fluorescent lamps at an intensity of 10 µmol $m^{-2}$ $s^{-1}$ PPFD for 3.5 months. PLB reaching diameter of 0.5 cm was used for the following experiments.

2.2.3.2 Encapsulation-dehydration method.

PLB were selected and encapsulated in an alginate matrix composed of 3% (w/v) Na-alginate plus 0.4 M sucrose in ND liquid medium, adjusted to pH 5.2. Drops of this solution were dispensed with a pipette into 100 mM $CaCl_2$ solution supplemented with 0.4 M sucrose, and kept for 30 minutes at 25±1°C. Encapsulated PLB (about 6-7 mm in diameter) were then removed from the solution and washed in sterile distilled water for 3 times and then were blotted dried with sterile filter paper. Encapsulated PLB were then cold-hardened for 4 day at 8±1°C. For osmotic desiccation, the cold-hardened encapsulated PLB were placed on a sterile filter paper laid in open Petri dishes (9 cm in diameter) and then exposed to sterile air-flow at 80 ft/min from the laminar air-flow cabinet at 25±1°C for 0-210 minutes. After dehydration, 10 dried beads were placed in a 2.0 ml cryotube, then directly plunged into LN for 1 day in the dark. The cryotubes were taken out of LN tank and rapidly warmed in a water bath at 38±2°C for 2 minutes. Cryopreserved PLB were cultured on regrowth medium and kept in the dark for 1 week. The cultures were then transferred to fresh regrowth medium and maintained at 25±1°C under a 16 hour photoperiod with light supplied by cool-white fluorescent lamps at an intensity of 10 µmol $m^{-2}$ $s^{-1}$ PPFD. For control, cold-hardened and dehydrated beads were cultured directly on regrowth medium for plantlet regeneration. Ten beads were used for each treatment with three replicates.

2.2.3.3 Water content determination.

Water content was determined for encapsulated PLB following dehydration in the laminar air-flow cabinet for 0-210 minutes. Dry weight of the beads was measured after drying in the oven at 60°C for 1 day. The percentage of water content was calculated on the basis of the fresh weight.

2.2.3.4 Regrowth rate.

After 2 months of culture, regrowth rate was calculated on the basis of PLB forming plantlets from encapsulated PLB.

2.2.3.5 Morphological study.

The plantlets with well-developed shoots and roots were selected and rinsed thoroughly with tap water to remove residual nutrients and agar from the plant tissue. The plantlets were transferred to plastic basket containing foams for 1

month and then transplanted to pots containing sphagnum moss. The plantlets were grown in the greenhouse, with about 60% shading and 80% relative humidity. The young plants were sprayed with water twice a day. After 0, 3 and 6 months of culture in the greenhouse, the survival rate, plantlet height, root length, number of roots and leaves /plantlet were recorded.

2.2.3.6 Ploidy stability analysis using flow cytometry.

To analyze their ploidy stability, 6-month-old plantlets developed from non-cryopreserved and cryopreserved PLB were collected and subjected to FCM analysis according to the method described by Ishigaki *et al*. (2009). Nuclei were analyzed in the same way as described in 2.1.6.

2.2.4 Study on the effects of sucrose pretreatment and dehydration time on survival rate of cryopreserved encapsulated PLB.

2.2.4.1 Plant materials.

PLB derived from one protocorm germinated from one seed on ½ MS medium supplemented with 0.1 mg/l NAA in combination with 0.1 mg/l TDZ and 3% sucrose, adjusted to pH 5.7 were used. The cultures were maintained at 25±1°C under a 16 hour photoperiod with light supplied by cool-white fluorescent lamps at an intensity of 10 µmol $m^{-2}$ $s^{-1}$ PPFD for 3.5 months. PLB reaching diameter of 0.5 cm was used for the following experiments.

2.2.4.2 Encapsulation-dehydration method.

PLB were selected and encapsulated in an alginate matrix composed of 3% (w/v) Na-alginate plus 0.4 M sucrose in ND liquid medium, adjusted to pH 5.2. Drops of this solution were dispensed with a pipette into 100 mM $CaCl_2$ solution supplemented with 0.4 M sucrose, and kept for 30 minutes at 25±1°C. Encapsulated PLB (about 6-7 mm in diameter) were then removed from the solution and washed in sterile distilled water for 3 times and then were blotted dried with sterile filter paper. The beads were precultured in ND liquid medium supplemented with different concentrations of sucrose (control, 0.1 M, 0.3 M) at 25±1°C, for 1 day keeping on a rotary shaker at 110 rpm. For osmotic desiccation, encapsulated PLB were placed on a sterile filter paper laid in open Petri-dishes (9 cm in diameter) and then exposed to sterile air-flow at 80 ft/min from the laminar air-flow cabinet at 25±1°C for 0-7 hours.

After dehydration, 10 dried beads were placed in a 2.0 ml cryotube, then directly plunged into LN for 1 day in the dark. The cryotubes were taken out of LN tank and rapidly warmed in a water bath at 38±2°C for 2 minutes. Cryopreserved PLB were cultured on regrowth medium and kept in the dark for 1 week. The cultures were then transferred to fresh regrowth medium and maintained at 25±1°C under a 16 hour photoperiod with light supplied by cool-white fluorescent lamps at an intensity of 10 µmol $m^{-2}$ $s^{-1}$ PPFD. For control, the precultured and dehydrated beads by the above procedures were cultured directly on regrowth medium for plantlet regeneration.

2.2.4.3 Water content determination.

Water content was determined for encapsulated PLB following dehydration in the laminar air-flow cabinet for 0-7 hour. Dry weights of the beads were measured after drying in the oven at 60°C for 1 day. The percentage of water content was calculated on the basis of the fresh weight.

2.2.4.4 Regrowth rate.

After 2 months of culture, regrowth rate was calculated on the basis of PLB forming plantlets from encapsulated PLB.

2.2.4.5 Morphological study.

The plantlets with well-developed shoots and roots were selected and rinsed thoroughly with tap water to remove residual nutrients and agar from the plant tissue. The plantlets were transferred to plastic basket containing foams for 1 month and then transplanted to pots containing sphagnum moss. The plantlets were grown in the greenhouse, with about 60% shading and 80% relative humidity. The young plants were sprayed with water twice a day. After 0, 3 and 6 months of culture in the greenhouse, the survival rate, plantlet height, root length, number of roots and leaves /plantlet were recorded.

2.2.4.6 Ploidy stability analysis using flow cytometry.

To analyze their ploidy stability, 6-month-old plantlets developed from non-cryopreserved and cryopreserved PLB were collected and subjected to FCM analysis according to the method described by Ishigaki *et al*. (2009). Nuclei were analyzed in the same way as described in 2.1.6

2.2.5 Experimental Design and Data Analysis.

Experiments were performed in a complete randomized design (CRD). The data were analyzed by ANOVA using SPSS version 11.5 and the mean values were separated using Duncan's multiple range test (DMRT) at a 5% probability level.

## 2.3 Alginate-encapsulation, short-term storage and plantlet regeneration from PLB of *Phalaenopsis cornu-cervi* (Breda) Blume & Rchb. f.

2.3.1 Study on the effects of different storage conditions and intervals on their conversion ability of *Phalaenopsis cornu-cervi* (Breda) Blume & Rchb. f.

PLB of *P. cornu-cervi* (Breda) Blume & Rchb. f. about 0.5 cm in diameter were isolated individually from 3-month-old proliferating PLB-clusters. Individual PLB were dipped and drenched in 3% (w/v) sodium-alginate solution containing ND liquid medium with 2% (w/v) sucrose, free of calcium and plant growth regulator for 10 minutes. Aliquots of the alginate solution, each containing one PLB, were aseptically pipette out and gently dropped individually with Pasteur pipette into 100 mM sterile calcium chloride solution. The droplets containing PLB were then allowed to polymerize for 30 minutes to achieve alginate beads. The resulting beads (7-9 mm in diameter) were washed in sterile distilled water for 3 times. The encapsulated PLB were then placed in sterile Petri-dishes (ten beads/plate), and in different storey of a refrigerator at temperature of 4±1°C, 8±1°C and 25±1°C to be stored for 180 days. About 30 beads from each set stored in each temperature regime were taken out and cultured on ND medium supplemented with 4% (w/v) sucrose with 0.2% (w/v) activated charcoal (AC) every 15 days. The encapsulated PLB grew out in the medium rupturing the beads and were maintained there for development into complete plantlets.

2.3.2 Study on the ploidy instability analysis using flow cytometry (FCM).

To analyze their ploidy stability of complete plantlets of *P. cornu-cervi* (Breda) Blume & Rchb. f. derived from different storage temperatures and time of

encapsulated PLB. Nuclei of young leaves taken from two-month-old plantlets from control plants or treated plants were isolated by chopping the leaves. Nuclei were analyzed in the same way as described in 2.1.6.

### 2.3.3 Experimental Design and Data Analysis.

Experiments were performed in a completely randomized design (CRD). The data were analyzed by ANOVA using SPSS version 11.5 and the mean values were separated using Duncan's multiple range test (DMRT) at a 5% probability level.

# CHAPTER 3

# RESULTS

## 3.1 Micropropagation of *Phalaenopsis cornu-cervi* (Breda) Blume & Rchb. f.

### 3.1.1 Seed germination, protocorm formation and plantlet development.

The hand-pollinated orchid flowers were observed regularly and after 6 months of pollination, the green, well-developed capsules were collected from the greenhouse-grown plants (Figure 2A). The seeds in the fruits remained light brown in color at this time. The seeds were immediately germinated on MS agar medium supplemented with 15% (v/v) coconut water (CW) (Figure 2B).

Seed germination was observed after culture for 49 days (Figure 2C). The germinating seeds increased in size, swelled and turned green (Figure 2D-E). After transferring to MS medium supplemented with 15% (v/v) CW and 0.2% (w/v) activated charcoal (AC) all protocorms developed into shoots and roots (Figure 2F). After five-months of culture, these plantlets developed 5-6 leaves and 3-4 roots and ready to be transferred to pots (Figure 2G-I).

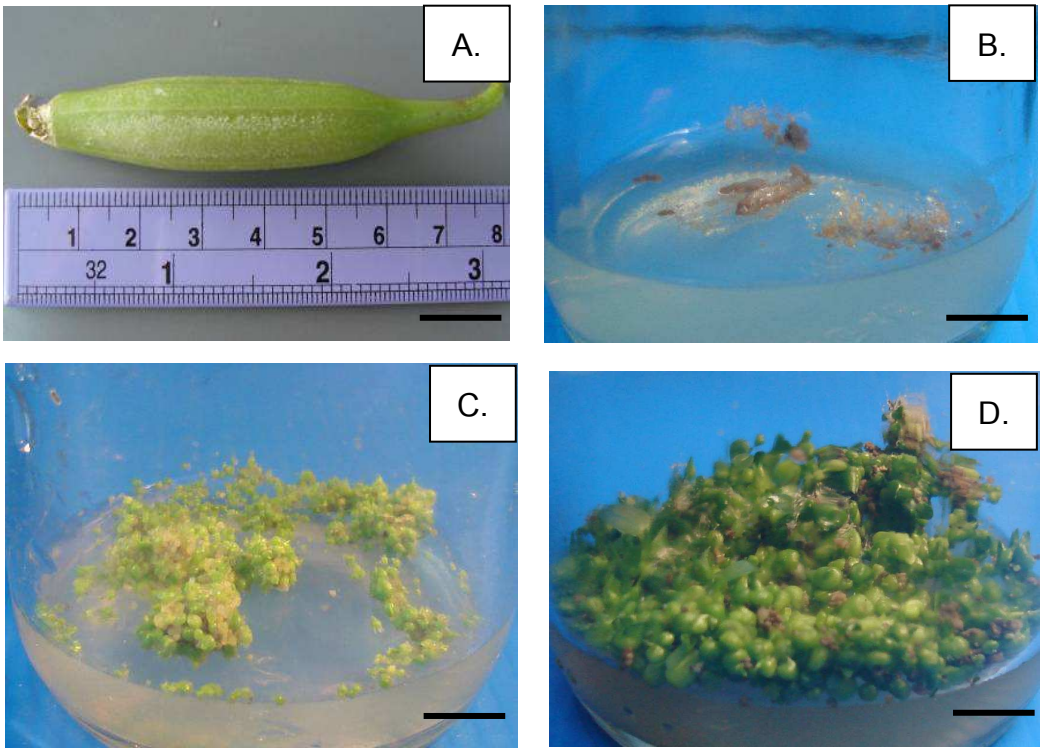



Figure 2. Development of plantlets from seeds of *P. cornu-cervi* (Breda) Blume & Rchb. f. germinated on MS medium. (Bar=1 cm)

A: An immature pod of *P. cornu-cervi* (Breda) Blume & Rchb. f. (6 MAP).

B: Seeds were sown on MS medium supplemented with 15% (v/v) coconut water (CW).

C: Asymbiotic germination of seeds after 49 days of culture on MS medium supplemented with 15% (v/v) CW.

D: Seeds germinated into protocorms after 2 months from sowing.

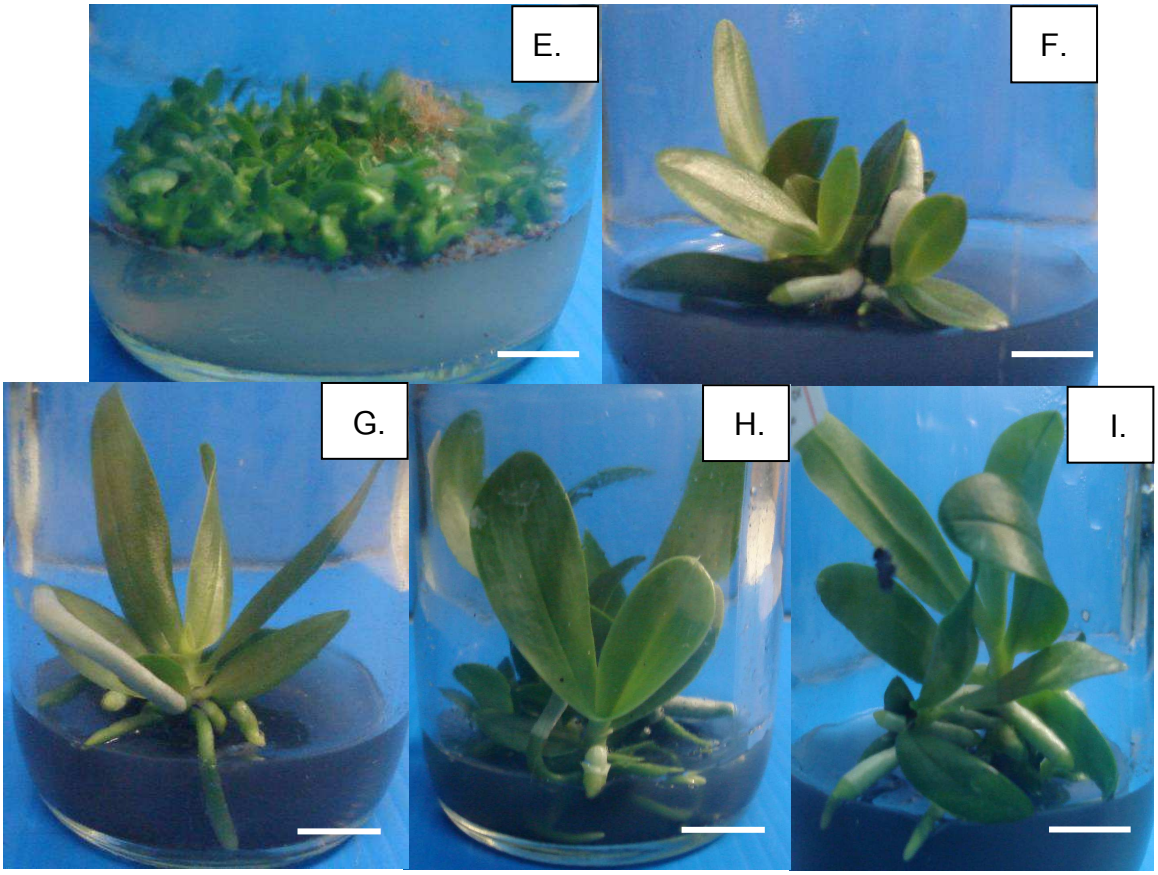



Figure 2. Development of plantlets from seeds of *P. cornu-cervi* (Breda) Blume & Rchb. f. germinated on MS medium. (Bar=1 cm)

E: Asymbiotic germination of seeds after 3 months of culture on MS medium supplemented with 15% (v/v) coconut water (CW).

F: Four-month-old seedlings on MS medium supplemented with 15% (v/v) CW and 0.2% (w/v) activated charcoal (AC).

G-I: Five-month-old seedlings on MS medium supplemented with 15% (v/v) CW and 0.2% (w/v) AC ready to be transferred to pots filled with sphagnum moss.

### 3.1.2 Effects of culture media on growth and PLB induction from protocorms *in vitro.*

After 3 weeks of culture, the highest growth of PLB or protocorms was observed in ND liquid medium with or without 15% (v/v) CW, followed by MS liquid medium with 15% (v/v) CW, MS liquid medium without CW, VW liquid medium with 15% (v/v) CW and VW liquid medium without CW, respectively (Figure 3). The protocorms were pale green to yellow in color when they were cultured in MS liquid medium without CW (Figure 3B). Similar results were found in VW liquid medium with 15% (v/v) CW or without CW (Figure 3C, D). However, these protocorms died after 4 weeks of culture.

At the fourth weeks of culture, protocorms derived from ND liquid medium with or without 15% (v/v) CW and MS liquid medium with 15% (v/v) CW were transferred to culture in ND liquid medium with 15% (v/v) CW or without CW or MS liquid medium with 15% (v/v) CW. All the culture media turned brown to black color due to the exudates released from cultured segments. According to this phenomenon, it is necessary to transfer all protocorms to new liquid ND medium supplemented with 15% (v/v) CW every 3 weeks for three times (Figure 4A, B). Upon transferring to the fresh culture medium, protocorms turned green and developed into young seedlings with 1-2 leaves and 1-2 roots after 8 weeks of culture (Figure 4C). Whole seedling cultures were transferred onto MS medium supplemented with 15% (v/v) CW and 0.2% (w/v) AC and kept under a 16-hour photoperiod. Under this condition, young seedlings continued developing, increased in size and further formed roots. Plantlets were obtained after three months of culture (Figure 5). These plantlets developed 5-6 leaves and 3-4 roots and ready to be transferred to plastic basket containing foams for acclimatization in the greenhouse (Figure 6). These plants grew vigorously, uniformly and finally developed into normal plants with 100% survival rate.

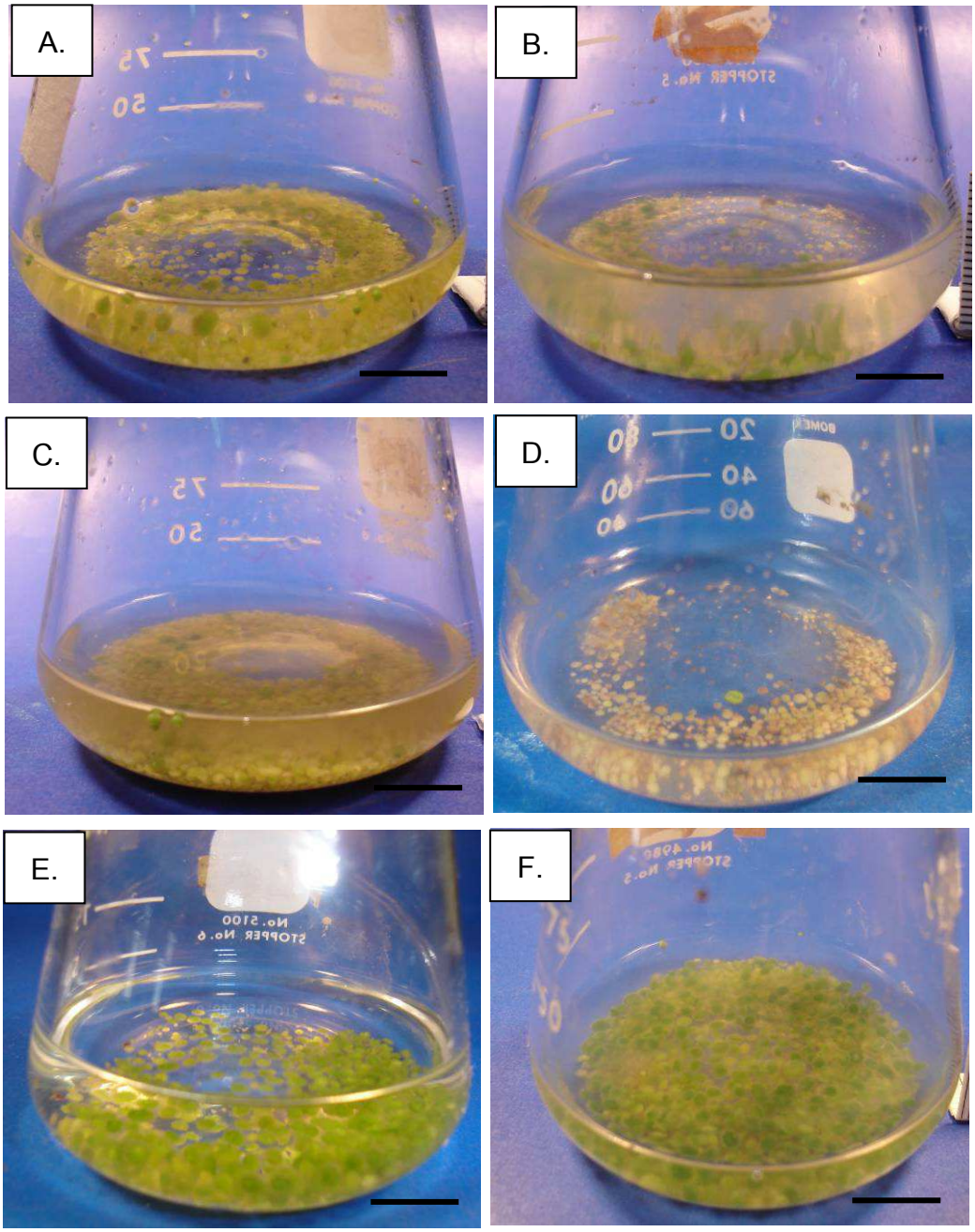



Figure 3. Development of protocorms in various liquid culture media with different components. (Bar = 1 cm)

A: MS medium with 15% (v/v) CW
B: MS medium without CW
C: VW medium with 15% (v/v) CW
D: VW medium without CW
E: ND medium with 15% (v/v) CW
F: ND medium without CW

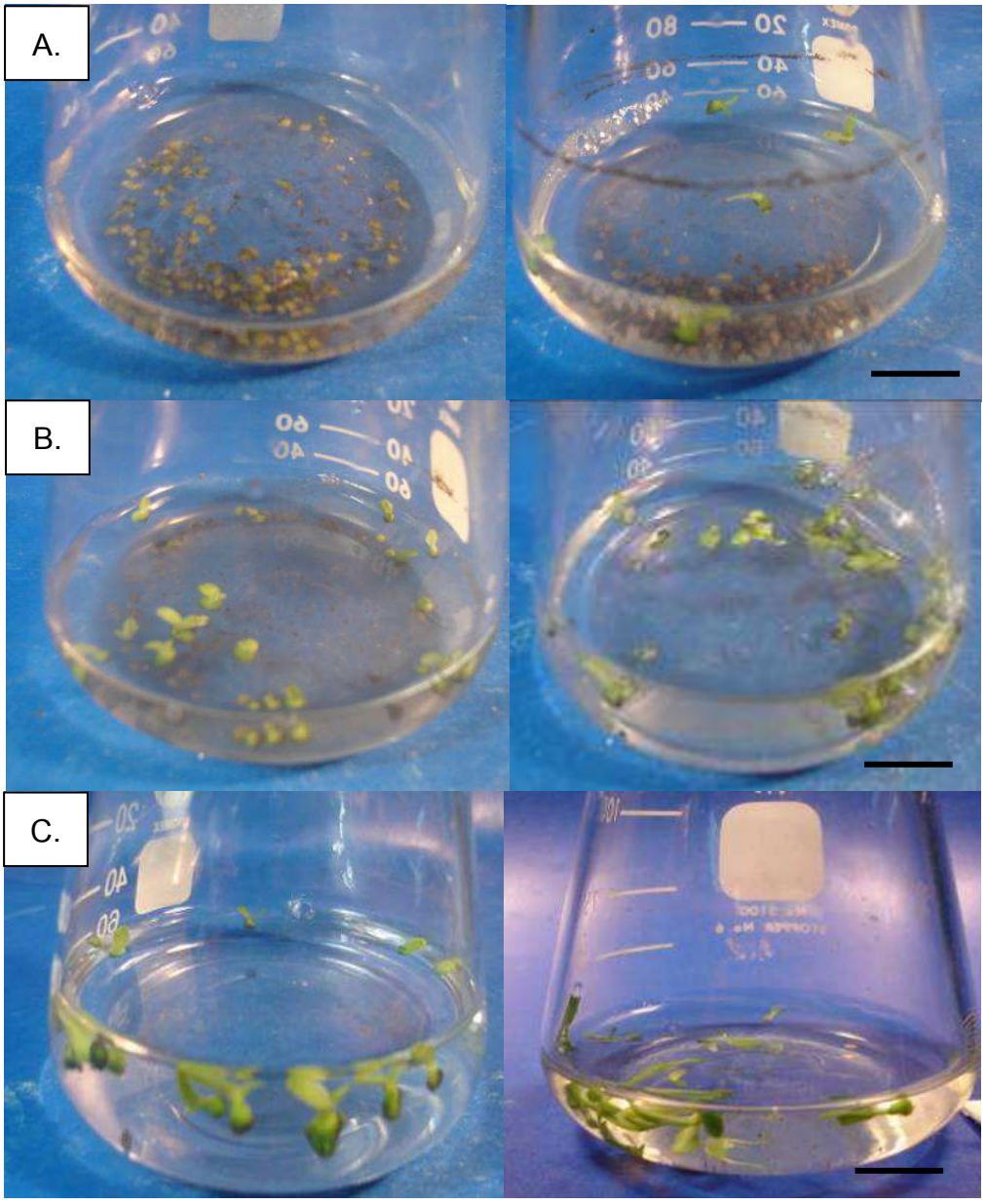



Figure 4. Development of protocorms in ND liquid medium with 15% (v/v) coconut water (CW) at different periods of culture. (Bar = 1 cm)

A: 5 weeks of culture B: 6 weeks of culture C: 8 weeks of culture

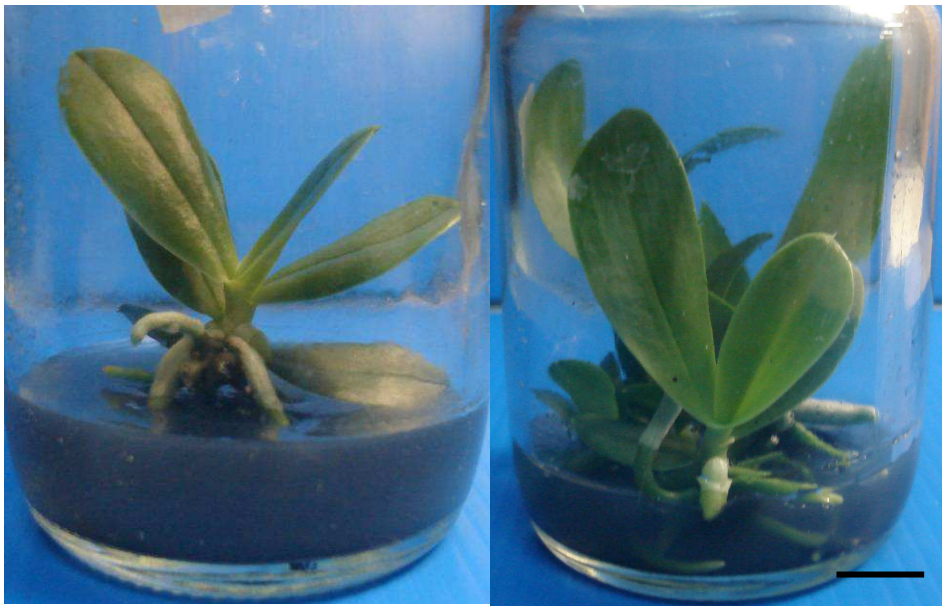


Figure 5. Three-month-old plantlets on MS medium supplemented with 15% (v/v) coconut water (CW) and 0.2% (w/v) activated charcoal (AC). (Bar = 1 cm)

Figure 6. Plantlets acclimatized in the greenhouse.

### 3.1.3 Effects of ascorbic acid and culture media on PLB induction from bisected and protocorms *in vitro.*

After culturing protocorms at GI3 (Arditti and Heaky, 1979) and bisected protocorms in ND or VW liquid media supplemented with ascorbic acid at various concentrations. The results showed that bisected protocorms became brown (Figure 7A), when cultured in ascorbic acid-free ND liquid medium. ND liquid medium supplemented with 100, 150 and 200 mg/l ascorbic acid, all protocorms turned black in all culture media (Figure 7B, C, D).

The bisected protocorms had pale green to yellow in color when they were cultured in VW liquid medium or VW liquid medium supplemented with 100 mg/l ascorbic acid (Figure 7E, 7F). VW liquid medium containing 150 mg/l ascorbic acid promoted PLB formation. This medium produced small PLB around the bisected protocorms (Figure 7G) and gave the highest fresh weight of new PLB at 0.1631 g/explant (Table 1). While all bisected protocorms turned black when cultured in VW liquid medium supplemented with 200 mg/l ascorbic acid (Figure 7H).

In case of culturing of protocorms, after 3 weeks of culture in ND liquid medium, protocorms at GI3 developed into young seedlings with 2 leaves and 2-3 white roots (Figure 8A). ND liquid medium supplemented with 100 mg/l ascorbic acid promoted the development of protocorms into young seedlings with 2 leaves consequently stopped growth at only GI5 (Arditti and Heaky, 1979). The base of protocorms turned to black (Figure 8B). While the ND liquid medium supplemented with 150 and 200 mg/l ascorbic acid promoted the development of protocorms into only GI4 (Arditti and Heaky, 1979) and after that all protocorms turned black and died (Figure 8C, D).

Protocorms cultured in VW liquid medium developed into GI3 (Arditti and Heaky, 1979) and proceeded through young seedlings with 2 leaves and 1-2 protuberance small white roots (Figure 8E).

Protocorms cultured in VW liquid medium containing 100 mg/l ascorbic acid grew in size and developed into only GI5 (Arditti and Heaky, 1979). The basal part of protocorms contained small tuber roots and the base of explants turned into black color (Figure 8F). Similar results were found in VW liquid medium supplemented with 150 and 200 mg/l ascorbic acid (Figure 8G, H). Protocorms proliferated into new

PLB around the base of explants. After another 50 days of culture, all bisected protocorms turned black when cultured in ND liquid medium containing all concentrations of ascorbic acid tested (Figure 9A-D). Bisected protocorms enlarged and more PLB were formed when cultured in VW liquid medium supplemented with 100 and 150 mg/l ascorbic acid (Figure 9F, G) while all bisected protocorms turned into black color when cultured in VW liquid medium supplemented with 200 mg/l ascorbic acid (Figure 9H).

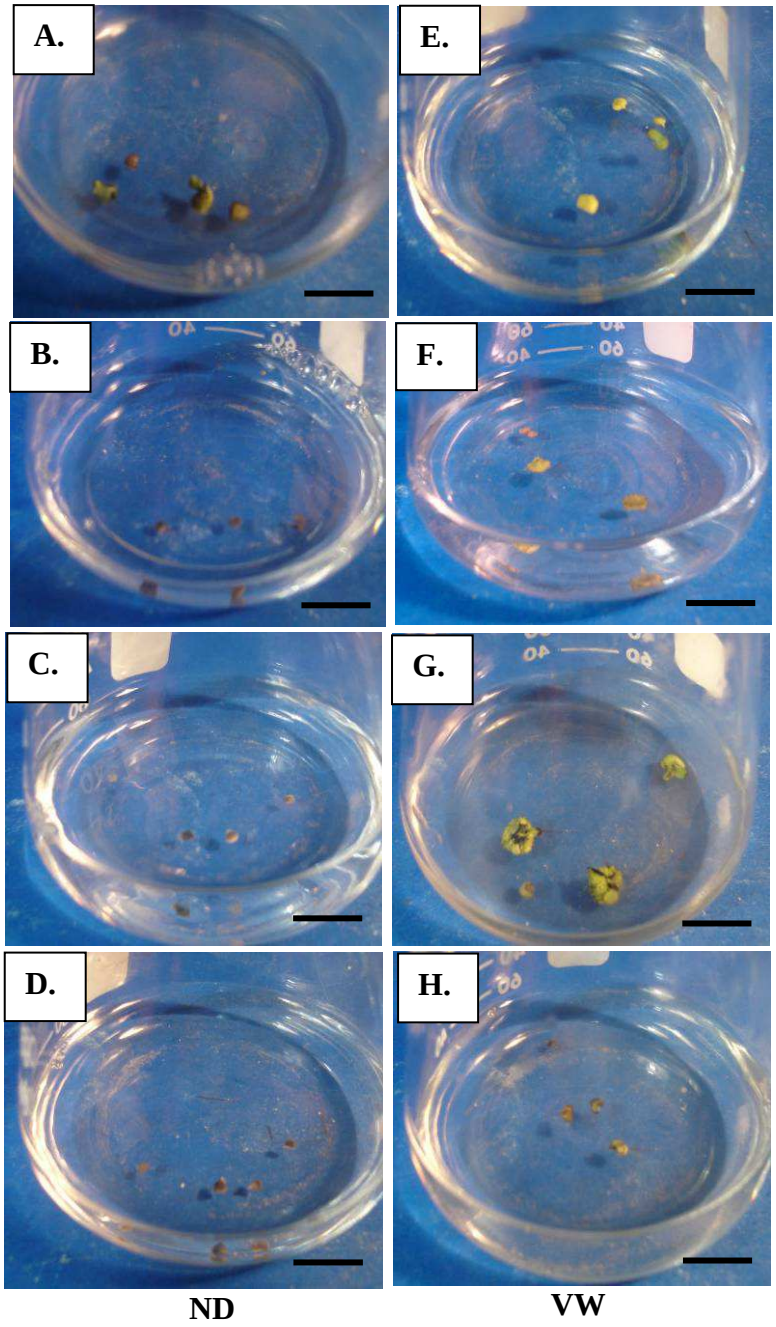



Figure 7. Growth of bisected protocorms cultured in various liquid culture media for 3 weeks. (Bar=1 cm)

A, E: Bisected protocorms in ND and VW liquid media without ascorbic acid.

B, F: Bisected protocorms in ND and VW liquid media supplemented with 100 mg/l ascorbic acid.

C, G: Bisected protocorms in ND and VW liquid media supplemented with 150 mg/l ascorbic acid.

D, H: Bisected protocorms in ND and VW liquid media supplemented with 200 mg/l ascorbic acid.

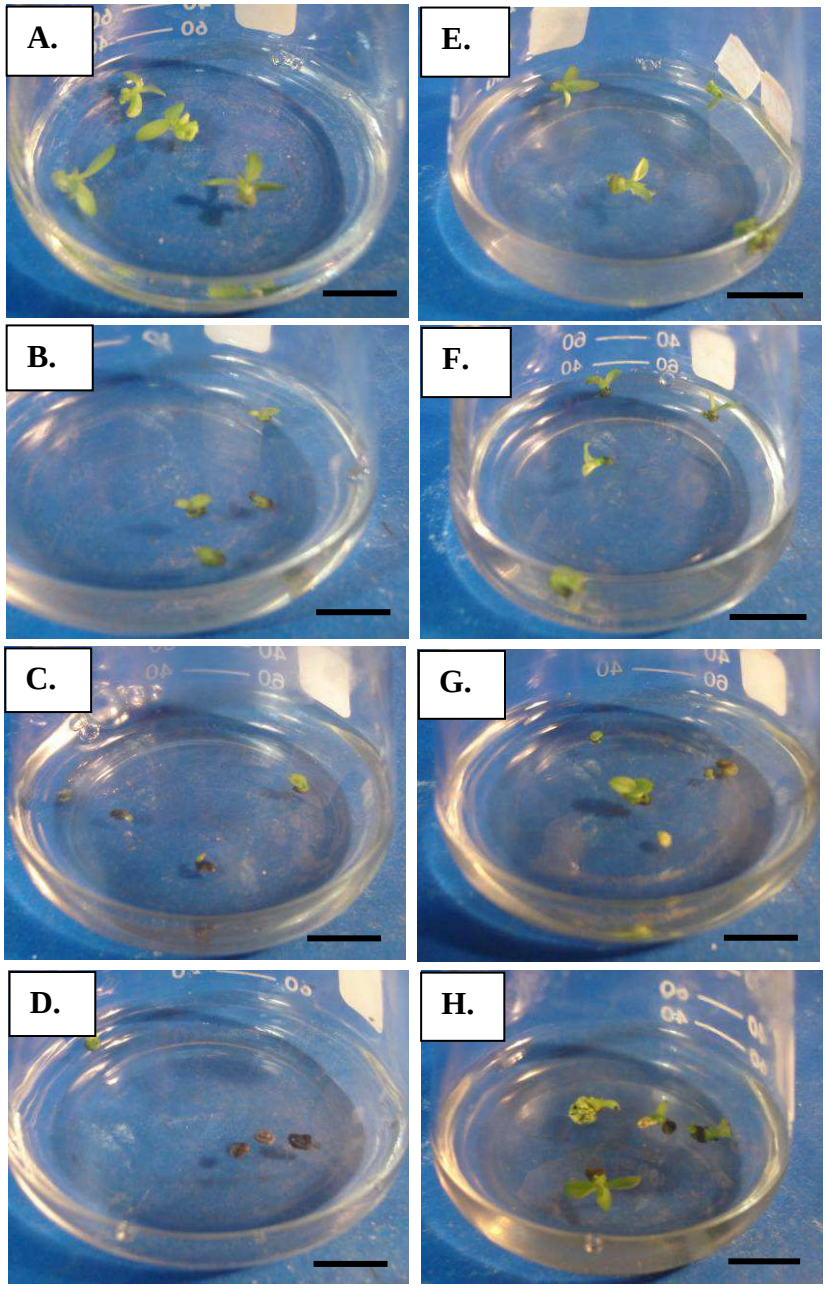



Figure 8. Growth and development of protocorms cultured in various liquid culture media for 3 weeks. (Bar=1 cm)

A, E: Protocorms in ND and VW liquid media without ascorbic acid.

B, F: Protocorms in ND and VW liquid media supplemented with 100 mg/l ascorbic acid.

C, G: Protocorms in ND and VW liquid media supplemented with 150 mg/l ascorbic acid.

D, H: Protocorms in ND and VW liquid media supplemented with 200 mg/l ascorbic acid.

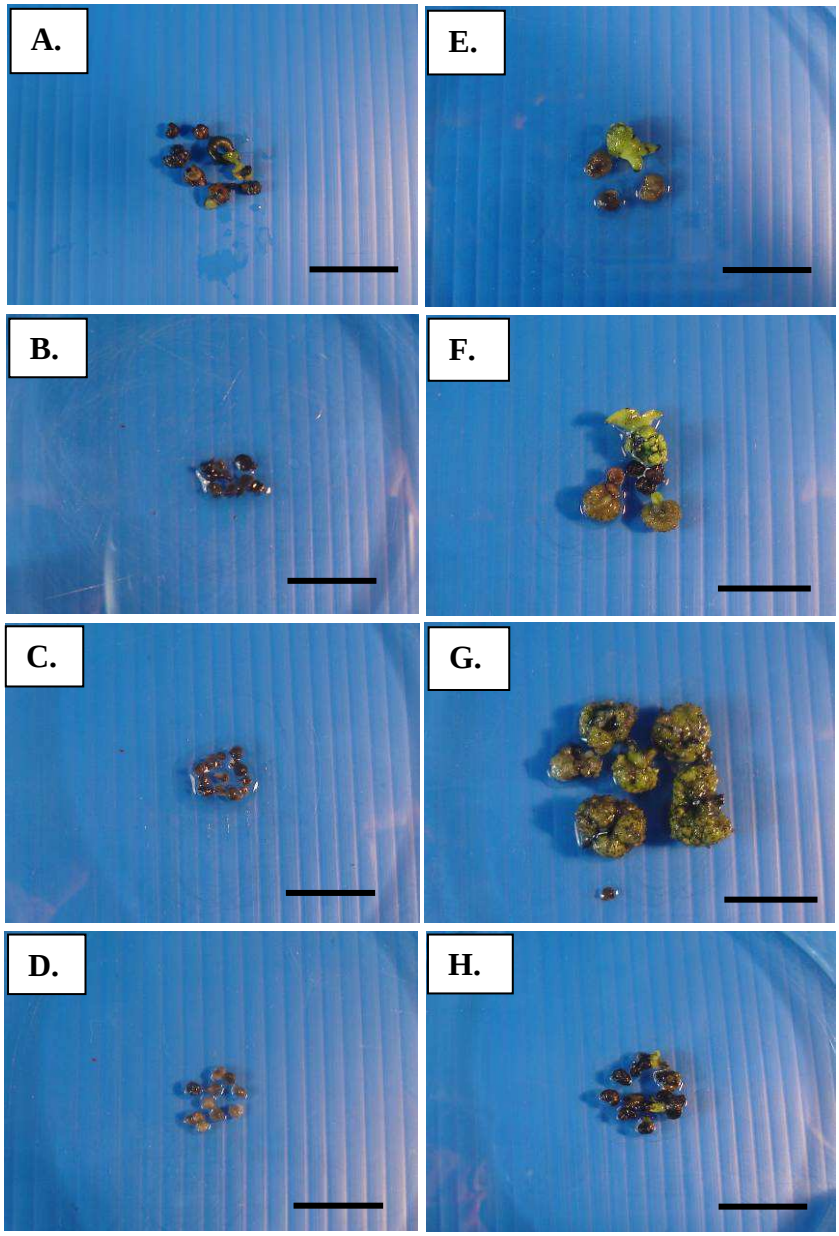



Figure 9. Growth and proliferation of bisected protocorms cultured in liquid media for 50 days. (Bar=1 cm)

A, E: Bisected protocorms in ND and VW liquid media without ascorbic acid.

B, F: Bisected protocorms in ND and VW liquid media supplemented with 100 mg/l ascorbic acid.

C, G: Bisected protocorms in ND and VW liquid media supplemented with 150 mg/l ascorbic acid.

D, H: Bisected protocorms in ND and VW liquid media supplemented with 200 mg/l ascorbic acid.

Protocorms increased in size and developed into seedlings when they were cultured in ND liquid medium without ascorbic acid (Figure 10A). All protocorms turned black and some protocorms died after being cultured in ND liquid medium supplemented with ascorbic acid at various concentrations (Figure 10B, C, D). Similar results were observed in VW medium. Protocorms developed into seedlings when they were cultured in VW liquid medium without ascorbic acid (Figure 10E). However, neoformation of PLB was formed in 100, 150 and 200 mg/l ascorbic acid containing VW liquid medium (Figure 10F, G, H).

Plantlets from both ND and VW liquid culture media caused browning or blackening of media after 50 days of culture (Figure 11). Obtaining results in this experiment indicated that ND medium supplemented with ascorbic acid at various concentrations was not effective in proliferation of bisected protocorms and protocorms of *P. cornu-cervi* (Breda) Blume & Rchb. f. (Table 1, Figure 9, 10). However, ascorbic acid containing VW medium at concentration 150 mg/l played a significant role in proliferation of the bisected protocorms (21 PLB/explants) and protocorms (14 PLB/explant) after being cultured for 50 days. At this stage of culture (50 days after being cultured) all protocorms produced healthy shoots.

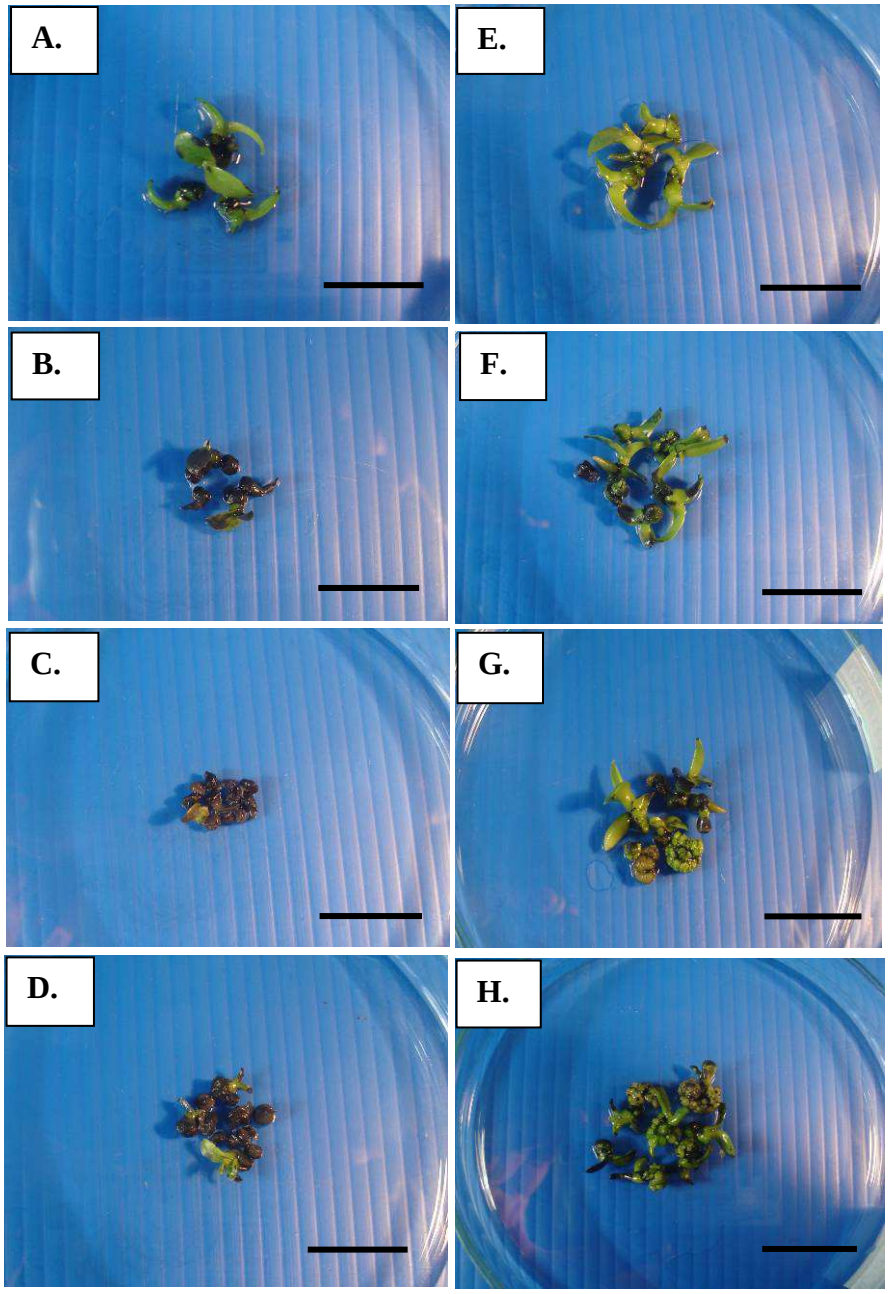



Figure 10. Protocorms were cultured in liquid media for 50 days. (Bar=1 cm)

A, E: Protocorms in ND and VW liquid media without ascorbic acid.

B, F: Protocorms in ND and VW liquid media supplemented with 100 mg/l ascorbic acid.

C, G: Protocorms in ND and VW liquid media supplemented with 150 mg/l ascorbic acid.

D, H: Protocorms in ND and VW liquid media supplemented with 200 mg/l ascorbic acid.

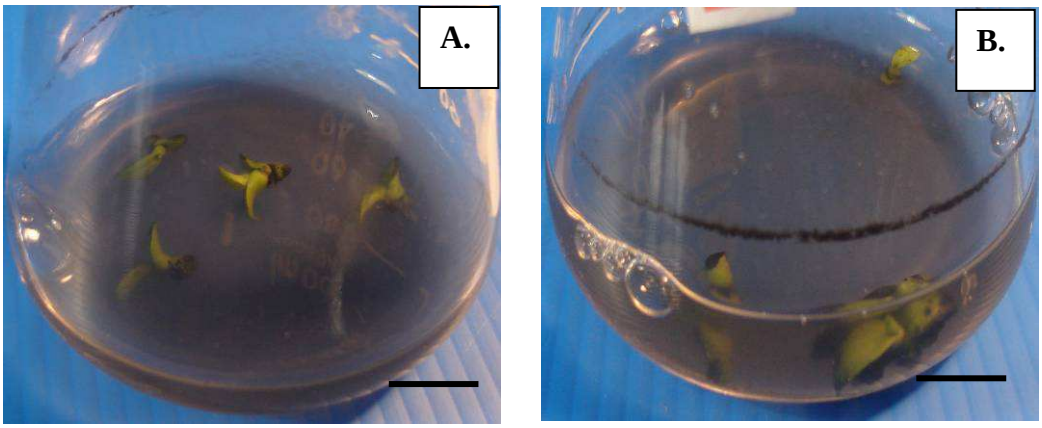



Figure 11. Seedling developed from protocorms after 50 days of culture. (Bar=1 cm)
A: ND liquid medium and B: VW liquid medium.

Table 1 Effects of culture media and ascorbic acid on PLB formation from protocorms and bisected protocorm segments of *P. cornu-cervi* (Breda) Blume & Rchb. f.

| Media + Ascorbic acid (As) (mg/l) | PLB formation (%) | No. of PLB per explant (Mean±S.E.) | Fresh weight (g) (Mean±S.E.) |
|---|---|---|---|
| Bisected protocorms | | | |
| ND | 0 | $0\pm0^{f}$ | $0\pm0^{h}$ |
| ND + As 100 | 0 | $0\pm0^{f}$ | $0\pm0^{h}$ |
| ND + As 150 | 0 | $0\pm0^{f}$ | $0\pm0^{h}$ |
| ND + As 200 | 0 | $0\pm0^{f}$ | $0\pm0^{h}$ |
| VW | 25 | $2.0\pm0.32^{e}$ | $0.0285\pm0^{f}$ |
| VW + As 100 | 50 | $5.4\pm0.27^{d}$ | $0.0340\pm0^{e}$ |
| VW + As 150 | 100 | $21.5\pm0.39^{a}$ | $0.1631\pm0^{a}$ |
| VW + As 200 | 0 | $0\pm0^{f}$ | $0\pm0^{h}$ |
| Protocorms | | | |
| ND | 0 | $0\pm0^{f}$ | $0\pm0^{h}$ |
| ND + As 100 | 0 | $0\pm0^{f}$ | $0\pm0^{h}$ |
| ND + As 150 | 0 | $0\pm0^{f}$ | $0\pm0^{h}$ |
| ND + As 200 | 0 | $0\pm0^{f}$ | $0\pm0^{h}$ |
| VW | 0 | $0\pm0^{f}$ | $0\pm0^{h}$ |
| VW + As 100 | 25 | $2.0\pm0.45^{e}$ | $0.0641\pm0^{d}$ |
| VW + As 150 | 50 | $14.4\pm0.45^{b}$ | $0.1269\pm0^{b}$ |
| VW + As 200 | 70 | $7.5\pm0.20^{c}$ | $0.0909\pm0^{c}$ |

The values followed by different letters within columns are significantly different from others at 5% level by DMRT.

3.1.4 Effects of starting explants and PGRs on PLB formation.

In the present study, seeds taken from the green pods were sown on the MS medium supplemented with 15% (v/v) coconut water (CW). Swelling and glistering of the embryos were first observed within 1.5 months after being cultured. The swelling of the embryo synthesized chloroplast, turned from yellow to yellowish green color and finally germinated into protocorms at GI4 stage (Figure 12A) and cluster of two-leaf seedlings (Figure 12B) were obtained after 2 to 3 months of culture on MS medium supplemented with 15% (v/v) CW and 0.2% (w/v) activated charcoal (AC). Both protocorms and seedlings were used as initial explants for proliferation of protocorm-like bodies (PLB) in the next experiment.

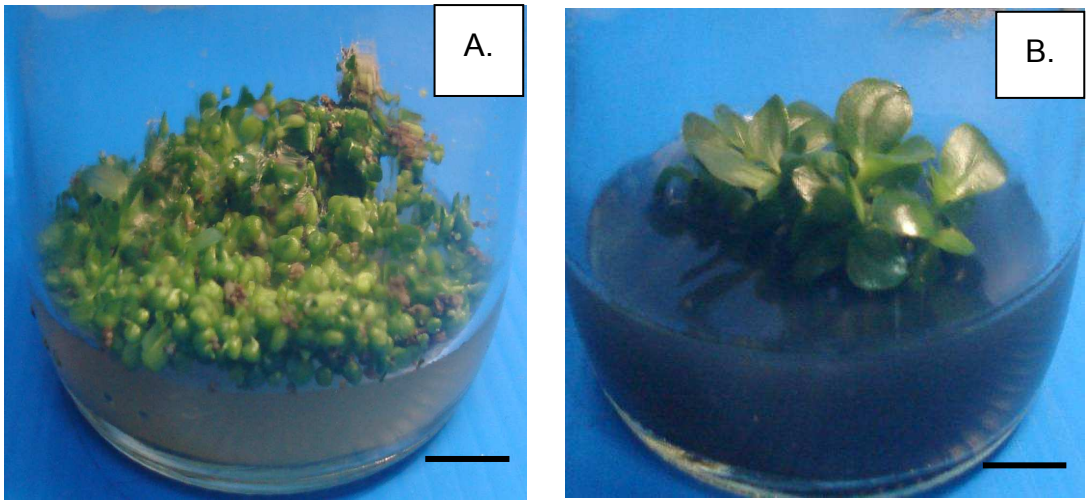



Figure 12. Aseptically germination of *P. cornu-cervi* (Breda) Blume & Rchb. f. from green pods at 6 months after pollination (MAP). (Bar=1 cm)

A: Asymbiotic germination of seeds after 2 months of culture on MS medium supplemented with 15% (v/v) coconut water (CW).

B: Three-month-old seedlings on MS medium supplemented with 15% (v/v) coconut water (CW) and 0.2% (w/v) activated charcoal (AC).

Among four different initial explants, bisected protocorms resulted in proliferation of new PLB and showed the highest percentage of protocorm browning as compared to the other explants (Table 3, Figure 14). Wounded protocorms resulted in proliferation of new PLB and showed the lowest percentage of protocorm browning as compared to other explants (Table 4). One cm in length of leaf explants swelled and remained green after 4 weeks of culture (Figure 16). Plant growth regulators played no important role in PLB induction from leaf segments (Table 5, Figure 16). After 6 weeks of culture, the leaf segments became necrotic and no PLB were formed. In the present study, leaf and bisected protocorm explants of *P. cornu-cervi* (Breda) Blume & Rchb. f. produced enormous phenolic compounds leading to the failure in PLB formation.

In our culture system, wounds caused by cutting played an important role in the formation of new protocorms. Wounding can bring about the production of a higher number of new protocorms at the basal part of initial wounded protocorms. However, in some cases, wounding caused the death of some of the wounded protocorms. In the PGR-free-half-strength MS medium, only about 30% of wounded protocorms developed into PLB (Table 4, Figure 15).

In the presence of TDZ or BA alone or TDZ in combination with NAA, PLB were directly formed from the surface of wounded protocorm segments after 4 weeks of culture. The response in PLB proliferation was improved considerably with the application of cytokinins, especially TDZ. Even TDZ alone at high concentration of 1.0 mg/l promoted a high percentage (100) and number of PLB formation (12.2 PLB/wounded protocorm) after 4 weeks of culture. Addition of NAA at 0.1 mg/l together with 0.1 mg/l TDZ gave the better number of all sizes of PLB (11-13 PLB/explants/wounded protocorm) (Table 4, Figure 15F).

After another 2 weeks of culture on the same PGR-containing media, the PLB enlarged and more PLB and shoots were formed (Figure 17A). When culturing wounded primary PLB on ½ MS medium supplemented with 0.1 mg/l NAA and 0.1 mg/l TDZ combination for 4-5 months, wounding can bring about the production of a higher number of new PLB on the original one (Figure 17B, C). Cytokinins at concentrations higher than 1.0 mg/l resulted in significant lower numbers of PLB (Table 4). TDZ was more effective than BA in inducing PLB formation.

Between the two cytokinins tested (BA, TDZ), TDZ was more efficient in the PLB induction from wounded protocorm segments than BA.

For plant regeneration, PLB from both the PGR-free medium and in the presence of NAA, TDZ or BA caused browning or blackening of media after 6 weeks of culture. Wounded primary PLB were cultured on ½ MS medium supplemented with 0.1 mg/l NAA and 0.1 mg/l TDZ combination for 4-5 months (Figure 17B, C). Upon transferring PLB to hormone-free medium supplemented with 15% (v/v) CW and 0.2% (w/v) AC and kept under a 16-h photoperiod for 5 months, PLB converted into healthy plants with well-developed 3-4 leaves and 3-4 roots per shoot (Figure 17D).

In this present study, AC in culture media seems to reduce exudates caused inhibition of proliferation because they enhanced protocorms development. The present study is the first report to show that PLB can be induced from wounded protocorm segments of *P. cornu-cervi* (Breda) Blume & Rchb. f. on media containing CW. The use of culture media supplemented with plant growth regulators and those solidified with agar were also effective for the efficient initiation of PLB and regeneration into plantlets. These protocols are simple, inexpensive and bring about the production of a large number of plantlets by germinating seeds and PLB induction in a short period of time. This research has demonstrated that propagation of *P. cornu-cervi* (Breda) Blume & Rchb. f. can be successfully carried out via PLB induction.

Table 2 Effects of NAA, TDZ and BA containing ½ MS medium on PLB formation from protocorms of *P. cornu-cervi* (Breda) Blume & Rchb. f. The frequency of embryo forming explants and the mean number of PLB per explant were scored after 45 days of culture.

| NAA | TDZ | BA | Browning | PLB formation | No. of PLB per explant | | |
|---|---|---|---|---|---|---|---|
| | | | | | Small Size | Medium size | Large size |
| (mg/l) | (mg/l) | (mg/l) | (%) | (%) | (Mean±S.E.) | (Mean±S.E.) | (Mean±S.E.) |
| Protocorms | | | | | | | |
| 0.0 | 0.0 | | 0 | 0 | 0.00±0.00$^{e}$ | 0.00±0.00$^{d}$ | 0 |
| | 0.1 | | 0 | 0 | 0.00±0.00$^{e}$ | 0.00±0.00$^{d}$ | 0 |
| | 1.0 | | 0 | 10 | 10.00±0.58$^{a}$ | 0.00±0.00$^{d}$ | 0 |
| | 3.0 | | 20 | 20 | 0.00±0.00$^{e}$ | 2.50±0.29$^{b}$ | 0 |
| 0.1 | 0 | | 0 | 0 | 0.00±0.00$^{e}$ | 0.00±0.00$^{d}$ | 0 |
| | 0.1 | | 0 | 20 | 2.00±0.00$^{c}$ | 2.00±0.00$^{c}$ | 0 |
| | 1.0 | | 0 | 20 | 2.00±0.00$^{c}$ | 0.00±0.00$^{d}$ | 0 |
| | 3.0 | | 20 | 10 | 3.00±0.58$^{b}$ | 0.00±0.00$^{d}$ | 0 |
| 1 | 0 | | 10 | 10 | 10.00±0.58$^{a}$ | 10.00±0.58$^{a}$ | 0 |
| | 0.1 | | 10 | 20 | 1.50±0.29$^{d}$ | 2.50±0.29$^{b}$ | 0 |
| | 1.0 | | 0 | 0 | 0.00±0.00$^{e}$ | 0.00±0.00$^{d}$ | 0 |
| | 3.0 | | 20 | 0 | 0.00±0.00$^{e}$ | 0.00±0.00$^{d}$ | 0 |
| 0 | 0 | 0.1 | 0 | 0 | 0.00±0.00$^{e}$ | 0.00±0.00$^{d}$ | 0 |
| | | 1.0 | 0 | 0 | 0.00±0.00$^{e}$ | 0.00±0.00$^{d}$ | 0 |
| | | 3.0 | 20 | 0 | 0.00±0.00$^{e}$ | 0.00±0.00$^{d}$ | 0 |
| | | 10.0 | 0 | 0 | 0.00±0.00$^{e}$ | 0.00±0.00$^{d}$ | 0 |

The values followed by different letters within columns are significantly different from others at 5% level by DMRT.

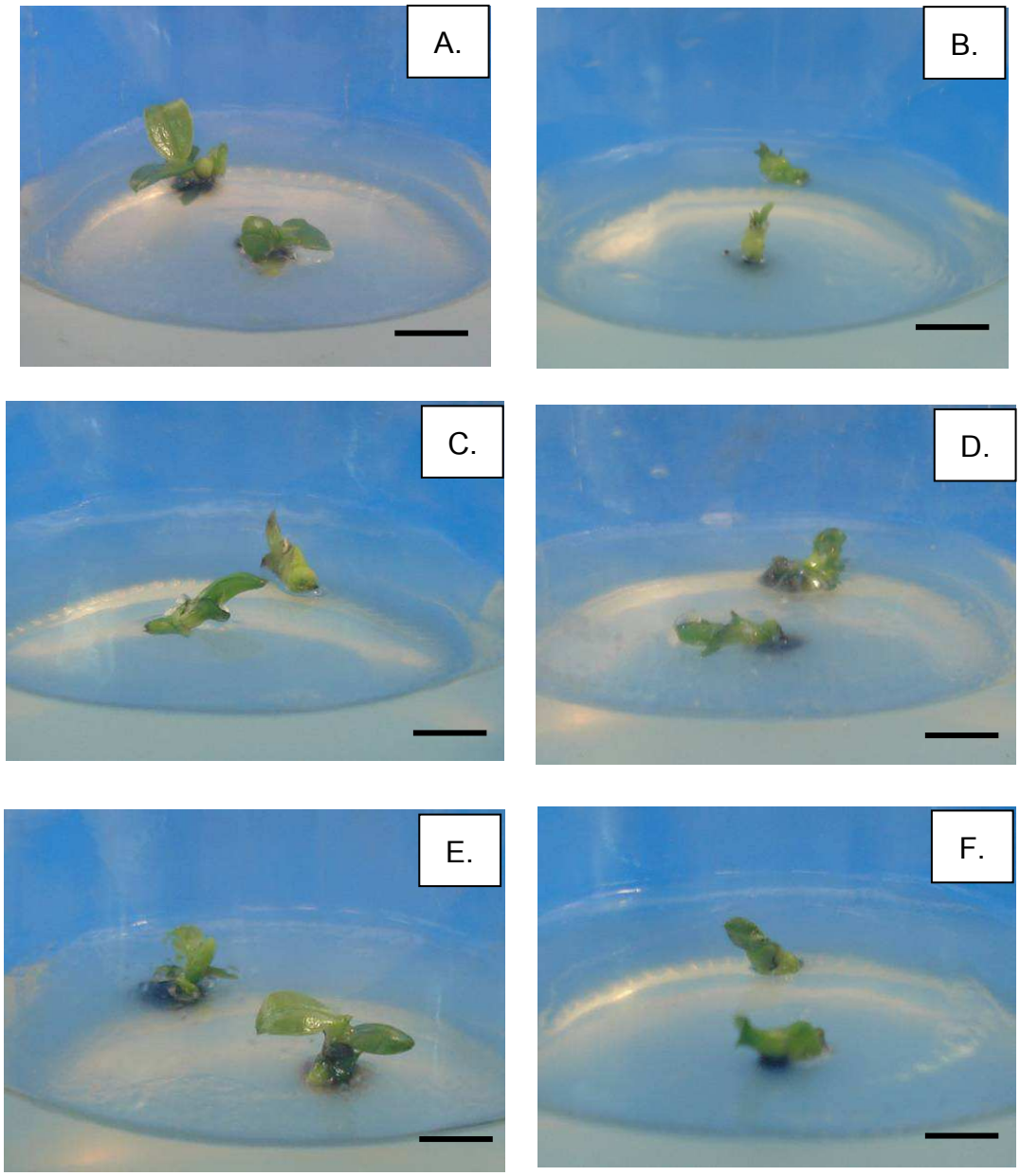



Figure 13. Development of new PLB from culturing protocorms on ½ MS medium containing PGRs at various concentrations. (Bar=1 cm)

A: 0 mg/l NAA + 0 mg/l TDZ

B: 0 mg/l NAA + 0.1 mg/l TDZ

C: 0 mg/l NAA + 1.0 mg/l TDZ

D: 0 mg/l NAA + 3.0 mg/l TDZ

E: 0.1 mg/l NAA + 0 mg/l TDZ

F: 0.1 mg/l NAA + 0.1 mg/l TDZ

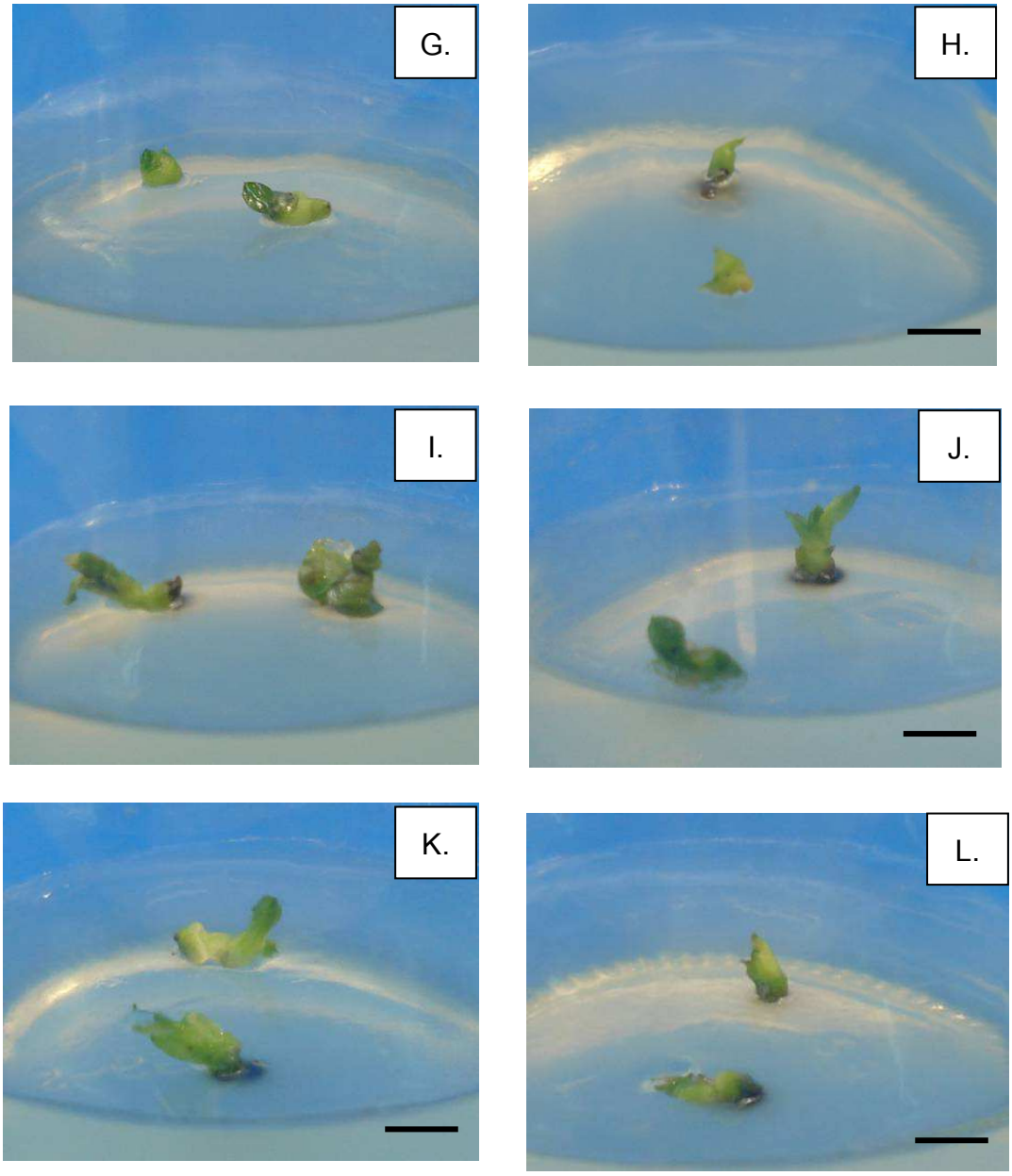



Figure 13. Development of new PLB from culturing protocorms on ½ MS medium containing PGRs at various concentrations. (Bar=1 cm)

G: 0.1 mg/l NAA + 1.0 mg/l TDZ

H: 0.1 mg/l NAA + 3.0 mg/l TDZ

I: 1.0 mg/l NAA + 0 mg/l TDZ

J: 1.0 mg/l NAA + 0.1 mg/l TDZ

K: 1.0 mg/l NAA + 1.0 mg/l TDZ

L: 1.0 mg/l NAA + 3.0 mg/l TDZ

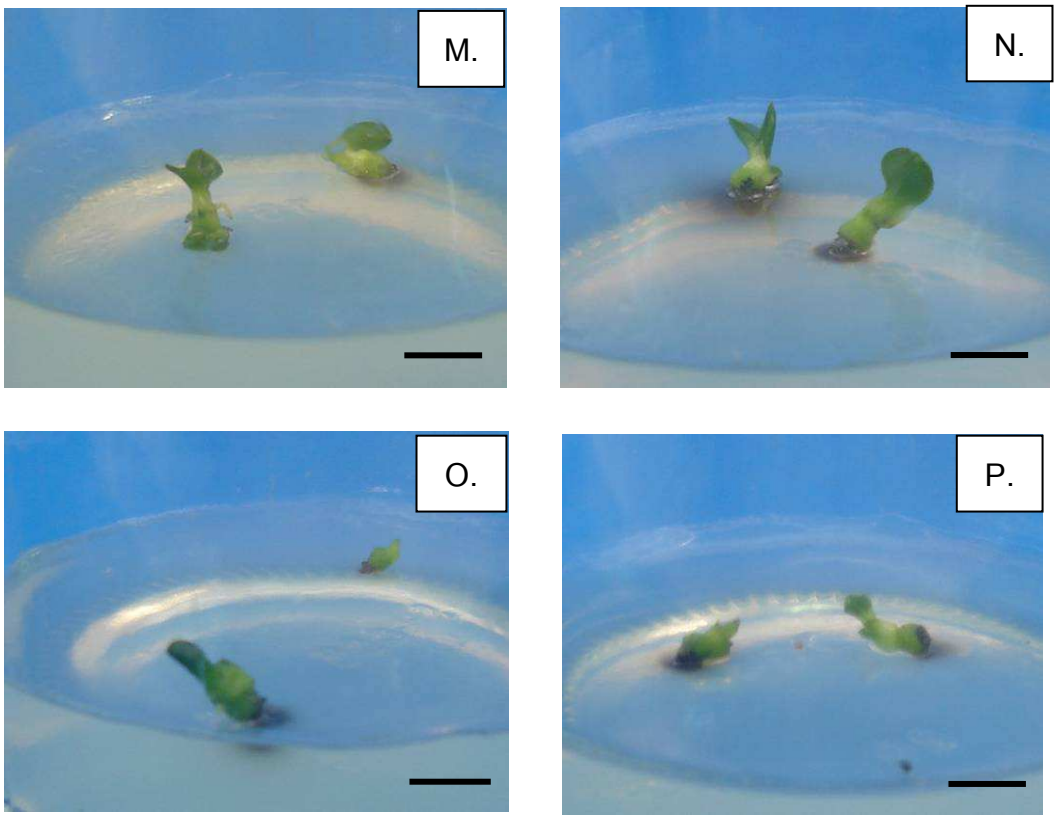



Figure 13. Development of new PLB from culturing protocorms on ½ MS medium containing PGRs at various concentrations. (Bar=1 cm)

M: 0.1 mg/l TDZ + 0.1 mg/l BA

N: 0.1 mg/l TDZ + 1.0 mg/l BA

O: 0.1 mg/l TDZ + 3.0 mg/l BA

P: 0.1 mg/l TDZ + 10.0 mg/l BA

Table 3 Effects of NAA, TDZ and BA containing ½ MS medium on PLB formation from bisected protocorms of *P. cornu-cervi* (Breda) Blume & Rchb. f. The frequency of embryo forming explants and the mean number of PLB per explant were scored after 45 days of culture.

| NAA | TDZ | BA | Browning | PLB formation | No. of PLB per explant | | |
|---|---|---|---|---|---|---|---|
| | | | | | Small Size | Medium size | Large size |
| (mg/l) | (mg/l) | (mg/l) | (%) | (%) | (Mean±S.E.) | (Mean±S.E.) | (Mean±S.E.) |
| Bisected protocorms | | | | | | | |
| 0 | 0 | | 50 | 30 | 3.33±0.33$^{de}$ | 2.33±0.58$^{e}$ | 0.67±0.67$^{gh}$ |
| | 0.1 | | 90 | 10 | 0.00±0.00$^{e}$ | 2.00±0.00$^{e}$ | 0.00±0.00$^{h}$ |
| | 1.0 | | 70 | 20 | 5.50±0.29$^{cd}$ | 0.00±0.00$^{e}$ | 0.00±0.00$^{h}$ |
| | 3.0 | | 70 | 20 | 8.00±4.62$^{bc}$ | 8.00±6.00$^{cd}$ | 5.00±2.89$^{ef}$ |
| 0.1 | 0 | | 50 | 40 | 0.00±0.00$^{e}$ | 0.00±0.00$^{e}$ | 10.25±0.25$^{cd}$ |
| | 0.1 | | 62.5 | 37.5 | 4.67±2.40$^{cd}$ | 8.00±5.00$^{cd}$ | 4.67±2.40$^{ef}$ |
| | 1.0 | | 70 | 30 | 16.00±2.31$^{a}$ | 16.33±2.89$^{a}$ | 16.00±2.00$^{a}$ |
| | 3.0 | | 70 | 30 | 11.00±2.65$^{b}$ | 10.33±2.52$^{bc}$ | 12.67±1.76$^{abc}$ |
| 1 | 0 | | 60 | 40 | 0.00±0.00$^{e}$ | 8.50±5.26$^{c}$ | 4.75±0.75$^{ef}$ |
| | 0.1 | | 40 | 60 | 4.25±0.63$^{cd}$ | 10.00±1.63$^{bc}$ | 2.75±0.48$^{fgh}$ |
| | 1.0 | | 50 | 40 | 16.33±0.33$^{a}$ | 13.67±2.94$^{ab}$ | 4.17±0.65$^{efg}$ |
| | 3.0 | | 90 | 10 | 3.00±0.00$^{de}$ | 4.00±1.00$^{de}$ | 8.00±0.58$^{de}$ |
| 0 | 0 | 0.1 | 90 | 10 | 2.00±0.00$^{de}$ | 2.00±0.00$^{e}$ | 3.00±0.00$^{fgh}$ |
| | | 1.0 | 70 | 30 | 3.00±1.73$^{de}$ | 3.00±0.00$^{e}$ | 5.00±2.89$^{ef}$ |
| | | 3.0 | 40 | 60 | 1.50±0.72$^{de}$ | 2.83±0.75$^{e}$ | 14.17±0.54$^{ab}$ |
| | | 10.0 | 70 | 30 | 4.67±0.67$^{cd}$ | 0.00±0.00$^{e}$ | 10.67±0.67$^{bcd}$ |

The values followed by different letters within columns are significantly different from others at 5% level by DMRT.

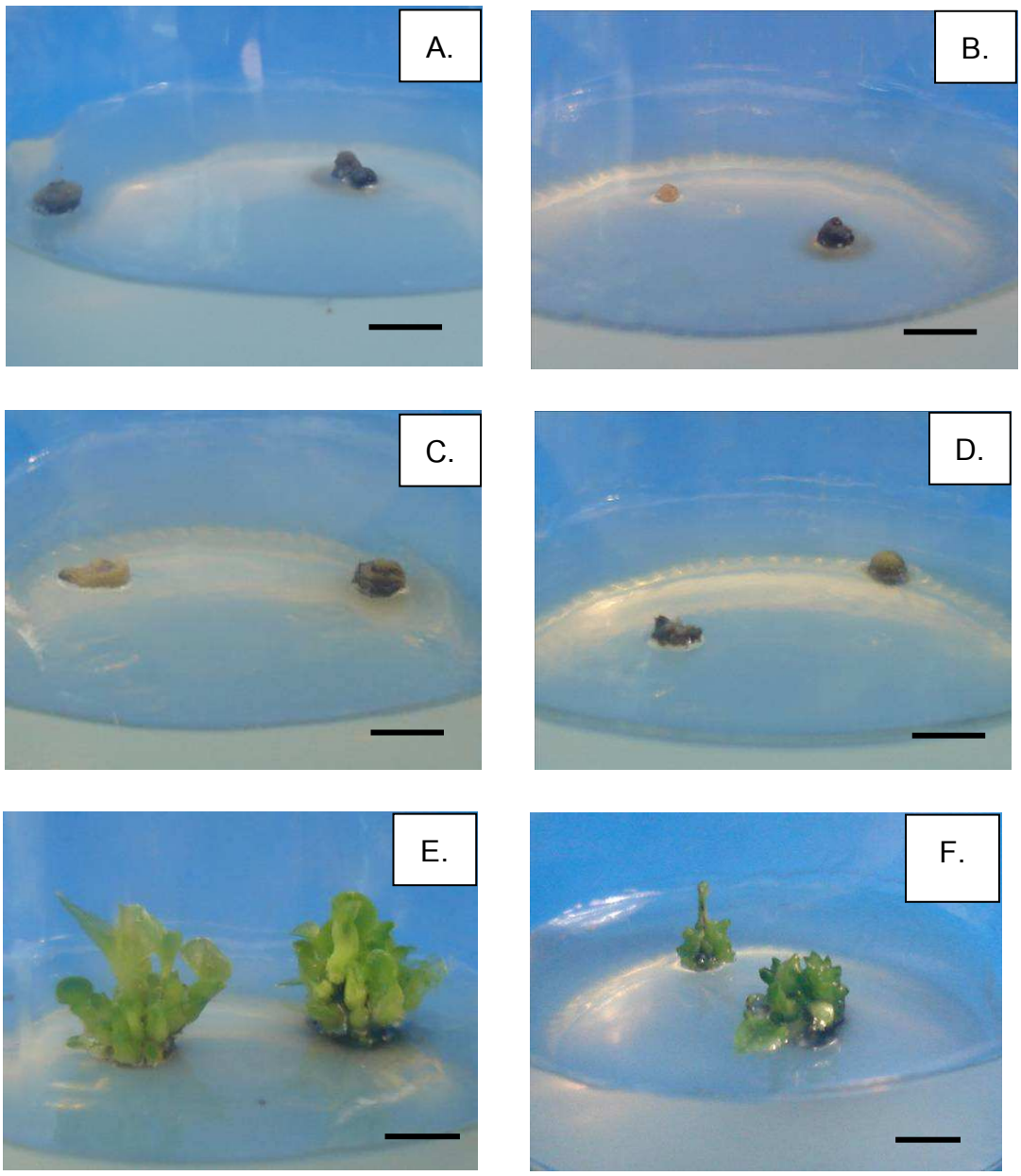



Figure 14. Development of new PLB from culturing bisected protocorms on ½ MS medium containing PGRs at various concentrations. (Bar=1 cm)

A: 0 mg/l NAA + 0 mg/l TDZ

B: 0 mg/l NAA + 0.1 mg/l TDZ

C: 0 mg/l NAA + 1.0 mg/l TDZ

D: 0 mg/l NAA + 3.0 mg/l TDZ

E: 0.1 mg/l NAA + 0 mg/l TDZ

F: 0.1 mg/l NAA + 0.1 mg/l TDZ

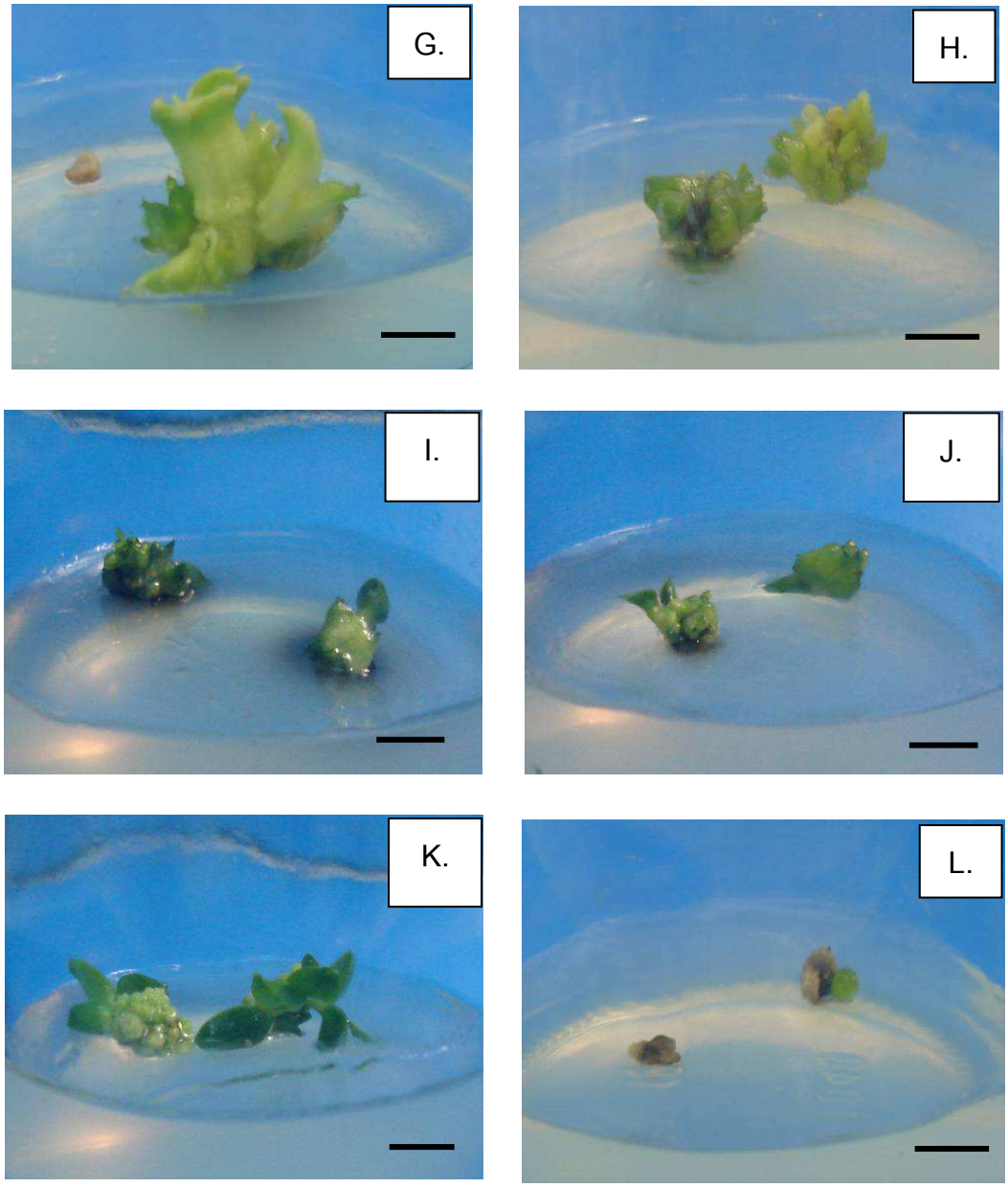



Figure 14. Development of new PLB from culturing bisected protocorms on ½ MS medium containing PGRs at various concentrations. (Bar=1 cm)

G: 0.1 mg/l NAA + 1.0 mg/l TDZ

H: 0.1 mg/l NAA + 3.0 mg/l TDZ

I: 1.0 mg/l NAA + 0 mg/l TDZ

J: 1.0 mg/l NAA + 0.1 mg/l TDZ

K: 1.0 mg/l NAA + 1.0 mg/l TDZ

L: 1.0 mg/l NAA + 3.0 mg/l TDZ

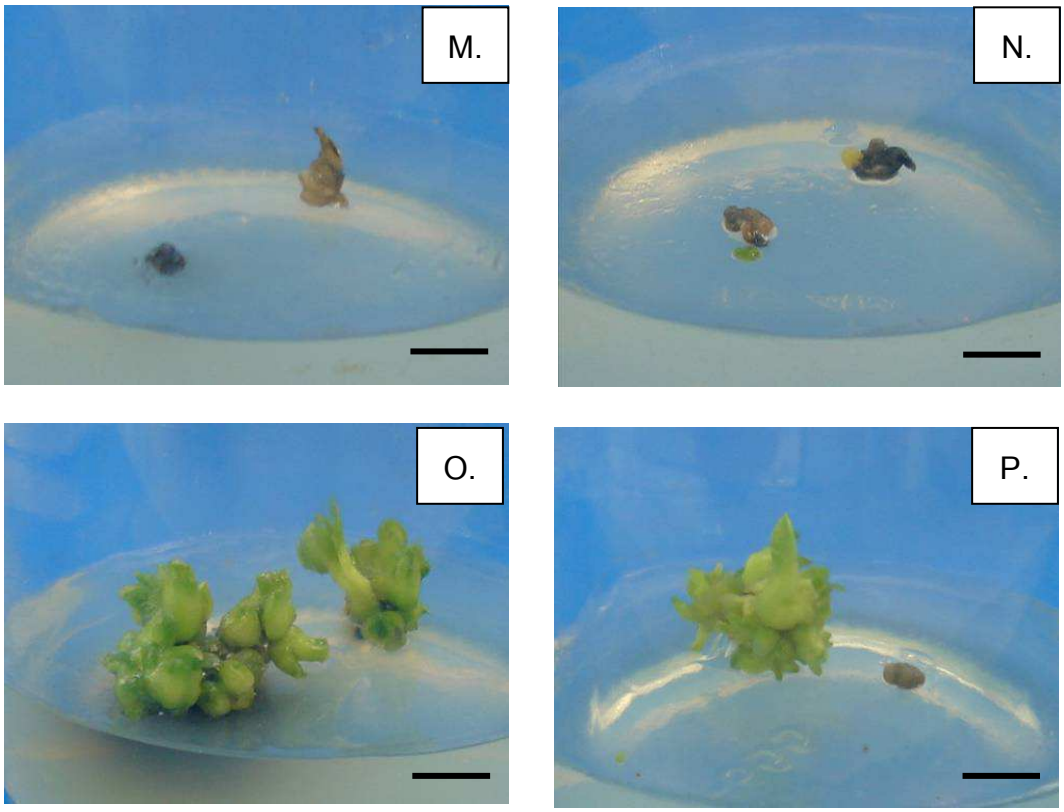



Figure 14. Development of new PLB from culturing bisected protocorms on ½ MS medium containing PGRs at various concentrations. (Bar=1 cm)

M: 0.1 mg/l TDZ + 0.1 mg/l BA

N: 0.1 mg/l TDZ + 1.0 mg/l BA

O: 0.1 mg/l TDZ + 3.0 mg/l BA

P: 0.1 mg/l TDZ + 10.0 mg/l BA

Table 4 Effects of NAA, TDZ and BA containing ½ MS medium on PLB formation from wounded protocorms of *P. cornu-cervi* (Breda) Blume & Rchb. f. The frequency of embryo forming explants and the mean number of PLB per explant were scored after 45 days of culture.

| NAA | TDZ | BA | Browning | PLB formation | No. of PLB per explant | | |
|---|---|---|---|---|---|---|---|
| | | | | | Small Size | Medium size | Large size |
| (mg/l) | (mg/l) | (mg/l) | (%) | (%) | (Mean±S.E.) | (Mean±S.E.) | (Mean±S.E.) |
| Wounded protocorms | | | | | | | |
| 0.0 | 0.0 | | 5 | 30 | 2.00±0.00$^{ef}$ | 3.00±0.37$^{cdef}$ | 3.30±0.55$^{i}$ |
| | 0.1 | | 5 | 90 | 8.80±4.10$^{abcde}$ | 4.60±0.37$^{bcd}$ | 5.10±0.82$^{fgh}$ |
| | 1.0 | | 0 | 100 | 12.20±0.39$^{abc}$ | 5.40±0.16$^{bcd}$ | 5.50±0.22$^{fgh}$ |
| | 3.0 | | 10 | 80 | 5.20±1.46$^{cdef}$ | 2.00±0.65$^{def}$ | 4.60±0.60$^{ghi}$ |
| 0.1 | 0.0 | | 5 | 90 | 8.30±2.54$^{abcde}$ | 5.30±1.45$^{bcd}$ | 8.00±0.53$^{de}$ |
| | 0.1 | | 0 | 100 | 13.90±4.16$^{a}$ | 10.70±3.20$^{a}$ | 11.00±0.75$^{ab}$ |
| | 1.0 | | 5 | 70 | 9.70±0.52$^{abcd}$ | 5.70±0.92$^{bcd}$ | 6.60±0.37$^{ef}$ |
| | 3.0 | | 0 | 100 | 9.20±3.04$^{abcde}$ | 6.20±1.40$^{bc}$ | 8.40±0.40$^{cd}$ |
| 1.0 | 0.0 | | 5 | 90 | 0.00±0.00$^{f}$ | 0.50±0.38$^{ef}$ | 6.00±0.65$^{fg}$ |
| | 0.1 | | 5 | 90 | 8.50±2.46$^{abcde}$ | 3.10±0.61$^{cdef}$ | 4.70±0.49$^{ghi}$ |
| | 1.0 | | 0 | 100 | 13.20±3.10$^{ab}$ | 8.00±0.51$^{ab}$ | 11.50±0.83$^{a}$ |
| | 3.0 | | 0 | 90 | 0.00±0.00$^{f}$ | 0.00±0.00$^{f}$ | 9.70±0.28$^{bc}$ |
| 0.0 | 0.0 | 0.1 | 5 | 90 | 3.60±0.33$^{def}$ | 4.00±0.44$^{cde}$ | 4.10±0.11$^{hi}$ |
| | | 1.0 | 0 | 100 | 3.10±0.74$^{def}$ | 2.60±0.27$^{cdef}$ | 5.20±0.13$^{fgh}$ |
| | | 3.0 | 10 | 80 | 2.80±0.64$^{def}$ | 2.50±0.33$^{cdef}$ | 4.50±0.27$^{ghi}$ |
| | | 10.0 | 10 | 80 | 6.00±1.41$^{bcdef}$ | 4.50±0.27$^{bcd}$ | 4.30±0.26$^{ghi}$ |

The values followed by different letters within columns are significantly different from others at 5% level by DMRT.

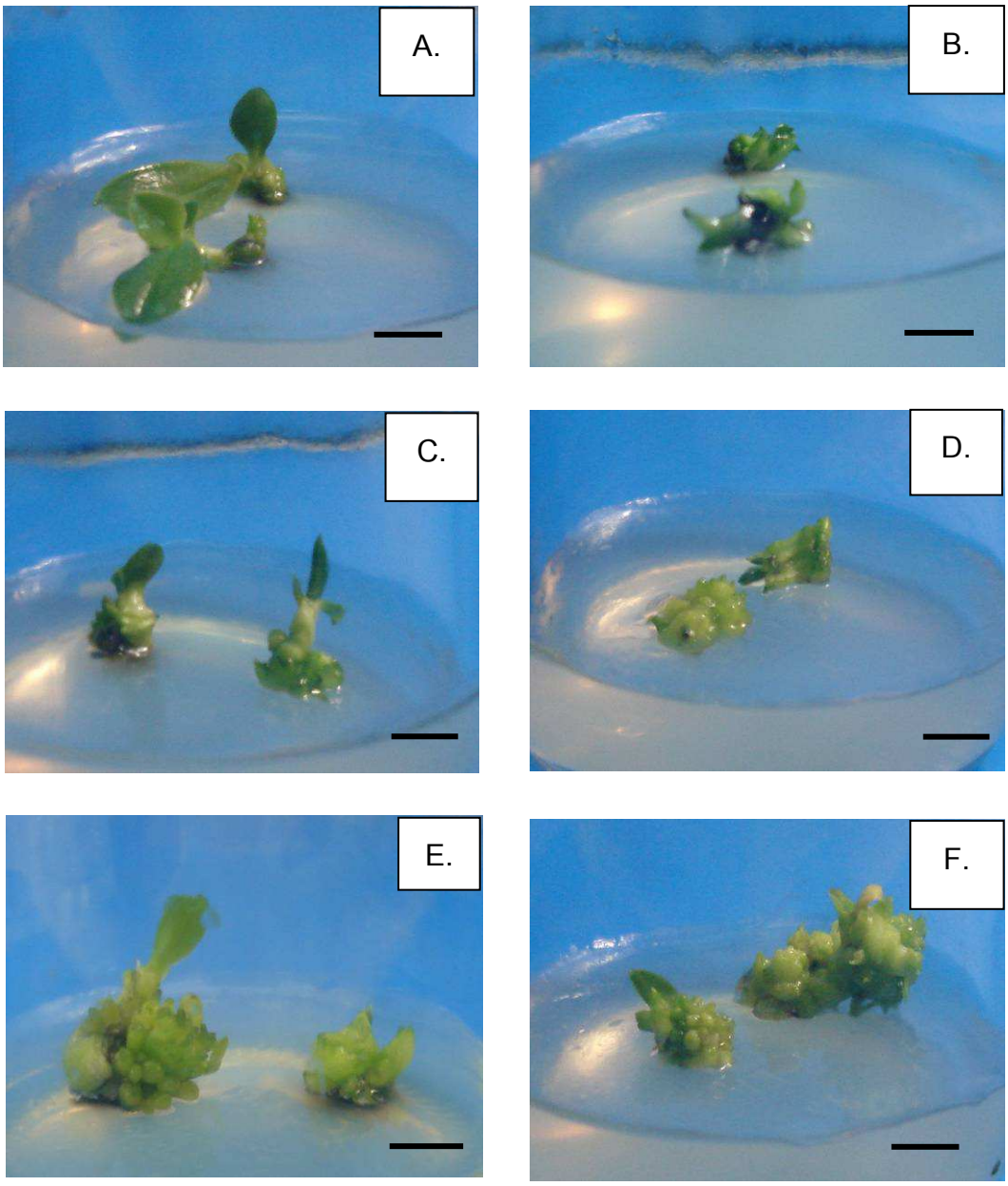



Figure 15. Development of new PLB from culturing wounded protocorms on ½ MS medium containing PGRs at various concentrations. (Bar=1 cm)

A: 0 mg/l NAA + 0 mg/l TDZ

B: 0 mg/l NAA + 0.1 mg/l TDZ

C: 0 mg/l NAA + 1.0 mg/l TDZ

D: 0 mg/l NAA + 3.0 mg/l TDZ

E: 0.1 mg/l NAA + 0 mg/l TDZ

F: 0.1 mg/l NAA + 0.1 mg/l TDZ

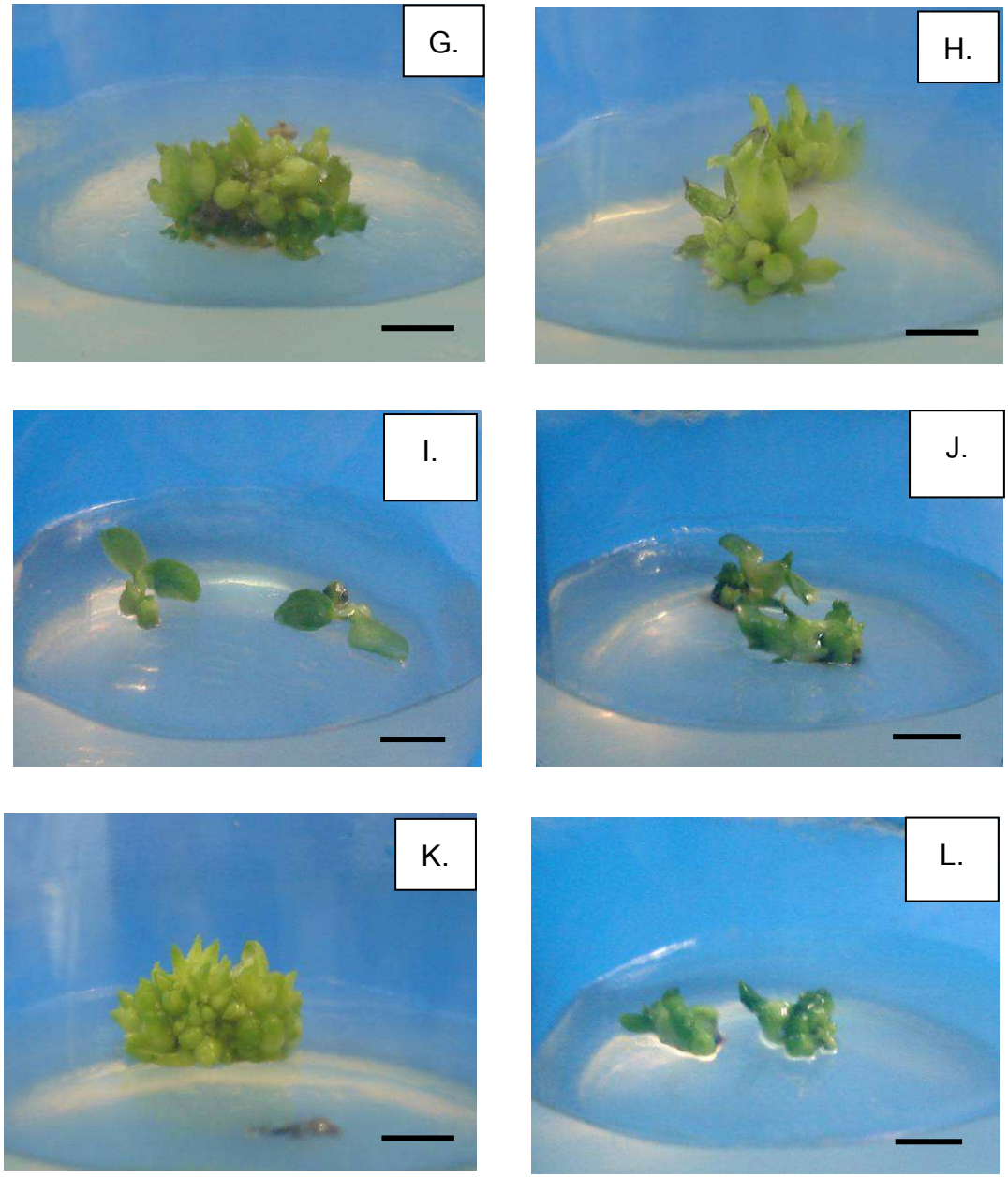



Figure 15. Development of new PLB from culturing wounded protocorms on ½ MS medium containing PGRs at various concentrations. (Bar=1 cm)

G: 0.1 mg/l NAA + 1.0 mg/l TDZ

H: 0.1 mg/l NAA + 3.0 mg/l TDZ

I: 1.0 mg/l NAA + 0 mg/l TDZ

J: 1.0 mg/l NAA + 0.1 mg/l TDZ

K: 1.0 mg/l NAA + 1.0 mg/l TDZ

L: 1.0 mg/l NAA + 3.0 mg/l TDZ

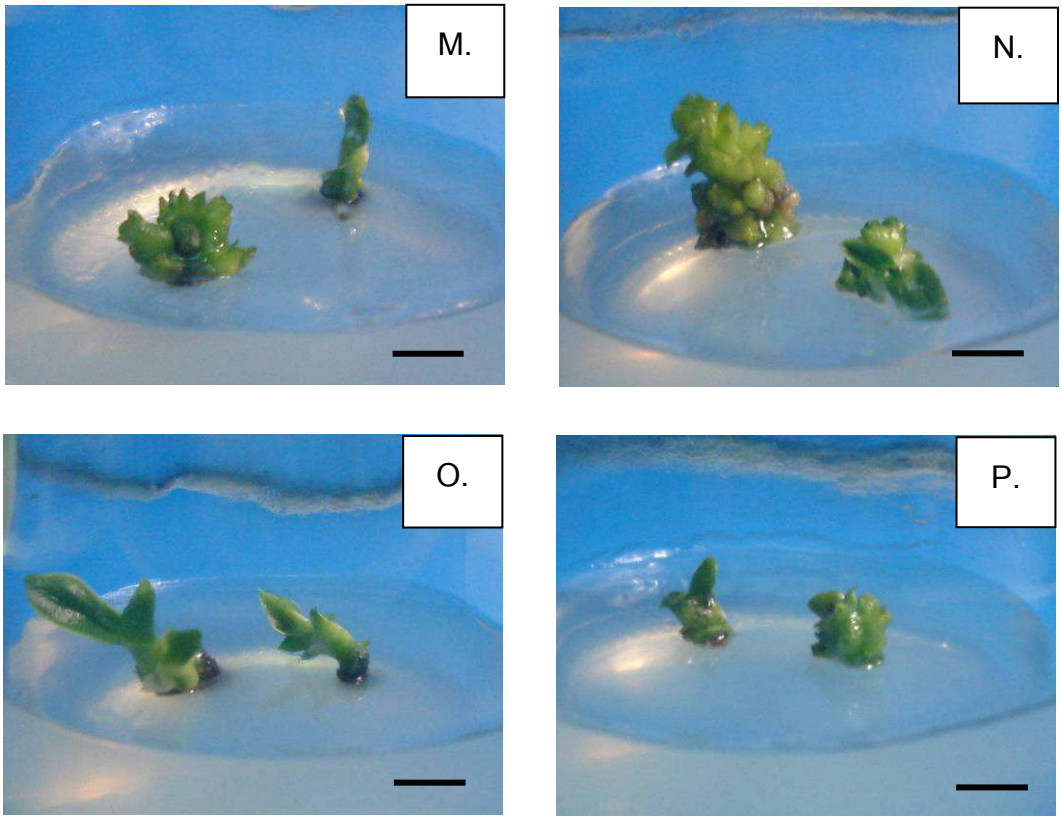



Figure 15. Development of new PLB from culturing wounded protocorms on ½ MS medium containing PGRs at various concentrations. (Bar=1 cm)

M: 0.1 mg/l TDZ + 0.1 mg/l BA

N: 0.1 mg/l TDZ + 1.0 mg/l BA

O: 0.1 mg/l TDZ + 3.0 mg/l BA

P: 0.1 mg/l TDZ + 10.0 mg/l BA

Table 5 Effects of NAA, TDZ and BA containing ½ MS medium on PLB formation from leaf segments of *P. cornu-cervi* (Breda) Blume & Rchb. f. The frequency of embryo forming explants and the mean number of PLB per explant were scored after 45 days of culture.

| NAA | TDZ | BA | Browning | PLB formation | No. of PLB per explant | | |
|---|---|---|---|---|---|---|---|
| | | | | | Small Size | Medium size | Large size |
| (mg/l) | (mg/l) | (mg/l) | (%) | (%) | (Mean±S.E.) | (Mean±S.E.) | (Mean±S.E.) |
| Leaf segments | | | | | | | |
| 0 | 0 | | 100 | 0 | 0 | 0 | 0 |
| | 0.1 | | 100 | 0 | 0 | 0 | 0 |
| | 1.0 | | 100 | 0 | 0 | 0 | 0 |
| | 3.0 | | 100 | 0 | 0 | 0 | 0 |
| 0.1 | 0 | | 100 | 0 | 0 | 0 | 0 |
| | 0.1 | | 100 | 0 | 0 | 0 | 0 |
| | 1.0 | | 100 | 0 | 0 | 0 | 0 |
| | 3.0 | | 100 | 0 | 0 | 0 | 0 |
| 1 | 0 | | 100 | 0 | 0 | 0 | 0 |
| | 0.1 | | 100 | 0 | 0 | 0 | 0 |
| | 1.0 | | 100 | 0 | 0 | 0 | 0 |
| | 3.0 | | 100 | 0 | 0 | 0 | 0 |
| 0 | 0 | 0.1 | 100 | 0 | 0 | 0 | 0 |
| | | 1.0 | 100 | 0 | 0 | 0 | 0 |
| | | 3.0 | 100 | 0 | 0 | 0 | 0 |
| | | 10.0 | 100 | 0 | 0 | 0 | 0 |

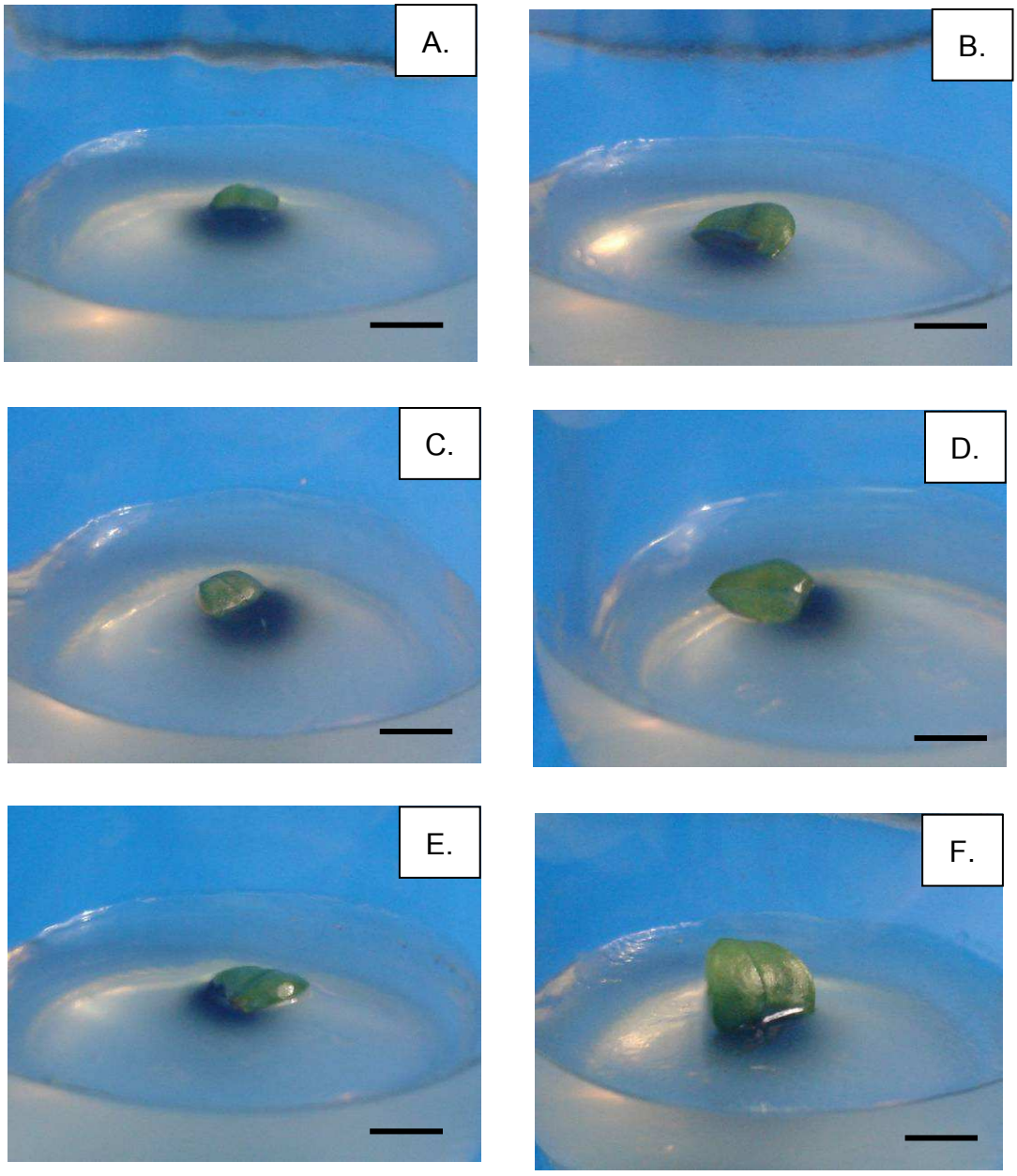



Figure 16. Development of leaf segments after being cultured on ½ MS medium supplemented with PGRs at various concentrations. (Bar=1 cm)

A: 0 mg/l NAA + 0 mg/l TDZ

B: 0 mg/l NAA + 0.1 mg/l TDZ

C: 0 mg/l NAA + 1.0 mg/l TDZ

D: 0 mg/l NAA + 3.0 mg/l TDZ

E: 0.1 mg/l NAA + 0 mg/l TDZ

F: 0.1 mg/l NAA + 0.1 mg/l TDZ

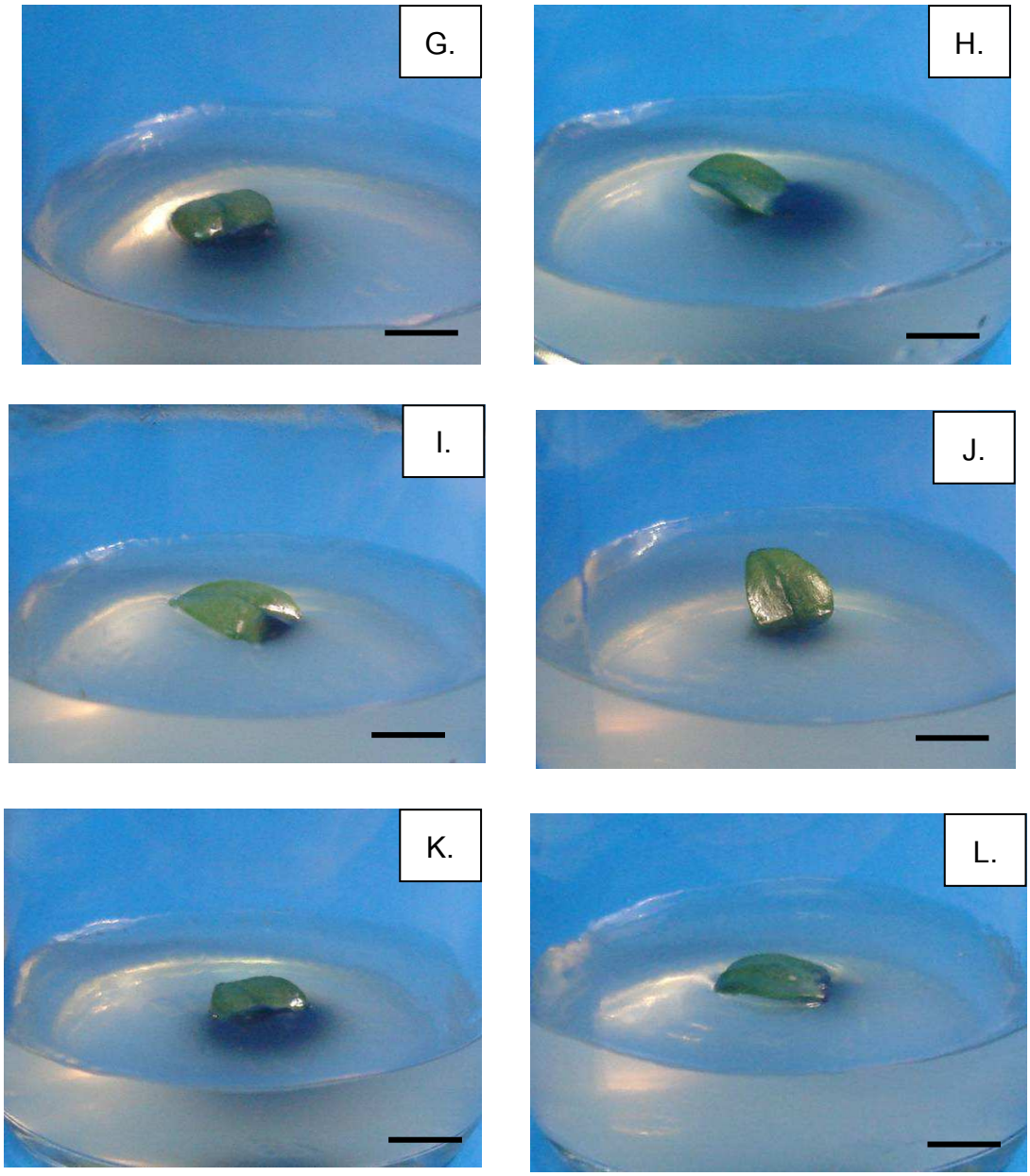



Figure 16. Development of leaf segments after being cultured on ½ MS medium supplemented with PGRs at various concentrations. (Bar=1 cm)

G: 0.1 mg/l NAA + 1.0 mg/l TDZ

H: 0.1 mg/l NAA + 3.0 mg/l TDZ

I: 1.0 mg/l NAA + 0 mg/l TDZ

J: 1.0 mg/l NAA + 0.1 mg/l TDZ

K: 1.0 mg/l NAA + 1.0 mg/l TDZ

L: 1.0 mg/l NAA + 3.0 mg/l TDZ

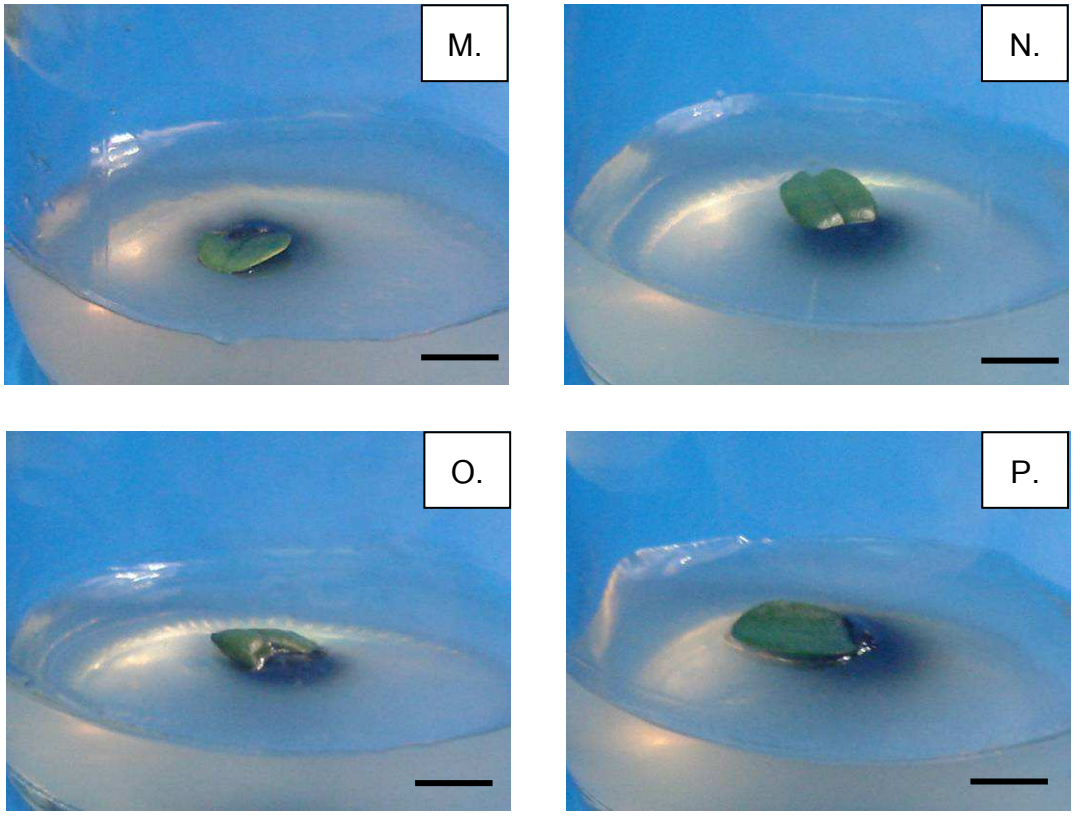



Figure 16. Development of leaf segments after being cultured on ½ MS medium supplemented with PGRs at various concentrations. (Bar=1 cm)

M: 0.1 mg/l TDZ + 0.1 mg/l BA

N: 0.1 mg/l TDZ + 1.0 mg/l BA

O: 0.1 mg/l TDZ + 3.0 mg/l BA

P: 0.1 mg/l TDZ + 10.0 mg/l BA

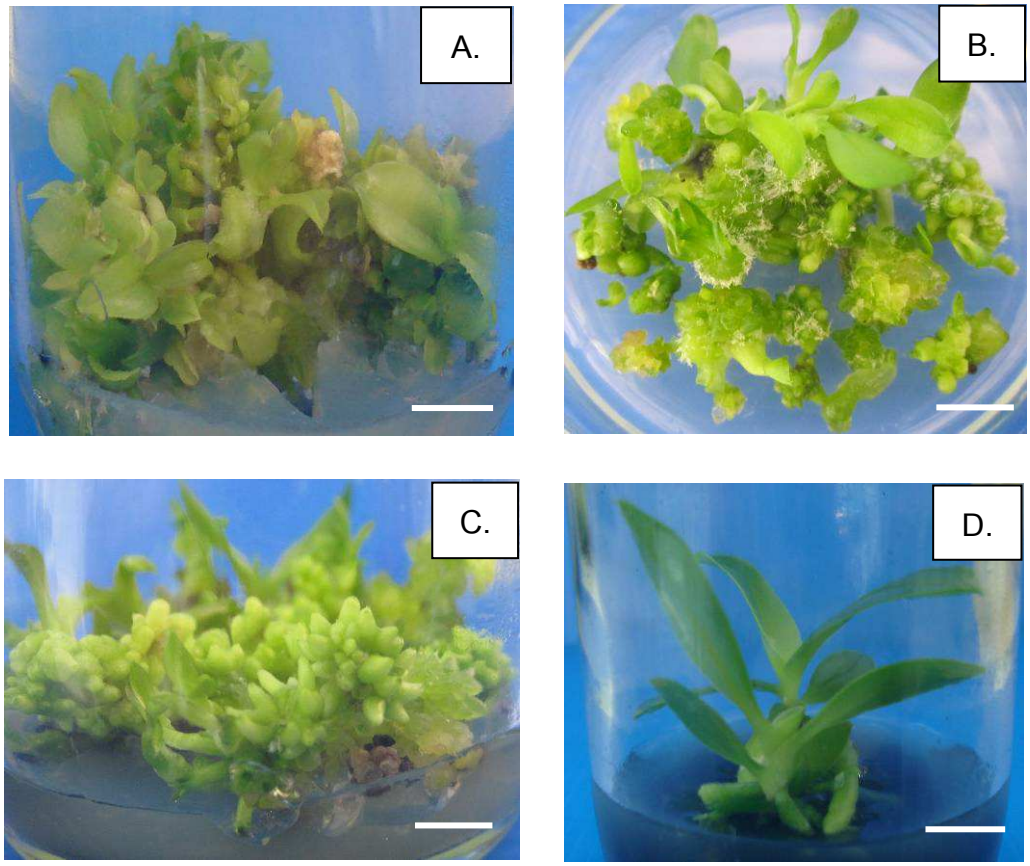



Figure 17. PLB development of *P. cornu-cervi* (Breda) Blume & Rchb. f. from culturing wounded protocorms on ½ MS medium containing 0.1 mg/l NAA and 0.1 mg/l TDZ. (Bar=1 cm)

A: Conversion of PLB to shoots.

B, C: New PLB derived from culturing wounded primary PLB on ½ MS medium supplemented with 0.1 mg/l NAA and 0.1 mg/l TDZ combination after 4 and 5 months, respectively.

D: PLB-derived plantlets on MS medium supplemented with 15% (v/v) coconut water (CW) and 0.2% (w/v) activated charcoal (AC).

3.1.5 Effects of culture media, concentrations of sucrose and activated charcoal on plantlet growth.

The PLB were excised and transferred to ND or MS media supplemented with various concentrations of sucrose (0, 2, 3 and 4%) with or without AC. The results revealed that in the presence of AC in culture media gave a better response than culture media without AC.

The PLB grew well on both ND and MS agar media. Significant difference was observed between culture media and concentrations of sucrose for survival rate, fresh weight, plantlet height (Table 6), number of leaves per plantlet, leaf length, leaf width, number of roots per plantlet and root length (Table 7).

PLB enlarged in their size after 2 months of culture, subsequent to the development into small plantlets after 3 months of culture. To study the type of culture media and carbon source on plantlet regeneration from PLB, different concentrations of sucrose were added in the medium. Higher concentration of sucrose gave a higher fresh weight than lower concentrations (Table 6).

In the present study, concentrations of sucrose affected the survival rate of plantlets from PLB in both culture media with or without AC. On the other hand, survival rate of plantlets on media without sucrose was lower (Table 6). However, a critical problem during culture on media without AC (Table 6) was that tissue browning resulting from phenolic compound accumulation occurred and this caused the loss of growth capacity (Table 7). This important problem could be solved using 0.2% (w/v) activated charcoal (AC) as a medium addendum.

Of the sixteen different culture media used in this experiment, the best result was obtained when PLB segments were cultured on ND medium supplemented with 4% (w/v) sucrose with AC (Table 6, 7). After 5 months of culture, sucrose at 0, 2, 3 or 4% showed significant difference on plantlet growth. On sugar containing medium, all PLB could germinate at 100% and seedling growth was comparatively faster than those obtained from culture medium without sugar. The survival rate of PLB cultured on ND and MS medium supplemented with AC was slightly higher than that obtained in the medium without AC. Survival rate of PLB obtained from AC containing medium was 95-100% (Table 6). Similar results were also found in plant height and fresh weight.

In this present study, ND medium supplemented with 4% (w/v) sucrose in the presence of AC gave the highest survival rate at 100%, plantlet height at 10.9 mm, fresh weight at 1.309 g, number of leaves per plantlet at 6.1 leaves, leaf length at 33.85 mm, leaf width at 13.45 mm, number of roots per plantlet at 8.9 roots and root length at 30.2 mm (Table 6, 7: Figure 18L). Therefore, this medium was suitable for the conversion of PLB into plantlets. It was also found that the concentration of sucrose in both culture media affected on survival rate, plantlet height and fresh weight of plantlets from PLB. On the other hand, development of plantlets on culture media with sucrose was better than culture media without sucrose.

At the first 2 months of culture, PLB produced a large quantity of phenolic compounds and exuded from the cut ends leading to browning of the surrounding ND or MS media (Figure 18C, G). However, this phenomenon did not inhibit the development of PLB which were cultured in AC containing media (Figure 18K, L, O).

In this present study, AC in culture media seems to reduce exudates released from wounds, thus, enhancing the development of PLB. PLB converted into healthy plantlets with well-developed leaves and roots when they were cultured on medium supplemented with AC and kept under a 16-h photoperiod for 5 months. In culture medium without AC, slow growth was observed. In this report the *P. cornu-cervi* (Breda) Blume & Rchb. f. plantlets raised on ND medium containing AC had greater height than all other treatments without AC. Obvious effects of AC was observed on the root formation. PLB developed into plantlets with well-developed 8.9 roots and 30.2 mm root length per plantlet when cultured on ND medium containing 4% (w/v) sucrose and 0.2% (w/v) AC (Figure 18L). The PLB easily developed into plantlets on this medium. The results suggested that AC might assist adsorbing phenolic compounds in modified ND or MS medium. Therefore, the survival rate of plantlets was increased when AC was added into ND or MS medium. Addition of AC in culture medium may cause better development of root and aerial parts of plants without any addition of exogenous auxins and/or cytokinins.

After 6 months of culture on ND medium supplemented with 4% (w/v) sucrose and 0.2% (w/v) AC, complete plantlets were formed. Fully developed plantlets rooted well *in vitro* with good shoot and root formation (Figure 18L).

Complete plantlets (Figure 19A) were removed from the bottle, washed twice with tap water to remove traces of agar and transplanted into pots filled with sphagnum moss. They were successfully acclimatized and grown in greenhouse under 60% shading and 80% relative humidity. After 2 months of being transferred, survival rate of plantlets at 100% (100 plantlets) was obtained (Figure 19B). There were no obvious differences in morphology of plantlets and no phenotypic variations were observed among them during vegetative period in the greenhouse. One plant flowered at 3 months after being transferred to greenhouse (Figure 19C).

The present study is the first report to show that PLB can be induced from wounded protocorm segments on ½ MS medium supplemented with 0.1 mg/l NAA and 0.1 mg/l TDZ combination. ND medium supplemented with 4% (w/v) sucrose and containing AC was suitable for conversion of PLB of *P. cornu-cervi* (Breda) Blume & Rchb. f. into complete plantlets when considering survival rate, plantlet height, fresh weight, number of leaves per plantlet, leaf length, leaf width, number of roots per plantlet and root length. The use of media supplemented with suitable sucrose concentration and containing AC was also effective for development of PLB into complete plantlets. One advantage of this medium is that it does not require any addition of hormones.

Table 6 Survival rate and plantlet growth from PLB of *P. cornu-cervi* (Breda) Blume & Rchb. f. after 5 months of culture on 2 different culture media with or without AC and 4 concentrations of sucrose.

| Media | Sucrose (%) | Survival rate (%) | Plantlet height (mm) (Mean± S.E.) | Fresh weight (g) (Mean± S.E.) |
|---|---|---|---|---|
| Without AC | | | | |
| ND | 0 | 70.00 | $5.1\pm0.10^{h1}$ | $0.260\pm0.01^{g}$ |
| | 2 | 80.00 | $6.3\pm0.15^{g}$ | $0.360\pm0.02^{fg}$ |
| | 3 | 100.00 | $7.1\pm0.10^{f}$ | $0.494\pm0.03^{def}$ |
| | 4 | 90.00 | $7.2\pm0.13^{f}$ | $0.561\pm0.04^{de}$ |
| MS | 0 | 75.00 | $4.3\pm0.21^{i}$ | $0.240\pm0.09^{g}$ |
| | 2 | 94.44 | $4.2\pm0.36^{i}$ | $0.248\pm0.02^{g}$ |
| | 3 | 92.86 | $3.7\pm0.15^{i}$ | $0.392\pm0.03^{efg}$ |
| | 4 | 90.00 | $4.2\pm0.20^{i}$ | $0.311\pm0.03^{fg}$ |
| With AC | | | | |
| ND | 0 | 95.00 | $9.4\pm0.16^{bc}$ | $0.601\pm0.05^{d}$ |
| | 2 | 95.00 | $10.1\pm0.18^{b}$ | $0.785\pm0.07^{c}$ |
| | 3 | 100.00 | $10.0\pm0.00^{b}$ | $0.998\pm0.07^{b}$ |
| | 4 | 100.00 | $10.9\pm0.31^{a}$ | $1.309\pm0.10^{a}$ |
| MS | 0 | 85.00 | $7.9\pm0.38^{ef}$ | $0.370\pm0.06^{efg}$ |
| | 2 | 100.00 | $7.4\pm0.37^{f}$ | $0.498\pm0.07^{def}$ |
| | 3 | 100.00 | $9.1\pm0.62^{cd}$ | $0.503\pm0.07^{def}$ |
| | 4 | 88.88 | $8.4\pm0.40^{de}$ | $0.595\pm0.13^{d}$ |

The values followed by different letters within columns are significantly different from others at 5% level by DMRT.

Table 7 Leaf and root growth from PLB of *P. cornu-cervi* (Breda) Blume & Rchb. f. after 5 months of culture on 2 different culture media with or without AC and 4 concentrations of sucrose.

| Media | Sucrose (%) | No.of leaves/ plantlet (Mean±S.E.) | leaf length (mm) (Mean±S.E.) | leaf width (mm) (Mean±S.E.) | No.of roots/ plantlet (Mean±S.E.) | root length (mm) (Mean±S.E.) |
|---|---|---|---|---|---|---|
| Without AC | | | | | | |
| ND | 0 | 5.70±0.30$^{bc1}$ | 16.95±0.73$^{def}$ | 6.90±0.29$^{hi}$ | 3.90±0.35$^{fgh}$ | 8.25±0.37$^{de}$ |
| | 2 | 6.20±0.36$^{abc}$ | 18.90±1.08$^{bcde}$ | 8.45±0.41$^{efg}$ | 5.10±0.31$^{efg}$ | 16.70±1.47$^{c}$ |
| | 3 | 6.90±0.23$^{ab}$ | 21.35±1.50$^{b}$ | 11.30±0.50$^{b}$ | 6.50±0.50$^{cd}$ | 24.40±2.77$^{b}$ |
| | 4 | 6.40±0.58$^{abc}$ | 17.10±1.09$^{cdef}$ | 10.00±0.45$^{bcd}$ | 5.70±0.42$^{de}$ | 25.75±2.21$^{b}$ |
| MS | 0 | 7.00±0.52$^{a}$ | 13.35±0.53$^{fg}$ | 6.70±0.32$^{i}$ | 3.70±0.30$^{gh}$ | 4.45±0.64$^{e}$ |
| | 2 | 6.30±0.26$^{abc}$ | 13.30±0.46$^{fg}$ | 7.05±0.34$^{ghi}$ | 4.00±0.37$^{fgh}$ | 6.45±0.65$^{e}$ |
| | 3 | 6.60±0.31$^{abc}$ | 16.00±0.75$^{ef}$ | 8.10±0.19$^{fghi}$ | 5.20±0.42$^{def}$ | 6.00±0.81$^{e}$ |
| | 4 | 6.40±0.31$^{abc}$ | 11.70±0.47$^{g}$ | 7.10±0.25$^{ghi}$ | 4.90±0.35$^{efg}$ | 4.15±0.64$^{e}$ |
| With AC | | | | | | |
| ND | 0 | 6.00±0.21$^{abc}$ | 20.90±1.45$^{bc}$ | 10.45±0.67$^{bc}$ | 4.40±0.22$^{efg}$ | 12.75±0.93$^{cd}$ |
| | 2 | 6.60±0.52$^{abc}$ | 31.55±1.87$^{a}$ | 14.05±0.65$^{a}$ | 7.10±0.43$^{bc}$ | 25.20±2.12$^{b}$ |
| | 3 | 6.40±0.31$^{abc}$ | 30.95±1.01$^{a}$ | 13.10±0.42$^{a}$ | 8.30±0.65$^{ab}$ | 21.35±2.14$^{b}$ |
| | 4 | 6.10±0.28$^{abc}$ | 33.85±1.63$^{a}$ | 13.45±0.39$^{a}$ | 8.90±0.50$^{a}$ | 30.20±1.85$^{a}$ |
| MS | 0 | 5.40±0.37$^{c}$ | 18.10±0.89$^{bcde}$ | 8.25±0.42$^{efgh}$ | 2.90±0.31$^{h}$ | 5.15±0.65$^{e}$ |
| | 2 | 7.00±0.33$^{a}$ | 21.80±1.77$^{b}$ | 9.65±0.56$^{cde}$ | 4.20±0.36$^{fgh}$ | 7.70±0.90$^{e}$ |
| | 3 | 6.80±0.29$^{ab}$ | 21.15±1.28$^{b}$ | 10.70±0.55$^{bc}$ | 4.90±0.38$^{efg}$ | 8.65±1.24$^{de}$ |
| | 4 | 7.20±0.42$^{a}$ | 20.10±1.95$^{bcd}$ | 9.00±0.66$^{def}$ | 5.30±0.83$^{def}$ | 8.90±2.12$^{de}$ |

The values followed by different letters within columns are significantly different from others at 5% level by DMRT.

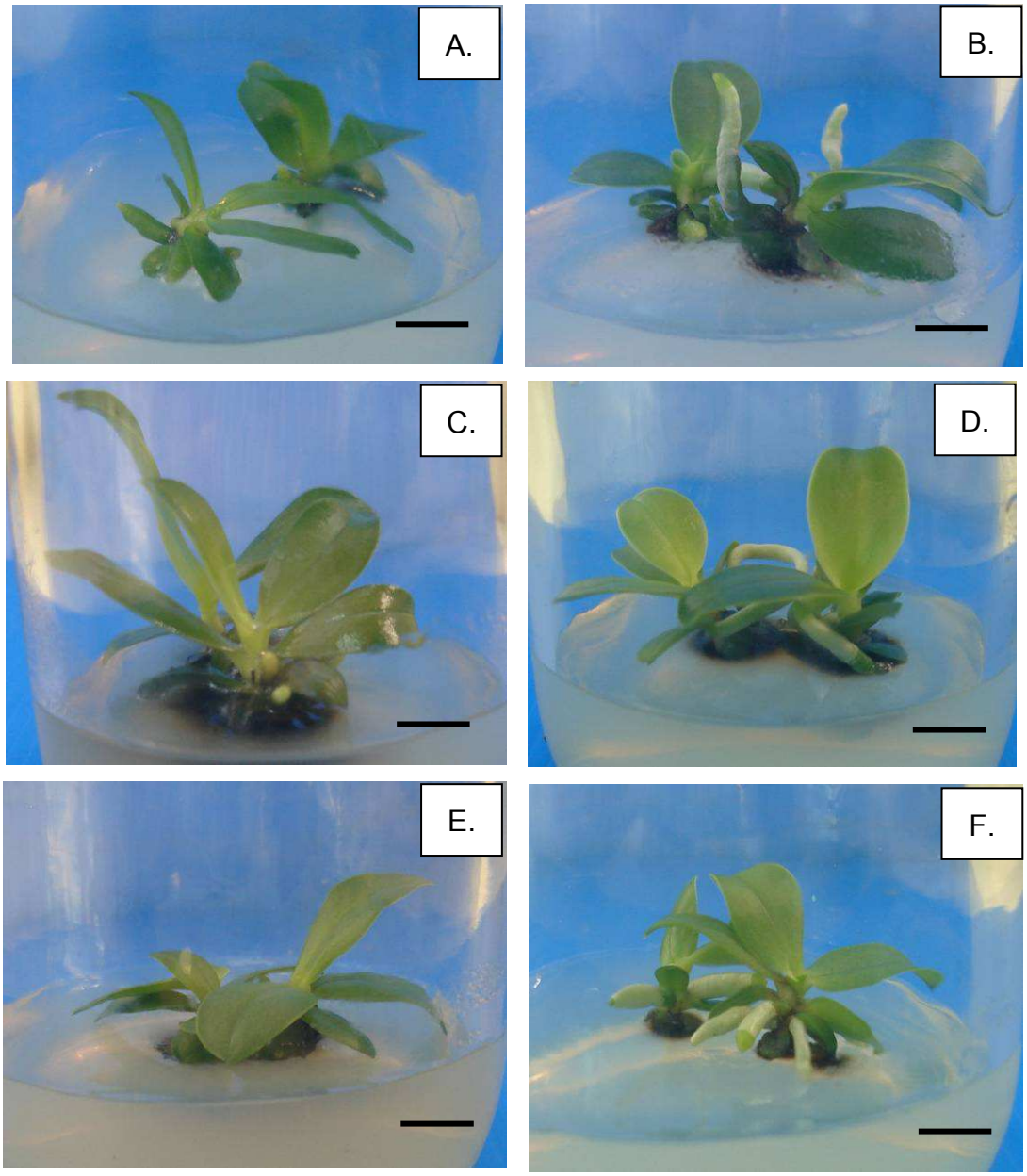



Figure 18. Conversion of protocorm-derived PLB into plantlets on 2 different culture media with 4 concentrations of sucrose without PGRs after 5 months of culture. (Bar=1 cm)

A: ND medium.

B: ND medium supplemented with 2% (w/v) sucrose.

C: ND medium supplemented with 3% (w/v) sucrose.

D: ND medium supplemented with 4% (w/v) sucrose.

E: MS medium.

F: MS medium supplemented with 2% (w/v) sucrose.

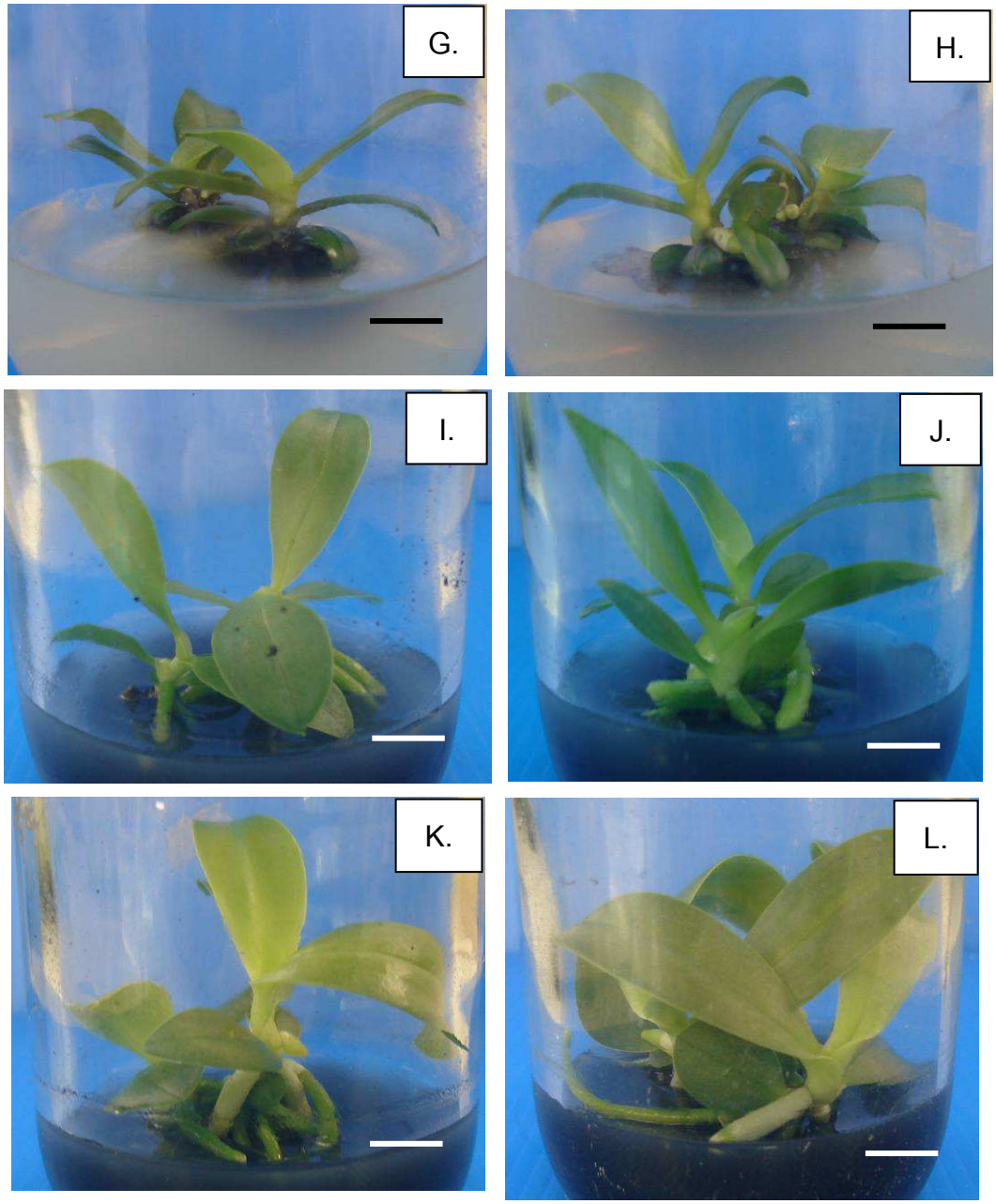



Figure 18. Conversion of protocorm-derived PLB into plantlets on 2 different culture media with 4 concentrations of sucrose without PGRs after 5 months of culture. (Bar=1 cm)

G: MS medium supplemented with 3% (w/v) sucrose.

H: MS medium supplemented with 4% (w/v) sucrose.

I: ND medium supplemented with 0.3% (w/v) AC.

J: ND medium supplemented with 0.2% (w/v) AC and 2% (w/v) sucrose.

K: ND medium supplemented with 0.2% (w/v) AC and 3% (w/v) sucrose.

L: ND medium supplemented with 0.2% (w/v) AC and 4% (w/v) sucrose.

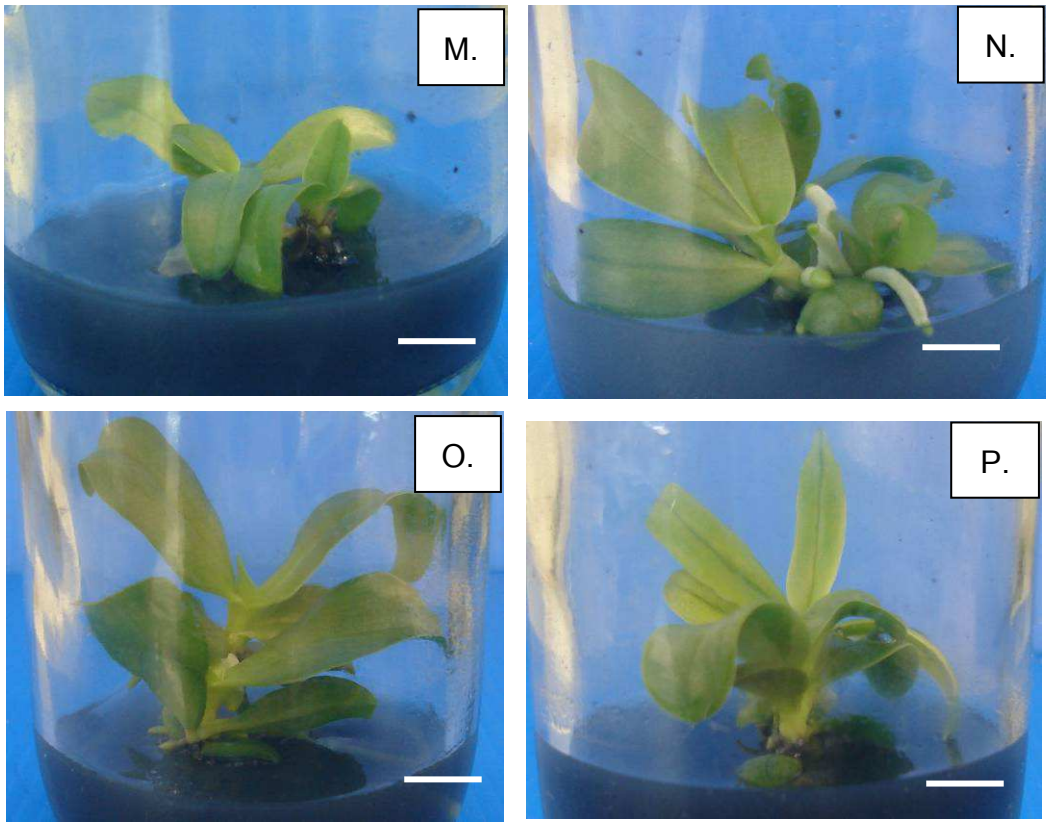



Figure 18. Conversion of protocorm-derived PLB into plantlets on 2 different culture media with 4 concentrations of sucrose without PGRs after 5 months of culture. (Bar=1 cm)

M:MS medium supplemented with 0.2% (w/v) AC.

N: MS medium supplemented with 0.2% (w/v) AC and 2% (w/v) sucrose.

O: MS medium supplemented with 0.2% (w/v) AC and 3% (w/v) sucrose.

P: MS medium supplemented with 0.2% (w/v) AC and 4% (w/v) sucrose.

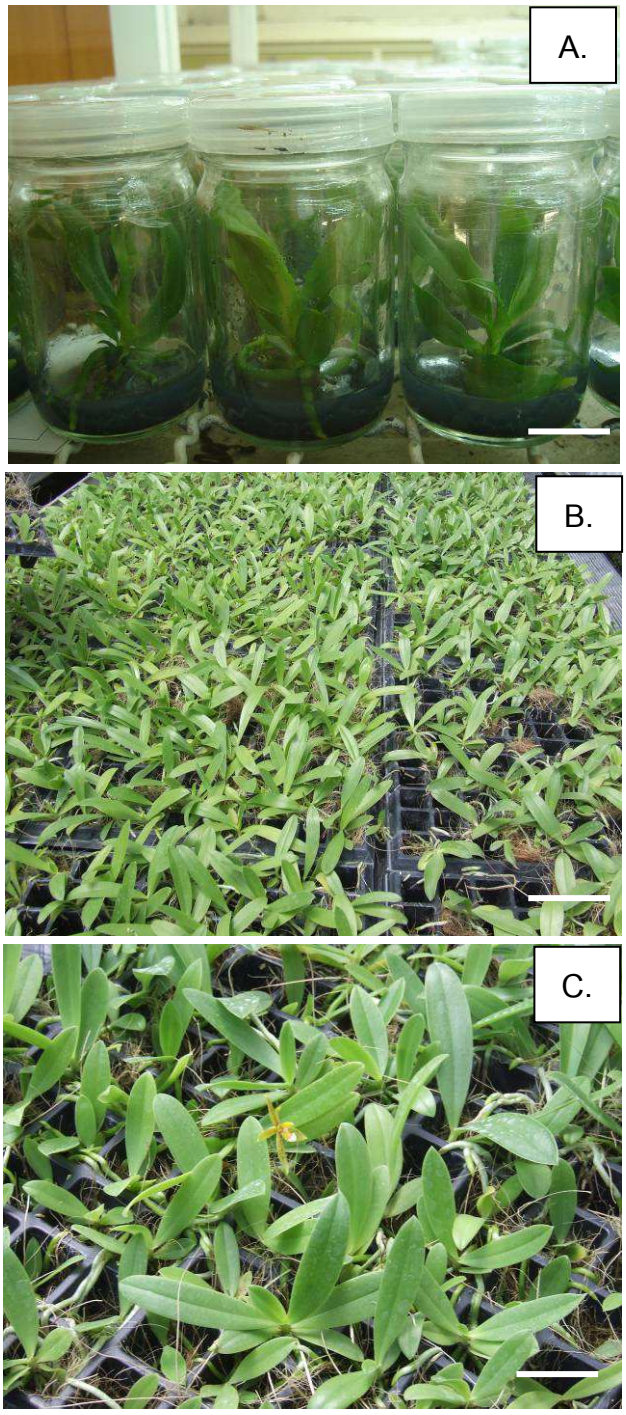


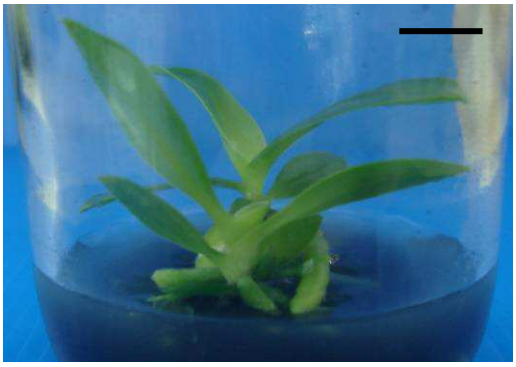

Figure 19. Plantlets of *P. cornu-cervi* (Breda) Blume & Rchb. f. (Bar=1cm)

A: Plantlets after 6 months of sowing seeds on ND medium supplemented with 4% (w/v) sucrose and containing 0.2% (w/v) AC.

B, C: 2-month-old and 3-month-old acclimatized plantlets grown in the greenhouse.

3.1.6 Ploidy instability analysis using flow cytometry (FCM).

Plantlets obtained from wounded protocorms which were cultured on ½ MS medium supplemented with different concentrations of PGRs showed the same peaks of relative DNA content indicated that they had the same ploidy level as control plantlets (Figure 21). In addition, PGRs, both auxins and cytokinins used in this investigation played non-significant role on instability of ploidy level of plantlets after 4 months of culture. There was no change in ploidy level of plantlets by this protocol. So, this technique is quite stable for mass propagation of *P. cornu-cervi* (Breda) Blume & Rchb. f.

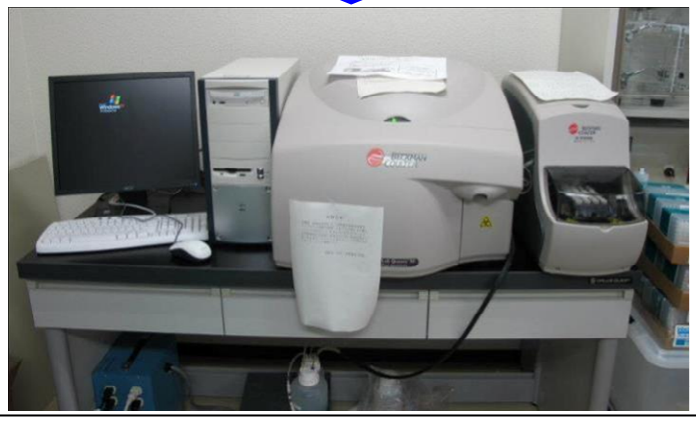


Young leaves of *P. cornu-cervi* (Breda) Blume & Rchb. f.

Leaf pieces were soaked with 1 ml of an extraction buffer for 5 minutes and chopped with a sharp razor blade

After mixing leaf tissue and buffer together it was filtered through 50-µm nylon mesh.


The nuclear suspension was stained with 100 µl of 0.1% PI solution and incubated for 5 minutes at room temperature.



Flow cytometer Beckman Coulter, Frontier Science Research Center University of Miyazaki, Miyazaki, Japan

Figure 20. Protocol for ploidy instability analysis using flow cytometry (FCM).

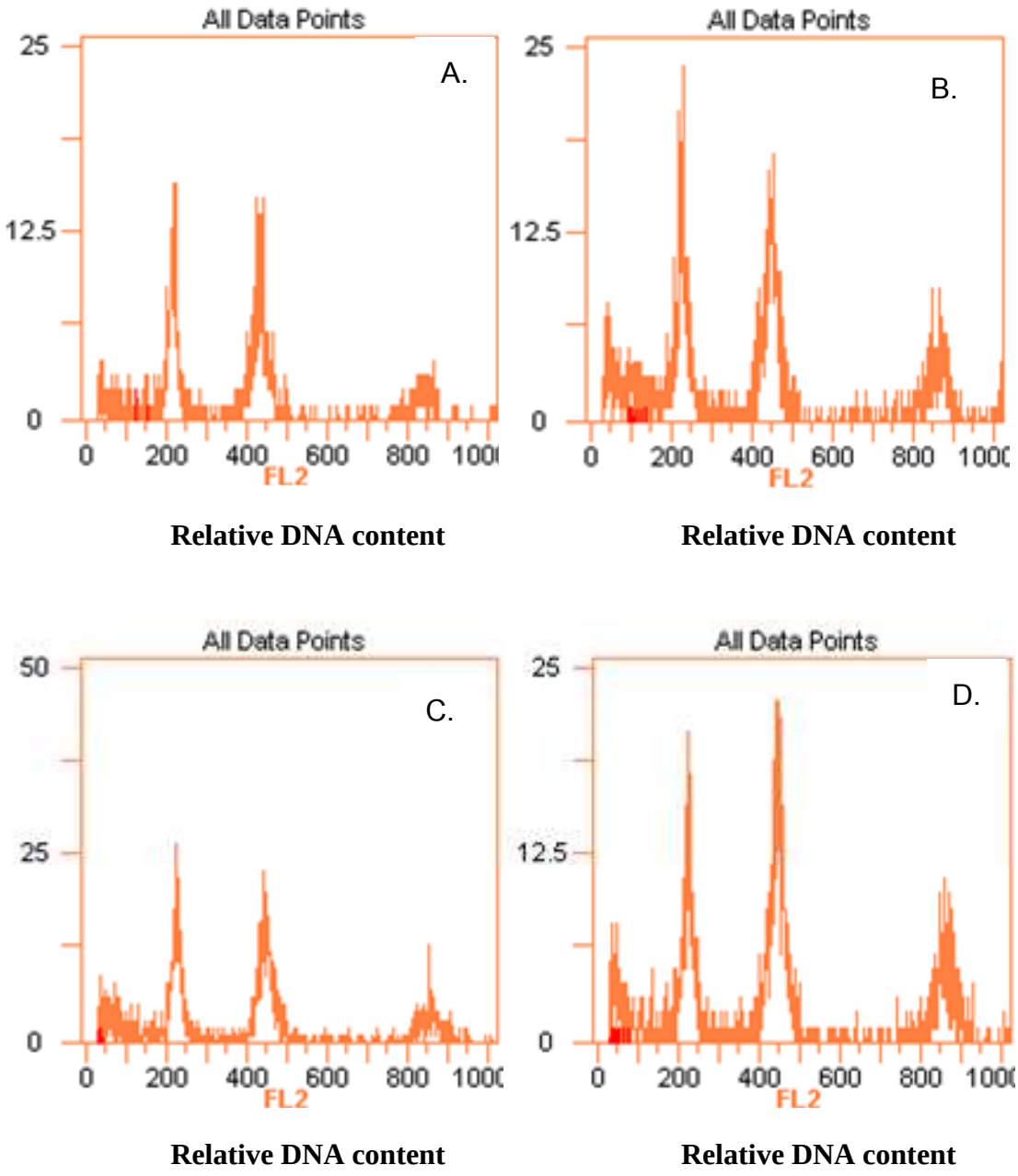



Figure 21. FCM analysis of nuclei isolated from young leaves of 5-month-old plantlets derived from culturing wounded protocorms on ½ MS medium containing PGRs at various concentrations.

A: 0 mg/l NAA + 0 mg/l TDZ (control)

B: 0 mg/l NAA + 0.1 mg/l TDZ

C: 0 mg/l NAA + 1.0 mg/l TDZ

D: 0 mg/l NAA + 3.0 mg/l TDZ

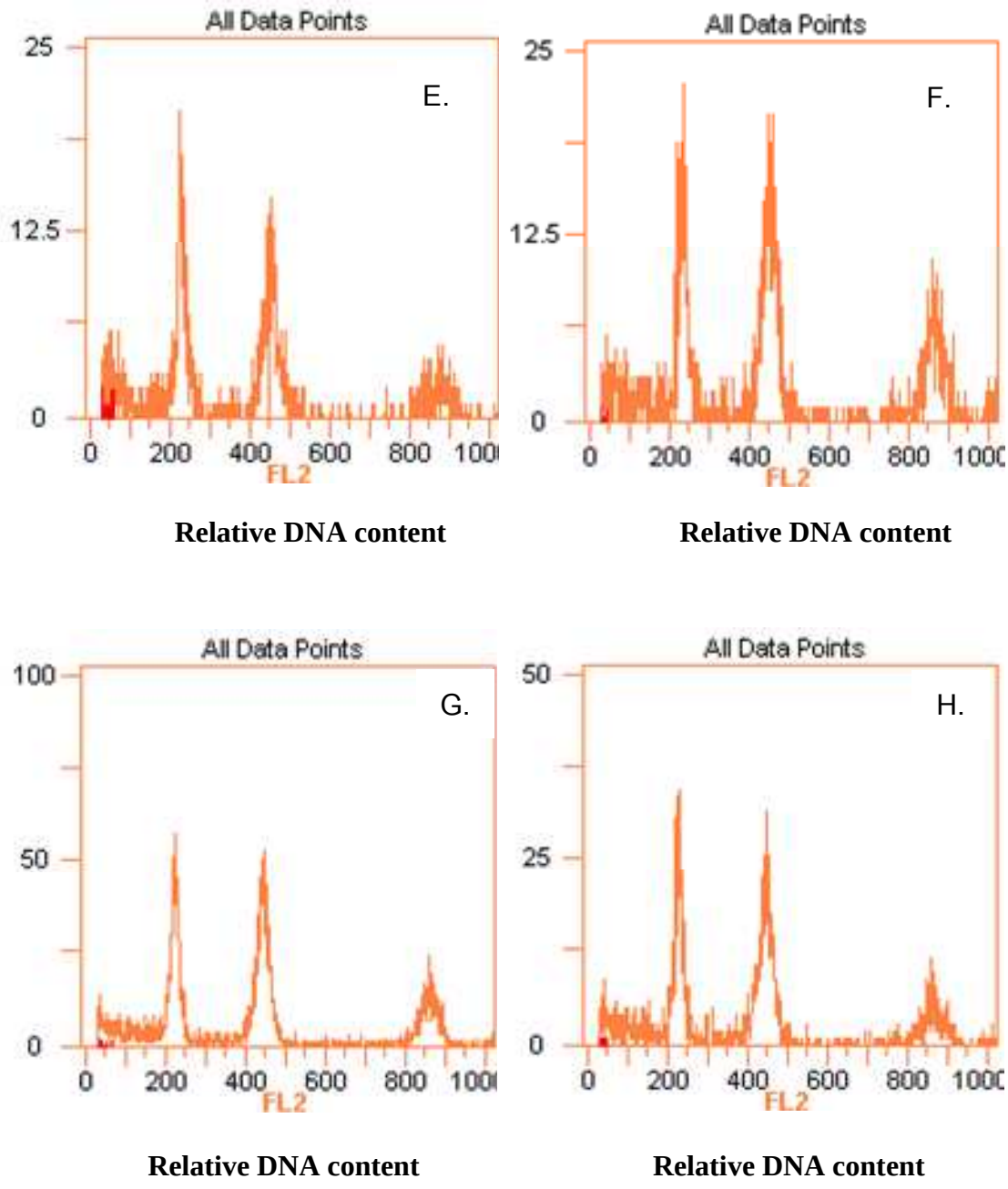



Figure 21. FCM analysis of nuclei isolated from young leaves of 5-month-old plantlets derived from culturing wounded protocorms on ½ MS medium containing PGRs at various concentrations.

E: 0.1 mg/l NAA + 0 mg/l TDZ

F: 0.1 mg/l NAA + 0.1 mg/l TDZ

G: 0.1 mg/l NAA + 1.0 mg/l TDZ

H: 0.1 mg/l NAA + 3.0 mg/l TDZ

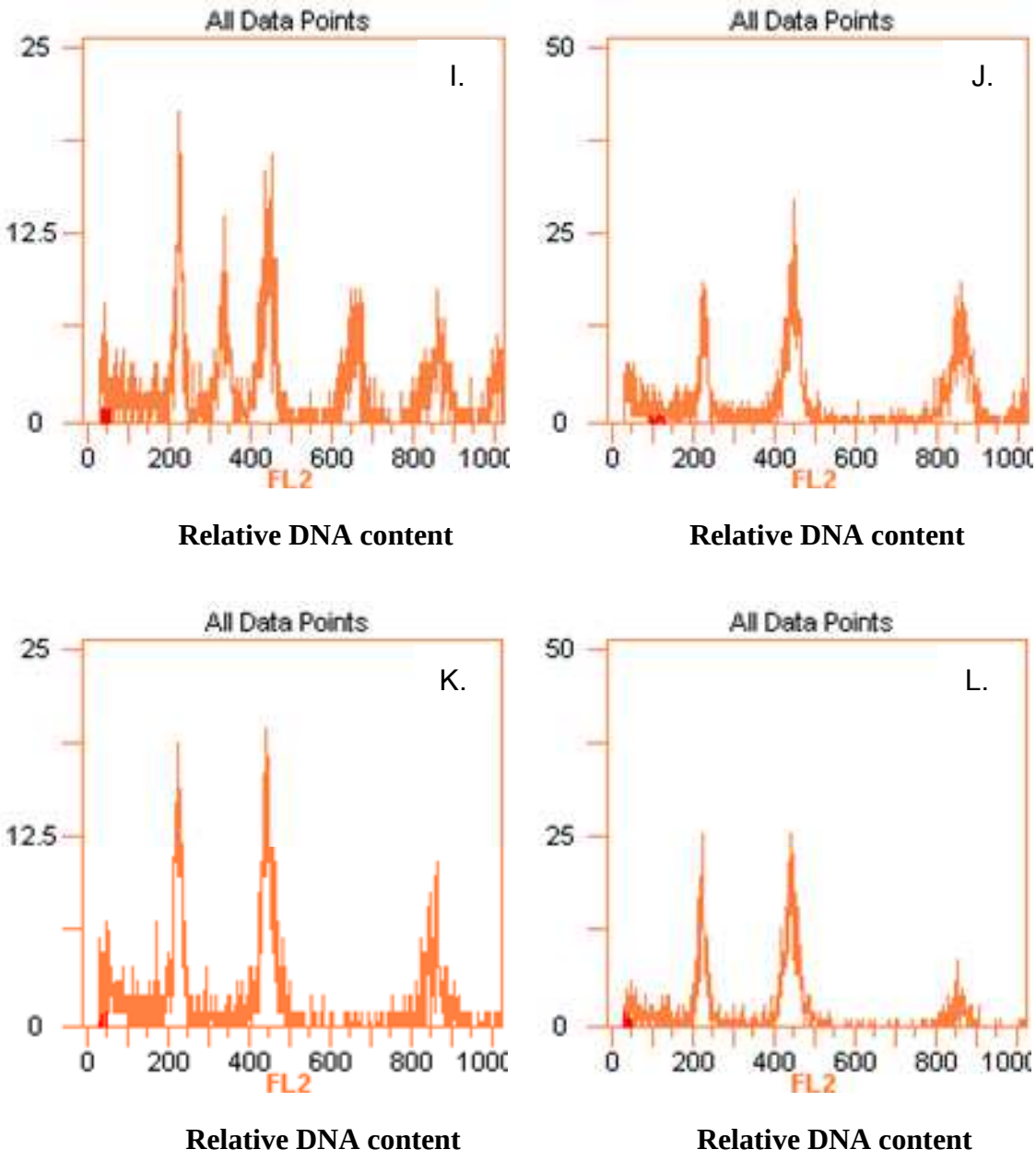



Figure 21. FCM analysis of nuclei isolated from young leaves of 5-month-old plantlets derived from culturing wounded protocorms on ½ MS medium containing PGRs at various concentrations.

I: 1.0 mg/l NAA + 0 mg/l TDZ

J: 1.0 mg/l NAA + 0.1 mg/l TDZ

K: 1.0 mg/l NAA + 1.0 mg/l TDZ

L: 1.0 mg/l NAA + 3.0 mg/l TDZ

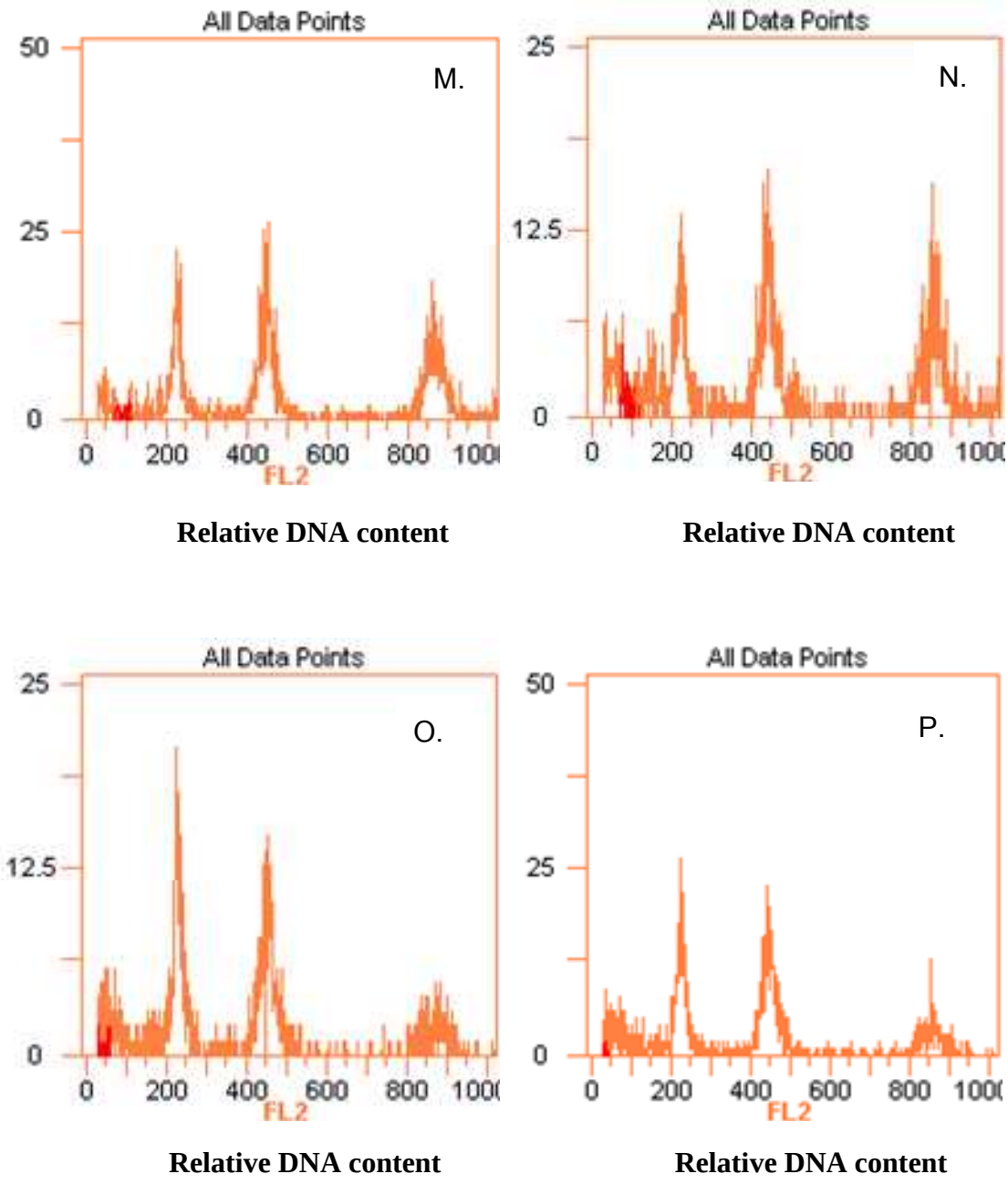



Figure 21. FCM analysis of nuclei isolated from young leaves of 5-month-old plantlets derived from culturing wounded protocorms on ½ MS medium containing PGRs at various concentrations.

M: 0.1 mg/l TDZ + 0.1 mg/l BA

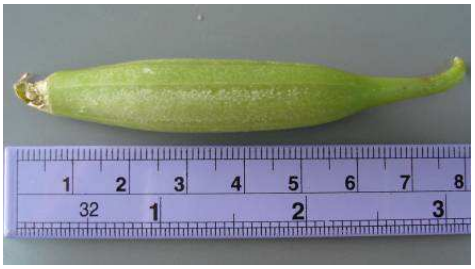
N: 0.1 mg/l TDZ + 1.0 mg/l BA

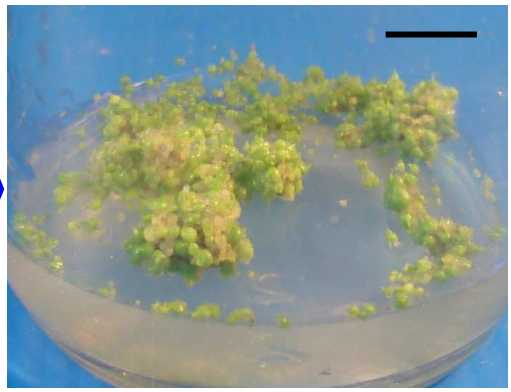
O: 0.1 mg/l TDZ + 3.0 mg/l BA

P: 0.1 mg/l TDZ + 10.0 mg/l BA

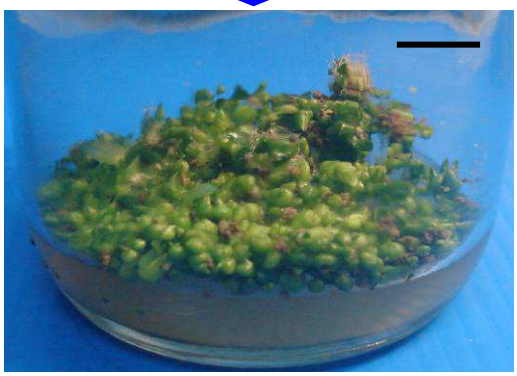
3.1.7 Plant regeneration and transfer to the greenhouse.

Survival rate of plantlets after 2 months of culture in the greenhouse was 100% (Figure 22).

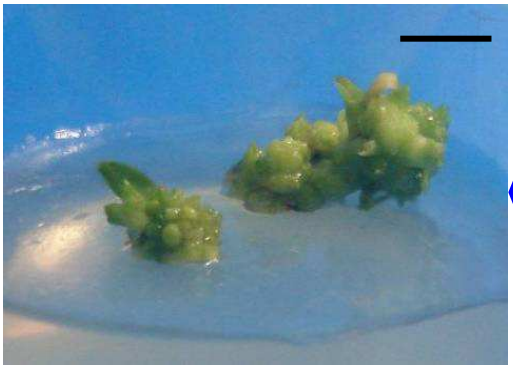




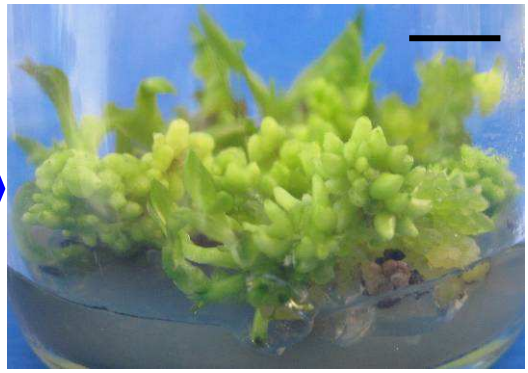
Asymbiotic germination of seeds after 49 days of culture on MS +15% CW.



Protocorms at 2 months after sowing.



PLB derived from culturing wounded protocorms on ½ MS+0.1 mg/l NAA and 0.1 mg/l TDZ combination.



New PLB formation from culturing wounded primary PLB on ½ MS medium supplemented with 0.1 mg/l NAA and 0.1 mg/l TDZ after 4 and 5 months of culture, respectively.

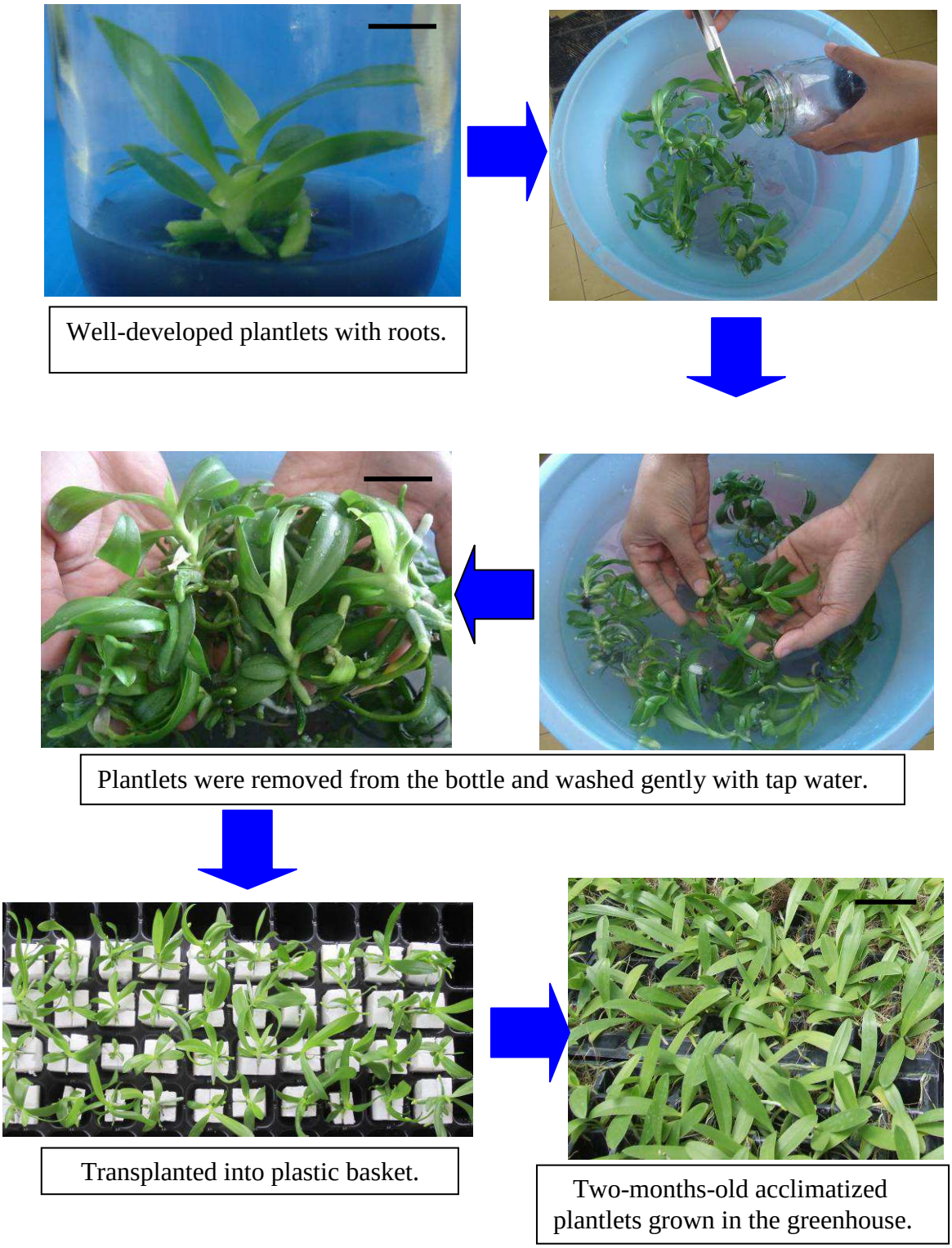



Figure 22. Establishment of micropropagation protocol of *P. cornu-cervi* (Breda) Blume & Rchb. f. through protocorms. (Bar = 1 cm)

## 3.2 Cryopreservation of *Phalaenopsis cornu-cervi* (Breda) Blume & Rchb. f.

### 3.2.1 Effects of cryopreservation methods and ages of protocorm on survival rate of cryopreserved protocorms.

Protocorms derived from 49 days and 2 months of culture (Figure 23) were cryopreserved by various methods, such as directly plunged into liquid nitrogen tank, encapsulation-vitrification method and encapsulation-dehydration method. The results showed that no survival protocorm was obtained from those methods of cryopreservation. However, encapsulation together with vitrification or dehydration protocorms without storing in LN could survive at 10 to 27%. Encapsulation with dehydration gave the better results than encapsulation with vitrification. Moreover, the older protocorms (2-month-old protocorms) gave the better results than younger protocorms (49-day-old protocorms) (Table 8).

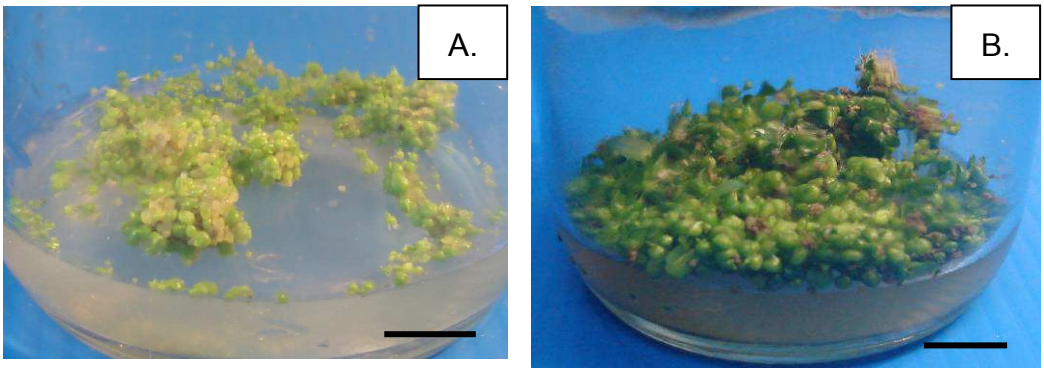



Figure 23. Asymbiotic germination of seeds of *P. cornu-cervi* (Breda) Blume & Rchb. f. on MS medium supplemented with 15% (v/v) CW. (Bar=1 cm)
A: 49 days of protocorms on germination medium.
B: 2 months of protocorms on germination medium.

Table 8 Effects of cryopreservation methods and ages of protocorm on survival rate of cryopreserved protocorms on regrowth medium after 2 months of culture.

| Cryopreservation methods | Survival rate (%) (Mean±S.E.) | |
|---|---|---|
| | (-LN) | (+LN) |
| Protocorms, 49 days old | 100.00±0.00[a] | 0 |
| Protocorms, 2 months old | 100.00±0.00[a] | 0 |
| Protocorms, 49 days old+encapsulation-vitrification 0°C. | 0.00±0.00[e] | 0 |
| Protocorms, 2 months old+encapsulation-vitrification 0°C. | 12.00±1.53[d] | 0 |
| Protocorms, 49 days old+encapsulation-vitrification 25°C. | 0.00±0.00[e] | 0 |
| Protocorms, 2 months old+encapsulation-vitrification 25°C. | 10.67±0.67[d] | 0 |
| Protocorms, 49 days old+encapsulation-dehydration. | 18.33±0.88[c] | 0 |
| Protocorms, 2 months old+encapsulation-dehydration | 26.67±1.67[b] | 0 |

Means followed by different letters within column are significantly different from others at 5% level by DMRT.

3.2.2 Effects of dehydration time on survival rate of cryopreserved encapsulated PLB.

The encapsulated PLB were dehydrated under sterile air-flow at 80 ft/min from the laminar air-flow cabinet at 25±1°C for various time resulted in continually decrease of water content (Figure 24, 25). Water content decreased to 12.36% after 7 hours of dehydration under the laminar air-flow cabinet. No survival rate was obtained from 0-7 hours of dehydration PLB after storing in LN for 1 day (Table 9).

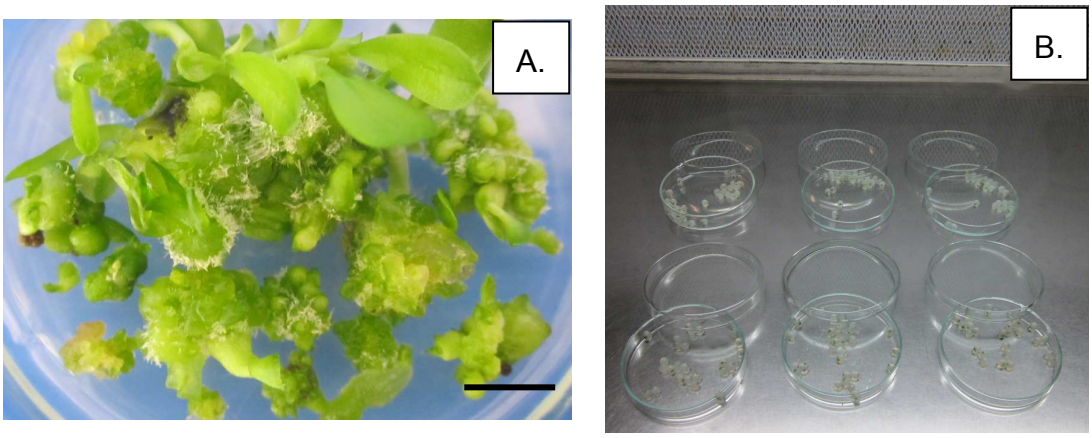



Figure 24. Explants were used to study the effects of dehydration time on survival rate of cryopreserved encapsulated PLB. (Bar=1 cm)

A: New PLB derived from culturing wounded primary PLB on ½ MS medium supplemented with 0.1 mg/l NAA and 0.1 mg/l TDZ for 4 months.

B: Encapsulated PLB placed on open Petri-dishes and exposed to sterile air-flow at 80 ft/min from the laminar air-flow cabinet at 25±1°C for 0-7 hours.

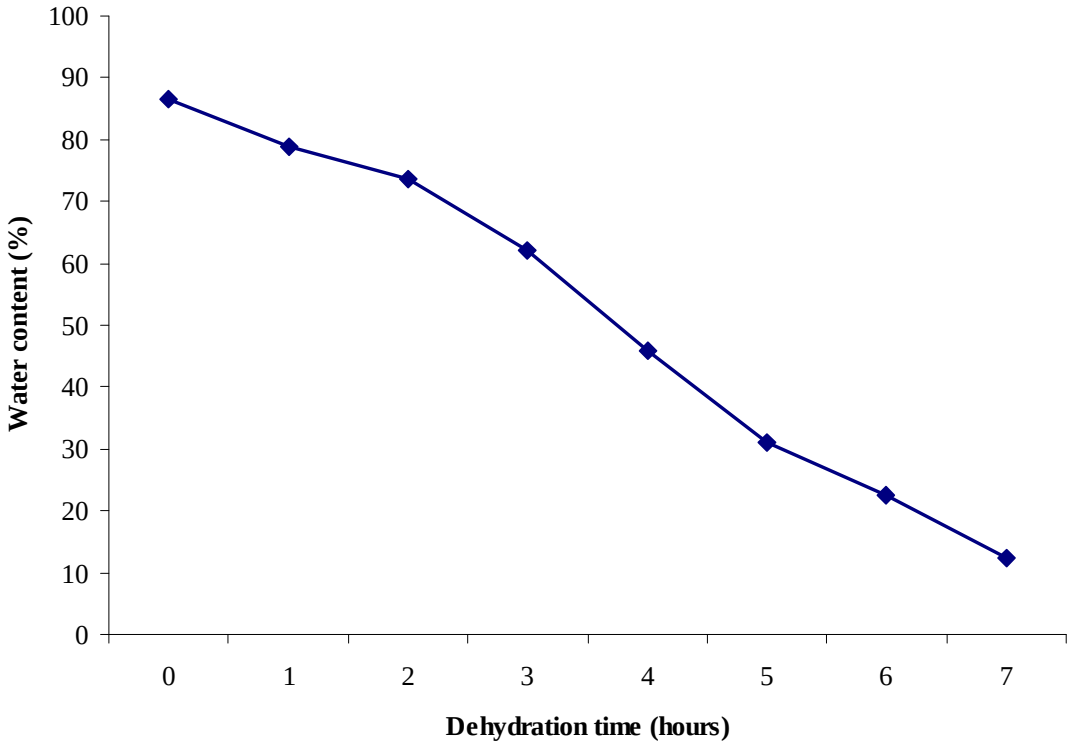



Figure 25. Water content of encapsulated PLB during various time of dehydration (0-7 hours) in a laminar air-flow cabinet at 25±1°C.

Table 9 Effects of dehydration time on survival rate of cryopreserved encapsulated PLB.

| Dehydration duration (Hours) | Survival rate (%) (Mean±S.E.) | |
|---|---|---|
| | (-LN) | (+LN) |
| 0 | 100.00±0.00[a] | 0 |
| 1 | 76.67±3.33[b] | 0 |
| 2 | 73.33±3.33[b] | 0 |
| 3 | 50.00±0.00[c] | 0 |
| 4 | 46.67±3.33[c] | 0 |
| 5 | 35.00±2.89[d] | 0 |
| 6 | 32.33±1.86[de] | 0 |
| 7 | 26.67±1.67[e] | 0 |

Means followed by different letters within column are significantly different from others at 5% level by DMRT.

3.2.3 Effects of cold-hardening and dehydration time on survival rate of cryopreserved encapsulated PLB.

Encapsulated PLB were cold-hardened for 4 days at 8±1°C. For osmotic desiccation, encapsulated PLB were placed on a sterile filter paper laid in open Petri dishes (9 cm in diameter) and then exposed to sterile air-flow at 80 ft/min from the laminar air-flow cabinet at 25±1°C for 0-210 minutes (Figure 26). Changes in the water content of the cold-hardening beads and survival rate of cryopreserved PLB are presented in Figure 27 and Table 10 respectively. The initial water content of the cold-hardening beads was 92.51% on a fresh weight basis (after cold-hardened for 4 days at 8±1°C). Water content decreased to 82.32% after 120 minutes of dehydration under laminar air-flow cabinet and was recorded to be 68.57% after 210 minutes. Survival rate was not obtained from 0-120 minutes of dehydration after cryopreservation (Table 10). This method was not suitable for cryopreservation.

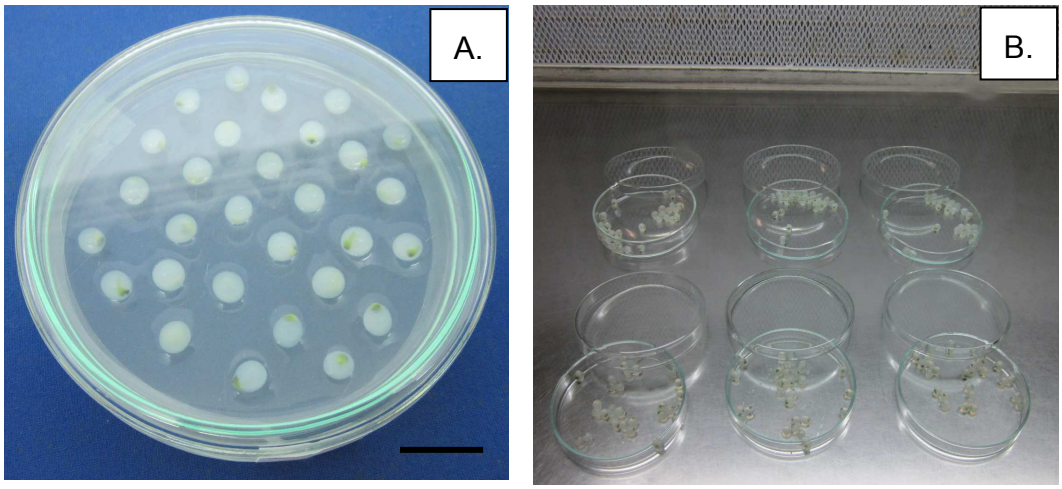



Figure 26. Explants were used to study the effects of cold-hardening and dehydration time on survival rate of cryopreserved encapsulated PLB. (Bar=1 cm)

A: Cold-hardened-encapsulated PLB for 4 days at 8±1°C.

B: Encapsulated PLB placed on open Petri-dishes and exposed to sterile air-flow at 80 ft/min from a laminar air-flow cabinet at 25±1°C for 0-210 minutes.

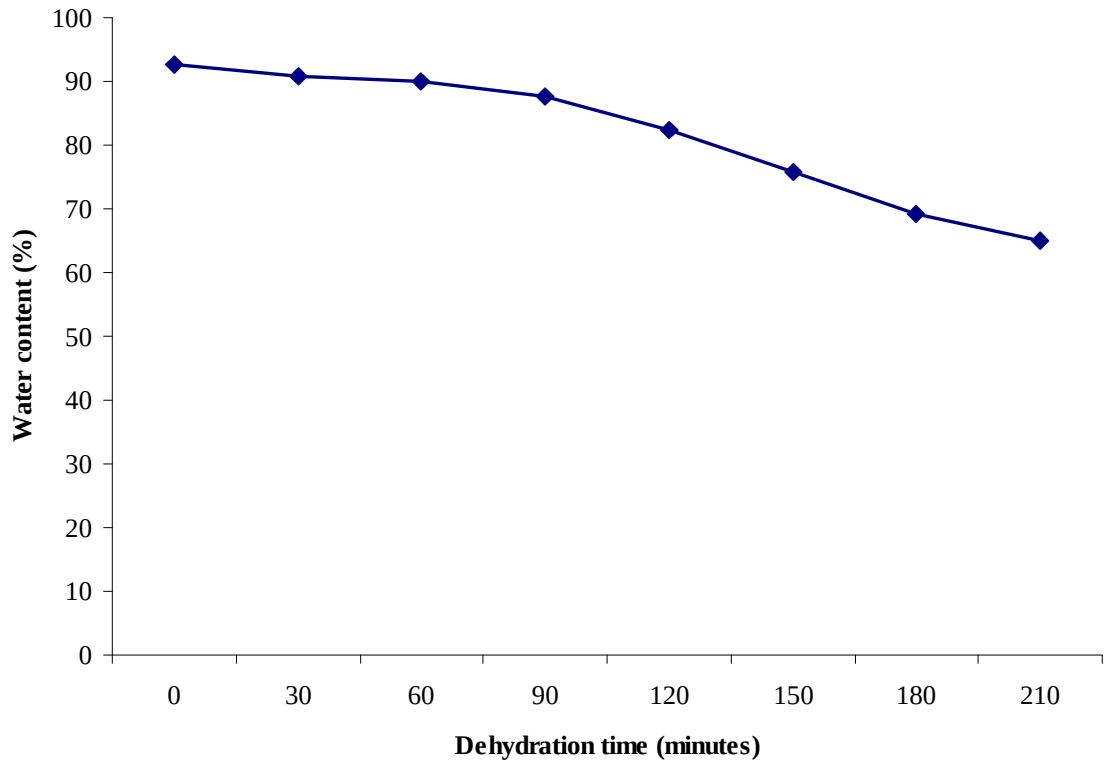



Figure 27. Water content of encapsulated PLB during various dehydration periods (0-210 minutes) in a laminar air-flow cabinet at 25±1°C.

Table 10 Effects of cold-hardening and dehydration time on survival rate of cryo-preserved encapsulated PLB.

| Dehydration duration (Minutes) | Survival rate (%) (Mean±S.E.) | | | |
|---|---|---|---|---|
| | Cold-hardened | | Non Cold-hardened | |
| | (-LN) | (+LN) | (-LN) | (+LN) |
| 0 | 73.33±1.67[a] | 0 | 100.00±0.00[a] | 0 |
| 30 | 63.33±1.67[b] | 0 | 88.33±1.67[b] | 0 |
| 60 | 48.33±1.67[c] | 0 | 73.33±1.67[c] | 0 |
| 90 | 38.33±1.67[d] | 0 | 53.33±3.33[d] | 0 |
| 120 | 36.67±1.67[d] | 0 | 50.00±0.00[de] | 0 |
| 150 | 30.00±2.89[e] | 0 | 43.33±3.33[ef] | 0 |
| 180 | 26.67±1.67[ef] | 0 | 36.67±3.33[fg] | 0 |
| 210 | 21.67±1.67[f] | 0 | 33.33±3.33[g] | 0 |

Means followed by different letters within column are significantly different from others at 5% level by DMRT.

3.2.4 Effects of sucrose pretreatment and dehydration time on survival rate of cryopreserved encapsulated PLB.

To determine the optimal duration of sucrose preculture, encapsulated PLB were precultured in ND liquid medium supplemented with different concentrations of sucrose (0, 0.1, 0.3 M) at 25±1°C for 1 day and then exposed to sterile air-flow at 80 ft/min from the laminar air-flow cabinet at 25±1°C for 0-7 hours. The results revealed that survival rate of PLB were not improved by this procedure. All encapsulated PLB died after cryopreservation in LN for 1 day (Table 11). Water content of encapsulated PLB after dehydration at various times was showed in Figure 28. Preculture of PLB in high concentration of sucrose (0.3 M) caused a gradually decrease in water content whereas low concentration (0.1 M) or without sucrose caused sharply decrease in water content after dehydration. Even though water content in PLB or bead could be adjusted to a low level, survival rate of PLB after storing in LN was not obtained.

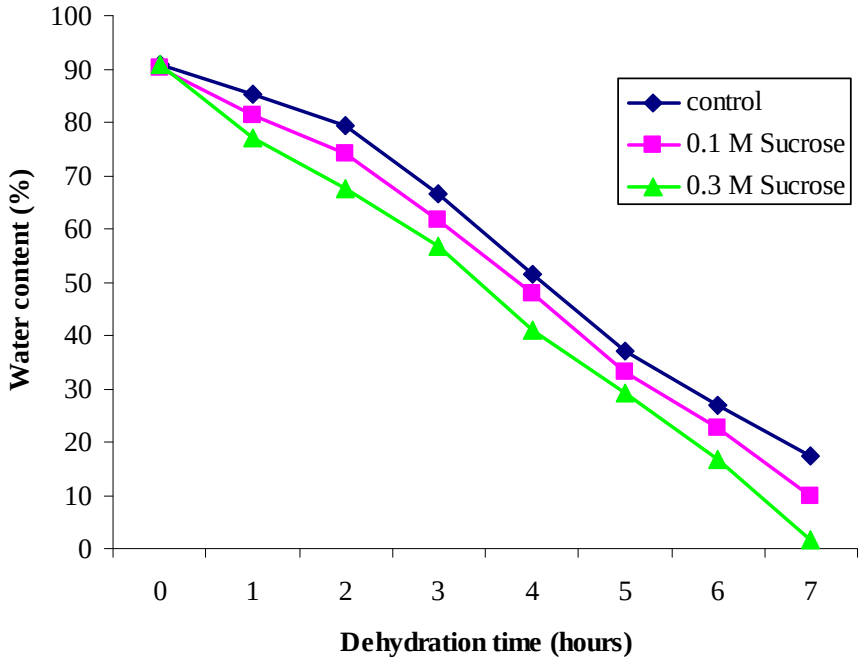



Figure 28. Water content of encapsulated PLB obtained from sucrose pretreatment and dehydration time (0-7 hours) in a laminar air-flow cabinet at 25±1°C.

Table 11 Effects of sucrose pretreatment and dehydration time on survival rate of cryopreserved encapsulated PLB.

| Dehydration times (hours) | Survival rate (%) (Mean±S.E.) | | | | | |
|---|---|---|---|---|---|---|
| | 0 M Sucrose | | 0.1 M Sucrose | | 0.3 M Sucrose | |
| | (-LN) | (+LN) | (-LN) | (+LN) | (-LN) | (+LN) |
| 0 | 100.00±0.00$^{a}$ | 0 | 76.67±3.33$^{a}$ | 0 | 66.67±3.33$^{a}$ | 0 |
| 1 | 76.67±3.33$^{b}$ | 0 | 60.00±5.77$^{b}$ | 0 | 53.33±3.33$^{b}$ | 0 |
| 2 | 73.33±3.33$^{b}$ | 0 | 46.67±3.33$^{c}$ | 0 | 36.67±6.67$^{c}$ | 0 |
| 3 | 56.67±3.33$^{c}$ | 0 | 36.67±6.67$^{cd}$ | 0 | 30.00±5.77$^{cd}$ | 0 |
| 4 | 53.33±6.67$^{cd}$ | 0 | 40.00±5.77$^{cd}$ | 0 | 23.33±3.33$^{d}$ | 0 |
| 5 | 46.67±3.33$^{cde}$ | 0 | 26.67±3.33$^{d}$ | 0 | 6.67±3.33$^{e}$ | 0 |
| 6 | 43.33±3.33$^{de}$ | 0 | 20.00±0.00$^{e}$ | 0 | 3.33±3.33$^{e}$ | 0 |
| 7 | 36.67±3.33$^{e}$ | 0 | 16.67±3.33$^{e}$ | 0 | 0.00±0.00$^{e}$ | 0 |

Means followed by different letters within column are significantly different from others at 5% level by DMRT.

## 3.3 Alginate-encapsulation, short-term storage and plantlet regeneration from PLB of *Phalaenopsis cornu-cervi* (Breda) Blume & Rchb. f.

### 3.3.1 Effects of different storage conditions and intervals on their conversion ability of *Phalaenopsis cornu-cervi* (Breda) Blume & Rchb. f.

*In vitro* derived PLB of *P. cornu-cervi* (Breda) Blume & Rchb. f. were encapsulated as synthetic seeds preparation by alginate encapsulation, and then stored in artificial endosperm solution at 4±1°C, 8±1°C and 25±1°C conditions in interaction with different storage intervals of 15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165 and 180 days to evaluate the comparative regrowth capacity of synthetic seeds.

Among the three temperature regimes, room temperature (25±1°C) storage gave promising results for germination of capsules. Conversion percentage of synthetic seeds decreased from 35% to 11.67% after 30 days of storage at 8±1°C. However, the PLB stored more than 30 days in such condition gave no germination at all (Table 12, Figure 29). Encapsulated PLB stored at 4±1°C lost their viability completely (Table 12).

Table 12 Effects of different storage temperatures and time on conversion of encapsulated PLB of *Phalaenopsis cornu-cervi* (Breda) Blume & Rchb. f.

| Storage temperature (°C) | Storage duration (days) | Conversion (Mean± S.E.) (%) |
|---|---|---|
| 4±1°C | 15 | 0.00±0.00[d] |
| | 30 | 0.00±0.00[d] |
| | 45 | 0.00±0.00[d] |
| | 60 | 0.00±0.00[d] |
| | 75 | 0.00±0.00[d] |
| | 90 | 0.00±0.00[d] |
| | 105 | 0.00±0.00[d] |
| | 120 | 0.00±0.00[d] |
| | 135 | 0.00±0.00[d] |
| | 150 | 0.00±0.00[d] |
| | 165 | 0.00±0.00[d] |
| | 180 | 0.00±0.00[d] |
| 8±1°C | 15 | 35.00±2.89[b] |
| | 30 | 11.67±1.67[c] |
| | 45 | 0.00±0.00[d] |
| | 60 | 0.00±0.00[d] |
| | 75 | 0.00±0.00[d] |
| | 90 | 0.00±0.00[d] |
| | 105 | 0.00±0.00[d] |
| | 120 | 0.00±0.00[d] |
| | 135 | 0.00±0.00[d] |
| | 150 | 0.00±0.00[d] |
| | 165 | 0.00±0.00[d] |
| | 180 | 0.00±0.00[d] |
| 25±1°C | 15 | 100±0.00[a] |
| | 30 | 100±0.00[a] |

Table 12 Effects of different storage temperatures and time on conversion of encapsulated PLB of *Phalaenopsis cornu-cervi* (Breda) Blume & Rchb. f.

| Storage temperature (°C) | Storage duration (days) | Conversion (Mean± S.E.) (%) |
|---|---|---|
| 25±1°C | 45 | * |
| | 60 | * |
| | 75 | * |
| | 90 | * |
| | 105 | * |
| | 120 | * |
| | 135 | * |
| | 150 | * |
| | 165 | * |
| | 180 | * |

* Encapsulated PLB germinated when storing in sterile Petri dishes.

Means followed by different letters within column are significantly different from others at 5% level by DMRT.

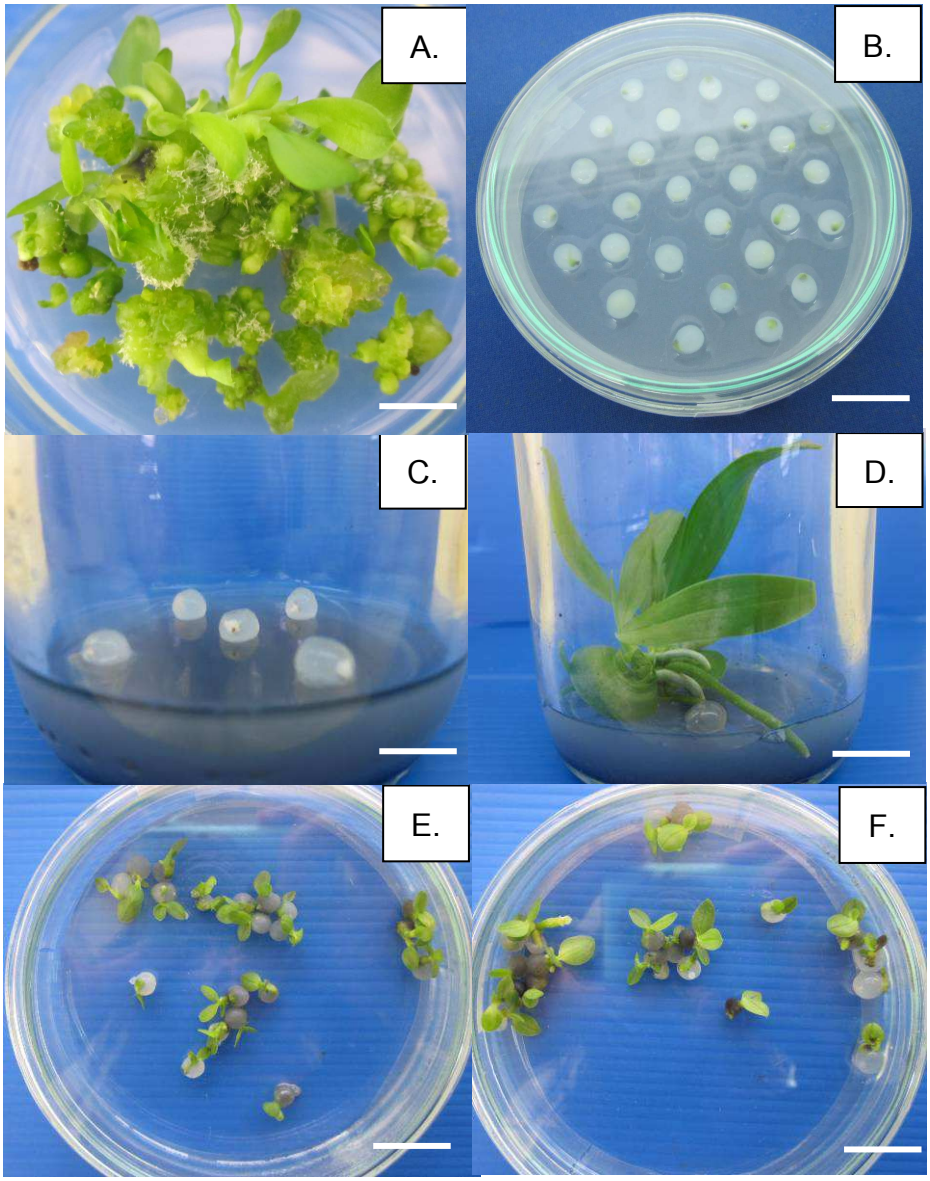



Figure 29. Short-term storage and regeneration of encapsulated PLB of *P. cornu-cervi* (Breda) Blume & Rchb. f. (Bar=1 cm)

A: New PLB derived from culturing wounded primary PLB on ½ MS Medium supplemented with 0.1 mg/l NAA and 0.1 mg/l TDZ combination after 4 months.

B: Calcium alginate bead with PLB of *P. cornu-cervi* (Breda) Blume & Rchb. f.

C: Encapsulated PLB stored at 4±1°C for 15 days lost their viability completely.

D: Plantlets with well-developed shoots and roots after 2.5 months of culture on ND medium supplemented with 4% (w/v) sucrose with activated charcoal, regenerated from encapsulated PLB after 15 days of storing at 8±1°C.

E, F: Conversion of PLB to shoots in sterile Petri dishes from encapsulated PLB after 45 and 60 days of storing at room temperature (25±1°C).

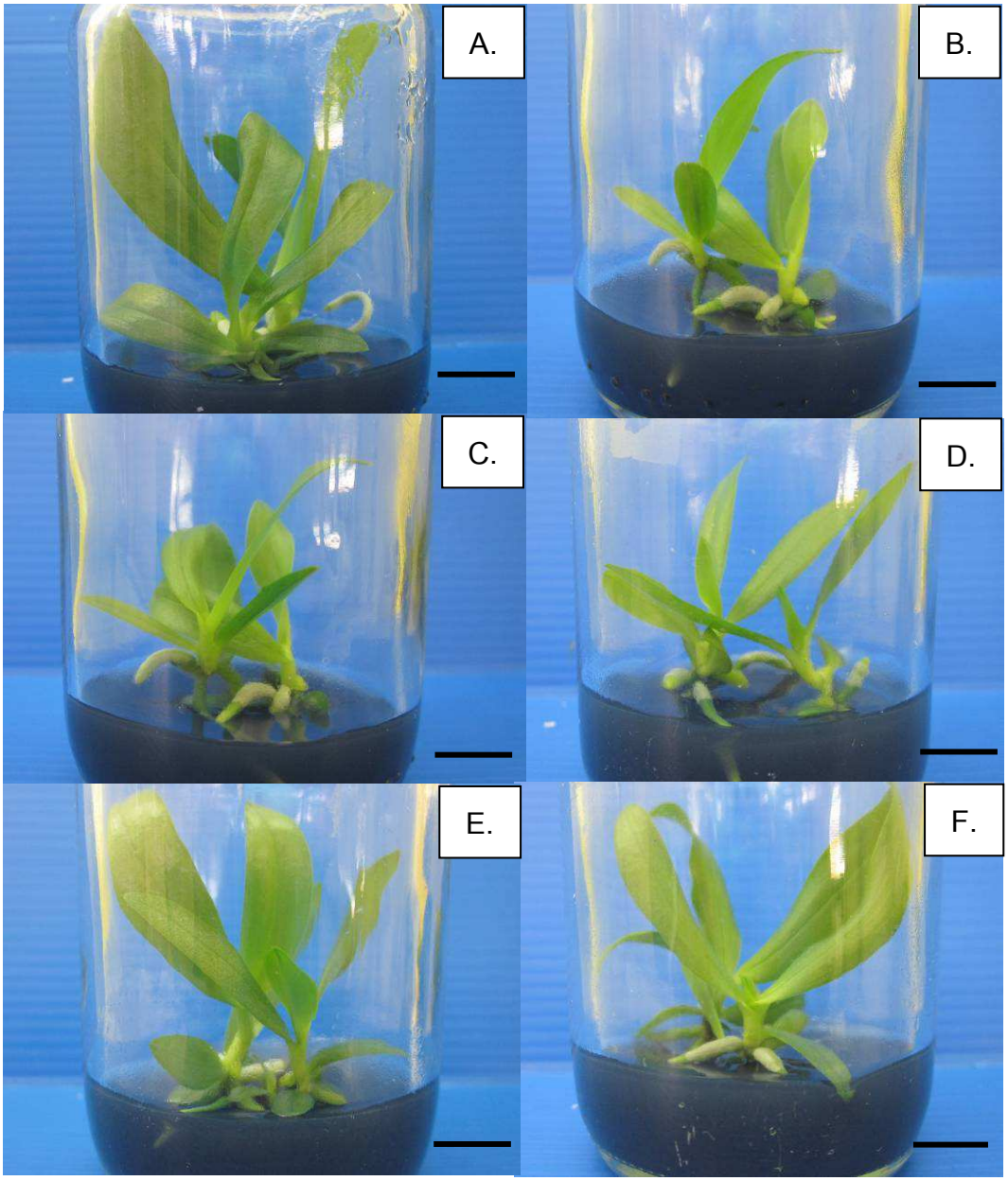



Figure 30. Healthy plantlets derived from different storage temperatures and time on germination of encapsulated PLB of *P. cornu-cervi* (Breda) Blume & Rchb. f. after 4 months of culture on ND medium supplemented with 4% (w/v) sucrose and 0.2% (w/v) activated charcoal. (Bar=1 cm)

A: Control plantlets

B: Plantlets derived from encapsulated PLB storing at 8±1°C for 15 days.

C: Plantlets derived from encapsulated PLB storing at 8±1°C for 30 days.

D: Plantlets derived from encapsulated PLB storing at 25±1°C for 15 days.

E: Plantlets derived from encapsulated PLB storing at 25±1°C for 30 days.

F: Plantlets derived from encapsulated PLB storing at 25±1°C for 45 days.

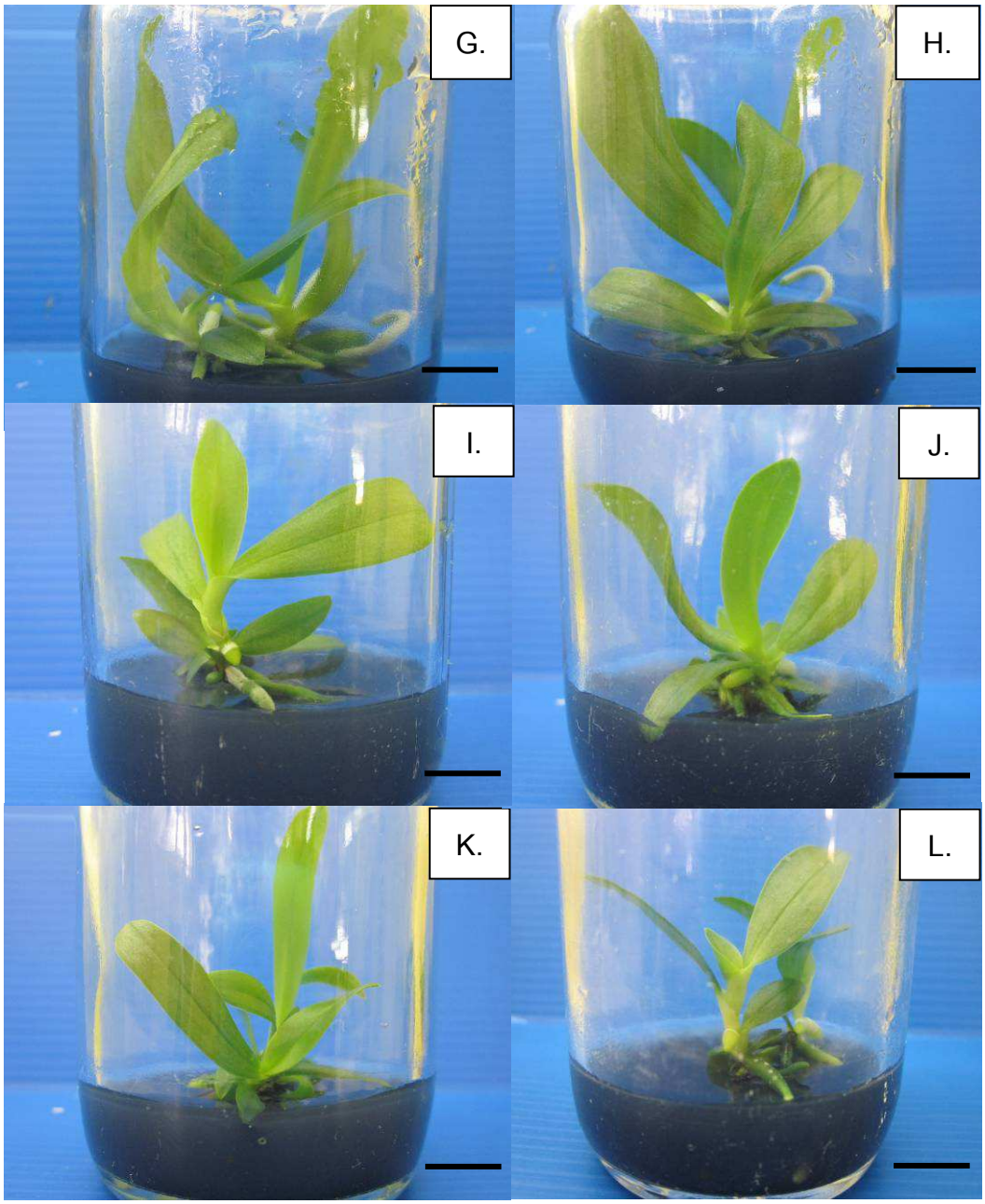



Figure 30. Healthy plantlets derived from different storage temperatures and time on germination of encapsulated PLB of *P. cornu-cervi* (Breda) Blume & Rchb. f. after 4 months of culture on ND medium supplemented with 4% (w/v) sucrose and 0.2% (w/v) activated charcoal. (Bar=1 cm)

G: Plantlets derived from encapsulated PLB storing at 25±1°C for 60 days.

H: Plantlets derived from encapsulated PLB storing at 25±1°C for 75 days.

I: Plantlets derived from encapsulated PLB storing at 25±1°C for 90 days.

J: Plantlets derived from encapsulated PLB storing at 25±1°C for 105 days.

K: Plantlets derived from encapsulated PLB storing at 25±1°C for 120 days.

L: Plantlets derived from encapsulated PLB storing at 25±1°C for 135 days.

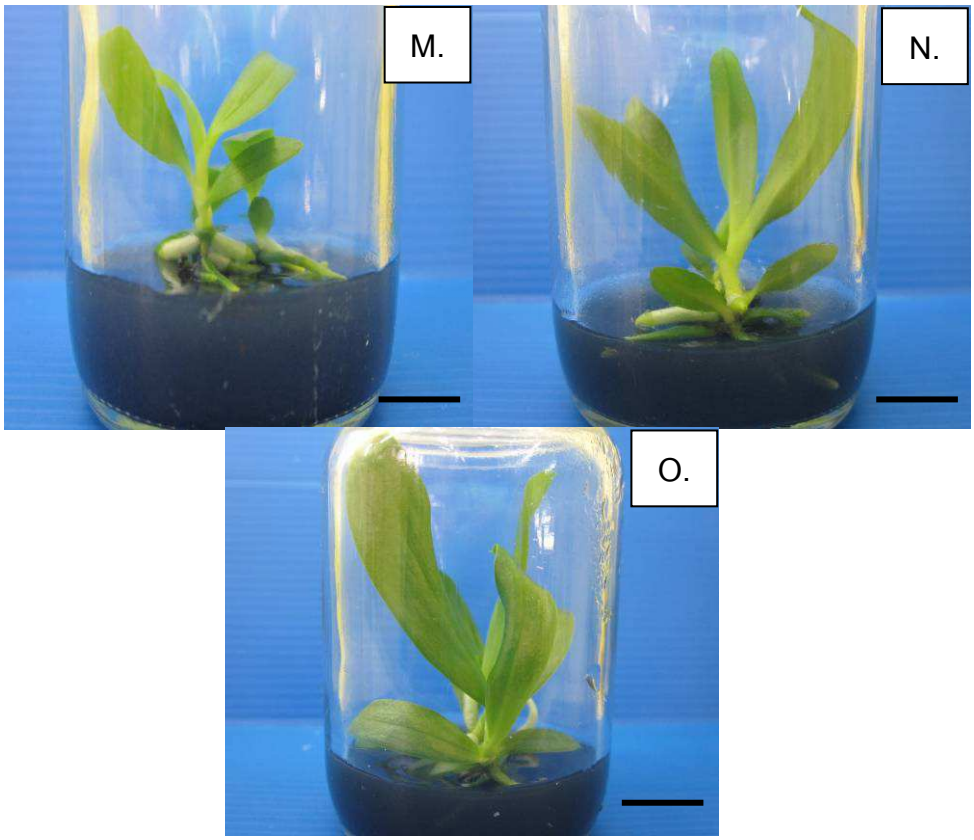



Figure 30. Healthy plantlets derived from different storage temperatures and time on germination of encapsulated PLB of *P. cornu-cervi* (Breda) Blume & Rchb. f. after 4 months of culture on ND medium supplemented with 4% (w/v) sucrose and 0.2% (w/v) activated charcoal. (Bar=1 cm)

M: Plantlets derived from encapsulated PLB storing at 25±1°C for 150 days.

N: Plantlets derived from encapsulated PLB storing at 25±1°C for 165 days.

O: Plantlets derived from encapsulated PLB storing at 25±1°C for 180 days.

3.3.2 Ploidy instability analysis using flow cytometry (FCM).

FCM analysis of plantlets at 5 months after culture initiation derived from either control or different storage temperatures showed the same pattern of relative DNA content peak. There was no change in the ploidy level was observed (Figure 31)**.**

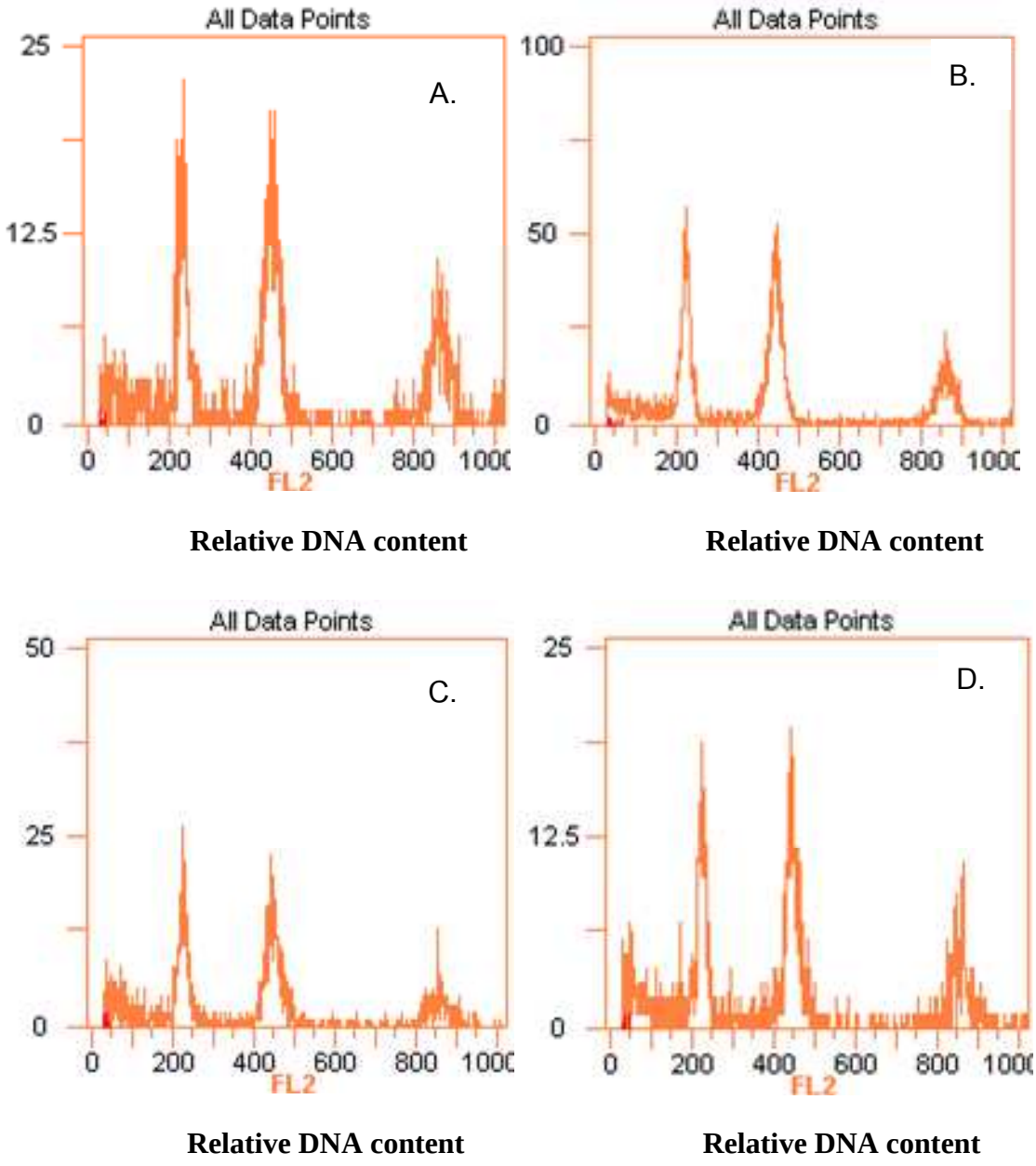



Figure 31. FCM analysis of nuclei isolated from young leaves of 5-month-old plantlets derived from encapsulated PLB which were stored at various temperatures and time.

A: Plantlets derived from culturing wounded primary PLB on ½ MS medium supplemented with 0.1 mg/l NAA and 0.1 mg/l TDZ (Control).

B: Plantlets derived from encapsulated PLB storing at 8±1°C for 15 days.

C: Plantlets derived from encapsulated PLB storing at 8±1°C for 30 days.

D: Plantlets derived from encapsulated PLB storing at 25±1°C for 30 days.

# CHAPTER 4

# DISCUSSION

## 4.1 Micropropagation of *Phalaenopsis cornu-cervi* (Breda) Blume & Rchb. f.

### 4.1.1 Seed germination, protocorm formation and plantlet development.

The seeds of *Phalaenopsis cornu-cervi* (Breda) Blume & Rchb. f. germinated on MS medium supplemented with 15% (v/v) coconut water (CW) within 49 days. Each species of orchid has been reported to use specific culture media for germination of seeds (Arditti and Ernst, 1984, 1993; Kauth *et al*., 2008). However, MS medium has been used by many investigators to asymbiotically germinate of orchid seeds of different genera including *Dendrobium* (Rangsayatorn, 2009; Vendrame *et al*., 2007). MS medium containing highly enriched macro- and micro-elements and vitamins (Murashige and Skoog, 1962). The advantage of using seeds from immature capsules is that embryos become viable and easy to surface-sterilize for *in vitro* seed germination (Yam and Weatherhead, 1988; Mitchell, 1989). The orchid seeds are very small and have a poor level of germination. Endosperm development does not occur and the embryo development stops at the globular stage. Since the endosperm is absent the seeds have limited food reserves, for example lipid droplets, and small amounts of proteins. Irrespective of these limitations the orchid seeds can germinate *in vitro* (Knudson, 1946; Arditii, 1967).

Formation of orchid seedlings from the seeds involves three successive phases: germination, formation of protocorm, and seedling development. Germination of orchid seeds started by passage of water through the testa of the seeds. Early germination is recognized by observing the color and shape of the seeds. The first visible signs of germination are swelling of the embryo followed by turning green and emergence of the breasted seed coats. This is called the spherical stage, and subsequently develops into the protocorm stage and later into the swelling stage. The formation of rhizoids in the protocorm is a unique feature of Orchidaceae. Subsequent cell division occurs in the apical and basal regions of the protocorm and in leaf

primordia; morphogenesis is started from the shoot meristem. Later, the first root is formed endogenously (Mitra *et al*., 1976).

Activated charcoal supplemented in culture medium promotes further development of the seedlings. In this present study, the seedlings showed faster growth on MS medium supplemented with 0.2% (w/v) activated charcoal. The positive properties of activated charcoal might adsorb unidentified morphogeneticaly active or toxic substances, 5-hydroxy-methylfurfural, which is produced by the dehydration of sucrose during autoclaving, especially inhibitory phenolics and carboxylic compounds produced by cut or damaged tissues, and excessive hormones and vitamins in the media like the report of Pan and van Staden (1998). Moreover, it plays a role in its positive effect on development of protocorms. However, the results from this study showed that activated charcoal is not necessary during the early stages of seedling growth but had a critical role in further development of the seedlings.

### 4.1.2 Effects of culture media on growth and PLB induction from protocorms *in vitro.*

This study was undertaken to evaluate the growth of *P. cornu-cervi* (Breda) Blume & Rchb. f. seeds as influenced by three commonly used culture media in orchid. Our results showed that ND and MS medium were superior to VW medium and both media containing 15% (v/v) coconut water (CW) gave better results than those without CW. CW is known to act like a cytokinin substance, hence promote cell division and growth of the protocorms (Bonga and Aderkas,1992). However, in this present study all culture media could not induce and multiply PLB. Beside seeds, other explants also showed a high potential for PLB induction, such as leaf explants of *P. amabilis* (Chen and Chang, 2006) and root tips of *Doritaenopsis*. (Park *et al*., 2003).

### 4.1.3 Effects of ascorbic acid and culture media on PLB induction from bisected and protocorms *in vitro.*

Bisected protocorms cultured in VW liquid medium supplemented with 150 mg/l ascorbic acid in the present study gave the best proliferation of PLB at 21 PLB/explant after being cultured for 50 days. The other explants in VW liquid culture media caused browning or blackening of media after 50 days of culture. Even bisected protocorms cultured in ND liquid medium containing all concentrations of ascorbic acid tested turned black and could not produce the new PLB. Tanaka and Sakanishi (1977) reported that phenolic exudation caused poor regeneration capacity in *Phalaenopsis* tissue culture. The tissue blackening or browning caused by the oxidation of phenolic compounds which are exuded from cut surfaces of explants of woody plants, fruits, and vegetables and caused a serious problem in establishing *in vitro* cultures (Yildiz *et al*., 2007). The exudates inhibit growth and eventually cause tissue necrosis. Enzymes, such as polyphenoloxidase (PPO) and peroxidase (POD) participate in blackening *in vitro* cultures (Pizzocaro *et al*., 1993; Down and Norton, 1995; Whitaker and Lee, 1995) and catalyze oxidation of phenolic compounds.

PPO-catalyzed tissue blackening can be prevented by chemical, enzymatic and physical treatments (Laurila *et al*., 1998). However, these treatments often cannot be used in *in vitro* culture. It has been recommended that activated charcoal, ascorbic acid, citric acid, and sodium chloride can be added to culture medium to limit *in vitro* tissue blackening (Pizzocaro *et al*., 1993). Attempts in propagation of those plant species through tissue culture technique have had limited success due to the oxidative browning of explants (Ziv and Halevy, 1983; Paiva *et al*., 2004; Kantharaju *et al*., 2008). The browning and subsequent death of cultured explants is a major problem that is usually dependent on the phenolic compounds and the quantity of total phenols (Ozyigit, 2008). Phenolic compounds occur as secondary metabolites in all plant species (Antolovich *et al*., 2000; Kefeli *et al*., 2003). The phenols are synthesized by the plants and in many cases excreted and then oxidized (Ozyigit, 2008). In tissue culture studies, phenolic substances, especially oxidized phenols generally affected *in vitro* development negatively (Arnaldos *et al*., 2001). Oxidized phenolic compounds may inhibit enzyme activity and result in the darkening of the culture medium and

subsequent lethal browning of explants (Compton and Preece, 1986; Laukkanen *et al*., 1999).

The antioxidant, ascorbic acid, was selected as it has been used successfully in the past to inhibit the exudation of phenols and to reduce oxidative browning in various plant species (Arditti and Ernst, 1993; George, 1996; Abdelwahd *et al*., 2008). Ascorbic acid is able to scavenge oxygen radicals produced when the plant tissue is wounded, therefore protecting the cells from oxidative injury. The oxidative browning of explants tissue is reduced by ascorbic acid detoxifying these free radicals (Titov *et al*., 2006). Thus, ascorbic acid is useful and effective in managing the problem of phenolics and improving plant growth *in vitro* (Abdelwahd *et al*., 2008). North *et al*. (2012) reported that activated charcoal was significantly found to reduce the total phenol content of media by 53%, compared with ascorbic acid. Furthermore, the wounding of explants significantly increased phenolic exudation.

Tissue injury stimulates the production of phenols (Dodds and Roberts, 1995) and phenolic exudation is exaggerated in response to wounding (George, 1993; Zeweldu and Ludders, 1998; Strosse *et al*., 2009). The deposition of phenolic acids in plant cell walls is an important defense mechanism (Bolwell *et al*., 1985; Pan and van Staden, 1998; Ndakidemi and Dakora, 2003), which exerts an inhibitory growth function when they are excreted from the plant (Kefeli *et al*., 2003). When cells are damaged, like the wounding performed in this study, the sub-cellular compartmentation is lost, enabling the contents of cytoplasm and vacuoles to mix and phenolic compounds readily become oxidized by air (Compton and Preece, 1986; Laukkanen *et al*., 1999). Phenol oxidation and exudation takes place in these scarred surface cells (Ozyigit, 2008). Oxidized phenolic compounds may inhibit enzyme activity and result in darkening of the culture medium and subsequent lethal browning of explants (Compton and Preece, 1986; Laukkanen *et al*., 1999).

Phenolic concentration is often affected by several internal and external factors (Zapprometov, 1989). Some nutrients (Lux-Endrich *et al*., 2000) and some stress factors like drought, water, radiation and pathogen infection from injured surfaces effect concentrations of the phenolics in plants (Zapprometov, 1989; Kefeli *et al*., 2003). The various PGRs concentrations may affect phenolic exudation as phenols are reactive compounds (Lux-Endrich *et al*., 2000).

In the future, ascorbic acid together with activated charcoal might be incorporated in culture media for a comparative study to elucidate the most effective in reducing phenolic compound exudation. Activated charcoal is commonly used in tissue culture media to improve cell growth and development (Pan and van Staden, 1998; Thomas, 2008). The beneficial effects of activated charcoal may be attributed to its irreversible adsorption of inhibitory compounds in the culture medium and substantially reduce the toxic metabolites, phenolic exudation and exudate accumulation (Fridborg *et al*., 1978; Thomas, 2008). This high adsorptive capacity is due to the structure of activated charcoal. It has a very fine network of pores with a large inner surface area on which many substances can be adsorbed (Pan and van Staden, 1998; Dąbrowski *et al*., 2005; Thomas, 2008).

### 4.1.4 Effects of starting explants and PGRs on PLB formation.

No effect of PGRs was observed on PLB induction from leaf segments of *P. cornu-cervi* (Breda) Blume & Rchb. f. However, Nayak *et al*. (1997a) reported that leaf segments of *Acampe praemorsa* could produce PLB. Chen and Chang (2006) also reported PLB formation from leaf explants of *P. amabilis* after 45 days of culture. The successful PLB induction from culturing leaf explants might depend upon genotype and PGRs containing in culture medium. For *P. cornu-cervi* (Breda) Blume & Rchb. f. in the present study, leaf explants produced enormous phenolic compounds leading to the failure in PLB formation. Thus, the next investigation should be concentrated on a higher concentration of plant growth regulators together with antioxidant, e.g. ascorbic acid, polyvinylpyrolidone (PVP) or activated charcoal alone or in combination.

Among four different explant types, the wounded protocorms were feasible. Culture method of wounded protocorm described here is an efficient *in vitro* technique for the rapid propagation of *P. cornu-cervi* (Breda) Blume & Rchb. f. In the PGR-free ½ MS medium, only about 30% of wounded protocorms developed into PLB. The results obtained in the present study were similar to those reported by Yam *et al*., (1991) and Park *et al*., (2000) which found that trimmed protocorms at the basal part promoted PLB multiplication in *Phalaenopsis*. In our culture system, wound treatment caused by the cutting process played an important role in the formation of new PLB.

Our experiments showed that wounding can bring about the production of a higher number of new PLB on the original protocorms or PLB, but it also caused the death of some of the wounded protocorms. Thus, it could be concluded that protocorms with wound produced new PLB at greater numbers than those with untrimmed or unwounded ones.

Between the two cytokinins tested (BA, TDZ), TDZ was effective in the PLB induction from wounded protocorm segments of *P. cornu-cervi* (Breda) Blume & Rchb. f. The types and concentrations of PGRs played an important role in *in vitro* propagation of many orchid species (Arditti and Ernst, 1993). In this present study, TDZ was more effective than BA for inducing PLB formation in *P. cornu-cervi* (Breda) Blume & Rchb. f. It was also clearly shown that TDZ alone was more effective than BA in PLB induction and proliferation. This result was also in agreement with the observations in *Cymbidium ensifolium* var. *misericors*. (Chang and Chang, 1998), *Phalaenopsis* and *Doritaenopsis* (Ernst, 1994), *Epidendrum radicans* (Chen *et al*., 2002), *Doritaenopsis* (Park *et al*., 2003), and *Phalaenopsis* (Kuo *et al*., 2005). Recently, TDZ has been used in orchid tissue culture for various purposes due to its remarkable ability to induce callus or organogenesis. TDZ induced callus formation from various explants, especially when it was used along with either NAA or 2, 4-D (Huan and Tanaka, 2004). Induction of organogenesis and somatic embryogenesis using TDZ has been reported in several orchid species (Ernst, 1994; Chen and Piluek, 1995; Nayak *et al*., 1997b; Chen and Chang, 2001). However, auxins in term of NAA are no longer need to combine with TDZ in this study. It might be possible that the species being studied could produce a large quantity of endogenous auxins itself. Thus, there is no need to add exogenous auxins. By addition of auxins, negative effect was observed. A high frequency of browning of protocorms was obtained leading to the failure of *in vitro* propagation of this orchid species.

Formation of PLB can be classified into two types. The first is the direct formation of PLB from protocorms, shoot tips, root tips, and stem segments through somatic embryogenesis (Luo *et al*., 2008; Mayer *et al*., 2010; Naing *et al*., 2011). The second is the formation of PLB through callus. (Hong *et al*., 2008; Huang and Chung, 2010; Ng and Saleh, 2011). In the present study, PLB of *P. cornu-cervi*

(Breda) Blume & Rchb.f. developed directly from the protocorms without callus formation.

PLB from both the PGR-free medium and in the presence of NAA, TDZ or BA caused browning or blackening of media after 6 weeks of culture. Upon transfer PLB to hormone-free medium supplemented with 15% (v/v) CW and 0.2% (w/v) AC, PLB converted into healthy plants. Browning or blackening of cultured explants caused by wounding. This activity promoted the formation of phenolic substances under the control of polyphenol oxidase. Tanaka and Sakanishi (1977) reported that phenolic exudation caused poor regeneration capacity in *Phalaenopsis* tissue culture. This inhibitory effect may be related to the size and differential sensitivity of various explants (Seeni and Latha, 1992). In this present study, AC in culture media seems to reduce exudates, thus enhanced PLB development. PLB converted into healthy plants with well-developed leaves and roots when cultured on culture medium supplemented with AC and kept under a 16-h photoperiod for 5 months. In plant tissue culture, AC has been widely used to stimulate rooting of micropropagated shoots since it can absorb both inhibitory substances and cytokinins in the culture medium. Moreover, it is suggested that the AC favors the establishment of a balance of endogenous auxins and cytokinins that facilitates root formation by decreasing decomposition of endogenous IAA under the light condition (Pan and van Staden, 1998). Eymar *et al*. (2000) observed that the addition of AC increased and maintained pH levels during culture, increased the nitrogen uptake and improved growth and visual aspects of the explants and reduced the inhibitory effect of exogenous cytokinin on root growth. In this present study, AC in culture media seems to reduce exudates, thus, enhanced frequency of PLB proliferation and further development of those PLB into healthy shoots or plants.

The present study is the first report to show that PLB can be induced and proliferated from wounded protocorm segments of *P. cornu-cervi* (Breda) Blume & Rchb. f. on culture media containing CW. However, the use of culture media supplemented with plant growth regulators solidified with agar also effective on initiation of PLB and eventually regeneration into plantlets. These protocols are simple, inexpensive and bring about the production of a large number of plantlets by germinating seeds and PLB induction in a short period of time. This research has

demonstrated that *P. cornu-cervi* (Breda) Blume & Rchb. f. can be successfully propagated via PLB induction.

4.1.5 Effects of culture media, concentrations of sucrose and activated charcoal on plantlet growth.

ND medium supplemented with 4% (w/v) sucrose in the presence of AC gave the highest survival rate, plantlet height, fresh weight, number of leaves per plantlet, leaf length, leaf width, number of roots per plantlet and root length. This medium was suitable for the conversion of PLB into plantlets. Generally, 2-4% (w/v) sucrose is used as carbon source in culture medium. The results from the present study suggest that the growth of monopodial orchid is influenced by sucrose. However, an optimum concentration of sucrose depended on species. Normally, *in vitro* condition has low $CO_2$ concentration and not sufficient light energy. So, carbon sources are very important components for *in vitro* growth and development. Sucrose is widely used but other sugars were reported for *in vitro* propagation of orchid, such as glucose, fructose, sorbitol, maltose and trehalose (Islam *et al*., 1998). In case of somatic embryogenesis, the carbohydrate source has also been reported to be an important parameter in the conversion of embryos into plantlets. Jheng *et al*. (2006) reported that higher concentrations (1 and 2%) of the different types of carbohydrate (sucrose, maltose and trehalose) were tested and the results revealed that high concentrations of those sugars gave higher fresh weight than that obtained from the lower concentration (0.5%). The source of carbon containing in culture media is a very important for *in vitro* micropropagation of orchid and other plant species. Carbon sources were added to the culture medium because of light energy deficiency and low $CO_2$ concentration present in *in vitro* conditions. Plants cultured *in vitro* often showed a low photosynthetic rate and incomplete autotrophy (Faria *et al*., 2004). Sugar acts as a carbon and energy source and also acts as an osmotic regulator in the induction medium. Sucrose is commonly used in tissue culture media. Faria *et al*. (2004) reported that the presence of 6% (w/v) sucrose in the medium was the most efficient treatment for increasing height and fresh weight of *Dendrobium nobile in vitro* culture.

The beneficial effects of AC could be due to positive stimulation of many developmental processes (van Winkle and Pullman, 2006) and its ability to

absorb the phenolic compounds, which can injure living tissues. Since AC is also known to adsorb gases, it is possible to speculate that some of its effects are ethylene adsorption. Good growth and development of *Phalaenopsis* plantlets *in vitro* were obtained when culture media were supplemented with 0.2% (w/v) AC (Hinnen *et al*., 1989; Ernst, 1994) or 0.5% (w/v) AC (Park *et al*., 2000). Similar observations were also reported in *Vanda coerulea* Griff ex. Lindl (Seeni and Latha, 2000). AC increased the number and the length of roots. AC has both beneficial and harmful effects in culture medium, depending upon the medium, explants, and plant growth regulators used. The beneficial effects of AC on tissue responses *in vitro* could be attributed to providing a dark environment by darkening the medium (Dumas and Monteuuis, 1995) adsorption of harmful substances produced by either culture media or explant (Fridborg and Eriksson, 1975; Fridborg *et al*., 1978), adsorption of plant growth regulators and other organic compounds (Nissen and Sutter, 1990; Weatherhead *et al*., 1978).

### 4.2 Cryopreservation of *Phalaenopsis cornu-cervi* (Breda) Blume & Rchb. f.

Usually conventional conservation of orchids *in vitro* entails high maintenance costs, risk of somaclonal variation and genetic instability. While, cryopreservation using liquid nitrogen played an important technique for long-term preservation, since it required small space and did not cause genetic alteration because the extremely ultra-low temperature (-196°C) cloud stop all biological activities.

Encapsulation-dehydration method is one of the vitrification methods based on cryopreservation techniques. Compare to other vitrification methods, the manipulation of encapsulated explants by this method is easy and non-toxic cryoprotectants are applied to protect the explants during dehydration (Sakai *et al*., 2000). Cryopreservation is an important technique for long-term preservation without genetic alteration. The encapsulation-dehydration method, encapsulated apices into alginate gel are osmoprotected with high sucrose before air-drying and plunge into liquid nitrogen.

4.2.1 Effects of cryopreservation methods and ages of protocorms on survival rate of cryopreserved protocorms.

There are several factors that can influence the post-thaw recovery of cryopreserved explants (Skerlep *et al*., 2008). In this reports, effects of cryo-preservation methods and ages of protocorms on survival rate of cryopreserved protocorms was evaluated. The results showed that no survival protocorms was obtained from those methods of cryopreservation. However, encapsulation together with vitrification or dehydration protocorms without storing in LN could survive at 10 to 27%. Encapsulation followed by dehydration gave the better results than encapsulation followed by vitrification. Moreover, older protocorms after germination (2-month-old protocorms, 3-4 mm protocorms size) gave the better results than younger protocorms (49-day-old protocorms, (1-2 mm protocorms size).

The developmental phase of plant material affected the success on cryopreservation (Li *et al*., 2009). Therefore, suitable physiological status of explants could be a key point to raise tolerance to liquid nitrogen treatment in cryopreservation (Takagi *et al*., 1997). In the present study, non-cryopreserved PLB at size of 3-4 mm gave the better survival rate in comparison with protocorms at size of 1-2 mm. Moreover, the survival rate was also based on culturing on regrowth medium. Explants which are too small often suffer with more mechanical injury (Boucaud *et al*., 2002). In cryopreservation of garlic shoot apices, explants with base diameter at 3 mm gave rise to more than 90% of post thaw regeneration (Baek *et al*., 2003). Khoddamzadeh *et al*. (2011) reported the success in cryopreserved protocorms of *Phalaenopsis* Blume at size ranging from 3 to 5 mm in diameter by encapsulating in alginate bead. Similar results were also obtained from this present study which showed that bigger explants (3-4 mm protocorms) resulted in the increment of survival rate in comparison with smaller explants (1-2 mm protocorms). This data supports the view that smaller explants (1-2 mm) subjected to higher mechanical injury during cryopreservation.

4.2.1.1 Encapsulation-vitrification

Vitrification refers to the physical process by which a highly concentrated aqueous solution solidifies into a glassy solid at sufficiently low

temperature without crystallization (Hong *et al*., 2009). Thus, it is essential to enhance the dehydration tolerance of plant tissues to the vitrification solution.

In the present study, protocorms derived from 49 days and 2 months of culture were precultured in ND liquid medium supplemented with 0.3 M sucrose for 3 days on a rotary shaker at 110 rpm (25±1°C) before dehydrating with PVS2, then encapsulation in algenate bead. Encapsulated the protocorms were loaded in a loading solution containing 2.0 M glycerol and 0.4 M sucrose for 60 minutes at 25±1°C, then directly dehydrated with PVS2 solution at either 25±1°C or 0°C for 60 minutes. Unfortunately, by those procedures, no survival rate of protocorms was obtained from those protocorms after storing in LN.

For cryopreservation protocols, direct exposure to the vitrification solution is harmful due to osmotic stress or chemical toxicity which has been described as a major hindrance to cryopreservation by vitrification (Matsumoto *et al*., 1994). In addition, osmotolerance is rarely achieved by preculture with sucrose alone. Therefore, a loading treatment with a load solution containing various amounts of sucrose and glycerol is commonly used (Xue *et al*., 2008). Loading treatment can be done either by mixing the loading solution in the alginate matrix as in case of *Dendrobium cariniferum* Rchb. f. or incubating the encapsulated beads in the loading solution for various duration before treating with PVS2 as in case of *D. candidum* PLB (Yin and Hong, 2009).

Ishikawa *et al*. (1997) reported that zygotic embryos of the Japanese terrestrial orchid, *Bletilla striata* incubated in a loading solution containing 2.0 M glycerol and 0.4 M sucrose for 15 minutes at 25°C gave the highest regeneration frequency of zygotic embryos at 60%. In case of *D. candidum*, loading treatment of a mixture of 2.0 M glycerol and 1.0 M sucrose enhanced survival and regeneration of PLB (Yin and Hong, 2009). Highest survival percentage of cryopreserved PLB of *D. nobile* Lindl. was obtained when encapsulated PLB were treated for 60 minutes with a loading solution prior to dehydration (Mohanty *et al*., 2012). Similar results were obtained in cryopreservation protocorms of *D. cariniferum* Rchb. f. by encapsulation-vitrification method (Pornchuti and Thammasiri, 2008). The reason of applying loading solution in the bead is to avoid the intracellular ice formation as the PLB remain in direct contact with the loading solution. But as the alginate bead itself contains a major

amount of water which may result in extra cellular ice crystals formation, a further treatment with loading solution for different duration is preferable.

Incubation period and temperature of the vitrification solution are two important factors affecting survival rate of cryopreserved plant tissues. Overexposure of plant tissues to the vitrification solution may lead to chemical toxicity and excessive osmotic stress. The optimal exposure time for PVS2 varies with plant species and depends on the temperature during exposure (Hong *et al*., 2009). In earlier reports the optimal exposure time to PVS2 at 25±2°C varied for different orchid species (Thammasiri, 2000; Pornchuti and Thammasiri, 2008; Yin and Hong, 2009). However, dehydration at 0°C also yields higher survival rate, with the incubation time largely extended, thus allowing for greater flexibility in handling large numbers of samples at the same time (Wang *et al*., 2002). A significance increase in survivability was noticed when PLB of *D. nobile* Lindl. were dehydrated in PVS2 solution at 0°C with a frequency of survival 78.1% for 115 minutes as compared to 25°C at percentage survival rate of 70.1% for 85 minutes (Mohanty *et al*., 2012). These findings are similar to Yin and Hong (2009) who reported that survival percentage of cryopreserved PLB of *D. candidum* dehydrated with PVS2 at 25°C for 120 minutes increased from 76.2 to 89.4% following dehydration at 0°C for 150 minutes. Interestingly, protocorms of *D. cariniferum* treated with PVS2 and subjected to cryopreservation by encapsulation-vitrification exhibited lower survival frequency of 15% (Thammasiri, 2008). Therefore, the survival percentage by encapsulation-vitrification method could be attributed to genotypic differences as well as differences in incubation periods and temperatures of the vitrification solution. However, survival and recovery rate of cryopreserved protocorms in this present study was not obtained. The cells treated with vitrification solution subjected to osmotic stress. In addition, some chemicals in vitrification solution might be up taken into the cells resulting in toxicity (Matsumoto *et al*., 1994; Sakai, 2000). Thus, exposures of the cells to vitrification solution need to be careful. It is suggested that the components of vitrification solution and their concentrations should be minimized under an optimization of time for dehydration (Charoensub *et al*., 1999; Tsukazaki *et al*., 2000). Direct exposure of germplasm to PVS2 was reported to reduce viability; however, stepwise increase in PVS2 concentration could reduce this toxic effect (Kobayashi *et al*., 2006).

4.2.1.2 Encapsulation-dehydration

***Effects of pretreatment***

Preculture of explants in suitable medium helps to increase tolerance to dehydration and subsequent freezing in LN. The addition of sucrose in the preculture culture medium helps in osmoprotection by stabilizing cellular membranes and maintaining turgor pressure (Valentovie *et al*., 2006). Higher concentrations of osmoticum protect plant cells from desiccation injury.

In the present study, it was found that all the PLB of *P. cornu-cervi* (Breda) Blume & Rchb. f. could not withstand a sucrose concentration at 0.3 M when precultured after encapsulation. The results revealed that survival rate of encapsulated PLB of this orchid were not improved by this procedure. All encapsulated PLB died after cryopreservation in LN for 1 day. However increase in sucrose concentration in the preculture medium resulted in the decrement of percentage survival rate of the encapsulated PLB. This might have been due to sucrose creating harmful osmotic stress in the treated explants promoting excessive dehydration of the PLB and hence incurring toxicity. It has long been reported that preculture duration influences upon the high survival percentage of different explants after cryopreservation in orchids (Ishikawa *et al*., 1997; Maneerattanarungroj *et al*., 2007). However, the survival percentage of cryopreserved protocorms of *D. virgineum* was reduced to 15%, when they were precultured in a modified liquid VW medium supplemented with 0.3 M sucrose for 3 days (Pornchuti and Thammasiri, 2008). Similarly, low regrowth (13.33%) was observed in encapsulated shoot tips of *Dendrobium* Walter Oumae which were precultured with 0.3 M sucrose in agar medium for 2 days (Lurswijidjarus and Thammasiri, 2004).

Preculturing in media containing sugar is a vital step in successful cryostorage of tissues using the vitrification procedure (Yin and Hong, 2009). High sugar content in the cytoplasm of the tissue aids in the establishment of vitrified state during cryopreservation and enables cells to tolerate dehydration that can cause freezing damage (Yin and Hong, 2009). Since, biological samples contain high amount of water which can cause mechanical injury due to intracellular and extracellular ice formation during freezing and thawing, thus, a reduction in water content is crucial prior to cryopreservation (Fabian *et al*., 2008; Yin and Hong, 2009). Preculturing with medium

containing high concentration of sucrose (0.3 M) was reported to be useful in improving survival rate after cryopreservation of mature (Ishikawa *et al*., 1997) and immature (Hirano *et al*., 2005a,b) orchid seeds. However, in cryopreservation of *D. candidum* Wall ex Lindl., preculture of PLB in 0.75 M sucrose was found to be optimum (Yin and Hong, 2009).

Our results with *P. cornu-cervi* (Breda) Blume & Rchb. f. in this present study revealed that application of a correct preculture duration and concentration of sucrose to encapsulated PLB is essential for maximum survivability. Therefore, all these different studies suggest that different orchid species exhibit varying levels of tolerance to high concentration of sucrose.

***Effect of dehydration***

The control of water content of plant samples before freezing is the key factor in successful in cryoprotection protocols (Zhang *et al*., 2001). The water content of the encapsulated beads was removed by both osmotic dehydration and sterile air-flow. Earlier studies suggested that insufficient dehydration of the cells or tissues caused intracellular ice resulting in cryoinjury during cold storage in liquid nitrogen. However, Bian *et al*. (2002) reported that over-dehydration of the cells or tissues promoted osmotic stress and damaging of those tissues.

In the present study, survival rate of PLB of *P. cornu-cervi* (Breda) Blume & Rchb. f. was not improved via cryopreservation after preculturing beads by dehydrating for 0-7 hours (reduction of water content to 90.81%). This might be the osmotic shock occured due to overdehydration (Maruyama *et al*., 1998). Jitsopakul *et al*. (2007) reported that the regrowth rate of non-cryopreserved and cryopreserved protocorms of *Vanda coerulea* depend on the water content of the precultured beads during dehydration. The optimal water content of alginate beads as well as survival rate is varied depends on plant species and explants (Suzuki *et al*., 1998; Gonzalez-Arnao *et al*., 2000; Padro *et al*., 2011). Therefore, the optimum water content of encapsulated explants should be carefully determined before any application in order to achieve the highest survival rate of a particular cultivar of a given plant species.

This study has demonstrated that PLB of *P. cornu-cervi* (Breda) Blume & Rchb. f. are not suitable for cryopreservation. There was markedly high water content in the plant tissue which might cause the ice crystal formation when all encapsulated PLB were stored into LN. In the future, seeds of *P. cornu-cervi* (Breda) Blume & Rchb. f. are an alternative choice for cryopreservation. Seeds of *P. cornu-cervi* (Breda) Blume & Rchb. f. are very small and can tolerate to cold and suitable for a long-term preservation. Therefore, the cryopreservation of seeds could be a feasible way of preserving their genetic resource and diversity.

Orchid seeds are mostly tolerant to desiccation and freezing and suitable for a long-term preservation. Such seeds are known as orthodox in their storage behavior. Cryopreservation is potentially of great value for the conservation of orchid germplasm. Furthermore, tropical, sub-tropical and temperate orchid seeds can be stored together. In addition, one pod has abundant seeds, possibly up to thousands of seeds. Terrestrial and epiphytic orchid seeds with moisture content lower than 14% can be conserved in LN (-196°C) (Pritchard, 1984, 1995). Seeds of *Encyclia vitellinum* survived after storage at -40°C for 35 days and those of *Cattleya* hybrids and *D. nobile* tolerated 465 day at -79°C (Seaton and Hailes, 1989). Seeds of *D. candidum* with less than 12% moisture content gave 95% survival rate when they were stored in LN (Wang *et al*., 1998).

There are some reports published on cryopreservation of orchids, such as zygotic embryos and immature seeds of *Bletilla striata* (Hirano *et al*., 2005a; Ishikawa *et al*., 1997), seeds of *Doritis pulcherrima* (Thammasiri, 2000), immature seeds of *Ponerorchis graminifolia* (Hirano *et al*., 2005b) and seeds of *Bratonia* hybrid (Popov, 2004). There is a report published on cryopreservation of some Thai orchid species, such as, seeds of *D. chrysotoxum* (99%, vitrification), *D. cruentum* (32%, vitrification), *D. draconis* (95%, vitrification), *D. hercoglossum* (80%, encapsulation-vitrification), *Doritis pulcherrima* (62%, vitrification), *Rhynchostylis coelestis* (85%, vitrification) and *Vanda coerulea* (67%, vitrification) (Thammasiri, 2008).

The desiccation characteristics of seed differ among species, being broadly grouped as desiccation-tolerant (orthodox), intermediate and desiccation sensitive (recalcitrant) behaviors (Towill and Bajaj, 2002). The seed longevity of the first group can be increased by lowering seed moisture and storage temperatures

(Towill, 2002). Orchid seeds are orthodox, very tiny, and without cotyledon and endosperm (Pritchard, 1984). The embryos are composed of homogeneous small parenchymatous cells with mostly lipid reserves (Hadley, 1982; Pritchard, 1984). Due to their minute size and homogeneous cells, the orchid seeds can be sufficiently dehydrated which lead to successful cryopreservation (Berjak *et al*., 1996).

## 4.3 Alginate-encapsulation, short-term storage and plantlet regeneration from PLB of *Phalaenopsis cornu-cervi* (Breda) Blume & Rchb. f.

### 4.3.1 Effects of different storage conditions and intervals on their conversion ability of *Phalaenopsis cornu-cervi* (Breda) Blume & Rchb. f.

Synthetic seed technology is an exciting and rapidly growing area of research as deals with *in vitro* conservation and storage of rare, endangered and desirable genotypes along with its easy handling and transportation (Kumaria and Tandon, 2001; Germana *et al*., 2011). *In vitro* conservation involves the maintenance of explants in a pathogen-free environment for short-to medium-or long-term (Engelmann and Engels, 2002). For short-term storage, the aim is to increase the interval between subcultures by reducing growth. Minimum growth condition for short- to medium-term storage can be followed in several ways, such as induction of osmotic stress with sucrose or mannitol (Wescott, 1981), reduced temperature and/or light (Withers, 1991) and incorporation of sub-lethal levels of growth retardant (Gupta, 2001). Storage through slow growth methods is reproducible and widely applicable among different plant species and genotypes for conservation of germplasm (Withers, 1991). Elite germplasms of various rare and endangered plant species like *Coffea arabica* (Nassar, 2003), *Rauvolfia tetraphylla* (Faisal *et al*., 2006), *Pterostylis saxicola* and *Diuris arenaria* (Sommrville *et al*., 2008) and *Pogostemon cablin* (Kumara Swamy *et al*., 2009) have been stored by *in vitro* methods using this slow growth technique. Roca *et al*. (1988) have successfully shown that nodal cuttings from meristem derived plantlets of cassava (*Maniht esculentum*) could be maintained for 2 years on a medium with low osmotic concentration and activated charcoal. In Garlic (*Allium sativum*), the shoot tips

could be stored for a period of 16 months following an increase in sucrose concentration to 10% (El-Gizawy and Ford-Llyod, 1987).

Though germplasm of many ornamental plants have also been successfully stored using this minimal growth technology, very few reports have been made for orchids, viz. *Vanilla planifolia* (Divakaran *et al*., 2006), *Vanda coerulea* (Sarmah *et al*., 2010), *Cymbidium devonianum* (Das *et al*., 2011). Dubus (1980a, b) reported preservation of *Cymbidium* protocorms by increasing the sucrose concentration; however, many other authors have reported maintaining the cultures at low temperatures for storage and preservation (Sharma *et al*., 1992; Corrie and Tandon, 1993; Datta *et al*., 1999; Das *et al*., 2008). Das *et al*. (2011) reported the increment in storage duration in *C. devonianum* by reduction nutrient strength in the encapsulated matrix as well as low temperature. However, the successful application of minimal growth technology requires the establishment of specific protocols for each type of explants and species (Watt *et al*., 2000).

This is the first report of synthetic seed production, storage and conversion of PLB in *P. cornu-cervi* (Breda) Blume & Rchb. f. The present study developed efficient methods for alginate-encapsulation, conversion and short-term storage of *P. cornu-cervi* (Breda) Blume & Rchb. f. capsules for germplasm exchange between laboratories. PLB, induced from protocorms segments of *P. cornu-cervi* (Breda) Blume & Rchb. f. were isolated from *in vitro* proliferating PLB clusters. Individual PLB were encapsulated in calcium alginate beads to manage mass propagation, short-term storage and germplasm sharing. The superior gel matrix for encapsulation was obtained using 3% sodium alginate and 100 mM calcium chloride ($CaCl_2.2H_2O$). Among the three temperature regimes of storage encapsulated PLB were successful stored at 25±1°C for 30 days with a maximum percentage of germination at 100 when culture on ND medium supplemented with 4% (w/v) sucrose with 0.2% (w/v) AC. The present investigation was to evaluate the encapsulated PLB of *P. cornu-cervi* (Breda) Blume & Rchb. f. for feasible study of their regeneration, followed by the application of this method to store in medium-term. In addition, this method can serve as an *ex situ* conservation method of this orchid. However, assortment of the suitable plant part as the preliminary experimental material and optimization of conversion process for plant recovery, are the two requirements which are essential to a successful

storage procedure via encapsulation. Apart from having enormous potential for plant development from pre-existing meristematic tissues, PLB are appropriate for encapsulation studies as they are excellent material for synthetic seeds production. Contrastingly, shoot tips or other non-embryogenic parts could not perform as efficiently as PLB due to their comparatively low regeneration ability. In addition, the use of PLB, induced directly from PLB, would guarantee genetic fidelity in the post-storage regenerant clones.

#### 4.3.1.1 Encapsulation of PLB

The alginate-beads containing PLB (capsules) were obtained using the gelling matrix of 3% sodium alginate with 100 mM $CaCl_2$ solution. The mean sizes of bulk beads achieved by this procedure were 7-9 mm in diameter. Successful use of calcium alginate encapsulation matrix for storage of synthetic seeds was reported by Singh (1991) using 21 days old *Spathoglottis plicata* PLB. Corrie and Tandon (1993) have also used alginate matrix to encapsulate PLB of *Cymbidium giganteum* for short-term storage. In our study, 3% solution of sodium alginate and 100 mM $CaCl_2$ presumably helped in optimal ion exchange between $Na^+$ and $Ca^{2+}$, producing firm, clear, isodiametric beads. Lower levels (1 and 2%) of sodium alginate were not appropriate (data not presented); since, the capsules were not in definite shape and excessively soft to grip; while at higher concentrations (4%) the pods were effectively firm to cause substantial delay in propagule emergence (data not presented). Similar results were also reported by Singh *et al*. (2009) in *Spilanthes acmella* (L.) Murr. where they evaluated the role of sodium alginate concentration, affecting the gel matrix/capsule quality and subsequent conversion of the capsules. According to Nagesh *et al*. (2009), lower concentration of sodium alginate might cause resistance of beads formed. Low resistance beads exposed for a long period (30 minutes in the present study) to $CaCl_2$ could adsorb large quantity of $CaCl_2$. Absorption is a surface phenomenon and higher accumulation of $CaCl_2$ might restrain the further development causing $CaCl_2$-toxicity (Nagesh *et al*., 2009). In contrast, higher resistance beads formed due to increase in concentration of sodium alginate (3%) and adsorb less quantity of $CaCl_2$. Therefore, toxicity due to $CaCl_2$ could be less, resulting in high frequency recovery. The influence of combinations between sodium alginate and $CaCl_2$ on bead

texture and consistency was also reported by Singh *et al*., (2010) in *Eclipta alba* (L.) Hassk, an important medicinal herb. Sodium alginate is a biodegradable and biocompatible copolymer comprised of L-glucuronic acid and D-mannuronic acid units and has been comprehensively studied due to its potential to form hydrogels in the presence of divalent cations. The inflexible structure and outsized pores of these gels, which are water-insoluble, makes them efficient for encapsulation of living plant cells (Bajaj, 1995), as they permit the swap of substances to and from the adjacent medium. The successful use of pipette to obtain desirable shape and size of capsules in the present study corresponds to the earlier report of Mallon *et al*., (2007). Alginate encapsulation is a technique that can be used for germplasm storage or for reducing the requirement of transferring and subculture, out of season (West *et al*., 2006). This would serve the purpose of the present study, where we need to arrest the continuous proliferation of PLB through storage, hence minimize the need for frequent subculture.

#### 4.3.1.2 Effects of different storage conditions and intervals on PLB conversion ability.

Among the three temperature regimes, room temperature (25±1°C) storage gave promising results for conversion of pods. Conversion percentage of synthetic seeds decreased from 35% to 11.67% after 30 days of storage at 8±1°C. However, the PLB stored more than 30 days in such condition gave no conversion at all (Table 12). Encapsulated PLB stored at 4±1°C lost their viability completely (Table 12). Contrastingly, capsules stored at 25±1°C for 30 days were green, with potential for conversion and intact bead consistency with 100% conversion. The percentage of conversion of encapsuled PLB into plantlets declined progressively with the increase in the duration of storage, both at 4±1°C and 8±1°C. However, encapsuled PLB stored at 25±1°C gave the highest conversion rate, significantly different ($p$<0.05) to those stored in 4±1°C and 8±1°C. The merit of 25±1°C storage over 4±1°C for encapsulated PLB in the present study was in agree with the earlier report of Hung and Trueman (2011) on *Khaya senegalensis* shoot tips, where they also highlighted that the ease of storage at 25°C is highly encouraging for germplasm exchange, because capsules can be maintained under laboratory conditions and then dispatched and processed

conveniently. Mishra *et al*. (2011) reported that storage of encapsulated microshoots of *Picrorhiza kurrooa,* a herbaceous plant**,** at 25±1°C was favorable.

The failure of prolonged storage in 4±1°C was also described in earlier reports (Redenbaugh *et al*., 1987) where, low temperature (4±1°C) storage of synthetic alfalfa seeds was rather short. Similarly, the germination rate of encapsulated embryos of *Asparagus cooperi* was reported to be low (Ghosh and Sen, 1994) under storage in this conditions. Likewise, the conversion of encapsulated nodal segments of *Punica granatum* L. also showed markedly decline, following storage at low temperature (Naik and Chand, 2006). However, the response of synthetic seeds to storage temperature appears to be species specific. Some responds to either 4°C (Saiprasad and Polisetty, 2003; Lisek and Olikowska, 2004; Singh *et al*., 2010; Sharma and Shahzad, 2012) or room temperature (Devi *et al*., 2000; Mohanraj *et al*., 2009; Hung and Trueman, 2011; Gantait *et al*., 2012).

Storage at room temperature (25±1°C) implemented in this study was effective for short-term storage and handling without refrigerated containers, and even storage up to 30 days gave considerable conversion (100%) in *P. cornu-cervi* (Breda) Blume & Rchb. f. Complete plantlets of *P. cornu-cervi* (Breda) Blume & Rchb. f. developed from each capsules on conversion medium, were successfully transferred to *ex vitro* conditions. The plantlets were healthy, green in color and morphologically uniform.

From the success of the present study, it seems that the storage of capsules in sterile Petri-dishes and sealed with Parafilm (though having limited supply of oxygen for respiration of capsules) proved to be suitable to protect loss of humidity which is essential for retention of viability of encapsulated PLB, since sodium alginate have been reported to succumb to rapid dehydration (Dainty *et al*., 1986). In addition, encapsulation of PLB of this orchid appears to be a promising tool for storage and on-demand supply of plant material for propagation or germplasm exchange. Similar use of the encapsulation method for storage have also been reported earlier in many other endemic and endangered orchids like *Renanthera imschootiana* (Chetia *et al*., 1998) and *Geodorum densiflorum* (Datta *et al*., 1999), *Ipsea malabarica* (Martin, 2003). The *in vitro* storage achieved for *P. cornu-cervi* (Breda) Blume & Rchb. f. in our study has the prospective to cut the cost for maintaining the continuous proliferating PLB cultures,

because of the abridged requirement for manual labor due to less frequent subculturing. According to Rai *et al*. (2008), an important feature of the encapsulated vegetative propagules is their capability to retain viability after storage for a sufficient period required for exchange of germplasm.

### 4.3.2 Ploidy instability analysis using flow cytometry (FCM).

Despite wide utilization of encapsulation technology in micro-propagation and storage of orchids, the genetic fidelity of synthetic seed-derived propagules have been completely unassessed. The escalating exploitation of synthetic seed technology for propagation and germplasm preservation obliges genetic stability assessment of propagules subsequent to their conservation (Dehmer, 2005). To date, methodical germplasm sampling and their molecular status assessment via DNA marker technology intervention has turned into an efficient practice (Mishra *et al*., 2011). Among the various available DNA uniformity/polymorphism detection systems, inter-simple sequence repeats (ISSR) has been proven to be simple, efficient and reproducible to detect clonal fidelity (Gantait *et al*., 2010).

To analyze their ploidy stability in the present study of complete plantlets of *P. cornu-cervi* (Breda) Blume & Rchb. f. derived from different storage temperatures and time of encapsulated PLB were subjected to FCM analysis. FCM analysis of plantlets showed the same pattern of relative DNA content peak. There was not change in the ploidy level of all tested plants**.** The high frequency of plantlet retrieval from encapsulated PLB of *P. cornu-cervi* (Breda) Blume & Rchb. f. after 30 days storage at 8±1°C and 25±1°C could be used as a delivery system for germplasm exchange and offers the possibility of using this method for *ex situ* conservation of this endangered orchid plant.

Findings of this study corroborate earlier reports of the genetic fidelity of synthetic seed derived plantlets (Gangopadhyay *et al*., 2005; Srivastava, *et al*., 2009; Mishra *et al*., 2011), cryopreserved tissues of various plant species (Jokipii *et al*., 2004; Bekheet *et al*., 2007) and micropropagated plants (Martins *et al*., 2004; Venkatachalam *et al*., 2007; Borchetia *et al*., 2009).

# CHAPTER 5

# CONCLUSIONS

## 5.1 Micropropagation of *Phalaenopsis cornu-cervi* (Breda) Blume & Rchb. f.

ND or MS medium supplemented with 15% (v/v) coconut water (CW) gave the best growth of protocorms while MS medium supplemented with 15% (v/v) coconut water (CW) and 0.2% (w/v) activated charcoal (AC) gave the best growth of seedlings.

Bisected protocorms enlarged and more PLB were formed when cultured in VW liquid medium supplemented with 100 and 150 mg/l ascorbic acid.

All PLB were formed from wounded protocorm segments cultured on medium containing NAA at 0.1 mg/l and TDZ at 0.1 mg/l. This culture medium was found to be the best for induction of PLB. After 6 weeks of culture, the highest percentage of PLB formation occurred and each explant produced small, medium and large size of PLB at 13.9, 10.7 and 11 PLB per culture explant, respectively.

ND medium supplemented with 4% (w/v) sucrose and containing AC is suitable for conversion of PLB of *P. cornu-cervi* (Breda) Blume & Rchb. f. into complete plantlets. This culture medium gave the highest survival rate (100%), plantlet height (10.9 mm), fresh weight (1.309 g), numbers of leaf per plantlet (6.1 leaves), leaf length (33.85 mm) leaf width (13.45 mm), numbers of root per plantlet (8.9 roots) and root length (30.2 mm). The incorporation of AC in the medium could reduce or inhibit the accumulation of phenolic compounds that caused tissue browning.

Plantlets obtained from wounded protocorms which were cultured on ½ MS supplemented with different concentrations of PGRs had the same ploidy level as control plantlets.

The regenerated plantlets grew normally when transplanted to pots containing sphagnum moss in the greenhouse with 100% survival rate.

## 5.2 Cryopreservation of *Phalaenopsis cornu-cervi* (Breda) Blume & Rchb. f.

Protocorms and PLB of *P. cornu-cervi* (Breda) Blume & Rchb. f. were not successfully cryopreserved using direct plunging into liquid nitrogen, encapsulation-vitrification, encapsulation-dehydration, encapsulation-dehydration with cold-hardening or encapsulation-dehydration with sucrose pretreatment.

## 5.3 Alginate-encapsulation, short-term storage and plantlet regeneration from PLB of *Phalaenopsis cornu-cervi* (Breda) Blume & Rchb. f.

Among the three temperature regimes of storage, encapsulated PLB stored at 25±1°C, for 30 days showed maximum percentage of germination at 100 when culture on ND medium supplemented with 4% (w/v) sucrose with 0.2% (w/v) AC.

Plantlets obtained from either control or different storage temperatures showed the same ploidy level as control plantlets. There was no change in ploidy level of plantlets by this protocol.

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# APPENDICES

# APPENDIX A

## Media for micropropagation and cryopreservation

1. Components of Murashige and Skoog (MS, 1962) medium.

| Components | mg/l |
|---|---|
| Macro elements | |
| $NH_4NO_3$ | 1,650.000 |
| $KNO_3$ | 1,900.000 |
| $KH_2PO_4$ | 170.000 |
| $CaCl_2.2H_2O$ | 440.000 |
| $MgSO_4.7H_2O$ | 370.000 |
| Micro elements | |
| KI | 0.830 |
| $H_3BO_3$ | 6.200 |
| $MnSO_4.H_2O$ | 16.900 |
| $ZnSO_4.7H_2O$ | 10.600 |
| $CuSO_4.5H_2O$ | 0.025 |
| $Na_2MoO_4.2H_2O$ | 0.250 |
| $CoCl_2.6H_2O$ | 0.025 |
| $FeSO_4.7H_2O$ | 27.800 |
| $Na_2EDTA$ | 37.300 |
| Organic compounds | |
| Myo-inositol | 100.000 |
| Nicotinic acid | 0.500 |
| Pyridoxine HCl | 0.500 |
| Thaiamine HCl | 0.100 |
| Glycine | 2.000 |
| Sucrose | 30,000.00 |
| Agar | 7,500.00 |
| pH | 5.7 |

2. Components of Vancin and Went (VW, 1949) medium.

| Components | mg/l |
|---|---|
| Stock I | |
| $NH_4NO_3$ | 525.00 |
| $KNO_3$ | 425.00 |
| Stock II | |
| $KH_2PO_4$ | 250.00 |
| $MnSO_4.H_2O$ | 7.50 |
| $MgSO_4.7H_2O$ | 250.00 |
| $(NH_4)_2SO_4$ | 500.00 |
| Stock III | |
| $FeSO_4.7H_20$ | 27.80 |
| $Na_2EDTA$ | 37.30 |
| $Ca_3(PO_4)_2$ | 200.00 |
| Sucrose | 20,000.00 |
| Agar | 6,000.00-7,000.00 |
| Coconut water | 150 ml |
| pH | 4.8-5.0 |

$Ca_3(PO_4)_2$ were dissolved in 10 ml of 1 N HCl.

3. Components of New Dogashima (ND, 1993) medium.

| Components | mg/l |
|---|---|
| Macro elements | |
| $NH_4NO_3$ | 480.00 |
| $KNO_3$ | 200.00 |
| $Ca(NO_3)_2.4H_2O$ | 470.00 |
| KCl | 150.00 |
| $MgSO_4.7H_2O$ | 250.00 |
| $KH_2PO_4$ | 550.00 |
| Micro elements | |
| $MnSO_4.4H_2O$ | 3.00 |
| $ZnSO_4.7H_2O$ | 0.5 |
| $H_3BO_3$ | 0.5 |
| $CuSO_4.5H_2O$ | 0.025 |
| $Na_2MoO_4.2H_2O$ | 0.025 |
| $CoCl_2.6H_2O$ | 0.025 |
| Concentrated $H_2SO_4$ | 0.5 µl |
| Organic compounds | |
| Myo-inositol | 100.00 |
| Nicotinic acid | 1.00 |
| Pyridoxine hydrochloride | 1.00 |
| Thiamine hydrochloride | 1.00 |
| Calcium pantothenate | 1.00 |
| Adenine | 1.00 |
| L-Cysteine | 1.00 |
| D-Biotin | 0.1 |
| Sucrose | 20,000 |
| Agar | 7,500.00 |
| pH 5.2 | |

# APPENDIX B

## Solutions for cryopreservation

1. Loading solution (LS)

| | | |
|---|---|---|
| Glycerol | 2 | M |
| Sucrose | 0.4 | M |

Dissolved glycerol in ND liquid medium supplemented with 0.4 M sucrose. After dissolving, sterilized in an autoclave at 121°C for 15 minutes and kept at 4°C.

2. Plant Vitrification Solution formula 2 (PVS2)

| | | |
|---|---|---|
| Glycerol | 30% | (w/v) |
| Ethylene glycol (EG) | 15% | (w/v) |
| Dimethyl sulphoxide (DMSO) | 15% | (w/v) |
| Sucrose | 0.4 | M |

Dissolved glycerol, ethylene glycol and dimethyl sulphoxide in ND liquid medium supplemented with 0.4 M sucrose. After dissolving, sterilized in an autoclave at 121°C for 15 minutes and kept at 4°C.

3. Na-alginate solution for protocorm and PLB cryopreservation

| | | |
|---|---|---|
| Na-alginate | 3% | (w/v) |
| Sucrose | 0.4 | M |

Slowly and carefully added 3% Na-alginate in ND liquid medium supplemented 0.4 M sucrose due to low solubility. After mixing homogeneous, sterilized in an autoclave at 121°C for 15 minutes and kept at 4°C.

4. Calcium chloride solution for protocorms and PLB cryopreservation

| | | |
|---|---|---|
| $CaCl_2$ | 100 | mM |
| Sucrose | 0.4 | M |

Dissolved $CaCl_2$ in ND liquid medium supplemented with 0.4 M sucrose. After dissolving, sterilized in an autoclave at 121°C for 15 minutes and kept at 4°C.

# APPENDIX C

## Solutions for artificial seed production

1. Na-alginate solution

| | | |
|---|---|---|
| Na-alginate | 3% | (w/v) |
| Sucrose | 2% | (w/v) |

Slowly and carefully added 3% Na-alginate in ND liquid medium supplemented 2% (w/v) sucrose due to low solubility. After mixing homogeneous, sterilized in an autoclave at 121°C for 15 minutes and kept at 4°C.

2. Calcium chloride solution

| | | |
|---|---|---|
| $CaCl_2$ | 100 | mM |

Dissolved $CaCl_2$ in ND liquid medium supplemented with 2% (w/v) sucrose. After dissolving, sterilized in an autoclave at 121°C for 15 minutes and kept at 4°C.

# VITAE

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**List of Publications and Proceedings**

**Publications**

Rittirat, S., Kanchanapoom, K., Thammasiri, K. and Te-chato, S. 2010. Effects of Culture media on Growth and PLBs induction from Protocorms of *Phalaenopsis cornu-cervi* (Breda) Blume & Rchb. f. *In Vitro*. Agricultural Science Journal. Vol. 41 (2): 361-364.

Rittirat, S., Te-chato, S., Kerdsuwan, N. and Kongruk, S. 2011. Micropropagation of Chang Daeng (*Rhynchostylis gigantean* var. Sagarik) by embryogenic callus. Songklanakarin Journal of Science and Technology. Vol. 33 (6): 659-663.

Rittirat, S., Thammasiri, K. and Te-chato, S. 2012. Effect of media and sucrose concentrations with or without activated charcoal on the plantlet growth of *P. cornu-cervi* (Breda) Blume & Rchb. f. International Journal of Agricultural Technology (IJAT). Vol. 8 (6): 2077-2087.

Rittirat, S., Kongruk, S. and Te-chato, S. 2012. Induction of Protocorm-like bodies (PLBs) and Plantlet Regeneration from Wounded Protocorms of *Phalaenopsis cornu-cervi* (Breda) Blume & Rchb. f. International Journal of Agricultural Technology (IJAT). Vol. 8 (7): 2397-2407.

**Proceedings**

Rittirat, S., Kanchanapoom, K., Te-chato, S, and Thammasiri, K. 2010. Effects of Culture media on Growth and PLBs induction from Protocorms of *Phalaenopsis cornu-cervi* (Breda) Blume & Rchb. f. *In Vitro*. The 9th National Horticultural Congress. Krungsri River Hotel, Phranakhon Si Ayutthaya, Thailand. 11-14 May 2010. pp. 117. (Poster presentation)

Rittirat, S. and Te-chato, S. 2010. Induction of Protocorm-like bodies (PLBs) and Plantlet Regeneration from Wounded Protocorms of *Phalaenopsis cornu-cervi* (Breda) Blume & Rchb. f. The 7th IMT-GT UNINET and The 3rd Joint International PSU-UNS Conferences on Bioscience for the future 2010. 60th Anniversary of His Majesty the King's Accession to the Throne International Convention Center, Prince of Songkla University, Hat Yai, Songkhla, Thailand. 7-8 October 2010. pp. 170. (Oral presentation)

Rittirat, S., Thammasiri, K. and Te-chato, S. 2011. Cryopreservation of Protocorm of *Phalaenopsis cornu-cervi* (Breda) Blume & Rchb. f. in Liquid Nitrogen by Vitrification Method. The 10th National Horticultural Congress. Miracle Grand Convention Hotel, Bangkok, Thailand. 18-20 May 2011. pp. 146. (Poster presentation)

Rittirat, S., Thammasiri, K. and Te-chato, S. 2011. Influence of Media and Sucrose Concentration on Conversion of Protocorm-Like Bodies (PLBs) of *Phalaenopsis cornu-cervi* (Breda) Blume & Rchb. f. to Complete Plantlets. The 37th congress on science and technology of Thailand (STT37). Centara Grand Hotel and Bangkok Convention Centre at Central World, Bangkok Thailand. 10-12 October 2011. pp. 301. (Oral presentation)

Rittirat, S., Sujjaritthurakarn, P. and Kanchanapoom, K. 2012. Induction of protocorm-like bodies from leaf segments of *Phalaenopsis cornu-cervi* (Breda) Blume & Rchb. f. Royal Flora Ratchaphruek 2012: The International Symposium on Orchids and Ornamental Plants. Imperial Mae Ping Hotel, Chiang Mai, Thailand. 9-12 January 2012. pp. 67. (Poster presentation)

Rittirat, S., Thammasiri, K. and Te-chato, S. 2012. Effect of culture media, sucrose and activated charcoal on seedling growth of *Phalaenopsis cornu-cervi* (Breda) Blume & Rchb. f. Royal Flora Ratchaphruek 2012: The International Symposium on Orchids and Ornamental Plants. Imperial Mae Ping Hotel, Chiang Mai, Thailand. 9-12 January 2012. pp. 62. (Poster presentation)