IN VITRO CLONAL PROPAGATION OF PHALAENOPSIS THROUGH FLOWER STALK NODE AND YOUNG LEAF

MAY KAUNG HSU THWE

AUGUST 2017

IN VITRO CLONAL PROPAGATION OF PHALAENOPSIS THROUGH FLOWER STALK NODE AND YOUNG LEAF

MAY KAUNG HSU THWE

A Thesis Submitted to the Post-Graduate Committee of the Yezin Agricultural University in Partial Fulfillment of the Requirements for the Degree of Master of Agricultural Science

Department of Horticulture and Agricultural Biotechnology
Yezin Agricultural University
Nay Pyi Taw, Myanmar

AUGUST 2017

The thesis attached hereto, entitled "***In Vitro* Clonal Propagation of *Phalaenopsis* through Flower Stalk Node and Young Leaf**" was prepared under the direction of chairperson of the candidate's supervisory committee and has been approved by all members of that committee and board of examiners as a partial fulfillment of the requirements for the degree of **MASTER OF AGRICULTURAL SCIENCE (HORTICULTURE AND AGRICULTURAL BIOTECHNOLOGY).**

Dr. Khin Thida Myint
Chairperson of Supervisory Committee
Professor and Head
Department of Horticulture and
Agricultural Biotechnology
Yezin Agricultural University

Dr. Aye Lae Lae Hlaing
External Examiner
Senior Research Assistant
Biotechnology Section
Department of Agricultural Research

Dr. Tin Tin Khaing
Member of Supervisory Committee
Professor and Principal
Yezin Agricultural University
(Magawe Campus)
Yezin Agricultural University

Daw Sabai Saw Shwe
Member of Supervisory Committee
Assistant Lecturer
Department of Horticulture and
Agricultural Biotechnology

Dr. Khin Thida Myint
Professor and Head
Department of Horticulture and Agricultural Biotechnology
Yezin Agricultural University

Date -----

This thesis was submitted to the Rector of the Yezin Agricultural University and was accepted as a partial fulfillment of the requirements for the degree of **MASTER OF AGRICULTURAL SCIENCE (HORTICULTURE AND AGRICULTURAL BIOTECHNOLOGY).**

Dr. Myo Kywe
Rector
Yezin Agricultural University
Nay Pyi Taw

Date -----

DECLARATION OF ORIGINALITY

This thesis represents the original work of the author, except where otherwise stated. It has not been submitted previously for a degree at any University.

May Kaung Hsu Thwe

Date -----

DEDICATED TO MY BELOVED PARENTS,
U KYAW SAN LINN AND DAW THEINGI KHAING

ACKNOWLEDGEMENTS

Firstly, I would like to express my heartfelt thanks to Dr. Myo Kywe, Rector, Yezin Agricultural University (YAU). I would like to deeply gratitude to Dr. Soe Soe Thein, Pro-rector (Academic), YAU and Dr. Nang Hseng Hom, Pro-rector (Administration), YAU for their valuable suggestions and comments to carry out this study.

I wish to express my deepest gratitude and thanks to my supervisor Dr. Khin Thida Myint, Professor and Head, Department of Horticulture and Agricultural Biotechnology, YAU, for her skillful supervision, constructive criticism, great understanding and encouragement for this research and critical checking of this manuscript.

I also would like to expend my warm thanks to Dr. Tin Tin Khaing, Professor and Principal, Magawe Campus, YAU, member of supervisory committee, for her keen interest, encourage, statistical support, valuable advice, careful reading and sharing of time for this manuscript.

I wish to express my warmly thanks to Dr. Aye Lae Lae Hlaing, Senior Research Assistant, Biotechnology Section, Department of Agricultural Research (DAR), for her valuable suggestion and encouragement to undertake for my study.

My sincere thanks are also due to Daw Sabai Saw Shwe, Assistant Lecture, Department of Horticulture and Agricultural Biotechnology, YAU, member of supervisory committee, for her valuable advice, encouragement, guidance on data analysis, careful reviewing and sharing of time in reading the manuscript.

Moreover, I wish to express indeed thanks to all of my teachers from the Department of Horticulture and Agricultural Biotechnology, YAU, for their helping and suggestions. I am very thankful to my colleagues and friends for giving their valuable time to assist in my research and effective support, suggesting and for their encouragement throughout my study.

I would like to express my thanks to Misubishi Group for their financial support to conduct this M.Agr.Sc research work.

Also my greatest and heartiest thanks to my beloved parents, U Kyaw San Linn and Daw Theingi Khaing who guide me to improve my life with supporting and encouragement and for their moral and financial supporting.

ABSTRACT

Phalaenopsis orchid, an elegant tropical orchid, has high price in flower markets around the world as well as Myanmar. It is difficult to propagate vegetatively in nature due to its growth habit. Nowadays, tissue culture technique has been widely used for most rare species and species that are difficult to propagate Therefore, tissue culture became an important method for propagation of *Phalaenopsis*. Tissue culture techniques, explant types, plant growth regulators and culture systems are mainly attributed to success. Two experiments were carried out by using different flower stalk nodes at different positions and *in vitro* young leaves. The objective of the research is to investigate the effect of plant growth regulator, culture systems and media in *in vitro* regeneration.

In flower stalk node culture, the effects of 1^{st}, 2^{nd}, 3^{rd}, 4^{th}, 5^{th} and 6^{th} nodal positions counted from base to top were tested for shoot regeneration. In leaf culture, two culture systems (solid and cotton support medium) and different concentrations and combinations of benzyladenine (BA) 2 and 4 $mg.L^{-1}$, thidiazuron (TDZ) 1 and 2 $mg.L^{-1}$, with and without naphthaleneacetic acid (NAA) 0.5 $mg.L^{-1}$ were tested for protocorm like bodies (PLBs) induction from leaf explant. In PLB proliferation stage, three culture systems (solid, liquid and cotton support liquid culture medium) were tested. Vecin and Went (VW) and Murashige & Skoog (MS) basal medium with and without 0.1 $mg.L^{-1}$ BA and 1 $mg.L^{-1}$ NAA were tested in rooting stage of shoot developed from leaf culture.

Among the flower stalk node positions, better responses were observed from 2^{nd} nodal position in shoot multiplication. 3^{rd} nodal position gave best results in root regeneration and hardening stages. Using flower stalk node culture for mass propagation, 2^{nd}, 3^{rd} and 4^{th} middle nodal positions should be used as the explants for production of *Phalaenopsis amabilis* orchid. In leaf culture, maximum number of PLBs per explant (7.1) was observed from medium supplemented with 2 $mg.L^{-1}$ BA. Culture systems used in initial culture were not statistically difference among each other. But solid culture showed better results than the cotton culture. The interaction effect between PGR and culture systems was observed in survival % and number of PLBs per explants. The results indicate the used of PGRs vary according to the culture systems. Therefore, BA should be used in cotton culture and TDZ in solid culture. Cotton culture gave the best result in PLBs proliferation on leaf explants. VW medium showed better results than MS medium in root formation of PLBs.

Key words: Flower stalk node, *In vitro* leaf, PLBs induction and proliferation, Culture systems, PGRs

CONTENTS

Page

LIST OF TABLES

Table **Page**

LIST OF PLATES

Plate **Page**

Plate **Page**

LIST OF APPENDICES

Appendix **Page**

CHAPTER I
INTRODUCTION

Orchid (orchidaceae) is the largest family among the most diverse families of flowering plants, with about 700-800 genera and 25000 species and more than 150,000 registered artificial hybrids (Nongdam and Chongtham 2011). Orchids are grown worldwide for commercial production and almost 8% shares in the world's floriculture trade (Asghar et al. 2011). A large number of genus such as *Arachnis, Ascocentrum, Cattleya, Cymbidium, Dendrobium, Laelia, Oncidium, Paphiopedilum, Phalaenopsis, Renanthera, Vanda* and their intergeneric hybrids are commercially cultivated around the world.

Phalaenopsis is commonly known as moth orchids which have long arching sprays. Most *Phalaenopsis* are epiphytes (grow on trees), but a few are lithophytes, (attach themselves to the surface of rocks). Its growth is a single vertical main stem which produces a series of thick, fleshy and distichously leaves. The flower is showy, membranous, white and the lip has three lobed, and the lip colors vary depending on the species and their hybrids eg. yellow, red and violet lip. It is able to bloom for a long period of time and can grow up to 10 cm in diameter and more. *Phalaenopsis* is one of the important genera from horticultural view point because of their big flowers, long flower stalk and vase life in which 62 species contained (Christenson 2001) and distributed throughout tropical and subtropical regions of Southeast Asia, from the Himalayan Mountains, southern China, and Taiwan to northern Australia (Chiba 2002). *Phalaenopsis* are grown for the commercial production as attractive cut flowers and potted plants throughout the world. Large scale production of *Phalaenopsis* is carried out in the Netherlands, Germany, China, Taiwan, the United States and Japan (Griesbach 2002).

Phalaenopsis is a monopodial epiphytic orchid, due to its nature, it is difficult to propagate vegetatively and the only mean for propagation is plant tissue culture. Therefore, *in vitro* propagation methods are required for commercial production. Micropropagation is widely used for commercial production that has gained the status of a multibillion-dollar industry throughout the world (Liao et al. 2011). Many *in vitro* culture protocols have been developed for *Phalaenopsis* regeneration, utilizing of flower-stalk nodes and internodes, cut ends of stems, protocorm-like body (PLB) sections, leaves and shoot tips (Balilashaki et al. 2015**)**.

Adventitious shoot proliferation from nodes of flower stalk and PLBs developed from leaf culture has been the main method for propagation of *Phalaenopsis*. Flower stalk node culture cannot damage the mother plant and can get many explants for regeneration. However, the response of flower stalk node culture varies according to the flower stalk node positions. Chang (2015) tested the effect of flower stalk node positions on shoot formation in *Tolumnia* GS248 orchid in which 3rd and 4th nodal positions of flower stalk nodes gave best response in shoot formation than 1st and 2nd node. But the informations related with the effect of nodal positions on plantlets regeneration is still weak. PLBs are important for orchid regeneration which can be induced directly from various explants. An efficient and rapid method of PLB induction is needed to develop for propagation of orchid. Mass clonal propagation was obtained from induction of PLBs from leaf sections by direct somatic embryogenesis that has been described for *Phalaenopsis* (Hsu and Chen 2003).

Culture medium is one of the most important factors that support the nutrients to the explants with gel designed, liquid and semi-liquid culture systems (Madigan and Martinko 2005). The physical consistency of culture media strongly influenced the *in vitro* growth of shoots and roots. Liquid medium supporting with cotton wool has been tested in *in vitro* multiplication of ginger, 50% survival rate was observed (Hussien et al. 2014**)**. Effect of culture systems on *in vitro* multiplication stage is still less information than culture media.

Concentration, combination and chemical properties of various kinds of growth regulators play important roles in *in vitro* propagation of many orchid species (Arditti and Ernst 1993) and depend mainly on the species of the plant, the tissue or organ cultured and the objective of the experiment. The most widely used types of plant growth regulators are auxin and cytokinin. The cytokinins and auxins are important in *in vitro* culture and mainly required in the media for growth of buds and shoot formation, the later they are concerned with root formation (North et al. 2012). The high concentration of auxins generally favors root formation, whereas the high concentration of cytokinins promotes shoot regeneration. Different proportions of growth regulators can break dormancy of bud and enhance shoot formation by suppressing apical dormance. Use of proper concentration and combination of PGRs, culture systems and explant types are important to get the efficient regeneration system of orchid.

In Myanmar, most orchids such as *Dendrobium*, *Vanda* and *Phalaenopsis* hybrid orchid species are imported from Thailand and China. Among them, *Phalaenopsis* is the high demand orchid specie and mainly cultivated for commercial production all over the world due to its diverse flower size, shape, color and long duration of inflorescence. It is commonly used as the cut flowers and potted plants in decorations at ceremonies, weddings and other festivals. Thus, large scale commercial production of *Phalaenopsis* orchid require in our country. Nowadays, tissue culture techniques are being developed and mostly have been used in many plant production processes. Local orchid growers and florists can get many benefits by using tissue culture techniques for mass propagation of *Phalaenopsis*. Using the tissue culture technique can improve not only the quality but also the quantity of orchid plantlets and that can produce many plantlets within the limited period. This technique will improve our orchid flower market and orchid growers can obtain many healthy and uniform plantlets with low cost. Therefore, this study was carried out with the following objectives;

- To study the effect of different nodal positions of flower stalk on shoot induction, multiplication and root regeneration *in vitro*
- To investigate the suitable plant growth regulators (PGRs) and culture systems for PLBs induction from leaf explants
- To compare different culture systems for PLBs proliferation and
- To find out the effect of media for root formation of shoot developed from PLBs.

CHAPTER II
LITERATURE REVIEW

2.1 Propagation of Orchids

The propagation methods used in orchid are division, backbulbs, serial cuttings, kiekies, micropropagation and seed culture. The uses of propagation methods can vary depend upon the growth habits of orchid, monopodial and sympodial. Conventionally, sympodial orchids like *Cattleya, Dendrobium, Paphiopedilum* and *Cymbidium* are multiplied through divisions whereas monopodials viz. *Vanda, Aerides, Arachnis, Mokara* etc. through cuttings. Among the orchids, *Phalaenopsis* are difficult to propagate vegetatively by conventional propagation. *Phalaenopsis* orchid reproduces asexually through cutting of kiekies from the flower stalk node in nature. They do not form offshoots as *Dendrobium* orchids.

2.2 Micropropagation of Orchids

In present day, orchid species become popular ornamental plants all over the world and mainly used in all aspects of the people. These are more needed to produce for commercial production due to the increasing demand of orchid. Therefore, propagation through tissue culture is desirable for production of *Phalaenopsis*. *In vitro* propagation has been used mainly for mass production. Various source of vegetative plant parts such as leaf, shoot, stem etc can be used as the explants in micropropagation of monopodial and sympodial orchid. Nowadays, researchers have been tested and established the effect of explants (vegetative or reproductive), PGRs (individual or combination) and other influence factors (culture systems, sterilization time or etc) on tissue culture according species to get the most effectient micropropagation. Jitsopakul (2013) tested the effect of PGRs and sucrose concentration on efficient adventitious shoot regeneration of *Vanda coerulea* in *in vitro* using shoot tip culture. Nongdam and Tikendra (2014) established an efficient *in vitro* regeneration protocol from seed culture in *Dendrobium chrysotoxum.* Kaur and Bhutani (2009) tested the effect of regenerative potential of leaf explants on *in vitro* mass propagation of *Vanda testacea.*

2.2.1 Micropropagation of *Phalaenopsis*

Phalaenopsis have been propagated by using the vegetative and reproductive parts of the plant in tissue culture. The availability of explants was limited since it is a

monopodial orchid and has very short nodes at the stem. Several kinds of tissue culture protocols have been developed for *in vitro* cloning such as flower stalk cutting (Tanaka et al. 1988), leaf segments (Park et al. 2002) and axillary buds of flower stalk node (Tokuhara and Mii 2003). Using the flower stalk of plant as experimental material is suitable way that cannot damage the mother plant and it becomes an effective organ for micropropagation of orchids. Node culture is one of the valuable procedures for propagation that produce shoots in culture. This technique becomes popular in commercial micropropagation because of the getting of clonal stability and this has less frequency of subculture or stages than the other techniques. Therefore, the risk of induced genetic irregularity is less in shoot multiplication stage (George and Debergh 2008). Due to this fact, node culture has been recommended as the method of least to induce somaclonal variation (Asthana et al. 2011). *Phalaenopsis* orchid tissue culture using flower stalk node was initiated by Rotor (1949). This technique has been found as the maximum application technique for mass propagation of monopodial orchid, *Phalaenopsis* species. The problem occurred during the flower stalk cultured was the different growth pattern of buds because some buds remained dormant, developed into flower stalks, or developed into vegetative shoot (Griesbach 1983). Based on these facts, different responses such as survival percent, multiple shoot production etc may occurred according to the different nodal positions as the donor plant, but it was not well documented. Hormone supplementation is also the important factor to success the flower stalk node culture. Balilashaki et al. (2014) presented the highest shoot formation from flower stalk node was occurred on MS basal medium supplemented with 4.4 $mg.L^{-1}$ BA and 1 $mg.L^{-1}$ NAA. Kosir et al. (2004) also showed that direct shoot formation from flower stalk node culture of *Phalaenopsis* orchid. Wu and Chen (2008) observed that 2 $mg.L^{-1}$ BA was found as the suitable hormone ratio for shoot formation of flower stalk node culture of *Phalaenopsis* and *Doritaenopsis*.

Another method which is mainly used for cloning is leaf culture. Direct induction of PLBs from somatic tissues such as shoot meristem, leaf, root tip, flower stalk and other vegetative plant parts have been developed in orchids. Leaf culture is one of the methods for effective PLB induction. Induction of PLBs from leaf sections has been described for *Phalaenopsis* (Hsu and Chen 2003). PLB induction was occurred in leaf tissue culture of *Phalaenopsis* by using the different kinds of leaf tissue from leaves developed from flower stalk node, leaves of seedling and adult

plants (Tanaka et al. 1975). Leaves from *in vitro* plantlets are suitable explants for *in vitro* cloning of *Phalaenopsis ambilis* that has no adverse effect on adult plant and not limited on availability of explant. It was generally successful to regenerate plants from PLBs which multiplied rapidly by division.

VW basal medium supplemented with 10mg.L^{-1} BA and 1mg.L^{-1} NAA was the effective ratio for *Phalaenopsis* species in induction of PLBs from leaf explant (Myint et al. 2001). Arditti and Ernst (1993) reported that modified MS medium supplemented with 1 mg.L^{-1} NAA and 10 mg.L^{-1} BA was suitable auxin-cytokinin ratio to induce PLBs from the mature leaf segments of *Phalaenopsis.* amabilis. Sinha et al. (2010) and Sinha and Jahan (2011) showed the highest PLBs induction was observed from ½ MS basal medium supplemented with 2 mg.L^{-1} BA and 1 mg.L^{-1} NAA in *Phalaenopsis* hybrids.

2.2.2 PLB proliferation

PLB was the effective somatic embryo for multiplication and regeneration stages. PLB can be found only in orchid *in vitro* culture among the plant. PLB is an early embryonic stage which is induced from explants either directly or indirectly. PLB can be obtained from *in vitro* culture of leaf, flower stalk node, protocorm. *In vitro* culture through PLBs was more effective than the other plant's parts. It can be divided and cultured on the proliferation medium can obtains large tissue stocks of clone within a relatively short time by repeating this process for rapid mass propagation (Yam and Arditti 2009). Luo et al. (2003) reported that micropropagation through PLBs is efficient because PLBs can be rapidly proliferated on solid or in liquid culture medium, and a large number of PLBs can be provided in a short period. PLBs are also general target tissue for genetic transformation studies in orchids because orchids can proliferate rapidly and can readily regenerate into complete plantlets (Sreeramanan et al. 2008).

2.2.3 Root formation from shoots

Rooting from shoots also an important part of tissue culture. Root formation needed for shoots obtained from various culture methods not only to form well plantlets but also for adaptation *in vivo*. For rooting, auxin is the most important hormone. Natural occurring auxin IAA and synthesis auxin IBA and NAA are mostly used for rooting. Wagner and Maguire (2007) reported that roots of *in vitro* shoots

were occurred on medium containing 5.4 mM NAA in *Doritaenopsis* Purple Gem 'Ching Hua'. Khatun et al. (2010) reported that 1.0 mg.L^{-1} each of BAP + NAA, BAP + IAA, BAP + IBA, and IAA + IBA were found as the best result for producing rooted plantlets with charcoal supplementation in *Dendrobium* orchid.

2.3 Plant Growth Regulators (PGRs)

Plant growth regulators play an essential role in plant tissue culture that determined the development pathway of cultured cells and tissues. The exogenous requirement of hormones depends on their levels of endogenous hormones in tissue, plant type and phase of plant growth. The effect of plant growth regulators on tissue or organ culture can vary according to the varieties of plant, physiological maturity of explants, culture type, etc. The major classes of plant growth regulators used in *in vitro* culture are auxin, cytokinin, gibberellin and abscisic acid.

2.3.1 Auxins

Auxins are widely used in plant propagation and tissue culture. The ability of auxins is to promote cell division and growth in explants and control the root initiation. Auxins have two groups, the first one is natural occurring auxin such as Indolacetic acid (IAA), and the second one is synthetic auxins such as naphthalene-1-acetic acid (NAA), 2, 4-dichlorophenoxyacetic acid (2,4-D) and Indole-3-butyric acid (IBA). The usage of hormones and their requirements vary according to the different variety and expected outcomes. Most auxins are used for root induction, but IBA or IAA was most effective in root induction than other auxin in many orchid species (Mohanty et al. 2012). Doods (1994) founded that VW medium supplemented with 2 mg.L^{-1} IBA and IAA medium produce roots from shoots of *Dendrobium* hybrids. Moreover, 1 – 2 mg.L^{-1} 2, 4-D in MS medium promoted callus formation from culturing apical meristem explants in *Dendrobium* (Anjum et al. 2006). Auxins also induced rhizome growth while inhibiting shoot production. In Rhizogenetic, NAA and IAA helped to sustain rhizome growth in Spathoglottis (Bapat and Narayanaswamy 1977) and in *Cymbidium* (Paek and Yeung 1991). Combined effect of NAA and BAP generated callus in *Dendrobium*, that are subcultured on hormone free media formed PLBs (Roy and Banerjee 2003). In *Oncidium* and *Cymbidium*, medium containing 2,4-D and TDZ hormones generated embryonic callus, which initiated PLBs after subculture on hormone-free media (Huan et al. 2004).

2.3.2 Cytokinins

Cytokinins such as 6-benzylaminopurine (BAP), Benzyladenine (BA), 2iP, Kinetin (KIN), Zeatin (ZEA) and Thidiazuron (TDZ) are commonly used in culture media. Cytokinins generally promote cell division and induce shoot formation and axillary shoot proliferation. Cytokinin can be divided into two groups; adenine type cytokinins and phenylurea cytokinins. KIN, ZEA and BA are adenine type while cytokinins, diphenylurea and TDZ includes phenulurea. Cytokinins are very effective in promoting direct or indirect shoot initiation and enhance the axillary shoot proliferation. A requirement of particular cytokinin is often noted according to the process of culture; for example in *Browallia viscosa* tissue culture, 2iP was required for adventitous shoot initiation but kinetin, BA and Zeatin were ineffective (Welsh and Sink 1981).

TDZ has been reported to be effective in the regeneration of a number of orchid species such as *Doritaenopsis*, *Phalaenopsis*, *Cymbidium*, *Oncidium*, and *Dendrobium* (Sujjaritthurakarn and Kanchanapoom 2011). Depending on TDZ concentration, exposure time, the cultured explant, and the species tested, activity of TDZ varies widely (Murthy et al. 1988). Myint et al. (2006) tested the effect of TDZ concentration on direct multiple shoot induction from stem culture of *Phalaenopsis* cv. Golden diamond. Chen and Chang (2006) reported that direct somatic embryogenesis was observed from leaf explants that cultured on media containing TDZ in *Phalaenopsis amabilis*. Sreeramanan et al. 2009 showed that 60 %, 70 % and 50 % of leaf segments responded and differentiated on medium supplemented with 0.1 $mg.L^{-1}$, 0.17 $mg.L^{-1}$ and 0.22 $mg.L^{-1}$ TDZ and 75 % of PLBs proliferation rate were observed on 0.1 $mg.L^{-1}$ TDZ in *Phalaenopsis violacea*. Similarly, highest number of PLBs per leaf explant was observed on medium with 3 $mg.L^{-1}$ TDZ in *Phalaenopsis amabilis* cv. Cool Breeze (Balilashaki et al. 2014).

The effect of BA has been founded in PLBs production from various explants of *Phalaenopsis* orchid (Park et al. 2002). BA and 2iP are also used for induction of embryo from leaf tissue culture of *Phalaenopsis* orchids. Gow (2005) demonstrated that 3 $mg.L^{-1}$ BA and 0.5 $mg.L^{-1}$ 2iP was the most effective combination for direct embryo formation from leaf in *Phalaenopsis amabilis* and *Phalaenopsis nebula.* Large number of rootless shoot were obtained from MS medium supplemented with 1.5 $mg.L^{-1}$ BAP in *Dendrobium officinallis* medicinal orchid (Moe 2014).

2.3.3 Auxin and cytokinin combination

Exogenous applications of cytokinins and auxins have been known to be important for shoot induction and elongation of many plant species *in vitro* (George 1993). Many aspects of cell growth, cell differentiation, organogenesis in tissue culture and organ culture have been found to be controlled by an interaction between auxin and cytokinin. In tissue culture, auxins are balanced with cytokinins for full morphogenesis. Cytokinins act in concert with auxin, having the opposite effects. Cytokinin alone has no effect on parenchyma cells. When auxin alone, explants grow large but do not divide. Tokuhara and Mii (1993) reported that the combination and appropriate concentration of NAA with BAP in the culture medium are important for micropropagation of *Phalaenopsis* on commercial scale. North et al. 2010 also reported that the combination and concentration of auxins and cytokinins in mediums is an important factor which determines successful plant regeneration. Park et al. 2002 demonstrated that 85 % of leaf explant response and 12 PLBs per explant were obtained from ½ MS medium supplemented with 12 $mg.L^{-1}$ BA and 1 $mg.L^{-1}$ NAA in *Phalaenopsis* orchid. PLBs growth rate were improved in the media supplemented with combinations of BAP and NAA compared to the growth of PLBs in individual PGRs on *Dendrobium Sonia* 28 (Julkiflee et al. 2014)

Auxin in combination with TDZ is suitable for PLBs formation from leaf sections of *in vitro* young plants (Niknejad et al. 2013). NAA is frequently used in combination with BAP or TDZ in many orchid species like *Vanda*, *Dendrobium* and *Phalaenopsis* (Zhao et al. 2008). However TDZ alone have positive effect for direct somatic embryogenesis from leaf explants of *Oncidium* (Chen and Chang 2001). Auxin can inhibit cytokinin-induced direct somatic embryogenesis in *Phalaenopsis* and *Oncidium* species (Kuo et al. 2005). Chen and Chang (2004) showed that effective inducing of somatic embryogenesis was found in TDZ alone medium and NAA inhibited direct embryo formation in *Phalaenopsis amabilis var*. *Formosa Shimadzu*. The requisite concentration of each hormones can varies according to the kind of plant, stage of plant and use of various plant parts.

2.4 Culture Medium

Culture medium is one of the most important factors that influence the morphogenesis growth and subsequence of cultured tissue. Concentration of nutrients supplied in medium strongly influenced on quality of morphogenetic responses and

how rapidly a tissue grows and the extent. Various culture media such as Murashige and Skoog (MS), Vacin and Went (VW), N6, New Dogashima medium (NDM), Knudson-C (KC), Hyponex, Gamborg (B5), Nitsch and Nitsch (NN) medium etc are used in tissue culture. Use of culture media can vary according to the establishment of protocols for specific purposes in tissue culture. Full strength of medium gave good results for several species but in some species, ½ or ¼ strength of medium gave better results than full concentration. For multiple shoot production of Kurai medicinal plant, full strength of MS medium was favorable *(H. antidysentrica)* (Mallikarjuna and Rajendrudu 2007). One half, one third, one fifth, or three fourth MS modified medium has been found effective in Gum arabic tree *(Acacia Senegal)* (Badji et al. 1993). For rice anther culture, N 6 basal medium has been mainly used. The simple media that used in seed germination such as Vacin and Went (VW) (Vacin and Went 1949), Knudson-C (KC) (Knudson 1946) or Hyponex (Kano 1965) are also employed for mericlone culture. More complex media such as Murashige and Skoog (MS) media used in some genera and some of the process such as seed germination, shoot induction and etc. Mayer et al. (2010) reported that modified MS medium was used in PLB induction from leaf culture of *Oncidium flexuosumsim*. The usage of ½ MS basal media has been described for Leaf thin-section culture PLB induction from leaf culture in *Phalaenopsis cornucervi* (Breda) Blume & Rchb. F (Rittirat et al. 2014). Myint et al. (2001) tested the effect of culture media and with and without PGRs (VW, MS, Hyponex, NDM) for rooting of *Phalaenopsis*.

2.5 Culture Systems

Culture system is one of the important factors to success the *in vitro* propagation. They support and maintain the nutrients to explant. Several culture systems such as solid, semi-solid and liquid can be used in plant tissue culture. Different culture systems have different abilities to maintain the nutrient and varied the cost of micropropagation. Alvard et al. (1992) stated that the cost of micropropagation reduced more in liquid culture than solid culture. Because solid culture requires labour intensive step including repeated sub-culturing. However the disadvantages of liquid culture were the rapid spread of contaminant and hyper-hydricity of the tissue. In solid culture, batch-to-batch variability, inhibition of growth, presence of impurities, impartment and impairment of vitrification were occurred due to the adverse effect of agar (Vyas et al. 2008). Liquid culture

supplemented with cotton, filter and other support material are also used. This type of medium also the best for culture due to the limitation in diffusion of water and nutrient, promoting the growth of explant due to the lack of agar, and reduce the toxic with phenol. The solid, liquid and semi-solid culture systems have different advantages and disadvantages. Different culture systems can vary the results obtained from tissue culture. Suitable culture systems are also important for multiple cloning because their usages are varying according to the stage of tissue culture. However, the researches related with the culture system are weak and a lot of report has not been observed.

CHAPTER III
MATERIALS AND METHODS

3.1 Experimental Site and Period

The experiment was carried out in tissue culture laboratory, Department of Horticulture and Agricultural Biotechnology, Yezin Agricultural University from January 2016 to 2017.

3.2 Experimental Materials

Phalaenopsis amabilis plants were obtained from Mayepadaythar Flower Garden, Yangon and *in vitro* plantlets were obtained from Mingalardone Tissue Culture Laboratory. *Phalaenopsis amabilis* at blooming stage with 3-4 flowers on flower stalk were used as explant materials for experiment 1 to observe the effect of different nodal positions of flower stalk (Plate 1. A) on shoot induction, multiplication and root regeneration.

In experiment 2, *in vitro* plantlets were used as explant materials for leaf culture (Plate 1. B) to observe the effect of culture systems, PGR and different media on plantlets regeneration of *Phalaenopsis* orchid.

Plate 1. Explant materials used in (A) flower stalk node culture and (B) *in vitro* young leaf culture of *Phalaenopsis amabilis*

3.3 Experiment 1. Effects of Different Nodal Positions of Flower Stalk on Shoot Induction and Multiplication

3.3.1 Effects of different nodal positions on shoot induction

3.3.1.1 Experiment design and treatment

The experiment was carried out a randomized complete block design with 8 replications. In initial culture, different nodal positions (1 to 6) of flower stalk were tested for shoot induction, multiplication and rooting (Plate 2).

3.3.1.2 Initial culture

Phalaenopsis amabilis orchid plants with three to six flowers containing at least six nodes were collected. Flower stalk was cut from the plant at the base and flower stalk nodes were used as the explants. From first node at the base of the flower stalk, flower stalk nodes were cut into 5 – 6 cm including a bud length and separate the nodal cutting as second, third, fourth, fifth and six nodes so that they were used as the explants (Plate 2). The selected flower stalk nodes were washed with tap water. These nodal cuttings were excised into 2 cm and sterilized with 70% ethanol for 30 seconds. Thereafter, single nodal segments were sterilized with 2% sodium hypochlorite solution for 15 minutes. Disinfected explants (1 cm) were washed three times with sterile distilled water.

MS medium supplemented with 2 $mg.L^{-1}$ BA and 0.5 $mg.L^{-1}$ NAA, agar 7 $g.L^{-1}$, sugar 20 $g.L^{-1}$ and coconut water 200 $ml.L^{-1}$ were used as the culture medium in this experiment. Media was adjusted at pH 5.57 before autoclaving. The volume medium 15 ml was dispensed into each test tube and sterilized with autoclave at 121º C for 15 minute.

After sterilization, the two sides of the node were cut about 0.5 cm above and below the bud and then removing the scales on the bud and then inoculated into the medium. Cultures were kept under the dark condition for two weeks. Two weeks after culture, cultures were kept under 25±1ºC at 16 hours photoperiod with 20-40 $\mu molm^2s^{-1}$ light intensity. Nine weeks after culture, 2 to 3 vegetative shoots with two to three leaves formed from a single node of a flower stalk (Plate 3).

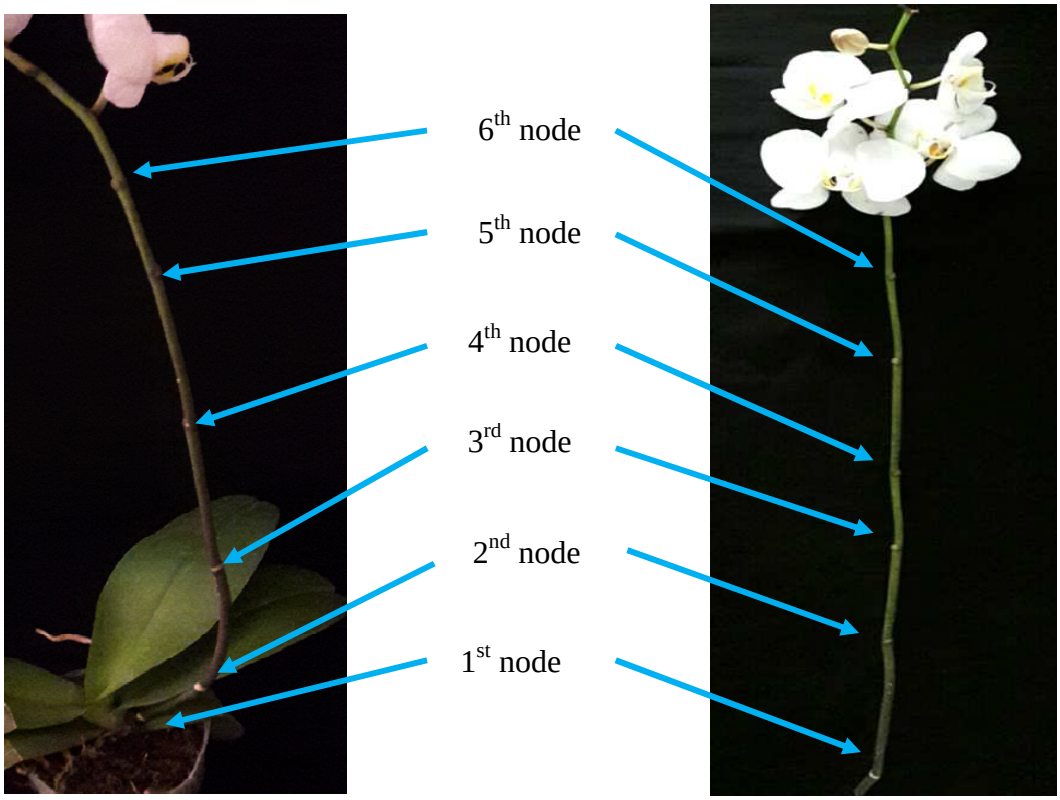
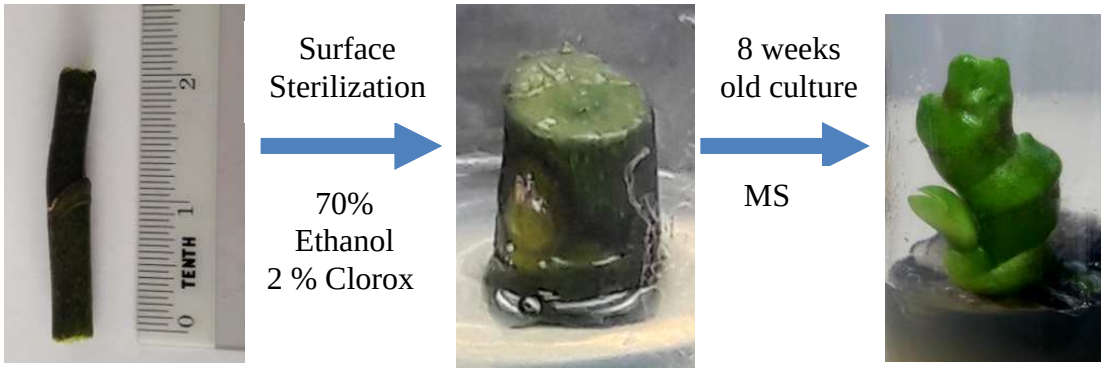


Plate 2. Nodal positions of flower stalk in *Phalaenopsis amabilis*



A nodal explants (5-6 cm length) from a flower stalk

Initial culture of nodal (1cm) explant

Shoot induction from nodal explants after 8 weeks culture

Plate 3. Procedure for initial culture of node culture

3.3.2 Effects of different nodal positions on shoot multiplication

Shoots obtained from node culture were used as the material for multiple shoots propagation (Plate 4. A). The tips of shoot and leaves were removed and the remaining part of the shoots were used as the secondary explant and that inocultaed into shoot multiplication medium (Plate 4. B).

The MS medium supplemented with 2 $mg.L^{-1}$ BA and 0.5 $mg.L^{-1}$ NAA, agar 7 $g.L^{-1}$, sucrose 20 $g.L^{-1}$ and coconut water 200 $ml.L^{-1}$ were used as the culture medium for shoot multiplication. Media was adjusted at pH 5.57 before autoclaving. The volume 40 ml medium was dispensed into the bottle and sterilized with autoclave at 121º C for 15 minute. An explant was inserted into each bottle. All explants were kept under darkness condition within 6 weeks for multiple shoots induction. After 6 weeks culture, cultures were kept under the low light intensity 1000-2000 Lux with 16 hours light and 8 hours dark photoperiod. After eight weeks culture, multiple shoots were observed on main shoot derived different nodal positions. Shoots were excised from multiple shoot clump and subculture on the MS medium without PGR for rooting by 3 weeks intervals.

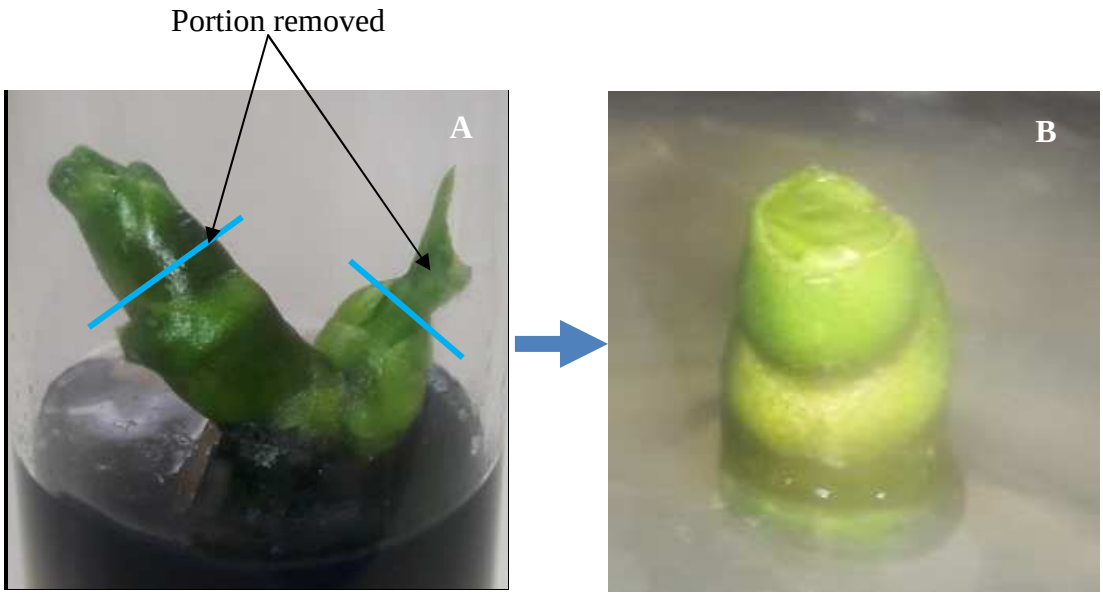


Plate 4. Materials for shoot multiplication of shoot developed from nodal culture of *Phalaenopsis amabilis* (A) 11 weeks old shoot from initial culture (B) Secondary explant ready to culture after topping

3.3.3 Effects of different nodal positions on root formation of shoots

Shoots obtained from shoot multiplication stage were cultured on MS basal medium supplemented with sugar 30 $g.L^{-1}$, 200 ml coconut water and agar 5.5 $g.L^{-1}$ for rooting (Plate 5. A). Media was adjusted at pH 5.56 before autoclaving. The volume 50 ml medium were dispensed into each bottle and sterilized with autoclave at 121º C for 15 minute. Single shoots of 3 to 5 cm in height were excised from multiple shoot clump and inoculated into each bottle (Plate 5. B). The cultures were kept under 15-25 $\mu molm^{-2}s^{-1}$ light intensity with 16 hours light and 8 hours dark photoperiod condition. Twelve weeks after culture, root induction response was occurred. Plantlets were observed at 23 weeks after culture.

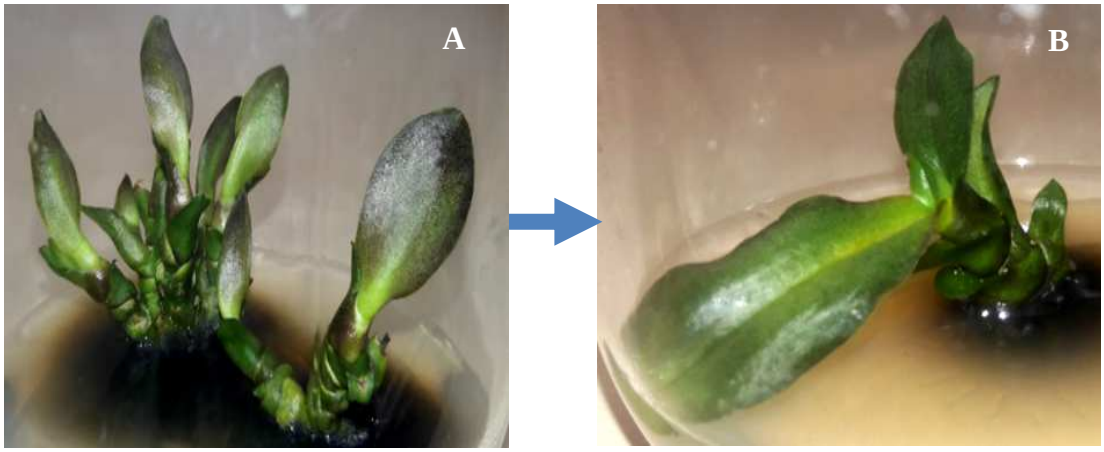


Plate 5. Root formation of shoots derived flower stalk node culture (A) 18 weeks old multiple shoots to be used for shoot multiplication (B) Separated into single shoot for root formation.

3.3.4 Hardening of plantlets obtained from flower stalk nodes at different positions on flower stalk

Two steps were carried out in hardening stage. In pre-hardening stage, complete plantlets obtained from flower stalk node culture were kept under the room temperature for two weeks. Cultured vessels were placed under the morning natural light for two hours and opened the culture vessels inside the laminar air flow cabinet to exchange gases. After two weeks, plantlets were taken out from the culture vessels and thoroughly washed with tap water to remove the agar medium completely without damaging the roots. These plantlets were cultured on the mixture of charcoal and moss substrate (1:1 ratio) supplemented with 1/10 MS medium for pre-hardening stage and maintained in *in vitro* for two weeks. And then, plantlets were taken out from the *in vitro* room for three weeks and the holes put on the cover of culture bottle to allow aeration. Five weeks after culture, plantlets were treated with diluted fungicide for 1 minute. They were cultured on the mixture of small charcoal pieces (1 – 2 cm diameter) and mosses (1:1 ratio) and kept under the natural light. Survival % was recorded after 2 months (Plate 6).

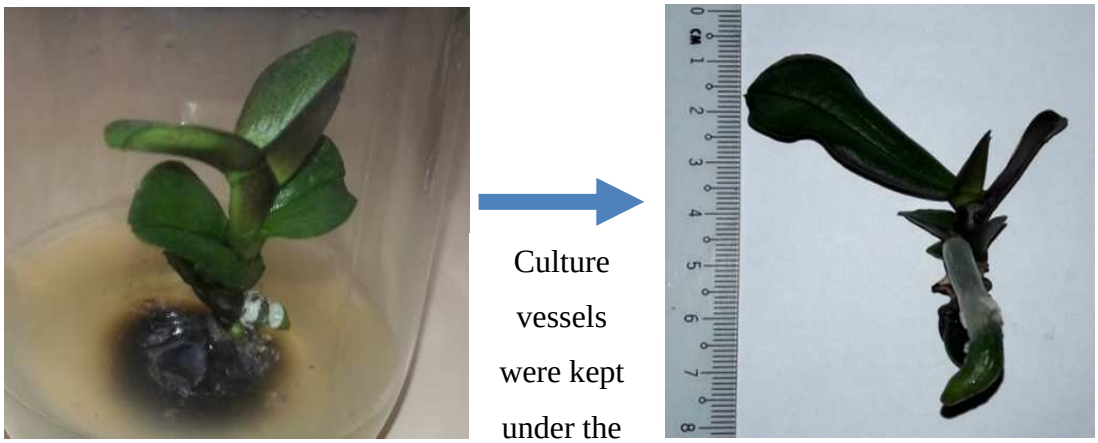


Plate 6. Procedure for hardening stage

3.3.5 Data collection (Experiment 1)

Effects of different nodal position on shoot induction

The following data were collected within 2 weeks to 11 weeks after culture for shoot induction from flower stalk node;

1. Survival %
2. Number of shoots developed per node
3. Days to buds swelling

Effects of different nodal position on shoot multiplication

The following data were collected within 6 weeks to 10 weeks after culture for shoot multiplication;

1. Survival %
2. Shoot regeneration %
3. Number of shoots per shoot clump

Effects of different nodal position on root formation

The following data were collected within 18 weeks to 25 weeks after culture for rooting of shoot obtained from flower stalk node;

1. Survival %
2. Days to induce root
3. Number of roots per shoot
4. Roots formation %

Hardening of plantlets obtained from different flower stalk nodes at different on flower stalk positions

Survival % data were collected within 8 weeks old hardening.

3.4 Experiment 2. Effects of Culture System, Plant Growth Regulators and their Combinations on Induction of Protocorm Like Bodies (PLBs) from *In vitro* Young Leaf

3.4.1 Effects of two culture systems and different PGRs regimes on PLBs induction from *in vitro* young leaf culture

The experiment was carried out with two factors factorial randomized complete block design with 4 replications. Two culture systems (solid culture and cotton support liquid culture) were applied as factor A and 8 different concentration and combination of PGRs (2 $mg.L^{-1}$ BA, 4 $mg.L^{-1}$ BA, 2 $mg.L^{-1}$ BA + 0.5 $mg.L^{-1}$ NAA, 4 $mg.L^{-1}$ BA + 0.5 $mg.L^{-1}$ NAA, 1 $mg.L^{-1}$ TDZ, 2 $mg.L^{-1}$ TDZ, 1 $mg.L^{-1}$ TDZ + 0.5 $mg.L^{-1}$ NAA and 2 $mg.L^{-1}$ TDZ + 0.5 $mg.L^{-1}$ NAA) were factor B. Sixteen treatments were tested in this stage. Three explants were cultured in each vessel. Twelve explants were cultured for each treatment per replication.

In vitro last emerging leaves were used as the explants (Plate 7. A). Young leaves (0.5 – 2 cm) were excised to get thin sections (1- 2 mm) explants (Plate 7. B). The explants were cultured on the ½ MS basal medium containing 20 $g.L^{-1}$ sucrose and supplemented with different concentrations of BA (2 and 4 $mg.L^{-1}$), TDZ (1 and 2 $mg.L^{-1}$) individually or in combination with 0.5 $mg.L^{-1}$ NAA,. The solidified medium supplemented with 7 $g.L^{-1}$ agar and liquid medium supported with cotton wool, 2 cm in thickness. The volume 30 ml medium was dispensed into each bottle for solid culture medium. Medium was adjusted at pH 5.7 before autoclaving. Cultures were incubated into darkness for 2 weeks to reduce phenol exudation, and then transferred to 16 h photoperiod, 20-40 $\mu molm^{-2}s^{-1}$ and temperature 25 ± 1 °C. Data were collected within 6 to 10 weeks. After 6 weeks culture, PLBs were emerged and developed.

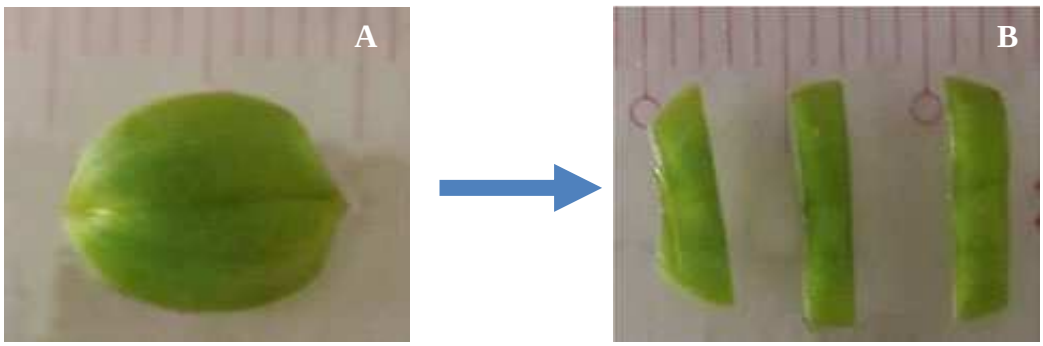


Plate 7. PLB induction of *in vitro* leaf culture (A) *In vitro* leaf material (B) 2 mm thin sections leaf explant

3.4.2 Effects of three culture systems on PLB proliferation

Two factors factorial CRD with 4 replications were used as the experimental design. Five PLB explants were cultured in each vessel. Fifteen explants were cultured for each treatment per replication. The basal ½ MS medium supplemented with 20 $g.L^{-1}$ sucrose, 100 ml coconut water and 150 $mg.L^{-1}$ L- glutamine was used. Treatments in this stage are solid culture, liquid culture and cotton support liquid culture.

PLBs obtained from leaf culture were used as the explants materials for PLBs proliferation (Plate 8). The PLBs clusters obtained from leaf culture were separated and approximately 5 mm size PLB was selected and divided into 4 pieces and cultured on the solid, liquid and cotton ½ MS medium supplemented with 20 $g.L^{-1}$ sucrose, 7 $g.L^{-1}$ agar, 100 ml coconut water and 150 $mg.L^{-1}$ L- glutamine for PLBs proliferation. The volume 40 ml medium was dispensed into the culture bottle and media was adjusted at pH 5.57 and autoclaved at 121º C for 15 minute. Five explants were cultured in each bottle. Cultures were kept under 16 h photoperiod, 20-40 $\mu molm^{-2}s^{-1}$ light intensity and temperature 25 ± 1 °C. Data were collected within 2 to 10 weeks. PLBs were developed to shoots within 12 weeks after culture.

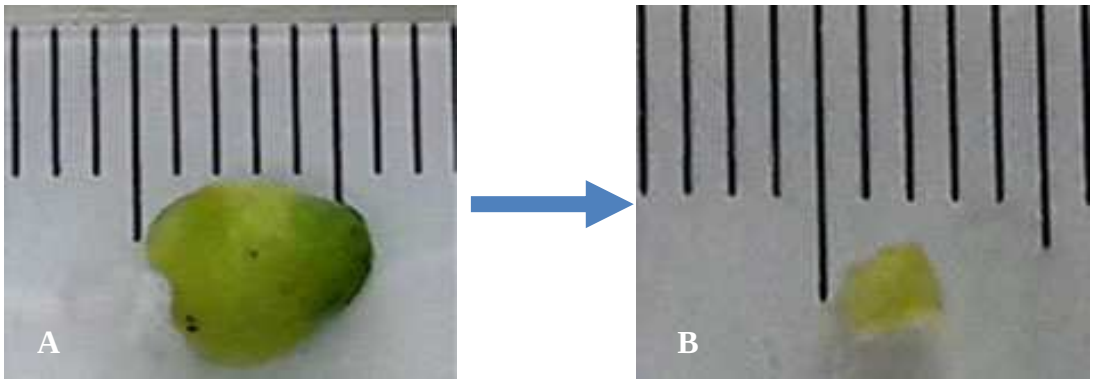


Plate 8. PLB proliferation of *in vitro* PLB derived from leaf culture (A) PLB obtained from initial culture and used as explant material for PLB proliferation (B) Excised PLB segment of 2 mm in length

3.4.3 Effects of two basal medium with and without PGRs on rooting of shoots developed from PLB

In this stage, effects of two basal media VW and MS media supplemented with and without plant growth regulators on rooting of shoots were compared. One or two shoots developed PLBs were selected and transferred into the media. Shoots with 2-3 leaves developed from PLBs were cultured on the VW and MS media containing with the combinations of 0.1 $mg.L^{-1}$ BA and 1 $mg.L^{-1}$ NAA and those without PGRs (Plate 9). Culture media were added 30 $g.L^{-1}$ sucrose, 5 $g.L^{-1}$ agar and 200 $ml.L^{-1}$ coconut water. Media were adjusted at pH 5.56 and the volume 30 ml media were dispensed in each bottle. And then these cultures were autoclaved at 121º C for 15 minute. Three explants were inoculated into each bottle. Nine explants were cultured for each replication. Randomized complete block design (RCBD) with 3 replications was used as the experimental design. Cultures were maintained under 16 hours photoperiod, 15-25 $\mu molm^{-2}s^{-1}$ and temperature 25 ± 1 °C. Data were collected within 2 to 20 weeks. Complete plantlets were observed within 20 weeks after culture.

Shoot developed from PLB

Plate 9. Plantlets regeneration of shoot developed from PLB

3.4.4 Data collection

Effects of two culture systems and different PGRs regimes on PLBs induction from *in vitro* young leaf culture

The following data were collected within 6 weeks to 10 weeks after culture for PLBs induction from *in vitro* leaf;

1. Survival %
2. PLBs formation %
3. Number of PLBs per leaf explant

Effects of three culture systems on PLB proliferation

The following data were collected within 2 weeks to 10 weeks after culture for PLB multiplication from PLBs derived *in vitro* leaf;

1. Survival %
2. PLBs formation %
3. Number of proliferated PLBs per PLB explant

Effects of Two Basal Medium with and without PGRs on Rooting of Shoots Developed from PLB

The following data were collected within 6 weeks to 14 weeks after culture for rooting of shoots developed PLB from leaf;

1. Survival %
2. Number of roots per shoot explant
3. Roots formation %

3.5 Data Analysis

The collected data were statistically analyzed by using the SAS 9.0 version. Treatments means were compared at 5% level of significant difference (LSD).

CHAPTER IV
RESULTS AND DISCUSSIONS

4.1 Experiment 1. Effects of Different Nodal Positions of flower Stalk on Shoot Induction, Multiplication and Root Regeneration

4.1.1 Effects of different nodal positions of flower stalk on shoot induction

The different responses of nodal positions were described in Table 1. Effects of different nodal positions (1– 6 nodal segments) of flower stalk were observed on days to bud swelling of node (Pr>F=0.009) and there were not significant difference among each other in survival and number of shoots per nodal explant parameters. Flower stalk buds became active and started vegetative growth and swelling at two weeks after culture. After 4 weeks culture, shoot emerged and fully developed with an elongated stalk. All buds were observed breaking within 10 to 30 days. Similar result was reported by Tisserat and Jones (1999) and they reported that nodal buds on flower stalk emerged and shoot induction was occurred within 10 to 30 days. Sixth nodal position from base to top of flower stalks showed earlier response in bud breaking (12 days) than the other nodal positions. It might be due to the capacity of active bud. The late response was observed from 2nd and 3rd nodal position (22 and 23 days). The bud swellings of 1st, 4th and 5th nodal positions were observed as 20, 18 and 15 days after culture, respectively (Table 1 and Plate 10).

The survival percent of nodes from all different nodal positions were not significantly different each other. However, the maximum survival percent (87.5 %) was observed from 2nd, 4th and 5th nodal positions of flower stalk and minimum survival percent (50%) from 3rd nodal position.

Effects of different nodal positions of flower stalk on number of shoots per node explant are shown in Table 1. Numbers of shoots per node explant obtained from different nodal positions were not statistically different among the explants but numbers of shoots per nodal explant were less in 1st, 2nd and 3rd nodal positions of flower stalk than the upper nodal segments (4th, 5th and 6th nodes). One or two shoots per node explant were observed from all nodal positions in shoot induction. Flower stalk node cultured show 3 growth types - dormant, vegetative shoot and reproductive shoot in *Phalaenopsis* orchid (Tsao et al. 2008). Similar result was observed in this study. Most of the vegetative shoots developed from the buds of lower nodal segments and reproductive shoots develop from upper nodal segments of flower stalk.

The same response was occurred due to the flowering stimulant that exists in stalk tissue. Tanaka et al. (1988) found in *Phalaenopsis* that upper nodal segments produced flower stem. Among the different nodal positions, upper nodal position that near the first flower showed more shoot induction from the base of the flower stem after 9 weeks cultured than lower nodal positions. Similar finding was observed in Tsao et al. 2008 they stated that nodal positions near the first flower more pronounced to induce the flower stem before producing the multiple shoots at the base of it.

In this experiment, all nodal positions of flower stalk showed bud break response in *phalaenopsis amabilis*. This is in contract to the most of the several reports that presented the lower 1st node was the dormant bud in *Phalaenopsis* orchid. Griesbach (1983) stated that dormant bud was present at the basal part of the inflorescence but that were used as the explants for *in vitro* propagation of *Phalaenopsis* orchid. However, it was observed that BA supplemented medium can break the dormancy of basal flower stalk node. Panwar et al. (2012) also stated that BA have beneficial effect for better response in bud breaking and shoot multiplication. Tsao et al. (2008; 2011) also reported that 5 – 10 $mg.L^{-1}$ BA was found as the suitable range for the good shoot induction and multiplication for lower nodal positions of flower stalk. While 2 $mg.L^{-1}$ BA level was observed as the best result for shoot induction than the other cytokinin treatments in *Phalaenopsis* and *Doritaenopsis* (Wu and Chen 2008). But, in this study, MS medium supplemented with the combination of 2 $mg.L^{-1}$ BA and 0.5 $mg.L^{-1}$ NAA was used as the basal medium for flower stalk node culture. This might be the reason for obtaining better shoot formation due to the synergistic effect of cytokinin and auxin in this study. Balilashaki et al. (2014) also found that the combination of 4.4 $mg.L^{-1}$ BA and 1 $mg.L^{-1}$ NAA supplemented MS medium showed highest shoot formation from flower stalk node culture of *Phalaenopsis amabilis* cv “Cool Breeze”. Similar result was observed from Devi et al. (2013) they reported that combined effect of 4 mg L^{-1} BAP and 2 mg L^{-1} NAA gave best response in number of shoot per explants from different explants of *Aeridesodorata* Lour. an endemic orchid.

Table 1. Effects of different nodal positions of flower stalk on survival %, number of shoots per explant and days to bud swelling in shoot induction

Positions of node on flower stalk	Survival %	Number of shoots per explant	Days to bud swelling
1st	62.5	1.00	20.0 ab
2nd	87.5	1.00	22.0 a
3rd	50.0	0.88	23.0 a
4th	87.5	1.31	18.0 abc
5th	87.5	1.68	15.0 bc
6th	62.5	1.25	12.0 c
LSD	1.3	0.86	6.5
Pr> F	ns	ns	**
CV %	45.9	71.5	35.4

Means followed by the same letter in each column are not significantly different at 5% level. Data were collected from 3 weeks to 11 weeks after cultured.
ns: No significant ** Significant at 1% level

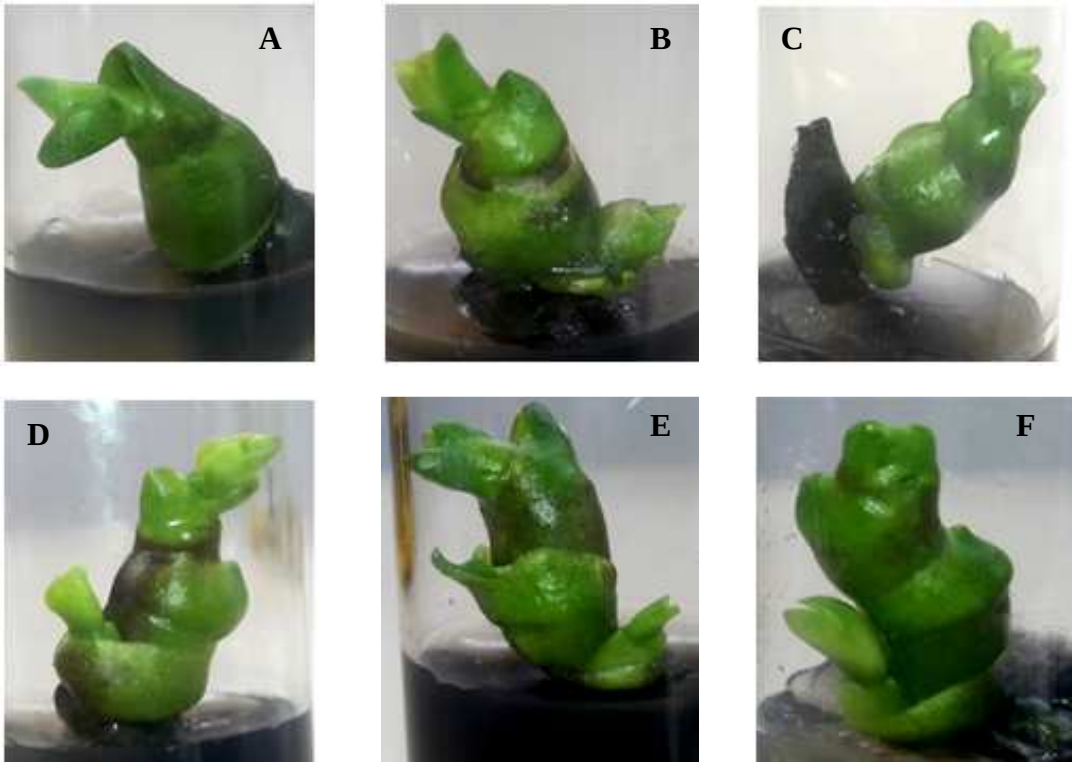


Plate 10. Shoot induction from different nodal positions of flower stalk *in vitro* 11 weeks after culture (A) Shoot of 1st node (B) Shoot of 2nd node (C) Shoot of 3rd node (D) Shoot of 4th node (E) Shoot of 5th node and (F) Shoot of 6th node

4.1.2 Effects of different nodal positions of flower stalk on shoot multiplication

Shoots obtained from different nodal positions of flower stalk were tested for regeneration (Table 2 and Plate 11). Different multiplication results were observed in 10 weeks after culture. The survival percent of explants from different nodal positions were not markedly different among each other. The buds on 2nd and 3rd nodes produced maximum survival percent (57 %) while minimum survival percent (42 % and 43 %) were observed from 4th and 1st nodal positions. Other nodal positions of flower stalk, 5th and 6th gave 53 % and 55 %, respectively.

Shoot regeneration percentage recorded from different nodal positions were not significantly different among each other. All nodal positions showed good response in multiplication stage. This might be influenced by the blooming situation of mother plant. (David and Bala 2012) reported that the stalks on which only a few flowers (3 to 6) blooming are found as the best choice to use for micropropagation.

Number of shoots per explants was significantly different among the treatments (Table 2). The maximum number of shoots per node explant (7.8) was resulted from 2nd nodal position followed by 3.3 in 4th nodal position, 2.5 in 3rd nodal position, 2.3 in 1st node, 2.00 in 6th node of flower stalk and minimum number of shoot 1.7 in 5th nodal position. Chang (2015) reported that lower node positions (1st, 2nd and 3rd) near the mother plant showed better shoot formation and multiplication in *Phalaenopsis* orchid. In *Phalaenopsis* orchid, flower stalk stem received assimilates from leaves and photosynthesized itself with weak CAM. During flowering time, all assimilates were transported to the inflorescence. According to the pressure flow hypothesis, photoassimilates from leaves (source) were moved within phloem by hydrostatic pressure to the flower (sink) across the flower stalk. During the transporting period, the situation of assimilate reached in flower stalk node can vary according to the nodal positions. Moreover, the first node seems to be latent bud than the 2nd and 3rd nodes and the upper nodes near the flower may have the flower stimulant. Therefore, explants from middle portion of flower stalk showed better shoot regeneration capacity.

Table 2. Effects of different nodal positions of flower stalk on survival %, shoot regeneration % and number of shoots per explant in shoot multiplication

Nodal positions	Survival %	Shoot regeneration %	Number of shoots per explant
1st	42.9	100.0	2.3 b
2nd	57.1	100.0	7.8 a
3rd	57.1	100.0	2.5 b
4th	42.1	100.0	3.3 b
5th	53.3	100.0	1.7 b
6th	55.6	80.0	2.0 b
LSD	0.3	0.3	1.9
Pr> F	ns	ns	*
CV %	33.1	33.1	119.0

Means followed by the same letter in each column are not significantly different at 5% level. Data were collected at 10 weeks after cultured.

ns: No significant * Significant at 5% level

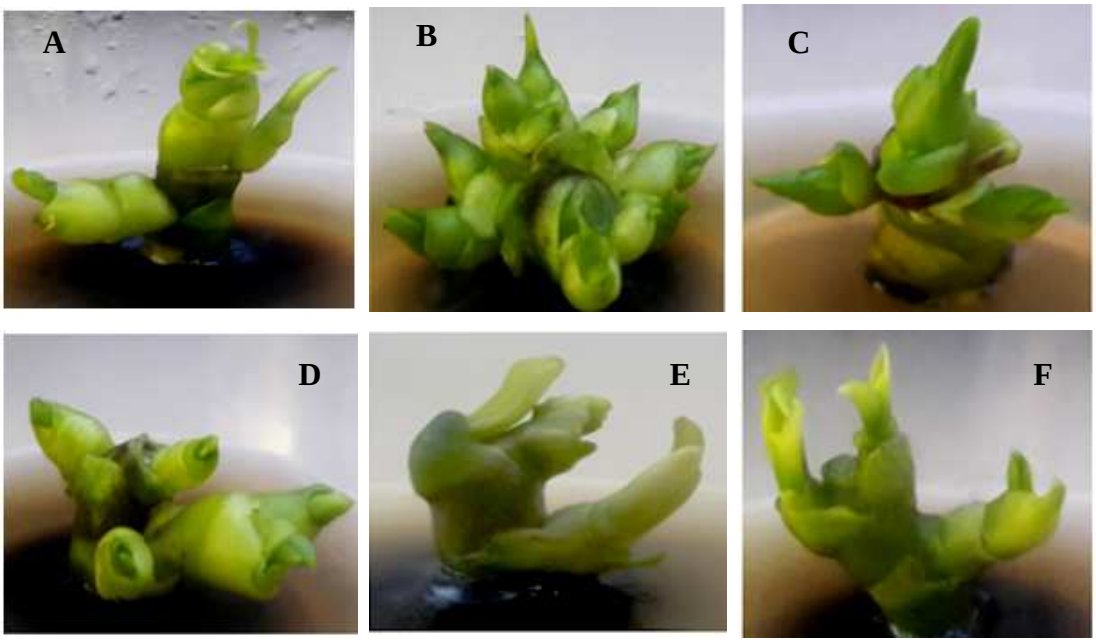


Plate 11. Shoot regeneration of *in vitro* shoot derived from flower stalk node culture 10 weeks after culture (A) 1st node (B) 2nd node (C) 3rd node (D) 4th node (E) 5th node (F) 6th node

4.1.3 Effects of different nodal positions of flower stalk on root regeneration

In rooting stage, shoots obtained from various nodal positions were cultured on the MS basal medium to test the effect of different node (Plate 12). The effect of node positions on survival percent, root formation percent, days to induce root and number of roots per explants were described in Table 3. Significant effects of nodal positions were observed in all parameters except survival percentage.

There were highly significant differences among the nodal positions in day to induce root and root formation percent parameters. The shoots develop from 3rd node (94 days), 4th node (98 days) and 5th node (96 days), receptively showed early response than the other treatments. The maximum root formation percentage (88.9 %) and 3.3 roots per explant were observed in shoots obtained from 3rd node and 60 % root formation with 1.6 roots per explant and 58.34 % root formation with 1.5 roots per explants were observed in shoots from 4th and 2nd node, respectively. It is point to be noted that root induction was not observed in shoots developed from 1st node.

Although 2nd node showed latest response in days to induce root, better results were observed in all parameters than the 5th and 6th flower nodes. It might be assumed that the growth habit of 2nd node that enhanced vegetative growth and take more time for shooting than the other node. Therefore, the 2nd nodes delay for plantlets formation. In this stage, shoots developed from 2nd nodal segment multiplied and they take time to induce root while 3rd, 4th, 5th and 6th nodal segments showed early response and complete plantlets. The shoots developed from 5th and 6th nodal positions showed early response and low root regeneration capacity than the shoots developed from 2nd, 3rd and 4th nodal positions. Therefore, the middle positions of flower stalk stem should be used as the explants for the commercial production of complete plantlets by using flower stalk node culture.

Table 3. Effects of different nodal positions on survival %, root formation % days to induce root and number of roots per explants in rooting of shoots

Positions of node	Survival %	Root formation%	Days to induce root	Number of roots per explant
1st	33.3 b	00.0 c	α	0.0 c
2nd	97.9 a	58.4 b	109.0 b	1.5 b
3rd	88.9 a	88.9 a	94.0 b	3.3 a
4th	93.3 a	60.0 ab	98.0 b	1.6 b
5th	44.4 ab	11.1 c	96.0 b	0.7 bc
6th	88.9 a	44.4 b	107.0 b	1.0 bc
CV%	41.5	7.9	8.6	59.5
Pr>F	ns	**	**	**
LSD	0.54	0.1	16.5	1.5

Means followed by the same letter in each column are not significantly different at 5% level. Data were collected at 21 weeks after cultured.

ns: No significant ** Significant at 1% level α = no root formation

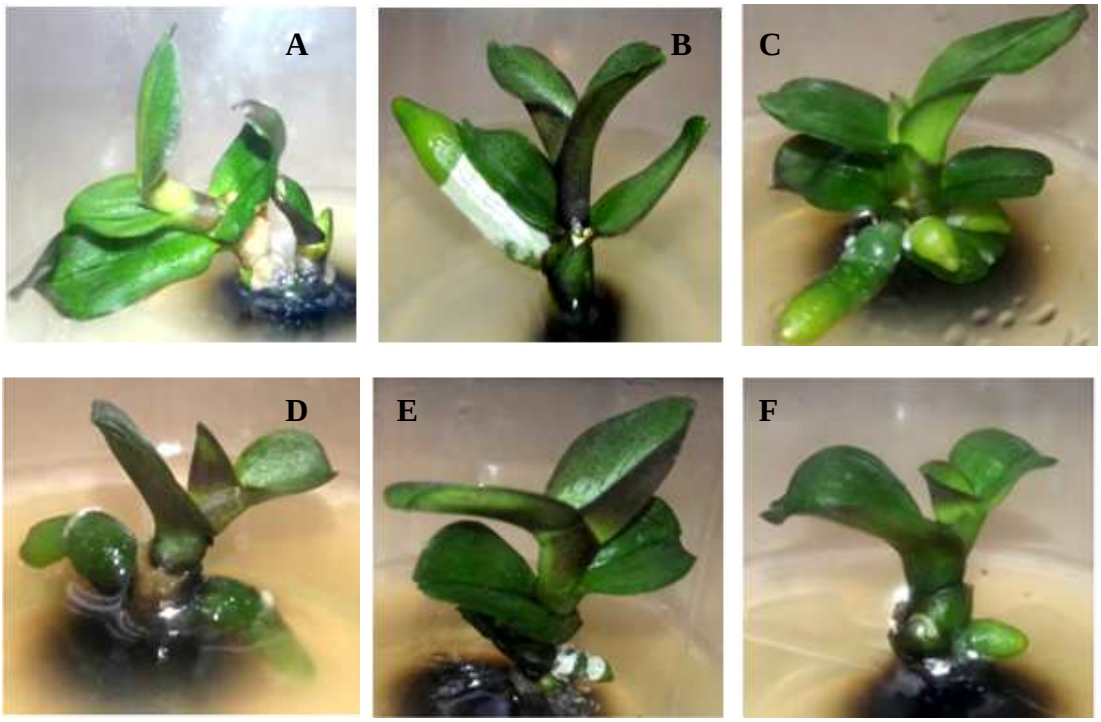


Plate 12. Root regeneration of plantlets from flower stalk node culture *in vitro* 20 weeks after culture (A) 1st node (B) 2nd node (C) 3rd node (D) 4th node (E) 5th node (F) 6th node

In experiment 1, survival percentage and number of shoots per explant in shoot induction stage were not markedly different in shoot induction of all nodal positions. All nodal positions showed similar response. However, upper (4^{th}, 5^{th} and 6^{th}) nodal positions showed earlier response than the lower (1^{st}, 2^{nd} and 3^{rd}) nodal positions. Although the latest response in days to bud swelling was observed in 2^{nd} and 3^{rd} node, the growth performances are good like other nodal positions. In shoot multiplication, survival and shoots regeneration percentage were not markedly different among all nodal positions. Nevertheless, 2^{nd}, 3^{rd} and 4^{th} nodal positions showed better shoot multiplication than the 1^{st}, 5^{th} and 6^{th} node. In rooting stage, 3^{rd} nodal position showed good result in all parameter. Plantlets obtained from 2^{nd} and 4^{th} nodal positions also give the better results than 1^{st}, 5^{th} and 6^{th} node.

Therefore, the middle parts of the flower stalk (2^{nd}, 3^{rd} and 4^{th}) nodal positions should be used for commercial plantlets production. Among the three nodal positions, 2^{nd} node showed significant response in all parameter in multiplication stage. Therefore, 2^{nd} nodal position (counted from base to top) can be assumed as the suitable explant for micropropagation of orchid.

4.1.4 Hardening of plantlets from flower stalk node culture

In pre- hardening stage, leaf color of the plantlets gradual turned to dark green and healthy due to the receiving of semi and alternate sun light and air. After 7 weeks, plantlets were transferred to the outside condition. Hundred percent survival was observed in hardening stage after 2 months of transplanting from culture vessel to outside environment (Plate 13).

Pre-hardening plantlets on moss and charcoal in culture bottle

Hardening plantlets on moss and charcoal in culture bottle

Plate 13. Hardening stages of plantlets obtained from flower stalk node culture

4.2 Experiment 2. Effects of PGRs and Culture Systems on PLBs Induction and Proliferation of *In Vitro* Leaf Culture

4.2.1 Protocorm like bodies (PLBs) induction

Table 4 shows the effects of PGRs and culture systems on survival percent of PLBs induction from *in vitro* leaf culture. After 42 days' culture, the explants produced protocorm like bodies. These PLBs grow and became green within 70 days after culture. There were significant differences among the survival percentages of explants grown on different plant growth regulator treatments but non significantly different was observed among the culture systems treatments. PLBs formation percentage resulted from PGR and culture systems were not significantly different. In number of PLBs per explants, highly significant difference was observed among the PGRs treatments but not found in culture systems (Pr>F= 0.006). In all parameters, differences between culture systems were not found.

Among the PGRs treatments, maximium survival percent (61.4 %) was obtained from 1 $mg.L^{-1}$ TDZ and 0.5 $mg.L^{-1}$ NAA supplemented medium and minimum (34.4 %) observed from 4 $mg.L^{-1}$ BA and 0.5 $mg.L^{-1}$ NAA contained medium. In survival %, all TDZ treatments showed better survival % than BA treatments. It would be due to the activity of TDZ hormone. Guo et al. (2011) reported that survival mechanisms of plant tissues used for asexual reproduction are triggered by TDZ hormone.

Maximum PLBs formation percent (81.2 %) was resulted from the medium supplemented with 2 $mg.L^{-1}$ BA and 0.5 $mg.L^{-1}$ NAA but number of PLBs per explant (4.8) was obtained (Plate 14). This finding is similar with the experiment in *Phalaenopsis amabilis* (L.) BL. cv. 'Golden Horizon' and *Phalaenopsis amabilis* (L.) Bl. cv. Lovely done by Sinha and Jahan (2011) and Sinha et al. (2010), they found that BA-NAA combinations increased the rate of PLB induction and the optimum concentration of BA and NAA was 2.0 and 0.5 $mg.L^{-1}$ in which 80 % of PLBs formation percent and 15 PLBs per explant were observed in *Phalaenopsis amabilis* (L.) BL. cv. 'Golden Horizon' and 75 % PLBs formation and 10 PLBs per explant found in *Phalaenopsis amabilis* (L.) Bl. cv. Lovely. However, number of PLBs was different from this study. In this fact, genotypes may be the main factor that can vary regeneration capacity. PLB formation percent (76.6 %) and maximum 7.1 PLBs per explant were obtained from 2 $mg.L^{-1}$ BA contained medium. Effects of TDZ and BA

on the protocorms induction in two orchid species, *Phalaenopsis cornu-cervi* and *Phalaenopsis aphrodite* spp. Formosana were studied by Feng and Chen (2014) and Rittirat et al. (2014). They observed that TDZ is more effective than BA in PLB induction but the current study was against with those results. It may be due to the possible effects of different genotypes (Mohammad et al. 2014**).** Balilashaki and Ghehsareh (2016) also reported that BAP was more effective than TDZ and maximum number of PLB 50.65 obtained from 15 $mg.L^{-1}$ BAP and 3 $mg.L^{-1}$ NAA in *Phalaenopsis amabilis* var. 'Manila'.

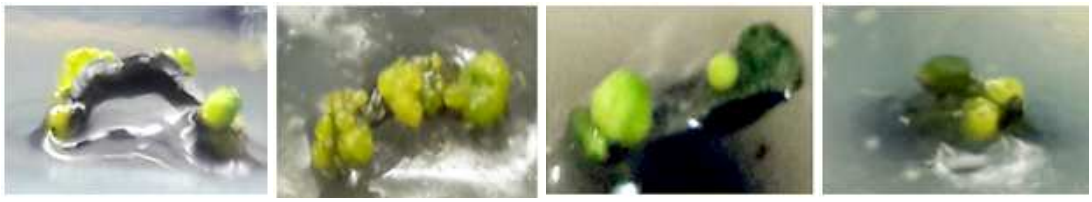
There was an interaction effect between plant growth regulators and culture systems in survival percent and number of PLBs per explants parameters (Table 4). It stated that the used of plant growth regulators can be varied according to the different culture systems. According to the finding of this study, TDZ was found as more effective than BA in solid culture while BA was more effective in cotton support liquid culture for PLBs formation. It can be assumed as not only the release effect of TDZ and BA but also the uptake nutrient effect of culture systems. Solid culture maintains nutrient uptake of explant because solid culture is the low release nutrient although TDZ is stable and it has the active release effect. Kuria et al. (2008) reported that solid media have lower ability to absorb the nutrients compared with liquid cultures. In cotton support liquid culture, there was the positive effect of BA due to the fast nutrient absorb of culture system and slow release of hormone. According to the interaction effect, there was no difference among the solid and cotton support liquid culture. However, cotton support liquid culture gave better result in PLBs formation than the solid culture for mass propagation. This finding was agreed with Park et al. (2002) who tested the solid, liquid ,cotton cultures and the maximum number of PLBs (20 per explant) were obtained from a single PLB in cotton culture.

Table 4. Effects of different PGRs and culture system on survival %, PLBs formation % and number of PLBs per explant in PLBs induction of leaf culture

Treatments	Survival %	PLBs formation %	Number of PLBs per explant
PGR ($mg.L^{-1}$)			
2.0 BA + 0.0 NAA	44.6 bcd	76.6 a	7.1 a
4.0 BA.+ 0.0 NAA	45.8 abcd	68.1 a	5.4 ab
2.0 BA.+ 0.5 NAA	41.6 cd	81.2 a	4.8 abc
4.0 BA + 0.5 NAA	34.4 d	65.0 ab	2.3 c
1.0 TDZ + 0.0 NAA	57.4 abc	37.4 b	2.6 c
2.0 TDZ + 0.0 NAA	49.0 abcd	58.4 ab	5.9 ab
1.0 TDZ + 0.5 NAA	61.4 a	57.9 ab	3.6 bc
2.0 TDZ + 0.5 NAA	59.3 ab	58.9 ab	2.3 c
LSD	16.5	28.9	2.6
Culture Systems (CS)			
Solid culture	51.5 a	61.7 a	4.6 a
Cotton culture	46.8 a	64.2 a	4.4 a
LSD	8.3	14.5	1.3
CV%	33.5	45.7	57.6
Pr>F			
PGRs	*	ns	**
CS	ns	ns	ns
PGRs * CS	*	ns	**

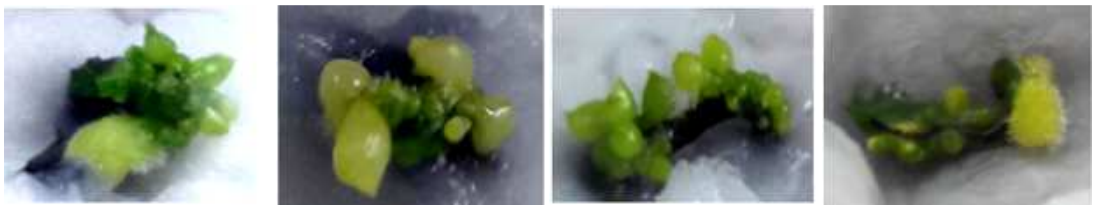
Means followed by the same letter in each column are not significantly different at 5% level. Data were collected from 6 weeks to 10 weeks after cultured.

ns: No significant ** Significant at 1% level * Significant at 5% level



BA 2 mg.L^{-1} | BA 4mg.L^{-1} | BA 2 mg.L^{-1}+ 0.5 mg.L^{-1} NAA | BA 4 mg.L^{-1}+ 0.5 mg.L^{-1} NAA

Solid MS basal medium supplemented with BA hormone



BA 2 mg.L^{-1} | BA 4mg.L^{-1} | BA 2 mg.L^{-1}+ 0.5 mg.L^{-1} NAA | BA 4 mg.L^{-1}+ 0.5 mg.L^{-1} NAA

Cotton MS basal medium supplemented with BA hormone

TDZ 1 mg.L^{-1} | TDZ 2 mg.L^{-1} | TDZ 1 mg.L^{-1}+ 0.5 mg.L^{-1} NAA | TDZ 2 mg.L^{-1}+ 0.5 mg.L^{-1} NAA

Solid MS basal medium supplemented with TDZ hormone

TDZ 1 mg.L^{-1} | TDZ 2 mg.L^{-1} | TDZ 1 mg.L^{-1}+ 0.5 mg.L^{-1} NAA | TDZ 2 mg.L^{-1}+ 0.5 mg.L^{-1}NAA

Cotton MS basal medium supplemented with TDZ hormone

Plate 14. PLBs induction from *in vitro* leaf culture in different treatments 8 weeks after culture

4.2.2 PLB proliferation of PLB developed from leaf culture

Table 5 shows the effect of three culture systems (solid, agitated liquid and cotton support liquid culture) on survival % of explants, PLBs formation percent and number of PLBs per explant in PLBs proliferation. Highly significant difference was observed in survival percent of cultured PLBs among the culture systems. Maximum survival percent (72.9 %) was obtained from cotton support liquid medium. PLBs formation percent and number of PLBs per explants were not statistically different among the treatments but maximum PLB formation 100 % and 10.8 PLBs per explants were resulted from the explants cultured on the cotton support medium. A hundred percent PLBs formation was observed in solid and cotton support culture medium. Number of PLBs per explants 9.5 was observed in solid culture while liquid agitate culture gave 8.1 PLBs per explants.

The result in this study was supported by the finding of Myint et al. (2001) who stated that cotton culture gives the better result in PLBs regeneration than solid culture. Liquid medium supporting with cotton affords better aeration to explant, limit the exposure to water and nutrient, and reduce the effect of phenol oxidation of explant. Most of PLBs resulted from solid culture showed pale in yellow color than the other culture systems in this research (Plate 15). Kuria et al. 2008 reported that lower development rate of plantlets and lower uptakes of nutrients were observed in solid media compared to liquid media. Most of the proliferated PLBs in liquid culture were found as suffering from hyper hydricity and more phenol exudation. Vyas et al. 2008 reported that explant cultured directly on liquid medium caused hyper-hydricity of tissue, rapid spread of contaminants, and plantlets asphyxiation because of the lack of agar. Therefore, it was observed that liquid culture system needs more frequent subculture than the solid and cotton support culture. All parameters of PLBs proliferation from leaf explants cultured on cotton support medium was better than those from the solid culture (Plate 15). Explants in solid medium directly expose to the phenol than the cotton culture systems. Therefore, cotton support liquid culture can be assumed as the suitable culture system for PLBs proliferation.

Table 5. Effects of culture system on survival %, PLBs formation % and number of PLBs per explant in PLB proliferation of leaf culture

Culture Systems	Survival %	PLBs formation %	Number of PLBs per explant
Agitated Liquid Culture	38.3 b	97.5	8.1
Solid Culture	34.6 b	100.0	9.5
Cotton Support Liquid Culture	72.9 a	100.0	10.8
LSD	1.4	0.2	4.7
CV%	12.7	1.5	30.9
Pr> F	**	ns	ns

Means followed by the same letter in each column are not significantly different at 5% level. Data were collected from 2 weeks to 10 weeks after cultured.

ns: No significant ** Significant at 1% level

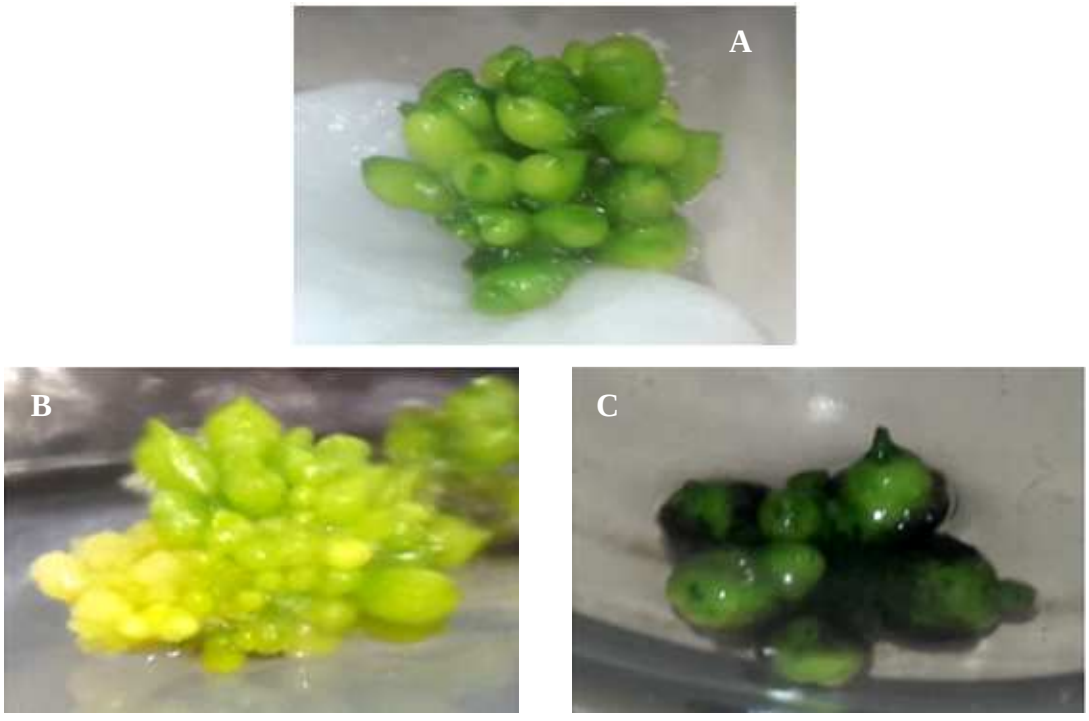


Plate 15. PLBs proliferation from developed PLBs 8 weeks after culture (A) PLB multiplication on liquid medium supported with cotton (B) PLB multiplication on solid medium (C) PLB multiplication on liquid medium

4.2.3 Root formation on shoot developed from PLB

The effects of MS and VW media with and without PGRs on rooting of shoots developed from PLBs derived leaf culture were studied in this stage. Table 6 and plate 16 shows the results of different media on survival percentage, root formation percentage and number of roots per explant. Interaction effect between plant growth regulator and culture media were not observed in all parameters. Highly significant differences were observed in all parameters among the media treatments. Maximum survival 98.2 % was observed in VW medium and minimum 59.2 % survival in MS media. In root formation percentage, modified VW medium was significantly higher than MS medium. Maximum root formation 49.9 % and 3.2 roots per explant were observed from VW medium while minimum 11 % and 1.1 roots per explant from MS medium. The results observed in all parameters of root induction on shoot developed from PLB showed that VW medium was better than MS (Plate 16). Similar result was reported by Myint et al. (2001). It was found that VW medium was better than the Hyponex (H), New Docashima Medium (NDM) media for rooting of *Phalaenopsis* species. In this study, it was observed that shoot developed from PLB cultured on the MS medium produced PLBs at the basal of the shoot and plant performance is not good. Shoot from VW medium showed good plant performance and root formation but PLBs were not observed (Plate 16 . A and B). This observation is against with the Prasongsom et al. (2016). They studied the effect of various medium on the shoot tip culture of *Rhynchostylis gigantea* (Lindl.) Ridl. (amethyst-purple), a rare Thai orchid specie. In this observation, modified VW medium did not produce plantlets, but induced PLB formation in root induction stage. It can be assumed that the nutrient requirements for orchid cultures that can be vary depend on the different among species (Kauth et al. 2008). Another factor influence to form adult plant is the chemical composition. VW medium used in this experiment is not contained ammonium nitrate and and MS medium contained high amount of ammonium nitrate and potassium phosphate. Kosir et al. 2004 reported that short and small shoot without root were observed on medium containing highest concentration of ammonium nitrate and potassium phosphate and adult plant were occurred in medium containing the lack of ammonium nitrate and the lower amounts of iron**.**

Table 6. Effects of media on survival %, root formation % and number of roots per explant in root regeneration of shoot developed from PLB derived leaf culture

Treatments	Survival %	Root formation %	Number of roots per explant
PGR ($mg.L^{-1}$)			
Free PGRs	79.6	27.7	2.2
0.1 BA + 1 NAA	77.7	33.3	2.2
LSD	16.6	15.4	0.9
Media (M)			
VW	98.2 a	49.9 a	3.2 a
MS	59.2 b	11.0 b	1.1 b
LSD	16.6	15.4	0.9
CV%	15.8	37.9	38.6
Pr ≥ F			
PGR	ns	ns	ns
M	**	**	**
PGR*M	ns	ns	ns

Means followed by the same letter in each column are not significantly different at 5% level. Data were collected at 17 weeks after cultured.

ns: No significant ** Significant at 1% level

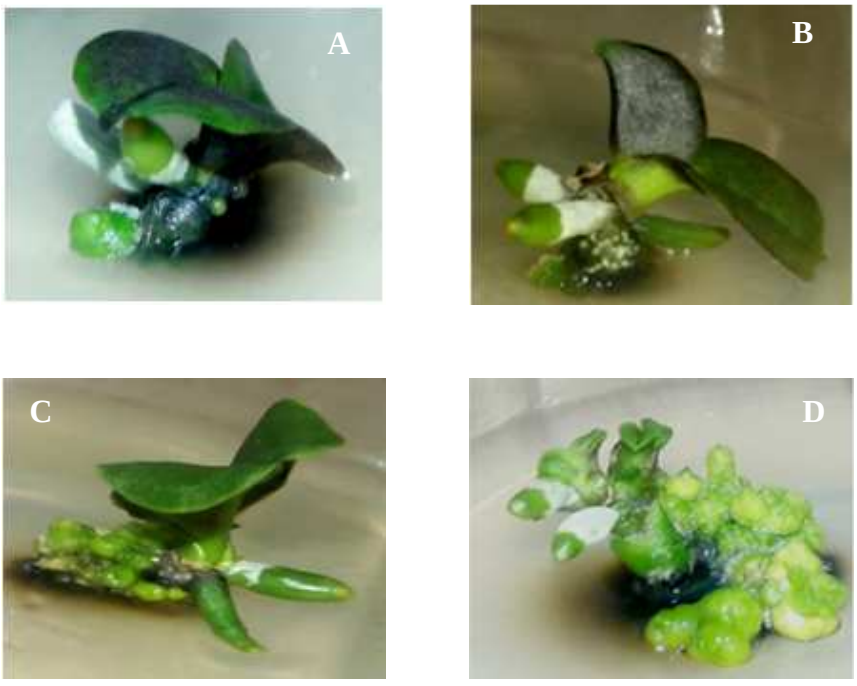


Plate 16. Root regeneration of shoot developed from proliferated PLBs (A) VW medium without PGRs (B) VW medium with PGRs (C) MS medium without PGRs (D) MS medium with PGRs

CHAPTER VI
CONCLUSION

Among the responses of different nodal positions of flower stalk node on shoot induction of *Phalaenopsis amabilis* in *in vitro* culture, 2nd, 4th and 5th nodal positions gave maximum survival percent and 5th nodal gave the maximum number of shoot per node explant that were cultured on the MS basal medium supplemented with 2 $mg.L^{-1}$ BA. However, 2nd nodal position gave highest frequency of multiplication in term of maximum number of shoots per node explants followed by 4th and 3rd than the other nodal positions in shoot to shoot multiplication. In rooting stage, 3rd nodal position showed better root formation performances than other treatments followed by 4th and 2nd node. In hardening stage, 100 % survival was observed from these middle positions of flower stalk. 1st node at the base, last node (just below the flower) should not be used as material in *in vitro* propagation. Therefore, it can be concluded that middle portions of flower stalk node are suitable for production of *Phalaenopsis amabilis* by using tissue culture.

In the effect of different concentration and combination of plant growth regulators and two culture systems on PLBs induction of *in vitro* leaf culture, 2 $mg.L^{-1}$ BA supplemented medium gave maximum number of PLBs from leaf explants. Interaction effect was also observed among the PGRs and culture systems. Cotton support liquid culture medium supplemented with 2 $mg.L^{-1}$ BA gave maximum PLBs formation percent and number of PLBs per explants. In solid culture, 2 $mg.L^{-1}$ TDZ showed maximum survival percent and number of PLBs per explants in PLBs induction. For PLB multiplication, cotton support liquid medium gave maximum survival percent, PLBs formation percent and number of PLBs per explants. Cotton support liquid medium was found as the most efficient medium for both PLB induction and multiplication. In rooting stage, shoots developed from PLBs cultured on the modified VW medium gave better result in root induction and plant performance.

Therefore, cotton support liquid medium is recommendable for PLB induction and multiplication in leaf tissue culture of *Phalaenopsis amabilis in vitro* cloning. PGRs should be chosen based on the different culture systems. TDZ hormone is suitable as the growth promoter in the solid culture medium while BA hormone is advisable in the cotton support liquid culture medium. Modified VW medium should

be used as the rooting medium for shoots developed from PLBs in *Phalaenopsis amabilis* orchid.

In this study, higher multiplication rate through PLBs was observed in leaf culture compared with shoot multiplication through node culture (Table 7). It was noticed that, plantlets obtained from flower stalk node culture were bigger in size and looks healthy. The plantlets obtained from leaf culture were small and need more time to transfer *ex vitro* condition. Therefore, it can be concluded that, flower stalk nodal culture should be used to obtain the healthy plantlets within the short period. Leaf culture should be used to obtain huge number of plantlets (Plate 17).

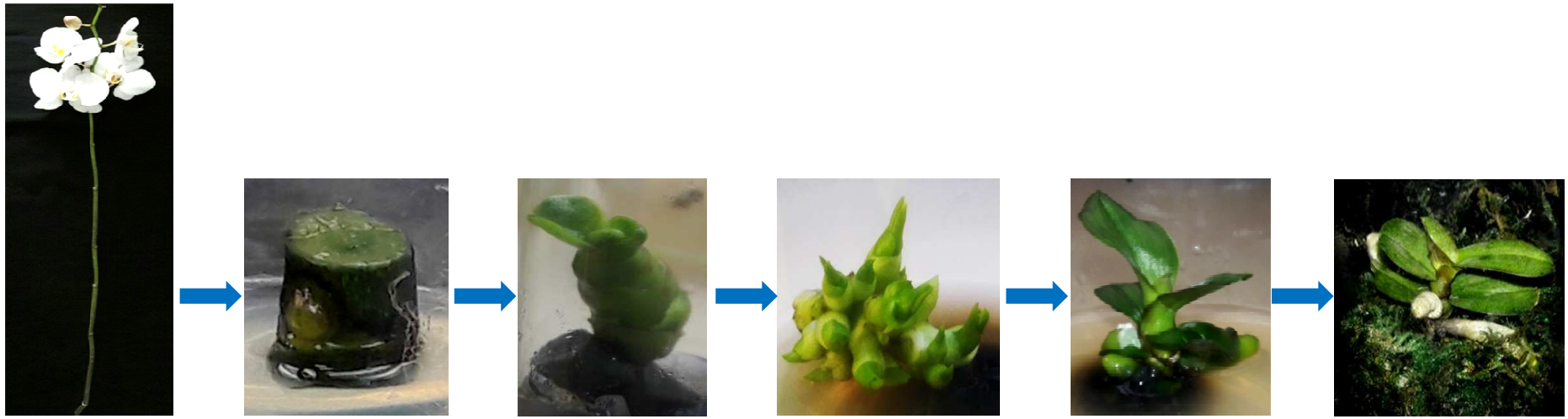


Plate 17. Protocol for regeneration of flower stalk node culture and *in vitro* leaf culture in *Phalaenopsis amabilis*

Table 7. Comparison between node and leaf culture in terms of time required to obtain plantlet and multiplication efficiency

Stages	**Flower Stalk Node Culture**		**Stages**	***In Vitro* Leaf Culture**	
	Time requirement	Multiplication efficiency		Time requirement	Multiplication efficiency
Initial stage	11 weeks	2 shoots per node explant	Initial stage	10 weeks	7 PLBs per explant
Multiplication stage	10 weeks	8 shoots per explant	Multiplication stage	11 weeks	10 PLBs per explant
Rooting stage	23 weeks	-	Rooting stage	20 weeks	-
Pre-hardening stage	7 weeks	-	Pre-hardening stage	-	-
Hardening	8 weeks	-	Hardening	-	-
Total	59 weeks (15 months)		Total		

REFERENCES

Alvard, D., F. Cote, and C. Tiesson. 1992. Comparison of methods of liquid medium culture for banana micropropagation. Plant Cell Tissue Organ Culture 32: 55-60.

Anjum, S., M. Zia, and M. F. Chaudhary. 2006. Investigations of different strategies for high frequency regeneration of *Dendrobium malones* 'Victory'. African Journal of Biotechnology 5 (19): 1738-1743.

Arditti, J. and R. Ernst. 1993. Micropropagation of Orchids (Jhon Wiley and Sons, New York). 467-520.

Asghar, S., T. Ahmad, I. A. Hafiz and M. Yaseen. 2011. *In vitro* propagation of orchid (*Dendrobium nobile*) var.Emma white. African Journal of Biotechnology 10 (16): 3097-3103.

Asthana, P., V. S. Jaiswal, and U. Jaiswal. 2011. Micropropagation of *Sapindus trifoliatus* L. and assessment of genetic fidelity of micropropagated plants using RAPD analysis. Acta Physiology Plant 33:1821–1829.

Badji, S., Y. Mairone, G. Merlin, P. Danthu, P. Neville, and J. P. Collonna. 1993. *In vitro* propagation of the gum Arabic tree (*Acacia senegal* (L.) Willd):Developing a rapid method for producing plants. Plant Cell Report 12: 629–633.

Balilashaki, K. and M. G. Ghehsareh. 2016. Micropropagation of *Phalaenopsis amabilis* var. 'Manila' by leaves obtained from *in vitro* culturing the nodes of flower stalks. University of Guilan. Department of Horticultural Science, Iran. Notulae Scientia Biologicae 8 (2):164-169.

Balilashaki, K., M. Vahedi, and R. Karimi. 2015. *In vitro* direct regeneration from node and leaf explants of *Phalaenopsis* cv. 'Surabaya'. University of Guilan. Department of Horticultural Science, Iran. Plant Tissue Culture and Biotechnology 25 (2): 193-205.

Balilashaki, K., R. Naderi, S. Kalantari, and M. Vahedi. 2014. Efficient *in vitro* Culture Protocols for Propagating *Phalaenopsis* 'Cool Breeze**'.** University of Tehran, Department of Horticultural Science, College of Agriculture and Natural Resources, Tehran, Iran. Plant Tissue Culture and Biotechnology 25 (2): 191-203.

Bapat, V. A. and S. Narayanaswamy. 1977. Rhizogenesis in a tissue culture of the orchid *Spathoglottis*. Torrey Botanical Club 104: 2-4.

Chang, C. 2015. Tissue culture and progeny selection of *Tolumnia* orchid. Master thesis.

Chen, J. T. and W. C. Chang. 2001. Effects of auxins and cytokinins on direct somatic embryogenesis on leaf explants of *Oncidium*'Gower Ramsey'. Plant Growth Regulators 34: 229-232.

Chen, J. T. and W. C. Chang. 2004 . Induction of repetitive embryogenesis from seed-derived protocorms of *Phalaenopsis amabilis var. Formosa Shimadzu*. *In Vitro* Cellular and Developmental Biology Plant 40: 290-293.

Chen, J. T. and W. C. Chang. 2006. Direct somatic embryogenesis and plant regeneration from leaf explants of *Phalaenopsis amabilis*. Journal of Biologia Plantarum 50 (2):169-173.

Chiba, M. 2002. *Phalaenopsis* species (In Japanese) Chiba Masaaki *Phalaenopsis Species*. Publishing Society, Saitama.

Christenson, E. A. 2001. *Phalaenopsis*: A Monograph. Timber Press, Portland.

David, R. and M. Bala. 2012. Preliminary results on the influence of growth hormones on the *in vitro* regeneration of *Phalaenopsis* flower stalks. Journal of Horticulture, Forestry and Biotechnology 16 (4): 24-27.

Devi, H. S., S. I. Devi and **T. D. Singh. 2013**. High frequency plant regeneration system of *Aerides odorata* Lour. Through foliar and shoot tip culture. Notulae Botanicae Horti Agrobotanici 41(1): 169 - 176.

Dodds, J. H. 1994. *In vitro* Methods for Conservation of Plant Genetic Resources. Chapman and Hall, London. In Fay, M. E (Eds). 4-5.

Feng, J. H. and J. T. Chen. 2014. A novel *in vitro* protocol for inducing direct somatic embryogenesis in *Phalaenopsis aphrodite* without taking explants. The Scientific World Journal 1-7.

George, E. F. 1993. Plant propagation by tissue culture, part I: the technology. Exegetics, Edington 1–574.

George, E. F. and P. C. Debergh. 2008. Micropropagation: uses and methods. plant propagation by tissue culture. 3rd Edition. In: George, E. F., Hall, M. A., De Klerk G-J (eds). 1.

Gow, W. P. 2005. Factors affecting direct somatic embryogenesis of *Phalaenopsis* leaf explants. Master thesis, Graduate Institute of Horticulture, National Taiwan University, Taiwan 1-79.

Griesbach, R. J. 1983. The use of indoleacetylamino acids in the *in vitro* propagation of *Phalaenopsis* orchids. Scientific Horticulture 19: 363-366.

Griesbach, R. J. 2002. Develpoment of *Phalaenopsis* Orchids for the Mass-Market. In: Janick, J. and Whipkey, A. (eds). Trends in new crops and new uses. American Society for Horticultural Science. USA 458-465.

Guo B., B. H. Abbasi, A. Zeb, L. L. X and Y. H. Wei. 2011. Thidiazuron: A multi-dimensional plant growth regulator. African Journal of Biotechnology 10(45), 8984-900.

Hsu, C. C. and F. C. Chen. 2003. Plant regeneration from protocorm-like-bodies induced in etiolated leaves of *Phalaenopsis Aphrodite* Rchb. f. *J.* Chin. Society for Horticultural Science. 49: 335-342.

Huan, L. V. T., T. Takamura and M. Tanaka. 2004. Callus formation and plant regeneration from callus through somatic embryo structures in *Cymbidium* orchid. Plant Science 166: 1443-9.

Hussien, F. A., M. A. Osman and T. I. M. Idris. 2014. The Influence of Liquid Media Support, Gelling Agents and Liquid overlays on performance of *in vitro* Cultures of Ginger (*Zingiber officinale*). International Journal of Scientific and Research Publications 4(10): 2250-3153.

Jitsopakula, N., K. Thammasirib and K. Ishikawa. 2013. Efficient adventitious shoot regeneration from shoot tip culture of *Vanda coerulea*, a Thai orchid. Journal of Science Asia 39 (2013): 449–455.

Julkiflee, A. L., J. Uddain, and S. Sreeramanan. 2014. Efficient micropropagation of *Dendrobium* sonia-28 for rapid PLBs proliferation. Emirates Journal of Food and Agriculture 26 (6): 545-551.

Kano, K. 1965. Studies on the media for orchid seed germination. Kagawa Mem Fac Agric Universty 20: 1–68.

Kaur, S. and K. K. Bhutani. 2009. *In vitro* propagation of *Vanda testacea* (Lindl.) Reichb .f. a rare orchid of high medicinal value. Plant Tissue Culture and Biotechnology 19 (1): 1-7.

Kauth, P. J., D. Dutra, T. R. Johnson, S. L. Stewart, W. Vendrame, and M. E. Kane. 2008. Technique and Applications of *In Vitro* Orchid Seed Germination. 1st Edition. Isleworth, Global Science Books, UK 375-91.

Khatun, M. M., H. Khatun, D. Khanam, and M. Al-Amin. 2010. *In vitro root* formation and plantlet development in *Dendrobium* orchid. Bangladesh Journal of Agricultural Research 35 (2): 257-265.

Knudson, L. 1946. A new nutrient solution for the germination of orchid seed. American Orchid Society Bull 14: 214–217

Kosir, P., S. Skof and Z. Luthar. 2004. Direct shoot regeneration from nodes of *Phalaenopsis* orchids. Acta Agriculturae Slovenica 83 – 2.

Kuo, H. L., J. T. Chen and W. C. Chang. 2005. Efficient plant regeneration through direct somatic embryogenesis from leaf explants of *Phalaenopsis* 'Little Steve'. *In Vitro* Cellular and Developmental Biology Plant 41: 453-6.

Kuria, P., P. Demo, A. Nyende, and E. Kahangi. 2008. Cassava starch as an alternative cheap gelling agent for the *in vitro* micro-propagation of potato (*Solanum tuberosum* L.). African Journal of Biotechnology 7 (3): 301-307.

Liao, Y. J., Y. C. Tsai, Y.W. Sun, R. S. Lin, and F. S. Wu. 2011. *In vitro* shoot induction and plant regeneration from flower buds in *Paphiopedilum* orchids. *In Vitro* Cellular and Developmental Biology Plant 47: 702–709.

Luo, J. P., X. Q. Zha and S. T. Jiang. 2003. Suspension culture of protocorm-like bodies from the endangered medicinal plant *Dendrobium huoshanenese*. China Journal of Chinese Materia Medica 28, 611–614.

Madigan, M. and J. Martinko. 2005. Brock Biology of Microorganisms. 11th ed. Prentice Hall.

Mallikarjuna, K. and G. Rajendurdu. 2007. High frequency *in vitro* propagation of *Holarrhena antidysentrica* from nodal buds of mature tree. Biology Plant 51:525–529.

Mayer, J. L. S., G. C. Stancato and A. D. G. R. Beatriz. 2010. Direct regeneration of protocorm-like bodies (PLBs) from leaf apices of *Oncidium flexuosum* Sims (Orchidaceae). Plant Cell Tissue Organ Culture. DOI 10.1007/s11240-010-9782-9

Moe, T. 2014. Effects of different explants and plant growth regulators on *in vitro* regeneration of a medicinal orchid *Dendrobium officinale*. Master thesis, Yezin Agricultural University, Yezin.

Mohammad M. A., A. Yadollahi, A. Shojaeiyan, S. Shokri and S. M., Ghojah. 2014. Effects of nutrient media, different cytokinin types and their concentrations on *in vitro* multiplication of G × N15 (hybrid of almond × peach) vegetative rootstock. Journal of Genetic Engineering and Biotechnology 12(2), 81-87.

Mohanty, P., M.C. Das, S. Kumaria and P. Tandon. 2012. High efficiency cryopreservation of the medicinal orchid *Dendrobium nobile* Lindl. Plant Cell Tissue Organ Culture 109:297-305.

Murthy, B. N. S., S. J. Murch and P. K. Saxena. 1988. Thidiazuron: a potent regulator of *in vitro* plant morphogenesis. *In Vitro* Cellular and Developmental Biology Plant 34:267-272

Myint, K. T., C. K. Kim, M. Y. Chung, J. S. Park and J. D. Chung. 2006. Direct induction of multiple shoots from stem tissue of *Phalaenopsis* hybrids. Flower Research Journal 14(2): 69-74.

Myint, K. T., M. Y. Chaung, J. D. Chung, and C. K. Kim. 2001. Propagation via *In vitro* culture of leaf tissue of *Phalaenopsis* seedlings. Journal of Korean Society for Horticultural Science 42(1): 1-5.

Niknejad, A., M. A. Kadir, and **S. B. Kadzimin. 2013.** *In vitro* plant regeneration from protocorms-like bodies (PLBs) and callus of *Phalaenopsis gigantea* (Epidendroideae: Orchidaceae). African Journal of Biotechnology 10 (56): 11808-11816.

Nongdam, P and L. Tikendra. 2014. Establishment of an efficient *in vitro* regeneration protocol for rapid and mass propagation of *Dendrobium chrysotoxum* Lindl. using seed culture. Scientific World Journal http://dx.doi.org/10.1155/2014/740150.

Nongdam, P. and N. Chongtham. 2011. *In vitro* rapid propagation of *Cymbidium aloifolium* (L.) Sw. A medicinally important orchid via seed culture. Journal of Biological Science 11 (3): 254-260.

North, J. J., P. A. Ndakidemi, and C. P. Laubscher. 2012. Effects of various medium compositions and wounding treatments on *in vitro* growth and regeneration of bird of paradise (*Strelitzia reginae*). Scientific Research and Essays *7* (10): 1118-1133.

North, J.J., P. A. Ndakidemi, and C. P. Laubscher. 2010. The potential of developing an *in vitro* method for propagating Strelitziaceae. African Journal of Biotechnology 9(45): 7583-7588.

Paek, K. Y. and E. C. Yeung. 1991. The effect of 1-naphthalene aceticacid and N6-benzyladenine on the growth of *Cymbidium forrestii* rhizomes *in vitro.* Plant Cell Tissue Organ Culture 24: 65-71.

Panwar, D., K. Ram and H. N. S Shekhawat. 2012. *In vitro* propagation of *Eulophianuda Lindl*., an endangered orchid. Scientia Horticulturae (Amsterdam) 139: 46-52.

Park, S.Y., H. N. Murthy and K.Y. Paek. 2002. Rapid propagation of *Phalaenopsis* from floral stalk-derived leaves. *In Vitro* Cellular and Developmental Biology Plant 38: 168-172.

Prasongsom, S., K. Thammasiri, and N. Chuenboonngarm. 2016. Efficient adventitious shoot regeneration from shoot tip culture of *Rhynchostylis gigantean* (Lindl.) Ridl. (amethyst-purple), a rare thai orchid species. Thailand. Walailak Journal of Science and Technology 13 (9): 757-767.

Rittirat, S., S. Klaocheed and K. Thammasiri. 2014. Enhanced Efficiency for Propagation of *Phalaenopsis cornu-cervi* (Breda) Blume & Rchb. F. Using Trimmed Leaf Technique,World Academy of Science, Engineering and Technology International Journal of Biological, Biomolecular, Agricultural, Food and Biotechnological Engineering 8-4.

Rotor, G. B. 1949. A method of vegetative propagation of *Phalaenopsis* stem cuttings. American Orchid Society Bulletin 18:738–739.

Roy, J. and N. Banerjee. 2003. Induction of callus and plant regeneration from shoot tip explants of *Dendrobium fimbriatum* Lindl Var. oculatum Hk. f. Scientific Horticulture (Amsterdam); 97:333-40

Sinha, P. and M. A. A. Jahan. 2011. Clonal propagation of *Phalaenopsis amabilis* (L.) BL. cv. 'Golden Horizon' through *In vitro* culture of leaf segments. Bangladesh Journal of Scientific and Industrial Research 46 (2):163-68.

Sinha, P., M. A. A. Jahan, J. L. Munshi, and R. Khatun. 2010. High frequency regeneration of *Phalaenopsis amabilis* (L.) Bl. cv. Lovely through *In*

vitro culture. Biological Research Division, BCSIR Laboratories, Bangladesh. Plant Tissue Culture and Biotechnology 20 (2): 185-193.

Sreeramanan S., B. Vinod, S. Sashi, and R. Xavier. 2008. Optimization of the transient-gusa gene transfer of *Phalaenopsis violacea* orchid via *Agrobacterium tumefaciens*: an assessment of factors influencing the efficiency of gene transfer mechanisms. Advances in Natural and Applied Sciences 2: 77-88.

Sreeramanan S., R. Xavier, P. Ranjetta and S. Umarani. 2009. Establishment of *in vitro Phalaenopsis Violacea.* plant cultures from flower-stalk cuttings. School of Biological Sciences, University Sains Malaysia (USM). Advances in Natural and Applied Sciences 3 (3): 432-437.

Sujjaritthurakarn, P. and K. Kanchanapoom. 2011. Efficient direct protocorm-like bodies induction of dwarf *Dendrobium* using thidiazuron**.** Prince of Songkla University, Department of Biology, Thailand. Notulae Scentiai Biologicae 3 (4): 88-92.

Tanaka, M., A. Hasegawa, and M. Goi. 1975. Studies on the clonal propagation of monopodial orchids by tissue culture formation of protocorm like bodies from leaf tissue in *Phalaenopsis* and *Vanda*. Journal of Japanense Society for Horticultural Science 44: 47-58.

Tanaka, M., M. Kumura, and M. Goi. 1988. Optimal conditions for shoot production from *Phalaenopsis* flower stalk cuttings cultured *in vitro*. Scientic Horticulture 35: 117-126.

Tisserat, B. and D. Jones. 1999. Clonal Propagation of Orchids. In: Hall, R.D. (Ed.): Plant Cell Culture Protocols. Methods in Molecular Biology, 111. Humana Press Inc., Totowa, NJ, USA 127-134.

Tokuhara, K. and M. Mii. 1993. Micropropagation of *Phalaenopsis* and *Doritaenopsis* by culturing shoot tips of flower stalk buds. Plant Cell Report 13: 7-11.

Tokuhara, K. and M. Mii. 2003. Highly efficient somatic embryogenesis from cell suspension cultures of *Phalaenopsis* orchids by adjusting carbohydrate sources. *In Vitro* Cell Developmental Biology Plant 39: 635-639.

Tsao, C. Y., U. C. Chen, and C. N. Hsia. 2011. Explant types derived from flower stalk culture and 6- benzyladenine concentration affects shoot

differentiation of *Phalaenopsis* hybrid in subculture. Journal of Taiwan Society Horticulture Science 57(1): 31-42.

Tsao, C. Y., U. C. Chen, M. T. Wu, and C. N. Hsia. 2008. Effect of flower stalk stage, nodal bud position and 6-benzyladenine concentration on the shoot induction in micropropagation of *Phalaenopsis* flower stalk node explant. Journal of Taiwan Society Horticulture Science 54 (3): 199-209.

Vacin, E. and F. W. Went. 1949. Some pH changes in nutrient solutions. Botanical Gazette 110: 605–613.

Vyas, S., M. S. RAO, R. K. Suthar and S. D. Purohit. 2008. Liquid culture system stimulates *in vitro* growth and shoot multiplication in four medicinally important plants. Plant Biotechnology Laboratory, Department of Botany, Mohanlal Sukhadia University, India.

Wagner A. Vendrame and I. Maguire. 2007. *In vitro* Propagation and Plantlet Regeneration from Doritaenopsis Purple Gem 'Ching Hua' Flower Explants. Tropical Research and Education Center, University of Florida, Horticulture Science 42 (5): 1256–1258.

Welsh, J. and K. C. Sink. 1981. Morphogenic responses of *Browallia* leaf sections and callus. Annals of Botany 48: 583-590.

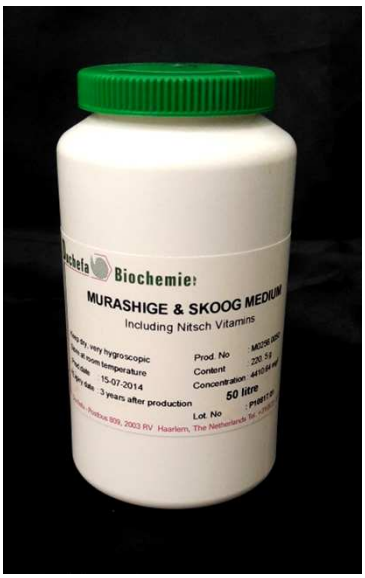
Wu, H. H. and F. C. Chen. 2008. Effect of plant growth regulators on shoot multiplication from flower stalk nodal buds of *Phalaenopsis* and *Doritaenopsis*. Journal of Taiwan Society Horticultural Science 54 (2): 151-159.

Yam, T. W. and J. Arditti. 2009. History of orchid propagation: a mirror of the history of biotechnology. Plant Biotechnology Rep 3: 1–56.

Zhao, P., F. Wu, F. S. Feng, and W. J. Wang. 2008. Protocorm like bodies PLBs formation and plant regeneration from their callus of *Dendrobium candidum wall* ex Lindl. *In Vitro* Cell Developmental Biology 44: 178-185.

APPENDICES

Appendix 1. MS powder, Plant growth regulators, Agar



MS powder

Agar

Plant growth regulators

Appendix 2. Chemical Composition of Vecin and Went (VW) Medium Used in Rooting on *In Vitro* Young Leaf of *Phalaenopsis amabilis*

Chemical	Concentration in medium	Concentration stock solution (1 liter)
NH_4SO_4	500.00 mg	50.00 g (100×)
KNO_3	500.00 mg	50.00 g (100×)
$MgSO_4$	250.00 mg	25.00 g (100×)
KH_2PO_4	250.00 mg	25.00 g (100×)
$FeSO_4$	27.80 mg	2.78 g (100×)
$MnSO_4$	7.50 mg	1.50 g (200×)
$Ca_3(PO_4)^3$	200.00 mg	40.00 g (200×)
Thamine HCL	0.40 mg	0.08 g (200×)

Appendix 3. Chemical Composition of Murashige & Skoog (MS) Medium Used in Rooting on *In Vitro* Young Leaf of *Phalaenopsis amabilis*

Chemical	Concentration in medium (1 liter)	Concentration stock solution (1 liter)
NH_4NO_3	1.650 g	165.00 g (100×)
KNO_3	1.900 g	190.00 g (100×)
$CaCl_2.2H_2O$	0.440 g	44.00 g (100×)
$MgSO_4.7H_2O$	0.370 g	37.00 g (100×)
KH_2PO_4	0.170 g	17.00 g (100×)
$FeSO_4.7H_2O$	27.880 mg	2.78 g (100×)
Na_2EDTA	37.300 mg	3.73g (100×)
H_3BO_4	6.200 mg	1240.00 mg (200×)
$MnSO_4.4H_2O$	22.300 mg	4460.00 mg (200×)
$ZnSO_4.4H_2O$	8.600 mg	1720.00 mg (200×)
KI	0.830 mg	166.00 mg (200×)
$NaMoO_4.2H_2O$	0.250 mg	50.00 mg (200×)
$CuSO_4.5H_2O$	0.025 mg	5.00 mg (200×)
$CoCl.6H_2O$	0.025 mg	5.00 mg (200×)
Nicotinic acid	0.500 mg	100.00 mg (200×)
Pyridoxin HCL	0.500 mg	100.00 mg (200×)
Thamine HCL	0.100 mg	20.00 mg (200×)
Glycine	2.000 mg	400.00 mg (200×)
I-inositol		100.00 mg