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Full Length Research Paper

In vitro plant regeneration from protocorms-like bodies (PLBs) and callus of *Phalaenopsis gigantea* (Epidendroideae: Orchidaceae)

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***Phalaenopsis*, with long arching sprays of flowers, are among the most beautiful flowers in the world. *Phalaenopsis* is an important genus and one of the most popular epiphytic monopodial orchids, grown commercially for the production of cut flowers and potted plants. Most of them have different and interesting morphological characteristics which have different value to the breeders. *Phalaenopsis gigantea* is one of the most difficult to grow and has the potential of producing beautiful hybrids. An efficient and reproducible method for large-scale propagation of *Ph. gigantea* using leaf sections has been developed. Leaf sections from *in vitro* young plants were cultured on New Dogashima Medium (NDM) supplemented with cytokinins (6-Benzylaminopurine (BAP), Thidiazuron (TDZ), and Kinetin (KIN), each at 0.01, 0.1, 0.5 and 1.0 mg/L) alone and in combinations with (auxins a-naphthaleneacetic acid (NAA), at 0.01, 0.1, 0.5 and 1.0 mg/L). The explants developed calli and protocorm-like-bodies (PLBs) within 6 weeks of culture. Treatment TDZ in combination with auxins was found to be the best for the induction of callus and PLBs. *In vitro* regeneration of *Ph. gigantea* PLB was achieved by exposure to light and transferring to hormone free NDM solid medium.**

Key words: ***Phalaenopsis***, PLBs, new Dogashima medium, regeneration.

INTRODUCTION

Tissue culture techniques have contributed to large scale production of plants of economic importance such as orchids and other ornamentals. The culture of meristems and other tissue culture techniques have produced plants that are virus free. The techniqnes are based on the principle of totipotency (Purohit, 2005). In orchids, the techniques have been widely used for mass production (Griesbach, 2002) of plants serving as an ***ex situ*** conservation strategy of species (Decruse et al., 2003). Many protocols have been developed including ***Cymbidium***, ***Vanda***, ***Phaphiopedilum*** and ***Phalaenopsis*** (Arditti and Ernst, 1993). Protocols for ***Phalaenopsis*** utilizing flower stalk buds (Tokuhara and Mii, 2001) entire shoots, shoot tips, stem nodes (Griesbach, 2002), leaf tissues/segment (Park et al., 2002) or root tips culture (Park et al., 2003) have been reported.

Moreover, these methods have been reported to be very difficult and inefficient (Chen et al., 2000; Murdad et al., 2006). ***Phalaenopsis*** species have been used in numerous breeding programs by both amateur and professional horticulturists (Huang et al., 2004). However, conventional breeding is slow and difficult as it requires 2 to 3 years to complete a life cycle. ***Phalaenopsis*** has a large genome size of 38 chromosomes with different lengths for different species, making cultivar improvement among commercial varieties difficult due to sexual incompatibility. Several sexual hybridizations are often required to improve even just only for one trait (Portia et al., 2005). ***Phalaenopsis gigantea*** is the largest species among the ***Phalaenopsis*** genus that is known as the

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Abbreviations: BAP, 6-Benzylaminopurine; **CW,** Coconut Water; **KIN,** Kinetin; **MS,** Murashige and Skoog; **NAA,** naphthalene Acetic Acid; **NDM,** New Dogashima Medium; **VW,** Vacin and Went; **TDZ,** Thidiazuran.

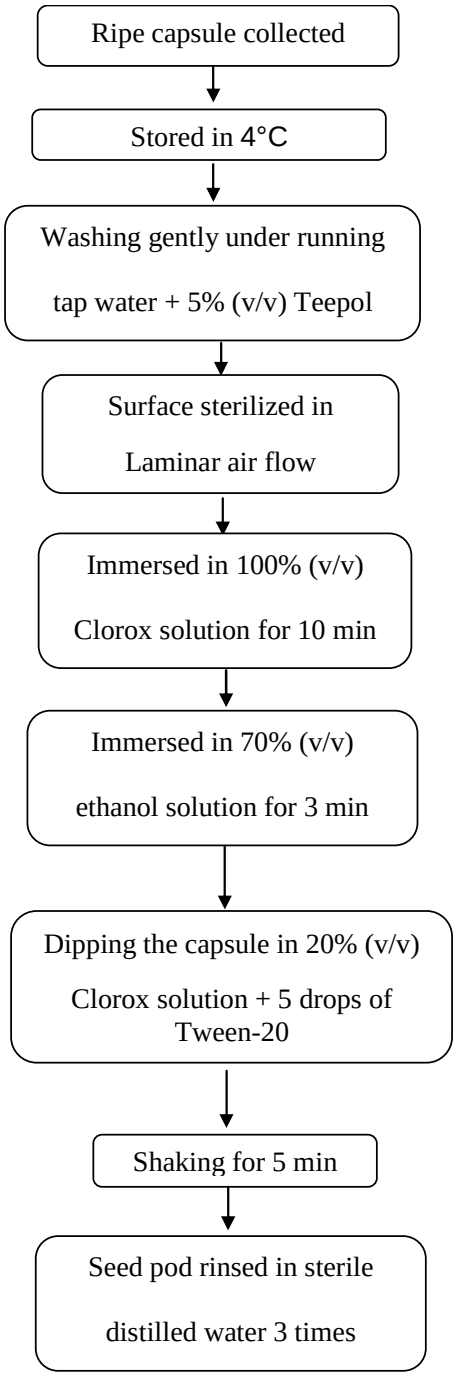

Figure 1. Sequence of procedures from seed capsule collection to culture of seeds.

Elephant Ear orchid because of its extremely large leaves, it is mainly found in Sabah and Kalimantan (Borneo) and has been declared as an endangered species in Appendix II of the Convention of International Trade of Endangered Species (C.I.T.E.S). This study reports on work accomplished in the ***in vitro*** plant regeneration from protocorms-like bodies (PLBs) and callus of ***Ph. gigantea***.

MATERIALS AND METHODS

Ripe capsule of ***Ph. gigantea*** was collected and sterilized. Seed pod was surface sterilized by washing gently under running tap water plus few drops of 5% (v/v) Teepol solution, transferred under Laminar air flow and immersed in 100% (v/v) Clorox solution for 10 min. The seed pod was immersed in 70% (v/v) ethanol solution for 3 min followed by dipping in 20% (v/v) Clorox solution containing 5 drops of (Tween-20) and with constant shaking for 5 min. Finally, the seed pod was gently rinsed 3 times with sterile distilled water (Figure 1). The sterilized capsule was cut with sterilized surgical blades and the seeds were carefully scooped out and sown on Vacin and Went (VW) medium (1949) (Table 1) basal medium containing coconut water (CW), 6-benzyladenin (BAP) and Kinetin (Kin) to enhance seed germination and subsequent development. Protocorm-like bodies (PLBs) were successfully induced and plantlets were produced after 60 days. BAP at 1 mgL^{-1} in combination with 2 mgL^{-1} KIN produced the highest number of plantlets followed by treatment 1 mgL^{-1} BAP and 1 mgL^{-1} KIN.

Germination was performed in darkness by wrapping each flask with two layers of aluminum foil to fully exclude light. The culture was maintained at 25±2°C, fluorescence lighting of 13.5-18 µmol m^{-2} s^{-1} in a 12 h regime. Young leaves from ***in vitro*** grown seedlings were used as explants source. Leaf tip segments (about 1 cm in length) were excised from leaves of donor plants and cultured on New Dogashima Medium (NDM) (Table 2) supplemented with same concentration of 0.01, 0.1, 0.5 and 1.0 mgL^{-1} for each of cytokinins, 6-benzylaminopurine (BAP), thidiazuron (TDZ), and kinetin (KIN), either alone or in combination with auxins, naphthalene acetic acid (NAA), at concentrations of 0.01, 0.1, 0.5 and 1.0 mgL^{-1} (Table 3). Protocorms-like bodies (PLBs) developed from leaf segments were sub cultured every 4 weeks.

In this study, callus and PLBs were observed to develop from leaf segments. In basal NDM medium supplemented with TDZ and NAA, within 6 to 8 weeks of culture gave the highest percentage of callus formation (100%) from treatment containing 1 mg/L NAA in combination with 0.1 mg/L TDZ (N1T0.1) followed by treatment supplemented with 1 mg/L NAA and 0.5 mg/L TDZ (N1T0.5) (Table 3, Figure 2). Out of the three cytokinins tested, TDZ was more effective in inducing PLBs and calli from the leaf sections when compared with BAP and KIN; TDZ at 1 mg/L proved to be beneficial in the production of PLB and callus in ***Ph. gigantea*** from leaf tips. BAP was also effective in inducing PLBs and calli but the explants did not give any response in medium supplemented with KIN. In this study, 1 mg/L of TDZ resulted in optimum PLB and callus induction.

However, the results were low in comparison to treatment containg 0.1 mg/L TDZ in combination with 1 mg/L NAA. All leaf explants responded and initiated PLBs and calli on medium supplemented with TDZ in combination with NAA. Combination design of TDZ and NAA (Table 3) were shown as complete factorial experiments, treatments were arranged such that all possible combination of two or more factors was examined simultaneously. TDZ as sole effect at 1 mg/L induced the highest percentage (47.81%) and it differed significantly compared to the rest of the treatments.

Finally, as interaction effect of TDZ and NAA, 1 mg/L NAA in combination with 0.1 mg/L TDZ induced the highest mean number of PLB (0.69) followed by 1 mg/L NAA and 0.5 mg/L TDZ with the mean number of 0.66 (Figure 2). However, treatment containing 1 mg/L NAA and 0.1 mg/L TDZ differed significantly compared to the control and rest of the treatments. All treatments without TDZ or with low concentration of TDZ produced no PLBs and no callus induction. Type, concentration and combination of growth regulators play important roles during ***in vitro*** propagation of many orchid species (Arditti and Ernst, 1993). BAP has also been found to play an important role in tissue cultures of other orchid's species (Murthy and Pyati, 2001).

Table 1. Chemical components for VW (Vacin and Went, 1949) liquid medium.

Component	Chemical formula	gL^{-1} Medium
Macro elements		
Calcium phosphate	$Ca_3(PO_4)_2$	0.2000
Potasium nitrate	KNO_3	0.5250
Monopotassium phosphate acid	KH_2PO_4	0.2500
Magnesium sulphate	$MgSO_4.7H_2O$	0.2500
Ammonium sulphate	$(NH_4)_2SO_4$	0.5000
Micro elements		
Manganese sulphate	$MnSO_4.2H_2$	0.0075
Ferum elements		
Ethlenediaminetetra acetate	$Na_2EDTA.2H_2O$	0.0373
Ferrous sulphate	$FeSO_4.7H_2O$	0.0278
Other additions		
Sucrose		20.0
Coconut water		200 ml

RESULTS AND DISCUSSION

Germination and seedling development in orchids are strikingly different from other flowering plants. A single orchid capsule is estimated to contain millions of seeds, which lack endosperm. In spite of a very large number, only few seeds germinate in nature. Currently, the horticultural trade depends on wild orchid population as a source of stock plants, but most are not propagated commercially.

In this study, an attempt was made to establish seed propagation of ***Ph. gigantea*** within a short span of time. BAP and Kin as cytokinin are known to accelerate germination and increase the rate of germination (Hicks, 2005; Nikolic, 2006). The seed pod sterilization procedures used in this study recorded no contamination of cultures by microorganisms. However, Chen and Chang (2004) reported the best response for plant rege-neration from seed-derived protocorms of ***Phalaenopsis amabilis*** var. ***formosa*** Shimadzu at 13.62 µM TDZ on modified half-strength MS basal medium after 45 days. In this study, the combination of BAP (1 mgL^{-1}) and Kin (2 mgL^{-1}) in VW supplemented with CW performed the best after 40 days (Figure 3). Seeds from immature capsules are suitable for ***in vitro*** germination, as embryos become viable and easy to surface-sterilize (Van Waes and Debergh, 1986). Orchid seeds are minute and exhibit poor level of differentiation. Endosperm development is surpassed and the embryo remains arrested at globular stage. They have limited food reserves especially lipid droplets and small amounts of proteins. Despite this poor organization and limited food reserves, they can germinate ***in vitro*** (Knudson, 1946; Arditti, 1967).

There has been a few information on the scientific literature of ***Phalaenopsis*** PLBs induction using leaf segments as explants. Early investigations on the clonal propagation of ***Phalaenopsis*** from leaf explants were carried out by Tanaka and collaborators in Japan, which were documented by Arditti and Ernst (1993). It was reported that modified MS medium supplemented with 1 mg/L NAA and 10 mg/L BA was suitable auxin-cytokinin ratio used to induce PLBs from the mature leaf segments of ***P.*** amabilis. This method showed that expanding the uppermost leaves of mature plants can be utilized in the clonal propagation of ***Phalaenopsis***; however, the yield is low (only two PLBs produced after 6 to 8 months incubation).

In another study conducted by Young et al. (2000), flower-stalk derived young leaf segments (eight weeks old) cultured on MS medium containing 1 mg/L NAA and 15 mg/L BA were capable to initiate ***Phalaenopsis*** PLBs, used for the PLBs mass multiplication via bioreactor system. However, the amount of PLBs induced from the leaf segments in this protocol was not reported. All these protocols had been examined in preliminary attempts to induce PLBs of ***Ph. gigantea***; however, it was found that either very low PLBs induction rate or no response was obtained. Considering the cause might be due to genotype dependence, this experiment, therefore, examined the different potential of auxin types and concentrations as a means to produce satisfactory quantity of PLBs to be used for further experiments. TDZ has also been reported to be effective in the regeneration of a number of orchid species and inducing direct somatic embryogenesis from leaf explants (Chen et al., 2000). Chen and Chang (2006) reported direct somatic embryogenesis from leaf explants of ***P. amabilis*** on media containing TDZ. They studied the effect of TDZ in

Table 2. Chemical components for New Dogashima Medium (NDM) (Tokuhara and Mii, 1993, 1998) for solid medium.

Component	Chemical formula	mg/L Medium
Macro elements		
Ammonium nitrate	NH_4NO_3	480
Potassium nitrate	KNO_3	200
Calcium nitrate	$Ca(NO_3)_2.4H_2O$	470
Potassium chloride	KCl	150
Magnesium sulphate	$MgSO_4$	250
Potassium dihydrogen phosphate	KH_2PO_4	550
Micro elements	Modified by Nitsch (1956)	
Manganese sulphate	$MnSO_4.4H_2O$	3
Zinc sulphate	$ZnSO_47H_2O$	0.5
Boric acid	H_3BO_3	0.5
Cuprum sulphate	$CuSO_4.5H_2O$	0.025
Sodium molybdate	$Na_2MoO_4.2H_2O$	0.025
Cobalt chloride	$CoCl_26H_2O$	0.025
Organics	Modified by Morel and Welmore (1951)	
Myo-inositol	$C_6H_{12}O_6$	100
Nicotinic acid	$C_6H_5NO_2$	1.0
Pyridoxine HCl	$C_8H_{11}NO_3$	1.0
Thiamine HCl	$C_{12}H_{17}ClN_4O_5$	1.0
Calcium pantothenate	$C_9H_{17}NO_5$	1.0
Adenine	$C_5H_5N_5$	1.0
I-Cystein	$C_3H_7NO_2S$	1.0
d-Biotin	$C_{10}H_{16}N_2O_3S$	0.1
Sucrose	$C_{12}H_{22}O_{11}$	20,000
Iron		
Fe-EDTA	$FeSO_4\ 7H_2O/Na_2EDTA$	21

combination with NAA.

The best result for their study was 0.03 mg/L at concentration of TDZ and followed by 0.03 (mg/L) in combination with 0.01 (mg/L) of NAA. Combined effects of cytokinin and auxin proved to be useful in PLB and callus induction in ***Vanda*** spp. (Vij and Pathak, 1990), ***Rhynchostylis gigantea*** (Bui et al., 1999), ***Dendrobium densiflorum*** (Luo et al., 2008). NAA is frequently used in combination with BAP or TDZ in many orchid species like ***Vanda*** and ***Dendrobium*** and ***Phalaenopsis*** (Park et al., 2002; Zhao, 2008). When TDZ was used alone, it was found to be more efficient than BAP in orchids such as ***Phalaenopsis*** and ***Doritaenopsis*** (Chang and Chang, 1998). ***Phalaenopsis*** is monopodial and is not easy to propagate vegetatively. Thus, rapid clonal propagation has long been desired. Adventitious shoot proliferation from shoot tips and nodes of flower spikes has so far been the main method for propagation of ***Phalaenopsis***. Callus and PLB initiation and subsequent plant regeneration had limited successes, and subcultures of the callus and long-term totipotency have not been well documented.

In this study, callus and PLB were induced from leaf segments of ***Phalaenopsis*** in NDM medium containing cytokinin (TDZ, BAP and combination of TDZ and NAA). Healthy plantlets without any apparent phenotypic abnormality were obtained in 2 months in NDM medium without hormones. As a result of the high frequency of somatic embryo formation and efficiency of embryo conversion into plants, somatic embryogenesis could be best accomplished through indirect PLB formation from callus. In this study, a regeneration system of ***P. gigantea*** was established. Calli and PLBs were induced from leaf segments under various conditions (Figures 4, 5, 6, and 7). Explants exhibited a high frequency in callus formation with 1 mg/L TDZ. The process of PLB regeneration from the leaf and their subsequent germination is independent of exogenous plant growth regulators, which is similar to the previous reports on the other orchid species such as ***Phalaenopsis*** Richard

Table 3. Different concentrations and combinations of TDZ and NAA.

Plant growth regulator		Code
NAA (mg/L)	TDZ (mg/L)	
0	0	N0T0
0.01	0	N0.01T0
0.1	0	N0.1T0
0.5	0	N0.5T0
1	0	N1T0
0	0.01	N0T0.01
0.01	0.01	N0.01T0.01
0.1	0.01	N0.1T0.01
0.5	0.01	N0.5T0.01
1	0.01	N1T0.01
0	0.1	N0T0.1
0.01	0.1	N0.01T0.1
0.1	0.1	N0.1T0.1
0.5	0.1	N0.5T0.1
1	0.1	N1T0.1
0	0.5	N0T0.5
0.01	0.5	N0.01T0.5
0.1	0.5	N0.1T0.5
0.5	0.5	N0.5T0.5
1	0.5	N1T0.5
0	1	N0T1
0.01	1	N0.01T1
0.1	1	N0.1T1
0.5	1	N0.5T1
1	1	N1T1

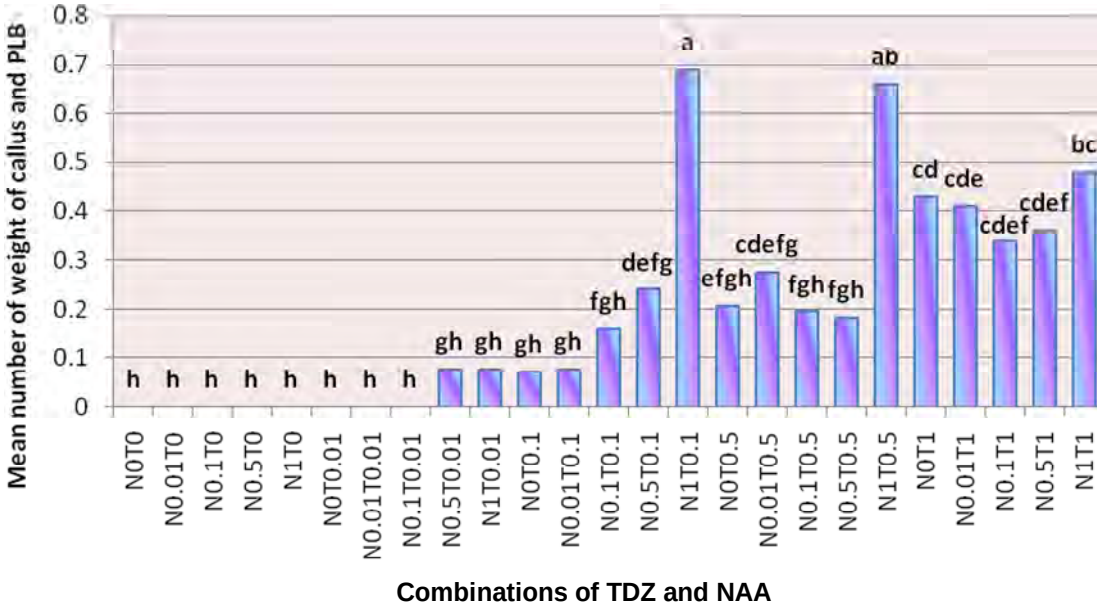

Figure 2. Effect of combination of TDZ (T) and NAA (N) (mg/L) in factorial as RCBD design on weight of callus and PLB induction. Means with the same letter were not significantly different at 0.05 probability level according to DNMRT test.

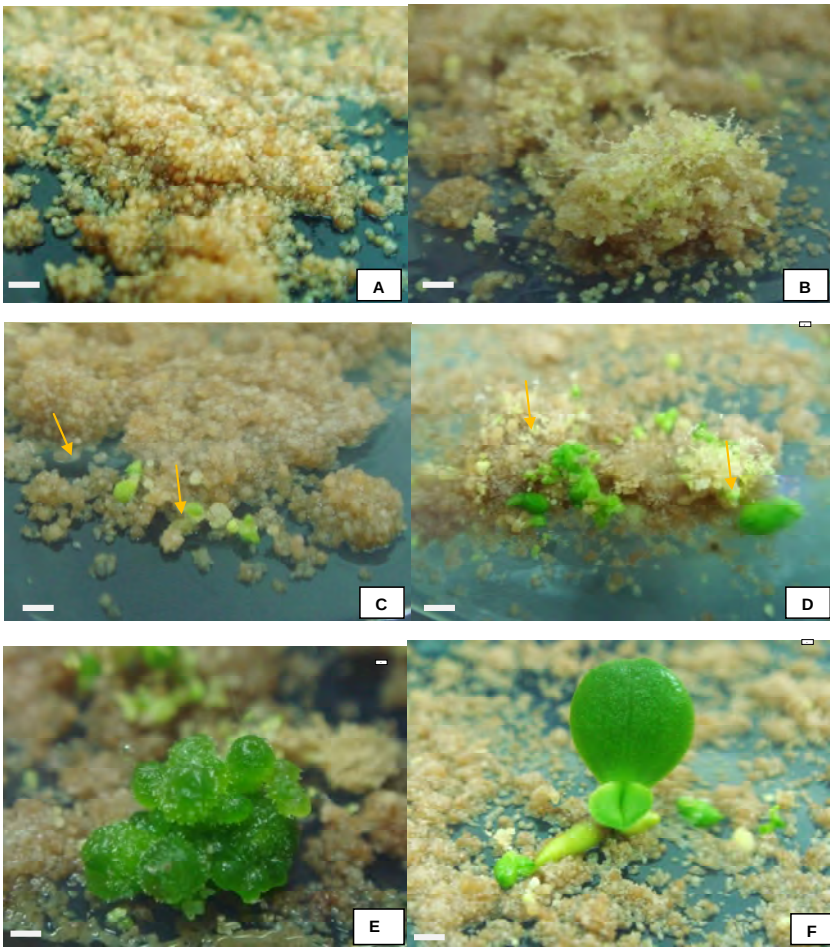

Figure 3. Seed germination in ***Phalaenopsis gigantea;*** (A) seed culture after one week culture, (B) seed culture after three weeks, (C) seed germination after four weeks, (D) seed germination after five weeks, (E) PLBs from seed after six weeks, (F) plantlet from seed germination on 1 BAP with 2 mgL^{-1} Kin after seven weeks of culture (bar = 2 mm).

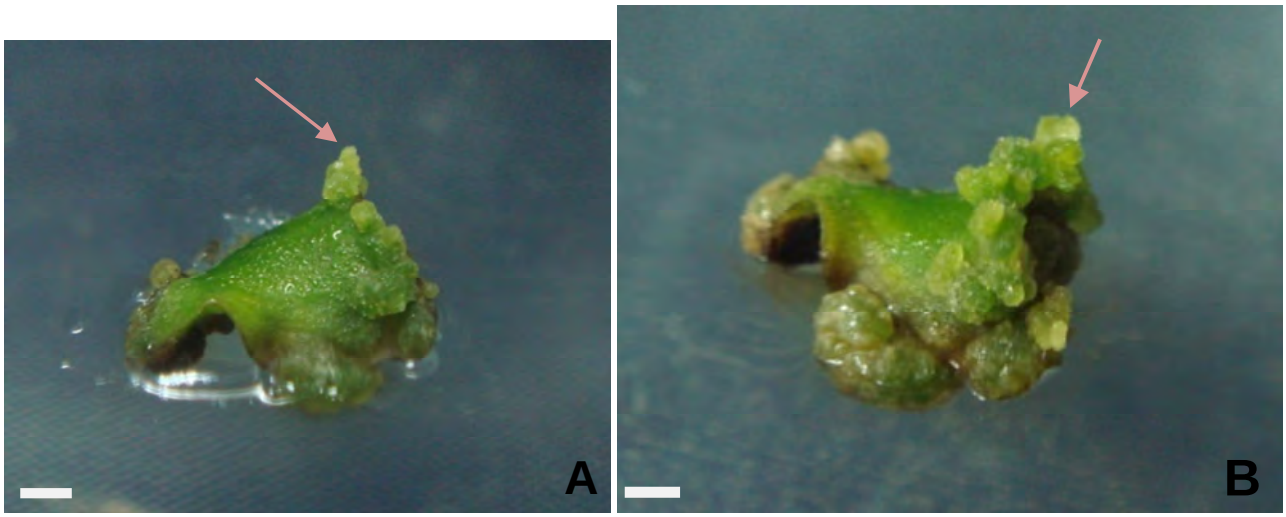

Figure 4. PLB Induction from leaf culture (A) After 6 weeks of culture (B) After 7 weeks of culture. Arrows indicate PLB on leaf culture (bar = 2 mm).

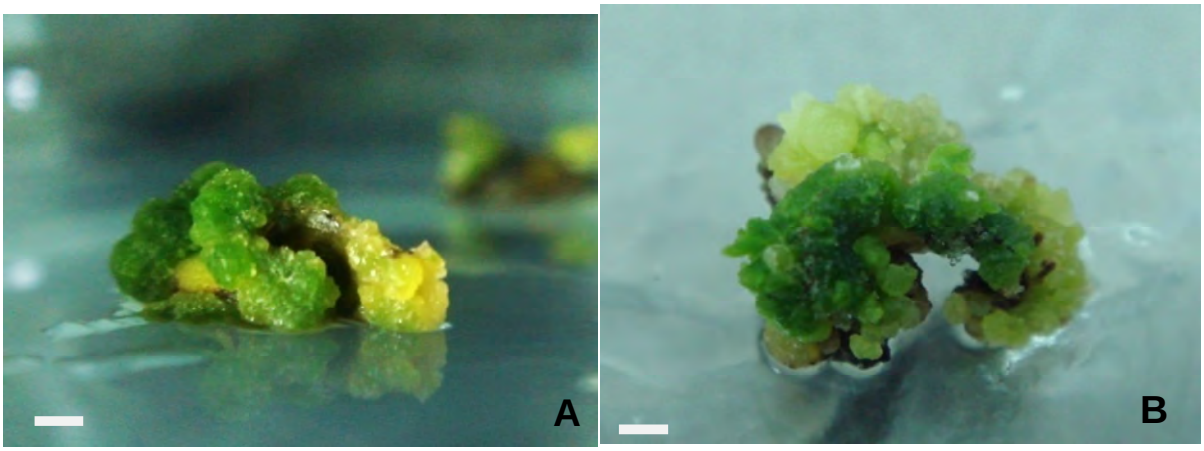

Figure 5. (A) Callus induction from leaf culture after 8 weeks and (B) PLB induction from leaf culture after 8 weeks (bar = 2 mm).

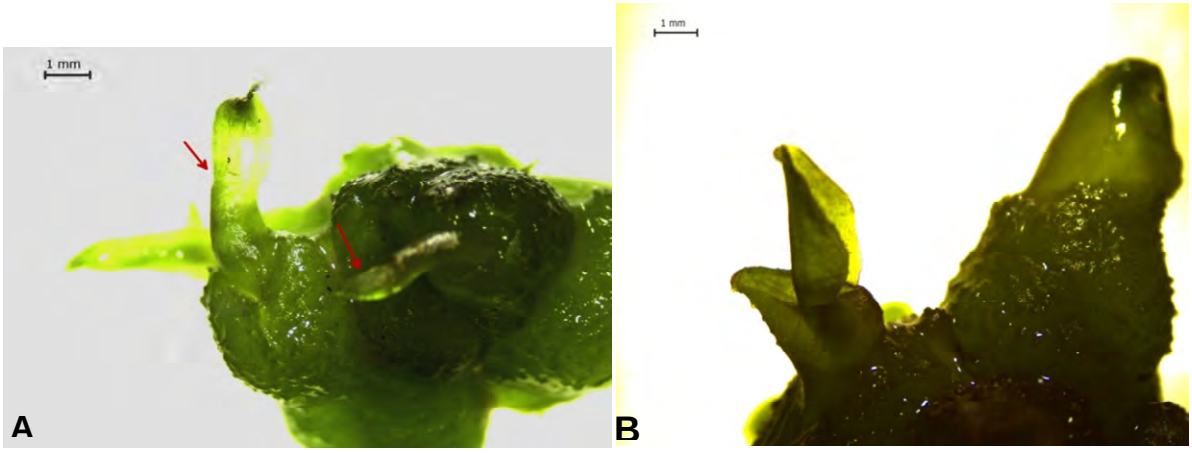

Figure 6. (A, B) Regeneration of PLBs from leaf culture after 8 weeks of culture on microscope, the arrows show leaf formed on PLB. Arrows indicate young leaf.

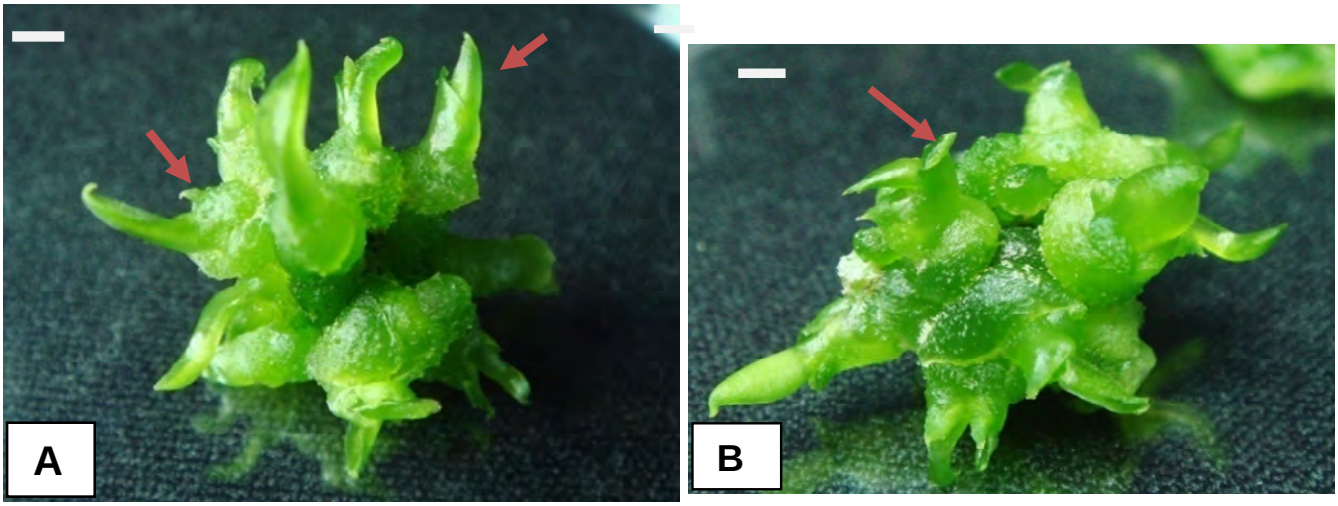

Figure 7. Regeneration of PLBs from leaf culture after 8 weeks. Arrows indicate young leaf (bar = 2 mm

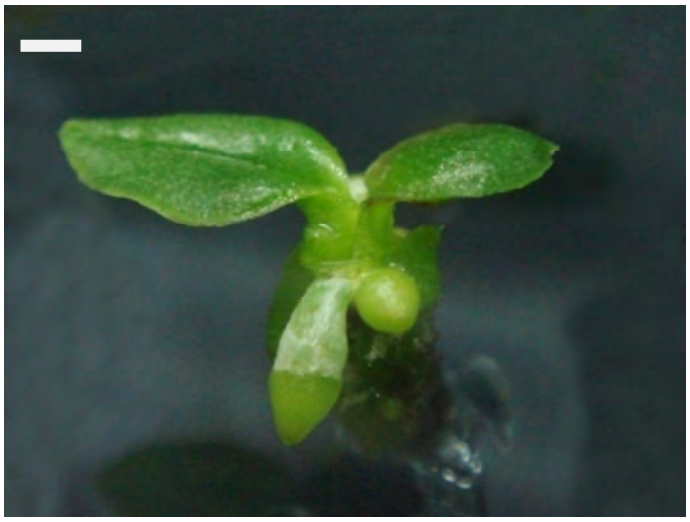

Figure 8. Plantlet produced from PLBs regenerated on solid medium (bar = 2mm).

Figure 9. High length of plantlet from PLB on hormone free NDM medium. After 12 weeks.

Shaffer 'Santa Cruz' (Ishii et al., 1998) and ***Dendrobium fimbriatum*** (Roy and Banerjee, 2003).

In vitro regeneration of ***Ph. gigantea*** PLB was achieved by exposure to light and transferring to a hormone free NDM solid medium. Although, the presence of plant growth regulators was essential for callus and PLB induction, PLB regeneration was achieved on NDM basal medium without any plant growth regulators (Figure 8). In ***Oncidium,*** Chen and Chang (2000) reported regeneration of PLBs when PLBs were placed on hormone free basal medium forming individual plantlets with both shoots and roots. In ***P. amabilis,*** Chen and Chang (2006) reported regeneration from leaf cultures with embryos/protocorms transferred onto hormone free medium and kept under a 16 h photoperiod.

Under this condition, protocorms continued developing and forming shoots, the protocorms continued to develop and consequently formed shoots. Plantlets were obtained after 8 weeks of culture. After four times of sub culturing at every 2 weeks interval, each plantlet developed 4 leaves with 4 roots. High length of regenerated plantlet from PLB was observed on hormone free NDM medium about 4.5 cm (Figure 9); however, the normal length after two months have been reported to be about 3 cm. Propagation of ***Ph. gigantea*** has been successfully carried out. This study show that leaf segments are potential explants that can be used to induce PLBs. Induction of PLBs from the leaf segments occurred only on NDM basal medium supplemented with cytokinin TDZ in low range concentration. However, TDZ in combination

with NAA was better than TDZ alone on PLBs induction, in terms of producing higher quantity and quality of PLBs.

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