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Genetic Stability of *In vitro* Multiplied *Phalaenopsis gigantea* Protocorm-like Bodies as Affected by Chitosan

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Abstract

Chitosan is a carbohydrate polymer derivative of chitin which presents in shell of crustaceans. This biopolymer is a non toxic and environmentally friendly, considered as a plant growth stimulator in some plant species. The present study investigates the effects of chitosan and media types on multiplication and genetic stability of *Phalaenopsis gigantea* protocorm-like bodies (PLBs). PLBs were inoculated in liquid New Dogashima Medium (NDM) and Vacin and Went (VW) supplemented with various concentrations of chitosan (0, 5, 10, 15, 20 and 25 mg/L). The highest PLB multiplication was observed on VW and NDM supplemented with 10 mg/L chitosan with mean number of PLBs 177 and 147, respectively. Chitosan promoted the formation of juvenile leaves and the highest number was observed in NDM supplemented with 20 mg/L chitosan with mean number of 66 leaves after 8 weeks of culture. Genetic stability was assessed among mother plant and secondary PLBs after 2, 4, 6, and 8 weeks of culture in liquid media. 8 out of 10 ISSR markers produced a total of 275 clear and reproducible bands with mean of 6.9 bands per primer. The secondary PLBs produced during sub-culturing process of chitosan treated liquid culture were genetically uniform and similar to mother plant.

Keywords: Chitin, Chitosan, ISSR, New Dogashima Medium, *Phalaenopsis gigantea*, PLBs

Introduction

The *Orchidaceae* family is considered as one of the most diverse flowering plant families, comprising of 25,000 species and more than 800 identified genera. Orchids contribute around 8% of global floriculture trade (Chugh *et al*., 2009). *Phalaenopsis gigantea* is one species found in the lowland forests of the state of Sabah, Malaysia. Deforestation and over-collections have resulted in near extinction of this species (Rodrigues and Kumar, 2009). This tropical orchid is commonly known as Elephant's Ear orchid with its enormous leaves. *Phalaenopsis gigantea* has the potential of producing beautiful hybrids. This species is usually propagated through the formation of new buds induced at the bases of mature plants. But the number of new buds initiated by a mature plant is very low (Shu-guo, 2008). The induction of protocorm-like bodies (PLBs) or callus from the protocorm using liquid media and shake cultures has become a reliable method for mass propagation due to the great number of PLBs that can be achieved with short labor time. The aeration system of liquid medium and the close contact of explants with medium may facilitate the uptake of oxygen, nutrients, phytohormones and consequently lead to increase the rate of plant regeneration (Sandal *et al*., 2001).

In attempts to accelerate PLBs and plantlet production, synthetic phytohormones, such as thidiazuron (TDZ), indole-3-butyric acid (IBA), 1-naphthalene acetic acid (NAA), and 6-benzyl amino purine (BAP), have been widely applied (Nayak *et al*., 1997; Roy and Banerjee, 2003; Saiprasad *et al*., 2004). However, applying synthetic hormones for increasing PLB multiplication leads to induce somaclonal variation (Araditti and Ernest, 1993). Molecular markers have been recently used as conventional tools for assessment of genetic stability of *in vitro* products. Inter-simple sequence repeat (ISSR) have also been used to assess genetic fidelity in several regenerated plants including banana (Lakshmanan *et al*., 2007), *Camellia sinensis* (Devarumath *et al*., 2002), and *Swertia chirayita* (Joshi and Dhawan, 2007).

Chitosan is a cationic polymer and N-deacetylated product derivative of chitin which is present in shells of crustaceans and cell wall of fungi (Devlieghere *et al.,* 2004). This component is an environmentally friendly carbohydrate polymer and has been reported to stimulate growth of some plant species, including orchids (Nge *et al*., 2006). It also has been reported that the supplementation of chitosan for *in vitro* regeneration of *Dendrobium 'Eiskul'* did not induce somaclonal variations (Pornpienpakdee *et al*., 2010). *Phalaenopsis gigantea* has produced outstanding novelty hybrids and has become a much sought after species that result in over-collection from its natural habitat leading to eventual extinction. This species is inherently difficult to propagate in the nature. This species is usually propagated through the formation of new buds at the bases of mature plants. However, this method

of propagation is very inefficient as the number of new buds produced by a plant is very low. In the current study efforts to enhance PLB multiplication using different liquid media supplemented with chitosan and assessment of genetic stability across regenerated PLBs and mother plant were undertaken.

Materials and methods

Plant materials and culture conditions

Young leaves from *in vitro* donor plant (mother plant) were used for induction of initial PLBs. The leaf tip segments (1.5 cm in length) were excised and cultured on semi solid New Dogashima medium (NDM) (Tokuhara and Mii 1993; 1998) supplemented with 0.1 mg/L thidiazuron (TDZ) and 1.0 mg/L naphthalene acetic acid (NAA). The protocol was selected based on an earlier report on *Phalaenopsis gigantea* (Niknejad *et al*., 2011). The cultures were placed under a 16-h photoperiod at an irradiance of 30 μmol m^2 s^1 and temperature of 25±2°C. The earliest morphological sign of PLB formation appeared as swellings on the adaxial side of leaf segments within 4-6 weeks of culture; small round bodies (small initial PLBs) were transferred to hormone free NDM and used as plant material (Fig. 1a).

Media and chitosan preparation

The interaction of different media types and chitosan concentrations on PLB multiplication of *P.gigantea* was investigated. For this purpose, liquid VW (Vacin and Went, 1949) and New Dogashima medium (NDM) (Tokuhara and Mii 1993; 1998) were used and each liquid medium was supplemented with 20% coconut water and different concentrations of chitosan (0, 5, 10, 15, 20 and 25 mg/L). Chitosan powder labeled as low molecular weight and 75% degree of deacetylation (Sigma Aldrich) was selected for the preparation of stock solution. One gram of chitosan powder was dissolved and stirred in 1% acetic acid and heated to 35°C, constantly agitating for 24 hours. The pH of chitosan stock was adjusted to 5.5 using 2 M NaOH and the solution was stirred for 4 hours. Ultimately, the pH of media was adjusted to 5.4 followed by autoclaving for 20 min at 121°C and 15 psi.

Proliferation conditions

For evaluating the multiplication of secondary PLB, five initial PLBs (0.25-0.3 g) induced from leaf segments of donor plant were cultured in 200 ml Erlenmeyer flasks containing 100 ml of growth media (VW and NDM). The PLBs produced in each flask were transferred to freshly prepared medium every 14 days for two months. The cultures were shaken at 60 rpm on a rotary shaker under 16-h photoperiods using fluorescence lighting of 30 μmol m^2 s^1 per day and the cultures were maintained at 25±2°C. The number of PLBs and their fresh weights were determined at the end of the experiment.

Experimental design and statistical analysis

The study on *in vitro* multiplication of PLB was laid out in a factorial combination of treatments based on randomized complete block design (RCBD). The recorded data were analyzed using the analysis of variance (ANOVA) and means separated using Duncan's New Multiple Range Test (DNMRT). All statistical data were tested at the 5% level of significance for comparison between treatment means. In this study 5 experimental units were considered for each replicated and the experiment consisted of 3 replicates. The results are presented as means ± the standard error.

DNA extraction

Leaves of *in vitro* mother plant were used to isolate genomic DNA for PCR amplification and termed as MP. To study the genetic stability among MP and regenerated PLBs after 2, 4, 6, and 8 weeks of culture, the secondary PLBs obtained at the end of every two weeks of sub-culturing (S1, S2, S3 and S4) were randomly used to isolate genomic DNA. Samples from MP, S1, S2, S3 and S4 were first washed with tap water and sterilized in 10% (v/v) Clorox® solution for 5 minutes. The PLBs were rinsed three times with distilled water, wrapped with aluminum foil and stored at -80°C for DNA extraction. Genomic DNA was extracted using cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1990) with minor modification. Quality and quantity of DNA was monitored by spectrophotometry and gel inspection.

PCR amplification and gel scoring

Eight primers were selected from a total of 10 ISSR primers for assessment of genetic fidelity (Tab. 1). PCR amplification was carried out in a total volume of 25 μL including DNA templates (20, 30, 40 and 50 ng/μL) and ISSR primers (1μM), 12.5 μL DreamTaq™ Green PCR Master Mix (Fermentas, Inc, Hanover, USA) and 10.4 μL nuclease free water. Amplification was performed in a programmable Thermal Controller (MJ Research Inc., USA) which was consisted of an initial denaturation step at 94°C for 5 minutes, followed by 35 cycles of 30 s denaturation at 94°C, annealing temperature at 54.8-60.5°C for 90 s (Tab. 1) and continued at 72°C for 90 s with a final extension at 72°C for 5 minutes. Amplified products were separated on 1.5 % (w/v) agarose gels. The molecular size of amplified PCR products were estimated using 1 kb DNA ladder (Fermentas, Inc, Hanover, USA). The digital image files were analyzed using UVIDoc software v.99.01 and fragment sizes were estimated based on DNA ladder. All the reactions were repeated three times. Only well-separated bands with high intensity were scored as present or absent for ISSR markers. The scoring of bands was done as 1 for presence and 0 for the absence of DNA bands in the gel. Electrophoretic DNA bands of low visual intensity that could not be readily differentiated as present or absent were considered ambiguous markers and were not scored.

Results

Effects of different chitosan concentrations and media types on PLBs proliferation

Initial response of primary PLBs to different chitosan concentrations and media types was observed 2 weeks after culturing and it was consisted of protocorms swellings (Fig. 1b). The nodular structure from the swelling regions was observed after 3-4 weeks of cultivation (Fig. 1c), and these nodular tissues ultimately developed further and formed more secondary PLBs. The results exhibited significant differences ($p \leq 0.05$) in PLB multiplication efficiency among different treatments (Fig. 2a). All treatments including the control were able to induce secondary PLBs with mean number of PLBs varying from 31 to 177 after 8 weeks of culturing. Regardless of media used, the best response was recorded at concentrations ranging from 5 to 15 mg/L of chitosan supplementation in both media and most PLBs were between 4-5 mm in diameter. Further increasing the amount of chitosan did not significantly improve the formation of secondary PLBs at 20 mg/L. Chitosan at a concentration of 10 mg/L produced the highest mean number of PLBs in VW (177) and NDM (147), showing no remarkable difference in terms of PLB mean number (Fig. 1d). Conversely, NDM at 0, 20 and 25 mg/L chitosan induced the lowest mean number of secondary PLBs compared to the other treatments, since only 40-32 PLBs per flask were observed after 8 weeks of cultivation. VW at 25 mg/L chitosan did not show further proliferation compared to the control. It was also observed that the effect of NDM at 15 mg/L chitosan on PLB proliferation was not statistically different ($p \leq 0.05$) with VW at 20 mg/L chitosan (Fig. 2a).

Effects of chitosan on leaf organogenesis

The earliest morphological changes and differentiation of PLBs occurred after 5 weeks of culturing. Some tiny secondary PLBs were converted to mature PLBs during the first five weeks of culturing and ultimately the small emerging leaves appeared on the apical region of differentiated PLBs after 7-8 weeks of cultivation (Fig. 1e). The highest differentiation potential of PLB and leaf formation were observed in liquid NDM at a chitosan concentration of 20 mg/L with a significantly larger mean number of developing leaves (66) compared to the other treatments during the same period of culture. Interestingly, there was no significant difference ($p \leq 0.05$) in mean number of leaves produced by NDM at 15 mg/L chitosan (42) and VW at 10 mg/L of supplemental chitosan (32). The highest number of new leaves in liquid VW treatments was observed at 10 mg/L chitosan supplementation (Tab. 2). Subsequently, the result showed that in the presence of chitosan in liquid culture, the success of leaf formation largely depended on the type of growth medium. The results showed that the interaction of NDM and chitosan was more effective on differentiation and conversion of mature PLBs to leaves when compared with the interaction of VW and chitosan.

Tab. 1. List of 10 ISSR primers used for PCR amplification and assessment of genetic stability across mother and secondary PLBs obtained during Sub-culturing process

Primer	Sequence (5'-3')	Tm (°C)	No. of bands	Size range (bp)
T06	AGAGAGAGAGAGAGAGT	56.9	6	400-1171
T05	CGTTGTGTGTGTGTGTGT	60.2	11	228-1537
UBC842	GAGAGAGAGAGAGAGAC/TGGGGG	60.2	6	935-3355
I2	ACACACACACACACACAT	54.8	7	382-1851
UBC812	GAGAGAGAGAGAGAGAA	54.8	5	384-2329
I65	AGAGAGAGAGAGAGAGCC	61.8	8	484-3074
UBC834	AGAGAGAGAGAGAGAGCT	59.1	7	420-2655
I74	ACTGACTGACTGACTG	54.2	5	297-2028
I25	ACACACACACACACACCA	57.6	-	-
C09	CAGATGGGAGTCAAGTCAAC	60.4	-	-

Tab. 2. Mean number of juvenile leaves after eight weeks of cultivation

Chitosan (mg/L)	Mean number of juvenile leaves (NDM)	Mean number of juvenile leaves (VW)
0	5±0.9 e	8±0.8 e
5	13±1.9 de	24±2.3 cd
10	17±2.03 de	32±1bc
15	42±3.07 b	18±2.9 de
20	66±13.5a	13±1.7de
25	8±1.53 e	4±1.3 e

According to analysis of variance (ANOVA), Values are exhibited as means ± standard errors (SE); different letters within a column represent significant differences at (p<0.05)

Effects of different chitosan concentrations on PLB growth

Varying the concentrations of chitosan in VW and NDM led to differences in total fresh weight. The increase in total fresh weight of PLBs and initiated leaves was registered after eight weeks of culture. The results showed that treatment with 10 mg/L chitosan induced the highest mean total fresh weight in VW (8.4 g) and NDM (7.3 g) compared to other treatments. After eight weeks of culture, liquid VW with 10 mg/L chitosan exhibited a stimulated 30-fold increase in total fresh weight in comparison with PLB weight (0.25-0.3 g) at the start of the experiment. Moreover, VW with 20 and 25 mg/L chitosan did not significantly increase mean fresh weight when compared with the chitosan free control. NDM with 25 mg/L chitosan and control cultures showed a non-stimulated increase in weight of 4-5-fold. In general, liquid VW alone or when supplemented with chitosan was more effective in enhancing the growth of PLBs when compared with liquid NDM (Fig. 2b).

Assessment of genetic stability using ISSR analysis

Optimization of ISSR protocol and selection of the primers exhibited that the ISSR bands were reproducible. Of the 10 random ISSR primers used for initial screening, only 8 primers gave more than four clear and scorable bands (Tab. 1). The genetic stability was assayed for PLBs obtained from each subculture stage in both media supplemented with 10 mg/L chitosan (optimal concentration). ISSR molecular technique generated 55 band classes and the number of bands for each primer varied from 5 to 11, with an average of 6.9 bands per ISSR primer. A total of 275 bands were generated by ISSR techniques, giving rise to monomorphic patterns across mother plant and secondary PLBs produced during the process of sub-culturing. Fig. 3 represents the monomorphic band classes generated by ISSR across mother plant and the PLBs achieved by the subculture stages of optimum chitosan (10 mg/L) in VW and NDM. Ultimately, the monomorphic banding model indicated that addition of 10 mg/L of chitosan in both media did not induce any detectable somaclonal variation.

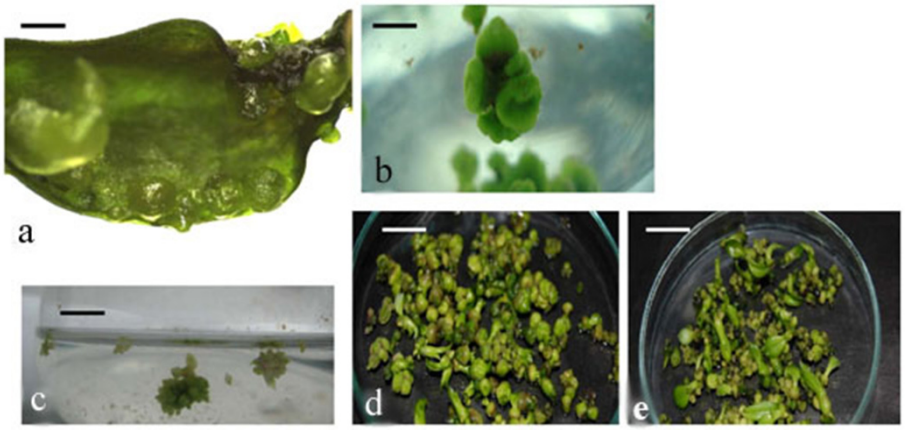

Fig. 1. *In vitro* micropropagation of *Phalaenopsis gigantea* PLBs. (a) PLB Induction from leaf segment cultured after 4 weeks of cultivation (bar=2 mm); (b) Swollen protocorm after 2 weeks of culturing (bar = 4 mm); (c) Secondary PLB formation after 4 weeks in liquid culture (bar = 20 mm); (d) PLB multiplication in liquid VW at 10 mg/L chitosan after 8 weeks of culturing (bar = 20 mm); (e) Leaf formation after 8 weeks of culture (bar = 20 mm)

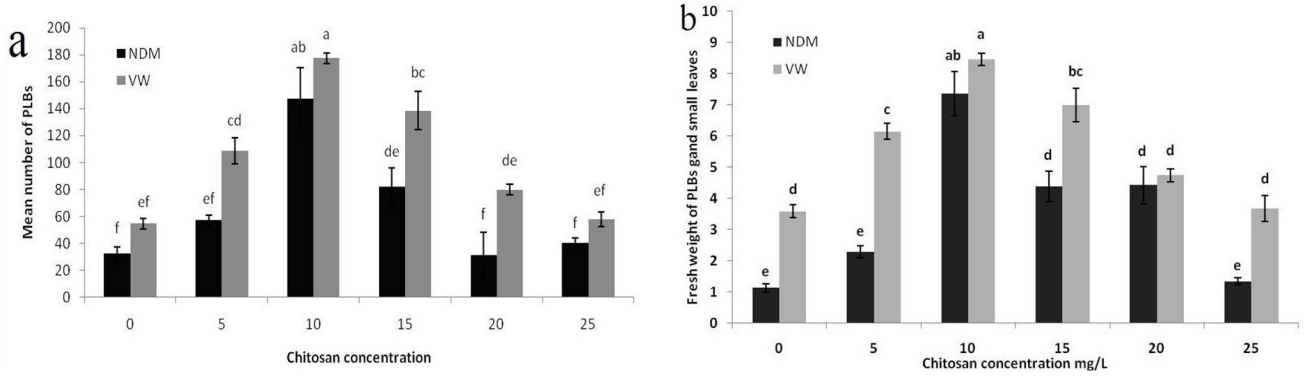

Fig. 2. (a) Effect of media types and chitosan concentrations (mg/L) on mean number of PLBs obtained after eight weeks of cultivation (b) Effect of media types and chitosan concentrations (mg/L) on total fresh weight of PLB's after eight weeks of cultivation [Standard Error was calculated from five independent experiments by one way ANOVA. Bars with different letters differ significantly ($p \leq 0.05$)]

Discussion

Effects of different chitosan concentrations and media types on PLBs proliferation

There have been only a few reports on *in vitro* effects of different chitosan types, molecular weights, deacetylation degree, polymerisation and concentration on orchid propagation (Nge *et al*., 2006; Pornpienpakdee *et al*., 2010). Various comparative analyses of plant responses to different chitosan concentrations have shown that the presence of chitosan in tissue culture medium induced higher frequencies of PLB formation and multiplication in different orchid species such as *Dendrobium phalaenopsis* (Nge *et al*., 2006), *Dendrobium 'Eiskul* (Pornpienpakdee *et al*., 2010) and *Grammatophyllum speciosum* (Sopalun *et al*., 2010). The study presented here is in conformity with other reports indicating that optimal PLB proliferation response was observed at 5-15 mg/L of chitosan range (Nge *et al*., 2006; Sopalun *et al*., 2010). Pornpienpakdee *et al*. (2010) observed that in VW medium supplemented with 10 mg/L polymeric chitosan of 70% (p-70) degree of decetylation, the average number of PLBs (541) was almost 2-fold higher than the number of PLBs (278) produced by chitosan free medium after 3 months of cultivation. PLB proliferation of *Grammatophyllum speciosum*, in ½MS liquid medium supplemented with 15 mg/L chitosan led to a 7-fold increase in PLB growth (Sopalun *et al*., 2010). Similarly, in the case of *Dendrobium phalaenopsis* the addition of 15 mg/L chitosan in liquid VW medium was optimal for PLB multiplication (Nge *et al*., 2006).

According to the above observations and the findings of the present study, the effectiveness of chitosan depended on molecular weight, frequency of application, the concentration, the ratio of sugar carbons to glucosamine and N-acetyl-glucosamine (Uthairatanakij *et al*., 2007). However, the PLB proliferation response to different chitosan concentrations seems to be variable from species to species. Regardless of the similar effects of 10 mg/L chitosan in both media on the mean number of secondary PLBs, the comparative analysis showed that the interaction of liquid VW and chitosan was more efficient for PLB proliferation in comparison with NDM supplemented with chitosan. Various media have been used for mass proliferation of orchid PLBs. Some reports showed that VW medium was more effective for *Phalaenopsis* and *Dendrobium* PLB proliferation (Baker *et al*., 1987; Kalpona *et al*., 2000). Ishii *et al*. (1998) also reported that VW with the addition of 20% CW was suitable for PLB multiplication in *Phalaenopsis*.

Effects of chitosan on leaf organogenesis

It has been previously reported that the presence of 20% CW in tissue culture media were effective for PLBs growth and plantlet regeneration (Ng and Saleh, 2010). This natural compound is rich in cytokinins that are mostly used for PLB multiplication in the plant tissue culture

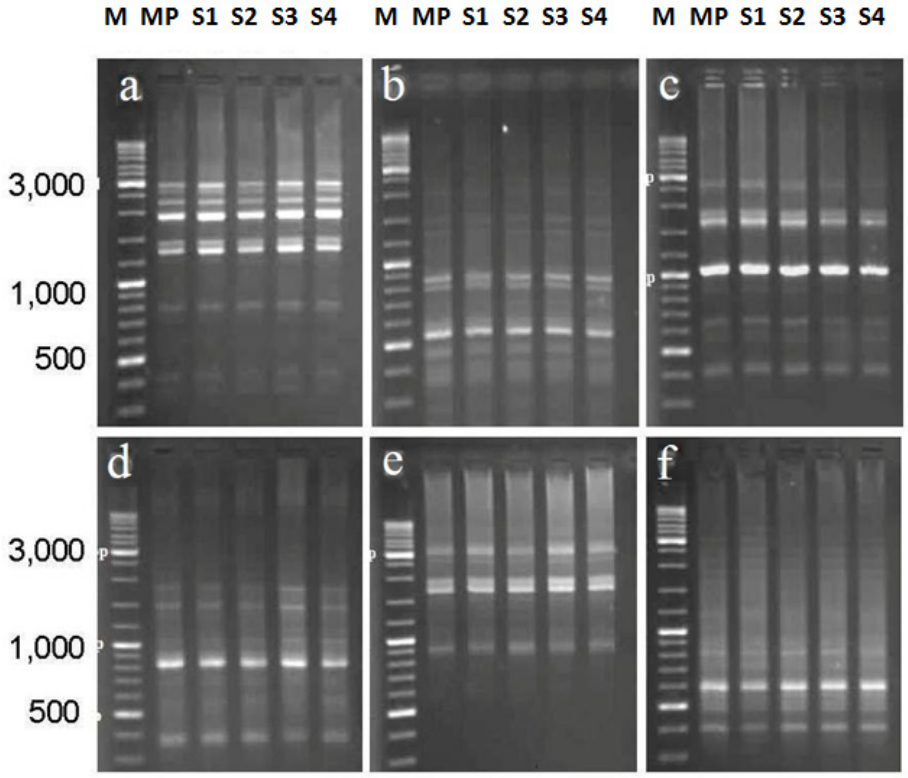

Fig. 3. ISSR banding pattern in multiplied PLBs and mother plants (lane MP is mother plant, lanes 1-4 are multiplied PLBs obtained after subcultures 1-4 with optimal chitosan). (A, B and C), Genetic stability of PLBs obtained in VW at 10 mg/L chitosan. (D, E and F), Genetic stability of PLBs in NDM at 10 mg/L chitosan

industry (Huan *et al*., 2004). However, the synthesis of cytokinins cannot completely substitute the effect of CW because other phytohormones such as auxins, gibberellins and undefined chemical components in CW may exert synergistic effects with cytokinin (Yong *et al*., 2009). After the formation of secondary PLBs and at the time of organogenesis, the endogenous PGR synthesis system may be activated and the increased levels of endogenous PGRs in PLBs may affect differentiation of the cells in the absence of exogenous PGRs (Smith and Krikorian, 1990). Nge *et al*. (2006) reported the formation of juvenile leaves during multiplication of *Dendrobium phalaenopsis* PLBs, in liquid tissue culture medium supplemented with chitosan and the initial PLB's from meristematic buds were treated with different chitosan concentrations. The optimum fresh weight of PLB's was reported at 15 ppm oligomer chitosan during the first three weeks of culturing. The excised meristem tissue initially increased in size and was converted into small round bodies, and the juvenile leaves appeared after five weeks of culturing. It has been previously indicated that in *D. formosum,* the effect of chitosan on growth and leaf induction depended on the growth medium composition (Limpanavech *et al*., 2003). The results of the present study showed that the liquid NDM supplemented with chitosan was more effective for leaf regeneration. Chitosan seems to be an appropriate growth stimulator for orchid micropropagation which may play a role in increasing the growth and development of explants by some signaling pathway similar to auxin biosynthesis via a tryptophan- independent pathway (Uthairatanakij *et al*., 2007).

Assessment of genetic stability using ISSR analysis

Most orchid researchers prefer to use PGR-free media to obtain genetically stable PLBs (Huan *et al*., 2004). As the propagation process does not involve constant exposure to exogenous PGRs, secondary PLBs with the lowest chance of somaclonal variation can be obtained. During *in vitro* culture of plants, variations can happen due to different reasons such as modifications in DNA methylation, gene amplification, chromosomal abnormality and point mutation (Saker *et al*., 2000). The use of synthetic plant growth regulators in growth media even at suboptimal concentrations was also found to induce somaclonal variations in some tissue cultured plants (Martins *et al*., 2004). The presence of CW in tissue culture media results in considerable plant cell multiplication without enhancing the number of undesirable mutations (Arditti, 2008). Pornpienpakdee *et al*. (2010) also reported that the addition of 10 and 20 mg/L chitosan in liquid media did not induce any somaclonal variation. Molecular analysis is being commonly used for monitoring genetic fidelity of *in vitro* raised plants. DNA based markers provide an effective procedure to determine tissue culture induced variations since these markers are not influenced by environmental factors (Peredo *et al.,* 2009). PCR-based techniques such as SSR, ISSR, RAPD and AFLP have been used for assessment of somaclonal variation and genetic fidelity of regenerants. Different studies have shown the genetic stability of *in vitro* raised plants such as gerbera regenerated from tissue culture of capitulum, leaf and shoot tips (Bhatia *et al.*, 2009), the plantlets generated from *in vitro* cultured banana regenerated from rhizomes (Lakshmanan *et al*., 2007), almond plantlets regenerated from axillary branches (Martins *et al*., 2004), a monopodial orchid hybrid with several shoots initiated from seedlings (Kishor and Devi, 2009) and *Cymbopogon flexuosus* initiated from somatic embryogenesis (rhizomatous explants) (Bhattacharya *et al*., 2008). The proliferation system reported here is efficient and capable of producing large numbers of genetically uniform *Phalaenopsis gigantea* PLBs within a relatively short period of time. The PLBs obtained using this procedure could be proliferated further in a large-scale bioreactor system.

Conclusions

In summary, the present report expresses the establishment of a promising *in vitro* culture system to stimulate PLBs proliferation without causing a somaclonal variation rate. VW medium supplemented with 10 mg/L chitosan was most ideal for multiplication of *phalaenopsis gigantea* PLB. The results of study showed that leaf formation from secondary PLBs in liquid culture supplemented with chitosan largely depends on medium composition. The present protocol of PLB multiplication, as outlined in this paper will be efficient means for commercial proliferation of *phalaenopsis gigantea* within a relatively short period of time (8 weeks).

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References

Araditti J, Ernest R (1993). Micropropagation of Orchid Wiley Publisher, New York JohnWiley and Son, 682 p.

Arditti J (2008). Micropropagation of Orchids. 2nd ed Blackwell Cambridge.

Baker KM, Mathes ML, Wallace BJ (1987). Germination of *Panthieva* and *Cattleya* seeds and development of *Phalaenopsis* protocorms. Lindleyana 2(2):77-83.

Bhatia R, Singh KP, Jhang T, Sharma TR (2009). Assessment of clonal fidelity of micropropagated gerbera plants by ISSR markers. Scientia Horticulturae 119(2):208-211.

Bhattacharya S, Dey T, Bandopadhyay TK, Ghosh PD (2008). Genetic polymorphism analysis of somatic embryo-derived plantlets of *Cymbopogon flexuosus* through RAPD assay. Plant Biotechnol Rep 2:245-252.

Chugh S, Guha S, Rao IU (2009). Micropropagation of orchids: A review on the potential of different explants. Scien-

tia Horticulturae 122(4):507-520.

Devarumath RM, Nandy S, Rani V, Marimuthu S, Muraleedharan N, Raina SN (2002). RAPD, ISSR and RFLP fingerprints as useful markers to evaluate genetic integrity of micropropagated plants of three diploid and triploid elite tea clones representing *Camellia sinensis* (China type) and *C. assamica* ssp. *Assamica* (Assam-India type). Plant Cell Reports 21:166-173.

Devlieghere F, Vermeulen A, Debevere J (2004). Chitosan: antimicrobial activity, interactions with food components and applicability as a coating on fruit and vegetables. Food Microbiol 26(6):703-714.

Doyle JJ, Doyle JL (1990). Isolation of plant DNA from fresh tissue. Focus 12:13-15.

Huan LVT, Takamura T, Tanaka M (2004). Callus formation and plant regeneration from callus through somatic embryo structures in *Cymbidium* orchid. Plant Sci 166:1443-1449.

Ishii Y, Takamura T, Goi M, Tanaka M (1998). Callus induction and somatic embryogenesis of *Phalaenopsis*. Plant Cell Report 17:446-450.

Joshi P, Dhawan V (2007). Assessment of genetic fidelity of micropropagated *Swertia chirayita* plantlets by ISSR marker assay. Biol Plantarum 51:22-26.

Kalpona S, Sathyanarayana BN, Sachdev K (2000). Effect of coconut water and banana pulp on *in vitro* culture of *Dendrobium*. J Plant Biol 29(2):209-210.

Kishor R, Devi H (2009). Induction of multiple shoots in a monopodial orchid hybrid (Aerides *vandarum* Reichb. f × *Vanda stangeana* Reichb. f) using thidiazuron and analysis of their genetic stability. Plant Cell, Tissue and Organ Culture 97:(2)121-129.

Lakshmanan V, Sreedhar RV, Bhagyalakshmi N (2007). Molecular analysis of genetic stability in long term micropropagated shoots of banana using RAPD and ISSR markers. Electron J Biotechnol 10:1-8.

Limpanavech P, Pichyangkura R, Khunwasi C, Chadchawan S, Lotrakul P, Bunjongrat P, Chaidee A, Akaraeakpanya T (2003). The effects of polymer type, concentration and %DD of bicatalyte modigied chitosan on flora production of *Dendrobium 'Eiskul'*, 60-64 p. In: National chitin-chitosan conference July 17-18, 2003, Chulalongkorn University Bangkok Thailand.

Martins M, Sarmento D, Oliveira MM (2004). Genetic stability of micropropagated almond plantlets as assessed by RAPD and ISSR markers. Plant Cell Rep 23:492-496.

Nayak NR, Rath SP, Patnaik S (1997). *In vitro* propagation of three epiphytic orchids, *Cymbidium aloifolium* (L.) Sw., *Dendrobium aphyllum* (Roxb.) Fisch. and *Dendrobium moschatum* (Buch-Ham) Sw. through thidiazuroninduced high frequency shoot proliferation. Scientia Hort 243-250.

Ng C-Y, Saleh N (2010). *In vitro* propagation of *Paphiopedilum* orchid through formation of protocorm-like bodies. Plant Cell, Tissue and Organ Culture 105(2):193-202.

Nge KL, Nwe N, Chandrkrachang S, Stevens WF (2006). Chitosan as a growth stimulator in orchid tissue culture. Plant Science 170(6):1185-1190.

Niknejad A, Kadir MA, Kadzimin SB (2011). *In vitro* plant regenreation from protocorms-like bodies (PLBs) and callus of *Phalaenopsis gigantea* (*Epidendroideae: Orchidaceae*) African Journal of Biotechnology 10(56):11808-11816.

Peredo EL, Arroyo-Garcia R, Revilla MA (2009). Epigenetic changes detected in micropropagated hop plants. J Plant Physiol 166(10):1101-1111.

Pornpienpakdee P, Singhasurasak R, Chaiyasap P, Pichyangkura R, Bunjongrat R, Chadchawan S, Limpanavech P (2010). Improving the micropropagation efficiency of hybrid *Dendrobium* orchids with chitosan. Scientia Horticulturae 124(4):490-499.

Rodrigues K, Kumar S (2009). Isolation and characterization of microsatellite loci in *Phalaenopsis gigantea*. Conservation Genetics 10(3):559-562.

Roy J, Banerjee M (2003). Induction of callus and plant regeneration from shoot-tip explants of *Dendrobium fimbriatum* Lindl. var. *oculatum* Hk. f. Sci Hortic 97:333-340.

Saiprasad GVS, Anand L, Ravishankar KV, Mythili JB, Nagesh M, Joshi R (2004). Isolation and characterization of mRNAs differentially expressed during ripening of mango fruits. Indian Journal of Biotechnology 3:533-537.

Saker MM, Bekheer SA, Taha HS, Fahmy AS, Moursy HA (2000). Detection of somaclonal variations in tissue cultured-derived date palm plants using iso-enzyme analysis and RAPD fingerprints. Biol Plantarum 43:347-351.

Sandal I, Bhattacharya A, Ahuja PS (2001). An efficient liquid culture system for tea shoot proliferation. Plant Cell Tissue and Organ Culture 65:75-80.

Shu-guo F (2008). Tissue culture system of *Phalaenopsis* in genetic transformation. Journal of Biotechnology 136:164-165.

Smith DL, Krikorian AD (1990). Low external pH replaces 2,4-D in maintaining and multiplying 2,4-D initiated embryogenic cells of carrot. Plant Physiol 72:329-336.

Sopalun K, Thammasiri K, Ishikawa K (2010). Micropropagation of the Thai orchid *Grammatophyllum speciosum* blume. Plant Cell, Tissue and Organ Culture 101(2):143-150.

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

Tokuhara K, Mii M (1998). Somaclonal variation in flower and inflorescence axis in micropropagated plants through flower stalk bud culture of Phalaenopsis and Doritaenopsis. Plant Biotechnol 15:23-28.

Uthairatanakij A, Teixeira da Silva JA, Obi Wan K (2007). Chitosan for improving orchid production and quality. Orchid Sci Biotechnol 1:1-5.

Vacin EF, Went FW (1949). Some pH changes in nutrient solutions. Bot Gaz 110:605-613

Yong JW, Ge L, Ng YF, Tan SN (2009). The Chemical Composition and Biological Properties of Coconut (*Cocos nucifera* L.) water. Molecules 14(12):5144-5164.