Pak. J. Biotechnol. Vol. 12 (2) 105-114 (2015) ISSN Print: 1812- 1837
ww.pjbt.org ISSN online: 2312-7791

OPTIMIZATION OF PROTOCORM-LIKE BODIES TISSUE CULTURE AND CLONAL PROPAGATION CONDITIONS FOR ENDANGERED LADY'S SLIPPER ORCHID (*Paphiopedilum niveum* (Rchb.f.) Stein)

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Article received August 6, 2015 Revised September 23, 2015 Accepted October 10, 2015

ABSTRACT:

Paphiopedilum niveum (Rchb. f.) Stein is an endangered orchid species and has been listed in the Convention on International Trade in Endangered Species (CITES) Appendix I. Its propagation has been limited due to the fact that the explants are difficult to maintain in culture. The present study was to evaluate clonal propagation of *P. niveum* from two explant types; protocorm-like bodies (PLBs) and callus. For PLB proliferation, PLB clumps were cultured on modified Vacin and Went (VW) medium supplemented with coconut water (CW) (0, 10%) and Phytagel (0%, 0.1% and 0.2%). The highest increase in fresh weight (159.40±5.40 mg) was obtained on VW medium containing 10% CW and 0.2% Phytagel. For induction of PLBs-derived callus, the PLBs clumps were cultured on modified VW solid medium supplemented with 2, 4-dichlorophenoxyacetic acid (2, 4-D) (0, 0.5 and 1.0 mg/L) in combination with thidiazuron (TDZ) (0, 0.1 and 0.5 mg/L) for 2 months. The highest increase in fresh weigh of callus (312.70±59.61 mg) was obtained on medium containing 0.5 mg/L 2, 4-D and 0.1 mg/L TDZ. Then, PLBs-derived callus were proliferated on VW solid medium supplemented with coconut water (CW) (0, 10 and 15%) and sucrose (0, 10 and 15 g/L). The highest callus growth index (0.56±0.06) was achieved from the medium containing 10% CW and 15 g/L sucrose. These proliferated PLBs-derived callus could be reinduced to form PLBs and eventually healthy plantlets.

Keywords: Callus, Clonal propagation, Paphiopedilum niveum, Protocorm-like bodies

INTRODUCTION

Paphiopedilum genus, known as "Lady's Slipper" orchid, is still popular in the international floricultural industry due to the variety in shapes, sizes, and colors of the flowers (Lee *et al*., 2011). However, all species of this genus have been listed in the Convention on International Trade in Endangered Species (CITES) Appendix I (Mcgough *et al*., 2006). Several species have been severely damaged by over-collection and by habitat destruction (Antonelli *et al*., 2011) and the reproduction of this *Paphiopedilum* is complicated by the fact that there is no reliable method for vegetative propagation (Arditti., 2008).Thus, the issue of conservation is necessary to maintain this orchid species and the propagation method is also a good way to keep them from extinction (Lee *et al*., 2011; Ng *et al*., 2011). Plant tissue culture techniques which have been practiced for more than a century are being used in order to produce the uniform clone in many plant species such as *Vicia faba* (Ismail et al., 2006), *Psidium guajava* (Abdullah *et al*., 2009) and *Saccharum officinarum* L. (Sughra *et al*., 2014). Protocorm-like bodies (PLBs) and callus play importance role in the rapid increasing number of *in vitro* mass propagation. PLBs could be proliferated rapidly and regenerated into complete plantlets. These PLBs are also the most general target tissue for genetic transformation studies in many orchids (Sujjaritthurakarn *et al*., 2011). However, at the same time, callus is a suitable plant material for cyto-differentiation, which is composed of fairly homogenous mass of cells and can be proliferated in large amount under known culture conditions (Razdan, 2003). In case of *Paphiopedilum*, a large number of *P. rothschildianum* PLBs could be induced from stem-derived callus when they were cultured on half-strength Murashige and Skoog (MS) (Murashige and Skoog, 1962) medium supplemented with 4.0 µM kinetin (Ng *et a*l., 2011).

Shoot multiplication of *P.callosum* were also induced form seedlings culturing on half strength MS medium supplemented with 10µM 6-benzyl-aminopurine (BAP) and 0.5µM thidiazuron (TDZ) (Wattanawikkit *et al*., 2011). Udomdee *et al* (2012) reported that shoots of *Paphiopedilum* Hsinying Ruby-web were induced on MS medium supplemented with 10g/L sucrose,50g/L potato extract,25g/L banana homogenate (BH) by *in vitro* cutting methods. A single young shoot of *Paphiopedilum* Alma Gavaert was also induced to form multiple shoots on modified half-strength MS medium containing 4.65 µM kinetin (Hong *et al*., 2008). In particular, plant-lets of *P. niveum* were obtained from callus-derived PLBs when these PLBs were cultured on modified MS medium supplemented with 50 g/L BH (Kaewubon *et al*., 2010). The aims of this study were to investigate the suitable conditions for the increasing number of *P. niveum* explants via micropropagation including PLB proliferation, callus induction through the PLB segments and proliferation of PLBs-derived callus. The increased in fresh weight of PLB proliferation, the increased fresh weight of PLBs-derived callus, the percentage of explants forming callus and the percentage of dead PLBs were recorded. The callus growth index (GI) and morphotype of PLBs-derived callus were also observed and determined.

MATERIALS AND METHODS

Plant material: PLBs of *P. niveum* (Figure 1,A) which were obtained from seed-derived callus were maintained in the culture room at light conditions. Eight-week-old PLBs (approximately 20 mg and 3-4 mm in size) consisting of both shoot apical meristem and root apical meristem were used as plant materials. They were cultured on modified Vacin and Went (VW) (Vacin and Went, 1949) solid medium supplemented with 10 g/L sucrose, 0.2% Phytagel and 2 g/L Activated Charcoal (AC). The pH of the medium was adjusted to 5.3 with 1N NaOH or HCl prior to auto claving at 121 °C for 20 min.

PLB proliferation: PLB clumps (approximately 20 mg) of *P. niveum* (Figure 1,B) were cultured on modified VW medium containing 10 g/L sucrose and 2 g/L AC (as described previously). This medium formula was supplemented with coconut water (CW) (0, 10%) at 3 culture states of Phytagel: 0%, 0.1%, and 0.2%, which were defined as liquid-state, semi-solid-state and solid-state cultures, respectively. The experiment was performed in a completely randomized design (CRD) with 12 replications and repeated thrice. After 8 weeks of culture, the increased fresh weight and the morphotype were measured and recorded. Photographs were taken using a Stereo Microscopes (Olympus-DP71).

Induction of PLBs-derived callus: PLB clumps (approximately 20mg) of *P. niveum* (Figure 1,C) were cultured on modified VW solid medium containing 15g/L sucrose, 0.1% Phytagel, 5.5g/L agar and 2g/L AC (prelim-nary experiment; data not shown). The modified VW medium containing a combination of various concentrations of 2, 4-D (0, 0.5 and 1 mg/L) and TDZ (0, 0.1 and 0.5 mg/L) were examined. The initial fresh weight was recorded and the cultures were incubated at 25±2 °C under dark conditions for 2 months. After that, they were transferred to maintain in light conditions. The increased fresh weight of PLBs-derived callus, the percentage of PLBs formed into callus, and the percentage of dead PLBs were recorded after 4 months of culture. Data were calculated using the following equations.

$$\text{Increased fresh weight} = \text{Final fresh weight of callus} - \text{initial fresh weight of PLBs}$$

$$\text{Percentage of PLBs formed into callus} = \frac{\text{Number of PLBs forming callus}}{\text{Total number of cultured PLBs}} \times 100$$

$$\text{Percentage of dead PLBs} = \frac{\text{Number of dead PLBs}}{\text{Total number of cultured PLBs}} \times 100$$

Cas proliferation:PLBs-derived callus (approxima -tely 20mg) of *P.niveum* (Figure-1D) was cultured on modified VW solid medium containing 0.1% Phytagel, 5.5g/L agar and 2g/L AC. The medium was supplemented with differ-rent concentrations of coconut water (CW) (0, 10 and 15%) in combi-

nation with different sucrose concentrations (0, 10 and 15 g/L). All cultures were maintained in the dark for a month, and then transferred to light conditions. Callus growth was represented with GI which was estimated according to Khater *et al* (2013). The increased fresh weight of callus, the value of GI and the callus color were observed and recorded at monthly interval for 2 months. Data were calculated using the following formulae.

$$\text{Increased fresh weight} = \text{Final fresh weight} - \text{Initial fresh weight of callus}$$

$$\text{Growth index} = \frac{\text{Final fresh weight of callus} - \text{Initial fresh weight of callus}}{\text{Initial fresh weight of callus}}$$

PLB reinduction from PLBs-derived callus to form PLBs: PLBs-derived callus of *P. niveum* (Figure-1E) was transferred to culture on modified VW medium. This medium was obtained from the best result of PLBs proliferation experiment (PLBs proliferation medium: PL medium). They were maintained in a culture room at light conditions.

Plant regeneration: PLB clumps (Figure 1,F) were transferred to modified MS medium supplemented with 20g/L sucrose, 50g/L BH, 6.8g/L agar and 2g/L AC for plantlet regeneration (Kaewubon et al., 2010). They were maintained in a culture room at the light conditions. The increased fresh weight and the PLB regeneration were observed after 4 months of culture.

Cultural conditions: All cultures in light conditions were maintained in a culture room (25±2°C) under light (intensity at 23 µmol/ m^2s) provided by Phillips white fluorescent light with a 16 h/ 8 h photoperiod.

Statistical analysis: Each experiment was performed in a completely randomized design (CRD) with 12 replications and repeated thrice. The means of increased fresh weight in all experiments, percentage of PLBs formed into callus, percentage of dead PLBs and callus growth index (GI) were subjected to an analysis of variance (ANOVA) and compared using Duncan's Multiple Range Tests (DMRT) at $P \leq 0.05$.

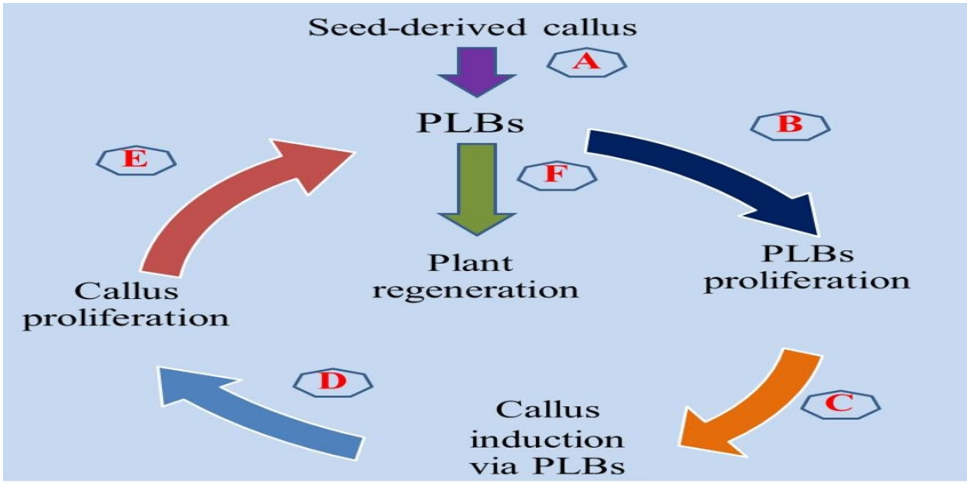

Figure-1: Diagrammatic representation of possible clonal propagation of *P. nevium* orchid. (A) PLBs were obtained from seed-derived callus (Kaewubon *et al*., 2010). (B) These PLBs were proliferated on different cultural states of modified VW medium containing different concentrations of CW. (C) PLBs-derived callus was induced on modified VW solid medium supplemented with various concentrations of 2, 4-D and TDZ. (D) The obtained callus was proliferated on modified VW medium supplemented with different concen-trations of CW and sucrose. (E) The callus was differentiated to form PLBs on PL medium. (F) PLBs were transferred to maintain on modified MS medium for plantlet regeneration (Kaewubon *et al*., 2010). PLBs: Protocorm-like bodies, VW medium: Vacin and Went medium, CW: coconut water, MS medium: Murashige and Skoog medium (Murashige and Skoog., 1962), PL medium: Proliferation medium and AC: Activated Charcoal.

RESULTS

Effects of CW and type of culture conditions on the PLB proliferation: Eight-week-old PLBs of *P. niveum* were cultured on modified VW medium supplemented with coconut water (CW) (0, 10%) at 3 cultural conditions of Phytagel: 0%, 0.1%, and 0.2%, which were defined as liquid-state, semi-solid-state and solid-state, respectively. After 8 weeks of culture, the highest increased fresh weight of PLBs was obtained from the treatment (T6) containing 10% CW combined with a solid-state (159.40±5.40) (Table 1). These PLBs on the solid-state supplemented with CW exhibited a green color and grew well (Figure 2E). The treatment (T4) that was supplemented with 10% CW combined with a semi-solid-state gave a high increase in fresh weight (109.37 ±4.11). PLBs which were inoculated on semi-solid medium within CW (T4) presented green-yellow PLBs and not healthy grown well (Figure 2C). However, PLBs on solidified medium still provided higher weight than those of semi-solid medium (Table 1). All treatments without CW which were in semi-solid (T3) and solid-state (T5) exhibited the low increasing fresh weight and there was no significant difference between these groups. Mean while, all treatments in liquid state gave low increase fresh weight (Table 1) and the browning of PLBs was observed in liquid-state for 8 weeks (Figure 2A-2B).

Table-1: Effect of coconut water (CW) and culture conditions on the PLB proliferation of *P. niveum*.

Treatment	Cultural states	CW (%)	Increased fresh weight of PLBs (mg)/ 100 mg of initial PLBs (means±SE)	Morphotype (color)
1	Liquid	0	13.28±9.99^{d}	Brown
2	Liquid	10	5.72±5.72^{d}	Brown
3	Semi-solid	0	52.48±10.79^{c}	Yellow
4	Semi-solid	10	109.37±4.11^{b}	Green-yellow
5	Solid	0	60.30±7.80^{c}	Yellow
6	Solid	10	159.40±5.40^{a}	Green-yellow

Data were scored after 8 weeks of culture and expressed as increased fresh weight of PLBs per 100 mg of initial PLBs. The different letters in a column are the significant differences at $P \leq 0.05$ with DMRT.

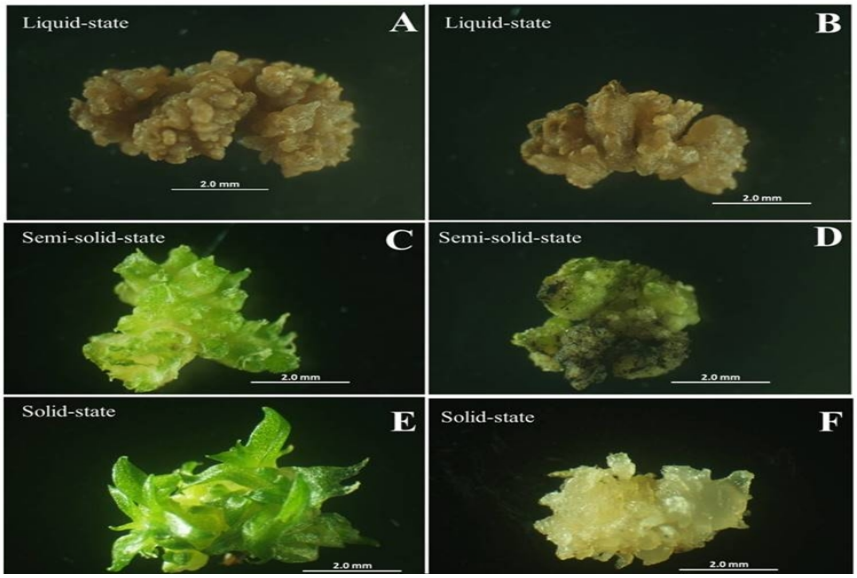

Figure-2: PLBs clumps of *P. niveum* cultured on modified VW medium supplemented with different concentrations of coconut water (CW) in combination with different culture states defined as liquid (0% Phytagel), semi-solid-state (0.1% Phytagel) and solid-state (0.2% Phytagel). (A, B) Browning PLBs on liquid cultural state. (C) PLBs on semi-solid-state in combination with 10%. (D) Yellow PLBs on semi-solid-state without CW. (E) PLBs clump on solid-state supplemented with 10% CW showing well grown shoot, (F) PLBs on solid-state with no CW exhibiting lower number of shoot than solid-state.

Effects of 2,4-D and TDZ on callus induction: The PLBs which were cultured on modified VW medium supplemented with 2, 4-D or TDZ alone exhibited the callus browning and eventually the callus death. These results were similar to that of control which PLBs were browning and death after 1 month of culture. However, callus formation was observed on the modified VW medium supplemented with 2,4-D in combination with TDZ. The highest increased fresh weight of callus (312.70 ±59.61 mg) was obtained from the treatment (T5) that PLBs was maintained on medium supplemented with 0.5 mg/L 2, 4-D and 0.1mg/L TDZ. This treatment also gave the highest percentage of callus formation (38.58±31.11%) and exhibited the lowest percentage of the dead PLBs (60.75±31.27) (Table 2). These PLBs formed into whitish yellow and compact callus at the base of PLBs (Figure 3A-3C). PLBs-derived callus after being transferred to light conditions for 8 weeks showed green and grew well (Figure 3D). Besides, the medium containing 0.5mg/L 2, 4-D in combination with 0.5 mg/L TDZ provided high increase in fresh weight (162.99±50.57mg/ 100mg of initial PLBs) (Table 2). The application of high level of 2, 4-D (1.0mg/L) and TDZ (0.5mg/L) (T9) exhibited the browning of PLBs and the callus death.

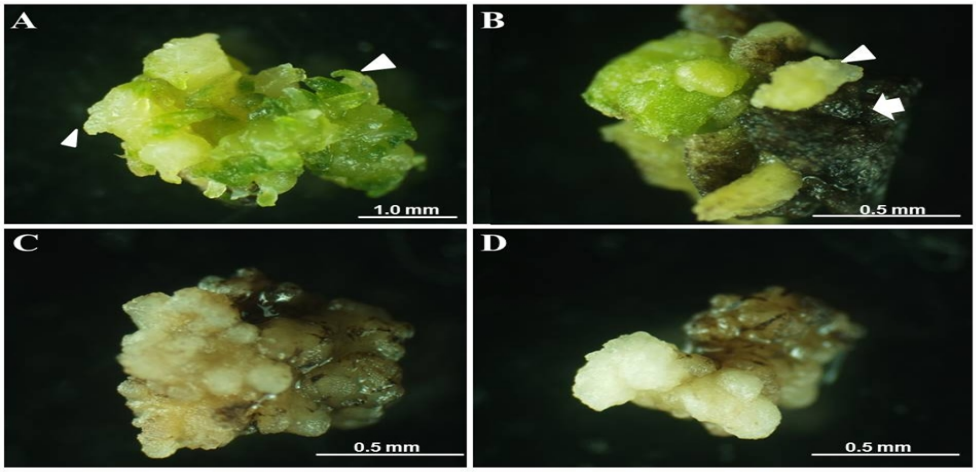

Figure -3: PLB-derived callus of *P. niveum* on modified VW solid medium supplemented with 0.5 mg/L 2, 4-D in combination with 0.1 mg/L TDZ. (A) Five-month-old PLBs with small shoots (arrow-heads) at the beginning of culture. (B) PLBs eventually died (arrow) after maintained in dark conditions for 4 months and appearance of new callus formation (arrow-head). (C) New white callus originated from the base of PLBs. (D) Well grown PLBs-derived callus after being transferred to light conditions for 6 months.

Table -2: Effects of 2, 4-D and TDZ on callus induction of *P. niveum*.

Treatment	2,4-D (mg/L)	TDZ (mg/L)	Increased fresh weight *(mg) (mean ± SE)	Percentage of explants formed into callus	Percentage of dead PLBs	Callus color
1	0	0	0.00±0.00^{c}	0.00±0.00^{c}	100±0.00^{a}	-
2	0	0.1	0.00±0.00^{c}	0.00±0.00^{c}	100±0.00^{a}	-
3	0	0.5	0.00±0.00^{c}	0.00±0.00^{c}	100±0.00^{a}	-
4	0.5	0	0.00±0.00^{c}	0.00±0.00^{c}	100±0.00^{a}	-
5	0.5	0.1	312.70±59.61^{a}	38.58±31.11^{a}	60.75±31.27^{c}	Green
6	0.5	0.5	162.99±50.57^{b}	19.25±22.06^{b}	80.25±22.53^{b}	Yellow -green
7	1.0	0	0.00±0.00^{c}	0.00±0.00^{c}	100±0.00^{a}	-
8	1.0	0.1	107.71±46.53^{b}	13.75±16.99^{b}	85.83±17.50^{b}	Yellow
9	1.0	0.5	0.00±0.00^{c}	0.00±0.00^{c}	100±0.00^{a}	-

- = Callus browning; Data were taken after PLBs were cultured on callus induction medium for 4 months.

* Increased fresh weight of callus/ 100 mg of initial PLBs. Each value represents mean ± SE.

Means followed by different letters in a column are significantly different at $P \leq 0.05$ according to one-way ANOVA with Duncan's Multiple Range Test.

Effect of CW and sucrose on callus proliferation: After the obtained callus was being transferred to callus proliferation medium and maintained in darkness for a month followed by a 16 h light/ 8 h dark photoperiod, Callus proliferation in term of the increased fresh weight of callus was then recorded after a culture for 2 months. The proliferated calli which were achieved from the medium supplemented with 10% CW and 15 g/L sucrose gave the highest increased fresh weight (160.52±25.02mg) and the highest GI (0.56±0.06) (Table 3). Morphologically, these proliferated calli exhibited a green color and grew very well in light conditions (Figure 4). However, the calli on medium supplemented with either CW or sucrose exhibited lower GI (Table 3).

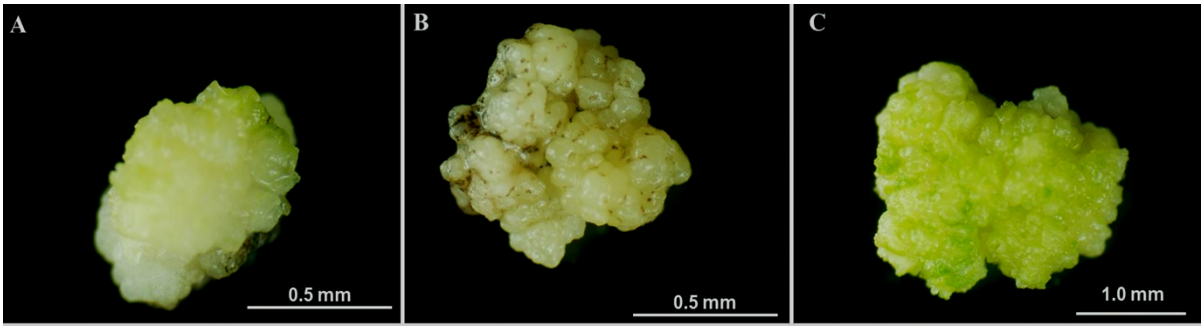

Figure- 4: Proliferated callus of *P. niveum* on modified VW solid medium supplemented with 10 % CW and 15 g/L sucrose. (A) Initial PLBs-derived callus obtained from callus induction. (B) Yellow callus after culture for a month. (C) A well growing, green callus after culture for 2 months**.**

Table-3: Effects of coconut water (CW) and sucrose on callus proliferation of *P. niveum.*

Treatment	CW (%)	Sucrose (g/L)	Increase fresh weight (mg) (means ± SE) *	Growth index	Callus color
1	0	0	5.93±1.87^{c}	0.05±0.02^{c}	Yellow - brown
2	10	0	4.94±1.32^{c}	0.05±0.01^{c}	Yellow - brown
3	15	0	6.74±1.87^{c}	0.06±0.02^{c}	Yellow - brown
4	0	10	7.43±2.21^{c}	0.07±0.02^{c}	Yellow - brown
5	10	10	83.32±11.31^{b}	0.43±0.04^{b}	Yellow - brown
6	15	10	103.09±21.39^{b}	0.42±0.08^{b}	Yellow
7	0	15	80.19±8.65^{b}	0.42±0.04^{b}	Yellow
8	10	15	160.52±25.02^{a}	0.56±0.06^{a}	Yellow - green
9	15	15	11.41±3.10^{c}	0.10±0.02 ^{c}	Yellow

Data were taken after a culture for 2 months.

*Increased fresh weight of callus per 100 mg of initial callus after culture for 2 months. Each value represents mean ± SE. Means followed by different letters in a column are significantly different at $P \leq 0.05$ according to ANOVA with DMRT analysis.

PLBs reinduction and plant regeneration: Proliferated PLBs-derived callus which were cultured on modified VW medium supplemented with 10 g/L sucrose 10% CW, 0.2% Phytagel and 2 g/L Activated Charcoal (AC) could be differentiated to form PLBs after culture for 2 months under light conditions (Figure 5A). Callus-derived PLBs presented multishoot occurring and healthy green PLBs after maintained in the light conditions for 3 months and 4 months, respectively (Figure 5B-5C). After that, those of callus-derived PLBs were transferred to plant regenerated medium which they were illustrated shoots occurring and healthy green plantlets after a culture for 7 months (Figure 5D). Eventually, plantlets could be well in the green house at the potted plant (Figure 6).

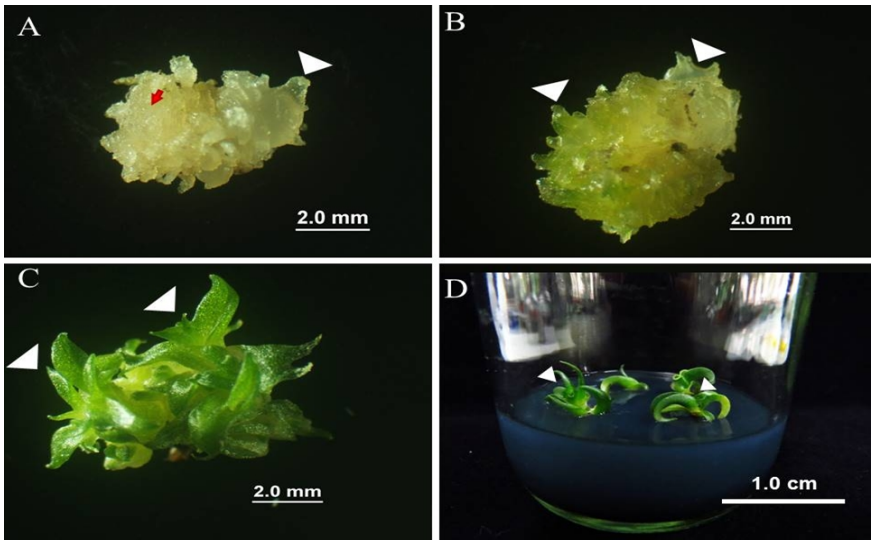

Figure-5: PLBs of *P. niveum* were reinduced from PLBs-derived callus explant culturing on modified VW medium supplemented with 10 g/L sucrose 10 % CW, 0.2 % Phytagel and 2 g/L AC. (A) Callus differentiated to form PLBs exhibiting shoot (arrow-head) after culturing for 2 months. (B) Callus-derived PLBs presented multishoots (arrow-heads) and (C) healthy green PLBs after a culturing for 3 months and 4 months, respectively. (D) Regenerated plantlets after a culture for 7 months on regeneration medium.

Figure- 6: Healthy green plantlet of *P. niveum* maintained in a greenhouse.

DISCUSSION

In order to increase number of *in vitro* mass propagation of *P. niveum* using PLBs explants. The highest increase in fresh weight of proliferated PLBs could be obtained on modified VW containing 10% CW and 0.2% Phytagel supplemented with 10 g/L sucrose and 2 g/L AC. (defined as solid culture state). Many reports revealed that the PLB proliferation of orchids was influenced by CW and culture state. The PLBs proliferation of *Vanda* Kasem's Delight could be obtained on modified VW supplemented with 15% CW (Gnasekaran *et al*., 2012). Nambiar *et al* (2012) also reported that CW was the most suitable carbohydrate source for PLB proliferation of *Dendrobium* Alya Pink. In addition, CW contains many types of biochemical compounds, for instance, sodium, potassium, iron, calcium, copper, magnesium, phosphorous, ascorbic acid and sulphur (Krishnankutty, 1987) as well as diphenyl urea which functions like a cytokinin

hormone that can enrich the growth of PLBs by encouraging cell division (Teixera da Silva *et al*., 2006; Gnasekaran *et al*., 2010). More-over, the solid state type of medium was the best for the PLB proliferation of *P. niveum*which was similar to that of *Dendrobium* Orchid (Aktar *et al*., 2008), *Vanda* Kasem's Delight (Gnasekaran *et al*., 2012) and *Cymbidium pendulum* (Kaur *et al*., 2012). Park *et al*., (2002) presented that PLBs of *Phalaenopsis* which were cultured on the solid medium gave higher proliferation rate than in the liquid medium. However, PLBs of *Dendrobium* Sonia-28 provided higher percentage of growth on the semi-solid medium than in the liquid medium (Julkiflee *et al*., 2014). Thus, plant materials cultured on the solid medium are upright position and they can absorb enough aeration and amounts of nutrients resulted in high biomass accumulation (Mbiyu *et al*., 2012). PLBs-derived callus of *P. niveum* was induced on modified VW medium containing 0.5 mg/L 2, 4-D (auxin) and 0.1 mg/L TDZ (cytokinin). The various ratios of auxin to cytokinin, or vice versa have been widely used for callus induction (Su *et al*., 2011; Li *et al*., 2006). The combination of 2, 4-D and TDZ was found in callus induction of *Swertia chirayita* (Kumar and Chandra., 2013), *Cordea uxiaedulis* (Seyoum and Mekbib,2014) and *Solanum tuberosum* (Abdelaleem, 2015). The effect of 2, 4-D on the callus induction was presented in many plant species such as *Saccharum officinarum* L. callus can be induced from leaf sheath roll by using the suitable concentration of 2, 4-D (5.0 mg/L) (Altaf *et al*., 2013). While, Khan *et al*. (2004) reported the best callus induction and proliferation was observed on modified MS medium containing 2 mg/L 2, 4-D. Raina (1989) reported that 2, 4-D was the most suitable auxin for callus induction of rice besides the optimal concentration of 2, 4-D varies depending on the explant sources and genotype. However, using 0.5 mg/L 2, 4-D alone in callus induction of wheat (*T. aestivum* L.) exhibited low callus induction yield (Zheng *et al*., 2001). TDZ, a high cytokinin activity, has also been used in callus induction of many plant species. It can promote growth of cytokinin-dependent callus cultures (Mok *et al*., 1982). Hong *et al.,* (2008) reported that TDZ had a positive effect on shoot regeneration in *Musa* sp. (Srangsam and Kanchanapoom., 2003) and *A. cariensis* (Erisen *et al*., 2011). However, it inhibited shoot proliferation and rooting of *Paphiopedilum* orchid

The highest increase in fresh weight of callus and the highest GI were obtained when callus was proliferated on medium supplemented with sucrose in combination with CW as organic additive. Rittirat *et al*. (2011) reported that callus of Chang Daeng (*Rhynchostylis gigantea* var. Sagarik) was proliferated on medium containing 15% CW and 2% sucrose. Both sucrose and CW play important roles on callus proliferation because CW contains complex organic compounds such as sugars, vitamins, minerals and amino acid (Yong et al., 2009) while sucrose is carbon source (Konate *et al*., 2013). After being transfer to modified MS medium, well grown PLBs developed to form healthy plantlets.

CONCLUSION

This is the first report investigating the optimization of PLBs tissue culture for *P. niveum* orchid. Clonal propagation of this orchid could be obtained from PLBs and PLB-derived callus explants. PLBs were proliferated on modified VW solid medium containing 10 g/L sucrose and 2g/L AC. This medium formula was supplemented with 10% CW and 0.2% Phytagel (PL medium). PLBs-derived callus which was induced on medium m containing 0.5mg/L 2, 4-D and 0.1mg/L TDZ exhibited the highest increase in fresh weight and the highest percentage of explants formed into callus. The obtained callus which was proliferated on the same basal medium containing 10% CW and 15g/L sucrose exhibitted the highest GI. PLBs-derived callus could be reinduced to form PLBs and presented well grown after being transferred to PL. Eventually, healthy green plantlets of *P. niveum* were well grown in the regeneration medium.

ACKNOWLEDGEMENTS: This work was supported by a grant from Graduate School and Prince of Songkla University Graduate Studies Grant (95000201), Prince of Songkla University, Thailand.

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