World Academy of Science, Engineering and Technology
International Journal of Agricultural and Biosystems Engineering
Vol:8, No:4, 2014

Enhanced Efficiency for Propagation of *Phalaenopsis cornu-cervi* (Breda) Blume & Rchb. F. Using Trimmed Leaf Technique

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***Abstract*—**The effects of thidiazuron (TDZ) and benzyladenine (BA) on protocorm-like bodies (PLBs) induction from leaf explants was investigated. It was found that TDZ was superior to BA. The highest percentage and number of PLBs per leaf explant at 30 and 5.3, respectively were obtained on ½ MS medium supplemented with 9μM TDZ. The regenerated plantlets were potted and acclimatized in the greenhouse. These plants grew well and developed into normal plants after 3 month of transplantation. The 100% survival of plantlets was achieved when planted on pots containing sphagnum moss.

***Keywords*—**Orchid, PLBs, sphagnum moss, thidiazuron.

I. Introduction

PHALAENOPSIS (*Orchidaceae*), commonly known as moth orchids, have long arching sprays. This genus distributed throughout Southeast Asia with a few species extending from Taiwan, Sikkhim to Australia and the Pacific. Most *Phalaenopsis* are epiphytes, meaning they grow on trees, but a few are lithophytes, meaning they attach themselves to the surface of rocks. In Thailand the genus *Phalaenopsis* comprises 2-3 epiphytic orchid species, such as *Phalaenopsis cornu-cervi* (Breda) Blume & Rchb. f. and *Phalaenopsis decumbens* Holtt.

This genus is a monopodial epiphytic orchid, which is difficult to propagate vegetatively and naturally slowly growth [1]. Tissue culture has been used widely for mass propagation of superior varieties of *Phalaenopsis* [2]. *Phalaenopsis*, a member in the family *Orchidaceae*, is a popular genus in horticultural and ornamental plants. It is a very important commercial plant for both flowering-potted plants and cut flower, which is widely propagated by tissue culture technique [2]. This technique can be used not only for rapid and large-scale propagation of the species but also for *ex situ* conservation.

P. cornu-cervi (Breda) Blume & Rchb. f. is an epiphytic orchid that belongs to the family Orchidaceae. Thai common name is Kao-Guang-Aon. *P. cornu-cervi* (Breda) Blume & Rchb. f. has been used as parents to produce yellow or striped *P. cornu-cervi* (Breda) Blume & Rchb. f. hybrids. *P. cornu-cervi* (Breda) Blume & Rchb. f. is a monopodial orchid which is difficult to propagate vegetatively and mass propagation of this species was limited. This species is a wild orchid and has economic value for potted plants. It has a scattered distribution in the southern of Thailand. However, to date, over-collection reduces their population and this species is at an endangered stage. Moreover, deforestation (habitat destruction) and climate change make this species to be at risk of extinction. There is no report on *in vitro* studies of this orchid. *P. cornu-cervi* (Breda) Blume & Rchb. f. is inherently slow-growing plants. Consequently, their clonally propagation is also relatively slow.

Therefore in this study, plantlet production in *P. cornu-cervi* (Breda) Blume & Rchb. f. via the culture of protocorms from seeds and protocorm-like bodies from whole leaf and leaf segments (proximal, middle and distal end) are detailed.

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II. Materials and Methods

A. Plant Materials

Six-month-old green pods by self-pollination were collected from 5-year-old plants of *P. cornu-cervi* (Breda) Blume & Rchb. f. (Fig. 1 (a)). Each pod was cleaned by washing with running tap water for a few minutes, subsequently soaked in 95% ethanol and flamed. The pods were aseptically cut longitudinally on a sterile Petri dish and the seeds were removed from the pods and sown on the surface of MS [3] medium with 3% sucrose and 0.75% agar in bottles each containing 25ml of medium. This medium was supplemented with 15% (v/v) coconut water (CW) to induce protocorms. All cultures were maintained at 25±1°C under a 16h photoperiod with light supplied by cool-white fluorescent lamps at an intensity of 20μmol m^{-2} s^{-1} photosynthetic photon flux density. After 2 months of culture, these seeds germinated into protocorms at GI4 (about 5mm-long) (Fig. 1 (b)). These protocorm segments were transferred to MS medium supplemented with 15% (v/v) CW for development into complete plantlets. Leaves were used as plant material for protocorm-like bodies (PLBs) induction.

B. Effects of Different Cytokinins on the Stimulation of *in vitro* PLBs Induction from Leaf Explants

Leaves (1cm in length) from 120-day old of *in vitro* grown plantlets were used as plant materials (Fig. 1 (c)). Whole leaf and leaf segments (proximal, middle and distal end) were

cultured on ½ MS basal medium supplemented with different concentrations of Benzyladenine (BA; 5, 9, 14, 23µM) or Thidiazuron (TDZ; 5, 9, 14, 23µM) for PLBs proliferation. All culture media were supplemented with 3% (w/v) sucrose, 15% (v/v) coconut water and solidified with 0.23% Gelrite. The pH of ½ MS basal medium was adjusted to 5.6 with 1 N KOH or HCl prior to autoclaving for 15min at 121°C. One segment was inoculated on each bottle and thirty bottles were considered for each treatment.

The cultures were incubated for 2 months under the same conditions mentioned earlier or incubated for 1 week in the dark then they were transferred to a 16/8 h photoperiod. The percentage of PLBs formation [% PLBs formation = (Number of leaf segments with formed PLBs /Number of total leaf segments inoculated) x 100] and number of PLBs per explant were evaluated. For PLBs regeneration, the regenerated PLBs with a cotyledon were transferred onto MS medium supplemented with 15% CW and 0.2% (w/v) activated charcoal (AC) for development into complete plantlets.

C. Ex vitro Plantlet Establishment

For *ex vitro* establishment, plantlets with three or four roots were washed with water for three times to remove the residual nutrient agar from the plant body and were dipped into fungicide solution for 5min. The plantlets were transplanted to plastic basket for 1 month and then transplanted to pots containing sphagnum moss for three-months. The seedlings were grown in the green house with controlling of temperature at 25-35°C and 60-70% relative humidity. The young plants were sprayed with water twice a day.

D. Statistical Analysis

Experiments were assigned in a completely randomized design (CRD). All data were analyzed statistically by ANOVA and means were compared by Duncan's multiple range test (DMRT) at $p<0.05$, with SPSS v. 13 software.

III. Results

No effect of plant growth regulators were observed in PLBs induction from proximal, middle and distal end segments. After 2 months of culture, these segments tend to be necrotic and no PLBs were found.

There was no PLBs formation in all leaf explants cultured on ½ MS medium without growth regulators under light conditions (Fig. 2 (a)). For the cytokinins tested, TDZ was more effective than BA. Within 2 months of culturing, white granules emerged from the surface of wounded leaf explants inoculated on ½ MS basal medium with different plant growth regulators and became light green granules. Of the two plant growth regulators tested, TDZ was found to be more effective than BA. PLBs induction was enhanced at 5 or 9 µM TDZ and reduced at 14 or 23µM TDZ (Table I).

The highest percentage of leaf explants producing PLBs (30%) and the best number of PLBs per leaf segment (5.3) were observed on ½ MS basal medium with 9µM TDZ ($p<0.05$) (Fig. 2 (b), Table I). The effectiveness of BA on PLBs induction from leaf explants was comparable to that of TDZ. The frequency of PLBs induction from leaf explants was 16.7% and number of PLBs per leaf explant at 2.2 on ½ MS basal medium with 23µM BA when incubated for 1 week in the dark then they were transferred to a 16h photoperiod (Table I). In all cases, cultures of explants under light conditions at the first period gave better response than keeping in the dark for one week. TDZ was more effective than BA in inducing PLBs formation. The regenerated plantlets with a pair of leaves and roots were obtained after 4 months of culture (Fig. 3 (a)). These plants grew normally when transplanted to pots containing sphagnum moss in the greenhouse with 100% survival rate (Fig. 3 (c)).

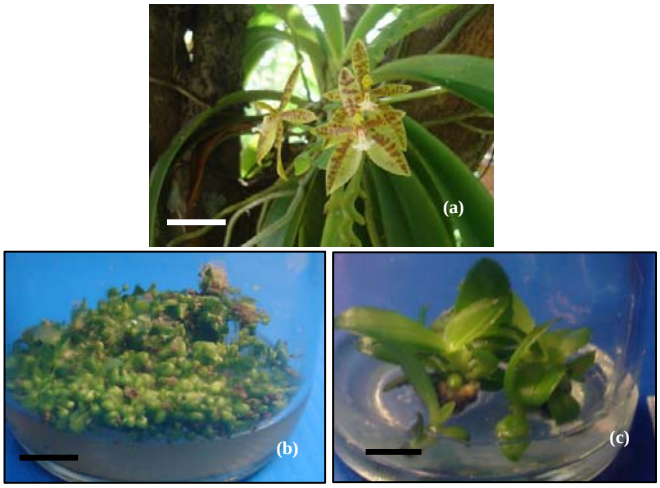

Fig. 1 (a) *P. cornu-cervi* (Breda) Blume & Rchb. f; (b) Asymbiotic germination of seeds from capsule after 2 months of culture on MS medium supplemented with 15% CW; (c) *in vitro* seed-derived plantlets used as leaf explants (bar=1 cm)

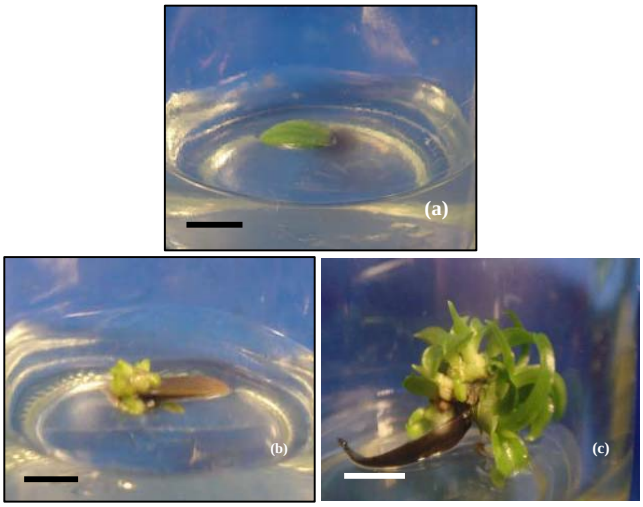

Fig. 2 (a) Leaf segments on ½ MS free medium under light condition after 3 months of culture; (b) PLBs formation on ½ MS basal medium supplemented with 9 µM TDZ under light condition for 2 months; (c) PLBs developed into plantlets on ½ MS basal medium supplemented with 9µM TDZ under light condition for 3 months; (bar=1cm)

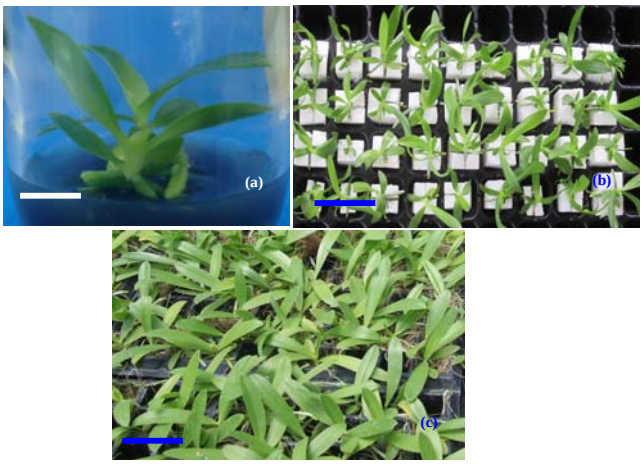

Fig. 3 (a) PLBs-derived plantlets on MS basal medium supplemented with 15% CW and 0.2% AC after 4 months of culture; (b) transplanted into plastic basket (one-month-old); (c) three-months-old acclimatized plantlets grown in the greenhouse (bar=1cm)

IV. Discussions

No effect of plant growth regulators were observed in PLBs induction from proximal, middle and distal end segments. After 2 months of culture, these segments tend to be necrotic and no PLBs were found. For *P. cornu-cervi* (Breda) Blume & Rchb. f. in the present study proximal, middle and distal end segments produced enormous phenolic compound leading to the failure in PLBs formation. Thus, the next investigation should be concentrated on a higher concentration of plant growth regulators together with antioxidant, e.g. ascorbic acid, polyvinylpyrolidone (PVP) or AC.

The highest percentage of leaf explants producing PLBs (30%) and the best number of PLBs per leaf segment (5.3) were observed on ½ MS basal medium with 9 µM TDZ ($p<0.05$). Similar results were also reported by Park et al. [4]. Leaf segments of *Acampe praemorsa* could produce PLBs [5]. PLBs formation from leaf explants of *P. amabilis* after 45 days of culture [6]. The successful on PLBs induction from culturing leaf explant might be depend upon genotype and PGRs containing in culture medium. Recently, TDZ has been used in orchid tissue culture for various purposes due to its remarkable ability to induce callus or organogenesis. TDZ was reported to be effective in regeneration for a number of orchid species [7]. TDZ was more effective than BA in inducing PLBs formation. TDZ used alone was more efficient than BA in *Phalaenopsis* and *Doritaenopsis* [7], [8].

TABLE I
Effects of Different Cytokinins on the Stimulation of *in vitro* PLBs Induction from Leaf Explants of *Phalaenopsis cornu-cervi* (Breda) Blume & Rchb. f. within 2 Months of Culture

Conditions	Cytokinin concentration (µM)		PLBs formation[1]	PLBs/explant[1]
	TDZ	BA	(%)	(Mean±S.E.)
Light 16/8	0	0	0.0±0.0 e	0.0±0.0 f
	5	0	13.3±3.3 bcd	1.3±0.3 ef
	9	0	30.0±1.0 a	5.3±0.2 a
	14	0	20.0±5.8 abc	4.2±1.2 ab
	23	0	10.0±5.8 cde	3.7±1.7 bc
	0	5	0.0±0.0 e	0.0±0.0 f
	0	9	6.7±3.3 de	1.5±0.5 def
	0	14	10.0±0.0 cde	1.7±0.3 def
	0	23	13.3±3.3 bcd	2.0±0.0 cde
Dark➡Light 16/8	0	0	0.0±0.0 e	0.0±0.0 f
	5	0	10.0±0.0 cde	1.0±0.0 ef
	9	0	23.3±3.3 ab	3.1±0.5 bcd
	14	0	16.7±3.3 bcd	2.8±0.4 bcde
	23	0	10.0±0.0 cde	2.7±0.3 bcde
	0	5	6.7±3.3 de	1.0±0.0 ef
	0	9	10.0±0.0 cde	1.7±0.3 def
	0	14	13.3±3.3 bcd	1.8±0.3 def
	0	23	16.7±3.3 bcd	2.2±0.2 cde

[1]Similar letters within columns mean no significant difference at $P\leq0.05$ by DMRT.

V. Conclusions

In conclusion, cultures of explants under light conditions at the first period gave better response than keeping in the dark for one week. Half-strength MS basal medium supplemented with 9 µM TDZ was more suitable for PLBs induction from leaf explants. The highest percentage and number of PLBs per leaf explant at 30 and 5.3 respectively were obtained on this medium and regenerated plantlets were acclimatized successfully with 100% survival rate in the greenhouse.

Acknowledgments

This work was financially supported by the Research and Development Institute of Nakhon Si Thammarat Rajabhat University, Nakhon Si Thammarat, Thailand, Prince of Songkla University, Pattani, Thailand and Mahidol University, Bangkok, Thailand.

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