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Induction of protocorm-like bodies (PLBs) and plantlet regeneration from wounded protocorms of *Phalaenopsis cornu-cervi* (Breda) Blume & Rchb. f.

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Seeds of orchid, *Phalaenopsis cornu-cervi* (Breda) Blume & Rchb. f. were aseptically germinated *in vitro* on MS (Murashige and Skoog, 1962) medium supplemented with 15% coconut water (CW). After 2 months of culture, the seeds germinated into protocorms at GI4. Protocorms, wounded protocorms, bisected protocorms and leaf segments were used as initial explants for PLBs induction. These explants were cultured on ½ MS medium supplemented with NAA (0, 0.1, 1.0 mg/l), TDZ (0, 0.1, 1.0, 3.0 mg/l) and BA (0.1, 1.0, 3.0, 10.0 mg/l). All PLBs were formed from wounded protocorm segments cultured on medium containing NAA at 0.1 mg/l and TDZ at 0.1 mg/l. This culture medium was found to be the best for induction of PLBs. After 6 weeks of culture, the highest percentage of PLBs formation occurred and each explant produced small, medium and large size of PLBs at 13.9, 10.7 and 11 PLBs per culture explant, respectively. The PLBs converted into plants with well-developed shoots and roots on MS medium supplemented with 15% CW and 0.2% activated charcoal, without plant growth regulators after about 4 months of culture. The regenerated plantlets grew normally when transplanted to pots containing sphagnum moss in the greenhouse with 100% survival rate. The simple protocol will be useful for large scale propagation and improvement by biotechnological methods of *Phalaenopsis cornu-cervi* (Breda) Blume & Rchb. f. in the future.

Key words: Orchid, micropropagation, activated charcoal, seedlings, explants, Phalaenopsis

Introduction

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 widely propagate by tissue culture technique (Tokuhara and Mii, 1993). This technique can be used not only for rapid and large-scale propagation of the species but also for *ex situ*

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conservation. Many authors have developed large-scale propagations through various explants of orchid species such as shoot tips (Martin and Madassery, 2006), leaf segments (Park *et al*., 2002; Teng *et al*., 2004; Sheelavanthmath *et al*., 2005) and root tips (Park *et al*., 2003). Unfortunately, these methods are very difficult to *Phalaenopsis*. Another problem encountered during the *in vitro* culture of *Phalaenopsis* is the long time period needed for the growth and multiplication of PLBs. *Phalaenopsis cornu-cervi* (Breda) Blume & Rchb. f. is a monopodial orchid which is difficult to propagate vegetatively. Mass propagation of this species was limited. Thus, in this study, we describe an efficient induction of PLBs from wounded protocorm segments of *P. cornu-cervi* (Breda) Blume & Rchb.f. and reduce the time required for the growth and multiplication of PLBs in order to mass propagation of this species.

Materials and methods

Plant material

Six-month-old green self-pollination pods were collected from 5-year-old plants of *P. cornu-cervi* (Breda) Blume & Rchb. f. (Figure 1). Each pod was cleaned by washing with running tap water for a few minutes, subsequently soaked in 95% ethanol and flamed. The pods were cut longitudinally into half on a sterile Petri dish and the seeds were aseptically sown on MS (Murashige and Skoog, 1962) medium supplemented with 15% coconut water (CW) to induce protocorms. After 2 to 3 months of culture, these seeds germinated into protocorms at GI4 (Figure 2A) and cluster of two-leaf seedlings (Figure 2B), respectively. Both protocorms and seedlings were used as starting explants for proliferation of protocorm-like bodies (PLBs).

Effect of starting explants on proliferation of PLBs

Four starting explants: protocorms, wounded protocorms, bisected protocorms and leaf segments were used as initiatial PLBs. The basal medium used in this study was half-strenght MS (Murashige and Skoog, 1962) supplemented with different concentrations of naphthaleneacetic acid (NAA) (0, 0.1, and 1.0 mg/l), thidiazuron (TDZ) (0, 0.1, 1.0 and 3.0 mg/l) and benzyladenine (BA) (0.1, 1.0, 3.0 and 10.0 mg/l), 15% coconut water, 2% sucrose. Those PGRs were used alone or in combination and added to culture medium prior to autoclaving. The culture medium was solidified with 0.75% agar. The pH of the media was adjusted to 5.7 with 1 N KOH or HCl prior to autoclaving for 15 min at 121 °C. Whole explants were placed on the surfaces of ½ MS medium and maintained at 25±1°C under a 16 h photoperiod with

light supplied by cool-white fluorescent lamps at an intensity of 10 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD). The percentage of PLBs formation and the mean number of PLBs per explant were scored and compared statistically after 45 days of culture.

Experimental Design and Data Analysis

Experiments were performed in a complete randomized design (CRD). The data were analyzed by ANOVA using SPSS version 11.5 and the mean values were compared using Duncan's multiple range test at a 5% probability level.

Results

Among four different explant types, only wounded protocorms resulted in proliferation of new PLBs. One cm long leaf explants were swelled and remained green color after 4 weeks of culture (Figure 2C). Plant growth regulators played non effective role in PLBs induction from leaf segments. After 6 weeks of culture, the leaf segments tended to be necrotic and no PLBs were form.

In our culture system, wound treatment caused by the cutting played an important role in proliferation of new PLBs. Wounding can bring about the production of a higher number of new PLBs on the original one, however, it also caused the death of some of the wounded protocorms. The efficiency of wounded protocorms producing PLBs was dependent on the types and concentration of PGRs in the medium. In the PGR-free half-strenght MS medium only about 30% of wounded protocorm developed into new PLBs (Table 1). Between the two cytokinins tested (BA, TDZ), TDZ was effective in the PLBs induction from wounded protocorm segments of *P. cornu-cervi* (Breda) Blume & Rchb.f. In the presence of TDZ or BA alone or TDZ in combination with NAA, PLBs were directly formed from the surface of wounded protocorm segments of *P. cornu-cervi* (Breda) Blume & Rchb.f. after 4 weeks of inoculation. Direct formation of PLBs was observed from protocorms without the intermediate formation of callus. The responsive in PLBs proliferation was improved considerably with the application of cytokinin, especially TDZ. Even high concentration of TDZ at 1.0 mg/l alone promoted a high percentage (100) and number of PLBs formation after 4 weeks of culture, addition of NAA (0.1 mg/l) together with 0.1 mg/l TDZ gave the better number of PLBs at all sizes (11-13 PLBs/explant) (Table 1, Figure 2D). Higher concentration of TDZ (>0.1 mg/l) yielded lower results in number of PLBs significantly (Table 1).

An early response of wounded protocorm segments of *P. cornu-cervi* (Breda) Blume & Rchb.f. was the production of phenolic exudates from the wounded protocorm segments and few browning of the surrounding medium during the 4 week observed. After another 2 weeks of culture on the same PGR-containing media, the PLBs enlarged, intense browning of the surrounding medium and more PLBs and shoots were formed (Figure 2E).

Fig. 1. (A) *P. cornu-cervi* (Breda) Blume & Rchb.f. (B) Flower of *P. cornu-cervi* (Breda) Blume & Rchb.f. (C) 6-month-old green capsule of *P. cornu-cervi* (Breda) Blume & Rchb.f. Bar = 1 cm (A to C).

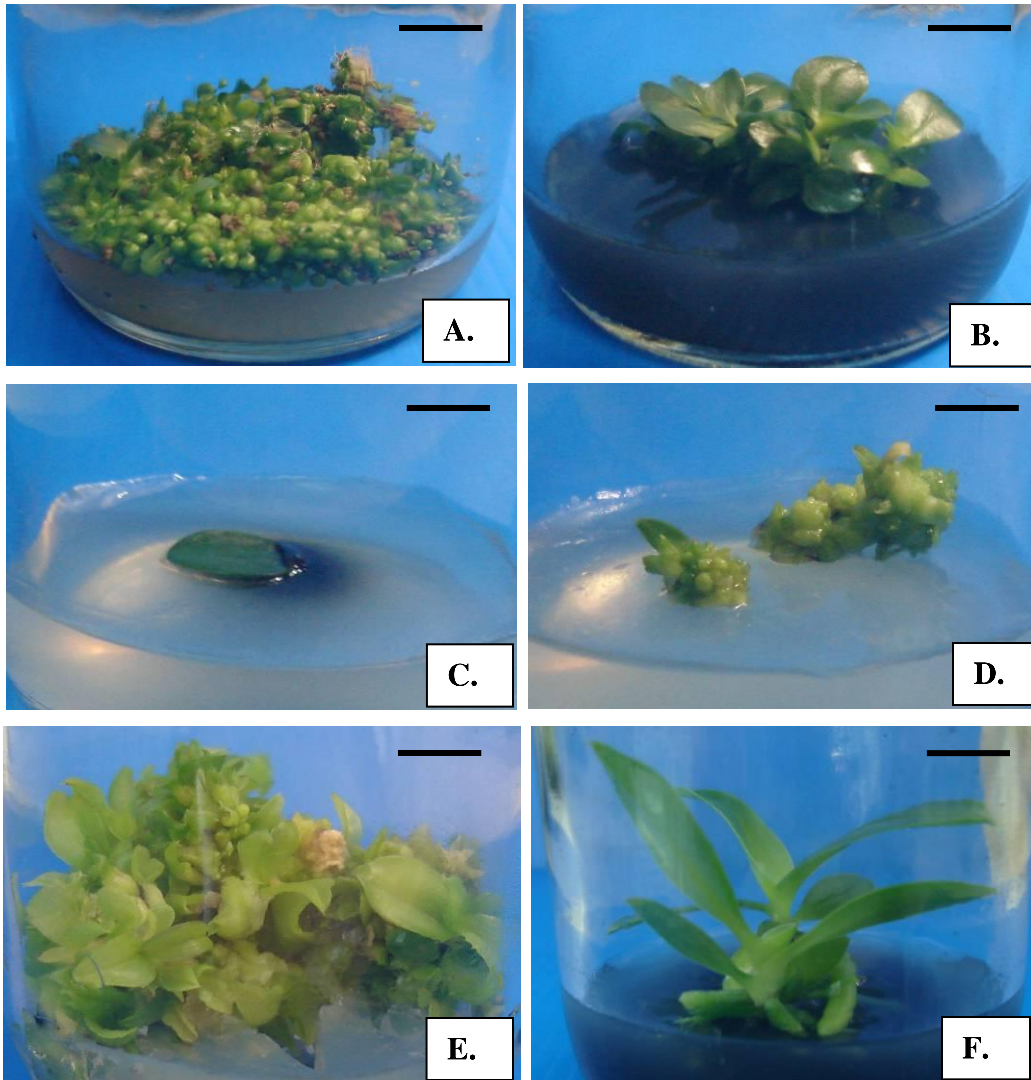

Fig. 2. Micropropagation of *P. cornu-cervi* (Breda) Blume & Rchb. f. through PLBs; (A) Asymbiotic germination of seeds from capsule after 2 months of culture on MS medium supplemented with 15% CW. (B) Three-month-old seedlings on MS medium supplemented with 15% CW and 0.2% AC. (C) Culture of leaf segment on ½ MS with or without PGR. (D) Development of new PLBs from culturing wounded protocorm on ½ MS medium containing 0.1 mg/l NAA and 0.1 mg/l TDZ. (E) Conversion of PLBs into shoots. (F) PLBs-derived plantlets on MS medium supplemented with 15% CW and 0.2% AC. Bar = 1 cm (A to F).

For plant regeneration PLBs from both the PGR-free medium and in the presence of NAA, TDZ or BA caused browning or blackening of media after 6 weeks of culture. However, this phenomenon did not inhibit the PLBs

development, which were transferred to hormone-free medium supplemented with 15% CW and 0.2% activated charcoal (AC) and kept under a 16-h photoperiod for 4 months. PLBs converted into healthy and vigorous plantlets with well-developed 3-4 leaves and 3-4 roots per shoot on the growth media (Figure 2F). The regenerated plantlets grew normally when transplanted to pots containing sphagnum moss in the net house with 100% survival rate (Figure 3). There were no obvious differences in morphology of TDZ-, NAA+TDZ- and BA-induced plantlets.

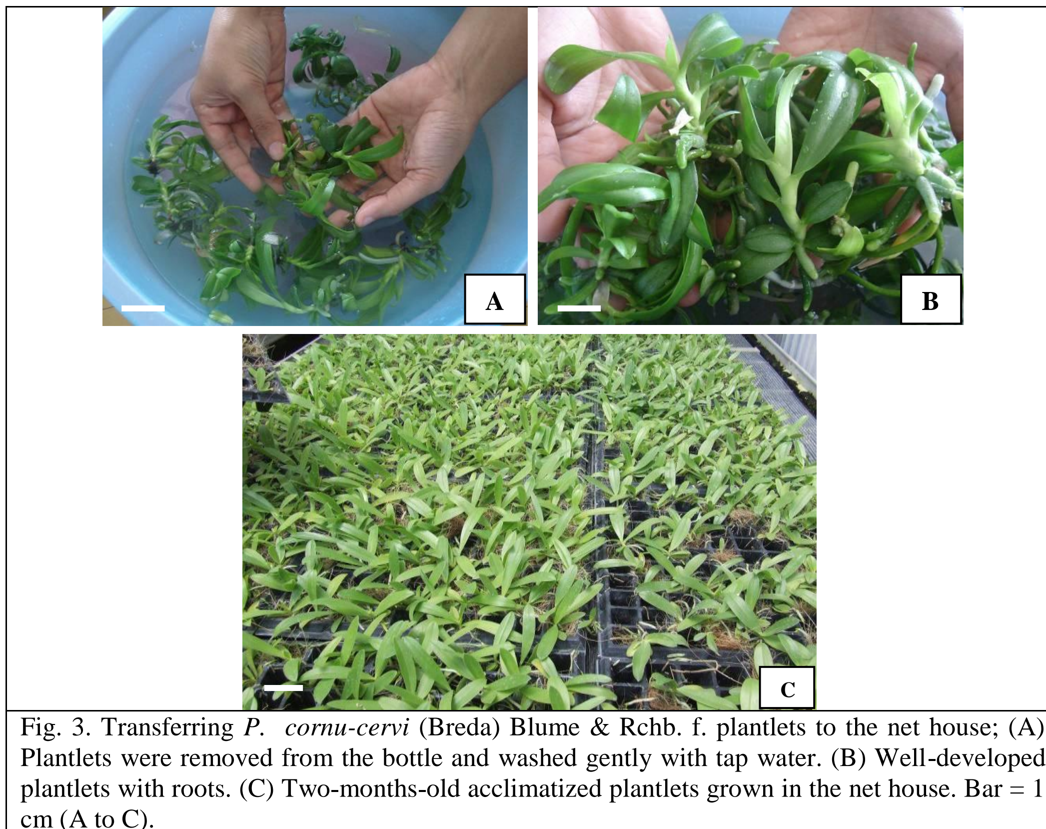

Fig. 3. Transferring *P. cornu-cervi* (Breda) Blume & Rchb. f. plantlets to the net house; (A) Plantlets were removed from the bottle and washed gently with tap water. (B) Well-developed plantlets with roots. (C) Two-months-old acclimatized plantlets grown in the net house. Bar = 1 cm (A to C).

Table 1. Effect of auxins and cytokinin on formation of PLBs in *P. cornu-cervi* (Breda) Blume & Rchb. f. The number of PLBs per explant was scored after 45 days after culture

***PGRs (mg/l)**			**Browning**	**PLBs formation**	**No. of PLBs per responding explant**		
NAA	TDZ	BA	(%)	(%)	S. size	M. size	L. size
0.0	0.0		5	30	2.0 ± 0.37^{ef}	3.0 ± 0.37^{cdef}	3.3 ± 0.55^{i}
	0.1		5	90	8.8 ± 4.11^{abcde}	4.6 ± 0.37^{bcd}	5.1 ± 0.82^{fgh}
	1.0		0	100	12.2 ± 0.39^{abc}	5.4 ± 0.16^{bcd}	5.5 ± 0.22^{fgh}
	3.0		10	80	5.2 ± 1.46^{cdef}	2.0 ± 0.65^{def}	4.6 ± 0.60^{ghi}
0.1	0.0		5	90	8.3 ± 2.54^{abcde}	5.3 ± 1.45^{bcd}	8.0 ± 0.53^{de}
	0.1		0	100	13.9 ± 4.16^{a}	10.7 ± 3.20^{a}	11.0 ± 0.74^{ab}
	1.0		5	70	9.7 ± 0.52^{abcd}	5.7 ± 0.92^{bcd}	6.6 ± 0.39^{ef}
	3.0		0	100	9.2 ± 3.04^{abcde}	6.2 ± 1.40^{bc}	8.4 ± 0.40^{cd}
1.0	0.0		5	90	0.0 ± 0.00^{f}	0.5 ± 0.38^{ef}	6.0 ± 0.64^{fg}
	0.1		5	90	8.5 ± 2.46^{abcde}	3.1 ± 0.61^{cdef}	4.7 ± 0.49^{ghi}
	1.0		0	100	13.2 ± 3.10^{ab}	8.0 ± 0.60^{ab}	11.5 ± 0.83^{a}
	3.0		0	90	0.0 ± 0.0^{f}	0.0 ± 0.0^{f}	9.7 ± 0.28^{bc}
0.0	0.0	0.1	5	90	3.6 ± 0.33^{def}	4.0 ± 0.44^{cde}	4.1 ± 0.11^{hi}
		1.0	0	100	3.1 ± 0.74^{def}	2.6 ± 0.27^{cdef}	5.2 ± 0.13^{fgh}
		3.0	10	80	2.8 ± 0.64^{def}	2.5 ± 0.33^{cdef}	4.5 ± 0.27^{ghi}
		10.0	10	80	6.0 ± 1.41^{bcdef}	4.5 ± 0.27^{bcd}	4.3 ± 0.26^{ghi}

*PGRs: Plant growth regulators. Values are presented as means ± SE. Different letters within a column indicate significant differences at $p \leq 0.05$ according to analysis of variance (ANOVA)

Discussion

No effect of plant growth regulators was observed in PLBs induction from leaf segments of *P. cornu-cervi* (Breda) Blume & Rchb. f. However, Nayak *et al*. (1997) reported that leaf segments of *Acampe praemorsa* could produce PLBs. Chen and Chang (2006) also reported PLBs formation from leaf explants of *P. amabilis* after 45 days of culture. The successful on PLBs induction from culturing leaf explant might be depend upon genotype and PGRs containing in culture medium. For *P. cornu-cervi* (Breda) Blume & Rchb. f. in the present study leaf explants 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.

Among four different explant types, the wounded protocorms culture method described here is an efficient *in vitro* technique for the rapid propagation of *P. cornu-cervi* (Breda) Blume & Rchb. f. In the PGR-free half-

strenght MS medium only about 30% of wounded protocorm developed into PLBs. The results obtained in the present study were similar to those reported by Yam *et al*., (1991) and Park *et al*., (2000) which found that trimmed protocorm at the basal part promoted PLBs multiplication in *Phalaenopsis* protocorms. In our culture system, wound treatment caused by the cutting process played an important role in the formation of new protocorms through proliferation. Our experiments show that wounding can bring about the production of a higher number of new protocorms on the original one, but it also caused the death of some of the wounded protocorms. It could be concluded that protocorms with wound produced new protocorms in greater numbers than those with untrimmed or unwounded bases.

Between the two cytokinins tested (BA, TDZ), TDZ was effective in the PLBs induction from wounded protocorm segments of *P. cornu-cervi* (Breda) Blume & Rchb.f. The types and concentrations of PGRs play an important role in *in vitro* propagation of many orchid species (Arditti and Ernst, 1993). TDZ was more effective than BA for inducing PLBs formation in *P. cornu-cervi* (Breda) Blume & Rchb.f. This study also clearly shows that TDZ alone was more effective than BA in PLBs induction and proliferation. This result is also in agreement with the observations in *Cymbidium ensifolium* var. *misericors.* (Chang and Chang, 1998), *Phalaenopsis* and *Doritaenopsis* (Ernst, 1994), *Epidendrum radicans* (Chen *et al*., 2002), *Doritaenopsis* (Park *et al*., 2003), and *Phalaenopsis* (Kuo *et al*., 2005). Recently, TDZ has been used in orchid tissue culture for various purposes due to its remarkable ability to induce callus or organogenesis. TDZ induced callus formation from various explants, especially when it was used along with either NAA or 2, 4-D (Huan and Tanaka, 2004). Induction of organogenesis and somatic embryogenesis using TDZ has been reported in several orchid species (Ernst, 1994; Chen and Piluek, 1995; Nayak *et al*., 1997; Chen and Chang, 2001).

Formation of PLBs can be classified into two types. The first is the direct formation of PLBs from protocorms, shoot tips, root tips, and stem segments through direct embryogenesis (Luo *et al*., 2008; Mayer *et al*., 2010; Naing *et al*., 2011). The second is the formation of PLBs through callus. (Hong *et al*., 2008; Huang and Chung, 2010; Ng and Saleh, 2011). In this present study, PLBs of *P. cornu-cervi* (Breda) Blume & Rchb.f. developed directly from the protocorms without callus formation.

PLBs from both the PGR-free medium and in the presence of NAA, TDZ or BA caused browning or blackening of media after 6 weeks of culture. Upon transfer PLBs to hormone-free medium supplemented with 15% CW and 0.2% AC, PLBs converted into healthy plants. Browning or blackening of cultured explants caused by wounding. This activity promoted the formation of phenolic

substances under the control of polyphenol oxidase. Tanaka and Sakanishi (1977) reported that phenolic exudation caused poor regeneration capacity in *Phalaenopsis* tissue culture. This inhibitory effect may be related to the size and differential sensitivity of various explants (Seeni and Latha, 1992). In plant tissue culture, AC was widely used to stimulate rooting of micropropagated shoots since it can absorb both inhibitory substances and cytokinins in the medium. Moreover, it is suggested that the AC favours the establishment of a balance of endogenous auxins and cytokinins that facilitates root formation by decreasing decomposition of endogenous IAA under the light condition (Pan and Staden, 1998). Eymar *et al*. (2000) observed that the addition of AC increased and maintained pH levels during culture, increased the nitrogen uptake and improved growth and visual aspects of the explants and reduced the inhibitory effect of exogenous cytokinin on root growth. In this present study, AC in media seems to reduce exudates, thus, enhanced protocorm development. The present study is the first report to show that PLBs can be induced and proliferated from wounded protocorm segments of *P. cornu-cervi* (Breda) Blume & Rchb.f. on culture media containing CW. However, the use of culture media supplemented with plant growth regulators solidified with agar also effective on initiation of PLBs and eventually regeneration into plantlets. These protocols are simple, inexpensive and bring about the production of a large number of plantlets by germinating seeds and PLBs induction in a short period of time. This research has demonstrated that of *P. cornu-cervi* (Breda) Blume & Rchb.f. can be successfully propagated via PLBs induction.

Conclusion

This is the first report on micropropagation of *P. cornu-cervi* (Breda) Blume & Rchb.f. The efficient initiation of PLBs and subsequently their conversion to plantlets by using 0.1 mg/l NAA in combination with 0.1 mg/l TDZ from wounded protocorm segments was successfully established. Well developed rooted plants using the AC containing PGRs free MS medium provided a simple and effective protocol for mass propagation of this valuable *Phalaenopsis* species.

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