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Effect of media and sucrose concentrations with or without activated charcoal on the plantlet growth of *P. cornu-cervi* (Breda) Blume&Rchb. f.

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Protocorm-like bodies (PLBs) of *Phalaenopsis cornu-cervi* (Breda) Blume & Rchbf. were derived from wounded protocorm segments culturing on ½ Murashige and Skoog, 1962 (MS) medium containing 0.1 mg/l NAA and 0.1 mg/l TDZ. When transferring these PLBs onto New Dogashima (ND) or MS medium supplemented with 0, 2, 3 or 4% sucrose with or without 0.2% activated charcoal (AC) for plant conversion. After 5 months of culture, the ND medium supplemented with 0.2% AC and 4% sucrose gave the best result, in term of fresh weight (1.309 g/explant), survival rate (100%), plantlet height (10.9 mm), leaf length (33.85 mm), leaf wide (13.45 mm), number of roots per explant (8.9) and root length (30.2 mm) without browning or necrotic tissues. Samples of the obtained plantlets had 100% of survival rate and grew well after transplanted into pots containing sphagnum moss, placed in a net house with about 60% shading and 80% relative humidity.

Keywords: Plant conversion, protocorm-like bodies, wounded protocorm, necrotic tissues, browning

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* grow on trees as epiphytes, but a few attach themselves to the surface of rocks as lithophytes. In Thailand, the genus *Phalaenopsis* comprises of 2-3 epiphytic orchid species, such as *Phalaenopsis cornu-cervi* (Breda)

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Blume & Rchb. f. and *Phalaenopsis decumbens* Holtt. This genus is a monopodial epiphytic orchid is difficult to propagate vegetatively (Kosir*et al*., 2004).

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. Tissue culture method acts as the powerful approach to propagate the number of plants. Micropropagation was different among plant species, types of explants and culture media. The source of carbon in culture medium is a very important component for proliferation of protocorm-like bodies (PLBs) in many orchids (Sopalun *et al*., 2010). Commonly used of carbon source is sucrose, glucose or fructose. Reports from several laboratories emphasize the importance of the source and concentration of sugars used to promote *in vitro* orchid seed germination, as well as plant growth (Ernest, 1967). However, browning of the PLBs of *P. cornu-cervi* (Breda) Blume & Rchb. f. is a problem during PLBs development. Browning is the result from the accumulation of phenolic compounds that causes loss of growth capacity and tissue death during culture. To solve these problems, activated charcoal (AC) is widely used in culture medium. AC has a very fine network of pores with large inner surface area on which many substances can be adsorbed. AC is often used in tissue culture to improve cell growth and development (Pan and Staden, 1998). It plays a critical role in orchid seed germination of *Cypripedium flavum* (Yan *et al*., 2006) etc. The addition of AC to both liquid and semi-solid media is a recognized practice and its influence in growth and development may be attributed mainly to the adsorption of inhibitory compounds in the culture medium and substancially decreasing the toxic metabolites, phenolic exudation and brown exudate accumulation. (Fridborg *et al*., 1978).

The aim of study was to evaluate the effect of media and sucrose concentrations with or without AC on the plantlet growth of *P. cornu-cervi* from PLBs.

Materials and methods

*Plant material*s

Six-month-old green pods by self-pollination were collected from 5-year-old plants of *P. cornu-cervi* (Fig. 1A). 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 aseptically half longitudinally on a sterile Petri dish and the seeds were aseptically sown on the surface of Murashige and Skoog, 1962 (MS) medium with 3% sucrose and 0.75% agar-agar (commercial grade) in bottles, each containing 25 ml 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 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). After 2 to 3 months of culture, these seeds germinated into protocorms at GI4 (about 5 mm-long) (Fig. 1B). These wounded protocorms segments were cultured on 1/2 MS medium supplemented with 0.1 mg/l NAA and 0.1 mg/l TDZ combination for induction of protocorm-like bodies (PLBs).

Effect of media formula and sucrose concentrations with or without activated charcoal on the plantlet growth of P. cornu-cervi

The PLBs (Fig. 1C) were excised and transferred to New Dogashima (ND) medium (Tokuhara and Mii, 1993) or MS medium supplemented with 0, 2, 3 or 4% sucrose with or without 0.2% (w/v) activated charcoal (AC) for plantlet regeneration. All culture media were added with 15% (v/v) coconut water (CW). The culture medium was solidified with 0.75% agar-agar (commercial grade). The pH of the ND and MS medium were adjusted to 5.2 and 5.6, respectively with 1 N KOH or 1 N HCl prior to autoclaving for 15 min at 121 °C. Whole explants were placed on the surfaces of these medium and the cultures were 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 survival percentage [% survival= (number of survival plantlets/number of total PLBs inoculated) x 100], fresh weight, plantlet height, number of leaves per plantlet, leaf length, leaf width, number of roots per plantlet and root length were calculated, recorded and statistically compared after 5 months of culture using completely randomized design (CRD).Analysis of variance and Duncan's multiple range test were used for comparison among treatment means. At least twenty cultures were raised for each treatment and all experiments were repeated two times.

Transplantation of plantlets

Plantlets were rinsed thoroughly with tap water to remove residual nutrients and agar from the plant body. The plantlets were then transplanted to pots containing sphagnum moss. The seedlings were grown in the net house with about 60% shading and 80% relative humidity. The young plants were sprayed with water twice a day.

Results

The PLBs were excised and transferred to ND or MS medium supplemented with various concentrations of sucrose (0, 2, 3 and 4%) with or without the presence of AC for development of plantlets. Here also the presence of AC in the medium resulted in a better response than medium containing sucrose alone.

The PLBs grow on both ND and MS agar medium. Significant differences were observed between culture media and concentrations of sucrose for survival rate, fresh weight, plantlet height (Table 1), number of leaves per plantlet, leaf length, leaf width, number of roots per plantlet and root length (Table 2). PLBs enlarged in their size after 2 month of cultured, subsequent to development into small plantlets after 3 month of culture. To study the type of media and effect of carbon sources on plant regeneration from PLBs, different concentrations of sucrose were added in the medium. Higher concentration of sucrose being tested gave a higher fresh weight than lower concentration (Table 1).

In our study, the sucrose concentration of media affected the survival rate of plantlets from PLBs in both media containing with or without AC. On the other hand, survival rate of plantlets on media without sucrose was decreased. However, a critical problem during culture is that tissue browning resulting from phenolic accumulation occurs and this causes loss of growth capacity (Table 1). This important problem could be solved using 0.2% AC as a medium addendum.

Of the sixteen different media used, the best result is obtained when PLBs segments were cultured on ND medium supplemented with 4% sucrose with AC (Table 1, 2). After 5 months of culture, sucrose at 0, 2, 3 or 4% concentration showed significant potency of plantlet growth in *P. cornu-cervi.* On this medium, 100% cultures responded and the seedlings showed comparatively faster growth. The survival rate of PLBs cultured on ND, MS, ND medium supplemented with AC or MS medium supplemented with AC was about 70-90%, 75-90%, 95-100% and 85-89% respectively.

In this present study, ND medium supplemented with 4% sucrose and containing AC gave the highest survival rate (100%), plantlet height (10.9 mm), fresh weight (1.309g), number of leaves per plantlet (6.1 leaves), leaf length (33.85 mm) leaf width (13.45 mm), number of roots per plantlet (8.9 roots) and root length (30.2 mm) (Figure 2D). Therefore, this medium was suitable for the conversion of PLBs into plantlets. The sucrose concentration in both culture media affected the survival rate, plantlet height and fresh weight of plantlets from PLBs. On the other hand, development of plantlets on culture media with sucrose was better than without.

An early response of PLBs was the production of phenolic exudates from the cut ends and intense browning of the surrounding ND or MS media (Fig. 2A-B), during the first 2 months. However, this did not inhibit the development of PLBs, which were embedded in AC containing media (Fig. 2C-E). In this present study, AC in media seems to reduce exudates caused inhibition because they enhanced PLBs development. PLBs converted into healthy plants with well-developed leaves and roots when cultured on medium supplemented with AC and kept under a 16-h photoperiod for 5 months. In treatments without AC, slow growth was observed. In this report the *P. cornu-cervi* plantlets had greater height on ND medium containing AC than all other treatments. Effect of AC was observed on the root formation. PLBs developed into plants with well-developed 8.9 roots and 30.2 mm root length per plantlet when cultured on ND medium containing 4% sucrose and supplemented with AC (Fig. 2D). The regenerated PLBs easily developed into plantlets on this medium. The results showed that AC adsorbed phenolic compound on modified ND or MS medium. The survival rate of plantlets was increased when AC was added into ND or MS medium. AC addition in culture medium of *P. cornu-cervi* plantlets may cause better development of root and aerial parts of plants without any addition of exogenous auxins and/or cytokinin.

After 6 months of culture on ND medium supplemented with 4% sucrose and containing AC, complete plantlets were formed. Fully developed plantlets rooted well *in vitro* with good shoot and root formation (Fig. 2D). Plantlets (Fig. 3A) were removed from the bottle, washed twice with tap water to remove traces of agar and transplanted into pots filled with sphagnum moss. They were successfully acclimatized and grown in net house with about 60% shading and 80% relative humidity. After 2 months, survival was 100% from 100 plantlets (Fig. 3B). There were no obvious differences in morphology of plantlets and no phenotypic variations were observed among them during their vegetative growth. There is a regenerated plant flowered normally 3 months after transfer to greenhouse (Fig. 3C).

The present study was the first report to show that PLBs can be induced from wounded protocorm segments of *P. cornu-cervi* on 1/2 MS medium supplemented with 0.1 mg/l NAA and 0.1 mg/l TDZ combination. The use of media supplemented with suitable sucrose and containing AC was also effective for development of PLBs into complete plantlets. One advantage of this medium is that it does not require any addition of hormones. This protocol is simple, inexpensive and brings about the production of plantlets. This research has demonstrated that of *P. cornu-cervi* can be successfully propagated via PLBs induction and development into complete plantlets.

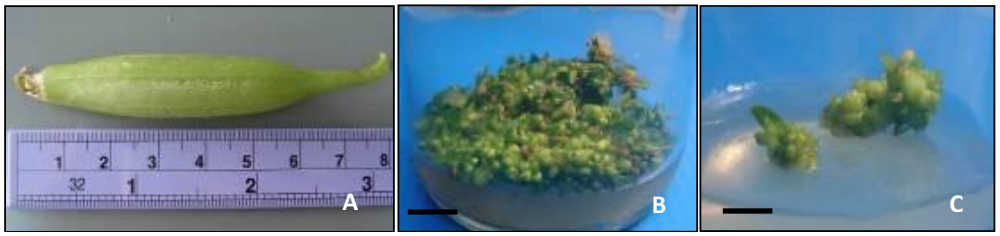

Fig. 1. A 6-month-old green pod of *P. cornu-cervi* ; A), asymbiotic germination of seeds from pod after 2 months of culture on MS medium supplemented with 15% CW (bar=1 cm); B), development of new PLBs from culturing wounded protocorm on ½ MS medium containing 0.1 mg/l NAA and 0.1 mg/l TDZ (bar=1 cm); C).

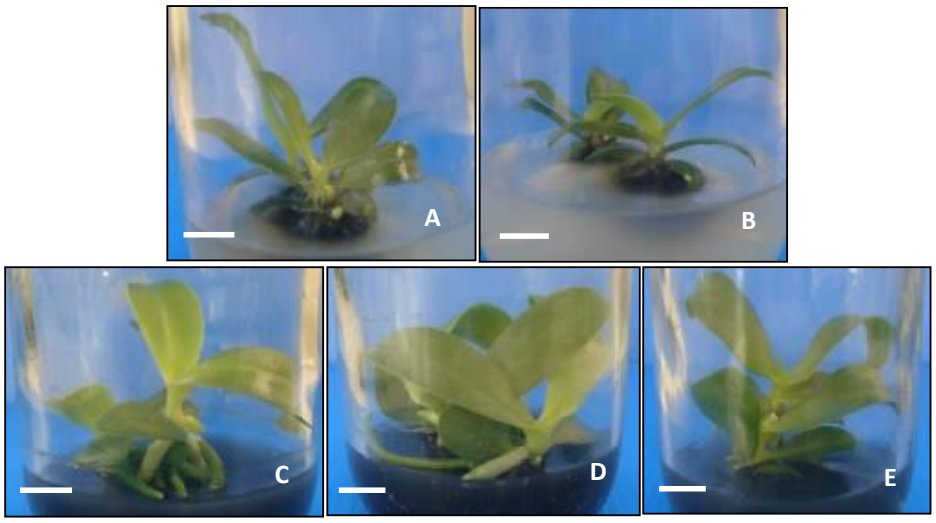

Fig. 2. Five-month-old plantlets converted from the protocorm-derived PLBs on ND medium supplemented with 3% sucrose; A), MS medium supplemented with 3% sucrose; B), ND medium containing 3% sucrose and supplemented with AC; C), ND medium containing 4% sucrose and supplemented with AC; D) and MS medium containing 3% sucrose and supplemented with AC; E) (bar=1 cm).

Fig. 3. Plantlets were obtained after 6 months of culture on ND medium supplemented with 4% sucrose and containing AC; A), 2-months-old; B) and 3-months-old acclimatized plantlets grown in the net house; C (bar=1 cm).

Table 1. Survival rate and plantlet growth from PLBs of *P. cornu-cervi* after 5 months of culture on 2 media with 4 levels of sucrose.

Media	**Sucrose (%)**	**Survival rate (%)**	**Plantlet height (mm) (Mean± S.E.)**	**Fresh weight (g) (Mean± S.E.)**
Without activated charcoal				
ND	0	70.00	5.1±0.10^{h}	0.260±0.01^{g}
	2	80.00	6.3±0.15^{g}	0.360±0.02^{fg}
	3	100.00	7.1±0.10^{f}	0.494±0.03^{def}
	4	90.00	7.2±0.13^{f}	0.561±0.04^{de}
MS	0	75.00	4.3±0.21^{i}	0.240±0.09^{g}
	2	94.44	4.2±0.36^{i}	0.248±0.02^{g}
	3	92.86	3.7±0.15^{i}	0.392±0.03^{efg}
	4	90.00	4.2±0.20^{i}	0.311±0.03^{fg}
With activated charcoal				
ND	0	95.00	9.4±0.16^{bc}	0.601±0.05^{d}
	2	95.00	10.1±0.18^{b}	0.785±0.07^{c}
	3	100.00	10.0±0.00^{b}	0.998±0.07^{b}
	4	100.00	10.9±0.31^{a}	1.309±0.10^{a}
MS	0	85.00	7.9±0.38^{ef}	0.370±0.06^{efg}
	2	100.00	7.4±0.37^{f}	0.498±0.07^{def}
	3	100.00	9.1±0.62^{cd}	0.503±0.07^{def}
	4	88.88	8.4±0.40^{de}	0.595±0.13^{d}

Similar letters within columns mean no significant difference at $P \leq 0.05$ by DMRT.

Table 2. Leaf and root growth from PLBs of *P. cornu-cervi* after 5 months of culture on 2 media with 4 levels of sucrose with or without activated charcoal

Media	Sucrose (%)	No.of leaves/ plantlet (Mean±S.E.)	leaf length (mm) (Mean±S.E.)	leaf width (mm) (Mean±S.E.)	No.of roots/ plantlet (Mean±S.E.)	root length (mm) (Mean±S.E.)
Without activated charcoal						
ND	0	5.7±0.30^{bc}	16.95±0.73^{def}	6.90±0.29^{hi}	3.9±0.35^{fgh}	8.25±0.37^{de}
	2	6.2±0.36^{abc}	18.90±1.08^{bcde}	8.45±0.41^{efg}	5.1±0.31^{efg}	16.70±1.47^{c}
	3	6.9±0.23^{ab}	21.35±1.50^{b}	11.30±0.50^{b}	6.5±0.50^{cd}	24.40±2.77^{b}
	4	6.4±0.58^{abc}	17.10±1.09^{cdef}	10.00±0.45^{bcd}	5.7±0.42^{de}	25.75±2.21^{b}
MS	0	7.0±0.52^{a}	13.35±0.53^{fg}	6.70±0.32^{i}	3.7±0.30^{gh}	4.45±0.64^{e}
	2	6.3±0.26^{abc}	13.30±0.46^{fg}	7.05±0.34^{ghi}	4.0±0.37^{fgh}	6.45±0.65^{e}
	3	6.6±0.31^{abc}	16.00±0.75^{ef}	8.10±0.19^{fghi}	5.2±0.42^{def}	6.00±0.81^{e}
	4	6.4±0.31^{abc}	11.70±0.47^{g}	7.10±0.25^{ghi}	4.9±0.35^{efg}	4.15±0.64^{e}
With activated charcoal						
ND	0	6.0±0.21^{abc}	20.90±1.45^{bc}	10.45±0.67^{bc}	4.4±0.22^{efg}	12.75±0.93^{cd}
	2	6.6±0.52^{abc}	31.55±1.87^{a}	14.05±0.65^{a}	7.1±0.43^{bc}	25.20±2.12^{b}
	3	6.4±0.31^{abc}	30.95±1.01^{a}	13.10±0.42^{a}	8.3±0.65^{ab}	21.35±2.14^{b}
	4	6.1±0.28^{abc}	33.85±1.63^{a}	13.45±0.39^{a}	8.9±0.50^{a}	30.20±1.85^{a}
MS	0	5.4±0.37^{c}	18.10±0.89^{bcde}	8.25±0.42^{efgh}	2.9±0.31^{h}	5.15±0.65^{e}
	2	7.0±0.33^{a}	21.80±1.77^{b}	9.65±0.56^{cde}	4.2±0.36^{fgh}	7.70±0.90^{e}
	3	6.8±0.29^{ab}	21.15±1.28^{b}	10.70±0.55^{bc}	4.9±0.38^{efg}	8.65±1.24^{de}
	4	7.2±0.42^{a}	20.10±1.95^{bcd}	9.00±0.66^{def}	5.3±0.83^{def}	8.90±2.12^{de}

Similar letters within columns mean no significant difference at $P \leq 0.05$ by DMRT.

Discussion

ND medium supplemented with 4% sucrose and containing AC gave the highest survival rate, plantlet height, fresh weight, number of leaves per plantlet, leaf length, leaf width, number of roots per plantlet and root length. This medium was suitable for the conversion of PLBs into plantlets. Generally, 2-4% w/v sucrose was used as carbon source in culture medium. These results suggest that the growth of monopodial orchid is influenced by sucrose and the optimal concentration of sucrose depended on species. The optimum medium composition was important for successful micropropagation. *In vitro* condition has low CO_2 concentration and not sufficient light energy. So carbon sources are very important components for *in vitro* culture. Sucrose is widely used but there are other sugars that are also used for orchid culture, such as glucose, fructose, sorbitol, maltose and trehalose (Islam *et al*., 1998). In addition to somatic embryogenesis, the carbohydrate source has also been reported to be an important parameter in the conversion of embryos to plantlets. Jheng *et al*. (2006) reported that higher concentrations (1 and 2%) of the different types of carbohydrate source (sucrose, maltose and trehalose) being tested gave a higher fresh weight than lower concentration (0.5%). The source of carbon is a very important component in *in vitro* culture media. Carbon sources are added to the

culture medium because of light energy deficiency and low CO_2 concentration present in *in vitro* conditions. Plants cultures *in vitro* often show a low photosynthetic rate and incomplete autotrophy (Faria *et al*., 2004). Sugar acts as a carbon and energy source and also acts as an osmotic regulator in the induction medium. Sucrose is commonly used in tissue culture media. Faria *et al*. (2004) reported that the presence of 6% sucrose in the medium was the most efficient treatment for increasing height and fresh weight of *Dendrobium nobile in vitro* culture.

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 this present study, AC in media seems to reduce exudates caused inhibition because they enhanced PLBs development. PLBs converted into healthy plants with well-developed leaves and roots when cultured on medium supplemented with AC and kept under a 16-h photoperiod for 5 months.

In plant tissue culture, AC was widely used to stimulate rooting of micropropagated shoots since it can adsorb both inhibitory substances and cytokinins in the medium. Moreover, it is suggested that the AC favors the establishment of a balance of endogenous auxins and cytokinins that facilitates root formation by decreasing decomposition of endogenous, IAA under light condition (Pan and Staden, 1998). The beneficial effects of AC could be due to positive stimulation of many development processes (Van Winkle and Pullman, 2006) and its ability to absorb the phenolics, which can injure living tissues. Since AC is also known to adsorb gases, it is possible to speculate that some of its effects are ethylene adsorption. Good growth and development of *Phalaenopsis* plantlets *in vitro* were obtained when culture media were supplemented with 0.2% (w/v) AC (Hinnen *et al*., 1989; Ernst, 1994) or 0.5% (v/v) AC (Park *et al*., 2000). Similar observations were also reported with *Vanda coerulea* Griff ex. Lindl (Seeni and Latha, 2000). AC increased the number and the length of roots. AC has beneficial and harmful effects in culture medium, depending upon the medium, explants, and plant growth regulators used. The beneficial effects of AC on tissue responses *in vitro* could be attributed to provide a dark environment by darkening the medium (Dumas and Monteuuis, 1995), adsorption of harmful substances produced by either the media or explant (Fridborg and Eriksson, 1975; Fridborg *et al*., 1978), adsorption of plant growth regulators and other organic compounds (Nissen and

Sutter, 1990; Weatherhead *et al*., 1978) or to release of substances naturally present in or adsorbed by AC (Ernst, 1975; Johansson *et al*., 1990).

Conclusion

ND medium supplemented with 4% sucrose and containing AC was suitable for conversion of PLBs of *P.cornu-cervi* into complete plantlets when considering survival rate, plantlet height, fresh weight, number of leaves per plantlet, leaf length, leaf width, number of roots per plantlet and root length.

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