Organic growth supplement stimulants for *in vitro* multiplication of *Cymbidium pendulum* (Roxb.) Sw.

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Abstract

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The aim of this study was to establish protocol for *in vitro* regeneration and multiplication of *Cymbidium pendulum* through protocorms, as explants using organic growth supplements such as banana homogenate, coconut water, and peptone. The effect of growth supplements was tested on multiplication (*neo*-formation of secondary protocorms from primary protocorms) of protocorms, development of maximum number of shoots and early plantlets in M medium (Mitra medium). Though the explants regenerated in the absence or presence of growth adjuncts in the medium; the regeneration frequency was found significantly higher in organic growth supplement-enriched medium than control. The protocorm segments (primary) regenerated into protocorm-like bodies (secondary protocorms) which eventually differentiated into shoots; no intervening callus stage was observed. Among the treatments, the highest regeneration frequency, robust shoots and root formation was recorded in banana homogenate (50 g/l); the roots were lengthy, thick, and covered all over the surface by root hairs. Higher concentration of banana homogenate (75 g/l) proved detrimental for the survival of cultures; the protocorm-like bodies showed necrosis and they perished soon after. Coconut water (10%) and peptone (2 g/l) supplemented medium proved optimum for profuse multiplication of protocorm-like bodies (PLBs) their early growth into plantlets. The treatments with organic growth supplements gave better and early plantlets as compared to control. Among these tested organic growth supplements, peptone (2 g/l) and coconut water (10%) proved beneficial for multiplication of regenerants, maximum number of shoots formation and early plantlet development; whereas banana homogenate (50 g/l) favoured the highest regeneration frequency and healthy growth of plantlets.

Keywords: banana homogenate; coconut water; orchids; peptone; protocorms; regeneration

Ever since Arditti (1967) suggested that complex growth supplements have the ability to influence *in vitro* regeneration, multiplication of protocorm-like body(s) (PLBs) and growth of orchid seedlings since than a variety of organic growth supplements such as apple juice, banana homogenate (BH), beaf extract, casein hydrolysate, coconut water (CW), corn extract, tomato juice, peptone, yeast extract etc. are tested for promoting multiplication, growth and development of *in vitro* cultures. Growth rate of the tissues can be increased by the addition of organic supplements and plant extracts (Fonnesbech 1972). Effects of organic growth additives are tested in a large number of orchids such as *Paphiopedilum* species (Flamee 1978), *Vanda* hybrids (Mathews, Rao 1980), *Acampe praemorsa* (Krishnamohan, Jorapur 1986), *Aranda* (Goh, Wang 1990), *Cattleya*, *Encyclia*, *Oncidium* and *Stanhopea* (Villolobus, Munoz 1994), *Dendrobium* species (Sudeep et al. 1997), *Geodorum densiflorum* (Bhadra, Hossain 2003), *Doritaenopsis*

(Chowdhury et al. 2003), *Cypripedium formosanum* (Lee, Lee 2003), *Dendrobium* hybrid (Lekha Rani et al. 2005), *Phalaenopsis gigantea* (Murdad et al. 2006), *Dendrobium* species (Aktar et al. 2008) and in *Zygopetalum mackayi* (Hong et al. 2010). Tissue culture techniques offer a viable system for true-to-type rapid mass multiplication and germplasm conservation of rare, endangered, threatened, floriculturally important, aromatic and medicinal plants (Arora, Bhojwani 1989; Sharma et al. 1991; Sudha, Seeni 1994; Sahoo, Chand 1998; Karuppusamy, Pullaiah 2007; Jawahar et al. 2008). Orchids are rare, endangered and threatened species all over the world. Several factors are responsible for their present condition such as deforestation, fragmentation of habitat especially in tropical regions, increased use of fertilizers, excessive exploitation of soil, and over collection (Znaniecka et al. 2005) and *C. pendulum* is no exception among orchids.

Cymbidium pendulum (Roxb.) Sw., (Orchidaceae) an epiphytic species of highly floriferous orchids is the most popular among the leading cut flower crops in the world. It has tremendous potential as a progenitor of meritorious hybrids of international repute. Being ornamental species for its delightful blossoms it is horticulturally important species, used in international floriculture trade. It is popularly used in home gardens and landscapes in most parts of the world. The species is also known for its therapeutic uses. It has been prescribed as herbal medicine for improvement and strengthening of weak lungs and relief of coughs (Chen, Tang 1982). Due to its high horticultural and therapeutic importance, the species has been subjected to commercial collection pressures which far exceed its natural regeneration. It is, therefore, strongly emphasized to multiply the species, through tissue culture techniques. Therefore, present studies were conducted with the aim to multiply the species *in vitro* using protocorm segments and testing the efficacy of growth supplements on *in vitro* multiplication of protocorms and early development of *C. pendulum* plantlets without the use of any growth regulators.

MATERIAL AND METHODS

Explant material, culture medium and procurement of growth supplements

Cymbidium pendulum protocorms (2.0 mm long) were procured from 28-week old *in vitro* cultures. The protocorms were maintained for 2 weeks on basal Mitra medium (M medium) (Mitra et al. 1976). Medium supplemented and 2.0% (w/v) sucrose (Daurala sugar works, Daurala, India) was used as source of nutrition and gelled with 0.9% (w/v) agar (Hi-Media, Mumbai, India) was used as control.

The complex growth supplements such as banana homogenate (25, 50 and 75 g/l w/v), coconut water (10, 20 and 30% v/v), peptone (1.0, 1.5 and 2.0 g/l) were used individually in the medium. Banana homogenate was obtained from ripened fruits purchased from market. Required quantities of pulp was weighed, homogenised in a blender and added to the medium. Coconut water, was collected from fresh green coconuts, filtered and used as such in the medium. Peptone (Hi-Media, Mumbai, India) was used in the medium.

The pH of medium was adjusted to 5.8 after adding the organic growth supplements. The medium was dispensed in test tubes of size (25 mm × 150 mm) and autoclaved at 121°C at pressure of 1.06 kg/cm^2 for 15 min. Autoclaved medium was kept at 37°C to check any further contamination.

Inoculations and incubation conditions

The inoculations were done under aseptic conditions in a laminar air flow cabinet. All the cultures were incubated at 25 ± 2°C under 12 h photoperiod of 3,500 lux light intensity (Fluorescent tubes 40 W; Philips India, Ltd., Mumbai, India). Subculturings were done as and when required. The experiment was repeated twice.

Observations and statistical analysis

The cultures were observed regularly under binocular microscope (Olympus SZX10, Tokyo, Japan) and data recorded accordingly. The results were tested using one-way ANOVA test and were analysed using the Tukey Multiple Comparison using SPSS (version 16) software package (SPSS Inc., Chicago, USA).

RESULTS

The efficacy of growth supplements was positively tested (Tables 1–3, Fig. 1a–c) on *in vitro* regeneration and multiplication of *Cymbidium pendulum* protocorm segments. In control (M medium) only

8.75% explants regenerated and developed into a single plantlet per explant after 18.75 weeks. Addition of organic growth supplements favoured early initiation of response in the cultures and multiplication of the regenerants. Banana homogenate (50 g/l) when added to basal medium showed a significant difference in terms of percentage of responding explant, up to 80.50% explants regenerated (Table 1). The resultant shoots were robust in size (Fig. 1a), the roots were lengthy, thick and covered all over the surface by hairy outgrowths at later stage of development. In this concentration, shoots and roots developed more uniformly to highest the average lengths. Higher concentration of BH (75 g/l) reduced the regeneration frequency, even the growth of regenerants was also retarded; the regenerated PLBs failed to differentiate further into shoots and roots (Table 1). They turned brown and perished soon after.

In coconut water (10%) enriched medium the explants regenerated with 73.75% frequency. The rate of PLB multiplication was higher in CW 10% as compared to control and other concentrations of CW used (Table 2). The regenerants multiplied profusely (Fig. 1b). Almost 9.0 shoots per explant were formed and the developmental processes leading to plantlet formation were also accelerated. Plantlets with 2–3 leaves and 1–2 roots were obtained within 11.25 ± 0.40 weeks. Likewise CW 10%, peptone (2 g/l) in the medium also favoured multiplication of neoformations (Fig. 1c) and early plantlet development within 11.75 weeks of culture; a maximum

Table 1. The effect of different quantity of banana homogenate (g/l) on *in vitro* multiplication of *C. pendulum* protocorms in M medium

Additive	Regeneration response (%)	Number of PLBs/explant	Number of shoots/explants	Complete plantlets (wks)
M	8.75 ± 1.2^{bcd}	1.00 ± 0.4^{c}	1.00 ± 0.4^{bcd}	18.75 ± 6.5^{d}
M + BH_{25}	21.75 ± 1.1^{ac}	1.75 ± 0.2^{c}	2.25 ± 0.2^{acd}	21.25 ± 0.7^{d}
M + BH_{50}	80.50 ± 2.1^{abd}	4.50 ± 0.28^{abd}	7.75 ± 0.2^{abd}	14.50 ± 1.0^{b}
M + BH_{75}	25.00 ± 2.0^{ac}	1.25 ± 0.2^{c}	0.00^{abc}	0.00^{abc}

M – Mitra medium; PLBs – protocorm-like bodies; BH – banana homogenate [concentration = g/l (w/v)]; Values in a column with similar superscripts are not significantly different at $p \leq 0.05$

Table 2. The effect of different concentrations of coconut water (%) on *in vitro* multiplication of *C. pendulum* protocorms in M medium

Additive	Regeneration response (%)	Number of PLBs/explant	Number of shoots/explant	Complete plantlets (wks)
M	8.75 ± 1.2^{bcd}	1.00 ± 0.4^{c}	1.00 ± 0.4^{bcd}	18.75 ± 6.5^{d}
M + $CW_{10\%}$	73.75 ± 1.20^{acd}	5.00 ± 0.40^{acd}	9.00 ± 0.70^{acd}	11.25 ± 0.40^{a}
M + $CW_{20\%}$	68.75 ± 1.25^{abd}	1.75 ± 0.47^{a}	2.50 ± 0.28^{ab}	21.25 ± 0.70^{d}
M + $CW_{30\%}$	61.25 ± 1.18^{abc}	1.75 ± 0.25^{a}	2.50 ± 0.20^{ab}	17.00 ± 0.40^{b}

M, PLBs – see Table 1; CW – coconut water [concentration = % (v/v)]; Values in a column with similar superscripts are not significantly different at $p \leq 0.05$

Table 3. The effect of different quantity of peptone (g/l) on *in vitro* multiplication of *C. pendulum* protocorms in M medium

Additives	Regeneration response (%)	Number of PLBs/explant	Number of shoots/explant	Complete plantlets (wks)
M	8.75 ± 1.2^{bcd}	1.00 ± 0.4^{c}	1.00 ± 0.4^{bcd}	18.75 ± 6.5^{d}
M + P_1	22.50 ± 1.4^{acd}	1.50 ± 0.20^{cd}	2.00 ± 0.4^{d}	19.25 ± 0.47^{cd}
M + $P_{1.5}$	31.25 ± 2.3^{abd}	2.75 ± 0.25^{abd}	3.25 ± 0.62^{ad}	15.25 ± 0.75^{abd}
M + $P_{2.0}$	71.25 ± 1.2^{abc}	4.75 ± 0.28^{abc}	9.00 ± 0.50^{abc}	11.75 ± 0.85^{abc}

M, PLBs – see Table 1; P – peptone [concentration = g/l (w/v)]; Values in a column with similar superscripts are not significantly different at $p \leq 0.05$

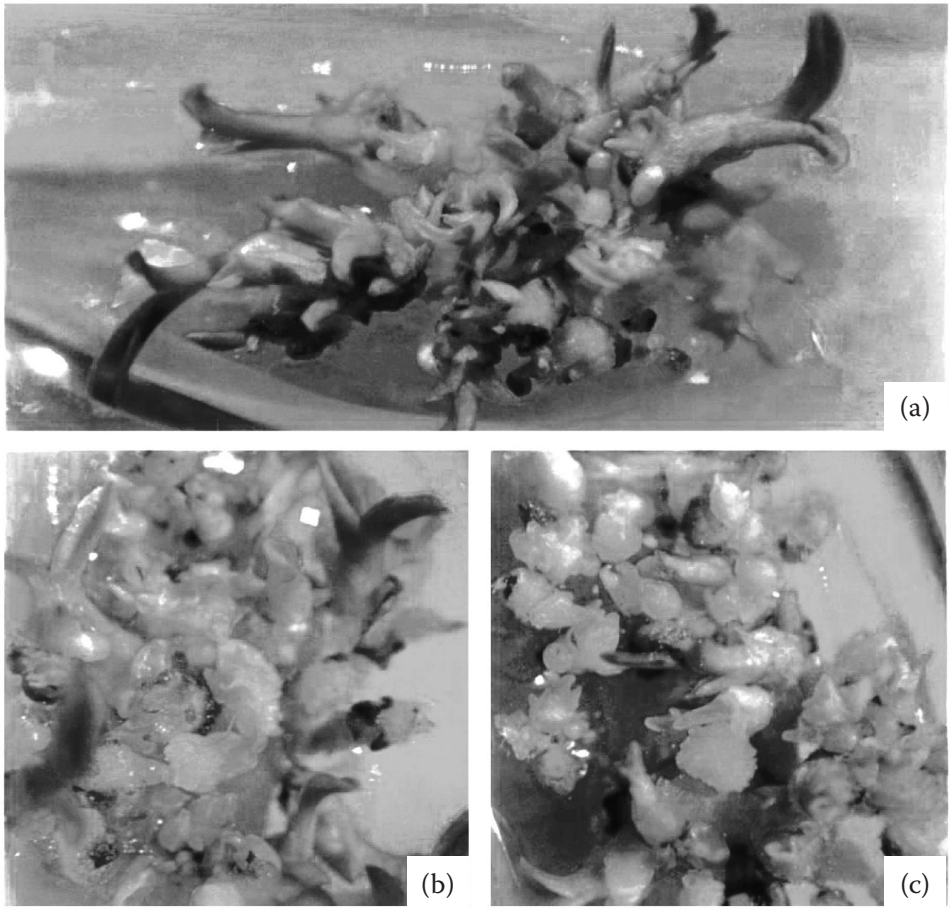

Fig. 1. Organic growth supplements and *in vitro* multiplication of *C. pendulum* protocorms in M medium. (a) development of healthy shoots in M + BH (50 g/l), (b) profuse multiplication of PLBs in M + CW (10%), (c) M + P (2 g/l)

of 9.0 shoots per explant developed in the cultures (Table 3).

DISCUSSION

Presently, the regeneration potential of protocorm explants (in terms of percentage of regeneration, average number of PLBs and shoots/explant) was markedly influenced by quality and quantity of organic growth supplements added to the medium. The explants elicited regeneration response in the basal medium with a very low regeneration frequency. The percentage of regeneration response was increased by addition of organic growth supplements in the cultures. Earlier Arditti (1979) made similar conclusions indicating the beneficial effects of organic growth supplements (BH, CW, peptone) on growth and differentiation of protocorms and further seedling development. It was also earlier reported by Chen and Chang (2002) that at an efficient concentration, organic and inorganic nitrogen sources can promote the growth of explants. In our experiment, 50 g/l of banana homogenate proved beneficial for development of healthy shoots growth of *C. pendulum*. The positive effects of BH (50 g/l), in the present cultures, in inducing the highest regeneration frequency and subsequent development of healthy shoot system (long and robust shoots) from PLBs, could be attributed to the higher content of sucrose concentrations in BH as earlier also suggested by Aktar et al. (2008) in enhancing *in vitro* regeneration of *Dendrobium* orchid PLBs. In *Vanda* species BH (10%) increased the shoot length. Beneficial effects of BH (10%) on leaf size of *Spathoglottis kimballianai* is reported by Minea et al. (2004). Banana homogenate significantly increased the number of leaves in *Dendrobium nobile* cultures (Sudeep et al. 1997). In our cultures, a higher concentration of BH 75 g/l proved detrimental to growth of cultures.

Coconut water (10%) and peptone (2 g/l) was found to be effective in multiplying PLBs and early plantlet development. Similar growth promoting

effects of these growth supplements in *Dendrobium* hybrid (Lekha Rani et al. 2005) is earlier reported. Growth promontory nature of CW is related to its ability of inducing cell divisions in non-dividing cells hence promoting early protocorm differentiation (Intuwong, Sagawa 1973). Addition of CW (15%) to the basal medium increased growth of the cultures and the shoots vigorously rooted in epiphytic orchids (McIntyre et al. 1974). Beneficial effect of CW in enhancing foliar growth of orchids *in vitro* grown seedlings is already on records and it is correlated to the fact that presence of a cytokinin (Kinetin) in coconut water is the probable cause of growth of cultures (Bhasker 1996). CW successfully initiated differentiation of shoots from PLBs of *Vanda teres* (Sinha, Roy 2004) and has helped in achieving high-frequency multiplication of PLBs in *Phalaenopsis gigantea* (Murdad et al. 2006).

A perusal of literature reveals that peptone being water soluble protein hydrolysate with very high amino acid content promotes growth of cultures. Similar effect of peptone was observed in our *Cymbidium pendulum* cultures. Literature studies report its beneficial effects in inducing protocorm multiplication in *Cymbidium macrorhizon* and *Cymbidium* species (Kusumoto, Furukuwa 1977). Peptone is also known to have stimulated callus growth in *Phalaenopsis*, *Doritaenopsis*, and *Neofinetia* (Ichihashi, Islam 1999). It also supported better seedling growth in *Paphiopedilum*, *Phaius*, and *Vanda* (Curtis 1947). In *Peristeria elata* peptone favoured early and healthy growth of seedlings (Bejoy et al. 2004).

Supplementations of organic growth adjuncts in orchid culture medium is simple, practical, beneficial and a conventional method to improve media used for commercial production (Ichihashi, Islam 1999). In the present studies, the used organic growth supplements contain amino acids, proteins, carbohydrates, vitamins, phenolic acids and organic compounds. Any of these component(s) could be responsible for promoting growth and development of the cultures. Further studies are required to determine which factor(s) is responsible for promotory effect of these organic additives.

Through the exploration of the effects of different organic growth supplements on the regeneration, shooting and multiplication of cultures, our results indicated that 2 g/l peptone or CW (10%) in M medium was the most sufficient for maximum shoot regeneration/explant and early plantlet development. Maximum frequency of regeneration was achieved in BH 50 g/l supplemented medium. Organic growth supplements proved beneficial for multiplication of the cultures besides enhancing growth of *Cymbidium pendulum* plantlets as compared to control. This is a simple and low-cost medium for *Cymbidium pendulum* tissue culture without the use of plant growth regulators.

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