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In vitro Mass Clonal Propagation of *Spathoglottis plicata* Blume

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Abstract

For *in vitro* clonal propagation of *Spathoglottis plicata* Blume nodal segments of young shoots were cultured on half strength of MS with 2% sucrose + 2.0 mg/l BA + 0.5 mg/l NAA + 2 g/l peptone + 15% (v/v) CW + 0.5 g/l AC, incubated at 24 ± 2°C under 3000 lux fluorescent light for a 16 hr photoperiod per day. About 19 micro-shoots were induced from the explants within 12 weeks. Subculture of micro-shoots for eight weeks on the same nutrient medium enhanced the number of micro-shoots up to 60. The clumps of the micro-shoots were dissected and cultured on half strength of MS with 2% sucrose + 2 g/l peptone + 15% (v/v) CW + 0.5 g/l AC + 200 mg/l L-glutamine. The micro-shoot sections elongated to form shoots, and new micro shoots were induced from the base within eight weeks of culture. For plantlet formation the best rooting medium was determined as half strength of MS with 2% sucrose + 2 g/l peptone + 15% (v/v) CW + 0.5 g/l AC + 50 g/l banana pulp. After rearing 25 g mixture of urea, TSP and MOP (2 : 1 : 1) were applied per plant at three months intervals. All the regenerated plants blossomed on the third year.

Introduction

Spathoglottis plicata Blume is a handsome terrestrial orchid species, with long, grassy leaves, and about 7.5 cm wide flowers on erect spikes, which are quite attractive having long blooming period. Its inflorescence is erect, about 75 cm long, densely 5 - 25 flowered, often with pinkish bracts. At maturity plants flowered almost throughout the year. It is distributed in Bangladesh, India and Indonesia (Mukherjee 2002). In Bangladesh the plant is naturally grown in Chittagong (Huda et al. 1999) but in the garden it is used as ornamental pot plant.

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Spathoglottis plicata is conventionally propagated through separation of pseudobulbs but the proliferation rate is very low. A more efficient approach for its regeneration is *in vitro* seed culture (Sarker and Roy 1993, Roy and Biswas 2004). For mass clonal propagation, regeneration from nodal segments and axillary bud explants is superior to seed culture due to availability of plant materials round the year, an exponential propagation rate and sustaining the parental characteristics in regenerated plants. Reports on the *in vitro* culture of *Spathoglottis plicata* are limited (Lim-Ho et al. 1984, Teng et al. 1997a, b; Barua and Bhadra 1999). The available information fail to provide a comprehensive protocol and understanding of micropropagation of *Spathoglottis plicata.* The present investigation was, therefore, undertaken to develop a suitable protocol for *in vitro* fast regeneration system of this indigenous terrestrial orchid.

Materials and Methods

Mature pot plants of *Spathoglottis plicata* with deep pink coloured flowers were collected from a local nursery and used as source materials. About 2 cm long nodal segments, each with one node and an axillary bud (Fig. 1A) were used as explants.

Surface sterilized explants were cultured aseptically, initially in KC (Knudson 1946), VW (Vacin and Went 1949), MS and half strength of MS media. Each medium was initially supplemented with 3% sucrose, 2.0 mg/l BA, 0.5 mg/l NAA, 10% CW, and 2 g/l peptone. The medium, which responded best, was used in subsequent experiments. The selected medium was supplemented with different concentrations and combinations of sucrose, auxins, cytokinins, peptone, coconut water, and activated charcoal to determine their optimum doses for getting highest number of PLBs/micro shoots. The cultures were gelled with 2.2 g/l gelrite (Duchefa, The Netherlands). The pH of the medium was adjusted to 5.6 before autoclaving at a pressure of 1.06 kg/cm^2 and 121ºC for 20 min. The medium was taken in 25 × 150 mm culture tubes containing 12 ml of medium. The cultures were kept at 24 ± 1ºC under fluorescence light intensity (2000 to 3000 lux) for 16 hr photoperiod per day.

After hardening the plants were reared in the nursery up to flowering. Experiments were performed in a completely randomized design and all experiments were repeated three times. Each treatment had 15 replicates. The morphogenetic response of explants for PLB/micro-shoot induction was evaluated after 12 weeks of culture. For PLB/micro shoot proliferation and plantlet regeneration, results were evaluated after eight weeks of culture. Morphogenetic response was expressed as percentage of explants with PLB/ micro-shoot in relation to the number of surviving explants. For acclimation 50

plantlets were taken for each treatment. Data were statistically analyzed and in some parameters means were compared using DMRT.

Results and Discussion

From the preliminary selection of defined nutrient medium, half strength of MS in combination with 2.0 mg/l BA, 0.5 mg/l NAA, 3% sucrose, 10% coconut water and 2 g/l peptone was found to be the most superior in all respects to other media and micro-shoots were directly induced from the explants without intervening PLBs. About 66.6 % cultures of the nodal segment explants induced 6 micro-shoots per culture within 60.5 days (Data are not shown).

For further development of the medium, half strength of MS was used in combination with different plant growth regulators (PGRs) and organic supplements for high frequency induction of micro-shoots. Effects of BA, Kn and TDZ individually and in combinations with NAA and IAA in different concentrations on micro-shoot induction were determined (Table 1). BA (2.0 mg/l) + NAA (0.5 mg/l) was the most suitable combination for micro-shoot induction from cultured explants. Maximum number (66.6%) of cultures produced micro-shoots in this particular medium composition. The highest number of micro-shoots per culture was 6 within 60 days of culture. So, for direct induction of micro-shoots from nodal explants the suitable PGRs combination as determined was 2.0 mg/l BA + 0.5 mg/l NAA in half strength of MS with 3% sucrose + 10% CW + 2 g/l peptone.

Teng et al. (1997a,b) obtained PLBs from nodal and leaf explants of eight-month-old seedlings cultured on MS supplemented with 5.37 μM NAA and 0.44 μM BA and 2 g/l charcoal and the best combination of PGRs for plantlet development was 2.69 μM NAA + 8.88 μMBA. Earlier researchers used modified MS (Teng et al. 1997a, b; Barua and Bhadra 1999) for induction of PLBs from the explants. In the present experiments, half strength of MS was found to be suitable in relation to number of micro-shoots formed per culture. Half strength MS was also found suitable for *in vitro* regeneration of other orchids, like *Epidendrum radicans* (Chen et al. 2002b), *Paphiopedilum* (Chen et al. 2002a), and *Phalaenopsis* (Park et al. 2002, Sinha et al. 2006, 2007a,b).

For further improvement of the medium for obtaining large number of micro shoots, different concentrations of sucrose, CW, peptone and activated charcoal were tried.

Sucrose (1 - 4%) was used in half strength of MS with 10% CW, 2 g/l peptone, 2.0 mg/l BA and 0.5 mg/l NAA. 2% sucrose in the medium induced highest number of micro-shoots from nodal segment explants, in which the number of micro-shoots induced from nodal segment was 12.2.

Table 1. Effects of different concentrations and combinations of auxins and cytokinins on micro-shoot induction of *Spathoglottis plicata*.

PGRs (mg/l)	% of culture induced micro-shoots	*Days required for micro-shoots induction	*No. of micro-shoots/explant
BA			
1.5	12.5	64.6 (4.7)	1.5 (0.4)
2.0	12.5	64.8 (4.8)	2.5 (0.5)
2.5	20.8	63.1 (4.6)	2.5 (0.5)
Kn			
1.5	8.3	62.5 (5.4)	2.5 (0.5)
2.0	12.5	62.8 (5.5)	3.0 (0.0)
2.5	12.5	63.5 (5.0)	3.0 (0.0)
TDZ			
1.5	18.5	65.8 (5.9)	1.4 (0.3)
2.0	32.6	57.3 (4.9)	1.5 (0.6)
2.5	21.6	67.4(5.4)	2.1 (0.3)
BA + NAA			
1.0 + 0.5	31.2	64.1 (4.7)	4.5 (0.8)
1.5 + 0.2	42.3	68.3 (5.0)	4.5 (0.5)
1.5 + 0.5	50.0	60.0 (5.5)	5.0 (0.5)
2.0 + 0.5	**66.6**	**60.5 (4.0)**	**6.0 (0.5)**
BA + IAA			
1.0 + 0.5	12.5	70.1 (4.7)	2.5 (0.2)
1.5 + 0.2	24.0	65.3 (4.0)	2.0 (0.5)
1.5 + 0.5	24.5	65.0 (5.0)	3.0 (0.5)
2.0 + 0.5	31.2	65.5 (4.5)	3.5 (0.5)
Kn + IAA			
1.0 + 0.5	12.5	74.1 (4.2)	2.5 (0.8)
1.5 + 0.2	31.2	68.3 (4.0)	2.5 (0.5)
1.5 + 0.5	31.2	65.0 (5.5)	3.0 (0.5)
2.0 + 0.5	31.2	65.5 (4.0)	3.0 (09)
Kn + NAA			
1.0 + 0.5	12.5	70.5 (4.6)	4.8 (0.8)
1.5 + 0.2	31.2	65.2 (4.7)	4.7 (0.5)
1.5 + 0.5	31.2	65.3 (5.9	4.5 (0.5)
2.0 + 0.5	42.3	60.7 (5.7)	4.8 (0.5)
TDZ + IAA			
1.0 + 0.5	10.5	72.1 (4.7)	2.2 (0.2)
1.5 + 0.2	20.0	68.3 (4.0)	2.0 (0.3)
1.5 + 0.5	21.5	69.0 (5.0)	3.0 (0.5)
2.0 + 0.5	11.2	75.5 (4.5)	3.5 (0.5)
TDZ + NAA			
1.0 + 0.5	12.2	74.1 (5.2)	2.5 (0.8)
1.5 + 0.2	31.2	68.3 (4.7)	2.5 (0.5)
1.5 + 0.5	31.2	65.0 (5.5)	3.0 (0.5)
2.0 + 0.5	21.2	75.5 (4.0)	3.0 (09)

*Data were recorded after 12 weeks. Results are mean of 12 cultures.

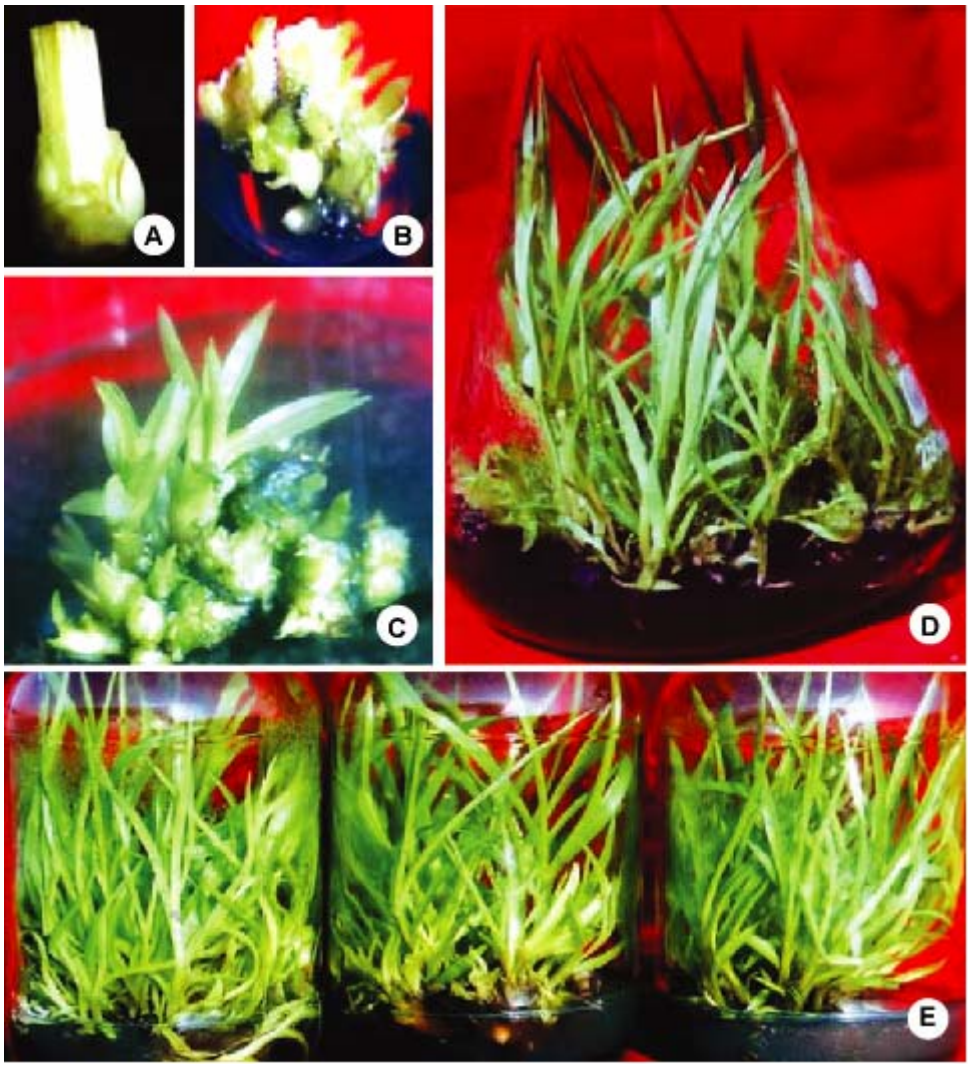
Effects of different concentrations of CW was studied by culturing the explants on half strength of MS with 2% sucrose + 2.0 mg/l BA + 0.5 mg/l NAA + 2 g/l peptone and CW (0 - 25% v/v). CW (13%) was the optimal concentration for

induction of highest number of micro-shoots from nodal segment. Effect of different concentrations of peptone was studied by culturing the explants on half strength of MS with 2% sucrose + 2.0 mg/l BA + 0.5 mg/l NAA + 15% (v/v) CW and peptone (0 - 3.0 g/l). It was observed that 2.0 g/l peptone was the optimal concentration for induction of highest number (16.5) of micro-shoots. To determine the effects of activated charcoal (AC) on induction of micro-shoots from explants. AC (0.5 - 2.0 g/l) was used in half strength of MS with 2% sucrose + 2.0 mg/l BA + 0.5 mg/l NAA + 2 g/l peptone + 15% (v/v) CW). In the present study it was observed that 0.5 g/l AC was the optimal concentration for induction of highest number (19.5) of micro-shoots from nodal segment explants (Figs. 1B). So, the medium as determined for high frequency induction of micro-shoots directly from the explants was half strength of MS with 2% sucrose + 2.0 mg/l BA + 0.5 mg/l NAA + 2 g/l peptone + 15% (v/v) CW + 0.5 g/l AC.

Teng et al. (1997a,b) used 3% sucrose in MS and obtained PLBs of *Spathoglottis plicata.* Bui *et al.* (1999) used 3% sucrose for obtaining shoot buds directly from explants of *Rhynchostylis gigantea.* Addition of 10% coconut water to the culture medium also enhanced proliferation of protocorms and stimulated shoot development in *Cymbidium* (Nayak et al. 2006) and *Phalaenopsis* (Park et al. 2002). Peptone and tryptone were used in culture medium as organic sources of nitrogen (Arditti 1982). Evers (1984) showed the promotive effect of charcoal on the growth and organogenesis. In the present study, besides PGR, peptone, CW and activated charcoal were added to the culture medium simultaneously, which successfully enhanced the number of micro-shoot induction per culture. The cumulative effects of these three additives certainly acted on high frequency induction of micro-shoot

Through the eight-week subculture the number of micro-shoots increased up to an average number of 60 (Fig. 1C). The micro-shoot clumps were then dissected longitudinally containing an average of 15 micro-shoots in each section. They were subcultured on the same nutrient medium without PGR, where within eight weeks the micro-shoots were proliferated into a huge number but they were not developed into proper shoots. In another experiment the micro-shoot clumps were subcultured on the medium mentioned above along with different concentrations (50 - 250 mg/l) of L-glutamine and casein hydrolysate (CH) individually for eight weeks. Results showed that in eight week of subculture on medium having 200 mg/l L-glutamine the micro-shoots developed into shoots and elongated properly (Fig. 1D). The mean height of the shoots (including leaves) was 70.7 mm (Table 2). Moreover, a large number of secondary micro-shoots were induced from the base of the developing shoots. On the other hand, the lengths of the shoots developed from the micro-shoots cultured on the medium having 150 mg/l CH was 53.6 mm. So, the medium as

determined, for shoots development and secondary micro-shoots induction was half strength of MS + 2% sucrose + 15% CW + 2 g/l peptone + 0.5 g/l activated charcoal + 200 mg/l L-glutamine.



Figs. 1. (A). Nodal segment explant. (B). Micro-shoots induction from nodal segment. (C). Multiplication and elongation of regenerated micro-shoots at eight-week. (D). Development of micro-shoots into shoots and formation of secondary micro-shoots at eight-week. (E). Induction of roots.

The high frequency induction of secondary micro-shoots was possible probably due to the synergistic effect of the organic compounds present in CW and L-glutamine. Moreover, subculture of clumps of micro-shoots is much easier and efficient in comparison with subculture of individual PLB sections as

determined in previous study (Teng et al. 1997 a,b). L-glutamine is most commonly used as organic nitrogen source (Pierik 1987).

Table 2. Effects of L-glutamine and casein hydrolysate (CH) individually on shoot development from micro-shoots.

L-glutamine/ CH (mg/l)	Nodal segment derived shoots	
	ΨShoot height (mm)	Remarks
L-glutamine		
0	20.5 ± 2.4 d	*
50	40.7 ± 2.5 c	*
100	43.3 ± 3.2 c	*
150	59.9 ± 3.4 b	*
200	70.7 ± 4.6 a	*
250	44.1 ± 3.1 c	*
CH		
0	21.2 ± 2.3 d	*
50	29.2 ± 2.0 d	*
100	36.2 ± 2.4 c	*
150	53.6 ± 3.3 b	*
200	41.5 ± 3.4 c	*
250	26.9 ± 2.9 d	*

ΨMean values ± SE followed by the same letter in a column are not significant at p = 0.05 by DMRT. *Shoot development with shoot growth and formation of secondary micro shoots. Data were recorded in eight weeks of culture. Results are mean ± SE of 15 cultures, repeated three times.

To obtain rooted plantlets the shoots were taken out from the culture vessels and subcultured on half strength of MS supplemented with 2% (w/v) sucrose + 15% (v/v) CW + 2 g/l peptone + 0.5 g/l AC along with banana pulp and potato homogenate (25 - 100 g/l) individually to observe their efficacy in root induction and growth. Adding of 50 g/l banana pulp in the medium induced an average of 6 roots in 100% cultures within eight weeks. The mean length of root was 68 mm (Table 3). Roots were also induced from the shoots cultured on medium containing potato homogenate within eight weeks but the frequency was not appreciable (Table 3). For rooting, Barua and Bhadra (1999) cultured regenerated shoots of *Spathoglottis plicata* on MS with 0.5 mg/l IAA, on which 3.2 roots were induced from each shoot. In the present study the addition of 50 g/l banana pulp to the medium induced an average of six roots in 100% shoots within eight weeks, as it is especially promotive for growth in orchid culture (Pierik 1987).

After hardening the plantlets were implanted in plastic basket containing coconut husk (10 mm^2). After 30 days the plantlets were implanted in clay pots (Fig. 2A) containing alluvial soil and compost (3 : 1) and kept under shade net. The plants were watered every seven days and they were fertilized with a mixture of urea, triple super phosphate (TSP) and muriate of potash (MOP)

(2 : 1 : 1) at 25 g/plant at three months intervals. In three years the plants increased in height, and blossomed in two - four inflorescence stalks per plant cluster (Fig. 2C).

Fig. 2. (A). Acclimated plantlets in pots. (B). Two-year-old plants. (C). Three-year-old plants with flowers.

As in the present study plants were directly regenerated through micro-shoot formation from explants without the intervention of callus, and the growth regulators (BA and NAA) were used in small doses only in micro-shoot

induction medium, there was no risk of somaclonal variation. In *in vitro* culture, the excessive use of growth regulators, and especially during the intermediary callus phase is undesirable as those are thought to be the main causes of variation in plants cultured *in vitro* (Prakash et al. 1996). In the present study it

Table 3. Effects of different concentrations of banana pulp (BP) and potato homogenate (PH) on root induction from shoots.

BP/ PH (g/l)	Nodal segment derived shoot		
	% shoots rooted	*Mean number of root ± SE	Mean length (mm) of root ± SE
BP			
25	53.42	3.2 ± 0.7 c	40.5 ± 4.2
50	100	6.3 ± 0.7 a	70.5 ± 4.3
75	81.43	4.3 ± 0.4 b	49.3 ± 4.5
100	38.56	4.3 ± 0.5 b	36.4 ± 3.1
PH			
25	47.34	3.2 ± 0.9 c	32.4 ± 2.6
50	61.91	4.2 ± 0.5 b	45.7 ± 5.5
75	51.49	3.0 ± 0.5 c	35.1 ± 3.4
100	30.43	3.41± 0.3 c	35.9 ± 2.8

Fifteen cultures were taken for each treatment and the experiments were repeated three times.
*Mean values followed by the same letter in a column are not significantly different at p = 0.05 by DMRT. Data were recorded in eight weeks after inoculation.

was avoided. The protocol as developed in the present study was through adjusting and determining the optimum concentrations and combinations of PGR, organic supplements and other medium adjuncts. The protocol is reproducible and would be utilized in high frequency regeneration of *Spathoglottis plicata* for commercial as well as conservation aspects.

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