BCSIR

Available online at www.banglajol.info

Bangladesh J. Sci. Ind. Res. **46(2)**, 163-68, 2011

BANGLADESH JOURNAL OF SCIENTIFIC AND INDUSTRIAL RESEARCH

E-mail: bjsir07@gmail.com

Clonal Propagation of *Phalaenopsis amabilis* (L.) BL. cv. 'Golden Horizon' Through *In vitro* Culture of Leaf Segments

P. Sinha* and M. A. A. Jahan

Biological Research Division, BCSIR Laboratories, Dhaka-1205, Bangladesh

Abstract

A protocol was established for mass clonal propagation of *Phalaenopsis amabilis* cv. 'Golden horizon' through *in vitro* culture of young leaf segments from mature plant. Explants were cultured on half strength Murashige and Skoog (1/2 MS) medium supplemented with N^6-benzyladenine (2.0 mg l^{-1}), a-naphthaleneaceetic acid (0.5 mg l^{-1}), 2% (w/v) sucrose, 10% (v/v) coconut water, 2 g l^{-1} peptone and 1 g l^{-1} activated charcoal. Each section of explant produced 15 protocorm-like bodies (PLBs) after 12 weeks of culture. When phytohormone was omitted from the medium and 150 mg l^{-1} L-glutamone was added PLBs were found to be enlarged with leafy shoots and new PLBs were induced from the base of the old ones. Leafy shoots rooted on half strength MS medium supplemented with 2 g l^{-1} peptone, 2% (w/v) sucrose, 10% (v/v) CW and 1 g l^{-1} activated charcoal, where 100% explants were developed into plantlets with roots within 8 weeks. The addition of 2.5 g l^{-1} banana pulp powder enhanced the number and length of roots. Within the first 32 weeks after initiation of culture about 1500 plantlets as well as a huge amount of PLBs were achieved from a single explant section. The plantlets were acclimatized in natural environment.

Key words: *Phalaenopsis orchid,* Leaf segments, Protocorm-like bodies, Micropropagation

Introduction

Orchids are outstanding among the floricultural plants in many ways, like diverse shapes, forms and colours. *Phalaenopsis,* a pot orchid, accounts for about 40 percent from an economic point of view (Debargh and Zimmerman 1991). There are many reports on *in vitro* regeneration protocols of *Phalaenopsis* (Reuter 1983, Tanaka and Sakanishi 1977, 1980, 1985, Tanaka 1987, 1992, Tokuhara and Mii 1993, Arditti and Earnst 1993). However, not all of these methods can be used for commercial micropropagation because of differences in survival rate, PLB formation, and plantlet regeneration. As *Phalaenopsis* has a high commercial value as a cut flower and as indoor pot plants throughout the world, a high frequency regeneration protocol is yet to be determined (Takuhara and Mii 2001). Tokuhara and Mii (2001, 2003) established an efficient system of micropropagation for *Phalaenopsis* through embryogenic callus induction and cell suspension culture, but callus mediated plant regeneration does not ensure plant homogeneity in any respect. Park and co-workers *et al.* (2002) established a two step *in vitro* propagation method for *Phalaenopsis* by using leaf explants, derived *in vitro* from flower stalk nodes. This paper reports an efficient and quick method for repetitive high frequency clonal propagation of purple coloured *Phalaenopsis amabilis* cv. 'Lovely' through *in vitro* culture of young leaf sections of mature plants.

Materials and Methods

The golden yellow cultivar of *Phalaenopsis amabilis* (L.) Bl. cv. 'Golden horizon' was selected for an explant source. Young emerging leaves were collected from the nursery and were used as explants. The explants were washed under running tap water followed by detergent, Tween 80 (5% v/v) for 5 min. After a thorough wash with double-distilled water, surface sterilization was done with 0.1% (w/v) mercuric chloride solution for 8-10 min. The explants were then washed thoroughly with sterile double-distilled water. The surface sterilized explants were then prepared for inoculation by cutting into pieces. For direct induction of PLBs, the explants were cultured on half-strength Murashige and Skoog (1962) (½ MS) medium supplemented with N^6-benzyladenine (BA, 0.5-2.5 mg l^{-1}) and Kinetin (Kn, 0.5-2.5 mg l^{-1})

* Corresponding author: E-mail; pinakiju@yahoo.com

individually and in combination with a-naphthaleneacetic acid (NAA, 0.2-0.5 mg l^{-1}) along with 2% sucrose, 10% (v/v) coconut water (CW), 2 g l^{-1} peptone and 1 g l^{-1} activated charcoal. The medium was gelled with 2.2 g l^{-1} gelrite (Duchefa, The Netherlands). The pH of the media was adjusted to 5.6 before autoclaving at 1.1 kg/cm^2 for 20 min at 121°C. All cultures were incubated at 24 ± 1°C, under cool white fluorescent light of 30 µ mol m-2 s-1 for 16 h per day.

For proliferation of PLBs and formation of shoots, each clump of PLBs developed from initial explants was cut into 4 pieces and subcultured on two different media, MS and half strength MS (½ MS) supplemented with 2% sucrose, 2 g l^{-1} peptone, 1 g l^{-1} activated charcoal and with or without coconut water (CW, 10%) and L-glutamin (50-300 mg l^{-1}). In all experiments explants were cultured in 250 ml conical flasks or disposed off jam bottles (86 x 120 mm) containing 40 ml medium.

In PLB proliferation medium, old PLBs were developed into leafy structures from the base of which new PLBs emerged. These leafy structures or shoots were subcultured on ½ MS medium with 2% sucrose, 2 g l^{-1} peptone, 10% CW and 1 g l^{-1} activated charcoal for plantlet formation. Banana pulp powder was also used to study its efficacy in root differentiation and growth.

For acclimatization the rooted plantlets (between 50 and 70 mm in height) were taken out from the media and washed with tap water to remove the gel adhered to the roots. These were then implanted in a plastic basket containing coconut husk. To maintain high humidity the plantlets were misted twice a day. After one month, plants were watered every alternate day and fertilized with 6.5N-4.5P-19K solution at 10-day intervals.

Experiments were performed in a randomized design and all experiments were repeated three times. In *in vitro* culture each treatment had 15 replicates. The morphogenetic response of explants for PLB induction was evaluated after 12 weeks of culture. For PLB proliferation and plantlet regeneration, results were evaluated within 8 weeks of culture.

Results and Discussion

About 15% explants responded in medium with BA or Kn alone to induce 2-3 PLBs per explant (data not shown). Kn-NAA combinations responded to PLB induction in 45-50% explants according to the concentration of Kn and NAA (data not shown). BA-NAA combinations increased the rate of PLB induction and the optimum concentration of BA and NAA for PLB induction was 2.0 and 0.5 mg l^{-1}, respectively, in which 80% of the cultures produced a maximum of 15 PLBs per explant (Table I). The explants showed small beaded structures on their surface after 4 weeks of culture, and they continued to develop and proliferate PLBs for an additional 8 weeks of culture (Fig. 1A). BA (10 mg l^{-1}) and NAA (1 mg l^{-1}) were also essential for induction of PLBs, which was 3.8 and 1.5 on leaf explants, cultured in modified Hyponex medium (Kano 1965) and MS medium, respectively (Tanaka and Sakanishi 1977, 1980). Similarly, use of NAA in combination with BA was needed in *in vitro* culture of *Aranda* (Lakshmanan *et al.* 1995) and *Dendrobium* (Sinha *et al.* 2003). In the present study we have optimized BA-NAA combination, and obtained the highest number of PLBs in leaf explants of *Phalaenopsis amabilis* cv. 'Golden horizon' cultured on ½ MS medium supplemented with 2.0 mg l^{-1} BA and 0.5 mg l^{-1} NAA along with 2% (w/v) sucrose, 10% CW, 2 g l^{-1} peptone and 1 g l^{-1} activated charcoal. In another experiments on leaf cultures of *Phalaenopsis*, 3% sucrose was used (Tanaka and Sakanishi 1977, 1980, Haas-von

Table I: Effects of BA and NAA on PLB induction from leaf section of *Phalaenopsis amabilis* cv. 'Golden horizon' after 12 weeks of culture on ½ MS medium supplemented with 2% sucrose, 10% CW, 2 g l^{-1} peptone and 1 g l^{-1} activated charcoal.

Treatments (mg l^{-1})		*Mean percentage of PLB-forming explants	*Mean number of PLBs per explant
BA	NAA		
0.0	0.0	0.0	0.0
0.5	0.2	0.0	0.0
1.0	0.2	45.6 (3.8)	3.5 (0.9)
1.5	0.2	60.5 (4.8)	4.3 (1.4)
2.0	0.2	64.0 (4.9)	2.4 (0.8)
2.5	0.2	43.5 (3.2)	2.5(0.8)
0.5	0.5	59.0 (4.8)	2.0 (0.5)
1.0	0.5	55.5 (4.7	4.6 (1.7)
1.5	0.5	68.5 (5.8)	5.5 (1.4)
2.0	0.5	80.5 (6.4)	15.0 (3.1)
2.5	0.5	41.6 (3.6)	3.3 (1.4)

*Standard error in the parenthesis

Fig. 1: (A-E): PLBs induction and regeneration of *Phalaenopsis amabilis* cv. 'Golden horizon' from young leaf explants.

A) PLBs induction from the explants cultured on ½ MS medium with 2.0 mg l^{-1} BA + 0.5 mg l^{-1} NAA + 2% (w/v) sucrose + 10% (v/v) CW + 2 g l^{-1} peptone + 1 g l^{-1} activated charcoal for 12 weeks,

B) Multiplication and development of leafy shoots from PLBs clump cultured on ½ MS medium with 2% (w/v) sucrose + 10% (v/v) CW + 2 g l^{-1} peptone + 150 mg l^{-1} L-glutamine + 1 g l^{-1} activated charcoal for 8 weeks,

C) Highly proliferated view of regenerated leafy shoots,

D) Plantlets with roots growing from leafy shoots cultured on ½ MS containing 2% (w/v) sucrose + 2 g l^{-1} peptone + 10% (v/v) CW + 1 g l^{-1} activated charcoal + 2.5 g l^{-1} banana pulp powder,

E) Acclimatized 2-month-old regenerated plants.

Schmude 1983, 1985). Park *et al.* (2002) tried different concentrations of sucrose during PLB induction from leaf sections and determined the optimum concentration of sucrose as 3% along with 10% CW for PLB induction, but in the present experiment, both the CW and 2 g l^{-1} peptone were used in ½ MS medium, which enhanced the PLB formation directly on the explants. It is documented that if coconut water is used with auxins it strongly induces cell division in tissues (Steward 1958). Coconut water contains 9-ß-D-ribofuranosylzeatin (a cytokinin) (Pierik 1987) and its presence (5-15% v/v) in the culture medium enhance the growth and proliferation of propagules especially in orchid culture (Park *et al*. 2002, Sinha *et al*. 2003) probably due to the additional amount of cytokinin.

Regeneration of *Phalaenopsis* was achieved through different experiments using different defined culture media. Solid and liquid VW media with 20% CW were used for proliferation of PLB (Tanaka and Sakanishi 1977, 1980). Other researchers (Haas-von Schmude 1983, 1985) used MS medium for proliferation and differentiation of PLBs. KC (Knudson 1946), VW (Vacin and Went 1949), MS (Murashige and Skoog 1962), LM (Lindemann *et al.* 1970), and modified Hyponex media (Kano 1965) were also used for proliferation and differentiation of PLBs, and it was observed that survival percentage of the explants and multiplication of PLBs was high on MS medium with 3% (w/v) potato homogenate (Park *et al.* 2002). In the present experiment initially induced PLBs clumps (number of PLBs 20) were dissected into four pieces. Each piece was subcultured on ½ MS medium in combination with 2% (w/v) sucrose + 2 g l^{-1} peptone + 1 g l^{-1} activated charcoal + 10% (v/v) CW and 150 mg l^{-1} L-glutamine. In this medium high-frequency proliferation (250 PLBs per explant) of PLBs was obtained within eight weeks of culture (Table II).

Park *et al.* (2002) obtained 20 PLBs from a single protocorm in raft culture. In the present study, the effect of L-glutamine on protocorm proliferation was evaluated and the highest number of PLBs per culture was obtained on media containing L-glutamine, and 250 PLBs developed from a single clump of 4-6 PLBs. So, the average number of PLBs developed from a single protocorm was 40-60, which was about two-three fold higher than that obtained in a previous experiment (Park *et al.* 2002). This high-frequency regeneration of PLBs was possible probably due to the cumulative effect of the organic compounds present in CW and L-glutamine. Moreover, subculture of clumps of PLBs is much easier and

Table II: Effect of MS and ½ MS media with/without CW and L-glutamine (along with 2% sucrose + 2 g l^{-1} peptone + 1 g l^{-1} activated charcoal) on PLB proliferation of *Phalaenopsis amabilis* cv. 'Golden horizon' after 8 weak of culture

MS			MS		
CW (%)	L-glutamine (mg l^{-1})	*Mean No. of PLBs per explant	CW (%)	L-glutamine (mg l^{-1})	*Mean No. of PLBs per explant
0	0	15.6 (1.3)	0	0	20.3 (3.2)
10	0	19.5 (1.9)	10	0	46.5 (4.2)
10	50	25.5 (2.6)	10	50	72.4 (4.6)
10	100	29.7 (2.7)	10	100	102.8 (5.9)
10	150	44.5 (3.2)	10	150	250.5 (8.9)
10	200	33.5 (4.7)	10	200	143.6 (6.8)
10	250	23.4 (1.9)	10	250	113.9 (5.4)
10	300	18.5 (1.3)	10	300	106.5 (4.3)
0	50	10.6 (0.7)	0	50	38.5 (1.3)
0	100	21.3 (1.4)	0	100	68.6 (2.3)
0	150	20.4 (1.3)	0	150	124.8 (5.4)
0	200	10.5 (1.0)	0	200	77.6 (3.2)
0	250	10.4 (0.8)	0	250	66.3 (3.7)
0	300	8.1 (0.7)	0	300	27.6 (2.6)

*Standard error in the parenthesis

efficient in comparison with subculture of individual PLB sections. Positive effects of CW and L-glutamine in culture medium on multiplication rates of somatic embryos in other plant species are commonly reported (Alam *et al.* 2006). After a further four weeks of culture (in the same medium) the PLBs were found to be enlarged with leafy shoots (Figs. 1B, 1C). The leafy shoots were subcultured for conversion into plantlets and the nonleafy bodies were subcultured in fresh PLB proliferation medium with the same constituents, for their development into leafy shoots and further multiplication. Thus, in each subculture profuse PLBs as well as leafy shoots were produced. Within the first 24 weeks after initiation of culture 1500 leafy shoots as well as a huge amount of protocorm-like bodies were achieved from a single explant section. The procedure is being repeated, so as to multiply the same continuously.

The leafy shoots were subcultured on ½ MS medium with 2 g l^{-1} peptone, 2% (w/v) sucrose, 10% CW and 1 g l^{-1} activated charcoal, upon which 100% explants developed into plantlets with stout roots within 8 weeks. The addition of 2.5 gl^{-1} banana pulp powder in the medium enhanced the growth and number of roots (Table III, Fig. 1D), as it is especially promotive for growth in orchid culture (Pierik 1987). Other researchers obtained plantlets from PLBs in modified Hyponex medium (Kano 1965) along with 2 g l^{-1} peptone, 3% sucrose, 0.05% activated charcoal and 3% (w/v) potato homogenate (Park *et al.* 2002). Modified KC and modified Hyponex medium with 3% sucrose and 2 g l^{-1} activated charcoal were also used (Tanaka and Sakanishi 1985, Tanaka 1987) for plantlets formation. In the present experiment identical leafy shoots were separated from the PLB proliferation medium and subcultured for their conversion into plantlets, which resulted in obtaining a large number of identical plantlets rapidly.

Within the first 32 weeks after initiation of culture, 1200 plantlets as well as a huge amount of PLBs were achieved from a single explant section. Repeating the subculture of protocorm-like bodies on proliferation medium and culturing leafy shoots on plantlet regeneration medium, could produce huge amount of plantlets every 32 weeks.

After acclimatization about 85% plantlets survived with full vigour and they are being maintained in 14 cm^3 plastic pots perforated at the bottom and containing coconut husk (20 mm^3) and charcoal (10 mm^3) (2 : 1), and they are being maintained in shady place at 30/25°C (Fig. 1E).

Table III: Effect of banana pulp powder on rooting of regenerated shoots of *Phalaenopsis amabilis* cv. 'Golden horizon' cultured on half strength MS medium with 2% sucrose + 10% CW + 2 g l^{-1} peptone + 1 g l^{-1} activated charcoal. Final data were recorded after 8 weeks of culture.

Weeks after culture	Banana powder (g l^{-1})	Shoots rooted (%)	Mean number of root ± SE	Mean length (mm) of root ± SE
2	0	0	0	0
	1.0	0	0	0
	2.5	12.44	2.6 ± 0.05	25.6 ± 1.5
	3.0	22.24	2.9 ± 0.08	26.6 ± 1.0
	4.0	14.54	2.2 ± 0.04	24.9 ± 1.4
4	0	25.50	3.4 ± 0.06	25.5 ± 1.5
	1.0	35.54	4.7 ± 0.89	34.6 ± 3.0
	2.5	65.54	5.6 ± 1.05	43.5 ± 3.5
	3.0	35.86	3.8 ± 0.05	24.7 ± 1.5
	4.0	24.64	2.4 ± 0.08	22.1 ± 1.8
6	0	45.74	4.0 ± 1.1	34.5 ± 3.1
	1.0	64.86	4.8 ± 1.0	35.2 ± 3.6
	2.5	100.00	6.9 ± 1.5	52.7 ± 4.0
	3.0	58.42	3.6 ± 0.4	26.5 ± 1.6
	4.0	28.48	3.2 ± 0.5	27.8 ± 1.5
8	0	100.00	5.2 ± 1.2	44.6 ± 2.9
	1.0	100.00	5.8 ± 1.0	47.5 ± 2.5
	2.5	100.00	9.7 ± 1.2	59.4 ± 5.6
	3.0	64.44	4.9 ± 0.5	24.1 ± 1.0
	4.0	32.63	3.6 ± 0.5	24.4 ± 1.1

In conclusion, an efficient protocol for reproducible high frequency regeneration of yellow cultivar of *Phalaenopsis amabilis* cv. 'Golden horizon' in a simple culture medium and with short culture period was established.

References

Alam M. F., Sinha P. and Hakim M. L. (2006). Mass clonal propagation of *Vanda teres* (Roxb.) Lindle through *in vitro* culture of nodal segments. *Propagation of Ornamental Plants*, **6:** 140-144.

Arditti J. and Ernst R. (1993). Phalaenopsis. *In:* Arditti J and

Ernst R (eds.). Micropropagation of orchids, pp. 469-520, Wiley, New York.

Debarg P. C. and Zimmerman R. H. (1991). Micropropagation: Technology and Application, p. 385, Kluwer Academic Publishers, The Hague.

Hass-Von Schmude N. F. (1983). Klonale Massenvemehrung von *Phalaenopsis.* Die Orchidee, **34:** 242-248.

Hass-Von Schmude N. F. (1985). Tissue culturing *Phalaenopsis* using leaves and leaf segments. *In*: Tan K (ed.). Proceedings of 11th Orchid Conference, p. 311, Miami.

Kano K. (1965). Studies on the media for seed germination. Memoir, Faculty of Agriculture, *Kagawa University, Japan,* **20:** 1-68.

Knudson L. (1946). A new nutrient solution for germination of orchid seed. *American Orchid Society Bulletin* **15**: 214-217.

Lakshmanan P., Loh C. S. and Goh C. J. (1995). An *in vitro* method for rapid regeneration of a monoploid orchid hybrid *Aranda* Deborah using thin section culture. *Plant Cell Rep.* **14:** 510- 514.

Lindemann E. G. P. Gunckel J. E. and Davidson O. W. (1970). Meristem culture of *Cattleya. American Orchid Society Bulletin* **39:** 100-127.

Murashige T. and Skoog F. (1962). A revised medium for rapid growth and bioassaya with tobacco tissue cultures. *Physiolgia Plantarum* **15**: 473-497.

Park S. Y. Murthy H. N. and Paek K. Y. (2002). Rapid propagation of *Phalaenopsis* from floral stalk-derived leaves. *In Vitro* Cellular and Developmental *Biology-Plant* **38:** 168-172.

Pierik R. L. M. (1987). *In Vitro* Culture of Higher Plants. P. 78, Martinus Nijhop Publishers, Dordrecht, Boston, Lancaster.

Reuter E. (1983). The importance of propagating *Phalaenopsis* by tissue culture. Orchid Review **91:** 199-201.

Sinha P. Fatema K. and Roy S. K. (2003). Micropropagation of *Dendrobium* cv. Thai beauty. *Bangladesh Journal of Life Science* **15:** 13-18.

Tanaka, M. (1987). Studies on the clonal propagation of *Phalaenopsis* through *in vitro* culture. Memoir, Facculty of Agriculture, Kagawa Univiversity, Japan, **49:** 1-85.

Tanaka M. (1992). Micropropagation of *Phalaenopsis* spp. In: Bajaj YPS (ed.). Biotechnology in agriculture and forestry, vol. 20, pp. 246-268, Berlin: Springer-Verlag, Berlin.

Tanaka M. and Sakanishi Y. (1980). Clonal propagation of *Phalaenopsis* through tissue culture. *In:* Kashemsanta MRS (ed.). Proceedings of 9th World Orchid conference, pp. 215-221, Bangkok.

Tanaka M. and Sakanishi Y. (1977). Clonal propagation of *Phalaenopsis* by leaf culture. American Orchid Society Bulletin, **46**: 733-737.

Tanaka M. and Sakanishi Y. (1985). Regeneration capacity of *in vitro* cultured leaf segment excised from mature *Phalaenopsis* plants. Bulletin, University of Osaka Prefecture, Series B, **37**: 1-4.

Tokuhara K. and Mii M. (1993). Micropropagation of *Phalaenopsis* and *Doritaenopsis* by culturing shoot tips of flower stalk buds. *Plant Cell Reports* **13**: 7-111.

Tokuhara K. and Mii M. (2001). Induction of embryogenic callus and cell suspension culture from shoot tips excised from flower stalk buds of *Phalaenopsis* (Orchidaceae). I*n Vitro Cellular* and *Developmental Biology-Plant* **37**: 457-461.

Tokuhara K. and Mii M. (2003). High-frequency somatic embryogenesis from cell suspension cultures of *Phalaenopsis* orchids by adjusting carbohydrate source. I*n Vitro Cellular and Developmental Biology-Plant* **39**: 635-631.

Vacin E. and Went F. (1949). Some pH changes in nutrient solutions. Botanical Gazette, **110**: 605-613.

Received :April 28, 2010;
Accepted : October 19, 2010