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High Frequency Regeneration of *Phalaenopsis amabilis* (L.) Bl. cv. Lovely through *In vitro* Culture

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Abstract

Young leaf explants from mature plant of *Phalaenopsis amabilis* cv. Lovely were cultured on half strength of MS supplemented with BA (2.0 mg/l), NAA (0.5 mg/l), 2% (w/v) sucrose, 10% (v/v) coconut water, 2 g/l peptone and 1 g/l activated charcoal. Each section of explant produced ten protocorm-like bodies (PLBs) after 12 weeks of culture. The PLBs were subcultured on the same nutrient medium but without phytohormone and addition of 150 mg/l L-glutamine, where PLBs were found to be enlarged with leafy shoots and new PLBs were induced from the base of the old ones. Plantlet development from leafy shoots was achieved on half strength MS supplemented with 2 g/l peptone, 2% (w/v) sucrose, 10% (v/v) CW and 1 g/l activated charcoal, where 100% explants were developed into plantlets with roots within eight weeks. The addition of 2.5 g/l banana powder enhanced the number and length of roots. Within the first 32 weeks after initiation of culture about 1200 plantlets as well as a huge amount of PLBs were achieved from a single explant section. The plantlets were acclimated in natural environment.

Introduction

Among the horticultural and floral crops, orchids are outstanding in many ways, like diverse shapes, forms and colours and *Phalaenopsis* accounts for about 40 per cent from an economic point of view (Debargh and Zimmerman 1991). It is called a pot orchid. 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

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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 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 continuous high frequency clonal propagation of purple coloured *P. amabilis* cv. 'Lovely' through *in vitro* culture of young leaf sections of mature plants.

Materials and Methods

Young emerging leaves of the purple cultivar of *Phalaenopsis amabilis* (L.) Bl. cv. 'Lovely' were collected from the nursery and used as explants. The young leaves 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) $HgCl_2$ solution for 8-10 min. The explants were again washed thoroughly with sterile double-distilled water. The surface sterilized leaves were then prepared for inoculation by cutting into pieces. For direct induction of PLBs, the explants were cultured on half strength of MS supplemented with BA (0.5 - 2.5 mg/l) and Kn (0.5 - 2.5 mg/l) individually and in combination with NAA (0.2 - 0.5 mg/l) along with 2% sucrose, 10% (v/v) coconut water (CW), 2 g/l peptone and 1 g/l activated charcoal. The medium was gelled with 2.2 g/l gelrite (Duchefa, The Netherlands). The pH of the media was adjusted to 5.6 before autoclaving at a pressure of 105 kPa for 20 min at 121°C. All cultures were incubated at 24 ± 1°C, under cool white fluorescent light of 30 μmol m^2/s for 16 hr per day.

For proliferation of PLBs and formation of shoots, each clump of PLBs developed from initial explants was cut into four pieces and subcultured on two different media, MS and half strength of MS supplemented with 2% sucrose, 2 g/l peptone, 1 g/l activated charcoal and with or without coconut water (10% CW) and L-glutamin (50 - 300 mg/l). In all experiments explants were cultured in 250 ml conical flasks or disposed off jam bottles (86 × 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 half strength of MS with 2% sucrose, 2 g/l peptone, 10% CW and 1 g/l activated charcoal for plantlet formation. Banana 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. They were then implanted in a plastic basket containing

coconut husk. To maintain high humidity the plantlets were misted twice a day. After acclimatization the plantlets were transplanted to 14 cm^3 plastic pots perforated at the bottom and containing coconut husk (20 mm^3) and charcoal (10 mm^3) (2 : 1) and maintained under shade at 30/25°C (day/night). Plants were watered every alternate day and fertilized with 6.5N-4.5P-19K solution at ten days 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 eight weeks of culture. Data were statistically analyzed and means were compared using DMRT (Duncan 1955).

Results and Discussion

In medium with BA or Kn alone 15% explants responded to induce two - three PLBs per explant (data not shown). Kn-NAA combinations responded to PLB induction in 40 - 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, respectively, in which 75% of cultures produced a maximum of ten PLBs per explant (Table 1). The explants showed small beaded structures on their surface after four weeks of culture, and they continued to develop PLBs for an additional eight weeks of culture (Fig. 1). BA (10 mg/l) and NAA (1 mg/l) 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, 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 the authors have optimized BA-NAA combination, and obtained the highest number of PLBs in leaf explants of *P. amabilis* cv. 'Lovely' cultured on half strength of MS supplemented with 2.0 mg/l BA and 0.5 mg/l NAA along with 2% (w/v) sucrose, 10% CW, 2 g/l peptone and 1 g/l activated charcoal. In another experiments on leaf cultures of *Phalaenopsis*, 3% sucrose was used (Tanaka and Sakanishi 1977, 1980, Haas-von 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 peptone were used in half strength of MS, 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-β-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.

Table 1. Effects of BA and NAA on PLB induction from leaf section of *P. amabilis* cv. 'Lovely' after 12 weeks of culture on half strength of MS supplemented with 2% sucrose, 10% CW, 2 g/l peptone and 1 g/l activated charcoal.

Treatments (mg/l)		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	39.3 d	5.5 c
1.5	0.2	59.4 b	4.3 b
2.0	0.2	62.3 b	3.5 d
2.5	0.2	41.4 d	3.3 d
0.5	0.5	53.0 c	3.2 d
1.0	0.5	51.5 c	7.1 b
1.5	0.5	64.1 b	7.7 b
2.0	0.5	75.5 a	10.0 a
2.5	0.5	38.7 d	5.4 c

Mean values followed by the same letter are not significant at $p \leq 0.05$ by DMRT.

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 for proliferation and differentiation of PLBs. KC (Knudson 1946), VW (Vacin and Went 1949), MS, 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 with 3% (w/v) potato homogenate (Park et al. 2002). In the present experiment initially induced PLBs clumps (20 PLBs) were dissected into four pieces. Each piece was subcultured on half strength of MS in combination with 2% (w/v) sucrose + 2 g/l peptone + 1 g/l activated charcoal + 10% (v/v) CW and 150 mg/l L-glutamine. In this medium high frequency proliferation (200 PLBs per explant) of PLBs was obtained within eight weeks of culture (Table 2).

Park et al. (2002) examined solid, liquid and raft cultures and the highest number of PLBs (20 per explant) were obtained 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

Figs 1-6: PLBs development and regeneration of *P. amabilis* cv. Lovely from young leaf explants. 1. PLBs development from the explants cultured for 12 weeks. 2. Multiplication and development of leafy shoots from PLBs clump cultured for eight weeks. 3. A magnified segment of proliferated PLBs clump (as shown in Fig. 2) showing leafy shoots and newly formed PLBs. 4. Highly proliferated view of regenerated leafy shoots. 5. Plantlets with roots growing from leafy shoots cultured. 6. Plants in pots.

obtained on media containing L-glutamine and 200 PLBs developed from a single clump of four - six PLBs. So, the average number of PLBs developed from a single protocorm was 35 - 50, which was twofold 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 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

Table 2. Effect of MS and half strength of MS with/without CW and L-glutamine (along with 2% sucrose + 2 g/l peptone + 1 g/l activated charcoal) on PLB proliferation of *P. amabilis* after eight weaks of culture.

MS			Half strength of MS		
CW (%)	L-glutamine (mg/l)	Mean No. of PLBs/explant	CW (%)	L-glutamine (mg/l)	Mean No. of PLBs/explant
0	0	9.9 f	0	0	22.4 g
10	0	14.4 e	10	0	36.5 f
10	50	21.0 d	10	50	71.6 e
10	100	25.6 c	10	100	102.8 d
10	150	40.5 a	10	150	200.5 a
10	200	31.7 b	10	200	156.6 b
10	250	22.2 d	10	250	127.9 c
10	300	14.9 e	10	300	98.4 d
0	50	10.0 f	0	50	23.6 g
0	100	22.7 d	0	100	67.0 e
0	150	22.5 d	0	150	104.7 d
0	200	9.7 f	0	200	72.6 e
0	250	9.6 f	0	250	66.3 e
0	300	9.0 f	0	300	24.6 g

Mean values followed by the same letter are not significant at $p \leq 0.05$ by DMRT.

(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 2 - 4). The leafy shoots were subcultured for conversion into plantlets and the non-leafy 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 1200 leafy shoots as well as a huge amount of PLBs 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 half strength of MS with 2 g/l peptone, 2% (w/v) sucrose, 10% CW and 1 g/l activated charcoal, upon which 100%

explants developed into plantlets with stout roots within eight weeks. The addition of 2.5 g/l banana powder in the medium enhanced the growth and number of roots (Table 3, Fig. 5) as it is especially promotive for growth in orchid culture

Table 3. Effect of banana powder on rooting of regenerated shoots of *P. amabilis* cv. 'Lovely' cultured on half strength MS with 2% sucrose + 10% CW + 2 g/l peptone + 1 g/l activated charcoal. Data were recorded after eight weeks of culture.

Weeks after culture	Banana powder (g/l)	% 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	2.4 ± 0.02 e	27.6 ± 1.7
	3.0	20.24	2.6 ± 0.06 e	22.6 ± 1.3
	4.0	14.54	2.1 ± 0.03 e	24.9 ± 1.5
4	0	25.50	3.1 ± 0.06 e	23.5 ± 1.1
	1.0	35.54	4.9 ± 0.89 c	33.6 ± 2.9
	2.5	65.54	5.9 ± 1.09 b	42.5 ± 3.6
	3.0	35.86	3.7 ± 0.03 d	22.7 ± 1.2
	4.0	24.64	2.2 ± 0.07 e	20.1 ± 1.3
6	0	45.74	4.9 ± 1.12 c	37.8 ± 3.00
	1.0	64.86	5.1 ± 1.04 c	33.1 ± 3.71
	2.5	100.00	7.8 ± 1.58 a	52.7 ± 4.75
	3.0	58.42	3.6 ± 0.47 d	28.9 ± 1.95
	4.0	28.48	3.5 ± 0.50 d	23.4 ± 1.54
8	0	100.00	5.0 ± 1.09 b	40.6 ± 2.42
	1.0	100.00	5.1 ± 1.00 b	45.5 ± 3.12
	2.5	100.00	8.8 ± 1.26 a	54.4 ± 5.32
	3.0	67.40	4.1 ± 0.45 c	24.1 ± 1.01
	4.0	35.53	3.0 ± 0.47 d	21.4 ± 1.12

Fifteen cultures were taken for each treatment and the experiments were repeated three times. *Mean values followed by the same letter are not significant at $p \leq 0.05$ by DMRT.

(Pierik 1987). Other researchers obtained plantlets from PLBs in modified Hyponex medium (Kano 1965) along with 2 g/l 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 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 PLBs on proliferation medium and culturing leafy shoots on plantlet regeneration medium, could produce huge amount of plantlets every 32 weeks.

After being acclimatization about 85% plantlets survived with full vigour and they are 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. 6).

In conclusion, an efficient protocol for continuous high frequency regeneration of pink cultivar of *P. amabilis* cv. 'Lovely' in a simple culture medium and short culture period was established. The authors have optimized the high frequency PLB proliferation medium (half strength of MS in combination with 2% (w/v) sucrose + 2 g/l peptone + 1 g/l activated charcoal + 10% (v/v) CW and 150 mg/l L-glutamine) and method (culture of PLB clump, instead of PLB section) and conversion of leafy shoots into plantlets in simple half MS. Likewise, through repeated subculture of PLB clumps, removing of leafy shoots and their subsequent subculture to plantlet regeneration medium and harvesting of regenerated plantlets, continuous high frequency production of plants could be maintained.

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