

# In vitro propagation of *Phalaenopsis* hybrid ‘Little gem’ by culturing apical part and axillary bud of flower stalk

Mi Young Chung · Aung Htay Naing · Khadiza Khatun · Hyung Geun Ahn · Ki Byung Lim · Chang Kil Kim

Received: 7 November 2016 / Revised: 15 November 2016 / Accepted: 15 November 2016  
© Korean Society for Plant Biotechnology

**Abstract** The in vitro propagation of the commercially important *Phalaenopsis* hybrid ‘Little gem’ was achieved by culturing the apical part and axillary buds excised from flower stalks. The explants were cultured on 5 different basal media: 3.0 g·L<sup>-1</sup> Hyponex and 4.0 g·L<sup>-1</sup> peptone (H<sub>3</sub>P<sub>4</sub>) and Murashige & Skoog (MS) media were shown to be suitable for shoot regeneration. The MS medium supplemented with 5.0 mg·L<sup>-1</sup> 6-benzylaminopurine (BA) was found to be more efficient for shoot regeneration. However, the number of shoots induced by axillary buds was higher than that induced by the apical part. Incubation of the apical part under darkness for one week, as well as of the explants in the same medium with activated charcoal (AC) 0.5 g·L<sup>-1</sup> promoted shoot regeneration and shoot growth; similar growth was not observed with axillary buds.

**Keywords** Activated charcoal, Dark treatment, Micropropagation, Plant growth regulator

## Introduction

*Phalaenopsis*, known as moth orchid, is considered as one of the most commercially important orchids due to its desirable

horticultural traits such as diverse flower colors, shapes, fragrance, compactness, and attractive potted plant types. In addition, as *Phalaenopsis* is a monopodial orchid type, it has longer blossom (Paek et al., 2011). The desirable characters increase its demand, and nearly 5 million plants were demanded in 2012, and the market is expected to reach 10 million in 2016. In general, most of the commercially cultivated *Phalaenopsis* cultivars are produced from seeds; they are thus heterozygous as well as showing phenotypic instability such as variation of plant growth, flowering time, and flower characteristics, which are dealing with its low market quality. Although it can be traditionally propagated by the cutting, these methods make the rate of multiplication slow and arrest the growth of the mother plant, resulting in ineffective for large scale production. Hence, propagation through tissue culture has been desired for commercial production.

Protocols for in vitro propagation of *Phalaenopsis* have been developed using various explants such as protocorm-like bodies (PLBs) (Park et al. 1996), flower stalks with axillary buds (Park et al. 2002), shoot tip of flower stalks explants (Tokuhara and Mii, 1993), leaf segments (Park et al., 2002) and root tips (Ichihashi, 1997). However, due to technical difficulties and low multiplication rate those protocols have been inadequate for large scale production of the orchid. Particularly, some of these studies could not achieve conversion of protocorms to plantlets, and developed plants were also physiologically weak. Some authors have reported direct regeneration without undesirable callus formation shortens the time period needed for regeneration and reduces the possibility of the occurrence of somaclonal variation. However, this requires precise cultural conditions such as types of media composition (Naing et al. 2011), additive compound (Naing et al. 2010), and growth regulators (Chen et al., 2000; Chen and Chang, 2004).

In the present study, we investigated the effects of basal medium, plant growth regulator, dark treatment, and activated charcoal on shoot regeneration and plant growth from different explants of the *Phalaenopsis* hybrid ‘Little gem’ in order to

---

M.Y. Chung  
Department of Agricultural Education, Suncheon National University, Suncheon 57922, Korea

K. Khatun  
Department of Agricultural Industry Economy and Education, Suncheon National University, Suncheon 57922, Korea

H. G. Ahn  
Science & Technology Policy Division, Government Complex-Sejong, Sejong 30110, Korea

A. H. Naing · K. B. Lim · C. K. Kim (✉)  
Department of Horticultural Science, Kyungpook National University, Daegu 41566, Korea  
e-mail: ckkim@knu.ac.kr

optimize an efficient and rapid propagation method for the orchid.

## Materials and Methods

### Plant material

The flower stalks at the first blossom stage with two to three open flowers, were used as explant source. The flower stalks were washed under running tap water, and were then sterilized with 70% ethanol for 10 s; followed by sterilizing them with 1 % NaOCl solution containing 2–3 drops Tween 20. Apical parts and axillary buds were excised and used as explants.

### Effects of basal media

To verify whether basal media affect in vitro regeneration, explants (apical parts and axillary buds) were cultured on the different basal media [ $3.0 \text{ g}\cdot\text{L}^{-1}$  Hyponex and  $2.0 \text{ g}\cdot\text{L}^{-1}$  peptone ( $\text{H}_3\text{P}_2$ ),  $3.0 \text{ g}\cdot\text{L}^{-1}$  Hyponex and  $4.0 \text{ g}\cdot\text{L}^{-1}$  peptone ( $\text{H}_3\text{P}_4$ ), Murashige and Skoog (MS), half strength Murashige and Skoog ( $\frac{1}{2}\text{MS}$ ), and Orchid mix. The culture were placed in a culture room with  $25\pm 2^\circ\text{C}$ , 16 h photoperiod, and cool white fluorescent lamps ( $50 \mu\text{mol}^{-1} \text{m}^{-2} \text{s}^{-1}$ ). After 8 weeks, the most appropriate media that gave optimal shoot regeneration (%) and number of shoot per explant were evaluated.

### Effects of plant growth regulator

From above experiment,  $\text{H}_3\text{P}_4$  and MS medium were found to be the most appropriate ones. For shoot regeneration, the explants were cultured on the media supplemented with different concentrations of 6-benzylaminopurine (BA). After 8 weeks of culture, optimal concentration of BA showing reasonable shoot regeneration (%), plant growth, and number of shoots per explant were evaluated. The culture condition was identical to that of above experiment.

### Effect of dark treatment

In above experiment, the explants cultured on the MS medium containing  $5.0 \text{ mg}\cdot\text{L}^{-1}$  BA exhibited better shoot regeneration parameters than other treatments, thus the media composition was selected for determination of dark treatment on shoot regeneration. To do so, the same explants were cultured on the selected medium and placed under the dark condition for different periods. The culture plates that were placed under

dark condition for designated periods were transferred to normal incubation described in above experiments. After 8 weeks of culture including initial dark period, shoot regeneration (%), shoot length, and number of shoots per explant were considered respectively.

### Effect of activated charcoal

In order to examine the effect of activated charcoal, explants were cultured on the MS medium containing  $5.0 \text{ mg}\cdot\text{L}^{-1}$  BA in combination with different concentrations of activated charcoal (AC). The culture plates were placed under the same condition. After 8 weeks, shoot regeneration (%), shoot length, and number of shoots per explant were considered respectively as done in dark treatment experiment.

### Experimental design and statistical design

The experiments were conducted using a Randomized Complete Block Design (RCB), and, PH, percentage of sucrose, and agar used in the experiments was 5.5, 3.0% and 0.7% respectively. There were ten explants per treatment with three replications. Data were statistically analyzed using DMRT ( $P \leq 0.005$ ).

## Results

### Effect of basal media

When apical parts or axillary buds from the flower stalks were cultured on the different basal media ( $\text{H}_3\text{P}_2$ ,  $\text{H}_3\text{P}_4$ , MS,  $\frac{1}{2}\text{MS}$ , or Orchid mix), in vitro responses such as shoot regeneration percentage and number of shoots per explant were differently observed (Table 1). In both explants, in vitro responses were observed to be likely superior in  $\text{H}_3\text{P}_4$  and MS to other media, in which axillary buds cultured on  $\text{H}_3\text{P}_2$  medium did not respond at all, while apical parts gave shoot regeneration (25.9%) and number of shoots per explant (1.4) in the same medium. On  $\text{H}_3\text{P}_2$ ,  $\frac{1}{2}\text{MS}$ , and Orchid mix, number of shoots induced by axillary bud was higher than that induced by apical parts. The similar responses were observed in  $\text{H}_3\text{P}_4$  and MS media, in which number of shoots induced by axillary buds was 2.5 per explant and higher than that (1.5) induced by apical parts.

### Effect of different concentrations of BA

According to the results of above experiment,  $\text{H}_3\text{P}_4$  and MS were found to be suitable for shoot regeneration from the explants (apical parts and axillary buds). In this study, when

**Table 1** Effects of different basal media on shoot regeneration from flower stalk of *Phalaenopsis* hybrid ‘Little gem’

Media	Flower stalk			
	Apical part		Axillary bud	
	Regeneration (%)	No. of shoots/explant	Regeneration (%)	No. of shoots/explant
H <sub>3</sub> P <sub>2</sub>	25.9b	1.4ab	0.0c	0.0c
H <sub>3</sub> P <sub>4</sub>	34.7a	1.5a	36.7a	2.5a
½MS	6.9c	1.0c	24.1b	2.3b
MS	34.5a	1.5a	34.5a	2.5a
Orchid mix	30.0ab	1.2bc	36.7a	2.3b

Means in the same column marked with same letter are not significantly different by DMRT.

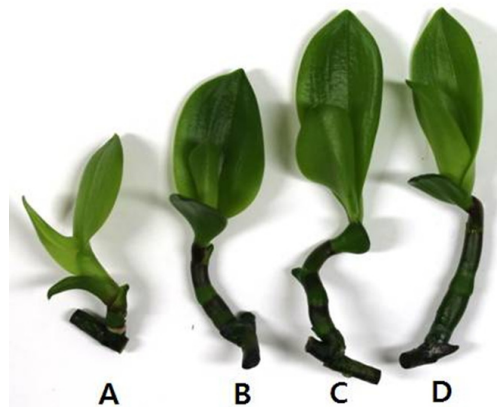
**Table 2** Effects of different concentrations of 6-benzylaminopurine (BA) on shoot regeneration from flower stalk of *Phalaenopsis* hybrid ‘Little gem’

Media	BA (mg·L <sup>-1</sup> )	Apical part		Axillary bud	
		Regeneration (%)	No. of shoots/explant	Regeneration (%)	No. of shoots/explant
H <sub>3</sub> P <sub>4</sub>	0	26.7d	1.5b	36.7a	2.5a
	3.0	69.7a	1.5b	29.1b	2.1b
	5.0	51.9b	2.1a	25.9bc	2.1b
	7.0	43.8c	2.1a	21.9c	2.1b
MS	0	34.5c	1.5b	34.5b	2.5ab
	3.0	83.3a	1.8ab	36.7b	2.5ab
	5.0	70.0b	2.1a	46.7a	2.9a
	7.0	65.6bc	2.1a	34.4b	2.1b

Means in the same column marked with same letter are not significantly different by DMRT.

different concentrations of BA were added to the media, it was found that in vitro responses distinctly depended on explant type, media, and BA concentrations. On H<sub>3</sub>P<sub>4</sub> medium, addition of BA distinctly increased shoot regeneration percentage of apical explants, while its negative effects on shoot regeneration were noticed from axillary buds (Table 2). In this medium, the apical parts cultured with 3.0 mg·L<sup>-1</sup> BA showed the highest regeneration percentage, but maximum number of shoots per explant (2.1) was observed from the explant cultured on 5.0 mg·L<sup>-1</sup> BA containing medium. In case of axillary bud, presence of BA linearly inhibited number shoot regeneration percentages, and number of shoots per explant was also lower than BA un-containing medium.

On MS medium, presence of BA positively promoted shoot regeneration as well as number of shoots per explant for both explants. Culture of the apical explants on the medium containing 3.0 mg·L<sup>-1</sup> BA increased shoot regeneration from 34.5% to 83.3%. Similarly, regeneration percentage of axillary buds cultured on 5.0 mg·L<sup>-1</sup> BA also reached to 46.7 from 34.5. In addition, shoot induced by 5.0 mg·L<sup>-1</sup> BA showed to be healthier than those induced by others (Fig. 1). Moreover,

**Fig. 1** Effects of different concentrations of BA on shoot regeneration from flower stalk culture of *Phalaenopsis* hybrid ‘Little gem’ (A: BA-free MS; B: 3.0 mg·l<sup>-1</sup> BA; C: 5.0 mg·l<sup>-1</sup> BA; D: 7.0 mg·l<sup>-1</sup> BA)

maximum number of shoots per explant (2.1 from apical part and 2.9 from axillary bud) were obtained on the medium containing 5.0 mg·L<sup>-1</sup> BA (Table 2).

**Table 3** Effects of incubation in the dark, on shoot regeneration from flower stalk of *Phaleonopsis* hybrid ‘Little gem’

Time in darkness (week)	Apical part		Axillary bud	
	Regeneration (%)	No. of shoots/explant	Regeneration (%)	No. of shoots/explant
0	70.0b	2.1a	46.7a	2.9a
1	80.0a	2.3a	44.3ab	3.3a
2	58c	2.0a	34.6b	3.0a
4	50d	2.0a	33.7b	3.0a

Means in the same column marked with same letter are not significantly different by DMRT.

**Table 4** Effects of activated charcoal (AC) on shoot regeneration from flower stalk of *Phaleonopsis* hybrid ‘Littlegem’

AC (g·L <sup>-1</sup> )	Shoot tip			Axillary bud		
	Regeneration %	Shoot length	No. of shoots/explant	Regeneration %	Shoot length	No. of buds/explant
0	70.0c	45.8b	2.1a	46.7a	31.7a	2.9a
0.5	90a	54.4a	1.8ab	18.7b	31.0a	2.9a
1.0	80b	54.2a	1.8ab	18.1b	31.0a	2.8a
2.0	80b	54.2a	1.8ab	17.4b	31.4a	2.3b

Means in the same column marked with same letter are not significantly different by DMRT.

#### Effect of dark treatment

Based on above experiment, the explants were cultured on the MS medium containing BA 5.0 mg·L<sup>-1</sup> and incubated at dark condition for different periods. We found that dark treatments also affected the shoot regeneration of the explants, however, its effects differed from explant to explant as well as treating periods (Table 3). In case of apical parts, culture of the explants at dark condition for 1 week increased shoot regeneration percentage, but increase of dark period longer than 1 week linearly reduced the shoot regeneration percentages. In contrast to apical part, even the dark treatment (1week) seemed to inhibit shoot regeneration from axillary buds. Interestingly, numbers of shoots induced by these explants were not varied among the dark treatments.

#### Effect of activated charcoal

When activated charcoal was added to the same medium, regeneration percentage as well as shoot length of the apical parts were better than those on the medium without AC (Table 4). However, presence of AC seemed to be slightly inhibiting number of shoots per explant. Unlike apical parts, the apopositive responses were observed in axillary buds, in which shoot regeneration and shoot length were found to be inhibited when concentration of AC was raised, however, there was no negative effect on number of shoots per explant (Table 4).

#### Discussion

The present study demonstrated effects of different factors (basal media, plant growth regulator, explants, dark period, and activated charcoal) on shoot regeneration of *Phaleonopsis* hybrid ‘Little gem’. Explants showed different responses when treated with different basal media, in which H<sub>3</sub>P<sub>4</sub> and MS were found to be the most appropriate and had been also reported as this in previous studies of orchid micropropagation (Chung et al 1985; Kosir et al. 2004). In this study, failure or less induction of shoots by ½MS and H<sub>3</sub>P<sub>2</sub> would be due to less amount of micro elements or peptone contained in the media. In addition, we found necessity of synergetic affects of explant and basal medium because H<sub>3</sub>P<sub>2</sub> medium could induce shoot from apical part while it could not do so in axillary buds. Likewise, ½MS could induce less percentage of shoot regeneration from apical bud, but it could induce reasonable percentage from axillary buds. Tokuhara and Mii 1993) also claimed that ½MS failed to induce shoot from flower stalk of *Phalaenopsis* and *Doritaenopsis*

When different concentrations of BA were added to the basal media (H<sub>3</sub>P<sub>4</sub> and MS), it negatively affected shoot regeneration from axillary buds, but its stimulatory effect on shoot regeneration was observed in apical parts. Comparatively, in both explants, addition of BA to MS medium was better in shoot regeneration and number of shoots per explant than adding to H<sub>3</sub>P<sub>4</sub> basal medium, It would be due to having less synergetic effect between BA and H<sub>3</sub>P<sub>4</sub> medium as compared

to MS medium. In addition, concentration of BA responded shoot regeneration differently among the explants, it might be due to different level of endogenous hormones contained in the explants. However, BA  $5.0 \text{ mg l}^{-1}$  was found to be suitable for both explants, whereas number of shoots induced by axillary bud was higher than that induced by apical part. Effect of BA alone on shoot regeneration from flower stalk had been reported by Chen and Piluek (1995), in which BA  $40 \text{ }\mu\text{M}$  could induce the number of shoots (6.0 per explant) from flower stalk of *Phalaenopsis*.

Influence of dark treatment in germination of orchid seeds had been reported in different species, in which its influences on seed germination varied depending on treatment periods and species (Arditti et al. 1981; Takahashi et al. 2000). Until now, however, its effect on shoot regeneration from flower stalk of *Phalaenopsis* has been limited. In this study, we found that dark treatment is likely to be important in shoot regeneration from the flower stalk, however, it is necessary to optimize the period giving optimal shoot regeneration; because the period shorter or longer than the optimal period seemed to be inhibiting percentage of shoot regeneration in this study.

Addition of AC to the medium apical parts showed increased percentage of shoot regeneration, but its opposite effect was observed when treated to axillary buds. In term of number of shoots, it was not increased in most treatments or even inhibited at higher concentration. However, the shoots induced by apical buds showed better and faster growth than those on the medium without AC while the growth of shoots induced by axillary buds was not affected by AC concentration. In apical part,  $0.5 \text{ g L}^{-1}$  AC was found to be a critical concentration controlling shoot growth than all other treatments, where the shoots attained a maximum height of 5.4 cm within 8 weeks, while AC did not affect growth of induced by on axillary bud. Thus, it can be suggested that ability of AC to promote in vitro shoot growth or shoot regeneration depends on explant types. The advantages of AC on in vitro seedling growth had been reported by Naing et al (2010), in which they claimed that positive effect of AC on in vitro seedling growth could be due to reduction of light at the base of explants, which promotes favorable environmental condition, or absorption of inhibitory substances such as polyphenols, which can be harmful to plant growth and proliferation (Fridborg and Eriksson, 1975).

In summary, MS medium containing  $5.0 \text{ mg L}^{-1}$  BA has shown to be suitable for shoot regeneration from apical part and axillary bud derived from flower stalk of *Phalaenopsis* hybrid 'Little gem'. In addition, incubation of the apical parts under dark condition for one week distinctly increased shoot regeneration percentage. Likewise, the explants cultured on the same medium containing  $0.5 \text{ g L}^{-1}$  AC also promoted shoot

regeneration and shoot growth.

In general, *Phalaenopsis* rarely produces offshoots in nature; in addition, vegetative propagation by stem cutting is also relatively low. Although shoot tip culture can be used as efficient technique for this kind of orchid, excision of the shoot tip damage the mother plants. Hence, utilization of flower stalk as explant sources would be a good choice for maintenance of the mother plants. Thus, protocol described herein could be useful for in vitro micropropagation and maintenance of this commercially important orchid.

### Acknowledgment

This work was supported by Export Promotion Technology Development Program, Ministry of Agriculture, Food and Rural Affairs, Republic of Korea. (No.IPET313009-4). This research was supported by Kyungpook National University Research Fund, 2015.

### References

- Arditti J (1967) Factors affecting the germination of orchid seeds. Bot. Rev. 33:1-97
- Chen Y and Piluek C (1995) Effects of thidiazuron and N6-benzylaminopurine on shoot regeneration of *Phalaenopsis*. Plant Growth Regulation 16:99-101
- Chen YC, Chang C, Chang WC (2000) A reliable protocol for plant regeneration from callus culture of *Phalaenopsis*. In Vitro Cell Dev Biol-Plant 36:420-423
- Chen JT and Chang WC (2004) Induction of repetitive embryogenesis from seed-derived protocorms of *Phalaenopsis amabilis* var. formosa Shimadzu. In vitro Cell Dev Biol-Plant 40:290-293
- Chung JD, Chun CK, Choi SO (1985) Asymbiotic germination of *Cymbidium ensifolium*. II. Effects of several supplements to the medium, pH values and light and/or dark culture periods on growth of rhizome and organogenesis for rhizome. J Korean Soc Hortic Sci 26:86-92
- Fridborg G and Eriksson T (1975) Effects of activated charcoal on growth and morphogenesis in cell cultures. Physiol. Plant. 34:306-308
- Ichihashi S (1997) Research on micropropagation of *Cymbidium*, nobile-type *Dendrobium*, and *Phalaenopsis* in Japan. In: Arditti, J. and A.M. Pridgeon (Eds.): Orchid Biology: Reviews and Perspectives, VII. Kluwer Academic Publishers, Dordrecht, Netherlands, p285-316
- Kosir P, Skof S, Luthar Z (2004) Direct shoot regeneration from nodes of *Phalaenopsis* orchid. Acta Agric Slov 83:233-242
- Naing AH, Park IS, Hwang YJ, Chung JD, Lim KB (2010) In vitro micropropagation and conservation of *Rhynchostylis retusa* BL. Hort Environ Biotechnol 51:440-444

- Naing AH, Chung JD, Park IS, Lim KB (2011) Efficient plant regeneration of the endangered medicinal orchid, *Coelogyne cristata* using protocorm-like bodies. *Acta Physiol Plant*. 33: 659-666
- Paek KY, Hahn EJ, Park SY (2011) Micropropagation of *Phalaenopsis* orchids via protocorms and protocorm-like bodies. In: Thorpe, T.A., Yeung, E.C. (Eds.), *Plant Embryo Culture: Methods and Protocols*. *Methods Mol Biol* 710:293-306
- Park SY, Murthy HN, Paek KY (2002) Rapid propagation of *Phalaenopsis* from floral stalk-derived leaves. *In Vitro Cell Dev Biol-Plant* 3:168-172
- Park YS, Kakuta S, Kano A, Okabe M (1996) Efficient propagation of protocorm-like bodies of *Phalaenopsis* in liquid medium. *Plant Cell Tiss Organ Cult* 45:79-85
- Takahashi K, Ogiwara I, Hakoda N (2000) Seed germination of *Habenaria (pecteilis) radiata* (Orchidaceae: Orchideae) in vitro. *Lindleyana* 15:59-63
- Tokuhara K and Mii M. (1993) Micropropagation of *Phalaenopsis* and *Doritaenopsis* by culturing shoot tips of flower stalk buds. *Plant Cell Rep* 13:7-11