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Micropropagation of two near threatened orchid. Part 2: *Phalaenopsis amabilis* Blume var. Grandiflora

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Key words: *in vitro* multiplication, orchid propagation, ornamentals, plant growth regulators.

Abstract: *Phalaenopsis* is one of the most popular orchids in the world, through the development of many artificial hybrids. In this research, a reliable and efficient protocol is presented for *in vitro* proliferation of *Phalaenopsis amabilis* Blume cv. Grandiflora. Protocorm-like bodies (PLBs) were cultured on Murashige and Skoog (MS) medium containing different concentrations of kinetin (Kn; 0.00, 0.50, 1.00, 2.00 and 3.00 mg l^{-1}) and indole-3-butyric acid (IBA; 0.00, 0.10, 0.20, 0.50 and 1.00 mg l^{-1}), either individually or in combination and activated charcoal (AC; 0.00, 0.50 and 1.00 g l^{-1}). A combination of 0.20 mg l^{-1} IBA and 2.00 mg l^{-1} Kn on medium containing 1.00 g l^{-1} AC was found to be suitable for maximum leaf number (6.16±0.503 per explant). The highest rooting frequency with 7.13±0.153 roots per explant was achieved on medium enriched with 0.50 mg l^{-1} IBA and 0.50 mg l^{-1} Kn on medium containing 1.00 g l^{-1} AC. The largest number of callus (9.10±0.611) was induced on explants cultured in medium containing 0.20 mg l^{-1} IBA and 0.50 mg l^{-1} Kn on medium without AC. The plantlets were successfully acclimatized in the greenhouse with a survival rate of 95% exhibiting normal developmental patterns.

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Data Availability Statement:
All relevant data are within the paper and its Supporting Information files.

Competing Interests:
The authors declare no competing interests.

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1. Introduction

Phalaenopsis Blume, known as moth orchid, is a genus of approximately 60 species native to tropical rainforests of South and South-East Asia, Australia and New Guinea (Winkelmann *et al*., 2006). *Phalaenopsis* as a cut and pot flowering plant is one of the most popular orchids in the trade and hobbyists through the development of many artificial hybrids. They are epiphytic plants, and consist of only a few leathery leaves (Sinha *et al*., 2010).

Large scale natural clonal propagation is not possible in *Phalaenopsis*. Therefore, the establishment of protocols for *in vitro* proliferation of orchids is the only method for high frequency regeneration of these plants. *In vitro* techniques can be used for storage of rare and endangered plant species and production of large number of plantlets in short period of time (Engelmann, 2011). *In vitro* multiplication of orchids deals with some problems such as high cost of production, low rate of shoot prolifer-

ation, poor rooting frequency and genetic variations (Bhattacharyya *et al*., 2016). Several methods for *in vitro* propagation of *Phalaenopsis* through callus induction and cell suspension culture were developed (Tanaka, 1992; Arditti and Ernst, 1993; Tokuhara and Mii, 2001; Sinha *et al*., 2010). Park *et al*. (2002) developed an efficient *in vitro* propagation method for *Phalaenopsis* by using protocorm-like bodies (PLBs) derived from leaf explants. Medium composition for *in vitro* culture of orchids by PLBs is species-specific and depends on several factors (Luo *et al*., 2009). Kuo *et al*. (2005) reported a protocol for regenerating a *Phalaenopsis* cultivar by direct somatic embryogenesis. This method was not so efficient and feasible for commercial propagation because of low frequency regeneration of different cultivars of *Phalaenopsis* hybrid. On the other hand, many protocols for *in vitro* propagation of orchids, especially those in danger of extinction, using PLBs as explants and various PGRs, have been reported (Sinha *et al*., 2010; Teixeira da Silva, 2006; Baker *et al*., 2014; Kaviani *et al*., 2017).

Various PGRs like α-naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA), 1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea (TDZ), 6-benzyle amino purine (BAP), 6-benzyladenine (BA) and kinetin (Kn) have been applied for micropropagation of rare and endangered orchids (Roy *et al*., 2011; Panwar *et al*., 2012; Zeng *et al*., 2012; Bhattacharyya *et al*., 2016; Kaviani *et al*., 2017). Many explants such as seed, leaf, node section, protocorm, PLB, tuber, shoot tip and inflorescence have been used for *in vitro* proliferation of endangered orchids (Sinha *et al*., 2010; Roy *et al*., 2011; Panwar *et al*., 2012; Zeng *et al*., 2012; Baker *et al*., 2014; Chen *et al*., 2015; Bhattacharyya *et al*., 2016). PLB is more efficient because of maximum multiplication in a short period of time (Luo *et al*. 2003). This study describes an efficient and reliable protocol for high frequency regeneration and callus induction of *Phalaenopsis amabilis* Blume var. Grandiflora, a rare and near endangered orchid species by PLBs.

2. Materials and Methods

Source of explant

Leaves (0.5-1 cm long) were excised from young *Phalaenopsis amabilis* Blume var. Grandiflora plants growing in the greenhouse of Hyrcan Agricultural Sciences and Biotechnology Research Institute, Amol, Iran. The leaves were washed under running tap water for 15-20 min and rinsed thoroughly with distilled water. These were surface sterilized with $HgCl_2$ (0.1% w/v) for 10 min followed by NaOCl (20%) for 15 min with 1 drop of Tween 20, then rinsed with sterile distilled water. Finally, leaves were sterilized in ethanol 75% for 1 min and washed 3-4 times with sterilized distilled water and finally excised to segments of 5-7 mm as primary explants for culture in Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 3% sucrose and 0.8% agar. The medium was supplemented with 0.20 mg l^{-1} NAA along with 3.00 mg l^{-1} BAP (appropriate types and concentrations of PGRs obtained before for maximum production of PLBs; data not shown). Healthy and sterilized PLBs (Fig. 1A) produced in the Plant Biotechnology Laboratory, Hyrcan Agricultural Sciences and Biotechnology Research Institute, Amol, Iran, were excised and used as secondary explants for *in vitro* propagation.

Culture medium and growth conditions

The explants (PLBs) were cultured in MS medium containing 3% sucrose and 0.8% agar. The medium was enriched with various PGRs and activated charcoal (AC). The pH of the medium was adjusted to 5.8 with 0.1 N NaOH or HCl prior to autoclaving. All media contained in culture bottles were autoclaved at 104 kPa and 121°C for 20 min.

To evaluate the effect of PGRs and AC on shoot multiplication (i.e. increasing the number of leaves and their development) and root induction, the explants were cultured on MS medium containing different concentrations of kn (0.00, 0.50, 1.00, 2.00 and 3.00 mg l^{-1}) and IBA (0.00, 0.10, 0.20, 0.50 and 1.00 mg l^{-1}), either individually or in combination. Medium was supplemented with or without activated charcoal (AC; 0.00, 0.50 and 1.00 g l^{-1}). For each treatment, three replicates and for each replicate, three specimens (explants) were taken (totally; 75 treatments, 225 replicates and 675 specimens or explants). Following establishment, cultures were maintained at 24±2°C, 70-80% RH, and 16-h photoperiod of 50-60 µmol m^{-2} s^{-1} irradiance provided by cool-white fluorescent tubes.

After 60 days, the effect of PGRs and AC on advanced PLBs development was assessed by measuring number of leaves per explant, leaf length, leaf width, number of roots per explant, root length, number of explants with callus and viability percentage.

Plant development and acclimatization

In vitro rooted plantlets were taken out from culture vessels and washed thoroughly under running tap water to remove adherent nutrient and transplanted to plastic pots (18 cm height × 12 cm diameter) filled

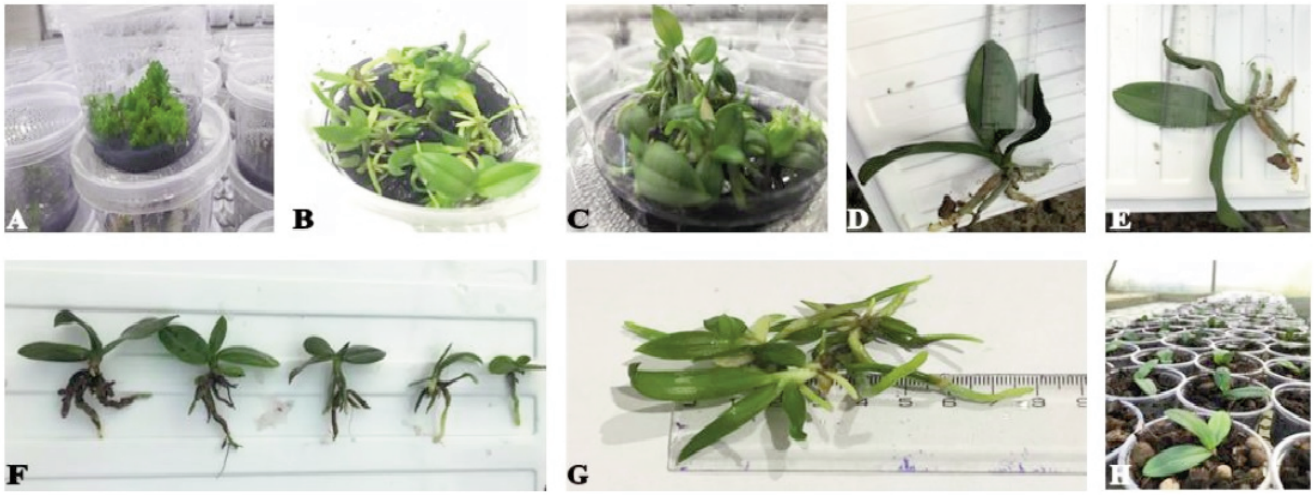

Fig. 1 - Micropropagation of *Phalaenopsis amabilis* Blume cv. Grandiflora through protocorm-like bodies (PLBs). (A) PLBs produced on MS medium containing 0.20 mg l^{-1} NAA + 3.00 mg l^{-1} BAP. (B) Micropropagated shoots from PLBs on medium containing 0.50 mg l^{-1} IBA + 2.00 mg l^{-1} Kn. (C) Plantlets produced on medium supplemented with 0.20 mg l^{-1} IBA + 2.00 mg l^{-1} Kn + 1.00 g l^{-1} AC. (D) Length of leaf obtained on medium containing 0.50 mg l^{-1} IBA together with 2.00 mg l^{-1} Kn and 1.00 g l^{-1} AC. (E) Width of leaf obtained on medium containing 0.50 mg l^{-1} IBA together with 1.00 mg l^{-1} Kn 1.00 g l^{-1} AC. (F) Plantlets produced on media containing 1.00 g l^{-1} AC together with different concentrations of IBA and Kn. From left to right: 0.10 mg l^{-1} IBA + 0.50 mg l^{-1} Kn, 0.50 mg l^{-1} IBA + 0.50 mg l^{-1} Kn, 0.20 mg l^{-1} IBA + 1.00 mg l^{-1} Kn, 0.10 mg l^{-1} IBA + 1.00 mg l^{-1} Kn and 0.50 mg l^{-1} Kn without IBA. (G) Number and length of roots obtained on medium enriched with 0.50 mg l^{-1} IBA plus 0.50 mg l^{-1} Kn and 1.00 g l^{-1}. (H) Greenhouse acclimatized plantlets in pots filled with leca, peat moss and perlite (in ratio of 1:1:1).

with a potting mixture of leca (Light Expanded Clay Aggregate), peat moss and perlite (1:1:1). All the pots were then transferred to the greenhouse with temperature of 24±2°C to 20±2°C day/night (light intensity of 3500 Lux, RH of 80-90% and 14-h photoperiod) for acclimatization. The pots were covered with polyethylene bags to retain moisture inside and were opened gradually during 2 weeks. Plantlets were initially covered with a polythene sheet to maintain relative humidity (90%). The number of surviving plants was recorded after 12 weeks of transfer.

Experimental design and data analysis

The experiments were established in a completely randomized design with three replicates per treatment (totally 675 explants). PGRs-free MS medium was used as control in the experiments. The results were expressed as mean±SD. Data were subjected to analysis of variance (ANOVA) (except for acclimatization records) and means were compared by the LSD test at $P<0.05$ using the SPSS ver. 17 (SPSS Inc., USA).

3. Results

The effect of PGRs and AC on the leaf growth (number, length and diameter) and root growth (number and length) of *Phalaenopsis amabilis* Blume var. Grandiflora is shown in Tables 1-4 and figure 1. PLBs produced on MS medium containing 0.20 mg l^{-1} NAA + 3.00 mg l^{-1} BAP (appropriate types and concentrations of PGRs obtained before for maximum production of PLBs; data not shown), were used as primary explants (Fig. 2A). These PLBs were produced after 60 days of culture of leaf explant on this medium (Fig. 2B). These PLBs were used as secondary explants and cultured on media supplemented with

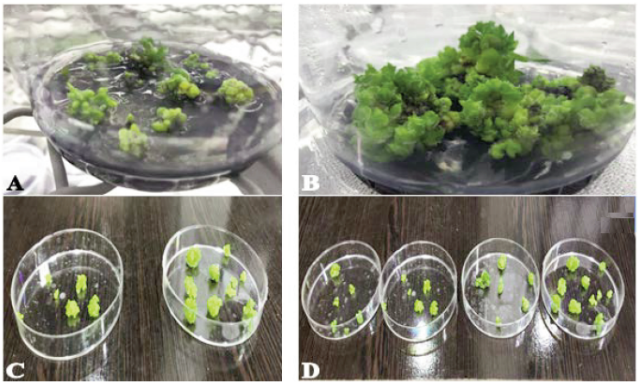

Fig. 2 - (A) PLBs and callus formation. (B) PLBs development and multiplication in MS medium containing 0.20 mg l^{-1} NAA + 3.00 mg l^{-1} BAP. (C) Callus formation from the explants cultured in media enriched with 0.50 mg l^{-1} IBA + 3.00 mg l^{-1} Kn (left) and 0.20 mg l^{-1} IBA and 0.50 mg l^{-1} Kn (right). (D) Callus formation from the explants cultured in media enriched with different concentrations of IBA and Kn (from left to right: 0.50 mg l^{-1} IBA + 3.00 mg l^{-1} Kn, control, 1.00 mg l^{-1} IBA + 1.00 mg l^{-1} Kn and 0.20 mg l^{-1} IBA and 0.50 mg l^{-1} Kn).

different concentrations of PGRs and AC. ANOVA showed significant differences between various concentrations of PGRs and AC on most measured parameters (Table 1).

Effect of PGRs and AC on multiplication parameters

Advanced shoot development was significantly affected by the composition of the medium. The media containing 0.20 mg l^{-1} IBA and 2.00 mg l^{-1} Kn along with 0.50 g l^{-1} AC, and without AC were suitable for leaf number (Tables 2, 3, Figs. 1B, C). The highest leaf length (4.66±0.702 cm per explant) and leaf width (3.13±0.603 cm per explant) were obtained in media enriched with 0.50 mg l^{-1} IBA along with 2.00 mg l^{-1} Kn and 0.50 mg l^{-1} IBA along with 1.00 mg l^{-1} Kn, respectively (Table 4, Figs. 1D, E). MS medium enriched with 0.20 mg l^{-1} IBA and 2.00 mg l^{-1} Kn along with 1.00 g l^{-1} AC was the most appropriate medium for leaf number (6.16±0.503 per explant) (Table 4). Among all concentrations of IBA, Kn and AC used

Table 1 - Effect of different concentrations of Kn, IBA and AC on the studied parameters of *in vitro* grown *Phalaenopsis amabilis* Blume cv. Grandiflora

Source of variations	df	Mean of squares						
		Leaf number	Leaf length	Leaf width	Root number	Root length	Callus number	Viability percentage
AC	2	6.526 **	5.563 **	6.881 **	6.38 **	5.87 **	3.402 ns	76.00 ns
IBA	4	14.71 **	8.818 **	4.708 **	23.50 **	48.60 **	5.224 **	1000 **
Kn	4	48.81 **	9.746 **	9.030 **	5.54 **	11.13 **	49.2 **	1026 **
AC × IBA	8	0.206 ns	1.139 **	0.417 *	3.29 **	2.35 **	9.66 **	291 **
AC × Kn	8	1.238 **	1.560 **	0.3782 ns	1.63 **	1.434 *	12.87 **	297 **
IBA × Kn	16	7.043 **	4.208 **	2.805 **	6.35 **	4.43 **	20.60 **	651 **
AC × IBA × Kn	32	1.326 **	1.587 **	0.697 **	2.89 **	2.066 **	10.90 **	662 **
Error	150	0.453	0.304	0.197	0.597	0.602	1.517	99.66
CV	–	19.09	20.83	25.01	15.79	17.6	25.47	12.47

*, **: Significant at the 0.05 and 0.01 probability level, respectively, NS: Not significant at p=0.05.

Table 2 - Effect of different concentrations of Kn and IBA without AC on the studied parameters of *in vitro* grown *Phalaenopsis amabilis* Blume cv. Grandiflora

PGRs (mg l^{-1})		Leaf number	Leaf length (cm)	Leaf width (cm)	Root number	Root length (cm)	Callus number	Viability percentage
IBA	Kn							
0.00	0.00	2.66±0.681 d-g	1.73±0.153 h	1.16±0.306 fg	4.03±0.513 bc	3.33±0.603 hi	4.76±2.454 cd	70.00±10.000 bc
0.00	0.50	3.76±0.624 bcd	2.03±0.208 e-h	0.93±0.153 g	4.33±0.929 a-c	3.10±0.624 i	5.03±0.854 cd	63.30±10.000 c
0.00	1.00	2.93±0.265 c-g	2.13±0.520 d-h	1.20±0.265 e-g	4.00±0.529 bc	3.53±1.206 f-i	5.10±1.539 cd	73.30±10.000 a-c
0.00	2.00	2.20±0.493 fg	2.86±0.503 b-e	1.56±0.153 d-g	4.86±1.358 a-c	4.73±0.400 bcdef	4.53±0.737 cd	90.00±15.275 a
0.00	3.00	2.83±0.208 d-g	2.40±0.208 c-h	2.00±0.100 c-e	3.76±0.416 c	3.30±0.231 hi	7.96±2.166 ab	80.00±10.000 ab
0.10	0.00	2.06±0.200 g	1.83±0.321 f-h	1.66±0.100 c-g	4.80±0.889 a-c	4.20±0.794 b-i	3.63±1.332 cd	96.60±15.275 a
0.10	0.50	3.20±0.929 c-g	2.30±0.802 c-h	1.56±0.361 d-g	4.56±0.100 a-c	3.93±0.493 c-i	3.16±0.954 d	80.00±15.275 ab
0.10	1.00	3.10± 0.643 c-g	2.43±0.781 c-h	2.43±0.265 a-c	5.16±0.800 a-c	5.13±0.702 bcd	5.73±2.452 bc	70.00±10.000 bc
0.10	2.00	4.10±0.624 a-c	2.26±0.153 c-h	2.86±0.794 ab	4.53±1.415 a-c	4.43±0.451 b-h	3.76±3.694 cd	73.30±20.000 a-c
0.10	3.00	3.00±0.300 c-g	1.80±0.153 gh	1.26±0.351 d-g	4.06±0.493 bc	4.20±1.012 b-i	4.60±1.193 cd	83.30±10.000 ab
0.20	0.00	2.56±0.300 efg	2.40±0.854 c-h	1.16±0.361 fg	4.96±1.450 a-c	5.30±0.351 b	3.56±0.781 cd	73.30±10.000 a-c
0.20	0.50	3.76±0.306 bcd	2.43±0.321 c-h	1.80±0.529 c-f	5.40±0.700 ab	4.20±0.462 b-i	9.10±0.611 a	73.30±10.000 a-c
0.20	1.00	3.73±0.723 b-e	3.10±0.700 a-d	2.03±0.500 cd	5.40±0.493 ab	6.60±0.458 a	4.76±1.858 cd	83.30±10.000 ab
0.20	2.00	5.10±0.889 a	2.76±0.493 b-g	3.06±0.153 a	5.63±1.015 a	5.10±0.586 bcd	3.40±0.473 d	80.00±10.000 ab
0.20	3.00	3.73±0.153 b-e	3.66±0.794 ab	1.96±0.529 c-f	4.90±0.651 a-c	4.96±1.365 bcde	4.60±0.651 cd	80.00±15.275 ab
0.50	0.00	2.83±0.153 d-g	2.16±0.306 d-h	1.33±0.814 d-g	4.90±0.954 a-c	5.23±0.361 b	4.73±0.896 cd	90.00±5.774 a
0.50	0.50	3.46±0.300 b-e	2.33±0.153 c-h	1.70±0.058 c-g	5.20±0.721 a-c	4.40±1.250 b-h	3.93±0.404 cd	80.00±10.000 ab
0.50	1.00	3.66±0.361 b-e	2.90±0.854 a-e	2.06±0.200 b-d	4.50±0.971 a-c	5.20±1.552 bc	4.86±1.973 cd	80.00±10.000 ab
0.50	2.00	4.60±0.361 ab	3.63±0.721 ab	1.33±0.351 d-g	4.56±0.635 a-c	4.66±0.961 b-g	3.40±0.833 d	80.00±10.000 ab
0.50	3.00	3.20±0.503 c-g	2.20±0.458 c-h	1.30±0.265 d-g	5.06±1.250 a-c	3.40±0.513 g-i	4.73±1.249 cd	80.00±10.000 ab
1.00	0.00	2.86±0.503 d-g	2.26±0.306 c-h	1.56±0.100 d-g	4.56±1.457 a-c	3.73±1.350 e-i	4.43±1.058 cd	80.00±10.000 ab
1.00	0.50	3.13±0.458 c-g	2.13±0.764 d-h	1.66±0.208 c-g	4.63±1.358 a-c	4.20±1.286 b-i	5.73±3.134 bc	90.00±10.000 a
1.00	1.00	3.03±0.833 c-g	2.83±0.306 b-f	1.96±0.751 c-f	4.33±0.681 a-c	3.86±0.987 d-i	4.93±1.102 cd	73.30±10.000 a-c
1.00	2.00	4.56±0.723 ab	3.20±0.351 a-c	1.63±0.800 c-g	4.70±0.651 a-c	3.86±1.845 d-i	4.03±2.364 cd	80.00±5.774 ab
1.00	3.00	3.26±0.854 c-e	3.90±0.208 a	1.40±0.850 d-g	4.56±0.404 a-c	4.06±0.416 b-i	4.20±0.513 cd	90.00±15.275 a

Means with different letters on the same column are significantly different (p<0.05) based on LSD test.

Table 3 - Effect of different concentrations of Kn and IBA along with 0.50 mg l^{-1} AC on the studied parameters of *in vitro* grown *Phalaenopsis amabilis* Blume cv. Grandiflora

PGRs (mg l^{-1})		Leaf number	Leaf length (cm)	Leaf width (cm)	Root number	Root length (cm)	Callus number	Viability percentage
IBA	Kn							
0.00	0.00	3.40±0.950 cd	1.66±0.872 f	1.73±1.210 b-e	3.86±0.907 gh	3.20±0.493 g	4.00±0.624d-g	90.00±10.000 a
0.00	0.50	2.66±0.907 d	1.76±0.252 ef	0.80±1.250 f	3.23±1.286 h	3.33±0.351 efg	5.60±1.054 bcd	80.00± 0.000 ab
0.00	1.00	3.63±0.819 bcd	2.60±0.265 a-f	1.30±0.208 c-f	4.10±0.751 f-h	3.16±0.346 g	6.70±1.136 abc	80.00±20.817 ab
0.00	2.00	2.90±1.106 d	3.10±0.306 abc	1.60±0.513 b-e	5.13±0.889 b-g	3.23±0.458 fg	3.00±0.666 g	70.00±15.275 b
0.00	3.00	2.90±0.917 d	2.33±0.961 b-f	1.33±0.513 c-f	4.80±0.907 b-g	3.33±0.954 efg	3.73±1.159 d-g	70.00±10.000 b
0.10	0.00	3.20±0.351 d	2.33±0.265 b-f	1.13±0.115 ef	5.80±1.002 a-e	5.06±1.212 a-d	3.26±1.332 fg	90.00±5.774 a
0.10	0.50	2.66±0.777 d	2.10±0.265 def	1.60±0.100 b-e	6.16±0.473 abc	3.76±0.751 d-g	5.50±0.721 bcd	90.00±10.000 a
0.10	1.00	2.86±0.702 d	2.43±0.721 b-f	2.06±0.208 abc	5.83±0.200 a-e	4.23±0.557 c-g	6.56±0.656 abc	90.00±10.000 a
0.10	2.00	5.13±0.666 a	2.10±0.611 def	2.30±0.265 a	5.96±1.682 a-d	3.86±0.100 c-g	3.40±1.353 efg	90.00±15.275 a
0.10	3.00	3.33±1.193 d	2.53±0.666 b-f	1.56±0.306 b-f	4.90±0.917 b-g	4.70±1.266 a-e	3.53±1.159 d-g	73.30±10.000 ab
0.20	0.00	2.96±1.277 d	2.90±0.458 a-d	1.20±0.265 def	3.73±0.520 gh	5.96±1.106 ab	3.90±0.656 d-g	70.00±10.000 b
0.20	0.50	3.26±0.473 d	2.63±0.436 a-f	1.93±0.451 a-d	6.10±1.411 abc	4.63±1.229 b-f	5.36±0.666 b-e	70.00±15.275 b
0.20	1.00	3.80±1.044 bcd	2.23±0.351 c-f	2.26±0.289 ab	7.06±0.777 a	6.06±1.710 a	5.43±1.514 b-e	76.60±20.817 ab
0.20	2.00	5.80±1.002 a	2.16±0.557 c-f	1.83±0.814 b-e	5.56±0.557 a-f	5.26±0.902 abc	8.56±3.029 a	70.00±15.275 b
0.20	3.00	3.83±0.800 bcd	3.23±0.611 ab	1.73±1.150 b-e	4.50±1.436 d-h	4.00±0.800 c-g	3.16±0.624 fg	90.00±10.000 a
0.50	0.00	3.10±1.539 d	2.10± 0.950 def	1.36±0.681 c-f	5.40±1.234 b-f	4.40±1.290 c-g	7.30±0.702 ab	70.00±10.000 b
0.50	0.50	4.63±1.629 abc	2.70±0.153 a-e	1.63±0.586 b-e	6.23±1.320 ab	3.80±2.022 d-g	4.33±0.643 d-g	73.30±10.000 ab
0.50	1.00	2.86±0.709 d	2.73±1.210 a-e	1.90±0.611 a-f	4.93±0.700 b-g	4.53±0.709 c-g	5.16±1.229 c-f	80.00±10.000 ab
0.50	2.00	4.76±0.473 ab	3.56±0.709 a	1.46±0.709 c-f	4.66±0.624 c-h	4.00±0.954 c-g	4.76±0.833 c-g	86.60±10.000 ab
0.50	3.00	3.53±0.361 bcd	2.93±1.595 a-d	1.70±0.458 b-e	4.13±1.007 f-h	4.56±0.900 b-g	4.90±0.436 c-g	86.6±10.000 ab
1.00	0.00	2.86±0.681 d	3.03±0.306 a-d	1.56±0.252 b-f	4.73±0.794 b-h	3.66±1.234 d-g	3.20±2.219 fg	70.00±10.000 b
1.00	0.50	3.36±0.900 cd	2.36±0.755 b-f	1.66±0.208 b-e	4.53±0.361 d-h	4.56±0.436 b-g	5.60±3.005 bcd	90.00±10.000 a
1.00	1.00	3.40±0.794 cd	2.93±0.300 a-d	1.93±0.755 a-d	4.43±0.987 e-h	4.53±1.405 c-g	4.63±3.233 c-g	70.00±15.275 b
1.00	2.00	4.73±0.379 ab	2.90±0.153 a-d	1.46±0.200 c-f	4.13±1.493 f-h	4.26±0.624 c-g	3.93±0.666 d-g	73.30±10.000 ab
1.00	3.00	3.76±0.907 bcd	2.73±0.100 a-e	1.63±0.451 b-e	4.20±1.795 f-h	4.20±0.755 c-g	3.93±0.153 d-g	90.00±11.547 a

Means with different letters on the same column are significantly different ($p<0.05$) based on LSD test.

individually, maximum leaf number (4.52±0.33 per explant) was produced in medium containing 2.00 mg l^{-1} Kn (data not shown). Production of leaf was relatively high by all PLBs grown on medium containing 2.00 mg l^{-1} Kn in combination with all concentrations of IBA with or without AC (Tables 2, 3, 4). Thus, the optimal concentration of Kn was 2.00 mg l^{-1}. Also, the optimal concentrations of IBA were 0.20 and 0.50 mg l^{-1}. These concentrations in combination with each other recorded maximum shoot and root production. Media supplemented with 1.00 g l^{-1} AC was most suitable for *in vitro* leaf growth since it resulted in the largest leaf number and development (Table 4, Figs. 1D, E, F).

Advanced root development was significantly affected by the composition of the medium, when measured through root length and root number. All treatments of PGRs and AC, individually and in combination had significant effects ($P<0.01$) on root growth (Table 1). Root length was highest (6.66±0.709 cm per explant) in presence of 0.20 mg l^{-1} IBA plus 2.00 mg l^{-1} Kn and 1.00 g l^{-1} AC medium (Table 4). However, no statistically significant difference in root length was detected between this and half-concentration of IBA and Kn (Fig. 1F). All other media differed significantly and gave lower root length growth rates. Among all treatments, 0.50 mg l^{-1} IBA plus 0.50 mg l^{-1} Kn and 1.00 g l^{-1} AC was found to be the most effective for root formation (7.13±0.153 per explant) (Table 4, Fig. 1G). However, the root number (7.06±0.777 per explant) produced in medium containing 0.20 mg l^{-1} IBA plus 1.00 mg l^{-1} Kn and 0.50 g l^{-1} AC was noticeable (Table 3). In most cases, minimum root number was recorded in media without IBA. Among all concentrations of IBA, Kn and AC used individually, maximum root number (5.28±0.564 per explant) was produced in medium containing 0.20 mg l^{-1} IBA (data not shown).

The *in vitro* rooted plantlets were successfully acclimatized in the greenhouse (Fig. 1H). Pots were filled with leca, peat moss and perlite (in ratio of 1:1:1). Acclimatization was achieved in 4-6 weeks, and at this stage plants attain the height of about 12-16 cm. Acclimatization of micropropagated plantlets to the natural conditions requires several anatomical, morphological and physiological changes especially in xylem, leaves and photosynthesis. The hardened plantlets were maintained in the Hyrcan Agricultural

Sciences and Biotechnology Research Institute, Amol, Iran with 95% field establishment rate.

Effect of PGRs and AC on viability percentage

Significant differences were found in viability percentage among the different concentrations of PGRs alone and in combination with each other, also with AC concentrations. The rate of produced plantlets was highest when IBA at 0.10 mg l^{-1} alone was added to the media (Table 2). Least viability percentage (63.30±10.00) was observed in PLBs cultured on media containing 0.50 mg l^{-1} Kn without AC and 1.00 mg l^{-1} IBA along with 1.00 mg l^{-1} Kn with 1.00 g l^{-1} AC (Tables 2, 4).

Effect of PGRs and AC on callus production

LSD test did not show significant differences among different concentrations of AC for callus production. A combination of 0.20 mg l^{-1} IBA and 0.50 mg l^{-1} Kn induced highest callus production (9.10±0.611) (Figs. 2C, D), which differed significantly from the other tested combinations, being this rate two or three-fold higher than in the other treatments (Tables 2, 3, 4). There was no any direct correlation between increasing PGRs and AC concentrations and increase in callus production. In most cases, minimum callus formation was observed in the explants cultured on media without IBA or Kn with or without AC (Tables 2, 3, 4, Figs. 2C, D).

4. Discussion and Conclusions

The present investigation demonstrated that the addition of external PGRs in proper concentrations induced leaf formation from the PLBs explants cultured in the MS medium. The regeneration of multiple shoots (leaves in some orchids like *Phalaenopsis amabilis*) has been reported to be closely related with the type and concentration of cytokinins used (Amoo *et al*., 2014). Development of multiple shoots from PLBs has been successfully achieved in some orchids such as *Cymbidium*, *Dendrobium*, *Catasetum*, *Phalanoepsis*, *Habeneria* and *Satyrium* (Talukdar, 2001; Sheelavanthmath and Murthy, 2001; Mahendran and Bai, 2009; Baker *et al*., 2014; Kaviani *et al*., 2017). In *Dendrobium huoshanense*, Kn was reported to be more effective for plantlet regeneration from PLBs than other cytokinins (Luo *et al*., 2009). Kn was also used for shoot multiplication of

Table 4 - Mean comparison of the effect of different concentrations of Kn and IBA on measured characters of *Catasetum pileatum* Alba grown *in vitro* condition

PGRs (mg l^{-1})		Leaf number	Leaf length (cm)	Leaf width (cm)	Root number	Root length (cm)	Callus number	Viability percentage
IBA	Kn							
0.00	0.00	3.30±0.896 e-h	2.30±0.529 ef	1.40±0.462 d	4.50±0.802 c-f	3.46±0.436 de	5.80±0.208 a-c	90.00±15.275 a
0.00	0.50	3.10±0.493 e-h	2.56cdef ± 0.656	1.60±0.451 d	3.90±0.529 f	3.20±1.153 e	4.56±1.422 bc	73.30±10.000 a-c
0.00	1.00	3.40±0.666 d-g	2.73±0.802 cdef	2.20±0.608 a-d	4.83±1.464 b-f	4.16±1.210 c-e	5.66±0.451 a-c	80.00±10.000 a-c
0.00	2.00	3.43±0.473 d-g	2.33±0.361 def	1.90±0.737 bcd	4.90±0.917 b-f	3.73±1.069 c-e	4.43±1.779 bc	70.00±11.547 bc
0.00	3.00	3.06±1.453 e-h	2.20±1.405 ef	2.30±0.153 a-d	4.33±0.321 def	4.53±0.208 b-e	3.86±0.265 c	73.30±10.000 a-c
0.10	0.00	3.00±0.611 e-h	2.30±0.416 ef	1.50±0.321 d	5.36±0.493 b-e	5.10±0.458 a-d	3.83±0.666 c	80.00±15.275 abc
0.10	0.50	2.83±0.945 gh	3.16±1.401 cde	2.00±0.208 a-d	4.73±0.764 b-f	4.53±0.643 b-e	6.90±1.007 ab	90.00±10.000 a
0.10	1.00	3.26±1.277 e-h	2.23±0.794 ef	2.10±0.200 a-d	5.86±0.451 abc	6.13±0.436 ab	5.80±1.097 a-c	73.30±10.000 a-c
0.10	2.00	5.03±1.290 ab	3.46±0.551 bc	2.63±0.400 ab	5.76±0.208 abcd	5.13±1.168 a-d	5.20±0.889 a-c	90.00±5.774 a
0.10	3.00	2.90±1.650 fgh	3.06±0.200 cdef	2.06±0.513 a-d	4.73±0.200 b-f	4.50±0.624 b-e	4.26±1.217 bc	90.00±10.000 a
0.20	0.00	2.26±0.850 h	2.90±0.208 cdef	1.43±0.351 d	6.10±0.950 ab	4.70±0.473 b-e	3.80±1.332 c	90.00±10.000 a
0.20	0.50	3.80±0.929 b-g	3.20±0.416 cde	1.90±0.416 bcd	4.40±0.473 def	4.06±0.666 c-e	6.90±0.300 ab	80.00±10.000 a-c
0.20	1.00	3.23±0.513 e-h	2.60±0.794 cdef	2.56±0.896 abc	6.10±0.700 ab	6.66±0.709 a	5.03±1.350 bc	76.60±10.000 a-c
0.20	2.00	6.16±0.503 a	2.73±0.907 cdef	2.86±0.058 b	4.46±0.643 c-f	5.40±0.361 a-c	4.36±1.790 bc	70.00±10.000 bc
0.20	3.00	4.20±0.208 b-f	3.43±0.666 bcd	2.00±0.208 a-d	4.93±0.850 b-f	5.20±0.850 a-c	3.80±1.836 c	90.00±10.000 a
0.50	0.00	3.00±0.153 e-h	2.20±0.651 ef	1.80±0.058 bcd	5.36±0.252 bcde	4.66±0.850 b-e	6.16±1.159 a-c	76.60±10.000 ac
0.50	0.50	4.63±0.702 bcd	2.70±0.306 cdef	1.56±0.987 d	7.13±0.153 a	4.40±1.124 c-e	5.26±0.586 a-c	86.60±11.547 ab
0.50	1.00	3.73±0.436 b-g	2.80±0.737 cdef	3.13±0.603 a	4.10±0.416 ef	3.86±0.493 c-e	4.46±1.909 bc	73.30±10.000 a-c
0.50	2.00	4.93±0.814 abc	4.66±0.702 a	1.43±0.551 d	4.76±0.681 b-f	4.36±0.751 c-e	4.03±0.889 c	80.00±5.774 a-c
0.50	3.00	3.66±0.404 c-g	2.90±0.300 cdef	1.76±0.306 c	4.50±0.854 c-f	4.40±0.252 cde	5.10±0.751 a-c	90.00±10.000 a
1.00	0.00	3.06±0.569 e-h	3.00±0.300 cdef	2.06±0.231 a-d	5.00±0.971 b-f	3.96±0.862 c-e	4.13±0.751 c	80.00±15.275 a-c
1.00	0.50	3.33±1.114 d-g	2.53±1.124 cdef	1.66±0.709 cd	4.86±0.757 b-f	5.06±0.529 a-d	7.73±1.210 a	70.00±10.000 bc
1.00	1.00	3.86±0.404 b-g	1.96±0.700 f	2.06±0.265 a-d	4.96±0.451 b-f	4.86±0.850 b-e	4.90± 1.253 bc	63.30±10.000 c
1.00	2.00	4.30±0.416 bcde	2.26±0.777 ef	1.86±0.115 bcd	4.76±0.557 b-f	4.30±0.361 c-e	4.56±0.929 bc	90.00±10.000 a
1.00	3.00	4.10±0.458 b-g	4.46±0.321 ab	1.70±0.458 bcd	4.83±0.651 b-f	3.80±0.624 c-e	3.80±0.458 c	90.00±10.000 a

Means with different letters on the same column are significantly different ($p<0.05$) based on LSD test.

some other orchids (Saiprasad *et al*., 2004; Malabadi *et al*., 2005; Panwar *et al*., 2012). In *Satyrium nepalense*, protocorm developed multiple shoots directly on the medium supplemented with cytokinins. In most of the orchids the presence of cytokinins alone promoted optimal shoot proliferation (Mahendran and Bai, 2009). BA is known to promote seedling leaf formation in *Paphiopedilum* (Huang *et al*., 2001; Chen *et al*., 2015). Bhattacharyya *et al*. (2016) reported that when the explants were grown in medium containing cytokinin and auxin, a higher rate of response frequency of shoot buds and PLBs was observed in all PGRs combinations. Also according to Seeni and Latha (2000) and Roy *et al*. (2011), PGRs in orchids act more efficiently when used in combination. Therefore, cytokinin and auxin are supposed to act synergistically.

Effectiveness of AC on shoot multiplication and leaf growth has been demonstrated in some orchids (George and Ravishankar, 1997; Thomas and Michael, 2007; Hossain *et al*., 2010; Roy *et al*., 2011; Zeng *et al*., 2012; Panwar *et al*., 2012). Study on *Paphiopedilum wardii* evidenced that the plantlet growth *in vitro* was significantly affected by AC along with PGRs (Zeng *et al*., 2012). Roy *et al*. (2011) showed that healthy plantlets of *Vanda coerulea* were induced from PLBs when cultured on medium fortified with 3.00 g l^{-1} AC, 5.36 µM NAA and 3.80 µM BAP. In our study, there was no difference between 0.50 and 1.00 g l^{-1} AC for leaf growth (Tables 3, 4). Supplementation of AC in the media significantly influenced plantlet growth (shoot multiplication and root growth) over the control (Roy *et al*., 2011). This finding confirmed our results on the effect of AC on leaf growth parameters. In fact, a combination of 0.20 mg l^{-1} IBA and 2.00 mg l^{-1} Kn on medium containing 0.50 and 1.00 g l^{-1} AC was found to be suitable for maximum leaf number.

This positive effect of AC on shoot multiplication has been attributed to the ability of AC to absorb phenolic compounds released by the plantlets into the media and regulate the pH level (Pan and van Staden, 1998; Eymar *et al*., 2000). A positive linear relationship was found between AC concentration and plantlet growth of *Vanda coerulea* Griff ex. Lindl. (Blue Vanda) (Roy *et al*., 2011). In *Paphiopedilum spicerianum*, 1.0 mg l^{-1} NAA, 10% banana homogenate and 0.50 g l^{-1} AC was the most effective to promote seedling formation (Chen *et al*., 2015). AC might also act as a growth promoter that inhibits harmful effects of some compounds produced during seedling formation (Roy *et al*., 2011).

Our study showed the positive effect of IBA on root formation. In *Satyrium nepalense* and *Dendrobium nobile*, IBA resulted in a better rooting efficiency over NAA in terms of rooting frequency and root number (Mahendran and Bai, 2009; Bhattacharyya *et al*., 2016). According to these authors, maximum rooting efficiency (86% or 5.4 roots/shoot) was obtained in medium fortified with 2.00 mg l^{-1} IBA in *Dendrobium nobile* (Bhattacharyya *et al*., 2016), while the highest number of roots per shoot (6.40) was achieved at 9.84 mM IBA in *Satyrium nepalense* D. Don. (Mahendran and Bai, 2009). The effectiveness of IBA in rooting has been shown for some other orchids like *Vanilla planifolia* (Giridhar *et al*., 2001), *Cymbidium aloifolium* (L.) SW. and *Dendrobium nobile* Lindl. (Nayak *et al*., 2002), *Cymbidium pendulum* (Nongdam *et al*., 2006), *Satyrium nepalense* (Mahendran and Bai, 2009), *Vanda teres* (Firoz Alam *et al*., 2010) and *Eulophia nuda* Lindl. (Panwar *et al*., 2012). A maximum 90% response for root formation and highest number of roots (5.50) with length of 5.30 cm per shoot was obtained on IBA (2.46 mM) treated shoots of *Eulophia nuda* Lindl. (Panwar *et al*., 2012). Study of Baker *et al*. (2014) on micropropagation of *Catasetum* demonstrated that the largest number of root (7.16) and root length (193.40 mm) were obtained on MS medium supplemented with 0.50 mg l^{-1} BA along with 0.50 mg l^{-1} NAA. Our results are in line with previous findings, as maximum root length and root number were obtained in medium containing both IBA and Kn.

Our study showed the positive effect of AC on root growth. Similarly, Roy *et al*. (2011) evidenced a positive influence of AC on root growth of *Vanda coerulea*. Some other researches demonstrated that the presence of AC in the media stimulated rooting in *Vanilla planifolia* (George and Ravishankar, 1997), *Cymbidium sinense* (Chang and Chang, 2000) and *Paphiopedilum spicerianum* (Chen *et al*., 2015). Addition of AC in rooting medium maintained the pH level, increased the nitrogen uptake and stimulated the rooting of *in vitro* shoots (Eymar *et al*., 2000; Panwar *et al*., 2012).

In the present work, minimum callus formation was obtained frequently on media without IBA or Kn with or without AC. In *Eulophia nuda* and on medium containing higher concentration of BA, the explants produced callus at the base of shoots while lesser number of shoots were differentiated on medium with lower BA concentration (Panwar *et al*., 2012). Also, in medium with higher concentration of BA

combined with Kn lower shoot production with more callus induction was observed.

In conclusion, *Phalaenopsis amabilis* Blume var. Grandiflora is a scarce and near threatened orchid. Many of orchid's species and cultivars are threatened, rare, vulnerable, endangered, indeterminate or in danger of extinction. Thus, it is necessary to develop convenient methods for the conservation and large scale production of these plants to be used for re-introduction, as well as commercial propagation.

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