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Micropropagation of two near threatened orchid. Part 1: *Catasetum pileatum* cv. Alba

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Abstract: Many orchid species are threatened. In this study, a reliable and efficient protocol was outlined for *in vitro* propagation of *Catasetum pileatum* cv. Alba, a near threatened orchid species with the proper usage of plant growth regulators (PGRs). Protocorms as explants were cultured on Murashige and Skoog (MS) medium containing different concentrations of kinetin (Kn; 0.00, 0.20, 0.50, 1.00, 2.00, 3.00 and 5.00 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. The frequency of protocorm-like bodies (PLBs) regeneration significantly relied on concentrations of PGRs used. A combination of 1.00 mg l^{-1} Kn and 1.00 mg l^{-1} IBA was found to be suitable for maximum PLB regeneration (8.63 per explant) and the largest number of leaf (12.70 per explant). The highest rooting frequency with 7.40 roots per explant was achieved on protocorms grown in medium enriched with 1.00 mg l^{-1} Kn and 0.50 mg l^{-1} IBA. Plantlets were transplanted to pots filled with a mixture of peat moss, leca and perlite (1:1:1) and transferred to the greenhouse. The plantlets were successfully acclimatized in the greenhouse with a survival rate of 80% exhibiting normal developmental patterns.

1. Introduction

Many orchid species in all over the world are threatened. These species have been listed in the red data book of the International Union for Conservation of Nature and Natural Resources (IUCN, 2011) because of immethodical collection, illegal trade and biodiversity loss and they have been included in Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), where the international trade is strictly controlled (Chugh *et al*., 2009; Swarts and Dixon, 2009; Reed *et al*., 2011). *Catasetum pileatum,* a rare and near threatened orchid, is a lowland species, where it occurs as a showy epiphyte. This ornamental species is an impressive plant even out of flower. Orchids are precious as pot and cut flowers not only because of their exotic beauty but also for their long shelf life (Chugh *et al*., 2009).

In vivo propagation of orchids is a slow process and resulted in traits segregation. Also, propagation of orchids by sexual means like seed caus-

es the production of heterozygous plants. Therefore, establishment of protocols for *in vitro* proliferation of orchids is important and a proper alternative procedure for propagation of orchids. *In vitro* techniques can be used for conservation of rare and endangered plant species and production of large number of plantlets in short period of time (Engelmann, 2011). Of course, micropropagation of orchids deals with some problems such as high cost of production, low rate of shoot proliferation, poor rooting frequency and phenotypic variations (Bhattacharyya *et al*., 2016).

Different PGRs such as α-naphthaleneacetic acid (NAA), IBA, 1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea (TDZ), 6-benzyle amino purine (BAP), 6-benzyladenine (BA) and Kn have been used for tissue culture of threatened and endangered orchids (Roy *et al*., 2011; Panwar *et al*., 2012; Zeng *et al*., 2012; Baker *et al*., 2014; Bhattacharyya *et al*., 2016; Kaviani *et al*., 2017). Medium composition for *in vitro* culture of orchids by PLBs is cultivar and species-specific and depends on several factors especially PGRs (Luo *et al*., 2009). Various explants such as seeds, leaves (foliar explants), nodes, PLBs, protocorms, tubers, shoot tips and floral stalk buds have been used for micropropagation of threatened and endangered orchids (Vij and Aggarwal, 2003; Roy *et al*., 2011; Panwar *et al*., 2012; Zeng *et al*., 2012; Baker *et al*., 2014; Chen *et al*., 2015; Bhattacharyya *et al*., 2016; Kaviani *et al*., 2017). Among all explants, PLBs and protocorms are more efficient because these can be rapidly multiplied on solid or liquid culture media, and maximum PLBs can be provided in a short period of time (Luo *et al*., 2003).

Asymbiotic seed germination and the use of PLBs induced from vegetative organs are two efficient propagation methods for large-scale propagation of orchids (Zeng *et al*., 2012). These protocols have been established for many orchid species, and different media, primary and secondary metabolites, and PGRs have been used for germination and propagation (Arditti and Ernst, 1993; Roy *et al*., 2011). Many protocols for *in vitro* propagation of orchids, especially those at risk of extinction, using PLBs as explants and various PGRs, have been reported (Teixeira da Silva *et al*., 2005, 2006; Firoz Alam *et al*., 2010; Sinha *et al*., 2010; Baker *et al*., 2014). This investigation is the first to report on *in vitro* multiplication of *Catasetum pileatum* cv. Alba, a near threatened orchid species, for developing a protocol by protocorms as explants and Kn and IBA as PGRs.

2. Materials and Methods

Plant material

Healthy and sterilized protocorms (0.7 cm long) of *Catasetum pileatum* cv. Alba grown on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) were prepared from the Plant Biotechnology Laboratory, Hyrcan Agricultural Sciences and Biotechnology Research Institute, Amol, Iran (Fig. 1A). The protocorms were used as explants and cul-

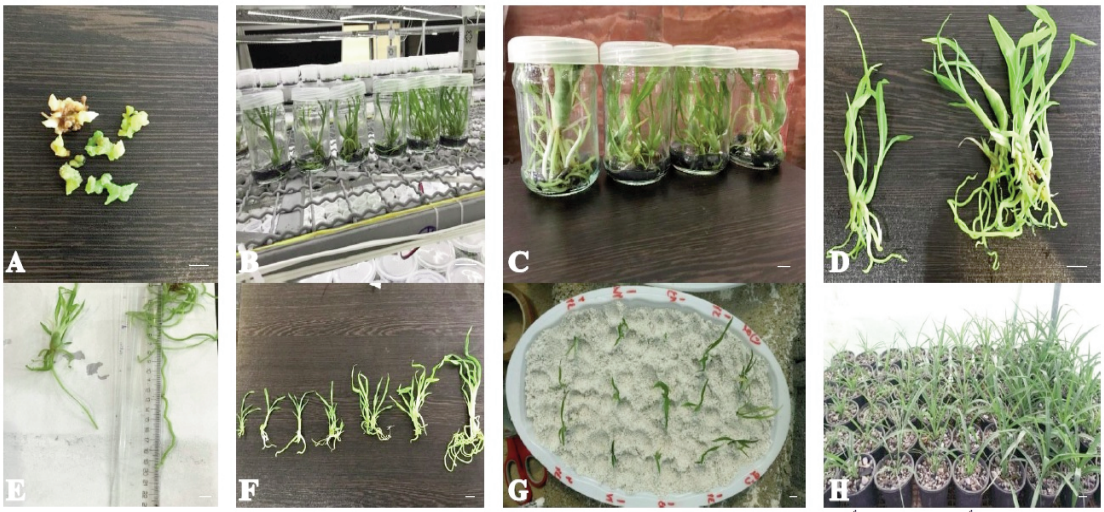

Fig. 1 - Micropropagation of *Catasetum pileatum* Alba through protocorms. (A) PLBs produced on medium containing 1.00 mg l^{-1} Kn + 1.00 mg l^{-1} IBA. (B) Developing PLBs on media enriched with different concentrations of Kn and IBA. (C) Micropropagated shoots from PLBs on medium containing Kn and IBA. (D) Leaves produced on PGRs-free medium (left) and medium containing 1.00 mg l^{-1} Kn + 1.00 mg l^{-1} IBA (right). (E) Roots produced on PGRs-free medium (left) and medium containing 0.50 mg l^{-1} Kn + 0.50 mg l^{-1} IBA. (F) Plantlets produced on media enriched with different concentrations of Kn and IBA. (G) Plantlets transplanted in pots filled with perlite. (H) Greenhouse acclimatized plantlets grown in pots filled with a mixture of leka, peat moss and perlite (in ratio of 1:1:1) (Scale bar = 1 cm).

tured on culture media poured into the culture bottles for *in vitro* propagation (Fig. 1B).

Culture medium and culture conditions

The explants were cultured on MS medium containing 3% sucrose and 0.8% Agar-agar. The medium was enriched with various PGRs. The pH of the medium was adjusted to 5.8 with 0.1 N NaOH or HCl prior to autoclaving. All media containing culture tubes were autoclaved at 104 kPa and 121°C for 20 min.

To evaluate the effect of PGRs on PLBs regeneration, shoot multiplication and root induction, the explants were cultured on MS medium containing different concentrations of kinetin (Kn; 0.00, 0.20, 0.50, 1.00, 2.00, 3.00 and 5.00 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. For each treatment, three replicates and for each replicate, three explants were taken (totally; 35 treatments, 105 replicates and 345 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.

Assessment of characteristics

After 60 days, the effect of PGRs on advanced PLBs development was assessed by using PLBs number, plantlet height, leaf number, root length, root number and viability percentage. PLBs germination percentage was calculated by the following formula:

$$\% \text{ sprouted PLBs} = \frac{\text{Number of sprouted PLBs}}{\text{Total PLBS}} \times 100$$

Hardening and acclimatization

In vitro rooted plantlets were taken out from culture vessels and washed thoroughly under running tap water to remove substrate residual and transplanted to plastic dishes containing perlite for 15-20 days (Fig. 1G). Then, plantlets were transferred to plastic pots (18 cm height × 12 cm diameter) filled with a potting mixture of Leca (Light Expanded Clay Aggregate), peat moss and perlite (in ratio of 1:1:1) (Fig. 1H). 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. Survival rate (%) was recorded after 60 days from transfer to greenhouse conditions. Plantlets were initially covered with a polythene sheet to maintain relative humidity (90%). The number of surviving plants was recorded after 4 weeks from transfer.

Experimental design and data analysis

The experiments were established in a completely randomized design with three replicates per treatment (totally; 345 explants). PGRs-free MS medium was used as control in the experiments. Data were subjected to analysis of variance (ANOVA) and means were compared by the LSD test at $P < 0.05$ using the SPSS ver. 17 (SPSS Inc., USA).

3. Results

Assessment of suitable conditions for PLBs regeneration

The main goal of this study was the maximum regeneration of PLBs during clonal proliferation. LSD test showed significant differences among different concentrations of Kn, also reciprocal effect of Kn and IBA for PLBs number ($p<0.01$). LSD also showed that the effect of IBA was no significant on PLBs number when applied individually (Table 1). The effects of Kn and IBA in the MS medium on the PLBs multiplication and growth are shown in figure 1 and Tables 2, 3 and 4. In the current study the formation of PLBs and shoot buds from the explants was observed within 45

Table 1 - Analysis of variance of the effect of different concentrations of Kn and IBA on measured characters of *Catasetum pileatum* Alba grown *in vitro* condition

Source of variations	df	Plantlets height	Number of PLBs per protocorm	Leaf number	Root number	Root length	Viability percentage
Kn	6	50.8**	10.61**	30.88**	0.870**	23.52**	457**
IBA	4	7.16 NS	1.66 NS	3.916**	15.57**	24.04**	258*
Kn × IBA	24	6.29**	2.60**	4.32**	0.647**	7.73**	167*
Error	70	3.42	1.126	1.72	0.241	0.973	83.8
cv (%)	-	21.5	19.97	20.2	9.714	10.91	11.01

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

days of culture establishment. The regeneration of PLBs are closely related with the concentration of both Kn and IBA when used in combination, however, Kn had more important role than IBA when these are applied individually. When the explants were cultured in medium supplemented with Kn individually, formation of more PLBs was observed than when explants were cultured in medium containing IBA individually (Tables 2 and 3). Among the tested Kn concentrations, 1.00 mg l^{-1} proved beneficial in inducing the highest frequency of 6.76 per explant generating PLBs (Table 2). When the explants were grown in medium enriched with Kn and IBA (1.00 mg l^{-1} from both of them) the largest number of PLBs (8.63 per explant) was observed (Table 4). Minimum PLBs regeneration (with average of 4.00 PLBs per explant) was obtained in media without Kn.

Assessment of suitable conditions for plantlets height and leaf number

The effect of PGRs in the MS medium on the plantlets growth (plantlets height and leaf number) is shown in figure 1C and Tables 1-4. After 60 days of culture of protocorms on media fortified with different concentrations of PGRs, plantlets height and leaf number were measured. Plantlets growth was significantly affected by the composition of the medium. Growth of plantlets from the explants without the addition of Kn to the culture medium was relatively poor. The plantlets growth rate ranged from 5.00 to 17.00 cm on the regeneration media (Table 4). MS medium fortified with 1.00 mg l^{-1} Kn and 0.50 mg l^{-1} IBA was the most appropriate medium for plantlet height (16.76 cm per explant) (Table 4). Treatment with 1.00 mg l^{-1} Kn in combination with 0.50 mg l^{-1} IBA produced plantlets with 13.36 cm long. Among all levels of Kn and IBA used individually, Kn at 1.00 mg l^{-1} was noted better induction of plantlet on explants than the other levels (Tables 2 and 3). The plantlets produced on medium without Kn was least (Tables 3 and 4).

MS medium fortified with 1.00 mg l^{-1} Kn and 1.00 mg l^{-1} IBA was the most appropriate medium for leaf number (12.70 per explant) (Fig. 1D, Table 4). The media containing 1.00 mg l^{-1} Kn and 0.50 mg l^{-1} IBA was suitable for leaf number, too (Table 4). Among all concentrations of IBA and Kn used individually (Tables 2 and 3), maximum leaf number (9.36 per explant) was produced in medium containing 1.00 mg l^{-1} Kn. Thus, the optimal concentration of Kn was

Table 2 - Mean comparison of the effect of different concentrations of Kn on measured characters of *Catasetum pileatum* Alba grown *in vitro* condition

Kn (mg l^{-1})	Plantlets height (cm)	Number of PLBs per protocorm	Leaf number	Root number	Root length (cm)	Viability percentage
0.00	6.22 d	4.01 d	4.75 d	5.32 a	8.68 bc	75.30 c
0.20	7.27 cd	4.80 c	5.58 cd	5.12 ab	10.28 a	85.30 b
0.50	9.72 b	5.41 bc	6.73 b	5.08 abc	10.68 a	84.00 b
1.00	11.84 a	6.76 a	9.36 a	5.38 a	9.14 b	93.30 a
2.00	9.02 b	5.18 bc	6.32 bc	4.95 bc	9.26 b	80.00 bc
3.00	8.38 bc	5.73 b	6.57 b	4.75 c	6.93 d	82.00 bc
5.00	7.62 c	5.27 bc	6.11 bc	4.81 bc	8.30 c	82.00 bc

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

Table 3 - Mean comparison of the effect of different concentrations of IBA on measured characters of *Catasetum pileatum* Alba grown *in vitro* condition

IBA (mg l^{-1})	Plantlets height (cm)	Number of PLBs per protocorm	Leaf number	Root number	Root length (cm)	Viability percentage
0.00	7.96 c	5.00 b	6.15 b	4.33 d	8.22 c	83.80 ab
0.10	8.66 abc	5.43 ab	6.20 ab	4.62 cd	9.32 b	88.09 a
0.20	9.15 ab	5.37 ab	6.17 b	4.80 bc	10.34 a	83.80 ab
0.50	9.14 ab	5.68 a	6.92 ab	6.53 a	9.62 b	81.40 b
1.00	8.00 bc	5.05 ab	7.00 a	5.00 b	7.70 c	78.50 b

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

1.00 mg l^{-1}. Also, the optimal concentrations of IBA were 1.00 and 0.50 mg l^{-1}. These concentrations in combination with each other recorded maximum leaf production. This means that the PGRs acted synergistically.

Assessment of suitable conditions for root characteristics and acclimatization

Advanced root development was significantly affected by the composition of the medium, when measured through root length and root number. All treatments of PGRs, individually and in combination had significant effects (P<0.01) on root growth (Table 1). Root length was highest (14.06 cm per explant) in 0.50 mg l^{-1} Kn along with 0.50 mg l^{-1} IBA (Fig. 1E), however, this medium was not significantly different compared to 0.50 mg l^{-1} Kn along with 0.20 mg l^{-1} IBA medium with induction of 12.90 cm long for root (Table 4). All other media were significantly different and gave lower root length growth rates.

Among all treatments, 1.00 mg l^{-1} Kn plus 0.50 mg l^{-1} IBA was found to be the most effective for root for-

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

Plant growth regulators (mg l^{-1})		Plantlets height (cm)	Number of PLBs per protocorm	Leaf number	Root number	Root length (cm)	Viability percentage
Kn	IBA						
0.00	0.00	5.63 jk	3.56 jk	5.20 f-h	5.26 d-g	5.73 o	73.33 e-g
0.00	0.10	6.60 h-k	3.83 ijk	4.46 h	4.13 k-n	6.83 mno	80.00 c-g
0.00	0.20	5.10 k	4.46 e-k	4.73 gh	4.93 e-j	11.23 cd	86.60 a-e
0.00	0.50	5.20 k	4.63 e-k	4.66 gh	7.00 a	11.80 bc	70.00 fg
0.00	1.00	6.60 h-k	3.56 jk	4.70 gh	5.30 c-g	7.83 k-m	66.60 g
0.20	0.00	6.50 i-k	5.36 c-h	5.40 e-h	4.56 g-n	10.90 c-g	90.00 a-d
0.20	0.10	8.73 c-i	5.93 b-f	5.56 d-h	4.43 h-n	12.93 ab	100.00 a
0.20	0.20	6.83 f-k	4.30 e-k	5.53 d-h	4.80 f-l	10.13 d-h	73.33 e-g
0.20	0.50	6.70 g-k	5.36 c-h	5.80 c-h	7.26 a	9.43 f-j	83.30 b-f
0.20	1.00	7.60 d-k	4.06 h-k	5.60 c-h	4.53 g-n	8.00 j-m	80.00 c-g
0.50	0.00	9.43 c-i	4.66 e-k	6.13 b-h	4.40 h-n	7.63 l-n	86.60 a-e
0.50	0.10	9.50 c-i	4.16 g-k	7.73 bc	5.10 d-i	9.76 d-i	83.30 b-f
0.50	0.20	10.30 cd	7.36 ab	6.46 b-h	4.30 j-n	12.90 ab	86.60 a
0.50	0.50	10.60 bc	5.73 b-h	6.13 b-h	6.63 ab	14.06 a	86.60 a-e
0.50	1.00	8.80 c-i	5.13 d-h	7.20 b-f	5.00 d-j	9.06 h-l	76.66 d-g
1.00	0.00	9.53 c-i	6.00 b-f	8.00 b	3.86 mn	9.33 g-k	93.30 abc
1.00	0.10	9.86c-e	6.63bcd	6.56b-h	4.86e-k	9.13h-l	96.60ab
1.00	0.20	13.36 b	5.53 c-h	7.40 b-e	5.63 cde	11.00 c-f	100.00 a
1.00	0.50	16.76 a	7.10 abc	12.16 a	7.40 a	8.23 i-m	96.60 ab
1.00	1.00	9.66 c-g	8.63 a	12.70 a	5.16 d-h	8.00 j-m	80.00 c-g
2.00	0.00	8.56 c-j	4.66 e-k	6.40 b-h	4.36 i-n	8.96 h-l	76.66 d-g
2.00	0.10	10.40 b-d	6.20 b-e	6.80 b-g	4.76 f-l	9.50 e-j	86.60 a-e
2.00	0.20	9.90 c-e	5.10 e-j	5.86 b-h	4.4 6 h-n	11.06 c-e	70.00 fg
2.00	0.50	7.50 d-k	5.33 c-h	5.90 b-h	5.76 cd	9.70 d-i	76.66 d-g
2.00	1.00	8.73 c-i	4.60 e-k	6.66 b-g	5.40 c-f	7.10 mno	90.00 a-d
3.00	0.00	7.83 c-k	5.50 c-h	5.86 b-h	3.83 n	7.66 l-n	76.66 d-g
3.00	0.10	7.23 e-k	5.93 b-e	6.80 b-g	4.46 h-n	7.60 l-n	90.00 a-d
3.00	0.20	9.40 c-i	6.93 a-c	7.66 bcd	4.56 g-n	6.20 no	80.00 c-g
3.00	0.50	9.60 c-h	5.90 b-g	6.56 b- h	6.06 bc	6.96 mno	86.60 a-e
3.00	1.00	7.86 c-k	4.40 f-k	5.96 b-h	4.83 f-k	6.23 no	76.660 d-g
5.00	0.00	8.23 c-k	5.26 c-j	6.10 b-h	4.03 l-n	7.33 mn	90.00 a-d
5.00	0.10	8.33 c-j	5.33 c-h	5.50 e-h	4.63 f-m	9.50 e-j	80.00 c-g
5.00	0.20	7.16 e-k	4.93 d-k	5.53 d-h	4.93 e-j	9.86 d-h	90.00 a-d
5.00	0.50	7.63 c-k	5.83 b-g	7.26 b-f	5.63 cde	7.16 mno	70.00 fg
5.00	1.00	6.76 g-k	5.00 e-k	6.16 b-h	4.83 f-k	7.66 l-n	80.00 c-g

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

mation (7.133 per explant) (Table 4). Root production in this medium was not significantly higher than following media. The root number (7.26 and 7.00 per explant) produced in media containing 0.20 mg l^{-1} Kn plus 0.50 mg l^{-1} IBA and 0.50 mg l^{-1} IBA without Kn, respectively was noticeable (Table 4). In most cases, minimum root number was recorded in media without IBA. Among all concentrations of IBA and Kn used singly (Tables 2 and 3), maximum root number (6.53 per explant) was produced in medium fortified with 0.50 mg l^{-1} IBA.

The *in vitro* rooted plantlets (Fig. 1F) were successfully acclimatized in the greenhouse. Pots were filled with leca, peat moss and perlite (in ratio of 1:1:1) (Figs. 1G and H). Acclimatization was achieved in 4-6 weeks, and at this stage plants attain the height of about 10-15 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 (Fig. 1H) are maintained in the Hyrcan Agricultural Sciences and Biotechnology Research Institute, Amol, Iran with 80% field establishment rate.

Assessment of suitable conditions for viability percentage

Significant difference was observed between the Kn ($P<0.01$), IBA and combination of Kn and IBA ($P<0.05$) levels and viability percentage of protocorms (Table 1). It has been observed that among the combinations and concentrations of PGRs, 0.20 mg l^{-1} Kn with 0.10 mg l^{-1} IBA and 1.00 mg l^{-1} with 0.20 mg l^{-1} IBA were the most effective for viability percentage (100.00) (Table 4). Viability percentage in these two media was significantly higher than that of other media. Least viability percentage (66.60) was observed in PLBs cultured on medium containing 1.00 mg l^{-1} IBA without Kn (Table 4).

4. Discussion and Conclusions

Current study revealed that the addition of external PGRs in appropriate concentrations induced PLBs formation from the protocorm explants cultured in the MS medium. In orchids, PLB regeneration from explants such as shoot and root tips and leaf and stem segments is a proper method of *in vitro* proliferation (Seeni and Latha, 2000; Dohling *et al*., 2012). Consonant with our findings, Roy *et al*. (2011) demonstrated that the frequency of PLBs regeneration of orchid *Vanda coerulea* significantly relied on kinds and concentrations of PGRs used. These researchers showed that a combination of 1.00 mg l^{-1} NAA and 0.85 mg l^{-1} BAP was found to be suitable for maximum PLB regeneration. Luo *et al*. (2008) showed that 0.50 mg l^{-1} Kn was proper for PLB formation of orchid *Dendrobium densiflorum*. The regeneration and proliferation of multiple PLBs are closely related with the type and concentration of cytokinins used. Among all cytokinins used for *in vitro* PLBs regeneration of orchids, BA, BAP, TDZ and Kn have the most application (Luo *et al*., 2008; Chugh *et al*., 2009; Firoz Alam *et al*., 2010; Roy *et al*., 2011; Panwar *et al*., 2012; Baker *et al*., 2014; Bhattacharyya *et al*., 2016; Kaviani *et al*., 2017). When the explants were grown in medium enriched with both of Kn and IBA the maximum PLBs was obtained. Contrary to our finding, BAP individually was better than in combination with NAA for the maximum PLBs formation of orchid *Oncidium* (Kalimuthu *et al*., 2007). Study on *Vanda coerulea* revealed that when the protocorms were cultured on BAP or NAA alone, at any concentration recorded low proliferation rate. This means that the PGRs acted synergistically (Roy *et al*., 2011). Baker *et al*. (2014) reported the highest PLBs regeneration of orchid *Catasetum* on MS medium containing a combination of 0.50 mg l^{-1} BA plus 0.50 mg l^{-1} NAA. Present study demonstrated that the production of more than seven PLBs was observed in the media supplemented with 0.50 mg l^{-1} kn along with 0.20 mg l^{-1} IBA and 1.00 mg l^{-1} Kn along with 0.50 mg l^{-1} IBA. Similar findings were found by Panwar *et al*. (2012) through study on orchid *Eulophia nuda*.

The combination, concentrations and the ratio between the PGRs are critically important for the formation of shoots and PLBs in orchids (Dohling *et al*., 2007; Baker *et al*., 2014; Bhattacharyya *et al*., 2016; Kaviani *et al*., 2017). Investigation on orchid *Eulophia nuda* showed that the maximum shoot multiplication was achieved on MS medium containing 2.00 mg l^{-1} BA and 1.00 mg l^{-1} Kn after 4 weeks of cultures (Panwar *et al*., 2012). The type, concentrations and different combinations of PGRs plays an important role during micropropagation of many orchid species (Arditti and Ernst, 1993; Panwar *et al*., 2012).

In orchids, the use of protocorm and PLB as the explants is the most appropriate and simplest method for *in vitro* propagation. Protocorm contains meristematic cells and can differentiate to a new shoot. Therefore, protocorm can be used to enhance proliferation and simultaneous production of orchid plantlets (Teixeira da Silva *et al*., 2005). Protocorms

are being applied by many researchers as explants for *in vitro* propagation of many rare and endangered orchid species (Deb and Temjensangba, 2006, Teixeira da Silva *et al*., 2006; Roy *et al*., 2011; Dohling *et al*., 2012; Baker *et al*., 2014; Bhattacharyya *et al*., 2016; Kaviani *et al*., 2017). PLB production was induced from many explants such as protocorm, shoot tip, node, root tip, leaf and stem segments during *in vitro* propagation of orchids (Dohling *et al*., 2012; Baker *et al*., 2014; Bhattacharyya *et al*., 2016; Kaviani *et al*., 2017).

The regeneration and propagation of multiple shoots are closely related with the type and concentration of cytokinins used (Amoo *et al*., 2014). The differentiation of multiple shoots from PLBs has been reported in some orchids such as *Cymbidium*, *Dendrobium*, *Catasetum*, *Phalanoepsis*, *Habeneria* and *Satyrium* (Talukdar, 2001; Sheelavanthmath and Murthy, 2001; Mahendran and Bai, 2009; Hossain *et al.*, 2010; Baker *et al*., 2014; Kaviani *et al*., 2017). Cytokinins have a wide range of functions including regulatory role on various physiological and developmental processes (Werner *et al*., 2001). In *Dendrobium huoshanense* C.Z. Tang et S.J. Cheng, Kn was reported to be more effective for plantlet regeneration from PLBs than BAP, N-benzyl-tetrahydropyranyladenine (BPA), isopentenyl adenine (2-iP), TDZ and Zeatin (Zt) (Luo *et al*., 2009). The best response appeared on the medium enriched with 4.50 mg l^{-1} Kn. Kn was also used for shoot proliferation of some other orchids (Saiprasad *et al*., 2004; Malabadi *et al*., 2005; Martin and Madassery, 2006; Panwar *et al*., 2012). Contrary to our findings, study on *Dendrobium nobile* revealed that when explants were cultured in MS medium supplemented with BAP alone, formation of PLBs was done but direct shoot formation was not observed (Bhattacharyya *et al*., 2016). Study of Mahendran and Bai (2009) demonstrated that among the cytokinins used for multiple shoot induction of *Satyrium nepalense* D. Don. TDZ was found to be superior. In this study, protocorm developed multiple shoots directly on the medium supplemented with cytokinins. In most of the orchids the presence of cytokinins singly promoted optimal shoot proliferation (Mahendran and Bai, 2009). In the present study, addition of external PGRs (both of Kn and IBA) in suitable concentrations induced plantlets growth and leaf formation from PLBs cultured in the MS medium without callus formation. The main advantage of direct organogenesis without an intervening callus phase is that somaclonal variation is reduced (Roy *et al*., 2011). When the media was supplemented with IBA singly the response was poor. Similar finding was reported by Roy *et al*. (2011) worked on *Vanda coerulea*. Bhattacharyya *et al*. (2016) showed that when the explants were grown in medium containing cytokinin and auxin, a higher rate of response frequency (92.6%) of shoot buds and PLBs was observed in all PGRs combinations. In *Eulophia nuda* Lindl., maximum shoot multiplication and elongation were obtained on MS medium containing 2.00 mg l^{-1} BA and 1.00 mg l^{-1} Kn (Panwar *et al*., 2012). PGRs in orchids act more efficiently when used in combination (Seeni and Latha, 2000; Roy *et al*., 2011).

Similar with our findings, IBA resulted in a better rooting efficiency over NAA in terms of rooting frequency and number of roots induced per shoot in *Satyrium nepalense* D.Don. and *Dendrobium nobile* (Mahendran and Bai, 2009; Bhattacharyya *et al*., 2016). As, maximum rooting efficiency (86% or 5.4 roots/shoot) was obtained in medium supplemented with 2.00 mg l^{-1} of IBA after 8 weeks of culture (Bhattacharyya *et al*., 2016). The highest number of roots per shoot (6.40) was achieved at 2.00 mg l^{-1} IBA (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 alofolium* (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 (5.30 cm) per shoot tubers was calculated on IBA (0.50 mg l^{-1}) 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 together with 0.50 mg l^{-1} NAA.

Orchids are among the most beautiful ornamental plants. *Catasetum pileatum* cv. Alba is a rare and near endangered orchid. Many of orchids are threatened, rare, vulnerable, endangered, indeterminate or in danger of extinction. Therefore, it is necessary to develop the suitable methods for conservation and large-scale production of these plants that can be used for their re-introduction.

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