In vitro propagation of orchid *Phalaenopsis amabilis* (L.) Blume var. Jawa

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Abstract

A protocol was developed for high frequency *in vitro* multiplication of an ornamental orchid, *Phalaenopsis amabilis* (L.) Blume var. Jawa, using plant growth regulators (PGRs). Protocorm-like bodies (PLBs), as explants were cultured on Murashige and Skoog (MS) medium fortified with various concentrations of kinetin (KIN) and indole-3-butyric acid (IBA), either individually or in combination. A combination of 1.00 mg l^{-1} KIN + 1.00 mg l^{-1} IBA was found to be suitable for regeneration of most measured characteristics especially maximum PLBs regeneration (30.40/plantlet), leaf number (5.93/plantlet), and root number (8.36/plantlet) from protocorm explants. The maximum number of plantlets (11.66) was calculated on MS medium supplemented with 1.00 mg l^{-1} KIN + 0.50 mg l^{-1} IBA, followed by 1.00 mg l^{-1} KIN + 1.00 mg l^{-1} IBA (10.33). Plantlets were transplanted to pots filled with cocochips and sphagnum moss (70:30) for acclimatization and transferred to the greenhouse. Upon *ex vitro* transfer, 90% of plants survived.

Keywords: micropropagation; ornamental plants; plant growth regulators; protocorm like bodies; tissue culture

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Introduction

Phalaenopsis amabilis (Orchidaceae), commonly known as moth orchid is a beautiful tropical plant distributed throughout Southeast Asia that can be successfully kept indoors (Chen and Chang, 2006). *Phalaenopsis* orchids have high economic value in the floriculture industry due to their large, colorful, and durable flowers as well as their wider adaptability to indoor conditions (Chugh et al., 2009). *Phalaenopsis* involves some species close to extinction. Orchids are one of the most diverse flowering plant families with 800 genera and 25000 species (Chugh et al., 2009). Orchids are grown as ornamentals and are valued as cut flowers not only because of their exotic beauty but also for their long shelf life (Chugh et al., 2009).

The characteristics of seedlings propagated by vegetative means are not uniform; therefore, lots of tissue culture protocols have been developed in this genus (Park et al., 2002; Chen and Chang, 2006). Large-scale multiplication of orchids using *in vitro* culture techniques has helped orchids occupy a position as one of the top ten cut flowers (Chugh et al., 2009). Orchid propagation by seeds results in the production of heterozygous plants. Thus, *in vitro* proliferation is a suitable alternative procedure for propagation

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of orchids. Different procedures have been established for *in vitro* proliferation of orchids species by various explants such as shoot tips, floral stalks, protocorm-like bodies (PLBs), leaf, inflorescence, and rhizome, as well as somatic embryos, callus, thin cell layer, and plantlets obtained from seed (Teixeira da Silva, 2003; Huan et al., 2004; Wang et al., 2004; Wu et al., 2004; Kalimuthu et al., 2006; Sinha et al., 2007; Janarthanam and Seshadri, 2008; Chugh et al., 2009; Penggow et al., 2010; Bali Lashaki and Ghasemi Ghehsareh, 2016). Protocorms are immature organs in orchids that can differentiate into new shoots. Protocorm cells are meristematic and have strong potential for totipotency; therefore, protocorms can be used for the proliferation of orchids and the production of plantlets (Teixeira da Silva et al., 2005). In comparison to plantlet development from seeds or adventitious shoots, the micropropagation through PLBs is more efficient because PLBs can be rapidly proliferated on solid or in liquid culture medium, and a large number of PLBs can be provided in a short period (Luo et al., 2003a). Many studies have revealed that the optimization of medium composition was an important approach to improve the micropropagation process of orchids by culturing PLBs that is species-specific (Shimura and Koda, 2004; Luo et al., 2009). In order to stimulate efficient micropropagation of PLBs, much effort has been done to modify the culture media, mainly by inclusion of plant growth regulators (PGRs) (Nayak et al., 2002; Nge et al., 2006) such as 6-benzylaminopurine (BA), α-naphthaleneacetic acid (NAA), thidiazuron (TDZ), N^6-benzylaminopurine (BAP), 3-indoleacetic acid (IAA) and gibberellic acid (GA_3) (Roy and Banerjee, 2003; Subramanium and Taha, 2003; Saiprasad et al., 2004; Malabadi et al., 2005; Roy et al., 2011). Cytokinins are the most important factors to improve the plant regeneration from PLBs (Nayak et al., 2002; Nasiruddin et al., 2003; Luo et al., 2009). Although the micropropagation of genus *Phalaenopsis* has been shown very good development, the wide spread application of micropropagation is still limited due to some problems such as contamination, the exudation of phenolic compounds and somaclonal variation (Zahara, 2017). This paper presents a simple and suitable protocol for *in vitro* multiplication of *Phalaenopsis amabilis* (L.) Blume var. Jawa using PGRs (KIN and IBA). Our findings showed that not much research has been done on var. Jawa.

Materials and Methods

Explant source

Healthy and sterilized PLBs of *Phalaenopsis amabilis* (L.) Blume var. Jawa were purchased from a plant tissue culture company in Austria (OrchidWire Co.) and used as a source of explants.

Culture media and conditions for protocorm germination

Once the micropropagation system was established, PLBs were cultured on Murashige and Skoog (1962, MS) medium supplemented with 3% (w/v) sucrose and 0.8% agar-agar. All media were adjusted to a pH of 5.7 ± 0.02 with HCl and NaOH prior to autoclaving at 121° C and 105 kg cm^{-2} for 20 min. All cultures were incubated at 24 ± 2° C under cool white fluorescent light (56 µmol m^{-2} s^{-1}) with a 16-h photoperiod.

Plant growth regulators and protocorm multiplication

The effect of PGRs (KIN and IBA) added to MS medium on PLBs multiplication and subsequent plantlets growth and development was evaluated. The PLBs were cultured in MS medium containing KIN (0.00, 0.50, 1.00, and 2.00 mg l^{-1}) and IBA (0.00, 0.50, 1.00, and 2.00 mg l^{-1}). Each treatment consisted of three Petri dishes and in each Petri dish four PLBs were inoculated. Explants secrete phenolic compounds into the media; therefore, 0.10 g l^{-1} activated charcoal was added to the media. Activated charcoal absorbs phenolic compound. Observations on PLBs regeneration (PLBs number), plantlet height, plantlet number, leaf length, leaf number, root

Table 1
Mean comparison of the effect of different concentrations of KIN and IBA on measured characters of *Phalaenopsis amabilis* grown *in vitro* condition

PGRs (mg l^{-1})	Mean comparison						
IBA + KIN	Corm number	Plantlet length (cm)	Plantlet number	Leaf length (cm)	Leaf number	Root length (cm)	Root number
0.00 + 0.00	14.20^{b}	0.66^{f}	4.00^{g}	0.30^{f}	1.60^{g}	0.27^{h}	2.80^{d}
0.00 + 0.50	15.96^{b}	1.38^{de}	4.33^{fg}	0.40^{ef}	2.40^{d-g}	0.55^{fgh}	4.66^{bcd}
0.00 + 1.00	15.86^{b}	1.40^{de}	5.00^{efg}	0.31^{ef}	4.36^{abc}	0.60^{fgh}	3.40^{cd}
0.00 + 2.00	15.53^{b}	1.18^{def}	9.00^{abc}	0.76^{d-f}	3.30^{c-f}	0.36^{gh}	4.36^{bcd}
0.50 + 0.00	15.60^{b}	2.13^{c}	5.00^{efg}	0.44^{ef}	1.63^{g}	0.95^{fgh}	6.80^{ab}
0.50 + 0.50	14.30^{b}	2.70^{b}	8.66^{abcd}	1.04^{c-f}	4.90^{abc}	1.28^{def}	5.10^{bcd}
0.50 + 1.00	15.30^{b}	2.08^{c}	11.66^{a}	2.56^{ab}	4.96^{ab}	1.04^{fgh}	6.66^{ab}
0.50 + 2.00	16.63^{b}	1.31^{de}	7.66^{bcdef}	2.15^{abc}	3.60^{b-e}	1.12^{efg}	6.06^{abc}
1.00 + 0.00	18.86^{b}	1.36^{de}	4.33^{fg}	0.79^{d-f}	2.10^{efg}	2.33^{bc}	5.46^{bcd}
1.00 + 0.50	13.40^{b}	1.61^{cd}	10.00^{ab}	1.63^{a-e}	3.63^{b-e}	2.00^{bcd}	5.20^{cd}
1.00 + 1.00	30.40^{a}	3.83^{a}	10.33^{ab}	2.76^{a}	5.93^{a}	2.60^{ab}	8.36^{a}
1.00 + 2.00	17.00^{b}	1.23^{de}	8.00^{bcde}	1.28^{c-f}	4.90^{abc}	3.16^{a}	5.36^{bcd}
2.00 + 0.00	16.83^{b}	1.06^{ef}	8.33^{abcde}	1.16^{c-f}	1.73^{fg}	0.42^{gh}	3.93^{cd}
2.00 + 0.50	17.76^{b}	1.06^{ef}	6.33^{cdefg}	1.45^{a-f}	3.40^{b-e}	1.33^{def}	5.13^{bcd}
2.00 + 1.00	17.06^{b}	2.05^{c}	5.66^{defg}	1.83^{a-d}	3.63^{b-e}	0.96^{fgh}	4.50^{bcd}
2.00 + 2.00	16.46^{b}	1.18^{def}	5.66^{defg}	0.98^{c-f}	3.83^{bcd}	1.80^{cde}	3.96^{cd}

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

length, root number, and pollution percentage were recorded 60 days after the culture initiation.

Plantlets acclimatization

In vitro rooted plantlets were washed with sterile water to remove adhered nutrient agar and cultivated in plastic pots containing coco chips and sphagnum moss (70:30). Plantlets were transferred to the greenhouse at 22 ± 2° C, 5000 lux photoperiod and 85% RH. These plantlets were watered every five days. Plantlets were exposed gradually to external environment.

Experimental design

The experimental units were setup in a completely randomized block design. Each experiment was carried out in three replicates and each replicate included four specimens (a total of 12 specimens for each treatment). Data on the measured characteristics were recorded from 192 explants after 60 days of culture.

Data Analysis

Statistical analysis was done using Microsoft Excel 2013 and Statistical Package for Social Sciences (SPSS) v 16.0. The analysis of variance (ANOVA) procedure for a factorial experiment was used to test for significant effect of treatments, followed by LSD test for comparisons of different means of different treatments.

Results

Influence of PGRs on PLBs regeneration

Results presented in Table 1 show that only a combination of IBA and KIN is effective for PLBs regeneration. Other treatments, individually or in combination with each other, did not show any significant differences on PLBs regeneration. These treatments also had no superiority to the control. A combination of 1.00 mg l^{-1} IBA along

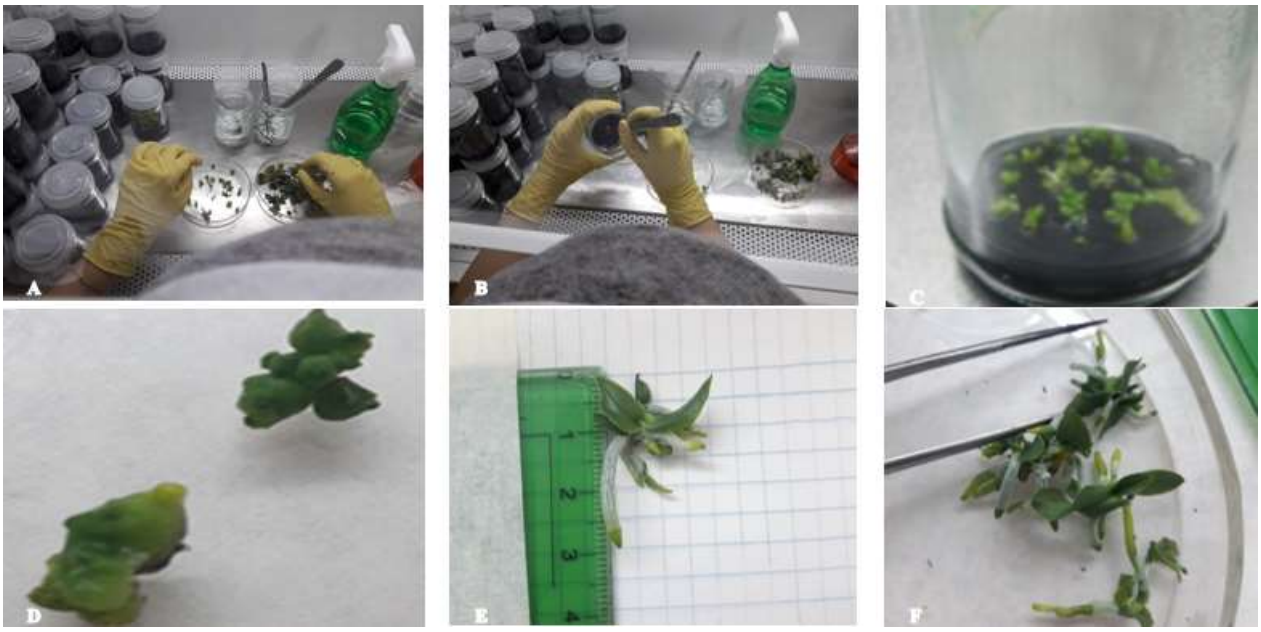

Fig. I. *In vitro* propagation of *Phalaenopsis amabilis* through protocorms-like bodies (PLBs); (A) PLBs formed from seeds used as explants; (B) PLBs cultured on MS medium containing plant growth regulators (PGRs) and activated charcoal (AC); (C) PLBs on medium containing PGRs and AC; (D) PLBs in development process; (E) Measurement of plantlet length; (F) Well-developed plantlets derived from PLBs

Table 2
Analysis of variance of the effect of different concentrations of KIN and IBA on measured characters of *Phalaenopsis amabilis* grown *in vitro* condition

		Mean Squares						
Source of variations	df	Corm number	Plantlet length	Plantlet number	Leaf length	Leaf number	Root length	Root number
Treatments	15	44.20**	1.80**	16.20**	1.82**	5.21**	2.20**	5.81*
Error	32	13.20	0.09	4.77	0.62	0.96	0.21	2.61
CV (%)	-	21.47	19.20	30.92	63.50	28.05	35.98	31.6

* and **: Significant at the 0.05 and 0.01 probability levels, respectively

with 1.00 mg l^{-1} KIN induced maximum PLBs regeneration (30.40 per plantlet) (Table 1, Fig. I). Among all concentrations of IBA and KIN used as alone, 1.00 mg l^{-1} was better than other concentrations. Minimum PLBs number was observed in the medium without PGRs (control). There was no positive correlation between the level of PGRs concentrations and PLBs number (Table 1). LSD test (Table 2) showed significant differences among different concentrations of IBA and KIN for PLBs number ($p \leq 0.01$). Pearson's correlation matrix revealed significant correlation between PLBs number and plantlet length ($p \leq 0.01$), leaf length ($p \leq 0.01$), root number ($p \leq 0.01$), and root length ($p \leq 0.05$) (Table 3).

Influence of PGRs on shoot regeneration

Analysis of variance (ANOVA) showed significant differences among different concentrations of IBA and KIN for plantlet lengths, plantlet numbers, leaf lengths and leaf numbers (all at $p \leq 0.01$, Table 2). The effect of IBA and KIN on the plantlet length was significant. In the current study, we did not find any difference among different concentrations of KIN (Table 1) in terms of their effect on enhancing the plantlet length. But, different concentrations of IBA showed significant differences in their effect on altering plantlets length. Maximum plantlet length (3.83 cm per plantlet) was achieved on the medium supplemented with 1.00 mg l^{-1} IBA plus

Table 3
Pearson's correlation matrix between the measured characteristics of *Phalaenopsis amabilis* grown *in vitro* condition

Measured characters	Plantlet number	Leaf length	Protocorm number	Plantlet length	Leaf number	Root number	Root length
Plantlet number	1						
Leaf length	0.310*	1					
Protocorm number	0.167	0.432**	1				
Plantlet length	0.339*	0.398**	0.530**	1			
Leaf number	0.441**	0.270	0.266	0.501**	1		
Root number	0.340*	0.354*	0.465**	0.522**	0.349*	1	
Root length	0.172	0.177	0.312*	0.321*	0.483**	0.265	1

Levels of significance: * p≤0.05, ** p≤0.01

1.00 mg l^{-1} KIN (Table 1). The medium containing 0.50 mg l^{-1} IBA plus 0.50 mg l^{-1} KIN was suitable for inducing the plant length. Minimum plantlet length (0.66 cm per plantlet) was seen in the medium without PGRs (control). Pearson's correlation matrix revealed significant correlation between plantlet length and plantlet number (p≤0.05), leaf length (p≤0.01), root number (p≤0.01), root length (p≤0.05), leaf number (p≤0.01), and protocorm number (p≤0.01) (Table 3).

Our results showed that PLBs in the medium enriched with 0.50 mg l^{-1} IBA in combination with 1.00 mg l^{-1} KIN were significantly different compared to other treatments, as they produced the maximum number of plantlets (11.66) (Table 1). The medium without supplementation of PGRs induced the minimum number of plantlets (4.00).

The study demonstrated that the treatments with 1.00 mg l^{-1} IBA in combination with 1.00 mg l^{-1} KIN and 1.00 mg l^{-1} IBA in combination with 0.50 mg l^{-1} KIN (with 10.33 and 10.00 plantlets, respectively) were more effective than other treatments except for 0.50 mg l^{-1} IBA in combination with 1.00 mg l^{-1} KIN. Pearson's correlation matrix demonstrated significant correlation between plantlet number and plantlet length (p≤0.05), leaf length (p≤0.05), root number (p≤0.05), and leaf number (p≤0.01) (Table 3).

Explants cultured in the presence of 1.00 mg l^{-1} IBA in combination with 1.00 mg l^{-1} KIN had the longest leaf length (2.76 cm per plantlet) and the largest number of leaves (5.93 per plantlet) was more than 8- and 4-fold higher than those found in explants grown in the media without PGRs (0.30 and 1.66 per plantlet, respectively) (Table 1). Leaf length (2.56 cm per plantlet) produced in the medium containing 0.50 mg l^{-1} IBA in combination with 1.00 mg l^{-1} KIN was also high. Table 1 reveals that the least number of leaves (less than 2.00 per plantlet) was induced in the media without KIN. Pearson's correlation matrix revealed significant correlation between leaf number and plantlet length (p≤0.01), plantlet number (p≤0.01), root number (p≤0.05), and root length (p≤0.01) (Table 3).

Influence of PGRs on root regeneration

Root initiation and growth were influenced by the levels of PGRs. We found that a combination of 1.00 mg l^{-1} IBA in combination with 2.00 mg l^{-1} KIN and 1.00 mg l^{-1} IBA in combination with 1.00 mg l^{-1} KIN provoked the highest root lengths (3.16 and 2.60 cm per plantlet, respectively). The treatment containing 1.00 mg l^{-1} IBA without KIN was suitable for induction of root length (2.33 cm per plantlet). The largest number of root (8.36 per plantlet) was recorded in the medium fortified with 1.00 mg l^{-1} IBA in combination with 1.00 mg l^{-1} KIN. The media containing 0.50 mg l^{-1} IBA without KIN or along with 1 and 2 mg l^{-1} KIN were found suitable as they resulted in the production of more than 6 shoots per explant. The minimum root number (2.80 per plantlet) and root length (0.27 cm per plantlet) were observed in the control medium (Table 1). There was no positive correlation between IBA and KIN concentration and root number and

length (Table 1). The data clearly showed that root number and root length were strongly affected by PGRs treatments ($p \leq 0.05$ and $p \leq 0.01$, respectively) (Table 1). Pearson's correlation matrix revealed significant correlation between root number and plantlet length ($p \leq 0.01$), plantlet number ($p \leq 0.05$), protocorm number ($p \leq 0.01$), leaf length ($p \leq 0.05$), and leaf number ($p \leq 0.05$) (Table 3).

Discussion

Protocorms are intermediate structures between the embryo and the plant and a superior explant for regeneration of PLB, an organ that resembles protocorms which also leads to plantlet formation like protocorm (Teixeira da Silva et al., 2006; Roy et al., 2011). Protocorms are highly meristematic and can be used for multiplication and production of orchid plantlets (Teixeira da Silva et al., 2005; Roy et al., 2011). Protocorms are obtained from many explants like leaf, shoot tip, seed, and thin cell layer (TCL) (Seeni and Latha, 2000; Sheelavantmath et al., 2000; Teixeira da Silva et al., 2006; Hossain et al., 2010; Roy et al., 2011). Since the seeds of orchid are without endosperm, they need specific nutritional and environmental conditions (Arditti et al., 1990). Protocorms are being applied by many researchers as explants for micropropagation of many orchid species (Seeni and Latha, 2000; Sheelavantmath et al., 2000; Nagaraju and Mani, 2005; Dev and Temjensangba, 2006; Teixeira da Silva et al., 2006; Hossain et al., 2010; Roy et al., 2011; Baker et al., 2014). Orchids need auxins and/or cytokinins for formation of PLBs and plantlet development (Roy et al., 2011). The type and concentration of PGRs play an important role in micropropagation of many plant species like orchids (Arditti and Ernst, 1993). The current study found that a combination of 1.00 mg l^{-1} IBA along with 1.00 mg l^{-1} KIN induced maximum PLBs regeneration (30.40 per plantlet). The other treatments, individually or in combination, did not show any significant differences on PLB regeneration. Several studies revealed that BAP is more effective for production of the largest number of protocorms (Nayak et al., 2002; Nagaraju et al., 2003; Kalimuthu et al., 2007; Roy et al., 2011). Auxins do not play an important role in formation of protocorms when applied alone, but in some orchid species they act effectively when applied in combination with cytokinins (Seeni and Latha, 2000; Roy et al., 2011). Stuies by Seeni and Latha (2000) and Roy et al. (2011) on *Vanda* spp. revealed that maximum PLB formation was obtained in the medium enriched with NAA and BAP. Baker et al. (2014) demonstrated that the addition of low concentrations of BA and NAA promoted protocorms multiplication and growth of *Orchis catasetum* plantlets. Luo et al. (2008) showed that 5.0 mg l^{-1} BAP was the best for induction of PLBs (15 per explant) per stem segment within 6 weeks. Also, 0.50 mg l^{-1} KIN was good for PLB formation. BAP plus NAA hve been suggested by some researchers to obtain the maximum number of PLBs (Kim and Kim, 2003; Puchooa, 2004; Bali Lashaki and Ghasemi Ghehsareh, 2016). The highest PLBs per explant (50.65) in *Phalaenopsis amabilis* var. 'Manila' were obtained in the medium supplemented with 15 mg l^{-1} BAP plus 3 mg l^{-1} NAA (Bali Lashaki and Ghasemi Ghehsareh, 2016). Our finding is in agreement with Bali Lashaki and Ghasemi Ghehsareh,(2016)but by using IBA and KIN. However, Luo et al. (2008) showed that NAA added to the medium containing optimal BAP did not significantly improve explants' response in *Dendrobium densiflorum* and even decreased production of PLBs at concentrations more than 1.0 mg l^{-1}. This is consistent with our finding. Appropriate combinations of cytokinins (BAP, TDZ) with NAA for PLB formation was reported in some orchids (Roy and Banerjee, 2003; Huan et al., 2004; Teixeira da Silva et al., 2007a, b; Malabadi et al., 2008).

Type and concentration of PGRs and how to use them, i.e. application of PGRs individually or in combination, have an important role in the success of shoot production. The effect of these factors depends on the type of plant species and varies from one species to another. This has been confirmed by various researchers studying on various orchid species and varieties. Applying exogenous auxins and/or cytokinins for regeneration of shoot buds and plantlet development has been reported for many orchid species (Arditti and Ernst, 1993). In fact, the combinations, concentrations, and the ratio between them are usually critically important

(Hossain et al., 2010). Moreover, endogenous PGRs level, type of explant, and tissue orientation are also different *in vitro* plants. Many researchers have recognized that the effect of a single PGR alone on shoot multiplication is better than its effect in combination with another PGRs in orchids (Martin and Madassery, 2006; Novotna et al., 2007; Zhao et al., 2007; Mahendran and Bai, 2009; Luo et al., 2009; Panwar et al., 2012; Parthibhan et al., 2015). These findings are in contrast with our findings, because maximum shoot number was produced in the media fortified with IBA in combination with KIN. Of course, a comparison between these two PGRs reveals that KIN is better than IBA. Kalimuthu et al. (2007) showed that the maximum number of shoots was observed in the MS medium supplemented with 2.0 mg l^{-1} BAP. BAP was better individually than in combination with NAA. Luo et al. (2009) in their study on micropropagation of *Dendrobium huoshanense* reported a higher frequency of shoot formation in the medium with 5-15 µM 2-iP compared to the medium without PGRs. Several studies demonstrated the positive effects of BAP, NAA, TDZ, and KIN for plantlet regeneration from PLBs (Nasiruddin et al., 2003; Luo et al., 2008; Chugh et al., 2009). Some other researchers showed superiority of PGRs in combination than singly for *in vitro* shoot proliferation of orchids (Seeni and Latha, 2000; Roy and Banerjee, 2003; Decruse et al., 2003; Huan et al., 2004; Teixeira da Silva et al., 2005; Malabadi et al., 2008; Baker et al., 2014). These are in agreement with our findings.

The ratio of auxin to cytokinin for shoot formation varies from species to species (Teng et al., 1997). A number of reports demonstrated that a combination of cytokinin and auxin at 2:1 is appropriate for shoot multiplication in some orchid species (Le et al., 1999; Seeni and Latha, 2000; Decruse et al., 2003; Roy and Banerjee, 2003; Huan et al., 2004; Teixeira da Silva et al., 2005; Malabadi et al., 2008). The present study fully confirms this and the highest number of shoots was achieved in the medium fortified with 1 mg l^{-1} KIN in combination with 0.50 mg l^{-1} IBA. Protocorms of *Dendrobium aqueum* produced a maximum of 9.4 shoots per explant in the medium containing 3 mg l^{-1} of NAA compared to the other treatments including different concentrations of cytokinins (BA, 2-iP, KIN and TDZ) and auxins (IBA, NAA and 2,4-D) (Parthibhan et al., 2015). In *Dactylorhiza incarnate*, the highest shoot growth rate was obtained in the medium containing 2-iP (Novotna et al., 2007). Bektas et al. (2013) introduced both BA and 2-iP for suitable shoot formation of protocorms in *Orchis coriophora*. An effective conversion of PLBs into shoots was obtained in *Dendrobium huoshanense* at 4 mg l^{-1} of KIN (Luo et al., 2009). In micropropagation of *Dendrobium huoshanense* through PLBs, the best results in terms of shoot development from PLBs occurred in the medium containing 20 mM KIN (Luo et al., 2009). Studies by Hossain et al. (2010) on *Cymbidium giganteum* Wall. ex Lindl. and Bali Lashaki and Ghasemi Ghehsareh (2016) on *Phalaenopsis amabilis* var. 'Manila' revealed multiple shoot formation in the medium supplemented with different concentrations and combinations of BAP plus NAA, and BA plus NAA, respectively. The frequency of shoot bud regeneration was greatly influenced by the specific BAP and NAA combination. The highest number of shoot buds (5.40 per explant) was recorded in the medium supplemented with 3.0 mg l^{-1} BAP while the lowest record of shoot bud regeneration was observed at higher concentrations of NAA. Panwar et al. (2012) reported that KIN increased the appropriate shoot multiplication in *Eulophia nuda* Lindl.

We found that the longest root and the largest number of root were generated in plantlets having the highest length of shoot, leaf and root also maximum number of leaf and root. In *Dendrobium aqueum* Lindley, a maximum number of 6.25 and 5.25 roots per explant were obtained on 7.00 mg l^{-1} and 3.00 mg l^{-1} of KIN along with an average length of 0.98 cm roots on KIN 5.00 mg l^{-1}. These findings show that KIN has a critical role on root growth and development (Parthibhan et al., 2015). The current study did not confirm these findings because the minimum root length and root number were seen in the media supplemented with KIN without IBA and control. In line with our results, maximum rooting efficiency (86%) with the highest rooting of 10.10 roots per shoot was obtained in *Dendrobium nobile* plants grown in the medium enriched with 2.00 mg l^{-1} of IBA. In orchid *Satyrium nepalense* D. Don., the highest numbers of roots (6.40 per shoot) were formed in the MS medium

supplemented with 9.84 mM IBA (Mahendran and Bai, 2009). These researchers also showed that the shoots cultured in MS medium containing different concentrations of NAA failed to produce healthy roots. The effectiveness of IBA in root induction and growth has been reported for some other orchids like *Vanilla planifolia* (Giridhar et al., 2001), *Cymbidium pendulum* (Nongdam et al., 2006), and *Satyrium nepalense* (Mahendran and Bai, 2009). Ram and Shekhawat (2011) and Panwar et al. (2012) used IBA for *ex vitro* rooting of orchid *Eulophia nuda* Lindl. Hossain at al. (2010) in *Cymbidium giganteum* Wall. ex Lindl. demonstrated that a solid root system was induced from PLBs and shoot buds when these were transferred to media enriched with 0.50 mg l^{-1} IAA. The best root induction (2.45 roots) in *Phalaenopsis amabilis* cv. Cool 'Breeze' was achieved with 1.00 mg l^{-1} IAA (Bali Lashaki et al., 2014). Similar to our results, Baker et al. (2014) showed that a combination of 0.50 mg l^{-1} BA and 0.50 mg l^{-1} NAA induced the largest number of roots (7.16 per plantlet) and the highest length of roots (19.34 mm per plantlet). These researchers also showed that a combination of 1.00 mg l^{-1} BA and 0.50 mg l^{-1} NAA was a suitable treatment for induction of root number (6.60 per plantlet) and root length (13.51 per plantlet). The developed shoots of *Dendrobium huoshanense* were rooted in growth regulator-free MS medium (Luo et al., 2009).

Conclusion

An efficient procedure for the micropropagation of *Phalaenopsis amabilis* (L.) Blume var. Jawa was presented by adjustment of IBA and KIN concentrations. This adjustment resulted in the enhancement of *in vitro* shoot multiplication and root induction. The study showed that the combination of IBA and KIN is the better for shoot multiplication and root induction. The best results were obtained for shoot and root induction in the same medium. The use of a medium for both shoot and root induction saves the costs in routine micropropagation and breeding programs.

References

Arditti, J. and **R. Ernst, R.** 1993. 'Micropropagation of orchids'. John Wiley and Sons, New York.

Arditti, J., R. Ernest, T. W. Yam and **C. Glabe.** 1990. 'The contribution to orchid mycorhizal fungi to seed germination: a speculative review'. *Lindleyana*, 5: 249-255.

Balilashaki, Kh. and **M. Ghasemi Ghehsareh.** 2016. 'Micropropagation of *Phalaenopsis amabilis* var. 'Manila' by leaves obtained from *in vitro* culturing the nodes of flower stalks'. *Notulae Scientia Biologicae*, 8 (2): 164-169.

Balilashaki, Kh., R. Naderi, S. Kalantari and **A. Soorni.** 2014. 'Micropropagation of *Phalaenopsis amabilis* cv. Cool 'Breeze' with using of flower stalk nodes and leaves of sterile obtained from node cultures'. *International Journal of Farming and Allied Sciences,* 3 (7): 823-829.

Baker, B., B. Kaviani, Gh. Nematzadeh and **N. Negahdar.** 2014. 'Micropropagation of *Orchis catasetum* – A rare and endangered orchid'. *Acta Scientiarum Polonorum-Hortorum Cultus,* 13 (2): 197-205.

Bektas, E., M. Cuce and **Sokmen, A.** 2013. '*In vitro* regeneration from protocorms in *Dendrobium aqueum* Lindley – An imperiled orchid'. *Turkish Journal of Botany*, 37: 336-342.

Chen, J. and **W. C. Chang.** 2006. 'Direct somatic embryogenensis and plant regeneration from leaf explants *Phalaenopsis amabilis*'. *Biologia plantarum,* 50 (2): 169-173.

Chugh, S., S. Guha and **U. Rao.** 2009. 'Micropropagation of orchids: A review on the potential of different explants'. *Scientia Horticulturae*, 122: 507-520.

Decruse, W., A. Gangaprasad, S. Seeni and **S. V. Menon.** 2003. 'A protocol for shoot multiplication from foliar meristem of *Vanda spathulata* (L.) Spreng'. *Indian Journal of Experimental Biology*, 41: 924-927.

Dev, C. R. and **Temjensangba,** 2006. '*In vitro* propagation of threatened terrestrial orchid *Malaxis khasiana* Soland ex. Swartz through immature seed culture'. *Indian Journal of Experimental Biology,* 44: 762-766.

Giridhar, P., B. Obul Reddy and **G. A. Ravishankar,** 2001. 'Silver nitrate influences

in vitro shoot multiplication and root formation in *Vanilla planifolia* Androl'. *Current Science*, 81: 1166–1170.

Hossain, M. M., M. Sharma, J. A. Teixeira da Silva and **P. Phthak,** 2010. 'Seed germination and tissue culture of *Cymbidium giganteum* Wall. Ex. Lindl'. *Scientia Horticulturae*, 123: 479-487.

Huan, L. V. T., T. Takamura and **M. Tanaka,** 2004. 'Callus formation and plant regeneration from callus through somatic embryo structures in *Cymbidium* orchid'. *Plant Science*, 166: 1443–1449.

Janarthanam, B. and **S. Seshadri,** 2008. 'Plantlet regeneration from leaf derived callus of *Vanilla planifolia* Androl'. *In vitro Cellular and Developmental Biology - Plant*, 44: 84-89.

Kalimuthu, K., R. Senthikumar and N. Murugalatha. 2006. 'Regeneration and mass multiplication of *Vanilla planifolia* Androl - a tropical orchid'. *Current Science*, 91: 1401-1403.

Kalimuthu, K., R. Senthikumar and **S. Vijayakumar,** 2007. '*In vitro* micropropagation of orchid, *Oncidium* sp. (Dancing Dolls)'. *African Journal of Biotechnology*, 6 (10): 1171-1174.

Kim, M. S. and J. Y. Kim, 2003. 'Micropropagation of *Dendrobium* hybrids through shoot tip culture'. *Acta Horticulturae*, 624: 527-533.

Le, B. V., N. T. Hang Phuong, L. T. Anh Hong and **K. T. Thanh Van,** 1999. 'High frequency shoot regeneration from *Rhynchostylis gigantea* (Orchidaceae) using thin cell layers'. *Plant Growth Regulation*, 28: 179–185.

Luo, J. P., Y. Wang, X. Q. Zha and **L. Huang,** 2008. 'Micropropagation of *Dendrobium densiflorum* Lindle. ex Wall. through protocorm-like bodies: effect of plant growth regulators and lanthanoids'. *Plant Cell, Tissue and Organ Culture,* 93: 333-340.

Luo, J. P., C. Wawrosch and **B. Kopp,** 2009. 'Enhanced micropropagation of *Dendrobium huoshanense* C.Z. Tang et S.J. Cheng through protocorm-like bodies: The effect of cytokinins, carbohydrate sources and cold pretreatment'. *Scientia Horticulturae*, 123: 258-262.

Luo, J. P., X. Q. Zha and **S. T. Jiang,** 2003a. 'Suspension culture of protocorm-like bodies from the endangered medicinal plant *Dendrobium huoshanenese*'. *China Journal of Chinese Materials Medicine*, 28: 611–614.

Mahendran, G. and **V. Narmatha Bai.** 2009. 'Mass propagation of *Satyrium nepalense* D. Don.—A medicinal orchid via seed culture'. *Scientia Horticulturae*, 119: 203–207.

Malabadi, R. B., G. S. Mulgund and **N. Kallappa.** 2005. 'Micropropagation of *Dendrobium nobile* from shoot tip sections'. *Journal of Plant Physiology*, 162: 473-478.

Malabadi, R. B., J. A. Teixeira da Silva, K. Nataraja and **G. S. Mulgund.** 2008. 'Shoot tip transverse thin cell layers and 2,4-epibrassinolide in the micropropagation of *Cymbidium bicolor* Lindl'. *Floriculture and Ornamental Biotechnology*, 2: 44–48.

Martin, K. P. and **J. Madassery.** 2006. 'Rapid *in vitro* propagation of *Dendrobium* hybrids through direct shoot formation from foliar explants, and protocorm-like bodies'. *Scientia Horticulturae*, 108: 95–99.

Murashige, T. and **F. Skoog.** 1962. 'A revised medium for rapid growth and bioassays with tobacco tissue cultures'. *Physiology Plant*. 15: 473–479.

Nagaraju, V., S. P. Das, P. C. Bhutia and **R. C. Upadhyaya.** 2003. 'Response of *Cymbidium lunavian* Atlas protocorms to media and benzyl aminopurine'. *Indian Journal of Horticulture*, 60 (1): 98–103.

Nagaraju, V. and **S. K. Mani.** 2005. Rapid *in vitro* propagation of orchid *Zygopetalum intermedium*.*Journal of Plant Biochemistry and Biotechnology*, 14: 27–32.

Nasiruddin, K. M., R. Begum and **S. Yasmin.** 2003. 'Protocorm-like bodies and plantlet regeneration from *Dendrobium formosum* leaf callus'. *Asian Journal of Plant Science*, 2: 955-957.

Nayak, N. R., S. Sahoo, S. Patnaik and **S. P. Rath.** 2002. 'Establishment of thin cross section (TCS) culture method for rapid micropropagation of *Cymbidium alofolium* (L.) SW. and *Dendrobium nobile* Lindl. (Orchidaceae)'. *Scientia Horticulturae*, 94: 107-116.

Nge, K. L., N. New, S. Chandrkrachang and **W. F. Stevens.** 2006. 'Chitosan as a growth

stimulator in orchid tissue culture'. *Plant Science*, 170: 1185-1190.

Nongdam, P., C. Nirmala and **R. Tewari.** 2006. '*In vitro* multiplication of *Cymbidium pendulum* orchids via embryo culture'. *Plant Cell Biotechnology and Molecular Biology*, 7: 145–150.

Novotna, K. W., H. Vejsadova and **P. Kindlemann.** 2007. 'Effect of sugars and growth regulators on *in vitro* growth of *Dactylorhiza* species. *Biologia Plantarum*, 51: 198-200.

Panwar, D., K. Ram and **H. N. S. Shekhawat.** 2012. '*In vitro* propagation of *Eulophia nuda* Lindl., an endangered orchid'. *Scientia Horticulturae*, 139: 46–52.

Park, S. Y., E. C. Yeung, D. Chakrabarty and **K. Y. Paek.** 2002. 'An efficient direct induction of protocorm- like bodies from leaf subepidermal cells of *Doritaenopsis* hybrid using thin-section culture'. *Plant Cell Report*, 21: 46–51.

Parthibhan, S., M. V. Rao and T. S. Kumar. 2015. '*In vitro* regeneration from protocorms in *Dendrobium aqueum* Lindley – An imperiled orchid'. *Journal of Genetic Engineering and Biotechnology*, 13: 227-233.

Puchooa, D. 2004. 'Comparison of different culture media for the *in vitro* culture of *Dendrobium* (Orchidaceae)'. *Indian Journal of Agriculture and Biology*, 6: 884-888.

Ram, K., N. S. Shekhawat. 2011. 'Micropropagation of commercially cultivated Henna (*Lawsonia inermis*) using nodal explants'. *Physiology and Molecular Biology of Plants*, 17: 281–289.

Roy, A. R., R. S. Patel, S. Sajeev and C. Deka. 2011. 'Asymbiotic seed germination, mass propagation and seedling development of *Vanda coerulea* Griff ex. Lindle. (Blue Vanda): An *in vitro* protocol for an endangered orchid'. *Scientia Horticulturae*, 128: 325-331.

Roy, J. and N. Banerjee. 2003.' Induction of callus and plant regeneration from shoot tip explants of *Dendrobium fimbriatum* Lindl. var. Oculatum H.K.f.' *Scientia Horticulturae*, 97: 333-340.

Saiprasad, G. V. S., P. Raghuveer, S. Khetarpal and R. Chandra. 2004. 'Effect of various polyamines on production of protocorm-like bodies in orchid- *Dendrobium* 'Sonia'. *Scientia Horticulturae*, 100: 161-168.

Seeni, S. and P. G. Latha. 2000. '*In vitro* multiplication and ecorehabilitation of the endangered Blue Vanda'. *Plant Cell, Tissue and Organ Culture*, 61: 1-8.

Sheelavantmath, S. S., H. N. Murthy, A. N. Pyati, H. G. Ashok Kumar and B. V. Ravishanker. 2000. '*In vitro* propagation of the endangered orchid, *Geodorum densiflorum* (Lam.) Schltr. through rhizome section culture'. *Plant Cell, Tissue and Organ Culture*, 60: 151-154.

Shimura, H. and Y. Koda. 2004. 'Micropropagation of *Cypripedium macranthos* var. Rebunerse through protocorm-like bodies derived from mature seed'. *Plant Cell, Tissue and Organ Culture*, 78: 273-276.

Sinha, P., M. K. Hakim and M. F. Alam. 2007. 'Efficient micropropagation of *Phalaenopsis amabilis* (L.) B.L. cv. 'Cool Breeze' using inflorescence axis thin section as explants'. *Propagation of Ornamental Plants*, 7: 9-15.

Subramanium, G. and R. M. Taha. 2003. 'Morphogenesis of *Cymbidium atropurpureum in vitro*'. *Malaysian Journal of Science*, 22: 1-5.

Teixeira da Silva, J. A. 2003. 'Thin cell layer technology in ornamental plant micropropagation and biotechnology'. *African Journal of Biotechnology*, 2: 683-691.

Teixeira da Silva, J. A., T. Yam, S. Fukai, N. Nayak and M. Tanaka, M. 2005. 'Establishment of optimum nutrient media for *in vitro* propagation of *Cymbidium* Sw (Orchidaceae) using protocorm-like body segments'. *Propagation of Ornamental Plants*, 5: 129-136.

Teixeira da Silva, J. A., N. Singh and M. Tanaka. 2006. 'Priming biotic factors for optimal protocorm-like body and callus induction in hybrid *Cymbidium* (Orchidaceae), and assessment of cytogenetic stability in regenerated plants'. *Plant Cell, Tissue and Organ Culture*, 84: 135-144.

Teixeira da Silva, J. A., D. T. T. Giang, M. T. Chan, A. Sanjaya, A. Norikane, M. L. Chai, J. Chico-Ruı´z, S. Penna, T. Granstro¨m and M. Tanaka. 2007a. 'The influence of different carbon sources, photohetero-, photoauto-

and photomixotrophic conditions on protocorm-like body organogenesis and callus formation in thin cell layer culture of hybrid *Cymbidium* (Orchidaceae)'. *Orchid Science and Biotechnology*, 1: 15–23.

Teixeira da Silva, J. A., A. Norikane and M. Tanaka. 2007b. '*Cymbidium*: successful *in vitro* growth and subsequent acclimatization'. *Acta Horticulturae*, 748: 207–214.

Teng, W. L., L. Nicholson and M. C. Teng. 1997. 'Micropropagation of *Spathoglottis plicata*'. *Plant Cell Report*, 16: 831–835.

Wang, Y. F., R. J. Lu, Y. F. Sun, R. M. Zhou and J. H. Huang. 2004. 'The induction and cultivation of cell-derived regenerates of *Dendrobium huoshanenese*'. *Acta Agriculturae Shanghai*, 20: 8-10.

Wu, I. F., J. T. Chen and W. C. Chang. 2004. 'Effect of auxins and cytokinins on embryo formation from root-derived callus of *Oncidium* 'Gower Ramsey'. *Plant Cell, Tissue and Organ Culture*, 77: 107-109.

Zahara, M. 2017. 'A review: Micropropagation of Phalaenopsis sp. from leaf and flower stalk explants'. *Jurnal Natural*, 17 (2): 91-95.

Zhao, P., W. Wang, S. F. Feng, F. Wu, J. Q. Yang and W. J. Wang. 2007. 'High-frequency shoot regeneration through transverse thin cell layer culture in *Dendrobium candidum* Wall ex Lind.' *Plant Cell, Tissue and Organ Culture*, 90: 131–139.