

Effect of Growth Regulators and Explant Orientation on Shoot Tip Culture of Borneo Endemic Orchid, *Dimorphorchis lowii*

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ABSTRACT

Multiple shoots were induced from the shoot tip explants derived from the *in vitro* grown seedlings of an endangered and horticultural important epiphyte orchid, *Dimorphorchis lowii*. Shoot tip explants were cultured vertically and/or horizontally on solidified Knudson C media (KC) added with various concentrations of Kinetin (Kn) and 6-Benzylaminopurine (BAP) for shoots multiplication. Shoots were initiated after 4 weeks of culture, and the highest number of healthy shoots (5.05 shoots per explant) was observed in 2.0 mg/l Kinetin (Kn), when the explant placed horizontally. Regenerated shoots were root-induced in KC medium with various concentrations and combinations of Naphthalene acetic acid (NAA), Indole acetic acid (IAA) and Indole butyric acid (IBA). Shoots cultured on medium with 1.0 mg/l IAA and 0.5 mg/l IBA was the most appropriate combination for rooting. Rooted plantlets were transferred in a medium mixture containing coco peat and sphagnum moss (2:1). After 2 months, 78% of plants survived when transferred to the glasshouse. This is the first report for *in vitro* propagation of *D. lowii* through shoot tip culture. The protocol developed can be utilized for both large-scale plant production and germplasm conservation of this species.

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Introduction

Orchid *Dimorphorchis lowii* is one of the species that gains much interest over the years because of its unique dimorphic flowers, which means it has two different flowers in one spike. It has the potential to be exported as an ornamental flower because of its one of its kind, beautiful and fragrant flowers. It is also one of the rarest epiphytic orchids found principally at the altitude of 600-1800m on Mount kinabalu, Mount Tombuyukon and Mount Trus Madi in Sabah (Crib *et al.*, 2008). Because of the increasing popularity, endangered (included in Appendix-II of Convention on International Trade in Endangered Species of Wild Fauna and Flora, CITES), thus difficult to locate, it is important to establish a technique for multiplication to produce a large number of plantlets and subsequently conserving this species. Panwar *et al.*, (2012) reported that many orchids species are now threatened and in fact almost the entire family is now included in CITES. The use of shoot tip explants has

become very popular due to its high efficiency in producing rapid mass propagation of some orchids (Pant & Swar, 2011), and it is genetically stable. It is also preferred because it is the most applicable and reliable method of uniformity and true-to-type regenerated plantlet (George & Debergh, 2008). Seeni & Latha (2000), and Chugh *et al.* (2009) reported that utilization of shoot tip culture technique has resulted in rapid multiplication of *Vanda coerulea* and successful establishment of the clonal plants in its natural habitat. Geetha & Shetty (2000) also reported in their study that approximately 100,000 *Vanilla* orchid plantlets regenerated in about 15 subcultures using shoot tips as an explant. Pant & Shrestha (2011) reported that commercial orchids are produced by tissue culture and this technique is used routinely in many countries for large-scale production of orchid seedlings. To date, there has been no documented report available on *in vitro* propagation of this species. Therefore this paper would be the first to describe the protocol for *in vitro* propagation of *D. lowii* using shoot tip explant.

Methodology

Plant materials and explants preparation.

The mother plant was collected early 2009 from a local nursery at Kg. Lobong-lobong, Kota Belud, Sabah. This plant was *ex situ* cultivated in the greenhouse conditions in orchid germplasm at Unit for Orchid Studies, Institute for Tropical Biology and Conservation, Universiti Malaysia Sabah. Matured capsules from hand-pollinated were harvested, cleansed and surface sterilized to provide millions of tiny seeds that were cultured in Vacin & Went (1949) medium added with 10% (w/v) potato homogenate. Protocorms developed from these cultures were then transferred to ½ strength MS medium (Murashige & Skoog, 1962) containing 2% (w/v) sucrose and 0.2% (w/v) yeast extract. This served as the staging medium for explants source. Shoots from these cultures were cut and separated individually and used as explants.

Shoot proliferation

Individual shoot was excised from shoot clumps (2–3mm in length) and transferred to medium containing different concentrations (0, 0.5, 1.0, 2.0, 3.0, 4.0 mg/l) of 6-Benzylaminopurin (BAP), and Kinetin (Kn), respectively. Explants were positioned vertically and horizontally on each side of the 100ml wide-mouth glass flask.

Rooting

Shoots (3-6mm in length), each with two to three expanded leaves were detached from the shoot clumps that were previously cultured in the staging medium. These shoot tips were cultured in medium containing different combinations of naphthalene acetic acid (NAA), indole acetic acid (IAA) and indole butyric acid (IBA) that are: 0, 1.0 mg/l IAA + 0.5 mg/l NAA, 2.0 mg/l IAA + 0.5 mg/l NAA, 1.0 mg/l IAA + 0.5 mg/l IBA and 2.0 mg/l IAA + 0.5 mg/l IBA.

Culture medium and culture conditions

All explants were cultured on KC medium (Knudson C, 1946) supplemented with 2% (w/v) sucrose.

The pH was adjusted to 5.3 ± 0.02 and solidified with 0.8% (w/v) agar (Sigma) prior to autoclaving for 20 min at 15 psi, 121°C . The cultures were maintained at $25 \pm 2^{\circ}\text{C}$ under a 24hd^{-1} photoperiod with a PPF of $20\text{--}50 \mu\text{mol m}^{-2}\text{s}^{-1}$ provided by cool white fluorescent tubes (Philips, Malaysia).

Acclimatization

After the formation of complete rooted plantlets, they were subjected to *ex vitro* hardening. These plantlets were removed from the culture flasks and were washed thoroughly with sterile double distilled water to remove residual medium agar. After that, they were treated fungicide solution (0.2%, w/v Ancom Thiram 80) for 1 min and rinsed again with sterile double distilled water. The rooted plantlets were then planted in the plastic pots containing coco peat and sphagnum moss (2:1). The plantlets were covered with clear plastic bags for 30 days and maintained under humidity. Plantlets became acclimated to a reduced relative humidity by gradually opening the plastic cover and after 50 days they were completely uncovered and hardened to greenhouse conditions.

Experimental design and statistical analysis

All the experiments were carried out in a completely randomized design (CRD) with ten replicates per treatment and each replicate contains four explants. Data were subjected to analysis of variance (ANOVA) and means were compared by the Duncan's multiple range test at $p < 0.05$ using the SPSS ver. 20 (SPSS Inc., USA).

Result and discussion

Effect of cytokinins and explant orientation on shoots proliferation

After 180 days of culture, both PGRs resulted in callus formation and PLB induction that developed into multiple shoots from the cut end of shoot tips with the percentage of shoot formation ranges from $2.50\% \pm 1.05$ to $80.00\% \pm 22.44$ (Table 1). However, the responses are dependent on the concentration and type of cytokinins. In control treatment (Figure 1A), a lower response of explant formed shoots ($37.50\% \pm 31.73$, producing less than 2 shoots) suggesting that cytokinin is required to induce multiple shoots formation. Result showed that Kn gave the best performances in number of shoots produced. A moderate level of 2.0 mg/L Kn with explants cultured horizontally (Figure 1B) triggered the highest percentage of shoot formation ($80.00\% \pm 22.44$). BAP supplemented medium produced percentages of survival equally as of Kn when explants were cultured vertically but overall it failed to promote shoot multiplication. A maximum of 100.00% was recorded on medium supplemented with 2.0 mg/L Kn when explants were placed vertically, but the number of shoots (2.86 ± 1.71) is lower as compared to horizontal orientation for the same growth regulator. In addition, the number of shoot produced and length of shoot were better than that of vertically placed explants grown in BAP supplemented medium. It can be seen on the mean number of shoots (5.05 ± 2.81) was significantly higher on medium supplemented with 2.0 mg/L Kn with explants cultured in horizontal position than that of BAP supplemented medium. This treatment also provided higher mean length of shoots ($9.13\text{mm} \pm 3.31$). Various workers on orchid micropropagation studied the effectiveness of Kn as a growth regulator. The successful use of Kn for shoot multiplication in this study may be accredited to the capability of

plant tissues to metabolize Kn more efficiently than BAP. For *D. lowii* orchid, Kn is important PGR for bud break and shoot differentiation due to their role in cell multiplication and the breakdown of apical dominance. According to Abassi *et al.* (2013) multiplication of shoots depends mainly on the number of buds and leaves on an explant. Specific plant hormones may arrest or hasten the growth and development of shoot buds. In this present study, it was indicated that Kn had a strong effect on promoting the growth of leaves and bud initials, causing a rise in the number of shoot buds that subsequently grew into multiple shoots. Martin *et al.* (2005) reported that a maximum number of shoots at 5.4 were produced on medium supplemented with 6.97 μ M Kn in *Dendrobium* hybrid. They stated that Kn was more effective in inducing bud break and the shoots showed better growth than on BAP supplemented medium. Asghar *et al.* (2011) reported that the proliferated shoots of *Dendrobium nobile* produced the longest shoot length of 4.19 cm when cultured on medium containing 1.5 mg/L Kn.

Table 1. The effect of different concentrations of BAP and Kn on shoot proliferation of *D. lowii* when placed vertically and or horizontally

Orientation of explant	Treatment (mg/l)		Percentage of survival	Percentage of explant forming shoots	Mean number of shoots per explant	Mean Length of shoot (mm)	
	BAP	Kn					
Vertical	0.0	0.0	67.50 \pm 37.36 ^{bcd}	2.50 \pm 1.05 ^e	1.03 \pm 0.10 ^c	3.96 \pm 2.46 ^c	
	0.5	0.0	85.00 \pm 24.15 ^{ab}	45.00 \pm 31.72 ^{bcd}	1.98 \pm 0.96 ^{bc}	5.02 \pm 1.68 ^{abc}	
	1.0	0.0	92.50 \pm 12.50 ^{ab}	50.00 \pm 22.22 ^{abc}	1.69 \pm 0.38 ^{bc}	4.16 \pm 1.68 ^{bc}	
	2.0	0.0	97.50 \pm 8.84 ^a	35.00 \pm 29.69 ^{bcd}	1.77 \pm 1.17 ^{bc}	4.73 \pm 1.47 ^{abc}	
	3.0	0.0	87.50 \pm 17.68 ^{ab}	15.00 \pm 12.24 ^{de}	1.03 \pm 0.55 ^c	3.27 \pm 1.64 ^c	
	4.0	0.0	95.00 \pm 8.33 ^a	42.50 \pm 38.36 ^{bcd}	1.66 \pm 0.94 ^{bc}	5.67 \pm 3.26 ^{abc}	
	0.0	0.5	67.50 \pm 27.95 ^{bcd}	30.00 \pm 22.89 ^{bcd}	1.58 \pm 0.81 ^{bc}	4.61 \pm 1.94 ^{bc}	
	0.0	1.0	75.00 \pm 21.65 ^{abc}	25.00 \pm 17.22 ^{cde}	1.58 \pm 0.95 ^{bc}	4.58 \pm 1.67 ^{bc}	
	0.0	2.0	100.00 ^a	52.50 \pm 34.11 ^{abc}	2.86 \pm 1.71 ^{bc}	7.86 \pm 3.64 ^{abc}	
	0.0	3.0	82.50 \pm 23.72 ^{ab}	22.50 \pm 16.72 ^{cde}	1.47 \pm 0.66 ^{bc}	5.15 \pm 1.93 ^{abc}	
	0.0	4.0	90.00 \pm 17.48 ^{ab}	40.00 \pm 26.37 ^{bcd}	1.62 \pm 0.56 ^{bc}	4.03 \pm 2.19 ^{bc}	
	Horizontal	0.0	0.0	50.00 \pm 23.57 ^{def}	37.50 \pm 31.73 ^{bcd}	1.67 \pm 1.00 ^{bc}	6.60 \pm 5.28 ^{abc}
		0.5	0.0	85.00 \pm 17.48 ^{ab}	55.00 \pm 33.85 ^{abc}	1.85 \pm 1.12 ^{bc}	4.38 \pm 2.31 ^{bc}
1.0		0.0	77.50 \pm 26.35 ^{abc}	65.00 \pm 19.29 ^{ab}	2.12 \pm 0.67 ^{bc}	4.49 \pm 1.59 ^{bc}	
2.0		0.0	75.00 \pm 26.73 ^{abcd}	50.00 \pm 37.65 ^{abc}	2.38 \pm 1.44 ^{bc}	4.26 \pm 2.02 ^{bc}	
3.0		0.0	70.00 \pm 22.97 ^{bcd}	40.00 \pm 35.09 ^{bcd}	1.93 \pm 1.14 ^{bc}	4.69 \pm 3.41 ^{abc}	
4.0		0.0	87.50 \pm 18.16 ^{ab}	50.00 \pm 38.96 ^{abc}	2.08 \pm 1.39 ^{bc}	7.20 \pm 5.81 ^{abc}	
0.0		0.5	45.00 \pm 30.05 ^{ef}	25.00 \pm 18.05 ^{cde}	3.66 \pm 3.01 ^{ab}	3.82 \pm 3.45 ^c	
0.0		1.0	52.50 \pm 19.54 ^{cdef}	52.50 \pm 43.66 ^{abc}	2.98 \pm 2.03 ^{bc}	8.04 \pm 7.09 ^{ab}	
0.0		2.0	82.50 \pm 25.00 ^{ab}	80.00 \pm 22.44 ^a	5.05 \pm 2.81 ^a	9.13 \pm 3.31 ^a	
0.0		3.0	67.50 \pm 31.29 ^{bcd}	32.50 \pm 30.03 ^{bcd}	1.80 \pm 1.44 ^{bc}	5.04 \pm 4.53 ^{abc}	
0.0		4.0	37.50 \pm 35.84 ^f	22.50 \pm 18.62 ^{cde}	1.67 \pm 1.03 ^{bc}	5.08 \pm 3.85 ^{abc}	

Results represent mean \pm standard deviation of ten replicated experiments after 180 days of culture. The different letters within a column indicates a significant difference at $p < 0.05$ level.

This study also proved that horizontally placement of explants produced better performances in percentage of shoot formation, number of shoots, as well as shoot lengths. Hence, horizontal placement of explant was more favorable than vertically placement of explant even though there was no significant different between these two orientations. A study performed by San-Jose *et al.* (1988) revealed that horizontal orientation as the most effective placement of explant in *Quercus robur*. In

their experiment, they reported that the horizontal position increased the proliferation capacity of this species probably due to larger contact of the explant tissue with the culture medium, hence increased nutrient absorption.

Effect of NAA, IAA and IBA on rooting

In rooting induction (Table 2), even though $82.50\% \pm 16.87$ explants survived in control treatment, none of them formed root (Figure 1C). This suggests that it is imperative for shoot tip explant to be cultured in medium enriched with auxins in order to induce root formation. Treatment with 1.0 mg/L IAA and 0.5 mg/L IBA produced up to $75.00\% \pm 29.78$ shoots responded by forming an average of 1.93 ± 0.96 roots with $13.02 \text{ mm} \pm 11.23$ length (Figure 1D). By increasing the level of IAA caused a steady decline in the rooting performance. At 2.0 mg/L IAA with 0.5 mg/L IBA, percentage of explant forming root decreased to $57.50\% \pm 23.19$, 1.19 ± 0.60 roots, and roots were mostly short and stunted (5.35 ± 4.16 mm, roots length). However, there were no significant differences observed between 1.0 mg/L and 2.0 mg/L IAA when combined with 0.5 mg/L IBA. In a slightly higher level of IAA (2.0 mg/L) with combination of NAA, swelling and callus formation was observed and caused an abnormal appearance of roots. The swelling exerted an inhibiting effect on the root growth and development. Similar observation was reported by Rai *et al.* (2009) when they used NAA to induce adventitious roots of guava shoots, a mass of callus was produced on the shoots base, decreased rooting frequency and formation of abnormal roots. In plants, auxins play a crucial role in regulating the growth and multiplication of plant organs and in lateral root formation (Woodward and Bartel, 2005; Mahmood *et al.*, 2009). In the present study, shoot explants absorbed IAA and IBA in combination better than combination with NAA. There were no significant differences between the combination of 1.0 mg/L IAA with 0.5 mg/L IBA and 2.0 mg/L IAA with 0.5 mg/L IBA. But fastest root formation was observed in the combination of 1.0 mg/L IAA with 0.5 mg/L IBA (20 days) as compared to in 2.0 mg/L IAA with 0.5 mg/L IBA (50 days). The combination of 1.0 mg/L IAA and 0.5 mg/L IBA showed better results in the overall performances. This is similar to result obtained by Khatun *et al.* (2010) where the highest number of roots (6.55 roots) was produced when shoot tips of *Dendrobium* orchid was rooted on medium containing 1.0 mg/L IAA and IBA. IBA is the strongest, most common, highly stable and least toxic rooting plant growth regulator among all auxins (Hartmann *et al.*, 2007; Abassi *et al.*, 2013). Husen & Pal (2007) reported that the application of IBA causes changes in the synthesis of protein and the production of RNA. These changes lead to a more rapid cell growth and division and, as a result, the number of roots increases. Asghar *et al.* (2011) reported that IBA at a level of 2.0 mg/L increased the rooting percentage (97.5%), number of roots (4.70) and root length (3.47 cm) of *Dendrobium nobile*. They also stated that IBA was more efficient than NAA and poor results of rooting occurred at higher concentrations (3.0 mg/L) of IBA and NAA. Auxin IAA is another common auxin used in *in vitro* propagation of orchid. Overvoorde *et al.* (2010) reported that IAA was important for root formation however at higher concentration, it could hindered

shoot elongation. In this study, a combination of 1.0 mg/L IAA and 0.5 mg/L IBA was chosen to be the best plant growth regulator (auxin).

Table 2. The effect of IAA, IBA and NAA on root formation from regenerated shoots of *D. lowii*

Type of auxin combinations (mg/L)	Percentage of survived explant	Percentage of root formation	Number of roots	Length of root(mm)
Control	82.50 ± 16.87 ^b	0 ^b	0 ^b	0 ^b
1.0 IAA+0.5 NAA	85.00 ± 19.67 ^b	70.00 ± 22.49 ^a	1.58 ± 0.89 ^a	10.29 ± 6.58 ^a
2.0 IAA+0.5 NAA	77.50 ± 21.89 ^b	62.50 ± 32.41 ^a	1.58 ± 1.00 ^a	6.57 ± 3.79 ^a
1.0 IAA+0.5 IBA	97.50 ± 7.91 ^a	75.00 ± 29.78 ^a	1.93 ± 0.96 ^a	13.02 ± 11.23 ^a
2.0 IAA+0.5 IBA	92.50 ± 12.50 ^{ab}	57.50 ± 23.19 ^a	1.19 ± 0.60 ^a	5.35 ± 4.16 ^a

Results represent mean ± standard deviation of ten replicated experiments after 120 days of culture. The different letters within a column indicates a significant difference at $p < 0.05$ level.

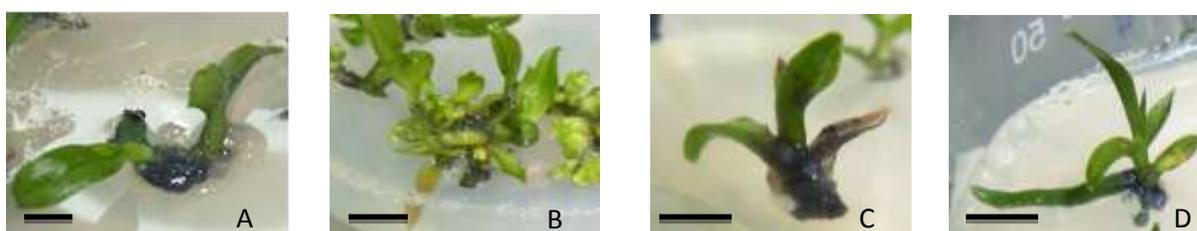


Figure 1. The proliferation of multiple shoots on (A) PGR-free medium and (B) medium containing 2.0 mg/l Kn. Root formation was absent in (C) PGR-free medium, but was observed in (D) medium containing 1.0 mg/l IAA + 0.5 mg/l IBA (Bar is 5 mm).

Plantlets Acclimatization

The *in vitro* well-developed rooted plantlets of *D. lowii* were successfully hardened on potting mixture containing coco peat and sphagnum moss (2:1). After 2 months, 78% of plants survived after transferred to the glasshouse (Figure 2). This work suggests that these mixtures are favorable for the acclimatization of epiphytic orchids. This simple and efficient procedure for regenerating a large number of plantlets from shoot tip cultures could be used for large-scale propagation and *ex situ* conservation of this endangered species.



Figure 2. Acclimatized plantlets after two months in the glass house.

Conclusion

A protocol was established for *in vitro* propagation of *D. lowii* using shoot tips as the starting material. Shoot multiplication was best seen in KC medium added with 2.0 mg/l Kinetin. The best rooting performance was observed in shoot tips that were cultured in KC medium added with 1.0 mg/l IAA

with 0.5 mg/l IBA. It is hoped that this successful protocol for micropropagation using shoot tip culture can be applied in a commercial scale with the aim to mass-produce and to re-introduce this species back to their natural habitat.

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