ISSN 1817-3721, E-ISSN 1818-8745

Plant Tissue Cult. & Biotech. **28**(2): 161-169, 2018 (December)
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PTC&B

In vitro Mass Propagation from Shoot Tip of *Dendrobium* Red Bull - An Endangered Epiphytic Orchid

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Key words: *Dendrobium*, Growth regulators, *In vitro* micropropagation, *Ex vitro* survivability

Abstract

An experiment was carried out to optimize the doses and combinations of growth regulators for *in vitro* propagation of *Dendrobium* Red Bull through shoot tip culture. The experiment was laid out according to completely randomized design with 15 treatments and three replications. For multiple shoot induction, newly grown shoot buds (keikies) were cultured on MS supplemented with various combinations of NAA and BAP. The regenerated plants were transferred to *ex vitro* in different substrates and acclimatized. Best response for the shoot length (21.19 mm) was obtained from the medium supplemented with 3.0 mg/l BAP with 1.5 mg/l NAA. Similarly maximum number of leaves was observed from the same combination at all the recorded days except 60 days, whereas the maximum shoot number (7.66) was obtained from the medium containing 3.0 mg/l BAP + 1.0 mg/l NAA. The shoots were then transferred to root inducing medium and well rooted plants were transferred to different substrates. Survivability of the regenerated plants varied from 60 to 92%. The substrate containing cocodust showed maximum survivability (92%) whereas minimum (60%) was observed from the substrate containing course sand. BAP and NAA proved to be good growth regulators for *in vitro* multiplication of *Dendrobium*.

Introduction

Orchid constitutes an order of royalty in the world of ornamental plants. The *Dendrobium* is the second genus of Orchidaceae, which is composed of approximately 1500 species scattered in the world, and only a few of these are used as ornamentals (Chen and Ji 1998). *Dendrobium* Red Bull is one of the so-called jewel orchids, which are planted originally for its beautiful flower. It

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DOI: https://doi.org/10.3329/ptcb.v28i2.39675

could be used as medicine against digestive, respiratory and ophthalmic diseases (Chen and Guo 2003). But nowadays this orchid is facing the threat of extinction owing to over collection from natural resources (Zhang et al. 2000). Orchid requires a combination of different factors for reproduction in nature. The propagation of this species through sexual means is a very slow process as its seeds lack endosperm and need fungal stimulant for germination in nature (Arditti et al. 1982). In nature, only 2 to 5% of seeds germinate (Rao 1997) even if they do so, the seeds take a long time for their germination and any disturbance in the habitat or physical environment destroys the whole population. Orchids are highly heterozygous and their vegetative propagation through division of clumps of rhizomes, bulbs or by the rooting of offshoots also takes long time and difficult to obtain desired number of plants. This difficulty in natural population drives the *Dendrobium* Red Bull to extinction. It is therefore important to take initiative for the mass propagation of this orchid and establish it in nature. Thus, tissue culture technique is a potential alternative method for mass scale propagation and conservation of rare, endangered and threatened orchids.

Micropropagation of plants through *in vitro* methods has become a significant and informative technique to reproduce and make the availability of crops, orchids and ornamental plants that are otherwise difficult to propagate. Commercial orchids are predominantly produced by tissue culture and this technique is used routinely in many countries for mass scale production of orchid plants. Establishment of a reliable cloning methodology for this orchid is important in terms of enabling the rapid propagation and production of a large number of high quality plants. Thus, the present study was taken up to optimize the physical and chemical conditions for mass *in vitro* propagation of *Dendrobium* Red Bull through shoot tip culture.

Materials and Methods

The experiment was conducted at Plant Breeding and Biotechnology Laboratory and Green House of Agrotechnology Discipline, Khulna University, Khulna. The experiment was laid out according to CRD with 3 replications and 15 treatments. The matured *Dendrobium* Red Bull plants were collected from Dipto Orchid Nursery, Valuka, Mymensingh and were established in a nethouse. Newly grown shoot buds (keikies) of about 1.0 - 1.5 cm long were excised from mother plants. The collected keikies were washed with running tap water for three times and then treated with 70% ethanol for 60 sec followed by treatment with 0.1% NaOCl solution for 8 min. The treated keikies were again washed with double distilled water. The surface sterilized explants were then cultured on agarified MS supplemented with different combinations and concentrations of BAP and

NAA for *in vitro* multiple shoot production. The cultures were subjected for five successive subcultures. For rooting, the *in vitro* grown shoots were cultured on agarified MS fortified with 2.0 mg/l IBA. The pH of the medium was adjusted to 5.8. The cultures were kept at 25 ± 1°C and 65% RH under 16 hrs photoperiod at 3000 lux. The regenerated plants with about 3 - 4 roots were then transferred to *ex vitro* small pots containing four different substrates *viz.* fine sand, course sand, cocodust and brick gravels after removal of agar attached with the roots and treated with antifugal solution Bavistin @ 0.1%. The pots were then covered with polythene after spraying water inside it and closed to check evapotranspiration and were kept in greenhouse for 7 days. Afterword the polythene were removed and the plantlets were kept in nethouse. Data were collected on *in vitro* and *ex vitro* parameters were analyzed for ANOVA. The differences between treatment means were compared by DMRT.

Results and Discussion

Newly grown shoot tips were used for shoot initiation and multiplication. Multiple shoots were observed without any intervening callus and protocorm-like-body (PLB) formation. The efficacy of multiple shoot formation differed with concentrations and combinations of BAP and NAA (Table 1). After 60 days the plants started producing multiple shoots which varied from 1.00 to 2.00 shoots/plant. The treatment combination 3.0 mg/l BAP and 1.0 mg/l NAA produced maximum number of shoots (3.33, 5.33 and 7.66, respectively). Minimum number of shoots were observed in the combination 1.00 mg/l BAP and 0.5 mg/l NAA at 90, 120 and 150 days of culture (1.33, 1.66 and 1.66, respectively).

Paudel et al. (2012) found that multiple shoot development from shoot tip section was significantly promoted by concentrations of BAP (0.5 - 2.0 mg/l) in combination with NAA (0.5 mg/l). Pant and Thapa (2012) reported the maximum number of rootless healthy shoots (4.5/culture) on MS fortified with BAP (1.5 mg/l) and NAA (0.5 mg/l).

Length of *in vitro* grown shoots of *Dendrobium* varied significantly with varying concentrations and combinations of growth regulators (Table 2). At each stage of culture, maximum shoot lengths were recorded from the medium supplemented with 3.0 mg/l BAP and 1.5 mg/l NAA followed by the combination of 3.0 mg/l BAP and 1.0 mg/l NAA. The regeneration media containing 1.0 mg/l BAP and 0.5 mg/l NAA produced the shortest shoot at every stages of culture.

Similar results were reported by Talukder et al. (2014) and observed the highest plant length (0.252 cm) on MS supplemented with 2.5 mg/l BAP along with 0.5 mg/l NAA. Bhattacharjee and Islam (2014) found that the combination of

1.0 mg/l NAA and 1.0 mg/l BAP as the best formulation for maximum shoot length (4.0 cm) of orchid *in vitro*.

Table 1. *In vitro* shoot production of *Dendrobium* Red Bull in varying levels of growth regulators.

Growth regulators (mg/l)		Shoot no. at (DAC)			
BAP	NAA	60	90	120	150
1	0.5	1.00	1.33b	1.66f	1.66e
1	1.0	1.66	2.66ab	3.66bcd	5.00bc
1	1.5	1.33	1.66b	2.00ef	2.66de
2	0.5	1.00	1.66b	2.66def	2.66de
2	1.0	1.33	2.66ab	4.00bc	6.00b
2	1.5	1.33	2.00ab	2.33ef	2.66de
3	0.5	1.33	2.00ab	3.00cde	4.00cd
3	1.0	2.00	3.33a	5.33 a	7.66a
3	1.5	1.33	2.33ab	3.66bcd	5.00bc
4	0.5	1.00	2.00ab	2.66def	4.66bc
4	1.0	1.66	2.66ab	4.33ab	6.00b
4	1.5	1.00	1.33b	2.33ef	3.00de
5	0.5	1.33	1.66b	2.66def	4.00cd
5	1.0	1.33	2.33ab	3.000cde	4.00cd
5	1.5	1.33	1.66b	2.66def	3.000 de
Level of significance		NS	*	**	**
LSD			1.207	1.067	1.232
CV (%)		36.23	32.98	19.87	17.03

DAC = Days after culture, NS = Not significant ($p > 0.05$), * = Significant at $p \leq 0.05$, ** = Significant at ≤ 0.01.

Due to the different combinations and concentrations of growth regulators significant variation was observed in leaf numbers also (Table 3). After 30 days of culture the shoots started to produce leaf and maximum (3.00) number of leaves was found in treatment 3.0 mg/l BAP + 1.5 mg/l NAA whereas at 60 days of culture maximum number of leaves (5.33) were obtained from the treatment 3.0 mg/l BAP + 1.0 mg/l NAA. Later, at 90 days of culture, maximum leaf number (7.33) was recorded from the media containing 3.0 mg/l BAP + 1.0 mg/l NAA and 2.0 mg/l BAP + 1.5 mg/l NAA as well as 3.0 BAP mg/l + 1.5 mg/l NAA. At 120 DAC, the maximum leaf (11.33) was found in combination of 3.0 mg/l BAP + 1.5 mg/l NAA. Finally, at 150 DAC, the medium supplemented with 3.0 mg/l BAP + 1.0 mg/l NAA and 3.0 mg/l BAP + 1.5 mg/l produced maximum number of leaves (13.33) followed by the medium having 2.0 mg/l BAP + 1.0 mg/l NAA (11.3).

Kabir and Al-amin (2010) found the highest leaf number from 1.0 mg/l of each BAP and NAA and the lowest from 0.5 mg/l each of BAP and NAA. Nasiruddin et al. (2003) found maximum number of leaves (4.42) per shoot at 2.5 mg/l BAP.

Table 2. Length of *in vitro* produced shoot at different DAC of *Dendrobium* Red Bull.

Growth regulators (mg/l)		Shoot length (mm) at (DAC)				
BAP	NAA	30	60	90	120	150
1	0.5	5.39g	6.79ef	7.75i	9.11g	12.24efgh
1	1.0	5.97efg	7.19def	8.37hi	10.04defg	11.44fgh
1	1.5	6.85cde	8.99c	11.62cde	12.68bc	14.46cd
2	0.5	6.05efg	6.98ef	8.35hi	9.36fg	10.90fgh
2	1.0	5.66fg	6.55f	7.99i	9.46fg	11.45fgh
2	1.5	7.55bc	10.72ab	12.68bc	16.30a	17.26b
3	0.5	5.98efg	7.02ef	8.38hi	9.56efg	10.43h
3	1.0	7.84b	10.89a	13.86ab	16.65a	20.98a
3	1.5	8.86a	12.06a	15.13a	18.05a	21.19a
4	0.5	6.43def	8.36cde	9.98efgh	11.67cdef	13.69de
4	1.0	7.01bcd	8.79cd	10.48defg	11.95bcde	12.49efg
4	1.5	7.05bcd	9.30bc	11.85cd	14.11b	15.60bc
5	0.5	7.13bcd	8.32cde	9.54fghi	10.99cdefg	11.89efgh
5	1.0	7.18bcd	7.80cdef	8.85ghi	9.80defg	10.56gh
5	1.5	6.52def	8.34cde	10.84def	12.17bcd	12.82def
Level of significance		**	**	**	**	**
LSD		0.8615	1.501	1.640	2.161	1.793
CV (%)		7.27	10.04	9.02	10.17	7.40

DAC = Days after culture, ** = Significant at $p \leq 0.01$.

The *in vitro* grown shoots of orchid were cultured in root inducing medium and effect of initial shoot inducing medium was observed on root induction (Table 4). Root number varied from 2.33 to 4.67. The shoot obtained from the cultures containing 4.0 mg/l BAP and 1.5 mg/l NAA produced maximum number of roots (4.67). The shoots from media having 1.0 mg/l BAP + 1.0 mg/l NAA, 1.0 mg/l BAP + 1.5 mg/l NAA, 2.0 mg/l BAP + 0.5 mg/l NAA and 2.0 mg/l BAP + 1.0 mg/l NAA produced least number of roots (3.00) in root induction media.

Paudel and Pant (2012) found a maximum number of roots (3.0 ± 0.40) on NAA (0.5 mg/l) and higher concentration of NAA showed the poor result of rooting in *Dendrobium.* Wang (2015) reported the higher number (4.6) of roots of *Dendrobium* from the medium containing 0.5 mg/l BAP and 2.0 mg/l NAA.

Ex vitro survivability of *in vitro* grown plantlets is a crucial aspect of *in vitro* propagation. A substantial number of micropropagated plants do not survive when transferred from *in vitro* conditions to greenhouse or field environment. In this study, the survivability of plantlets on different substrates *viz.* fine sand, course sand, cocodust and bricks gravels showed variable results (Fig. 1). The *ex vitro* survivability of the plantlets varied from 60 to 92%. The maximum survivability (92%) was observed on cocodust and that of minimum (60%) from substrate containing course sand.

Table 3. Leaf production efficiency of *in vitro* grown shoot tips of *Dendrobium* Red Bull.

Growth regulators (mg/l)		Leaf number at (DAC)				
BAP	NAA	30	60	90	120	150
1	0.5	2.33	3.33bc	5.00bc	6.00d	6.66d
1	1.0	2.33	3.66bc	5.00bc	6.66d	8.00cd
1	1.5	2.33	3.33c	5.00bc	6.33d	8.00cd
2	0.5	2.00	3.00c	3.66c	6.66d	8.66bcd
2	1.0	2.00	2.66c	5.33abc	7.33cd	9.66bc
2	1.5	3.00	5.00 a	7.33a	9.66abc	11.33ab
3	0.5	2.33	3.66bc	5.66abc	7.33cd	9.33bcd
3	1.0	2.66	5.33a	7.33a	10.33ab	13.33a
3	1.5	3.00	4.66ab	7.33a	11.33a	13.33a
4	0.5	2.00	2.66c	4.66bc	6.66d	9.33bcd
4	1.0	2.33	3.66bc	5.33abc	6.66d	8.00cd
4	1.5	2.66	3.66bc	6.00ab	8.33bcd	9.66bc
5	0.5	2.00	3.33c	5.33abc	6.66d	8.33cd
5	1.0	2.00	3.00c	5.00bc	7.00d	9.33bcd
5	1.5	2.33	3.66bc	6.00ab	7.66cd	10.33bc
Level of significance		NS	**	**	**	**
LSD			1.176	1.880	2.161	2.362
CV (%)		17.98	18.42	19.17	16.90	14.78

DAC = Days after culture, NS = Not significant ($p > 0.05$),* = Significant at $p \leq 0.05$, ** = Significant at ≤ 0.01.

Rao and Barman (2014) reported 88% survivability of *in vitro* grown shoots of orchid when they were transferred to sterile mixture of brick and charcoal and vermicompost. Rahman et al. (2009) reported that well rooted micropropagated plantlets were hardened successfully in the potting mixture containing coconut husk, charcoal and brick pieces in the ratio of 2 : 1 : 1.

Table 4. Root induction efficiency of grown shoots of *Dendrobium* Red Bull *in vitro.*

Media	Growth regulators		Root number
	BAP	NAA	
MS	1	0.5	3.33
MS	1	1.0	3.00
MS	1	1.5	3.00
MS	2	0.5	3.00
MS	2	1.0	3.00
MS	2	1.5	4.00
MS	3	0.5	3.66
MS	3	1.0	2.33
MS	3	1.5	3.33
MS	4	0.5	2.66
MS	4	1.0	3.33
MS	4	1.5	4.67
MS	5	0.5	4.00
MS	5	1.0	4.00
MS	5	1.5	2.67
Level of significance			NS
CV (%)			44.79%

NS = Not significant ($p > 0.05$).

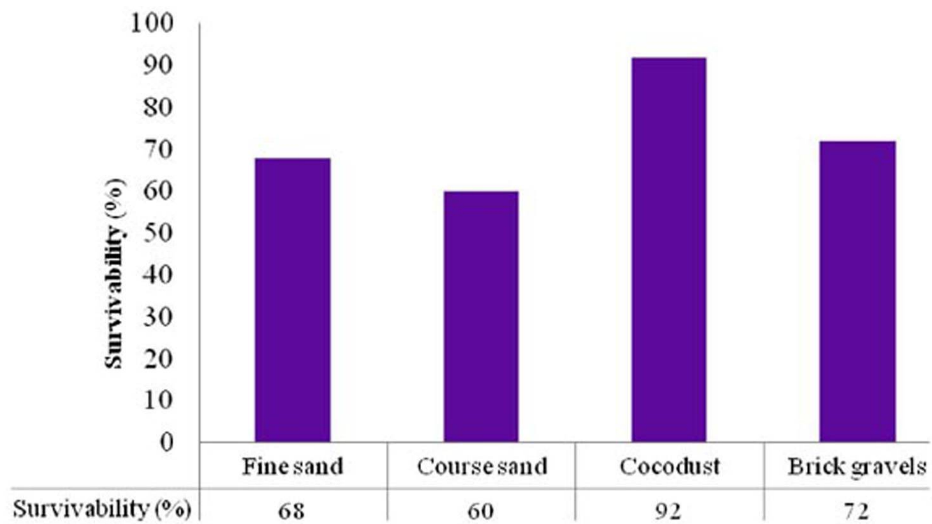

Fig. 1. Diagrammatic representation of *ex vitro* survivability rate of *in vitro* grown orchid.

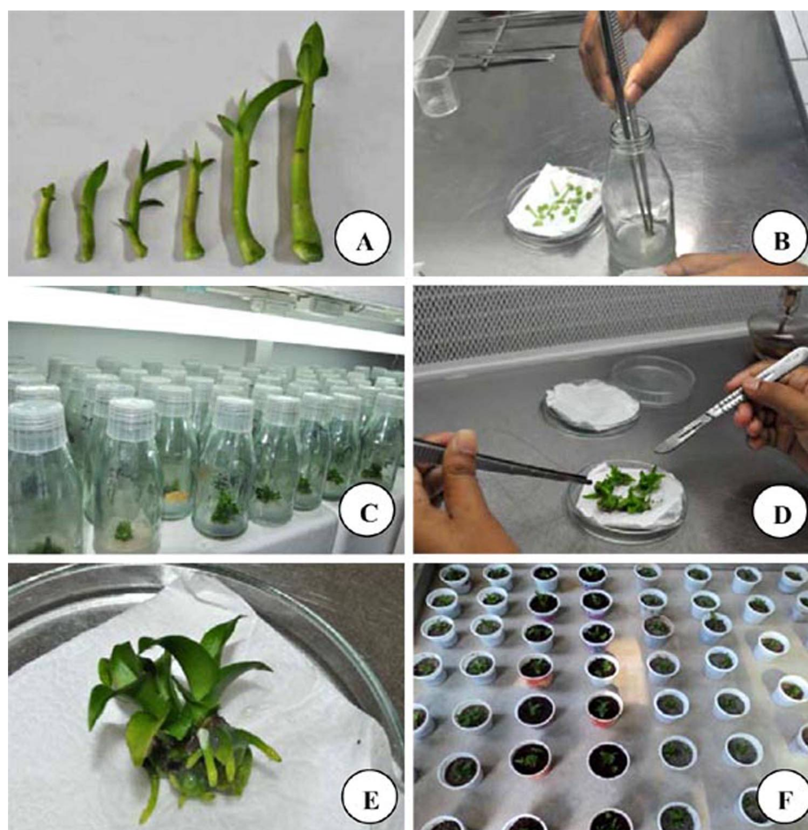

Fig. 2. Different stages of *in vitro* micropropagation of *Dendrobium* on growth regulators supplemented MS. A. Excised shoots. B. Culturing of shoot tip for multiplication. C. Multiple shoots *in vitro*. D. Multiple shoots culturing for rooting. E. Well rooted plantlets. F. *Ex vitro* transferred hardened plants.

In *in vitro* micropropagation of *Dendrobium* Red Bull, maximum shoots were obtained from 3.0 mg/l BAP + 1.0 mg/l NAA. The highest shoot length as well as number of leaves were recorded from 3.0 mg/l BAP + 1.5 mg/l NAA and 3. mg/l BAP + 1.0 mg/l NAA. The maximum root numbers were observed for the shoots obtained from 4.0 mg/l BAP + 1.0 mg/l NAA. In the present study, the best combination was 3.0 mg/l BAP + 1.0 mg/l NAA and 3.0 mg/l BAP + 1.5 mg/l NAA for shoot production, shoot length and leaf numbers.

Acknowledgement

The authors are grateful to Khulna University Research Cell, Khulna University, Khulna-9208, Bangladesh for financial assistance under Research Grants Program (RGP) to carry out the research work.

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