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Full Length Research Paper

In vitro mass propagation of an epiphytic orchid, *Dendrobium primulinum* Lindl. through shoot tip culture

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The present study develops a protocol for rapid *in vitro* micropropagation of a critically endangered and floriculturally most important epiphytic orchid, *Dendrobium primulinum* Lindl. through the culture of small shoot tip explants (0.3 to 0.5mm) derived from *in vitro* grown seedlings. The shoot tip explants cultured on solidified Murashige and Skoog (MS) basal medium and MS medium alone or supplemented with combination of various concentrations of growth regulators; α-naphthalene acetic acid (NAA) and 6-benzylaminopurine (BAP), produced shoots and multiple shoots. The maximum numbers of rootless healthy shoots were observed on MS medium fortified with BAP 1.5 mgl^{-1} with an average value of 4.5 shoots per culture where shoot multiplication was initiated after 5 weeks of culture of shoot tip. Among the different tested combination, MS medium with BAP (1.5 mg l^{-1}) and NAA (0.5 mg l^{-1}) were most effective for the shoot multiplication. MS medium supplemented with various concentrations of rooting hormones viz. NAA, IAA and IBA showed positive response in development of roots, except NAA 0.5 mgl^{-1}. The rooting was observed after 3 weeks of culture of shoot tip. The various concentrations of IAA and IBA were found to be effective hormone for rooting of *D. primulinum* in comparison to NAA. The best rooting response was observed on MS medium with exogenous supply of IAA 0.5 mgl^{-1}. The well developed *in vitro* rooted plantlets were hardened successfully in the potting mixture containing cocopeat and sphagnum moss in the ratio of 2:1. Nearly 70% of plantlets survived.

Key words: *Dendrobium primulinum*, shoot tip, micropropagation, growth regulator, *in vitro*, shoot multiplication, acclimatization.

INTRODUCTION

The genus *Dendrobium* is a highly evolved and diversified sympodial group of orchids, comprising more than 1100 plant species and widely distributed from Southeast Asia to New Guinea and Australia (Yin and Hong, 2009). In Nepal, Raskoti (2009) enumerated about 27 species of Dendrobium. *Dendrobium primulinum* Lindl is one of the critically endangered and floriculturally important epiphytic orchids of Nepal, found on tree trunks. It is commonly known as "primrose yellow *Dendrobium*" and is reported in central part of the country in deciduous tropical and subtropical forest at an elevation of 400 to 1200 m (Raskoti, 2009). Apart from the floricultural value, it has some traditional medicinal properties. The dried stem is commonly used as immune system enhancer. Due to its indiscriminate collection by orchid traders to meet the increasing demand for horticultural purposes, habitat destruction and natural calamities the population of *D. primulinum* Lindl. have been largely destroyed in their natural habitat and are categorized as rare and endangered species.

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Abbreviations: BAP, 6-Benzylaminopurine; **IBA,** indole butyric acid; **NAA,** α-naphthalene acetic acid; **IAA,** indole-3 acetic acid; **ANOVA,** analysis of variance; **BM,** basal medium.

Orchid requires a combination of multiplicity of factor for continued 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; the fungus is believed to augment the carbohydrate, auxin and vitamin transport in the orchid (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 off shoots also takes long time and difficult to obtain desired number of orchids. This difficulty in natural population drives the *D. primulinum* 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 orchid.

Shoot tip culture is an efficient system for the production of large number of plantlets in a short period of time. The propagation and cultivation of orchid was revolutionized after the discovery of Knudson (1922). He first developed the asymbiotic germination of orchid seeds while the credit of developing protocol for *in vitro* micropropagation goes to Morel (1960). Since then, commercial orchids are predominantly produced by tissue culture and this technique is used routinely in many countries for mass scale production of orchid seedlings. A perusal of available literature reveals that regeneration of plantlet has been achieved by only using stem section and *in vitro* flowering through immature embryo in *D. primulinum.* However, there is no report on clonal propagation of *D. primulinum* using different explants sources. 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 investigation was undertaken to develop an efficient protocol for *in vitro* micropropagation of *D. primulinum* Lindl. through shoot tip culture and to introduce this species in natural habitat.

MATERIALS AND METHODS

The materials used for the present investigation were small shoot tips of *D. primulinum* Lindl. which were sourced from 20 weeks old *in vitro* grown seedlings. This experiment was assessed on Murashige and Skoog (MS) (1962) static medium supplemented with or without different concentration and combination of various growth regulators to compare and investigate the effect of hormone concentration on shoot multiplication and rooting of *D. primulinum* Lindl. The medium was supplemented with 3% sucrose as carbon source and solidified with 0.8% agar. The pH of the medium was adjusted to 5.8 before autoclaving. The media was autoclaved at pressure of 15 psi and temperature of 121°C for 20 min.

Shoot tip culture and regeneration of multiple shoots

For shoot multiplication, the shoot tip as explants of size 0.3 to 0.5 mm were cut aseptically with the help of surgical blade and single shoot tip was inoculated on MS basal media and MS media alone or supplemented with combination of various concentration of growth regulators: 6-Benzylaminopurine, BAP (0.5 to 2.0 mgl^{-1}) and α-naphthalene acetic acid, NAA (0.5 to 1.0 mgl^{-1}). At every time of isolation and inoculation of explants, the blade and forceps were flamed later and dipped in spirit. After inoculation, the cultures were kept in control room and exposed to artificial light (fluorescent light) with a light/dark cycle of 16/8 h at 25±2°C. The observation was taken at regular intervals of one week up to the 24 weeks and the obtained result was recorded. The rootless multiple shoot were developed in the entire tested media except in MS with NAA 1.0 mgl^{-1}.

Rooting of regenerated shoots

For the induction of root, the regenerated multiple shoots of *D. primulinum* were excised and a single shoot was cultured on MS media containing various concentrations of rooting hormone viz. IAA, IBA and NAA (0.5 to 2 mgl^{-1}). The culture tubes were maintained in the culture room under the same condition as used for shoot multiplication. The observation was taken at regular intervals of one week up to 16 weeks of culture and the obtained root number and their length were recorded.

Acclimatization

After the formation of complete rooted plantlet, they were subjected to *ex vitro* hardening. The plantlets were removed from the culture tubes and were washed thoroughly with sterile double distilled water to remove any trace of the medium. They were then treated with 0.1% (w/v) Bavistin (fungicide) and again washed with sterilized double distilled water. The rooted plantlets were planted in the potting mixture containing cocopeat and Sphagnum moss in the ratio of 2:1. The plants were covered with plastic bags for 30 days and maintained under humidity. Plants became acclimatized to a reduced relative humidity by gradually opening the plastic cover and after 50 days, they were completely uncovered and hardened to greenhouse conditions.

Statistical analysis

Significance of treatment effects on shoot multiplication and rooting of shoot tip were analyzed using one way analysis of variance (ANOVA, $p \leq 0.05$) and comparison between mean values of treatments were made by Tukey HSD test. All statistical analysis was performed using R development core team.

RESULT AND DISCUSSION

In the present investigation, multiple shoots and shoots were observed without any intervening callus and protocorm-like-body (PLB) formation on all the tested MS basal media and MS media alone or supplemented with various concentrations and combination of BAP and NAA but there were quite differences between and within media in terms of number of shoot and their length. The highest number of rootless multiple shoots (an average

Table 1. Effect of BAP and NAA in MS media on the growth and proliferation of shoot through shoot tip explants after 24 weeks of culture.

Media	Growth hormone (BAP mg/l)	Concentration of hormone (NAA mg/l)	Mean number of shoots (±S.E)	Mean length of Shoot (cm) (±S.E.)	Mean number of roots (±S.E.)	Mean root length (cm) (±S.E.)
MS	0BM	-	2.0±0.17	1.0±0.07	0	0
MS	0.5	-	1.5±0.10	0.92±0.04	0	0
MS	1.0	-	2.25±0.11	1.13±0.06	0	0
MS	1.5	-	4.5±0.37	1.62±0.10	0	0
MS	2.0	-	2.5±0.11	1.28±0.04	0	0
MS	0.5	-	2.25±0.10	1.05±0.05	0	0
MS	1.0	-	2.5±0.12	1.25±0.03	1.25±0.14	0.42±0.04
MS	0.5	0.5	2.0±0.17	1.14±0.08	0	0
MS	1.0	0.5	3.25±0.21	1.38±0.04	0	0
MS	1.5	0.5	3.50±0.25	1.07±0.05	0	0
MS	2.0	0.5	2.5±0.28	1.34±0.06	0	0
MS	0.5	1.0	2.0±0.11	1.39±0.03	0	0
MS	1.0	1.0	1.75±0.17	1.21±0.07	0	0
MS	1.5	1.0	2.75±0.27	1.37±0.11	0	0
MS	2.0+1.0	1.0	2.75±0.36	1.26±0.05	0	0

Culture conditions: MS medium, 25±2°C, 24 weeks of primary culture, 4 replicates were used in each combination.

value of 4.5 shoots per explants) were obtained in MS medium supplemented with 1.5 mgl^{-1} BAP in which the longest shoots were also observed. The shoot multiplication was initiated after 5 weeks of culture. As the concentration of BAP from 0.5 to 1.5 mgl^{-1} considerably increased, the multiple shoot production, not the shoot formation was retarded on BAP 2 mgl^{-1}. The least number of shoot was observed in MS media fortified with 0.5 mgl^{-1} BAP (Table 1).

Rootless multiple shoots were obtained on all the tested media except on MS medium supplemented with NAA 1.0 mgl^{-1}. It may be due to the difference in genetic constituent of explants, culture condition and the presence of different endogenous growth substance in explants. During the present investigation, it was found that MS medium alone was not so effective for induction of multiple shoots. Similar result was obtained in *Dendrobium* species (Yasugi et al., 1994). This revealed that the addition of plant growth regulators in nutrient medium might be essential for further growth, development and proliferation of shoot tip explants.

In this experiment, among all the media tested alone or with BAP and NAA, MS medium with 1.5 mgl^{-1} BAP was found to be most effective for shoot multiplication which indicates that MS medium alone or with BAP might be suitable for shoot proliferation. This result was also supported by previous work of several researchers on *Dendrobium densiflorum* (Luo et al., 2006), *Geodorum densiflorum* (Bhadra and Hossain, 2003), *Cymbidium* and *Cattleya* (Nagarju et al., 2003).

Among the different combinations tested in this study, BAP (1.5 mg l^{-1}) and NAA (0.5 mg l^{-1}) were found to be effective for the shoot multiplication. The obtained result showed that combination of BAP and NAA is also suitable for shoot multiplication. The previous work of several researchers also showed that the high concentration of BAP and low concentration of NAA was favorable for the induction of multiple shoots. Similar results were obtained by Talukdar et al. (2003) in *Denrobium* orchid, Sunitibala and Kishor (2009) in *Dendrobium transparens*, *Aerides odorata* (Pant and Gurung, 2005), *Cymbidium aloifolium* (Rajkarnikar, 2011), *Cymbidium forestii* and *Cymbidium kanran* (Chung et al., 1998), *Dendrobium fimbriatum* (Rajkarnikar and Niraula, 1994). Shiau et al. (2005) cultured the axenic nodal segments of *in vitro* seedling of *Dendrobium candidum on* MS medium with 2 mgl^{-1} BA and 0.1 mgl^{-1} NAA and they obtained 73.2% shoots within 75 days. Hence, the regeneration potential of explants is markedly influenced by their physiological status and chemical stimulus present in the nutrient pool. The quality, quantity and nature of growth regulators have foremost effect on the regeneration capacity of the shoot tip.

For the rooting of shoots, MS medium fortified with different concentrations of rooting hormone viz. IAA, IBA and NAA were used. All tested media positively responded to development of roots, except MS media with NAA 0.5 mgl^{-1}. Statistically, both root number and

Table 2. Effect of IAA, IBA and NAA containing MS media on rooting of shoots of *D. primulinum* Lindl. after 15 weeks of culture.

MS + hormone	Concentration (mg/l)	Mean root nnumber (±S.E)	Mean root length (cm) (±S.E)
IAA	0.5	3.5 ±0.21	1.18±0.05
	1.0	2.25±0.21	1.11±0.02
	1.5	2.0±0.17	0.88±0.03
	2.0	1.5 ±0.12	0.8±0.03
IBA	0.5	2.25 ±0.11	1.0±0.04
	1.0	1.5±0.11	0.72±0.04
	1.5	3.0 ±0.31	1.30±0.08
	2.0	2.75 ±0.21	0.85±0.05
NAA	0.5	0.0 ±0.0	0.0±0.0
	1.0	1.25±0.14	0.4±0.03
	1.5	0.75 ±0.16	0.27±0.05
	2.0	0.50 ±0.08	0.22±0.04

Culture condition: MS medium, 25±2°C, 15 weeks of primary culture, 4 replicates were used in each combination.

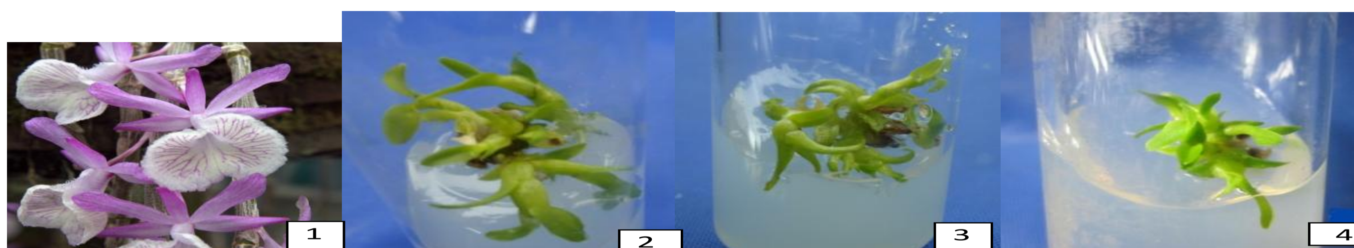

Figure 1. Flower of *D. primulinum* Lindl. (1). *In vitro* shoot multiplication of *D. primulinum* using shoot tip as explants (2 to 4). MS medium with 1.5 mgl^{-1} of BAP (2); MS medium with combination of 1.5 mgl^{-1} BAP and 0.5 mgl^{-1}NAA (3); MS medium with 1.0 mgl^{-1} of NAA (4).

root length of *D. primulinum* Lindl. were found to be highly significant at 5% level of significance. Among them, the large numbers of roots were observed on MS medium supplemented with IAA 0.5 mgl^{-1} with an average value of 3.5 roots per explants. In this condition, the rooting started after 3 weeks of culture. The root length ranged from 0.3 to 1.1 cm after 15 weeks of culture. The obtained roots were slightly whitish green and normal and most of them were aerial in nature but are highest in number in comparison to other concentrations. Thus, this medium was found to be most effective for the rooting of shoots of *D. primulinum*. It may be due to the presence of enhanced level of auxin (IAA) and related compounds in the medium which has strong absorption of inhibitory compounds. This result was similar to the previous findings of rooting of *Aerides odorata* (Pant and Gurung, 2005) and *Micropera pallida* (Bhadra and Hossain, 2004). Sunitibala and Kishor (2009) obtained highest level of rooting response of *Dendrobium transparens* on half strength MS medium with exogenous supply of IAA 1 mgl^{-1}. The longest root observed was about 0.4 to 1.5cm in MS medium supplemented with IBA 1.5 mgl^{-1}. In this condition, the root number was also observed to be the second highest among all the tested medium with an average value of 3.0 roots per culture. Similar result was obtained by the Pradhan (2007). In this investigation, maximum number and length of roots were observed on MS medium with the addition of IBA 1.5 mgl^{-1} in *D. densiflorum.* The various concentrations of IAA and IBA were observed better for the rooting of *D. primulinum* in comparison to the NAA (Table 2). Asghar et al. (2011) also obtained maximum number of roots on modified MS medium fortified with various concentration of IBA than in NAA in *Dendrobium nobile.*

The *in vitro* well developed rooted plantlets of *D. primulinum* were successfully hardened on potting mixture containing cocopeat and Sphagnum moss in the ratio of 2:1. Nearly 70% of plantlets survival was recorded. This work suggests that the mixture of cocopeat and moss will be favorable for the acclimatization of epiphytic orchids as in *D. primulinm* Lindl. (Figures 1 and 2) Hence, the ability of shoot tip in orchids to regenerate

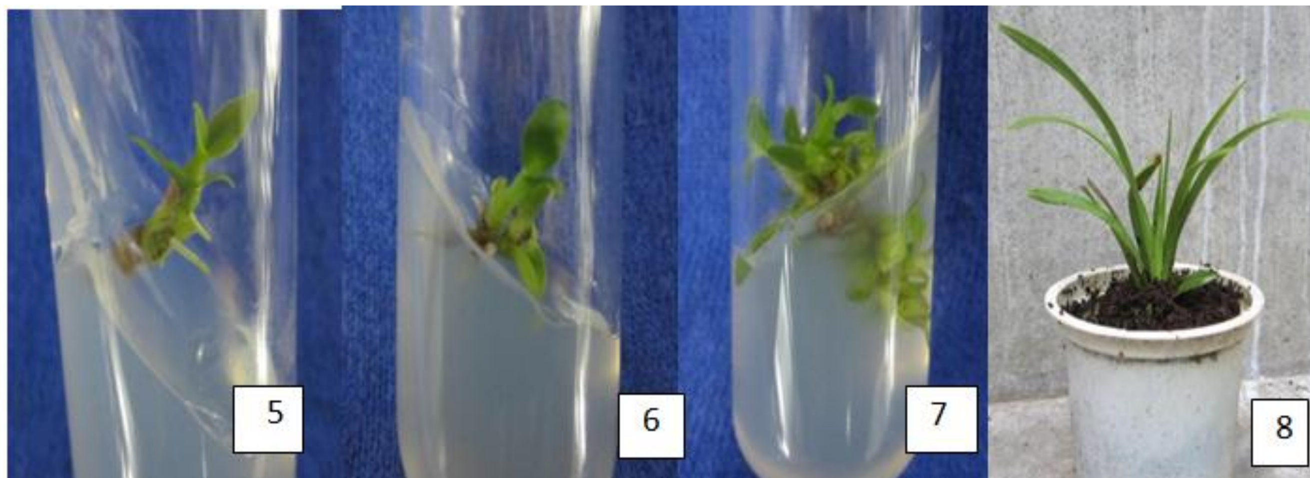

Figure 2. *In vitro* rooting of *D. primulinum* shoots (5 to 8). In Ms medium with IAA 0.5 mgl^{-1}(5); in MS medium with IBA 1.5 mgl^{-1}(6), in MS medium with IBA 2.0 mgl^{-1}; (7), a hardened plantlet on potting mixture (8).

multiple shoot buds and their further development into complete rooted plantlets suggest that shoot tip culture can be successfully employed for rapid multiplication by suitably adjusting the nutrient environment. Thus, the developed protocol will significantly contribute to the mass propagation, conservation as well meet the commercial demand of this wild endangered orchid, *D. primulinum* Lindl.

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