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Full Length Research Paper

In vitro seed germination and seedling growth of the orchid Dendrobium primulinum Lindl.

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***Dendrobium primulinum* Lindl. (*D. Primulinum* L.) is one of the important epiphytic orchid species for horticultural and commercial use. It is listed as a rare and critically endangered species of orchid. The present study is intended to conserve the orchid species through the micropropagation technique. The *in vitro* seed germination and seedling growth was carried out by taking a mature pod of *D. Primulinum* L. in Murashige and Skoog (MS) media, and the MS media supplemented with a varied concentration (0.5, 1.0, 1.5 and 2 mg/L) of 6-benzylaminopurine (BAP), and a fixed concentration (0.5 mg/L) of α-naphthalene acetic acid (NAA). Sequential phases of seed germination, protocorm formation, and seedling development in the presence of growth regulators were determined in the study. The significance of hormonal effects was determined by using One-Way Analysis of Variance (ANOVA, p≤0.05). The seed germination started after two weeks of culture in the media supplemented with BAP. The maximum seedling growth was obtained in the media supplemented with 1.5 mg/L of BAP. Although the hormone-free basal medium revealed an ideal condition for seed germination and spherules formation, the presence of an appropriate concentration of growth regulators such as 0.5 mg/L NAA or a combined 0.5 mg/L BAP + 0.5 mg/L NAA expressed a synergistic effect to enhance the protocorm formation and seedling development.**

Key words: *In vitro, Dendrobium primulinum*, 6-benzylaminopurine (BAP), α-Naphthalene acetic acid (NAA).

INTRODUCTION

Orchids have great ornamental, medicinal as well as edible values. Orchids are the most fascinating group of plants among the angiosperms. Beautiful flowers made them a treasured species among the ornamental plants. Orchids are especially vulnerable to illegal trafficking due to their beauty and popularity in wide applications, which led them to the verge of extinction. According to the Mongabay news series on Global Forests (Parker, 2018), nearly 30,000 species of orchids makeup roughly 10% of the earth's plant species, yet represent more than 70% of the species listed on the Convention on International Trade in Endangered Species (CITES).

In South-east Asia, especially in the lap of the Himalayas of Nepal, a total of 90 species of orchids have been recognized to have medicinal values (Pant and Rascoti, 2013). Hara et al. (1978) enumerated 26 species

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of orchids in Nepal at an elevation of 500 m to 2900 m. The genus Dendrobium comprises approximately 1500-2000 species distributed in the tropical and subtropical regions of Asia and North Australia (Hou et al., 2017). *Dendrobium primulinum* is one of the important Dendrobium species, which is commonly called Primrose yellow Dendrobium *Callista primulina*, or *Dendrobium nobile var pallidiflorum;* it is a beautiful species of the genus Dendrobium. It is relatively large-sized (25-45 cm in height), pendant epiphyte, clustered, and lanceolate to oval-shaped leaves found hanging on deciduous trees. Its flowering period starts in April. The fragrant flowers arise from the nodes along with the leafless cane. The species has been reported to have a natural habitat at an elevation of 1200 m -1400 m (Ghimire, 2008). Cheng et al. (2019) reported a comprehensive investigation of the diversity of Dendrobium species used in Traditional Chinese medicine, their supply and demand, as well as the development of Dendrobium industry.

Almost all orchids, including *D. primulinum*, are myco-heterotrophic at some points in their life cycle. Mycorrhizal fungi are required to germinate the orchid seed naturally via a symbiotic relationship. Therefore, orchid mycorrhizae play a crucial role during the orchid seed germination as the seed contains virtually no reserved energy. Hence, the germination rate of orchid seed in nature is only 2-5% (Rao, 1977). Germinating seeds via *in vitro* propagation is a breakthrough technology in orchid multiplication (Fay, 1994). In this method, seeds are cultured aseptically in a nutrient medium supplemented with simple carbon sources like sucrose (Tan et al., 1998). Currently, several studies have been made to optimize the seed germination and seedlings development of rare and endangered orchid species via *in vitro* technique and the effectiveness of the growth regulators. The growth regulators are chemical substances that influence the growth and differentiation of plant cells, tissues, and organs. Auxins, cytokinins, Gibberellins, and Abscisic acid (ABA) are well-known growth regulators used in tissue culture. Out of these, Auxin and cytokinins are often used together to obtain a synergistic effect for both root initiation and shoot bud formation during *in vitro* tissue culture from immature/matured seeds.

Hence, the present study is aimed to develop an efficient protocol for the *in vitro* propagation of *D. primulinum* species of orchid through mature seeds. Different concentrations of 6-benzylaminopurine (BAP), and α-Naphthalene acetic acid (NAA) growth regulators were implemented to investigate their effectiveness for a sequential growth and development of spherules, protocorms, shoot, and seedling.

MATERIALS AND METHODS

D. primulinum L. capsule (pod) containing mature seeds were taken from the natural habitat of the species in the Dolakha District of Nepal at an altitude of 1300 m. A matured capsule of *D. Primulinum* L. was used to study the *in vitro* propagation in this research. *In vitro* propagation by using a matured capsule is believed to take a relatively short time to germinate. The most appropriate MS medium was selected based on the time taken for seed germination and the number of seedlings formed. The MS medium is also known as the most common medium for plant tissue culture.

The capsule was sterilized by dipping in the detergent for 15-20 min and washing in running tap water for 1h until the water became clear and transparent. The capsule was then rinsed with 70% ethyl alcohol for 2 min and dipped in 1% sodium hypochlorite solution for 10 min. Finally, the capsule was rinsed again with distilled water for 5 min.

The basal medium for the tissue culture was chosen to be the modified Murashige and Skoog (MS) medium (Murashige and Skoog, 1962). Different concentrations of cytokinin (6-benzylaminopurine (BAP)) and a fixed concentration of Auxin (α-Naphthalene acetic acid (NAA)) as growth-regulating hormones were used to inoculate the seeds. The pH of the medium was adjusted to 5.8. The medium was then quasi-solidified with 0.8% w/v Difco Bacto Agar. About 20 ml of the prepared culture medium was taken into the culture tube, and the tube was tightly covered with Aluminium (Al) foil. The culture tubes containing medium were then autoclaved at 121°C under 15 lb/sq. inch pressure for 20 min. After cooling down, the culture tubes were taken out and kept in the culture room at a slanting position.

The sterilized capsule was then transferred to the pre-sterilized laminar airflow cabinet and dissected longitudinally into two halves using a sterile surgical blade (Figure 1). The seeds were then placed on the surface of MS medium and the MS medium containing different concentrations of growth regulators; BAP, and BAP + NAA, using sterile forceps. The cultures were incubated at 25±2°C with a photoperiod of 12-15 h. A regular observation was done to study seed germination and seedling development. The experiments were set-up in a completely randomized design. The significance of hormonal treatment effects was determined using One-Way Analysis of Variance (ANOVA, $p \leq 0.05$). A comparison between mean values was made by Tukey's test.

RESULTS AND DISCUSSION

The result showed that the initiation of seed germination and spherules formation was significantly ($P \leq 0.05$) observed after 2 weeks of culture in the MS basal medium without any hormonal supplements (Table 1). This observation was also supported by the studies carried out by Karki et al. (2005) in *Vanilla planifolia,* Pant and Gurung (2005) in *Aerides Odorata, and* Julkiflee et al. (2014) in *Dendrobium sonia-28* where the MS basal medium revealed its effectiveness in seed germination, growth rate percentage, as well as protocorm-like-bodies (PLBs) formation.

It is interesting to note that the use of hormonal supplements (BAP and NAA) in the MS medium caused a delay in spherules formation; especially, when they were used separately. The spherulation response was delayed by 2-3 weeks, depending on the concentration of additives, as compared to the MS basal medium. Spherules formation response was obtained in the medium containing a combination of 0.5 mg/L BAP and 0.5 mg/l NAA. However, the most effective germination response of *D. primulinum* L. (with the development of

Figure 1. Mature capsule of *D. primulinum* Lind. dissected longitudinally into two halves showing Yellowish tiny seeds.

Table 1. Effect of growth regulators on seed germination and seedling growth of orchid *D. primulinum* Lindl.

PGRs		Spherules formation (days)			Protocorm formation (days)			First shoot formation (days)		
BAP (mg/L)	NAA (mg/L)	Mean	SD	SE	Mean	SD	SE	Mean	SD	SE
0	0	17.5^{d}	2.08167	1.04083	45.5^{d}	1.91485	0.95743	108^{c}	2.70801	1.35401
0.5	0	17.5^{d}	1.73205	0.86603	45.5^{d}	1.29099	0.6455	118^{b}	2.94392	1.47196
1.0	0	38.5^{a}	2.38048	1.19024	66.5^{a}	2.38048	1.19024	118^{bc}	4.08248	2.04124
1.5	0	31.5^{b}	3.10913	1.55456	59.5^{ab}	4.50925	2.25462	137^{a}	4.08248	2.04124
2.0	0	38.5^{a}	2.38048	1.19024	66.5^{a}	2.64575	1.32288	137^{a}	2.94392	1.47196
0	0.5	24.5^{c}	1.73205	0.86603	52.5^{c}	1.91485	0.95743	113^{c}	3.91578	1.95789
0.5	0.5	24.5^{c}	1.0	0.5	52.5^{c}	0.57735	0.28868	118^{b}	2.16025	1.08012
1.0	0.5	31.5^{b}	2.38048	1.19024	59.5^{b}	1.91485	0.95743	118^{b}	1.41421	0.70711
1.5	0.5	31.5^{b}	0.57735	0.28868	59.5^{b}	1.29099	0.6455	118^{b}	2.16025	1.08012
2.0	0.5	38.5^{a}	3.10913	1.55456	66.5^{a}	1.73205	0.86603	118^{b}	1.41421	0.70711

Culture condition:-MS medium, 25 ± 2^{0}c, 137 days, 12-15h of photoperiod, 4 replicates were used in each combination. Means with different letters are significantly different at $p \leq 0.05$ as indicated by Tukey's test. Abbreviations: SD=Standard Deviation, SE=Standard error.

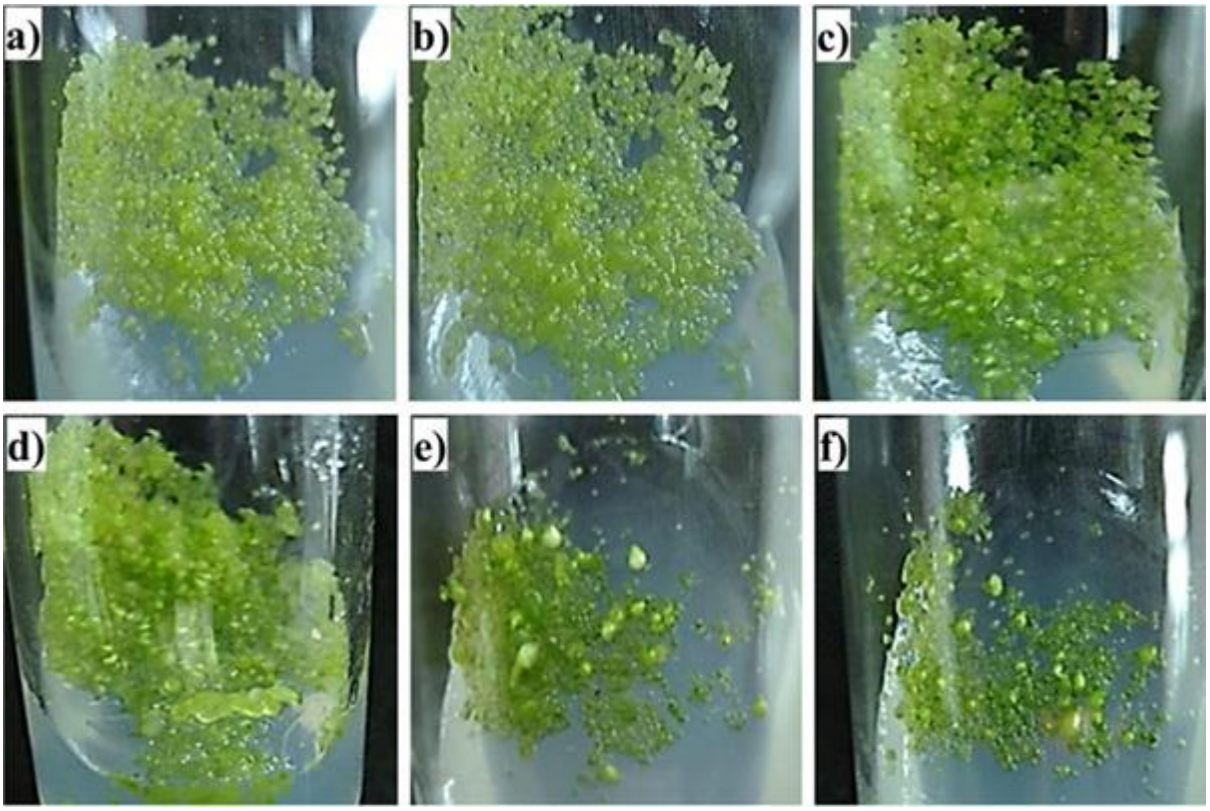

Figure 2. Spherules formation of *D. primulinum* Lindl. in (a) MS basal medium, and MS basal media containing; (b) 0.5 mg/L BAP, (c) 0.5 mg/L NAA, (d) 0.5 mg/L BAP+ 0.5 mg/L NAA, (e) 1.5 mg/L BAP, and (f) 1.5 mg/L BAP + 0.5 mg/L NAA hormonal supplements.

maximum numbers) was found with 1.5 mg/L BAP and a combination of 2 mg/L BAP + 0.5 mg/L NAA, as shown in Figure 2. On the other hand, the MS media supplemented with 1.0 and 2.0 mg/L of BAP or the combination of 2.0 mg/L BAP with 0.5 mg/L NAA have taken maximum weeks for spherules formation (Figure 3).

Protocorms were obtained after 6 weeks of culture in the MS basal medium while it took 7 weeks or more to observe protocorm formation in the BAP/NAA supplemented media depending on the concentration used (Figure 3). Similar findings were obtained by Basker and Narmatha Bai (2010) in the seed germination of *Eria bambusifolia* which took 7 weeks for protocorms formation while Pant et al. (2011) reported 9 weeks for *Phaius tancarvilleae*, and *Cymbidium eburneum* took 9 weeks for protocorms formation as reported by Gogoi et al. (2012). Figure 4 showed that protocorms produced in medium without or with different concentrations of hormonal supplements. The leafy structure of the protocorms has been observed in the MS basal medium without any hormonal supplements. On the other hand, by the addition of a small amount of BAP + NAA (0.5 mg/L each), the protocorms obtained show a significant enhancement to that of separately added nutrients, as shown in Figure 4a-d. The protocorms tend to transform into a well-differentiated shoot and leafy structure (Figure 4d). The proliferation of seedlings after subculture was observed in MS media supplemented with 1.5 mg/L BAP, where a maximum number of seedlings growth was observed.

Shoot initiation was observed in 16 weeks of culture (Table 1 and Figure 5). The quality, quantity, and nature of growth regulators have a foremost effect on the germination of seeds and regeneration capacity of the shoot. The maximum seedling growth was observed in 1.5 mg/L BAP (Figure 5c), and 0.5 mg/L BAP + 0.5 mg/L NAA (Figure 5d). The previous works of several researchers also showed that the high concentration of BAP and low concentration of NAA are suitable for shoot multiplication. Sunitibala and Kishor (2009) revealed the best response for shoot multiplication on the half-strength MS basal medium supplemented with 2.0 mg/L of BAP and 1.0 mg/L of NAA in the orchid *Dendrobium transparens* L. from axenic pseudobulb segments. Likewise, Talukder et al. (2003), and Jang et al. (2019) reported that the use of different concentrations of BAP and NAA had a significant effect on shoot proliferation of Dendrobium species. Furthermore, the combined use of BAP and NAA showed a better shoot proliferation instead of their single-use in their study. Dharma et al. (2013)

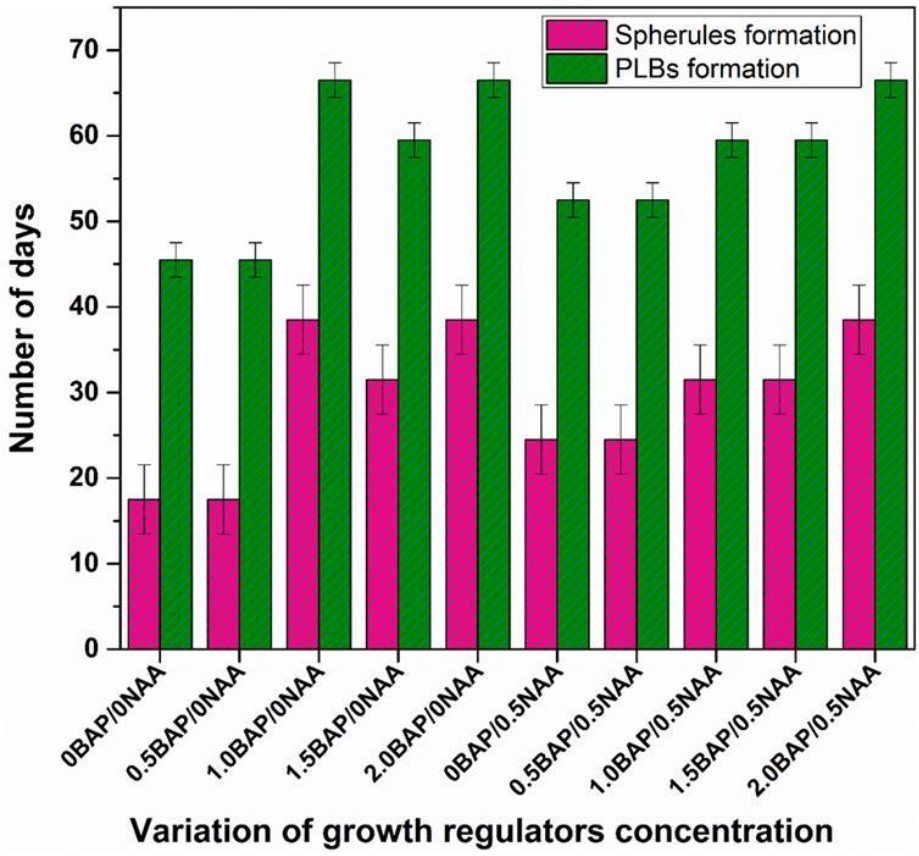

Figure 3. Time taken for spherules formation and initiation of protocorm like bodies (PLBs) with respect to different hormonal supplements.

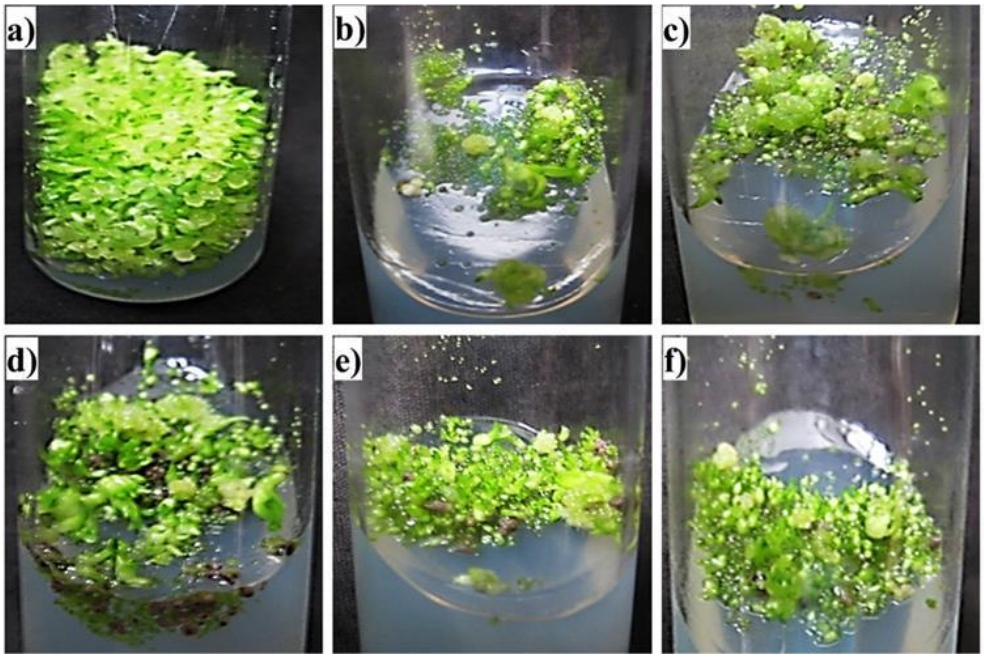

Figure 4. Protocorms formation of *D. primulinum* Lindl. in (a) MS basal medium, and MS basal media containing; (b) 0.5 mg/L BAP, c) 0.5 mg/L NAA, (d) 0.5 mg/L BAP+ 0.5 mg/L NAA, (e) 1.5 mg/L BAP, and (f) 1.5 mg/L BAP + 0.5 mg/L NAA hormonal supplements.

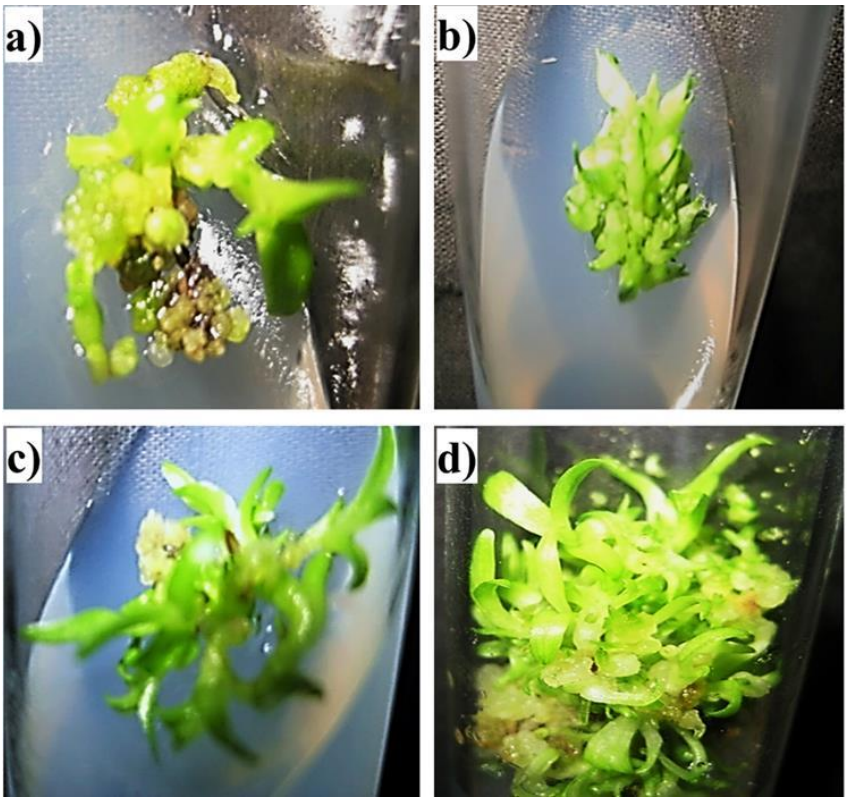

Figure 5. Callus induction and shoot initiation of *D. primulinum* Lindl. in different MS media; (a) MS basal medium, (b) 0.5 mg/L BAP, (c) 1.5 mg/L BAP and (d) 0.5 mg/L BAP + 0.5 mg/L NAA hormonal supplement media, respectively.

Table 2. Effect of BAP and NAA hormonal supplements on seedling development and shoots formation on *D. primulinum* Lindl.

BAP (mg/L)	NAA (mg/L)	Number of protocorms inoculated	Capability of seedling formation	Capabilities of seedling formation		
				Mean*	SD	SE
0	0	4	+++	3.75^{b}	0.95743	0.47871
0.5	0	4	++	3.0^{c}	0.8165	0.40825
1.0	0	4	++	2.75^{cd}	1.25831	0.62915
1.5	0	4	++++	4.5^{a}	1.29099	0.6455
2.0	0	4	++	3.0^{c}	0.8165	0.40825
0	0.5	4	++	2.75^{cd}	1.70783	0.85391
0.5	0.5	4	+++	3.0^{c}	1.41421	0.70711
1.0	0.5	4	+	2.25^{e}	0.5	0.25
1.5	0.5	4	++	2.75^{cd}	0.5	0.25
2.0	0.5	4	+	2.5^{d}	0.57735	0.28868

reported similar findings on *Coelogyne fuscescens* Lindl, where shoot initiation was obtained in 13 weeks of culture.

The product of orchid seedling from the seed involves sequential phases of germination. First, spherules are formed, followed by protocorms and seedling development. In the present investigation, the same sequence of seedling development was observed with the improved growth rate percentages in hormonal supplemented media. Effect of BAP and NAA on seedling development and shoots formation capability on *D. primulinum Lindl* is presented in Table 2. It clearly shows that the addition of 1.5 mg/L of BAP revealed a better capability for shoot formation as compared to the hormonal free medium and all other combinations.

Nevertheless, hormonal free basal medium and the medium containing a combined low concentration of BAP + NAA (0.5 mg/L each) have also shown strong capability for shoot formation. In comparison to the germination of other angiosperm seeds, the germination of orchid seed is difficult because orchid seed lacks endosperm, radical, and leaf rudiments. In nature, it requires mycorrhizal symbiosis for the germination and takes a very long time. So, tissue culture technique is the reliable process for mass propagation and conservation of endangered orchid species like *D. primulinum* Lindl. There is a significant influence of several factors like nutrients media, quality and quantity of plant growth regulators, age of seeds, and organic carbon source in the *in vitro* seed germination of orchids (Pongener and Deb, 2010).

In the present investigation, the combined effect of BAP and NAA has proved to be beneficial for seed germination and shoot development. The lower concentration of BAP and NAA showed fast germination of seeds to form spherules, protocorm, and first shoot formation, whereas the higher concentration took a longer time. The hormonal condition well favored the seed germination, and the fast spherules formation was obtained in 0.5 mg/l BAP, which took only 2 weeks after inoculation of seed. However, the MS basal medium was also found to be the suitable media for spherules formation.

This result was interesting in first shoot formation where media supplemented with 0.5-2.0 mg/L BAP + 0.5 mg/L NAA showed fast growth of shoots. The spherules formation, protocorm formation as well as growth and development of seedlings vary with the species and medium employed (Reddy et al., 1992; Basker and Narmatha Bai, 2010).

Conclusion

D. primulinum Lindl. is one of the most beautiful and endangered orchids, highly exploited for ornamental purposes and medicinal value. The phytohormones play a vital role in seed germination, and seedling growth of *D. primulinum* L. MS medium containing a combined 0.5 mg/l BAP and 0.5 mg/l NAA revealed that it had an effect on spherules, protocorm as well as first shoot formation. On the other hand, the most effective media for shoot formation was the one containing 1.5 mg/L BAP concentration based on the number of seedlings formed. Similarly, the media containing 0.5 mg/L BAP, and MS free basal medium were also found to be effective in terms of shoot multiplication. The present investigation was carried out for the conservation of rare and endangered orchid species by mass propagation. Hence, this *in vitro* seed germination technique by using hormonal supplement might be beneficial for the *ex-situ* conservation of this orchid species and fulfilling its commercial demand.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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