Advances in Forestry Science
Short Communication

In vitro seed germination and seedling development of *Coelogyne flaccida* Lindl. (Orchidaceae)

Gaurav Parmar[1*] Bijaya Pant[2]

[1] National Herbarium and Plant Laboratories, Godawari, Lalitpur, Nepal.
[2] Plant Biotechnology and Plant Biochemistry Unit, Central Department of Botany, Tribhuvan University, Kirtipur, Nepal.

[*] Author for correspondence: gaurav_mascot4u@yahoo.com
Received: 22 July 2015 / Accepted: 29 August 2015 / Published: 19 December 2015

Abstract
Coelogyne flaccida Lindl., an epiphytic orchid native to Nepal, has high ornamental and medicinal values and is found at elevations ranging from 900 to 1100 m. In this work *in vitro* seed germination and seedling development of this orchid was carried out on 0.8% (w/v) agar solidified Murashige and Skoog (MS) medium supplemented with different combinations of α-Naphthalene acetic acid (NAA) and 6-Benzylaminopurine (BAP). MS medium supplemented with 0.5 mg/L BAP and 0.5 mg/L NAA was found to be the ideal condition for the complete development of the seedlings. The germination started after six weeks of culture and developed seedlings were obtained after 22 weeks of culture on the medium supplemented with 0.5 mg/L BAP and 0.5 mg/L NAA. In the medium without hormone application, germination started after five weeks, but roots were not developed even after 32 weeks of culture, suggesting the usefulness of NAA in root induction. The present study has provided useful information that both phytohormones, BAP and NAA are necessary for the fast growth and development of the *in vitro* grown seedlings.
Key words: Multiplication; Micropropagation; MS culture medium; BAP; NAA.

Introduction

Nepal harbours 451 species of orchids belonging to 107 genera (Rajbhandari 2015). Orchids as a whole are cited under Appendix II of CITES except *Paphiopedilum insigne* and *Paphiopedilum venustum* in Nepal (DPR 2012). They are important aesthetically, medicinally and also regarded as ecological indicator (Joshi et al. 2009). They are very popular worldwide due to their various shapes, sizes, habits, habitats, colorful flowers, long lasting bloom, shinning green leaves and variously shaped pseudobulbs. A total of 90 species of orchids of Nepal have been reported to have medicinal value by Pant and Raskoti (2013). Whole plants as well as their different parts, viz., roots, rhizomes, pseudobulbs, stems and leaves are used as medicinal products. These are used for treatment of different diseases such as general debility, stomachache, bone fractures, colds, wound healing, general weakness and to cure various other diseases (Subedi et al. 2013; Pant and Raskoti 2013).

Coelogyne flaccida Lindl., commonly known as 'The Loose Coelogyne Pseudobulbs' are epiphyte on tree trunks at elevations of 900-1,100 m (Raskoti 2009; Rajbhandari 2015). It has high aesthetic value so is used as an ornamental plant in different gardens, nurseries, and hotels. Its medicinal value resides on the paste of its pseudobulb, which is used for headache treatment; and the juice is convenient for indigestion relief (Manandhar 2002). Its high market price in the national and international markets has led to its indiscriminate harvesting from its natural habitat, restricting natural populations to narrow areas.

Orchid seeds lack functional endosperm so the germination of seeds requires aiding a suitable fungus. The germination rate of orchid seeds in nature is only 2-5% (Rao 1977) even if they do so, seeds take a long time for their germination and any disturbance in the habitat or physical environment destroys the whole population. Also, the seedlings take 12 years to become an adult plant (Basker and Narmatha Bai 2006). Vegetative propagation of orchids through division of clumps of rhizomes, bulbs or by the rooting of off-shoots is slow and difficult to obtain a desired number of orchids. As the species is severely threatened to extinction, strategies for its conservation and sustainable management are of prime interest. *Ex situ* conservation strategies available comprise vegetative propagation through several approaches. It seems one of the best strategies for this orchid might be the tissue culture, in order to proceed with large scale propagation for both the market and the conservation of the species itself.

This orchid species is rapidly vanishing from its natural habitat due to its reckless collection, illegal trade, deforestation, habitat destruction and high market price in the national and international markets. Nevertheless, no tissue culture protocol for this orchid has been developed using its immature seed. So, from both conservation and commercial point of view it is imperative to develop *in vitro* mass propagation of this orchid using standard method.

Therefore, the present study was undertaken to develop an efficient protocol for *in vitro* micropropagation of *C. flaccida* through immature seeds.

Material and methods

For this study, eight weeks old immature capsules of *Coelogyne flaccida* collected from the orchid house of National Botanical Garden, Godawari, Lalitpur were used in the research.

The capsule of *C. flaccida* was sterilized by washing under running tap water added by 2-3 drops of tween 20 solution for 50 minutes until the water became totally clear. The capsule was then rinsed in 70% ethyl alcohol for two minutes and 1% solution of sodium hypochlorite for 10 minutes. Finally, it was rinsed with sterile water for five times.

Murashige and Skoog (1962) medium was used in similar conditions as the original, and added by different combinations of 6-Benzylaminopurine and α-Naphthalene acetic acid (Table 1). The medium was supplemented with 3% sucrose. The pH of the medium was adjusted to 5.8 before autoclaving and solidified with 0.8% (w/v) agar. The medium was autoclaved at 121°C for 15 minutes.

The sterilized capsule was then dissected longitudinally using sterile surgical blade inside pre-sterilized laminar air flow cabinet. The seeds were then inoculated on the surface of MS medium alone and in different combinations of BAP and NAA using sterile forceps. The cultures were incubated at 25±2°C under photoperiod of 16/8 hours light/dark cycle.

Results and discussion

The germination of seeds and growth and development of seedlings were markedly influenced by the presence of growth regulators in the medium. After inoculation of seeds, different medium combinations gave different response and at different time. Seedling development showed different developmental stages, viz., seed germination, protocorm formation, shoot formation and root formation. Seed germination started after five weeks of inoculation on hormone free MS medium and by the eighth weeks, it was observed on MS medium supplemented with 1.5 mg/L BAP, the last medium combination to show seed germination. Similarly, protocorms started to develop after seven weeks of culture in seven different combinations simultaneously. And, again the MS medium supplemented with 1.5 mg/L BAP was the last medium combination to develop protocorms, observed after 11 weeks.

The first shoot was observed simultaneously on the hormone free MS medium, MS medium supplemented with 1 mg/L NAA, and MS medium supplemented with 1 mg/L BAP and 1 mg/L NAA after nine weeks of culture. But, the first root development was observed on MS medium supplemented with 0.5 mg/L BAP and 0.5 mg/L NAA after 22 weeks of culture followed simultaneously by MS medium supplemented with 1 mg/L BAP and 1 mg/L NAA, and MS medium supplemented with 0.5 mg/L BAP and 1 mg/L NAA after 23 weeks (Figs. 1A-F). The medium combination, MS medium supplemented with 0.5 mg/L BAP and 0.5 mg/L NAA, which produced the first well developed seedling, was regarded as the best combination. The result of research is presented in the Table 1.

Table 1. Effect of plant growth regulators supplemented to MS medium on seed germination and seedling growth of *Coelogyne flaccida* Lindl.

BAP (mg/L)	NAA (mg/L)	Observation taken in weeks[(1)]			
		IG	PF	SF	RF
–	–	5	7	9	–
0.5	–	6	7	10	–
1.0	–	7	10	15	–
1.5	–	8	11	14	–
2.0	–	7	9	14	–
–	0.5	7	9	13	–
0.5	0.5	6	8	10	22
1.0	0.5	6	7	10	24
1.5	0.5	7	8	11	–
2.0	0.5	7	8	11	–
–	1.0	6	7	9	24
0.5	1.0	7	8	10	23
1.0	1.0	6	7	9	23
1.5	1.0	6	7	10	25
2.0	1.0	6	7	10	25

[(1)] Culture conditions: 25± 2°C, 32 weeks, 16 hours photoperiod and 6 replicates were used in each combination. IG = Initiation of germination, PF = Protocorm formation, SF = 1st shoot formation, RF = 1st root formation.

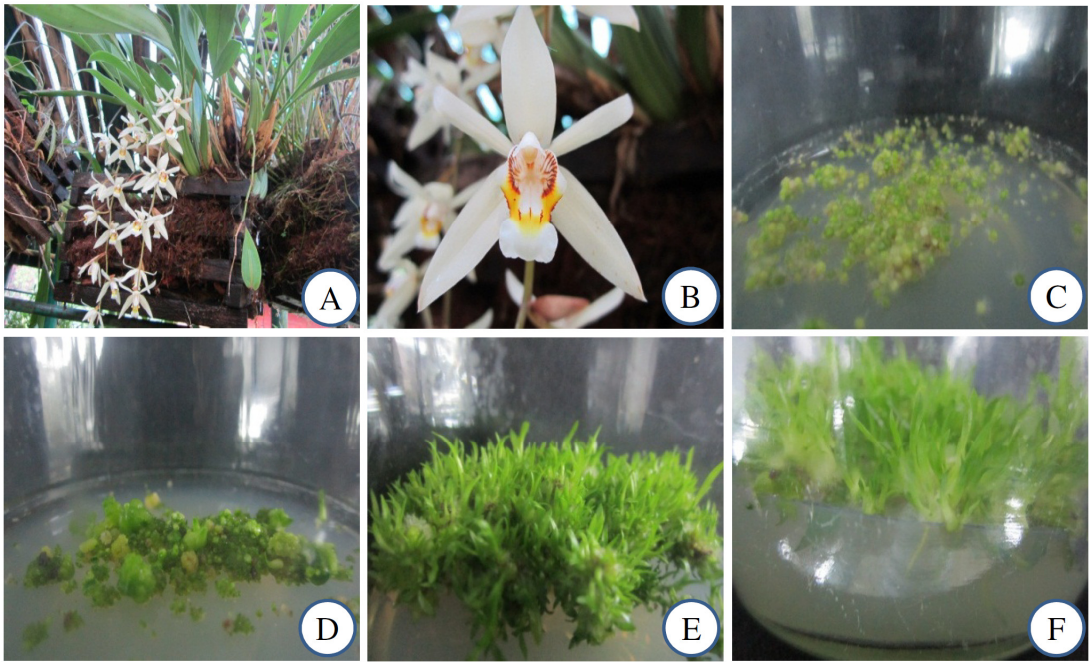

Figure 1. *In vitro* culture of *Coelogyne flaccida* Lindl. (A) Plant of with immature capsules and flowers; (B) A flower; (C) Formation of protocorms on hormone-free MS medium after 7 weeks of culture; (D) Swelling of protocorms and initiation of shoots on MS medium supplemented with 0.5 mg/L BAP and 0.5 mg/L NAA after 10 weeks of culture; (E) Shoots on MS medium supplemented with 1 mg/L BAP and 1 mg/L NAA after 15 weeks of culture; (F) Development of root on MS medium supplemented with 0.5 mg/L BAP and 1 mg/L NAA after 24 weeks of *in vitro* culture.

Immature capsules were selected for this research as it shows better germination response and saves time (Pant 2006). The effective germination response for *C. flaccida* was found to be on MS medium supplemented with BAP (0.5 mg/L) and NAA (0.5 mg/L). The quantity and nature of plant growth regulators have significant effect on the germination of orchid seeds (Arditti 1992). The most appropriate medium was selected on the basis of time taken for germination of seeds and their growth and development. Initiation of seed germination was observed after five weeks of culture.

This was supported by the findings of Reddy et al. (1992), who studied the seed germination and seedling growth in four different species of orchids (*Cymbidium aloifolium*, *Dendrobium crepidatum*, *Epidendrum radicans* and *Spathoglottis plicata*). It was also supported by the findings of Hoshi et al. (1994) on the seed germination of four species of *Cypripedium* and Pradhan and Pant (2009) on *Dendrobium densiflorum*.

Protocorms were obtained after seven weeks of culture. It was supported by the finding of Basker and Narmatha Bai (2010) in the seed germination of *Eria bambusifolia* which

took seven weeks for protocorms formation. Similar findings were reported by Nongdam and Chongtham (2011) on *Cymbidium aloifolium* which took five to six weeks and Pradhan and Pant (2009) on *Dendrobium densiflorum* which took six weeks for protocorm formation.

The first shoot initial was obtained after nine weeks of culture. This was supported by the similar findings of Pradhan and Pant (2009) on *Dendrobium densiflorum* which took eight weeks for the first shoot formation. The first root initial was obtained after 22 weeks of culture. Pant et al. (2011) in the seed germination of *Phaius tancarvilleae* found 18 weeks needed for the first root formation. Similarly, Pradhan and Pant (2009) on *Dendrobium densiflorum* took 19 weeks for the root initiation.

Complete plantlet of *C. flaccida* was obtained after 22 weeks of culture. This was supported by the findings of Pant et al. (2011) on *Phaius tancarvilleae* which took 24 weeks to develop into complete plantlets and Paudel et al. (2012) on *Esmeralda clarkei* which took 25 weeks.

Phytohormone NAA was found to be indispensable for root initiation as the medium combination lacking NAA did not develop root even after 32 weeks of culture (as given in Table 1). It may be due to genetic constitution of explants and the endogenous growth regulators present in them. According to Yam et al. (1989), the nutritional requirements of germinating orchid seeds vary with their physiological state and this may be species specific and according to Arditti and Ernst (1984), the nutrient requirement of orchid seeds in terms of quantity as well as form may vary at different stages of development for various species. This also revealed that the addition of root hormone NAA might be vital in the nutrient medium for the successful growth and development of roots.

This study revealed that though hormone free MS medium to be efficient for the fast seed germination, protocorm formation and shoot development, but it could not develop root even after 32 weeks of culture suggesting that root hormone NAA to be essential for the root development of this orchid. Thus, the MS medium supplemented with BAP (0.5 mg/L) and NAA (0.5 mg/L) was found to be the optimum combination for *in vitro* seedlings growth and development of *C. flaccida*. Therefore, the developed protocol will significantly contribute to the mass propagation, conservation as well meet the commercial demand of this orchid.

MS medium supplemented with BAP (0.5 mg/L) and NAA (0.5 mg/L) was found to be the best for *in vitro* seed germination and seedlings growth and development of *Coelogyne flaccida*, suggesting that the phytohormones - BAP and NAA - both are necessary for its fast growth and development. Phytohormone NAA was found to be indispensable for root initiation as the medium combination lacking NAA did not develop root even after 32 weeks of culture. There was no occurrence of intermediate callus in any tested media suggesting the possible homogeneity of the *in vitro* propagated seedlings.

Acknowledgements

The authors would like to express sincere gratitude to Prof. Dr. Pramod K. Jha, Head, Central Department of Botany, Tribhuvan University, Kirtipur, Nepal and Dr. Sushim Ranjan Baral, former Chief, National Herbarium and Plant Laboratories, Godawari, Nepal for arranging and providing necessary laboratory facilities to accomplish this research. We would also like to thank National Botanical Garden, Godawari, Lalitpur, Nepal for providing the orchid capsules.

References

Arditti J (1992) *Fundamentals of orchid biology*. New York: John Wiley & Sons. 704p.

Arditti J, Ernst R (1984) Physiology of orchid seed germination. In: Arditti J (ed) *Orchid biology*: reviews and perspectives. v.3. New York: Cornell University Press. p.177-222.

Basker S, Narmatha Bai V (2006) Micropropagation of *Coelogyne stricta* (D. Don) Schltr. via pseudobulb segment cultures. *Tropical and Subtropical Agroecosystems*, 6(1):31-35.

Basker S, Narmatha Bai V (2010) *In vitro* propagation of an epiphytic and rare orchid *Eria bambusifolia* Lindl. *Research in Biotechnology*, 1(1):15-20.

DPR. Department of Plant Resources (2012) *Plants of Nepal*: fact sheet. Nepal: Kathmandu. 15p.

Hoshi Y, Kondo K, Hamatani S (1994) *In vitro* seed germination of four Asiatic taxa and notes on the nodal micropropagation of American *Cypripedium montanum*. *Landline*, 9:93-97.

Joshi GC, Tewari LM, Lohani N, Upreti K, Jalal JS, Tewari G (2009) Diversity of orchids in Uttarakhand and their conservation strategy with special reference to their medicinal importance. *Report and Opinion*, 1(3):47-52.

Manandhar NP (2002) *Plants and people of Nepal*. Oregon, USA: Timber Press, Portland. 599p.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiologia Plantarum*, 15(3):473-497. doi: 10.1111/j.1399-3054.1962.tb08052.x

Nongdam P, Chongtham N (2011) *In vitro* rapid propagation of *Cymbidium aloifolium* (L.) Sw.: a medicinally important orchid via seed culture. *Journal of Biological Sciences*, 11(3):254-260. doi: 10.3923/jbs.2011.254.260

Pant B (2006) Biotechnology in orchid conservation. In: Karmacharya SB, Dhakal MR, Jha SN, Mandal TN, Chettri MK, Subba BR, Koirala U, Niroula B, Limbu KP (eds) *Natural resource management*. Nepal: Biratnagar. p.221-224.

Pant B, Raskoti BB (2013) *Medicinal orchids of Nepal*. Kathmandu, Nepal: Himalayan Map House (P.) Ltd. 104p.

Pant B, Shrestha S, Pradhan S (2011) *In vitro* seed germination and seedling development of *Phaius tancarvilleae* (L'Her.) Blume. *Scientific World*, 9(9):50-52. doi: 10.3126/sw.v9i9.5518

Paudel M, Pradhan S, Pant B (2012) *In vitro* seed germination and seedling development of *Esmeralda clarkei* Rchb.f. (Orchidaceae). *Plant Tissue Culture and Biotechnology*, 22(2):107-111. doi: 10.3329/ptcb.v22i2.14197

Pradhan S, Pant B (2009) *In vitro* seed germination in *Cymbidium elegans* Lindl. and *Dendrobium densiflorum* Lindl. ex Wall. (*Orchidaceae*). *Botanica Orientalis: Journal of Plant Science*, 6:100-102. doi: 10.3126/botor.v6i0.2917

Rajbhandari KR (2015) *A handbook of the orchids of Nepal*. Thapathali, Kathmandu, Nepal: Department of Plant Resources. 168p.

Rao AN (1977) Tissue culture in the orchid industry. In: Reinert J, Bajaj YPS (eds) *Applied and fundamental aspects of plant cell, tissue and organ culture*. Berlin: Springer Verlag. p.44-69.

Raskoti BB (2009) *The orchids of Nepal*. Nepal: Bhakta Bahadur Raskoti and Rita Ale Publication. 252p.

Reddy PV, Nanjan K, Shanmugavelu KG (1992) *In vitro* studies in tropical orchids: seed germination and seedling growth. *Journal of the Orchid Society of India*, 6(1/2):75-78.

Subedi A, Kunwar B, Choi Y, Dai Y, Andel TV, Chaudhary RP, Boer HJ, Gravendeel B (2013) Collection and trade of wild-harvested orchids in Nepal. *Journal of Ethnobiology and Ethnomedicine*, 9:64. doi: 10.1186/1746-4269-9-64

Yam TW, Arditti J, Weatherhead MA (1989) The use of darkening agents in seed germination and tissue culture media for orchids: a review. *Journal of the Orchid Society of India*, 3(1/2):35-39.