Journal of Nepal Biotechnology Association, 5 (1), 2024: 1-7 ISSN: 1728-1628
DOI: https://doi.org/10.3126/jnba/v5i1/63739

Research Article

Ex-situ Conservation of *Bulbophyllum leopardinum*, A Threatened Medicinal Orchid of Nepal

Bir Bahadur Thapa[1], Krishna Chand[1], Laxmi Sen Thakuri[1], Manju Kanu Baniya[2], Bijaya Pant[1,2]

[1] Annapurna Research Center, Maitighar, Kathmandu, Nepal
[2] Central Department of Botany, Tribhuvan University, Kirtipur, Kathmandu, Nepal

ARTICLE INFO

Submission: 10/06/2023
Acceptance: 15/12/2023
Published: 15/03/2024

CORRESPONDENCE

Bijaya Pant

Central Department of Botany, Tribhuvan University, Kirtipur, Kathmandu, Nepal

Email: b.pant@cdbtu.edu.np

0000-0001-5614-6031

Abstract

A successful micropropagation method was developed via the in-vitro seed germination and seedling growth of the epiphytic and/or lithophytic orchid *Bulbophyllum leopardinum*, a species having horticultural and therapeutic significance. To enhance seed germination, several quantities and combinations of naphthalene acetic acid (NAA), 6-benzyl amino purine (BAP), indole acetic acid (IAA), gibberellic acid (GA_3), and coconut water (CW) were added to 0.8% (w/v) agar-solidified MS medium. Half-strength MS medium has been experimented with alone and in combination with BAP, Kinetin (Kn), and GA_3 to promote shoot development. In-vitro-developed healthy shoots were chosen to establish roots in a half-strength MS (HMS) medium supplemented with various auxins. The best and earliest seed germination with the greenest protocorms (96.3±0.5% in 7 weeks) was achieved on HMS medium fortified with 15% CW (H15C). Further tests for the shoot as well as root development were continued with an H15C medium. H15C with 1 mg/l BAP and 1.5 mg/l Kinetin was most effective for early in vitro development and differentiation into seedlings with the many long shoots (9.3±0.1 shoots and 2.4±0.1 cm per culture) within 12 weeks of sub-culture. The most suitable rooting hormone was 1 mg/l NAA (4.2±0.26 roots per culture). This medium also produced the longest roots (1.9±0.09 cm per culture). By successfully developing a protocol for the mass propagation of *B. leopardinum*, this research has enhanced both the cultivation and the commercialization potential of this species.

Keywords: *Bulbophyllum leopardinum*, Micropropagation, PGRs, Seed germination, Shoot proliferation

Introduction

Orchidaceae, known as the "pandas of the plant world", is the second-largest family in the world and includes more than 28,000 species (Christenhusz & Byng, 2016) and 6–11% of all seed plants (Pillon & Chase, 2007). Despite being widespread, orchids are among the most fragile plants in nature (Fay, 2018). The Orchidaceae family has a significant economic impact because of its exotic beauty and long-lasting flowers, and they are especially valued in the horticulture industry. Their importance is also being acknowledged more and more in the pharmaceutical and fragrance sectors (Pant, 2013).

The tiny orchid seeds, which resemble dust, contain a tiny embryo that is encased in a single layer of protective cells. Its life cycle is complicated since it has little to no food stores. It requires at least a fungal acquaintance for germination and certain pollinators for pollination (Selosse, 2014). Because they are the most advanced blooming plants, orchids are extremely site-specific and require ideal circumstances to survive in any habitat. It makes sense that every orchid species is listed in CITES Appendix I and II.

The orchid mycorrhizal fungus (OMF) is essential for germination in nature (Pant et al., 2017; Shah et al., 2019a; Shah et al. 2019b). In contrast, an in vitro microenvironment can construct where asymbiotic orchid seed germination may occur (Knudson, 1951; Li et. al., 2018). Tissue culture is a cutting-edge method for growing plants on a large scale and may be used as a substitute source for naturally threatened species like orchids. However, it is difficult to design the procedure and methodology for mass propagation using tissue culture techniques.

The wet temperate forests of the eastern Himalayas, which lie in the nations of India, Nepal, Bhutan, Sikkim, Myanmar, and Thailand, are the natural habitat of *Bulbophyllum leopardinum* (Wall.) Lindl. It is an epiphyte and/or lithophyte, has one sharp deep-green leaf and an ovoid pseudobulb, and flourishes at elevations of 1,300-3,300 meters (eFloras, 2008). The popular name for this sympodial orchid, leopard-spotted orchid, comes from its light green blooms with red dots (Figure 1).

Figure 1: *Bulbophyllum leopardinum* in nature. **(a)** Epiphytic habitat, **(b)** Flower close-up, **(c)** Capsules close-up.

B. leopardinum is classified as a population of Least Concern by the IUCN Red List, but the ongoing decline in population and fragmentation brought on by rising rates of urbanization as well as the unauthorized and unrestrained felling, logging, and wood harvesting of its terrestrial host plants pose a threat (Cockel, 2013). Both people and animals eat this species, which is also used as herbal medicine, compost, and cow bedding (Teoh, 2016; Rajbhandari & Bhattarai, 2001; Pant & Raskoti, 2013). The present investigation has initiated the ex-situ conservation of this species by developing an efficient micropropagation protocol.

Materials and Methods

Plant material and aseptic sowing

Immature and non-dehisced pods (Figure 1c) were collected from Godawari, Lalitpur, Nepal located at 2,000 m above sea level. Collected pods were surface-sterilized by tap rinsing for 30 min, and washed with 90% ethanol for 50 sec and 1% sodium hypochlorite for 15 min before a final wash in sterile distilled water. The sterilized pods were longitudinally opened (Figure 2a) with a sterilized surgical blade and seeds were scooped out with a sterilized spatula. The seeds were spread over the surface of the medium supplemented with or without BAP and NAA or CW in culture tubes of 20 mL (Borosil Glass Works Ltd., India).

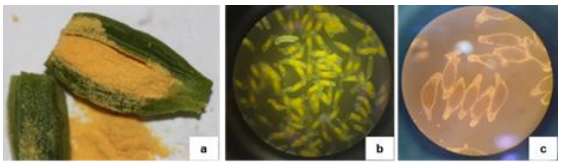

Figure 2: Microscopic seeds of *Bulbophyllum leopardinum*. **(a)** Longitudinally sectioned capsule, **(b)** Seeds under X80 of an inverted microscope exhibiting golden colour, **(c)** Seeds under X200 of an inverted microscope showing dark embryo at the centre.

Culture medium and culture conditions

MS medium of three strengths; full (FMS), half (HMS) and a quarter (QMS) were used with 3% (w/v) sucrose as a carbon source and solidified with 0.8% agar. The pH was adjusted to 5.7 in the final prepared medium, and the medium was autoclaved at 121 ˚C for 20 min at 1.05 Kg cm^2. The cultures were maintained at 25 ± 2 ˚C under a 16/8 h photoperiod (light/dark) provided by Philips white fluorescent lamps of 3,000-4,000 lux intensity.

Seed viability

A suspension of seeds in 200 µl of distilled water was observed in an inverted microscope (Olympus) under X80 magnification and seeds with and without embryos were counted. Seeds with embryos were

counted as viable while those without them were considered unviable. Ten repetitions of this microscopic field reading were averaged for the final analysis.

Seed germination and protocorm development

Different strengths of MS medium supplemented with or without NAA and BAP (0, 0.5, 1, 1.5 and 2 mg/ml) and coconut water (100 and 150 ml/l) as growth additives were used for in-vitro seed germination. About 100 g of seeds were spread over the surface of each medium. Eighteen treatments, each with six replicates, were arranged in a completely randomized design (CRD).

Shoot development

For shoot multiplication, HMS medium with 15% CW (H15C) as a basal medium was fortified with cytokinins (BAP, Kn and GA_3 at concentrations of 0.5, 1, 1.5 and 2 mg/l) alone or in combination with NAA (0.25, 0.50, 0.75 and 1.0 mg/l). Shoot development data were collected every four weeks. The 17 treatments each with six independent replicates were arranged in CRD.

Root development

Similarly, for root proliferation, HMS medium as a basal medium was fortified with auxins (NAA, IBA and IAA at the concentrations of 0.5, 1 and 1.5 mg/ml). Root development data were collected every four weeks until the 12 weeks. There were ten treatments, each with six replicates, arranged in CRD.

Statistical analysis

The average percentage of seeds that germinated was calculated for in-vitro seed germination. The data for shooting and rooting were displayed as the average of their corresponding lengths and numbers with a standard error.

Results and Discussion

Seed viability

Seeds were counted at X80 magnification under an inverted microscope. After averaging the counts of six microscopic fields, it was found that 96.04±0.33% of seeds had embryos and were therefore viable (Figure 2b).

Asymbiotic seed germination

Many researchers have used immature capsules for ease, effectiveness and speed (Pant et al., 2018; Thokchom et al., 2017; Pradhan et al. 2016; De et al., 2013). Organic supplements have been used to stimulate the growth and development and promote the shoot regeneration of various orchid species by Aktar et al., 2008 (*Dendrobium*), Kaur & Bhutani, 2012 (*Cymbidium pendulum*), Obsuwan & Thepsithar, 2014, and Huh et al., 2016 (*Cypripedium macranthos*).

The percentage of germinated seeds in various strengths of MS medium (FMS, HMS and QMS) with the adjuncts BAP, NAA and CW in which the yellowish powdery seeds of *B. leopardinum* were inoculated was recorded weekly. The initial stages of germination are typical for most orchids (Arditti, 1977). The seeds started to germinate after five weeks of inoculation. The tiny yellowish seed metamorphosed into greenish spherules (Figure 3a) after seven weeks of germination. After nine weeks, the maximum number (>90%) of seeds had germinated, except for QMS which took a minimum of 13 weeks.

In this study, more than 96% of seeds were germinated in HMS medium supplemented with 15% CW (H15C) (Figure 4) and the least seeds were germinated in a QMS with 1 mg/L BAP (Q1B). After nine weeks, the photosynthetic leaves emerged from the protocorms in an FMS medium with 1 mg/L NAA (F1N) (Figure 3b).

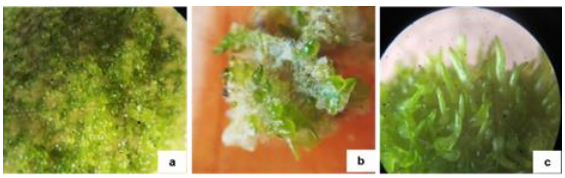

Figure 3: Diffèrent stages of *Bulbophyllum leopardinum* seeds germination. **(a)** Protocorms after 7 weeks of seeds inoculation, **(b)** Leaf primordia initiation after 9 weeks of seeds inoculation, **(c)** Shoot différentiation.

In many orchids like *Vanda pumila* (Maharjan et al., 2019), *B. nipondhii* (Pakum et al., 2016), *Bulbophyllum affine* (Maneerattanarungroj et al., 2013) and *Dendrobium nobile* (Asghar et al., 2011) introduced CW as a growth additive favoured both

germination and protocorm growth. In all these species, 150 ml/l of CW was found to be optimal. Although introducing 1 mg/l NAA to FMS resulted in the good growth and development of *B. leopardinum* plantlets (Figure 3c). In 150 ml/l CW-supplemented HMS medium, protocorm development was seen early and healthy. This result indicates that the growth-promoting effect observed in *B. leopardinum* is attributable to the supply of exogenous amino acids present in the HMS medium supplemented with CW. In contrast with our result where QMS failed to germinate for a long time (13 weeks), Lee & Yeung (2010) studied *Bulbophyllum fascinator* and observed desirable germination percentages (around 90%) among three different concentrations (1/2, 1/4 and 1/10) of MS medium indicating that seed germination of this species can adapt to a wild range of inorganic salt concentrations.

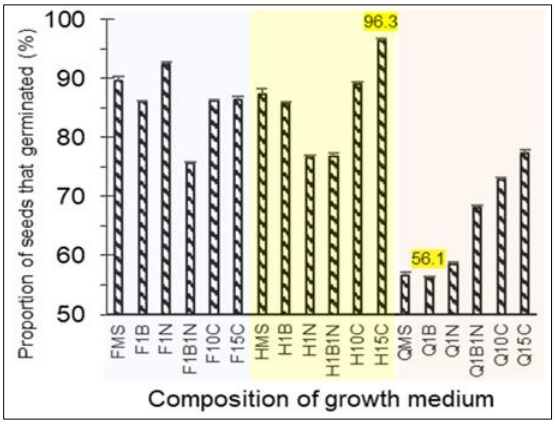

Figure 4: The percentage of seed germination in 7 weeks from inoculation.

Shoot proliferation from protocorms

H15C supplemented with GA_3 (0.5-2 mg/l) plus Kn (0.5-2 mg/l) promoted less number of shoot development than H15C supplemented with BAP (0.5-2 mg/l) after green protocorms and PLBs were transferred (Table 1). Roy & Banarjee (2001) observed that the addition of BAP to the HMS medium stimulated protocorm development and shoot bud initiation in *Geodorum densiflorum*. Watthana & Srimuang (2017) confirmed positive effect of CW along with potato extract and banana homogenate in shoot and root development.

The H15C medium with 1 mg/l BAP (H15C1B) induced protocorm multiplication and seedling growth in *B. leopardinum.* The greatest number (8.0±0.4) and longest shoots (1.8±0.3 cm) were observed (Figure 5a) in the H15C1B medium. Combined with 1.5 mg/l Kn (H15C1B1.5K), BAP induced even greater seedling development than on its own: on average there were 9.3±0.1 shoots and each was 2.4±0.1 cm long. In *Phaius tankervilleae*, MS medium supplemented with BAP stimulated protocorm formation and differentiation (Pant et al., 2011).

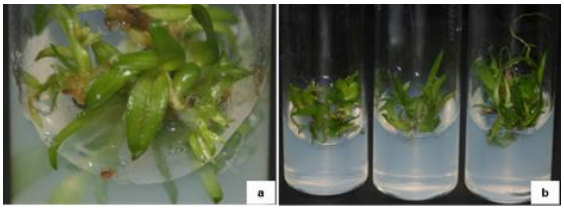

Figure 5: Shoot development in H15C1B0.75N, **(b)** Root development in H15C1N media (3rd one) compared with HMS (1st) and H15C (2nd) medium.

Root initiation and proliferation

Well-shooted plantlets were removed from the culture tubes and transferred to the rooting medium. In our study, 90% of the shoots were rooted well after being transferred to an H15C medium with or without auxins. The H15C medium supplemented with 1 mg/l NAA (Figure 5b) produced the most (4.2±0.26) and the longest (1.9±0.01 cm) roots. Rooting also took place in the two un-supplemented media but took longer than in the hormone-supplemented media.

To induce a good root system, multiple and fully developed shoot buds of *B. leopardinum* were transferred into different combinations of auxin-supplemented media. HMS medium fortified with different combinations of plant growth hormones (NAA, IAA and IBA at concentrations of 0.5 mg/l, 1 mg/l and 1.5 mg/l). HMS medium with 15% CW plus 1NAA (H15C1N) was found to be the most suitable combination for root proliferation where most (4.2 on average) and longest (1.9 cm on average) roots were found (Figure 6).

The roots of *B. leopardinum* were all thread-like; no large, thick and erect roots were observed during the research period. Mohammed et al. (2013) reported that HMS medium with auxin helps to increase the root systems of *B. lilacinum* and *Cymbidium aloifolium*. Gupta et al. (1998) and Bhadra et al. (2002) reported similar findings in *C. aloifolium* species.

Table 1: The effect of cytokinins (BAP, Kn & GA_3) with/without NAA in shoot proliferation of *Bulbophyllum leopardinum*.

Code	MS	Hormones (mg/L)				Observed after 12 weeks of subculture	
		BAP	Kn	GA3	NAA	Shoot No. ±S.E.	Shoot Length ±S.E.
H15C	H15C	0	0	0	0	4.8±0.8	1.1±0.1
H15C0.5B	H15C	0.5	0	0	0	2.4±0.1	1±0.2
H15C1B	H15C	1	0	0	0	8±0.2	1.8±0.1
H15C1.5B	H15C	1.5	0	0	0	3.3±0.3	1±0.1
H15C2B	H15C	2	0	0	0	3.6±0.1	0.8±0.2
H15C0.5K	H15C	0	0.5	0	0	3.5±0.6	1.1±0.3
H15C1K	H15C	0	1	0	0	4.5±0.3	1.3±0.2
H15C1.5K	H15C	0	1.5	0	0	3.4±0.4	1.6±0.2
H15C2K	H15C	0	2	0	0	3.3±0.2	1.5±0.2
H15C0.5G	H15C	0	0	0.5	0	5.1±0.1	1.3±0.3
H15C1G	H15C	0	0	1	0	3.8±0.1	1.7±0.2
H15C1.5G	H15C	0	0	1.5	0	6±0.1	1.4±0.1
H15C2G	H15C	0	0	2	0	6.4±0.3	1.3±0.3
H15C1B0.25N	H15C	1	0	0	0.25	5.2±0.2	1.4±0.2
H15C1B0.5N	H15C	1	0	0	0.5	5±0.1	1.8±0.1
H15C1B0.75N	**H15C**	**1**	**0**	**0**	**0.75**	**9.3±0.4**	**2.4±0.1**
H15C1B1N	H15C	1	0	0	1	4.9±0.5	1.8±0.5

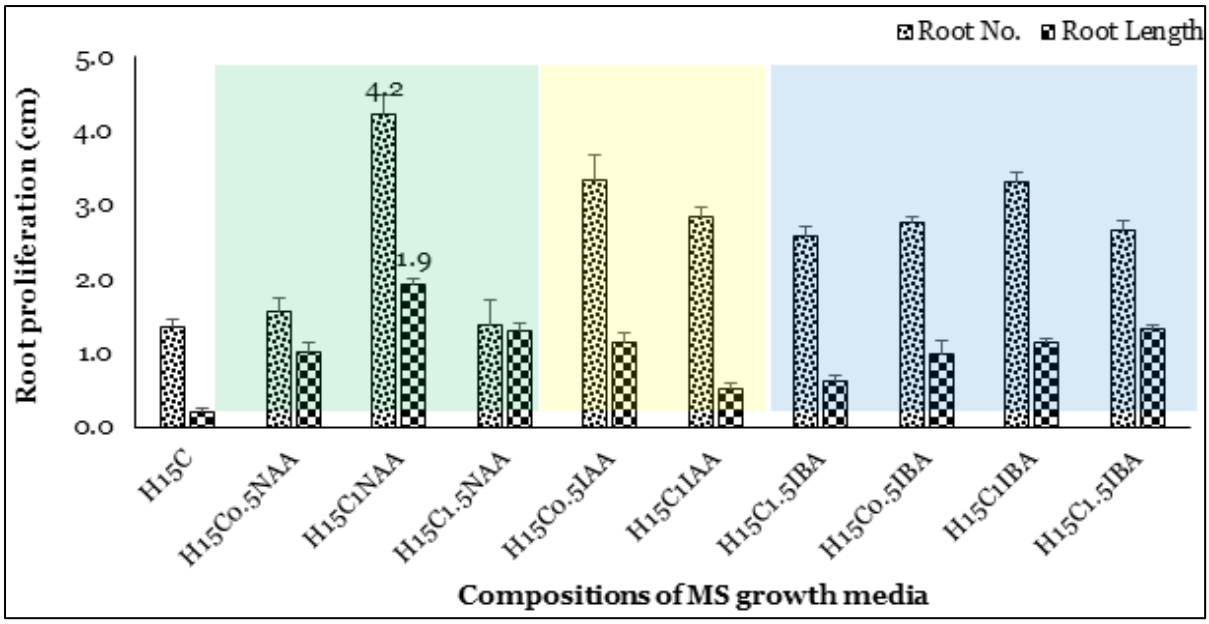

Figure 6: Root development in different root development media.

Conclusion

The results demonstrated that many cultures of the studied orchid can be produced in vitro from seeds using an asymbiotic in vitro seed germination. *B. leopardinum* (Wall.) Lindl. is a CITES-listed orchid, which has been placed on the IUCN Red List as having a decreasing and fragmented population because of the destruction and fragmentation of its habitat due, in part, to increasing urbanization. This multipurpose orchid is also traded illegally to use for compost, cattle-bedding, herbal medicine, animal feed, and food. To conserve and commercialize orchids like *B. leopardinum*, in vitro techniques of micropropagation can be a helpful tool. There is no

other information regarding the in vitro seed germination of *B. leopardinum* to our knowledge, so this investigation may provide a crucial alternative to the ex-situ conservation of the horticulturally and medicinally important *B. leopardinum* species.

Acknowledgements

The authors would like to thank the Taiwan Orchid Growers Association (TOGA) for providing financial support. We are also thankful to The Annapurna Research Center for providing the research facilities.

References

Aktar, S., Nasiruddin, K. M., & Hossain, K. (2008). Effects of different media and organic additives interaction on in vitro regeneration of Dendrobium orchid. *Journal of Agriculture & Rural Development*, *6*(1), 69-74.

Arditti, J. (1977). Clonal propagation of orchids by means of tissue culture-a manual. *Orchid biology: Review and perspective*, 203-293.

Asghar, S., Ahmad, T., Hafiz, I. A., & Yaseen, M. (2011). In vitro propagation of orchid (Dendrobium nobile) var. Emma white. *African Journal of Biotechnology*, *10*(16), 3097-3103.

Christenhusz, M. J., & Byng, J. W. (2016). The number of known plants species in the world and its annual increase. *Phytotaxa*, *261*(3), 201-217.

Cockel, C. 2013. *Bulbophyllum leopardinum*. The IUCN Red List of Threatened Species 2013: e.T44392371A44532711.

De, K. K., Majumdar, S., Sharma, R., & Sharma, B. (2013). Green pod culture and rapid micropropagation of Dendrobium chrysanthum Wall.–a horticultural and medicinal orchid. *Folia Horticulturae*, *25*(2), 169-169.

eFloras (2008). Published on the Internet http://www.efloras.org [accessed 05 February 2022] Missouri Botanical Garden, St. Louis, MO and Harvard University Herbaria, Cambridge, MA.

Fay, M. F. (2018). Orchid conservation: how can we meet the challenges in the twenty-first century?. *Botanical studies*, *59*(1), 1-6.

Gupta, P. D., & Bhadra, S. K. (1998). In vitro production of seedlings in Cymbidium aloifolium L. SW. *Plant Tissue Culture*, *8*(2), 177-182.

Huh, Y. S., Lee, J. K., Nam, S. Y., Paek, K. Y., & Suh, G. U. (2016). Improvement of asymbiotic seed germination and seedling development of Cypripedium macranthos Sw. with organic additives. *Journal of Plant Biotechnology*, *43*(1), 138-145.

Kaur, S., & Bhutani, K. K. (2012). Organic growth supplement stimulants for in vitro multiplication of Cymbidium pendulum (Roxb.) Sw. *Horticultural Science*, *39*(1), 47-52.

Knudson, L. (1951). Nutrient solutions for orchids. *Botanical Gazette*, *112*(4), 528-532.

Lee, Y. I., & Yeung, E. C. (2010). Embryo development and in vitro seed germination of Bulbophyllum fascinator. In *I International Orchid Symposium 878* (pp. 243-250).

Li, Y. Y., Chan, C., Stahl, C., & Yeung, E. C. (2018). Recent advances in orchid seed germination and micropropagation. *Orchid propagation: from laboratories to greenhouses—methods and protocols*, 497-520.

Maharjan, S., Pradhan, S., Thapa, B. B., & Pant, B. (2019). In vitro propagation of endangered orchid, Vanda pumila Hook. f. through protocorms culture. *American Journal of Plant Sciences*, *10*(07), 1220.

Maneerattanarungroj, C., Laywisadkul, S., & Kongbangkerd, A. (2010). Tissue culture of Bulbophyllum affine Lindl. *International Journal of Science*, *7*(2), 45-59.

Obsuwan, K., & Thepsithar, C. (2014). An effect of organic supplements on stimulating growth of Vanda and Mokara seedlings in tissue culture. *International Journal of Bioengineering and Life Sciences*, *8*(7), 696-698.

Pakum, W., Watthana, S., orn Srimuang, K., & Kongbangkerd, A. (2016). Influence of Medium Component on In vitro Propagation of Thai s Endangered Orchid: Bulbophyllum nipondhii Seidenf. *Plant Tissue Culture and Biotechnology*, *26*(1), 37-46.

Pant, B. (2013). Medicinal orchids and their uses: Tissue culture a potential alternative for

conservation. *African Journal of plant science*, *7*(10), 448-467.

Pant, B., & Raskoti, B. B. (2013). *Medicinal orchids of Nepal*. Himalayan Map House, Nepal.

Pant, B., Pradhan, S., Paudel, M. R., Shah, S., Pandey, S., & Joshi, P. R. (2018). Various culture techniques for the mass propagation of medicinal orchids from Nepal. In *III International Orchid Symposium 1262* (pp. 109-124).

Pant, B., Shah, S., Shrestha, R., Pandey, S., & Joshi, P. R. (2017). An overview on orchid endophytes. *Mycorrhiza-nutrient uptake, biocontrol, ecorestoration*, A. Varma, R. Prasad, N. Tuteja, eds, Springer, Cham 503-524.

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.

Pillon, Y., & Chase, M. W. (2007). Taxonomic exaggeration and its effects on orchid conservation. *Conservation Biology*, *21*(1), 263-265.

Pradhan, S., Regmi, T., Ranjit, M., & Pant, B. (2016). Production of virus-free orchid Cymbidium aloifolium (L.) Sw. by various tissue culture techniques. *Heliyon*, *2*(10), e00176.

Rajbhandari, K. R., & Bhattarai, S. (2001). *Beautiful Orchids of Nepal*. Keshab R. Rajbhandari.

Roy, J., & Banerjee, N. (2001). Cultural requirements for in vitro seed germination, protocorm growth and seedling development of Geodorum densiflorum (Lam.) Schltr. *Indian Journal of Experimental Biology*, *39*, 1041-1047.

Selosse, M. A. (2014). The latest news from biological interactions in orchids: in love, head to toe. *New Phytologist*, *202*(2), 337-340.

Shah, S., Shrestha, R., Maharjan, S., Selosse, M. A., & Pant, B. (2018). Isolation and characterization of plant growth-promoting endophytic fungi from the roots of Dendrobium moniliforme. *Plants*, *8*(1), 5.

Shah, S., Thapa, B. B., Chand, K., Pradhan, S., Singh, A., Varma, A., Thakuri, L. S., Joshi, P. R., & Pant, B. (2019). Piriformospora indica promotes the growth of the in-vitro-raised Cymbidium aloifolium plantlet and their acclimatization. *Plant Signaling & Behavior*, *14*(6), 1596716.

Teoh, E. S. (2016). Secondary metabolites of plants. In *Medicinal Orchids of Asia* (pp. 59-73). Springer, Cham.

Thokchom, R., Maitra, S., & Sharma, S. (2017). In vitro Mass Propagation of Endangered Terrestrial Orchid *Phaius tankervilliae* (L'Her.) Blume through Green Seed Pod Culture. *International Journal of Current Microbiology and Applied Sciences*, *6*(5), 722-728.

Watthana, S., & Srimuang, K. O. (2017). Influence of organic supplements on growth and development of in vitro shoots of Bulbophyllum dhaninivatii Seidenf. In *Applied Mechanics and Materials* (Vol. 855, pp. 42-46). Trans Tech Publications Ltd.