

IN VITRO PROPAGATION OF NEPALESE ORCHIDS: A REVIEW

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ABSTRACT

Nepalese orchids are made up of 458 taxa. Despite a ban on the collection and trade of all orchid species in Nepal, numerous anthropogenic factors are leading to the rapid loss of natural stands of germplasm. Biotechnology, specifically *in vitro* propagation, may be the only viable solution for preserving and reintroducing endangered germplasm back into the wild. Despite the large germplasm base, only tissue culture studies have been conducted, and most have focused almost exclusively on *in vitro* seed germination, the bulk of which have been conducted in the past few years. No other biotechnological advances have yet been made. This brief review provides a short synopsis of the advances made thus far in the *in vitro* propagation of Nepalese orchids.

Key words: *in vitro*; Nepal; Orchidaceae; seed germination; tissue culture

The use of tissue culture for Nepalese orchids

The Orchidaceae has an estimated 870 described genera and in excess of 25 000 species (Dressler 1993; Swarts & Dixon 2009). An estimated 458 taxa of orchids are found in Nepal (Rokaya et al. 2013), “including 104 genera, 437 species, 16 varieties, 3 subspecies and 2 forma and 18 endemic species.” Such detailed checklists are important to ensure that a solid botanical base of information exists to develop a structured biotechnological and preservation programme. Such a programme would involve the use of tissue culture to preserve and multiply rare germplasm, genetic transformation and molecular breeding to improve weak germplasm by introducing novel or strengthening characters, and traditional to obtain progeny with introduced characters. Wild Nepalese orchids are popularly known by their vernacular names: *Sunakhari*, *Sungava*, *Jiwanti*, *Bankera*, *Thur* or *Thurjo*. Orchids in Nepal have ornamental and medicinal uses (Acharya & Rokaya 2010; Pant 2013; Pant & Raskoti 2013; Subedi et al. 2013). Pant and Raskoti (2013) indicated that 90 orchid genera from Nepal have medicinal value.

Most orchids in Nepal are under threat due to overexploitation, illegal trade, habitat loss, deforestation and other anthropogenic-induced stresses (White & Sharma 2000; Rajbhandari & Bhattarai 2001; Bajracharya & Shakya 2002; Acharya et al. 2011; Rokaya et al. 2013). The Nepal Forest Act (1993), Forest Regulations (1995) and its amendment (2001) ban orchids from being collected and traded. Furthermore, Nepal has been a member nation of The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) since 1975 (<http://www.cites.org/eng/disc/parties/alphabet.php>) and there is ban on collection and trade of all species of orchids which are included in CITES Appendix II. However, these laws, regulations and policies are not effectively implemented since collection and trade are widely reported without restriction, sometimes even sold in open markets of Kathmandu for medicinal use (Acharya & Rokaya 2005). Contrary to these laws are regulations, such as a bill recently passed by The Government of Nepal on 7th March 2013, which permit the collection of wild orchids for sale and commercial cultivation (MOFSC 2013).

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Table 1 Tissue culture-related studies pertaining to Nepalese orchids

Species	Purpose of study	Method employed	Key results obtained	Reference(s)
<i>Cymbidium giganteum</i> Wall Ex Lindl.	Meristem culture; PLB induction	Young shoots (8 cm long) of greenhouse plants washed with RTW for 30 min; 10-min wash in 10% Teepol and 3-4 rinses with DW; 5 s in EtOH then 10% CaCl ₂ for 20 min and 5X rinses in SDW. Apical meristems (0.25-0.5 mm) cultured on MS + 1.0 g.l ⁻¹ casein acid hydrolysate + 10% CW ¹ ; 25 °C; 16-h PP; 3000 lux; pH 5.5; agar NR. 5.0 mg.l ⁻¹ BA + 1.0 mg.l ⁻¹ NAA to induce PLBs ² (same medium for rooting, with subcultures).	PLBs ² formed after 6 w, rooting within 2 mo and seedling formation within 6-8 mo. Acclimatization not performed. No supporting figures, tables, graphs or data.	Shrestha & Rajbhandary 1988
<i>Otochilus alba</i> Lindl.	Pseudobulb multiplication and production of pseudobulbs from runners	Apical shoots (5-6 mm long) of <i>ex vitro</i> plants surface sterilized in 0.1% HgCl ₂ for 6 min; 5X rinses in SDW. Apical 2-3 mm shoot tips cultured on MS (half-strength macronutrients) + 2% sucrose + 10% CW; 22-24 °C; 16-h PP; 3000 lux; pH 5.7; 0.7% agar. 0.2 mg.l ⁻¹ IAA or NAA to induce pseudobulbs and at 0.5 mg.l ⁻¹ to multiply pseudobulbs in liquid culture. Phytamax + 0.5 mg.l ⁻¹ IAA + 2.0 mg.l ⁻¹ 2iP (SIM), KC + 1.0 mg.l ⁻¹ IBA (RIM).	Pseudobulbs induced from shoot tips in 2 w. 0.5-1 mg.l ⁻¹ NAA resulted in callus formation in 18-22 d. Maximum of 28% of pseudobulbs formed shoots. Roots formed within 22-23 d. Acclimatization not performed.	Mukhopadhyay & Roy 1994
<i>Cymbidium longifolium</i> D-Don	Meristem culture; PLB induction	Young shoots (8 cm long) washed with RTW for 30 min; 2-min wash in 10% Teepol and 4X rinses with DW; 5 s in EtOH then 0.1% MgCl ₂ for 5 min and 5-6 rinses in SDW. Apical meristems cultured on MS + 1 g.l ⁻¹ casein acid hydrolysate + 10% CW; 25±4 °C; 16-h PP; 3000 lux; pH 5.5; agar NR. 2.2 mg.l ⁻¹ BA + 1.8 mg.l ⁻¹ NAA to induce PLBs ² . PLB subculture and proliferation, as well as rooting, on MS + 1.0 mg.l ⁻¹ BA + 1.5 mg.l ⁻¹ Kn + 10.0 mg.l ⁻¹ Ads.	PLBs ² formed after 6 w, rooting within 2 mo and seedling formation within 6-8 mo. Acclimatization performed, but not described in detail. No supporting figures, tables, graphs or data.	Shrestha & Rajbhandary 1994
<i>Cymbidium iridioides</i> D. Don	<i>In vitro</i> seed germination and micropropagation	Young green pods washed in tap water + a few drops Teepol for a few min; RTW for 30 min; 1% NaOCl for 10 min; 70% EtOH for 1 min; 3X rinses with SDW. 25±2 °C; 16-h PP; 200-300 lux; pH 5.7; 0.8% agar; carbohydrate source NR. MS + 1.0 mg.l ⁻¹ BA + 1.0 mg.l ⁻¹ NAA (seed germination). 0.5 mg.l ⁻¹ BA (SIM). 1.0 mg.l ⁻¹ IBA (RIM).	A maximum of 8.25 shoots formed per germinating seedling. Roots formed within 2 mo. Acclimatization not performed.	Swar & Pant 2004; Pant & Swar 2011
<i>Aerides odorata</i> Lour.	<i>In vitro</i> seed germination; seedling development	Immature green pods washed in detergent + Tween-20; RTW for 30 min; 1% NaOCl for 10 min; 70% EtOH for 1 min; 3X rinses with SDW. 25±2 °C; 16-h PP; 200-300 lux; pH 5.7; 0.8% agar; carbohydrate source NR. MS + no PGRs (all stages).	Under optimal conditions, 14, 17 and 24 w required to form the first leaf, the first root and a fully developed seedling, respectively. No other growth quantification. Acclimatization not performed.	Pant & Gurrung 2005
<i>Coelogyne cristata</i> Lindl.	<i>In vitro</i> seed germination; seedling development	Immature capsules washed in RTW; sterilized in 1% NaOCl for 10 min; 70% EtOH for 2 min; 3X rinses with SDW. 25±2 °C; 16-h PP; pH 5.7; 0.8% agar; PPF and carbohydrate source NR. MS/B ₅ + 0.5 mg.l ⁻¹ NAA + 1.0 mg.l ⁻¹ BA (seed germination, SIM). 1.0 mg.l ⁻¹ IBA (RIM).	Under optimal conditions, 9,25 shoots formed in 24 w. No other growth quantification. Acclimatization not performed.	Pant et al. 2008
<i>Dendrobium densiflorum</i> Lindl.	<i>In vitro</i> seed germination; seedling development; shoot tip culture	For 2009 study, immature capsules sterilized in 70% EtOH for 2 min; 1% NaOCl for 15 min; 5X rinses with SDW. For 2013a study, same protocol as for <i>Cymbidium elegans</i> . 25±2 °C; 16-h PP; pH 5.8; 0.8% agar; PPF and carbohydrate source NR (PPFD for 2013a = 350-500 lux). MS + 1.0 mg.l ⁻¹ BA (seed germination). MS + 0.5 mg.l ⁻¹ NAA + 2.0 mg.l ⁻¹ BA (SIM). 1.5 mg.l ⁻¹ IBA (RIM).	Fastest germination in 5 w, protocorm formation in 6 w and first shoot formation in 8 w. 85% of plantlets acclimatized in cocopeat + litter + clay (2:1:1) above sphagnum moss, but no visual proof provided.	Pradhan & Pant 2009; Pradhan et al. 2013a
<i>Cymbidium elegans</i> Lindl.	<i>In vitro</i> seed germination and micropropagation	Green immature capsules washed in RTW for 30 min; 1% NaOCl for 1-5 min; 70% EtOH for 2 min; 3X rinses with SDW. 25±2 °C; 16-h PP; pH 5.8; 0.8% agar; PPF and carbohydrate source NR. MS + 1.0 mg.l ⁻¹ BA (seed germination). MS + 0.5 mg.l ⁻¹ NAA then MS + 0.5 mg.l ⁻¹ NAA + 1.0 mg.l ⁻¹ BA (PLB multiplication).	Almost 100% seed germination possible within 9 w, protocorm formation in 10 w and first shoot formation in 31 w. 2.5 cm plantlets formed within 90 d. Seedlings acclimatized in coco-peat + moss.	Pradhan & Pant 2009; Pant & Pradhan 2010
<i>Phaius tancarvilleae</i> (L'Her.) Blume	<i>In vitro</i> seed germination; seedling development; shoot tip culture	Pod sterilization (as for Pant and Swar 2011, but NaOCl and EtOH steps inverted). 25±2 °C; 16-h PP; PPF and carbohydrate source NR for seed germination, but 3% sucrose for shoot tip culture; pH 5.8; 0.8% agar. MS + 0.5 mg.l ⁻¹ BA (all developmental stages from germination to shoot formation), or 1.0 mg.l ⁻¹ BA (from shoot tips). Sub-cultures every 8 weeks. 0.5 mg.l ⁻¹ NAA (RIM).	Germination after 7 w; complete seedlings after 24 w. Germination not quantified. 13.3 shoots/culture in 20 w. Acclimatization not performed for seed-derived plantlets, but shoot tip-derived plantlets acclimatized in cocopeat + sphagnum moss (2:1).	Pant et al. 2011; Pant & Shrestha 2011
<i>Cymbidium</i>	<i>In vitro</i> seed	The capsule was flamed then placed, after slicing longitudinally in two halves, cut side	Protocorms ³ formed after 8 w, rooting 2 mo after.	Rajkarnikar 2011

<i>alofolium</i> (L.) Sw.	germination; seedling development	down on germination medium. 25±2 °C; 16-h PP; PPF NR; 3% sucrose for shoot tip culture; pH 5.5; 0.8% agar. MS + 0.1% casein hydrolysate + 10% CW ¹ (protocorm ² induction) then onto MS + 10% CW ¹ + 1.0-1.5 mg·l ⁻¹ NAA + 1.0-2.0 mg·l ⁻¹ BA (PLB multiplication).	Acclimatization performed, but not described in detail. No supporting figures, tables, graphs or data.	
<i>Dendrobium primulinum</i> Lindl.	<i>In vitro</i> shoot tip culture and micropropagation	Explants were shoot tips (0.3-0.5 mm long) from 20-week-old seed-derived seedlings. 25±2 °C; 16-h PP; PPF NR; pH 5.8; 0.8% agar; 3% sucrose. MS + 1.5 mg·l ⁻¹ BA (SIM). MS + 1.5 mg·l ⁻¹ BA + 0.5 mg·l ⁻¹ NAA (SMM). 0.5 mg·l ⁻¹ IAA (RIM).	4.5 new shoots formed per shoot tip after 5 w. 70% survival after acclimatization in cocopeat + sphagnum moss (2:1).	Pant & Thapa 2012
<i>Esmeralda clarkei</i> Rehb.f.	<i>In vitro</i> seed germination and micropropagation	Immature green capsules rinsed in RTW for 1-2 h; 70% EtOH for 2 min; 1% NaOCl for 10 min; rinse with SDW. Explants were shoot tips 3-5 mm long from 5-month-old <i>in vitro</i> seedlings (2012a) or protocorms (2012b). 25±2 °C; 16-h PP; PPF and carbohydrate source NR; pH 5.8; 0.8% agar. MS + 0.5-2.0 mg·l ⁻¹ BA (SIM). MS + 0.5 mg·l ⁻¹ NAA (RIM).	9.5-11 or 11-14 shoots/treatment in 2012a and 2012b, respectively. 85.5% of plantlets acclimatized in soil + sand + saw dust (1:1:1) (2012a, 2013).	Paudel & Pant 2012a, 2012b, 2013; Paudel et al. 2012
<i>Coelogyne fuscescens</i> Lindl.	<i>In vitro</i> seed germination and plantlet regeneration	Capsules sterilized as follows: soapy water for a few minutes; RTW for 1 h; rinse with SDW; 70% EtOH for 2 min then 1% NaOCl for 15 min; 5X rinses with SDW. Optimal seed germination on MS + 1.0 mg·l ⁻¹ BA + 0.5 mg·l ⁻¹ NAA and further development on the same BA-free medium. 25 °C; 16-h PP; PPF; carbohydrate source and pH NR.	90% of seeds started to germinate after about 6 w, protocorms after 10 w, shoots after 13 w, and roots after 23 w. Acclimatization not performed.	Koirala et al. 2013
<i>Cymbidium alofolium</i> (L.) Sw.	<i>In vitro</i> seed germination; seedling development	Immature green pods washed in detergent + Tween-20; RTW for 30 min; 70% EtOH for 2 min; 1% NaOCl for 10 min; 3X rinses with SDW. 25±2 °C; 16-h PP; 350-500 lux; pH 5.8; 0.8% agar; 3% sucrose. MS or KC + 0.5 mg·l ⁻¹ BA + 0.5 mg·l ⁻¹ NAA (all stages). 0.5 mg·l ⁻¹ IAA (RIM).	Full-strength medium was better than half- or quarter-strength (MS or KC). >80% of seeds germinated. Protocorms formed after 10 w, leaf primordia after 17 w, roots after 23 w, and seedlings by 30 w. Acclimatization not performed.	Pradhan et al. 2013b
<i>Cymbidium devonianum</i> Paxton	<i>In vitro</i> seed germination; seedling development	Capsules washed in RTW + 2-3 drops Tween-20 for 50 min; 70% ethyl alcohol for 2 min; 1% NaOCl for 10 min; SDW for 5 min. Seed germination on MS + 2.0 mg·l ⁻¹ BA + 0.5 mg·l ⁻¹ NAA + 10% CW ¹ ; 3% sucrose; pH 5.8; 0.8% agar; 25±2 °C; 16-h PP; PPF NR. PLB induction and development on MS + 1.0 mg·l ⁻¹ BA + 1.5 mg·l ⁻¹ NAA + 10% CW ¹ . MS + 1.0 mg·l ⁻¹ BA + 1.5 mg·l ⁻¹ NAA + 1.5 mg·l ⁻¹ Kn + 10% CW (SIM)	Seeds germinated within 10 w, PLB ³ formation within 11 w, and shoot initials within 23 w. Acclimatization not performed.	Parmar 2014
<i>Dendrobium amoenum</i> Wall. ex. Lindl.	<i>In vitro</i> shoot tip culture and micropropagation	Sprouting shoot tips (1-2 cm long) washed in RTW for 1 h; 5 min with DW; sterilized with 0.1% HgCl ₂ for 5 min; shoot tips cultured on MS + 0.1% casein acid hydrolysate + 3% sucrose + 8% agar; pH 5.5; 25±2 °C; 16-h PP; PPF NR. MS medium + 1.0 mg·l ⁻¹ BA + 1.5 mg·l ⁻¹ Kn or 1.0 mg·l ⁻¹ NAA + 10% CW ¹ to induce PLBs and shoots. Smaller PLBs (<2.0 cm) cultured on MS with BA + Kn + 10% CW or BA + NAA + 10% CW and larger PLBs (2.0-2.5 cm long) cultured on MS + NAA. MS + 0.5 mg·l ⁻¹ NAA (RIM)	PLBs and shoots formed within 6-8 w. Maximum of 12-15 PLBs/explant. Acclimatization not performed. No quantitative data.	Rajkarnikar 2014

2IP, N⁶-isopentenyladenine or 6-*z*,*z*-dimethylallylamino purine; AdS, adenine sulphate; B₅ medium, (Gamborg et al 1968); BA, N⁶-benzyladenine (BA is used throughout even though BAP (6-benzylamino purine) may have been used in the original, according to Teixeira da Silva, 2012; CW, coconut water; d, days; DW, distilled water; EtOH, ethanol; h, hour; HgCl₂, mercury chloride; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; KC, Knudson's C medium (Knudson 1946); Kn, kinetin; min, minute(s); mo, months; MS, Murashige and Skoog (1962) medium; NAA, α -naphthaleneacetic acid; NaOCl, sodium hypochlorite; NR, not reported; PLB, protocorm-like body; PP, photoperiod; PPF, photosynthetic photon flux density; RIM, root induction medium; RTW, running tap water; SDW, sterile distilled water; SIM, shoot induction medium; SMM, shoot multiplication medium; VW, Vacin and Went (1949) medium; w, week(s)¹ reported as protocorms, but should be PLBs; ² reported as protocorms, but should be PLBs; ³ reported as PLBs, but should be protocorms. Medium solidifying agent (e.g. agar and carbohydrate source (e.g. sucrose) reported as a % (w/v).

To meet the demand for various uses and to protect endangered Nepalese orchid species, new technologies need to be developed for mass propagation. Biotechnology, including tissue culture, is an essential tool for the preservation of rare or endangered Nepalese orchids with ornamental and medicinal value, and improve their characteristics (Acharya & Rokaya 2010; Pant 2013, 2014). However, very few studies, outlined herein, have been carried out in this field in Nepal, and most studies have been published in local or relatively unknown journals. The aim of this review is to provide an overview of the biotechnological techniques used to grow and/or preserve Nepalese orchids and to provide some future perspectives.

Conclusions and future vision for Nepalese orchid biotechnology

The use of tissue culture for the preservation of Nepalese orchid germplasm has a very recent history, which began in 1988 but intensified after 2009. Table 1 indicates that tissue culture has been applied to 15 species (in 7 genera), about 3% of the total of 437 species of Nepalese orchids. Unlike orchids from other locations around the world, including neighbouring India, which already have a rich history of tissue culture and other biotechnological applications (Hossain et al. 2012; Teixeira da Silva 2013), the application of tissue culture to better understanding Nepalese orchids, and preserving or commercializing them, remains relatively unexplored. Table 1 reveals that most studies to date have dealt with *in vitro* seed germination and seedling production often followed by micropropagation initiated with a seedling explant. Almost all of the studies involve a sterilization procedure for the green pods or capsules and culture on Murashige and Skoog (1962) – MS basal medium in the light. Subsequent development of the protocorms and the induction of protocorm-like bodies in the presence of plant growth regulators characterize most of the studies listed in Table 1. Applied aspects such as synthetic seed technology for cryopreservation (Sharma et al. 2013), genetic transformation (Teixeira da Silva et al. 2011), the use of thin cell layers (Teixeira da Silva 2013) and *in vitro* flowering (Teixeira da Silva et al. 2014) are all aspects that are unexplored and that would enrich the focus

given to this group of as yet poorly understood orchids desperately in need of conservation. The government institution, The Department of Plant Resources (DPR) under Ministry of Forest and Soil Conservation (MOFSC) in coordination with the Central Department of Botany (CDB), Tribhuvan University, have initiated tissue culture of native orchids (KP Acharya, personal observation). However, this type of program needs to be implemented on a larger scale in order to cover the wide germplasm base that is under threat.

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