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Research Article

In vitro conservation of rare, medicinally important species *Dendrobum nobile* Lindl. (Orchidaceae)

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Abstract: A comparative study was made to access the regeneration response of the stem-node discs of *Dendrobium nobile* procured from *in vivo* (greenhouse) grown plants and *in vitro* grown cultures. The response of stem-node discs, procured both from *in vivo* and *in vitro* source, was successfully tested in M (Mitra *et al.,* 1976) medium alone and in combinations with cytokinins [N6-benzyladenine (6-BA - 0.5, 1.0 mg l^{-1}), furfurylaminopurine (Kn - 0.5, 1.0 mg l^{-1}) and auxin [α-naphthalene acetic acid (NAA - 0.5, 1.0 mg l^{-1})]. The explants regenerated via shoot bud initiation in the cultures. Juvenility of the tissues and chemical stimulus were the major factors in initiating the response in the explants. The initiation of regeneration response in *in vitro* derived explants was not obligatory to a treatment with growth regulators as compared to those derived from *in vivo* ones. Cytokinins at 1.0 mg l^{-1} favoured optimum regeneration percentage in the explants and invoked additional supernumerary loci in the regenerants. The regenerants from *in vitro* derived explants developed plantlets early in cytokinins enriched combinations.

Key words: *in vitro*, micropropagation, rare, shoot buds, stem-discs, plant growth regulators.

Introduction

World over, conservation of flora is one of the major issues. For smooth functioning of an ecosystem, continuous existence of each species is necessary. Presently, efforts have been made to develop a protocol to conserve a threatened species of orchids i.e. *Dendrobium nobile* a highly medicinal orchid in the *in vitro* environment. *D. nobile* is an epiphytic, semi-evergreen species. Biogeographically, this sympodial plant extends towards south China to Laos and Thailand. It is scattered in the tropical to temperate climates, in the North-Eastern Indian Himalayas, at an altitude of 200-2000 meters. it is medicinally valuable as well. *D. nobile* is one of the 50 fundamental herbs used in the traditional Chinese medicine where it has the name shi hu or shi hu lan. The drug shih-hu made from *D. nobile* has been valued greatly (Lawler, 1984). The drug nourishes the yin system of body; used as a tonic and strengthening medicine and impart longevity and serve as an aphrodisiac. The stems are useful in alleviating thirst, to calm restlessness, accelerating convalescence and reducing dryness of the mouth (Bensky *et al.,* 1986). Besides being a medicinal herb, *D. nobile* has a great potential as an ornamental plant due to the long shelf life of nearly 2-3 months of its appealing floral displays (http://www.plantsrescue.com). *D. nobile* commands a high demand among the floriculturally significant genotypes which are used as garden and landscape material. It is a progenitor of various commendable hybrids of international eminence. Being highly versatile in its ornamental and medicinal properties, its wild populations are continuously and swiftly getting rare due to unabated collections, which far exceed its natural regeneration. Several other factors which add up to the current conservation status of the species are fragmentation of habitat especially in the tropical regions as a result of deforestation, increased use of fertilizers, excessive exploitation of the soil and pollution. As a consequence, the genus *Dendrodium* is included in the Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES, 2017). Multidisciplinary approaches should be forced into action to save populations (*in situ* and *ex situ*) of the species from getting extinct in nature. Hence, through this communication, it is emphasized to conserve, propagate and multiply the species, through biotechnological techniques thus assisting with the conservation of wild populations of the species undertaken.

Since the conventional propagation (vegetative) through stem-cuttings in the nursery-beds, is a time consuming process and not even economically reliable; *in vitro* techniques offer an alternative for a large-scale production of the seedlings/plantlets within short span of time. In clonal propagation, it is immensely important to maintain genetic uniformity in the *in vitro* raised progenies. In out-breeding taxa like orchids, seed raised populations are extremely heterozygous and to maintain genetic stability of the regenerants, it is important to identify appropriate explants for *in vitro* propagation protocol. The utility of stem-node segments, as an effective alternative for micropropagating orchids is a successful approach in this direction as this

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method provides opportunities to produce a large number of true - to - type plantlets of interest.

The stem-segments of several medicinal, aromatic, and horticulturally important plant species are successfully utilized for genetic transformation studies (Rastogi and Dwivedi, 2006). In a few orchid species, this technique has been used as a reliable method for regeneration and multiplication (Arditti and Ernst,1993; George and Ravishankar, 1997;Vij and Kaur, 1998; Kanjilal *et al.,* 1999; Gangaprasad *et al.,* 2000; Pyati *et al.,* 2002; Decruse *et al.,* 2003; Basker and Narmatha, 2006; Martin 2007; Zhao *et al.,* 2007; Janarthanam and Sheshadri, 2008; Medina *et al.,* 2009; Rangsayatorn, 2009; Hong *et al.,* 2010; Kaur and Bhutani 2010; 2013).

Highlights

- The species is medicinally as well as floriculturally important, and categorized as endangered species
- *In vivo* sourced explants successfully initiated cultures *in vitro*
- *In vitro* sourced explants responded better than those obtained from *in vivo* ones
- Explants regenerated via direct shoot bud development

Currently, an attempt was made to explore the regenerative potential of *in vivo* and *in vitro* derived explants by establishing an efficient regeneration system using stem node as explants by (i) assessing the comparative regenerative potential of *in vitro* and *in vivo* derived explants (ii) checking the influence of different levels of growth regulators individually and in combination on regeneration percentage and successive development into plantlets. This step would definitely assist with the conservation of medicinally and floriculturally important *D. nobile* species thus saving its populations from getting extinct in nature.

Materials and Methods

Collection of plant material

D. nobile plants were collected from a commercial grower of Darjeeling district, West Bengal, India (latitude range: 26°31' - 27°13' N; longitude range: 87°59' - 88°53' E). The plants were replanted in the pots (size 27.5 cm × 22.4 cm) containing epiphytic substrate composed of bark pieces: brick pieces: charcoal pieces: (1:1:1). The surface of potting mix was covered with sphagnum moss to retain moisture of the compost. Plants were maintained in the greenhouse under natural light conditions with 70% relative humidity and 25/20°C day/night temperature.

Explants and culture medium

Uninodal stem-discs (<0.5-0.6 cm long) procured from in vivo grown plants and 30 weeks old cultures were used as explants. These were inoculated on Mitra *et al.,* 1976 medium and its combinations with growth regulators such as cytokinins [6-benzyl aminopurine (BAP), furfurylaminopurine (Kn) and auxin [α-naphthalene acetic acid (NAA)] at 0.5 and 1.0 mg l^{-1})]. Sucrose 2% (wv^{-1}) was invariably used as carbon source. The medium was gelled with 0.9% Agar powder (Qualigens). Activated charcoal (AC - 0.2%) was also used in another set of experiment.The pH of the medium was adjusted to 5.8 after adding the growth regulators and organic growth supplement. The media were gelled with 0.9% (M) agar powder (Hi - Media, Mumbai, India) and dispensed in test tubes (25 mm × 150 mm). The media were autoclaved at 121°C at pressure of 1.06 kg cm^{-2} for 15 min. Autoclaved medium was kept at 37°C to check any further contamination.

Surface sterilization

Uninodal stem-discs (<0.5- 1.0 cm long) procured from *in vivo* grown plants were collected. They were first scrubbed with a soft brush in flowing tap water. Gently, they were again scrubbed using dish wash liquid detergent to get rid of any debris left over the surface of stem discs. These were rinsed thoroughly in water. Later, they were surface sterilized with 0.1% mercuric chloride ($HgCl_2$; Qualigens, Mumbai, India) in an aqueous solution containing 1-2 drops of liquid soap as a wetting agent for 3-4 minutes. The aqueous solution of mercuric chloride was decanted and the stem-discs were rinsed 2-3 times with sterilised distilled water to remove any traces of mercuric chloride left on their surface. Thereafter, these sterilised explants were placed in a petri dish and very carefully their ends on both sides were severed-off and inoculated into M medium individually and its supplementations with various growth adjuncts.

Explants from *in vitro* grown seedlings

The stem-discs (0.5 cm – 0.6 cm long) were procured from 30 weeks old aseptic cultures. Inside a laminar air-flow cabinet, under aseptic conditions, the seedlings were removed from the culture vessels and their leaves and roots were severed-off. The explants with uninodal discs were prepared and inoculated on Mitra medium and its combinations with growth adjuncts.

Inoculations and culture conditions

The inoculations were done under aseptic conditions in a laminar air-flow cabinet. The cultures vessels with inoculated explants were incubated in a culture room at 25 ± 2°C under a 12/12 hrs light / dark photoperiod at 40 µ mol $m^{-2}s^{-1}$ light intensity provided by white fluorescent tubes (Fluorescent tubes, Philips India Ltd., Mumbai, India).To check the reproducibility of the protocol, the experiment was repeated twice. Sub-culturing was done as and when required. The result was expressed on minimum of eight replicates. The

cultures were observed regularly under binocular microscope and data recorded accordingly.

Histological studies

To make histological observations, the responding explants were collected from the culture vessels. Free hand sections were cut by placing them in the potato pith. Thin sections floating at the surface of water were selected and placed in a drop of water and observed under the microscope. The photographs were captured using a digital camera (Nikon Digital Sight, DS, Ri1 Nikon Corporation, Japan).

Results

The regeneration response of uninodal-discs (0.5-0.6cm long) varied with their source and chemical stimulus in the nutrient pool. Shoot buds were invariably induced in the responding explants and transformed into shoots. The results are summarised and illustrated (Tables - 1 and 2; Fig. 1a-g). Some of the pertinent findings are as follows:

Table 1. *In vitro* regeneration response of *in vitro* derived stem node segments of *D. nobile* in M medium and its combination with growth adjuncts

Additives	% explants responded	Initiation of response (wk)	No. of meristematic loci	No. of shoot buds/explant	Complete plantlets (wks)
-	25.02±0.01^{b}	5.20±0.03^{d}	1.00±0.00^{b}	1.00±0.00^{b}	12.32±0.2^{d}
AC	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
BAP $_{(0.5)}$	49.10±0.50^{e}	4.01±0.00^{cd}	1.00±0.00^{b}	1.02±0.00^{b}	10.05±0.37^{b}
BAP$_{(0.5)}$ +AC	48.08±0.91^{e}	4.00±0.50^{cd}	1.00±0.00^{b}	1.00±0.00^{b}	10.00±0.00^{b}
BAP $_{(1.0)}$	76.09±1.00^{d}	3.25±0.18^{c}	2.04±0.38^{bc}	2.00±0.00^{bc}	10.00±0.00^{b}
BAP$_{(1.0)}$ +AC	75.02±0.98^{d}	2.00±0.00^{b}	2.00±0.63^{bc}	2.00±0.00^{bc}	9.15±0.48^{b}
Kn $_{(0.5)}$	49.04±0.00^{e}	4.05±0.90^{cd}	1.00±0.00^{b}	1.10±0.00^{bc}	11.00±0.02^{bc}
Kn$_{(0.5)}$ +AC	48.23±0.05^{e}	4.47±0.36^{cd}	1.00±0.00^{b}	1.00±0.00^{b}	10.20±0.00^{b}
Kn$_{(1.0)}$	75.03±0.07^{d}	3.00±0.23^{c}	1.00±0.00^{b}	1.00±0.00^{b}	10.43±0.26^{b}
Kn$_{(1.0)}$ +AC	75.00±0.06^{d}	2.00±0.48^{b}	1.00±0.00^{b}	1.00±0.00^{b}	9.00±0.00^{b}
NAA$_{(1.0)}$	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
NAA$_{(1.0)}$ +AC	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}

Concentration of growth regulators used at 0.5 and 1.0 mg l^{-1}. Values in a column with similar superscripts are not significantly different at $p \leq 0.05$ according to Tukey's test.

Table 2. *In vitro* regeneration response of *in vivo* derived stem node segments of *D. nobile* in M medium and its combination with growth adjuncts

Additives	% explants responded	Initiation of response	No. of meristematic loci	No. of shoot buds /explant	Complete plantlets	Remarks
-	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	
AC	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	
BAP$_{(0.5)}$	25.12±0.10^{b}	10.01±0.07^{c}	1.00±0.00^{b}	1.00±0.00^{b}	25.00±0.12^{c}	
BAP$_{(0.5)}$ +AC	22.05±0.08^{b}	10.27±0.50^{c}	1.00±0.00 ^{b}	1.00±0.00^{b}	26.00±0.00^{a}	
BAP$_{(1.0)}$	50.00±1.25^{c}	6.20±0.15^{b}	1.00±0.00^{b}	2.00±0.00^{ab}	20.10±0.50^{b}	
BAP$_{(1.0)}$ +AC	50.00±0.47^{c}	6.25±1.00^{b}	1.00±0.00^{b}	2.00±0.00^{ab}	-	Plantlets were developed after 22.00±0.00^{a} week of culture upon a shift to AC starved BAP enriched medium.
NAA$_{(1.0)}$	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	
NAA$_{(1.0)}$ +AC	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	

Concentration of growth regulators used at 0.5 and 1.0 mg l^{-1}. Values in a column with similar superscripts are not significantly different at $p \leq 0.05$ according to Tukey's test.

The response in the *in vivo* sourced explants was obligatory to a treatment with BAP but the frequency with which the explants responded varied with the level of BAP in the medium. In BAP without and with AC at 0.5 mg l^{-1}, nearly 25% explants responded within 10 week of culture by sprouting a shoot bud. The response frequency was elevated up to 50% in combinations containing 1.0 mg l^{-1} BAP (Fig. a). Activated charcoal remained inhibitory to rhizogenesis (Fig. b); the cultures developed leafy shoots only in BAP 1.0 mg l^{-1}. Plantlets developed after 22 week of culture upon a shift to AC starved BAP enriched medium. The plantlets multiplied best in BAP/+AC containing medium. The plantlets had lengthy shoots bearing thin leaves and elongated roots in BAP at 0.5 mg l^{-1} supplemented medium (Fig. c). In activated charcoal enriched medium, the cultures developed thick leathery leaves in the cultures.

The response in the *in vitro* sourced explants was not obligatory to the use of growth stimulus but the frequency of regeneration was markedly influenced by the type of plant growth regulator in the medium. In the basal medium, nearly 25% explants responded to regeneration via bud break within 5 week of culture (Fig. d). Plantlets developed after 12 wks of culture. Activated charcoal did not initiate

any response in the cultures. A treatment with either of BAP and Kn at 0.5 mg l^{-1} favoured bud break in 50 per cent explants and it was elevated to 76 per cent when cytokinins were used at 1.0 mg l^{-1}. The plantlets were obtained within 9 week of culture. The cultures multiplied profusely by initiating several shoots and developed hairy, elongated roots in BAP 1.0 mg l^{-1} enriched medium (Fig. e). Additional activated charcoal favoured luxuriant growth of the plantlets (Fig. f). NAA proved detrimental to regeneration.

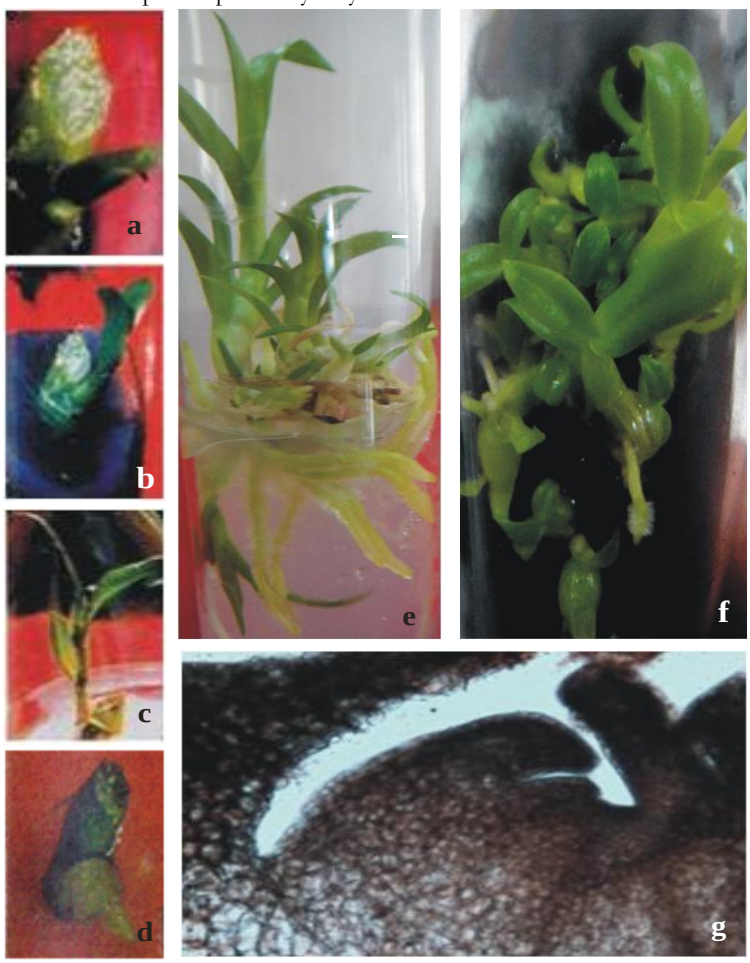

Figure 1a-g. Regeneration response of stem-node discs of *D. nobile* derived from *in vivo* and *in vitro* sourced explants in M medium and its combinations in growth adjuncts. a Sprouting of shootbud in *in vivo* sourced explant in M+ BAP $_{(1.0\ mg\ l^{-1})}$ + AC containing medium; b. Formation of leafy shoot with no root development in M + BAP $_{(1.0\ mg\ l^{-1})}$ +AC; c. Plantlet development in *in vivo* sourced stem disc in M + BAP $_{(0.5\ mg\ l^{-1})}$; d. Bud break in *in vitro* sourced explant in M medium, e. Profuse multiplication of shoots and rooting in M+ BAP$_{(0.5\ mg\ l^{-1})}$, f. Luxurient growth of plantlets in M + BAP + AC medium; g. Histological section revealed the meristemoids to be a shoot bud with leaf primordia and shoot apex.

Histological studies were conducted by slicing thin free-hand sections. These sections revealed a multicellular, dermal origin of the meristemoids. The actively dividing cells, formed meristematic zone. These zones developed into prominent epidermal bulges which formed primary shoot primordial and had vascular connections with the surrounding tissue. Later these formed shoot bud (Fig. g).

Discussion

Presently, the regenerative competence of stem-node segments of *D. nobile*. was successfully tested in M medium. The regeneration response in the explants, obtained from *in vivo* grown plants was obligatory to the use of growth adjuncts in the nutrient regime, whereas the explants procured from *in vitro* grown cultures responded readily in the basal medium itself. Moreover, the explants from *in vitro* grown cultures responded better than those procured from *in vivo* ones, in terms of time taken for initiation of response, induction of multiple meristematic loci; time taken in weeks to form complete plantlets, due probably to their habituated nature and juvenility of the tissues. The juvenility of the tissues as an important factor controlling cell proliferations has earlier been pointed in various orchid species such as *Bulbophyllum careyanum* (Vij *et al.,* 2000), *Malaxis acuminata* (Kaur and Bhutani, 2010), *Coelogyne flaccida* (Kaur and Bhutani 2013).

In the present study, NAA did not prove productive in the cultures; it failed to initiate response in the segments. The results are in accord with similar earlier findings where NAA affected regeneration in *Bulbophyllum careyanum* (Vij *et al.,* 2000) and had little or no effect in inducing neo-formations in *Dendrobium moschatum* (Kanjilal *et al.,* 1999) and in *Malaxis acuminata* also NAA impaired the percentage of responding explants and delayed plantlet development (Kaur and Bhutani, 2010).

A treatment with cytokinins BAP/Kn (1.0 mg l^{-1}) successfully initiated regeneration response in 75 per cent explants, induced additional meristematic loci in the regenerants, and also favoured early development of plantlets within 9 week of culture. The efficacy of BAP in inducing multiple shoot buds is already on records in Bulbophyllum careyanum (Vij *et al.,* 2000).

Incidentally, additional activated charcoal in the combinations did not prove beneficial, contrary to the earlier reports where AC remained efficient in enlarging the protocorm size, intense development of leaf and maximizing the aerial root growth in *Zygopetalum grandiflora* cultures (Moraes *et al.,* 2005). Literature studies also reveal that activated charcoal has promotory effect in seed germination and seedling development of *Paphiopedilum* and *Phalaenopsis* cultures Butcher D, Marlow, 1989; Hicks and Lynn, 2010; Kaur and Bhutani, 2012).

In the explants, the shoot bud development was observed to be, invariably, a direct organogenetic phenomenon in all the cultures irrespective of the media treatment. The result is similar to those reported earlier in *Coelogyne flaccida* (Kaur and Bhutani, 2013). George (1993) highlighted the importance of modifying the endogenous growth hormones ratio (auxin/cytokinin) by the application of exogenous growth regulators for the initiation of new shoots by activating the apical and lateral buds into shoots or inducting the multiple shoots from these meristematic areas. In this study, the responding explants, irrespective of the plant growth regulator treatments, invariably followed organogenetic pathway of regeneration i.e. shoot bud formation whereas the type of regeneration pathway which is to be followed by regenerants strongly depends upon the type and concentration of plant growth regulators to which they are subjected (Terzi and Schiavo,1990).

Conclusion

Therefore, from this study it is concluded that initiation of regeneration response is directly related to the juvenility of the explants and growth stimulus in the nutrient regime. *In vivo* sourced explants can be utilized to initiate cultures *in vitro*. A combination of M + BAP/Kn should be used to initiate, multiply and maintain the healthy plantlets. This simple one step protocol has the potential to mass propagate and conserve this rare orchid of medicinal and ornamental importance. Further focus of the current study is on acclimatization of *in vitro* raised cultures of *D. nobile* and restoring them back in their natural habitat. Thus, the simple protocol devised here will definitely contribute to the mass propagation and conservation of *D. nobile*.

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