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IN VITRO REGENERATION OF SHOOTS FROM NODAL EXPLANTS OF *DENDROBIUM CHRYSOTOXUM* LINDL.

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ABSTRACT

Transverse sections (2 mm thickness) of stem-nodes from *in vitro* raised seedlings had morphogenic potential on semisolid and liquid Murashige and Skoog medium supplemented with cytokinins N^6-benzyladenine (BA 4.44 μM), furfurylaminopurine (KIN 4.65 μM) and auxin α-naphthalene acetic acid (NAA 5.37 μM) individually and in combinations. The regeneration response was influenced by both the type of growth regulator and physical state of the medium. The explants produced either shoot buds on cytokinin-containing media or protocorm-like bodies (PLBs) on NAA containing media both solid and liquid. More *neo*-formations were produced on liquid media, especially those containing only NAA. They were formed at nodal and inter-nodal regions. The secondary buds were produced on the surface of primary PLBs. The plantlets were developed on MS medium containing banana homogenate 50 $g \cdot dm^{-3}$. The current study is the first ever report on successful regeneration of *Dendrobium chrysotoxum* from stem-node segments.

Key words: *Dendrobium chrysotoxum*, endangered species, species preservation, protocorm-like body, nodal explants

INTRODUCTION

Dendrobium chrysotoxum belongs to one of the largest group of angiosperms consisting of 25 000 species. It is an evergreen, epiphytic sympodial orchid species possessing spikes of 15-20 deep-yellow colored aromatic blossoms. The species grows in the tropical to sub-tropical climates at an altitude of 400-1000 meters in north-eastern Indian Himalayas, Bhutan, China, Thailand, and Laos in evergreen semi-deciduous forests (Shukla et al. 1998). *D. chrysotoxum* also known as golden orchid, and is very popular in floriculture market owing to its charismatic bright yellow-colored flowers. as cut-flower for floral arrangements, corsages, bouquets and as pot plants. The blossoms stay fresh for several days. Apart from its high floricultural value, the species is widely used for medicinal purposes. It is rich in biologically active chemical compounds showing potent antioxidant, immune stimulating, anti-hyperglycemic (Zhao et al. 2007), anti-angiogenic and anti-tumoral activity (Gong et al. 2004). Whole plants are used to extract cytotoxic compound, i.e. 1,4,5-trihydroxy-7-methoxy-9*H*-fluoren-9-one which is highly anti-carcinogenic (Chen et al. 2008). The fresh or dried stems of this species are used to treat loss of appetite with nausea and impaired vision (Yang et al. 2004).

Owing to its diverse worth (horticultural and therapeutic), entire plants of *D. chrysotoxum* are the target of massive commercial collections, which far exceeds their natural regeneration. As a result, its populations are vanishing swiftly in the wild and has become threatened. As a consequence, species is included in appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES 2017), along with other orchid species. Therefore, integrated multi-disciplinary approaches are required to save the ever-decreasing populations of *D. chrysotoxum*. Present efforts are a step forward to propagate the species through tissue culture techniques. *In vitro* technology offers

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an opportunity for the conservation of gene pools by mass propagating within a short time period. However, the micropropagation techniques for *D. chrysotoxum* at commercial level do not exist due to lack of efficient and reliable protocols with using different explants. The vegetative propagation through stem-cuttings in nursery beds is time-consuming and not even profitable. Moreover, seed-raised progenies are extremely heterozygous and express undesirable genetic variations in the populations. In this condition, use of alternative explants helps in raising innumerable genetically identical clones. So far, the regenerative potential of stem-nodes has been positively tested in some orchid species (Arditti & Ernst 1993; George & Ravishankar 1997; Vij & Kaur 1998; Kanjilal et al. 1999; Gangaprasad et al. 2000; Pyati et al. 2002; Decruse et al. 2003; Basker & Narmatha Bai 2006; Martin 2007; Zhao et al. 2007; Janarthanam & Seshadri 2008; Medina et al. 2009; Rangsayatorn 2009; Hong et al. 2010; Kaur & Bhutani 2010).Till date, *in vitro* propagation of *D. chrysotoxum* was reported only through seeds (immature/mature), shoot-tips and protocorm segments culture (Xu et al. 2001; Roy et al. 2007; Kaur & Bhutani 2011).The objective of this study was to access the regeneration competence of stem-nodes to multiply the *D. chrysotoxum* by subjecting them to varying physical and chemical stimulus in the medium.

MATERIALS AND METHODS

Dendrobium chrysotoxum plants were obtained from a commercial grower. The plants were replanted in the pots containing the epiphytic substrate such as charcoal, brick, and bark pieces in the ratio of 1 : 1 : 1. The top of the potting mix was covered with sphagnum moss to retain the moisture. The plants were maintained in the a greenhouse under natural light conditions with 70% relative humidity, and 25/20 °C day and night temperature. A voucher specimen (Herbarium number NIP – 155) has been deposited in the herbarium of Department of Natural Products Niper, Mohali, India.

The stem-node segments (uninodal, 2-3 mm thick), procured from 22 weeks old aseptic cultures (raised through asymbiotic seed germination technique) were used as explants. The segments were placed in the Murashige and Skoog (MS) (1962) agarised or in a liquid medium individually and supplemented with growth regulators such as cytokinins [6-benzylaminopurine (BA – 4.44μM) or furfurylaminopurine (KIN – 4.65 μM)], and auxin [α-naphthalene acetic acid (NAA – 5.37 μM)] (Hi-Media, Mumbai, India) individually and in combinations. The media were supplemented with 3 $g \cdot dm^{-3}$ sucrose (Hi-media, Mumbai, India) and gelled with 0.8% agar powder (Hi-Media, Mumbai, India).

D. chrysotoxum plantlets (2-3 cm in length) consisting of one to two nodes were sub-cultured on MS medium containing as organic growth supplement 50 g $dm^{-3} \cdot$(w/v) banana homogenate prepared according to Kaur & Bhutani (2014). The pH of the medium was adjusted to 5.7 after adding growth regulators and organic growth supplement. The agarised media were dispensed in the test tubes (25 × 150 mm) and liquid in 250 ml flasks (Borosil, India) and autoclaved at 121 °C at the pressure of 1.06 $kg \cdot cm^{-2}$ for 15 min. Sterile media were kept at 37 °C for 2 days to check any further contamination.

The cultures were incubated at 25 ± 2 ºC under 12 h photoperiod of 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light intensity provided by white fluorescent tubes (Fluorescent tubes; Philips India Ltd, Mumbai, India). The liquid cultures were rotated on an orbital shaker at 120 rpm. Eight replicates were used for each experiment. To check the reproducibility of the protocol, the experiments were repeated twice and data were compiled.

Observations and statistical analysis

The experiment was designed following complete randomized design (CRD) with eight replicates per treatment. The effect of medium composition on percentage of regeneration, time of initiation of regeneration response, average number of regenerates induced per explant, and time taken in weeks to form complete plantlets was tested applying Tukey's multiple comparison test ($P \leq 0.05$) in one way ANOVA to separate of significantly different groups. The statistical analyses were performed using the SPSS (version 17) software package. (SPSS Inc., Chicago, USA).The results are expressed as mean ± SD of eight replicates.

Histological studies

Histological studies were conducted to trace the origin of the meristemoids. Free hand sections of the responding segments were cut by placing them in a potato pith. Very thin sections, which were able to float on the surface of water, were selected. They were stained with safranine and observed under stereoscopic microscope (Nikon, H600L, Japan).The photographs were taken using a digital camera (Nikon Digital Sight, DS, Ri1 Nikon Corporation, Japan).

RESULTS AND DISCUSSION

The percentage of regeneration, nature of regenerants and their number varied with the type of growth regulator and physical state of the medium. On the agarised media regenerated from 25 to 75% of explants (Table 1), whereas regeneration in liquid medium was from 25 to 100% (Table 2). A survey of literature reveals that liquid cultures are more efficient in inducing proliferations of *neo*-formations (Levin et al. 1997; Ziv et al. 1998). In comparison to the solid medium, in the liquid explants stay in close contact with the medium which makes it possible for the uptake of nutrient components more effectively through a whole surface and leads to enhanced shoot and root growth (Smith & Spomer 1994; Sandal et al. 2001). Moreover, continuous shaking leads to mechanical separation of neo-formations usually developed as clusters (Levin et al. 1997; Ziv et al. 1998). Liquid-agitated cultures are more efficient in promoting growth and multiplication of the shoots by enhancing forced aeration thus providing sufficient oxygen supply to the tissues and nutrient uptake as earlier indicated by Puchooa (2004) in *Dendrobium* and *Malaxis acuminata* (Kaur & Bhutani 2010). In this study, also comparison was made between liquid-agitated medium and liquid static medium of the same composition but it was not included to the presented experiment. In this comparison liquid agitated medium supported better regeneration percentage as compared to liquid static medium.

Table 1. *In vitro* regeneration response of stem-node segments of *D. chrysotoxum* in MS agarised medium and its combinations with growth regulators

Additives	Regeneration response (%)	Initiation of response (wk)	Number of regenerates	Development of plantlets (wk)
NAA	50.00 ± 0.57^{b}	2.50 ± 0.14^{a}	$3.25 \pm 0.50^{d*}$	12.15 ± 0.19^{a}
BA	25.00 ± 0.50^{a}	7.17 ± 0.17^{cd}	$1.75 \pm 0.50^{b\dagger}$	17.65 ± 0.23^{c}
KIN	75.00 ± 0.50^{c}	4.17 ± 0.17^{b}	$2.00 \pm 0.00^{bc\dagger}$	13.57 ± 0.20^{bc}
BA + NAA	25.00 ± 0.00^{a}	6.05 ± 0.12^{bc}	$2.00 \pm 0.00^{bc\dagger}$	13.47 ± 0.09^{bc}
KIN + NAA	25.00 ± 0.00^{a}	7.05 ± 0.17^{cd}	$1.00 \pm 0.00^{a\dagger}$	19.10 ± 0.08^{cd}

Note: BA – 4.44 μM, KIN – 5.65 μM, NAA – 5.37 μM,* protocorm-like bodies, † = shoot buds. 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 stem-node segments of *D. chrysotoxum* in MS liquid (agitated) medium and its combinations with growth regulators

Additives	Regeneration response (%)	Initiation of response (wk)	Number of regenerates	Development of plantlets (wk)
NAA	100.00 ± 0.00^{d}	2.02 ± 0.00^{a}	$20.70 \pm 0.00^{d*}$	9.00 ± 0.00^{a} (PLBs were transferred to agar-gelled MS medium containing NAA and fortified with banana homogenate (50 $g \cdot dm^{-3}$)
BA	25.00 ± 0.50^{a}	6.90 ± 0.00^{c}	$3.75 \pm 0.00^{bc\dagger}$	18.00 ± 0.91^{d}
KIN	25.00 ± 0.50^{a}	7.15 ± 0.19^{d}	$1.75 \pm 0.50^{a\dagger}$	12.07 ± 0.81^{b}
BA + NAA	50.00 ± 0.57^{bc}	4.00 ± 0.00^{bc}	$1.25 \pm 0.50^{a\dagger}$	13.00 ± 0.11^{bc}
KIN + NAA	37.45 ± 0.49^{b}	3.77 ± 0.48^{bc}	$2.50 \pm 1.00^{b\dagger}$	18.31 ± 0.00^{d}

Note: see Table 1

The addition of growth regulators to the medium was obligatory for the initiation of regeneration in this study. These results are in contrast with those reported earlier in *Malaxis acuminata* (Kaur & Bhutani 2010) and *C. flaccida* (Kaur & Bhutani 2013) where the explants regenerated readily in the basal medium.

The kind and number of neo-formations in the segments depended on the growth regulator added to a medium. On the media containing cytokinin, either agarised and liquid only shoot buds were formed (Fig. 1b). On the media both agarised and liquid, supplemented with NAA, only protocorm like bodies (PLB) were formed (Table 1 & 2). PLBs formed earlier in liquid than in solid media.

In the agar-gelled medium, NAA alone stimulated regeneration response within 2.5 weeks of culture in 50% initial explants at axillary and extra-axillary (adventive) position (Fig. 1a). In liquid MS medium NAA proved highly productive in eliciting 100 per cent regeneration inducing maximum number of supernumerary loci at node and cut surfaces within 2 weeks of culture (Figs. 1c, d). On this medium a maximum of 20 PLBs per explants were regenerated whereas on solid medium only 3.75. The *neo*-formations multiplied covering almost entire segment. The PLBs did not differentiate if left in the liquid medium and started turning hyper hydrous. Therefore, immediate transfer to agarised medium of respective combination containing banana homogenate was done. After another 3 weeks of culture, PLBs multiplied profusely transforming into shoots (Figs. 1e, f). These shoots were separated and transferred to another vessel containing fresh medium where they formed into plantlets. After 4 weeks of culture, small globular masses were noticed developing at the base of the shoots (Fig. 1g). Also, PLB multiplication was also observed through budding at the surface of preformed PLB (Fig. 1h). The shoots rooted well in the same medium and developed into robust plantlets after 9 weeks of culture (Fig. 1i). The results are in accord with similar earlier findings in *Dendrobium longicornu* where on the shoot segments PLB-mediated regeneration occurred (Dohling et al. 2012). The suitability of auxins for PLB induction has earlier been reported in stem-node segments in *Dendrobium moschatum* (Kanjilal et al. 1999).

Among cytokinins, kinetin proved beneficial in inducing maximum shoot buds in 75% of explants (Table 1) through bud break (Fig. 1b). They grew into small protuberances and developed into shoots. Plantlets were formed after another 9 weeks of culture, similar to those reported earlier in *Dendrobium longicornu* cultures, in which the responding segments followed shoot bud-mediated regeneration (Vij et al. 2000; Košir et al. 2004; Dohling et al. 2012).

The combinations BA and KIN with NAA could initiate regeneration response only in 25% segments after 6 and 7 weeks of culture respectively in semisolid medium. In liquid medium, NAA with BA could enhance the regeneration frequency up to 50 per cent (Table 2).

In an earlier study, also the combination of NAA with BAP induced shoot buds in *Malaxis acuminata* stem-nodes (Vij & Kaur 1998), *Phalaenopsis* and *Doritaenopsis* (Tokuhara & Mii 1993). According to Roy & Banerjee (2003) the combination of auxin and cytokinin invoke shoot buds in *Dendrobium fimbriatum* var. *occulatum*. Cytokinins remained less effective as they could initiate response in 25 per cent of the segments by inducing single shoots per explant after 6 and 7 weeks of culture. The segments in static liquid MS medium and its combinations with growth regulators turned brown whereas those in liquid-agitated medium, responded to regeneration.

The histological observations of the responding explant revealed meristemoids to be hypodermal in origin. The cells with dense cytoplasm and conspicuous nuclei were involved in formation of the globules (Fig 2a), which is an important characteristic feature of the embryogenic cells as earlier studied in many species belonging to genus *Allium cepa* (Eady et al. 1998); *Lycium barbarum* (Li et al. 2001); *Agave sisalana* (Nikam et al. 2003) and in orchid *Malaxis acuminate* (Kaur & Bhutani 2010). These globules gradually grew in size, having no vascular connections with the surrounding peripheral tissues and eventually transformed into PLBs. The PLBs proliferated further to produce secondary PLBs as reported earlier (Zhao et al. 2008).

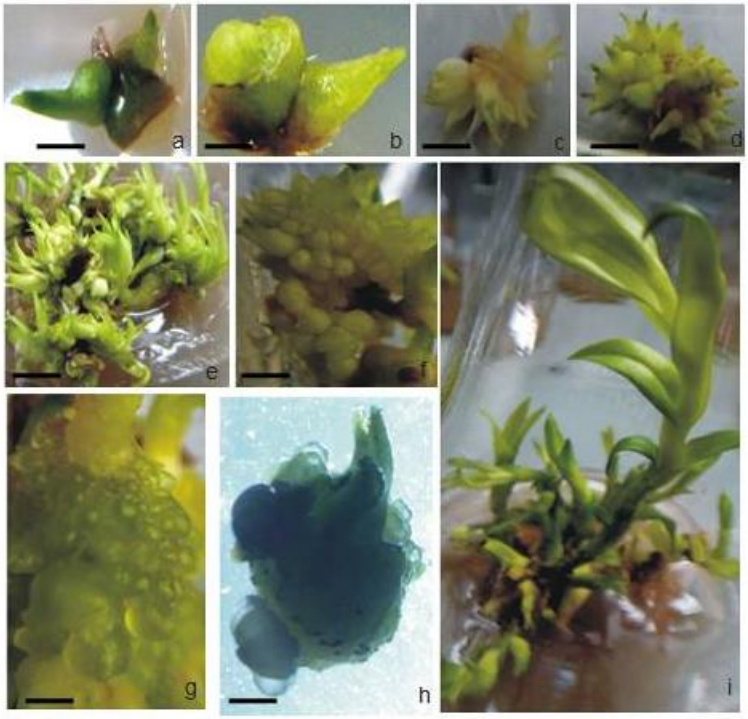

Fig. 1. *In vitro* propagation of *Dendrobium chrysotoxum* from stem-node segments in MS medium with growth regulators: (a) PLBs development in the segments at axillary and extra axillary position (adventive) in MS agar + NAA medium after 2.5 weeks (bar = 3.82 mm), (b) shoot bud development in MS + BA agar medium after 7.0 weeks (bar = 5.53 mm), (c, d**)** formation of PLBs in MS liquid + NAA medium at the cut surface after 2 weeks (bar = 6.62 mm), (e) growth of shoot buds from PLBs in MS agar + NAA medium + banana homogenate (50 $g \cdot dm^{-3}$) after 5 weeks (bar = 5.25 m), (f) development of globular PLBs or somatic embryos at the base of plantlets in MS agar + NAA medium + banana homogenate (50 $g \cdot dm^{-3}$) after 6 weeks (bar = 5.82 mm), (g) PLBs multiplication through budding at the surface (bar = 5.95 mm), (h) development of shoots in the cultures (bar = 4.51 mm), (i) development of healthy plantlets after 9 weeks

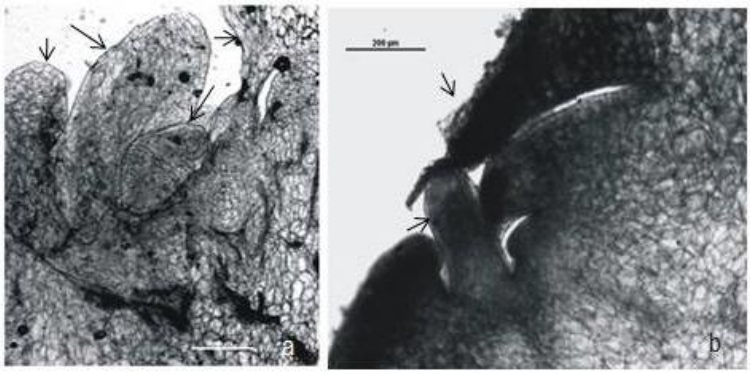

Fig. 2. Histological studies: (a) globular structures developed onto PLBs (arrow) (bar = 200 µm), (b) shoot apex with leaf primordial (arrow) (bar = 200 µm)

Literature survey also indicates that somatic embryogenesis is an initial step of PLB regeneration in orchids (Huang et al. 2004). On the other hand, the cells divided mitotically and formed meristematic zones that developed into prominent epidermal bulges (Fig. 2a) and subsequently formed shoot bud. Shoot apex was clearly evident (Fig 2b) as similar to that observed earlier in *Bulbophyllum careyanum* (Vij et al. 2000) and *Malaxis acuminata* (Kaur & Bhutani 2010). In the segments, initiation of *neo*-formations was observed as direct organogenesis process at the nodal and extra-nodal regions. In this

study, the explants underwent both PLB and shoot buds mediated pathways of regeneration. The induction of several meristematic clusters composed of densely packed meristematic cells, forming new meristemoids on the outer surface of the explants is already on records (Ziv et al. 1998; Young et al. 2000). Arditti (1980) indicated the advantages of utilizing organic growth supplements, such as banana homogenate, coconut water and peptone on promoting growth, differentiation of protocorms, and seedling development. In the present study, 50 $g \cdot dm^{-3}$ banana homogenate supported healthy (robust) growth of plantlets. Similar effects was also reported for other orchids – *Dendrobium* and *Spathoglottis kimballiana* (Minea et al. 2004), *Coelogyne flaccida* (Kaur & Bhutani 2014) and *Dendrobium chrysotoxum* initiated from seeds (Kaur & Bhutani 2011). The promotory effect of Bh could be related to high content of carbohydrates in bananas.

Further focus will be on the acclimatization of *in vitro* raised plantlets of *D. chrysotoxum* and restoring these in their natural habitat. Thus, an easy protocol devised here can contribute to the mass propagation and *ex-situ* conservation of *D. chrysotoxum.*

CONCLUSION

Liquid-agitated MS medium supplemented with NAA caused PLB formation on nodal slices of *D. chrysotoxum.* PLBs developed into robust plantlets on agarised MS medium containing banana homogenate. Addition of cytokinin to MS medium caused shoot bud formation but this process was less effective. The protocol presented can be used for conserving this endangered and medicinally important orchid species.

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