



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 N⁶-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·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-9H-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 – 4.44 μM) 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·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·(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·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 °C under 12 h photoperiod of 40 μmol·m⁻²·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 safranin 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 ± 0.50 ^{d*} | 12.15 ± 0.19 ^a |
| BA | 25.00 ± 0.50 ^a | 7.17 ± 0.17 ^{cd} | 1.75 ± 0.50 ^{b†} | 17.65 ± 0.23 ^c |
| KIN | 75.00 ± 0.50 ^c | 4.17 ± 0.17 ^b | 2.00 ± 0.00 ^{bc†} | 13.57 ± 0.20 ^{bc} |
| BA + NAA | 25.00 ± 0.00 ^a | 6.05 ± 0.12 ^{bc} | 2.00 ± 0.00 ^{bc†} | 13.47 ± 0.09 ^{bc} |
| KIN + NAA | 25.00 ± 0.00 ^a | 7.05 ± 0.17 ^{cd} | 1.00 ± 0.00 ^{a†} | 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 ± 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·dm ⁻³)) |
| BA | 25.00 ± 0.50 ^a | 6.90 ± 0.00 ^c | 3.75 ± 0.00 ^{bc†} | 18.00 ± 0.91 ^d |
| KIN | 25.00 ± 0.50 ^a | 7.15 ± 0.19 ^d | 1.75 ± 0.50 ^{a†} | 12.07 ± 0.81 ^b |
| BA + NAA | 50.00 ± 0.57 ^{bc} | 4.00 ± 0.00 ^{bc} | 1.25 ± 0.50 ^{a†} | 13.00 ± 0.11 ^{bc} |
| KIN + NAA | 37.45 ± 0.49 ^b | 3.77 ± 0.48 ^{bc} | 2.50 ± 1.00 ^{b†} | 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 acuminata* (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).

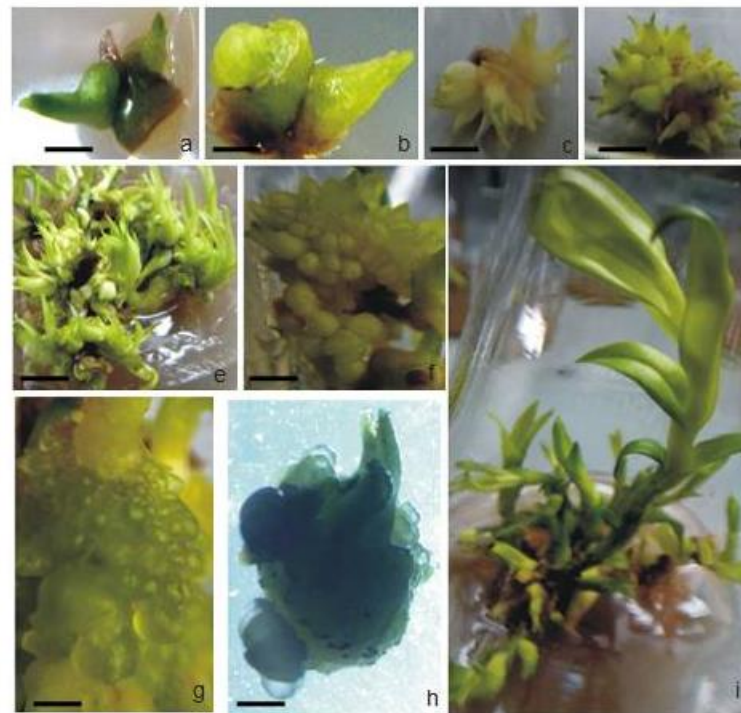


Fig. 1. *In vitro* propagation of *Dendrobium chrysotoxum* from stem-node segments in MS medium with growth regulators: (a) PLBs development in the segments at axillary and extra axillary position (adventive) in MS agar + NAA medium after 2.5 weeks (bar = 3.82 mm), (b) shoot bud development in MS + BA 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 ($50 \text{ g} \cdot \text{dm}^{-3}$) 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 ($50 \text{ g} \cdot \text{dm}^{-3}$) 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

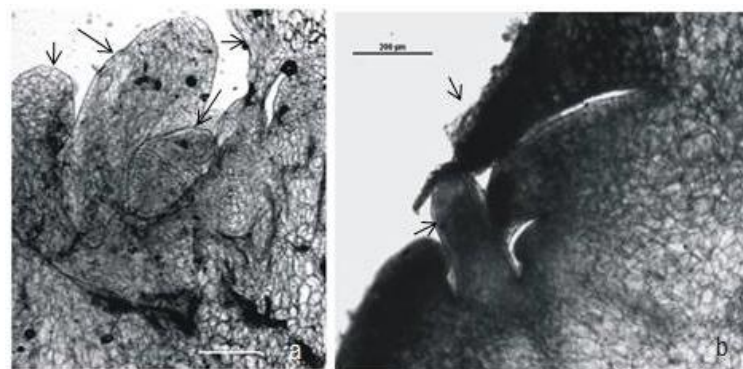


Fig. 2. Histological studies: (a) globular structures developed onto PLBs (arrow) (bar = 200 μm), (b) shoot apex with leaf primordia (arrow) (bar = 200 μm)

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·dm⁻³ banana homogenate supported healthy (robust) growth of plantlets. Similar effects was also reported for other orchids – *Dendrobium* and *Spathoglottis kimbaliiana* (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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REFERENCES

- Arditti J. 1980. Aspects of physiology of orchids. *Advances in Botanical Research* 7: 421–655 DOI: 10.1016/S0065-2296(08)60091-9.
- Arditti J., Ernst R. 1993. *Micropropagation of orchids*. John Wiley and Sons, New York, USA, 682 p.
- Basker S., Narmatha Bai V. 2006. Micropropagation of *Coelogyne stricta* (D. Don) Schltr. via pseudobulb segment cultures. *Tropical and Subtropical Agroecosystems* 6: 31–35.
- Chen Y., Li Y., Qing C., Zhang Y., Wang L., Liu Y. 2008. 1,4,5-Trihydroxy-7-methoxy-9H-fluoren-9-one, a new cytotoxic compound from *Dendrobium chrysotoxum*. *Food Chemistry* 108: 973–976. DOI: 10.1016/j.foodchem.2007.12.007.
- CITES 2017. Convention on International Trade in Endangered Species of Wild Fauna and Flora, Appendices I and II. <http://www.cites.org/eng/app/appendices.php>
- Decruse S.W., Gangaprasad A., Seeni S., Menon S.V. 2003. Micropropagation and ecorestoration of *Vanda spathulata*, an exquisite orchid. *Plant Cell, Tissue and Organ Culture* 72: 199–202. DOI: 10.1023/A:1022267009531.
- Dohling S., Kumaria S., Tandon P. 2012. Multiple shoot induction from axillary bud cultures of the medicinal orchid, *Dendrobium longicornu*. *AoB Plants*, Pls 032. DOI: 10.1093/aobpla/pls032.
- Eady C.C., Butler R.C., Suo Y. 1998. Somatic embryogenesis and plant regeneration from immature embryo cultures of onion (*Allium cepa* L.). *Plant Cell Reports* 18: 111–116. DOI: 10.1007/s002990050541.
- Gangaprasad A., Latha P.G., Seeni S. 2000. Micropropagation of terrestrial orchids, *Anoectochilus sikkimensis* and *Anoectochilus regalis*. *Indian Journal of Experimental Biology* 38: 149–154.
- George P.S., Ravishankar G.A. 1997. *In vitro* multiplication of *Vanilla planifolia* using axillary bud explants. *Plant Cell Reports* 16: 490–494. DOI: 10.1007/BF01092772.
- Gong Y.-Q., Fan Y., Wu D.-Z., Yang H., Hu Z.-B., Wang Z.-T. 2004. *In vivo* and *in vitro* evaluation of erianin, a novel anti-angiogenic agent. *European Journal of Cancer* 40: 1554–1565. DOI: 10.1016/j.ejca.2004.01.041.
- Hong P.-I., Chen J.-T., Chang W.-C. 2010. Shoot development and plant regeneration from protocorm-like bodies of *Zygopetalum mackayi*. *In Vitro Cellular and Developmental Biology – Plant* 46: 306–311. DOI: 10.1007/s11627-009-9262-2.
- Huang P.L., Doy Y., Jheng F.Y. 2004. Research and development on the establishment of transformation systems for *Phalaenopsis* orchids. *Proceedings of 8th Asia Pacific Orchid Conference*, Tainan, Taiwan Orchid Growers Association, pp. 416–426.

- Janarthanam B., Seshadri S. 2008. Plantlet regeneration from leaf derived callus of *Vanilla planifolia* Andr. In *Vitro Cellular and Developmental Biology – Plant* 44: 84–89. DOI: 10.1007/s11627-008-9123-4.
- Kanjilal B., De Sarker D., Mitra J., Datta K.B. 1999. Stem disc culture: development of a rapid mass propagation method for *Dendrobium moschatum* (Buch.-Ham.) Swartz – an endangered orchid. *Current Science* 77: 497–500.
- Kaur S., Bhutani K.K. 2010. Micropropagation of *Malaxis acuminata* D. Don: a rare orchid of high therapeutic value. *Open Access Journal of Medicinal and Aromatic Plants* 1: 29–33.
- Kaur S., Bhutani K.K. 2011. *In vitro* propagation of *Dendrobium chrysotoxum* (Lindl.). *Floriculture and Ornamental Biotechnology* 5: 50–56.
- Kaur S., Bhutani K.K. 2013. *In vitro* mass propagation of ornamentally and medicinally important *Coelogyne flaccida* Lindl. through pseudobulb segments. *Plant Tissue Culture and Biotechnology* 23: 39–47. DOI: 10.3329/ptcb.v23i1.15558.
- Kaur S., Bhutani K.K. 2014. *In vitro* conservation and asymbiotic propagation of *Coelogyne flaccida* (Lindl.) – a threatened orchid. *Plant Biosystems* 148: 935–944. DOI: 10.1080/11263504.2013.801368.
- Košir P., Škof S., Luthar Z. 2004. Direct shoot regeneration from nodes of *Phalaenopsis* orchids. *Acta Agriculturae Slovenica* 83: 233–242.
- Levin R., Stav R., Alper Y., Watad A.A. 1997. A technique for repeated non-axenic subculture of plant tissues in a bioreactor on liquid medium containing sucrose. *Plant Tissue Culture and Biotechnology* 3: 41–45.
- Martin K.P. 2007. Micropropagation of the bamboo orchid (*Arundina graminifolia* (D. Don) Hochr.) through protocorm-like bodies using node explants. *Propagation of Ornamental Plants* 7: 97–100.
- Medina R.D., Flachsland E.A., Gonzalez A.M., Terada G., Faloci M.M., Mroginski L.A. 2009. In vitro tuberization and plant regeneration from multinodal segment culture of *Habenaria bractescens* Lindl., an Argentinean wetland orchid. *Plant Cell, Tissue and Organ Culture* 97: 91–101. DOI: 10.1007/s11240-009-9505-2.
- Minea M., Piluek C., Menakanit A., Tantiwiwat S. 2004. A study on seed germination and seedling development of *Spathoglottis* Bl. orchids. *Kasetsart Journal – Natural Science* 38: 141–156.
- Murashige T., Skoog F. 1962. A revised medium of rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum* 15: 473–497. DOI: 10.1111/j.1399-3054.1962.tb08052.x.
- Nikam T.D., Bansude G.M., Aneesh Kumar K.C. 2003. Somatic embryogenesis in sisal (*Agave sisalana* Perr. ex. Engelm). *Plant Cell Reports* 22: 188–194. DOI: 10.1007/s00299-003-0675-9.
- Puchooa D. 2004. Comparison of different culture media for the *in vitro* culture of *Dendrobium* (Orchidaceae). *International Journal of Agriculture and Biology* 6: 884–888.
- Pyati A.N., Murthy H.N., Hahn E.J., Paek K.Y. 2002. *In vitro* propagation of *Dendrobium macrostachyum* Lindl. – a threatened orchid. *Indian Journal of Experimental Biology* 40: 620–623.
- Rangsayatorn N. 2009. Micropropagation of *Dendrobium draconis* Rchb. f. from thin cross-section culture. *Scientia Horticulturae* 122: 662–665. DOI: 10.1016/j.scienta.2009.06.037.
- Roy J., Banerjee N. 2003. Induction of callus and plant regeneration from shoot-tip explants of *Dendrobium fimbriatum* Lindl. var. *oculatum* Hk. f. *Scientia Horticulturae* 97: 333–340. DOI: 10.1016/S0304-4238(02)00156-5.
- Roy J., Naha S., Majumdar M., Banerjee N. 2007. Direct and callus-mediated protocorm-like body induction from shoot-tips of *Dendrobium chrysotoxum* Lindl. (Orchidaceae). *Plant Cell, Tissue and Organ Culture* 90: 31–39. DOI: 10.1007/s11240-007-9244-1.
- Sandal I., Bhattacharya A., Ahuja P.S. 2001. An efficient liquid culture system for tea shoot proliferation. *Plant Cell, Tissue and Organ Culture* 65: 75–80. DOI: 10.1023/A:1010662306067.
- Shukla B.K., Singh P., Chauhan A.S. 1998. Orchid diversity in Sikkim and its conservation. *Journal of the Orchid Society of India* 12: 53–76.
- Smith M.A.L., Spomer L.A. 1994. Vessels, gels, liquid media, and support systems. In: Aitken-Christie J. et al. (Eds.). *Automation and Environmental Control in Plant Tissue Culture*, Kluwer Academic Publishers, Dordrecht, pp. 371–404. DOI: 10.1007/978-94-015-8461-6_16.
- Tokuhara K., Mii M. 1993. Micropropagation of *Phalaenopsis* and *Doritaenopsis* by culturing shoot tips of flower stalk buds. *Plant Cell Reports* 13: 7–11. DOI: 10.1007/BF00232306.
- Vij S.P., Kaur P. 1998. Micropropagation of therapeutically important orchids: *Malaxis acuminata* D. don. *Journal of the Orchid Society of India* 12: 89–93.
- Vij S.P., Kher A., Pathak P. 2000. Micropropagation of *Bulbophyllum careyanum* (Hook.) Spreng. using pseudobulb segments. *Journal of the Orchid Society of India* 14: 47–56.

- Xu H., Liu J., Wang Z-T., Xu D-R., Ding J-Y. 2001. Studies on tissue culture of *Dendrobium chrysotoxum* Lindl *in vitro*. *Zhongguo Zhong Yao Za Zhi* 26: 378–381. [in Chinese with English abstract]
- Yang H., Chou G-X., Wang Z-T., Guo Y-W., Hu Z-B., Xu L-S. 2004. Two new compounds from *Dendrobium chrysotoxum*. *Helvetica Chimica Acta* 87: 394–399. DOI: 10.1002/hlca.200490037.
- Young P.S., Murthy H.N., Paek K.Y. 2000. Mass multiplication of protocorm-like bodies using bioreactor system and subsequent plant regeneration in *Phalaenopsis*. *Plant Cell, Tissue and Organ Culture* 63: 67–72. DOI: 10.1023/A:1006420116883.
- Zhao Y., Son Y.-O., Kim S.-S., Jang Y.-S., Lee J.-C. 2007. Antioxidant and anti-hypoglycemic activity of polysaccharide isolated from *Dendrobium chrysotoxum* Lindl. *Journal of Biochemistry and Molecular Biology* 40: 670–677. DOI: 10.5483/bmb-rep.2007.40.5.670.
- Zhao P., Wu F., Feng F-S., Wang W-J. 2008. Protocorm-like body (PLB) formation and plant regeneration from the callus culture of *Dendrobium candidum* Wall ex Lindl. *In Vitro Cellular and Developmental Biology – Plant* 44: 178–185. DOI: 10.1007/s11627-007-9101-2.
- Ziv M., Ronen G., Raviv M. 1998. Proliferation of meristematic clusters in disposable presterilized plastic bioreactors for large-scale micropropagation of plants. *In Vitro Cellular and Developmental Biology – Plant* 34: 152–158. DOI: 10.1007/BF02822781.