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Research Article – Botany

In vitro studies on *Dendrobium fimbriatum* Hk.F – An endangered orchid

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Abstract

A protocol for *in vitro* propagation from protocorms of *Dendrobium fimbriatum* a distributed in all over world and highly appreciated as an ornamental, was developed. Two different explants, entire protocorms and longitudinal halves of protocorms, were used. In addition, the effect of two different culture media, Murashige and Skoog (MS) and modified Knudson (KCm), supplemented with N6- benzyladenine (BA) (0, 0.5, 1, 2, 3, and 5 mg_L–1) and/or a-naphthalene acetic acid (NAA) at 0, 0.1, and 0.5 mg_L–1 was investigated. Adventitious shoot formation by direct organogenesis was obtained in all treatments; in some cases, the formation of protocorm like bodies was induced. Shoot formation was greater for entire protocorms; the best treatment was MS medium containing at BA 1 to 2 mg_L–1 in combination with at NAA 0.1 mg_L–1. The average height of shoots was three times greater in MS medium than in KC m medium. Sub culturing individual shoots in MS medium without plant growth regulators, but with 1 g_L–1 activated charcoal, allowed further development and rooting. An *ex vitro* survival rate of almost 100% was achieved. This study represents a comprehensive application for propagation, conservation, and sustainable use of this valuable natural resource

Key words: *In vitro*, propagation medium, *Dendrobium fimbriatum*

Introduction

Orchidaceae is the most species-rich plant family in the world, with estimate 17500 to 35000 species (Maridass, 2006). Orchids can be found from the tropics to the arctic regions, as epiphytes (typically tropical), terrestrials (typically temperate), and even include subterranean forms (Cribb *et al.,* 2003). Orchids are experiencing a steady decline in tropical countries due to the destruction of natural forest areas. A large proportion of orchid species are rare and endangered as a direct or indirect result of human activities, including collection, habitat destruction and degradation, and loss of pollinators and fungal partners (Batty *et al.,* 2002; Cribb *et al.,* 2003; Zettler *et al.,* 2003). The greatest potential for loss of orchid species is in the tropics, where species diversity is the highest, and this is the area in which orchids have been studied least (Batty *et al.,* 2002; Cribb *et al.,* 2003). However, orchids also exhibit considerable diversity and many species are threatened. Although there has been extensive research on these species (Batty *et al.,* 2002;Cribb *et al.,* 2003), much more research work is needed before they can be effectively conserved.It is essential to take measures for the conservation and propagation of these endangered orchid species. Hence, *In vitro* propagation of endangered plants can offer considerable benefits for the rapid cultivation of species that are at risk, that have limited reproductive capacity and exist in threatened habitats (Fay, 1992). *In vitro* propagation methods are essential components of plant genetic resource management and they are becoming increasingly important for conservation of rare and endangered plant species (Sudha *et al.,*1998; Benson *et al.,* 2000; Iankova *et al.,* 2001; Bhatia *et al.,* 2002).The *Dendrobium fimbriatum* is the second largest genus of Orchidaceae, which is composed of approximately 1500 species scattered in the world, and there is the most popular and highly valued orchids in the market (Chen and Ji, 1998). *In vitro* propagation of *Dendrobium fimbriatum*. sp has been investigated with variable results depending on the tissue culture techniques, the species or cultivar and explants as a source. In micropropagation, various parts of the plant such as shoot tip, flower stalk, root, and leaf have been utilized as a explants (Yasugi and Shinto, 1994). A tissue culture procedure for clonal propagation of *Dendrobium fimbriatum*. was developed by Morel in 1960. There is no information about regarding micropropagation of wild *Dendrobium fimbriatum*. sp. Moreover, for mass propagation of Orchid, protocorm propagation from seed and/or nodal explants of adult plants was an effective method, and it was superior to shoot tip on account of an exponential propagation rate (Wang *et al.,*1995). The present study has resulted in high frequency of shoot multiplication and rooting, starting from explants obtained from wild population of *Dendrobium fimbriatum*. in the South India and the micropropaged explants were successfully re introduced into their natural habitat.

Materials and Methods

Collection of explants

Dendrobium fimbriatum was collected in yercaud regionTamilnadu and was used as source of explants. This was maintained under standard greenhouse conditions

General Description of Dendrobium fimbriatum

The most commonly cultivated form of *Dendrobium fimbriatum*, variety *oculata* is distinguished by its maroon-blotched floral lip. This long-stemmed, semi-deciduous orchid is native to humid temperate upland forests from India to Vietnam. An epiphyte, it typically grows on tree trunks and rock ledges in the wild. This frost-tender

perennial bears leathery, lance-shaped leaves on long, erect to arching, spindle-shaped stems. Yellow to orange blooms with fringed, darker-hued lips appear in drooping, 6- to 15-flowered clusters from late winter to late spring. The lip has two maroon-brown blotches at its center. The flowers are very fragrant. Sporadic rebloom may occur. Fringed *Dendrobium* requires partial shade, mild summers, and relatively cool, dry winters. Plant it in a hanging basket or wall plaque in a coarse, highly organic potting mix, or fix it to a tree limb. Water freely from spring to fall, and sparingly in winter. It makes an excellent subject for cool greenhouses and frost-free gardens, combining well with bromeliads and other orchids.

Sterilization

Seed sterilization seeds of *O. tigrinum*, produced through hand-pollination, were obtained from the Francisco Javier Clavijero Botanical Garden, Xalapa, Veracruz. After ripe capsules opened, the seeds were collected and stored in paper envelopes for 15 d at 5 °C before use. Seeds were surface-sterilized by placing them in a 5-mL sterile syringe; 5 ml of 3% hydrogen peroxide (Kurak_) was added and the syringe was shaken vigorously for 10 min. Three milli liters of hydrogen peroxidewas then decanted; one to two drops of hydrogen peroxide (containing seeds) were introduced into a 125-mL baby food jar containing 25 mL of medium. Two media were used: 1) inorganic salts and vitamins of MS medium (Murashige and Skoog, 1962) augmented with 2 mg_L^{-1} glycine, 100 mg_L^{-1} myo-inositol, and 30 g_L^{-1} sucrose (MS medium); and 2) the same basal medium but with 1 g_L^{-1} activated charcoal added. The pH of each culture medium was adjusted to a pHof 5.7 ± 0.1 with 0.1NNaOH and 0.1NHCl before adding 8 g_L^{-1} agar (plant TC/ micropropagation grade; Phytotechnology Laboratories _), then autoclaving at 1.2 kg_cm–2 and 120 _C for 17 min. All cultures were incubated in a growth chamber at 25 ± 1 °C under a 16-h photoperiod provided by cool white fluorescent lamps (50 mmol_m^{-2}_s^{-1}). Induction stage. Germinated protocorms with a diameter of 2 to 3 mm were selected as explants. Two kinds of explants were used: entire protocorms and longitudinal halves of protocorms. The explants were transferred to two different treatment media: 1) MS medium; and 2) modified KC medium (Knudson, 1946) augmented with the micronutrients used in MS medium (KCm). Both media were supplemented with a combination of BA at concentrations of 0, 0.5, 1, 2, 3, or 5 mg_L^{-1} and NAA at concentrations of 0, 0.1, or 0.5 mg_L^{-1}. There were 20 replicate jars with three explants per jar (i.e., 60 explants per treatment). The longitudinal halves of protocorms were cultured with the wound surface in contact with culture media. The induction period was 90 days. The young rhizomes were disinfected with 70% ethanol for 30 sec followed by surface sterilization with 3% sodium hypochlorite (supplemented 2–3 drops of Tween 80 each 500 ml) for 20 min and then washed for 4–5 times in sterile distilled water. The rhizome was cut into small pieces (5mm), and the pieces were washed with sterile water.

Culture

The explants were placed onto cultured on the basal media of MS medium, (Murashige and Skoog,1962), supplemented with various concentrations of auxins, NAA (naphthalene acetic acid), IAA (indole acetic acid), IBA (indole butyric acid) and cytokinins BAP (benzyl-amino purine) and kinetin. The auxins concentrations were 0.5, 1.0,2.0,5.0,10.0 and 2.0μM/L, while the cytokinins were 0.5, 1.0,2.0,5.0,10.0 and 2.0μM/L. The pH of all media were adjusted to 5.8 with 0.1*M* KOH before sterilization. All media with 0.7% agar and 2% sucrose were autoclaved at 121°C for 20 min. The explants were cultured in 220 ml glass jars containing 30ml medium, which were closed with semi permeable plastic caps. All the cultures were kept in the culture room at 26 °C and 10/14h photoperiod under 1000-2000 lux light intensity. Regenerating shoots of 2–2.5cm in height were separated from calli and cultured onto solid rooting medium for vigorous growth and rooting. After 30 days of culture, these were well rooted and shoots were transferred to pots and shifted green house of 25°C with low natural light, and with 95% relative humidity. These plantlets were acclimatized in outside conditions for 15days. The rooted plantlets (3-5cm in height) were washed with water to remove residual media, and dipped into 50% 800× carbendazim for 15min, then they were directly transplanted to substrate viz. vermiculite, sawdust and humus: sawdust (1:1) under high relative humidity. Survival rate was recorded after 30 days of transplantation

Statistical analysis

Experiments design was thoroughly randomized and they were repeated three times. Every treatment had three replications. Observations on the number and height of shoots were recorded after 30 days of culture. All the growth of roots and shoots length values are statistically (mean ± error) calculated by SPSS- 11.0 version.

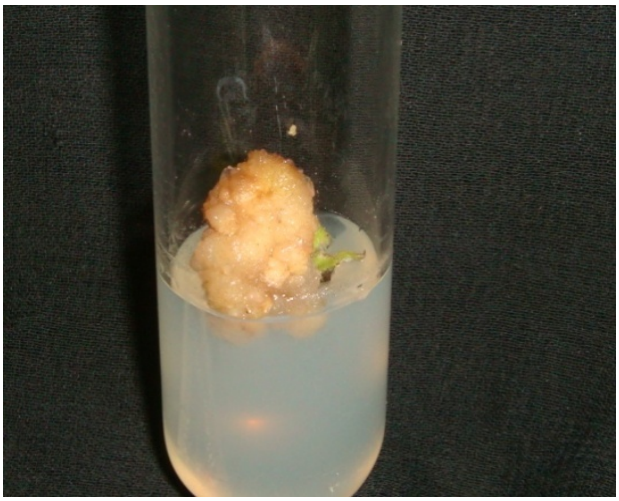

Fig. 1. Explant of and shoot and root formation of *Dendrobium fimbriatum*

Results and discussion

The seeds of *D. nobile* germinated within 7 weeks of culture in nutrient medium. The protocorms differentiated into seedlings after 4 weeks of germination (Fig. 1). Clonal propagation through PLBs or shoot buds from different explants of dendrobes has been used for the propagation protocols. In such cases, PLB induction under controlled conditions has been used to study the influence of abiotic and biotic factors (Teixeira da Silva *et al.,* 2006), medium constituents, carbon source, PGR, etc. (Kishor and Devi, 2009; Nasiruddin *et al.,* 2003). In the present study, TDZ incorporated in MS medium was used for PLB induction and subsequent organogenesis in the segments of pseudostem

explants (Table 1). Depending upon the concentrations of TDZ in the medium, the pseudostem explants produced compact masses of green PLBs. Initially, small projections resulted from the explants within 4 weeks of culture which proliferated eventually into multiple shoots and plantlets within 8 weeks of the culture (Fig. 3).

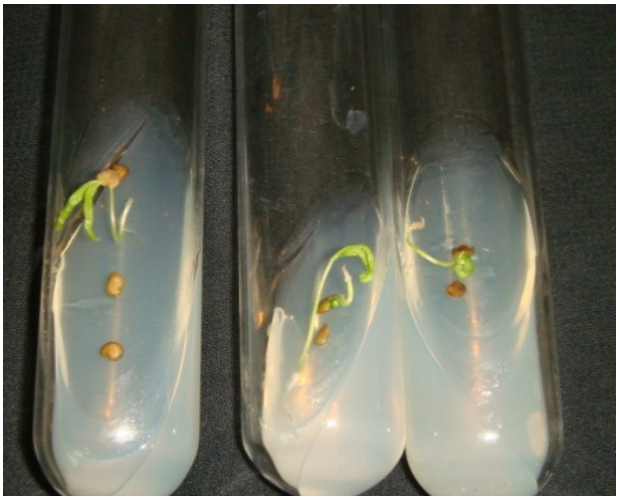

Fig. 2. Callus formation of *Dendrobium fimbriatum*

Fig. 3. Mother plant of *Dendrobium fimbriatum*

Table 1. Effect of germination on *Dendrobium fimbriatum*

Days of pollination	Seed condition	Percentage of germination
160	Bulk-like	08
200	Bulk-like	09
210	Bulk-like	08
270	Powdery	08
280	Powdery	90.92±1.80
230	Powdery	88.60±1.30

The highest explant response was 94.1% at 1.5 mg/l of TDZ. The synergistic effect of TDZ, in the present study, has been observed for efficient PLB induction from *D. nobile* explants so as to detect the immediate and long term effects of TDZ on the clonally propagated plants of this orchid species. In most of the orchids, cytokines either singly or in combination with auxins have been shown to induce PLBs (Bhattacharyya *et al.,* 2013; Dohling *et al.,* 2012; Miyazawa *et al.,* 1997; Nasiruddin *et al.,* 2003; Zhao *et al.,* 2008). The present study reconfirms the efficiency of TDZ as a substitute for auxin– cytokinin combination. The results presented in this work shows that rhizome buds of *Dendrobium fimbriatum* was growing in the field of cultured *in vitro* in a MS basal medium supplemented with BAP - enriched media to induce shoot and root formation (Fig.1). In previous reports different basal media supplemented with natural organic compounds have been used to enhance *in vitro* shoot and root development of several orchid species *Cymbidium kanran* (Paek *et al.,*1990; Chung *et al.,* 1985), *Cymbidium faberi* (Hasegawa *et al.,* 1985), *Cymbidium naveomagenatam*, *C.goeringii* (Paek and Kazoi,1998) and *Cymbidium forrestii* (Paek and Yeung,1991), *Dendrobium candium*,*D. loddigesi*, *D. waggi* and *D. moniliforme* (Zeng *et al.,*1998; Shiau *et al.,*2005.)

Table 2. Effect of hormones and protocorm growth status of *Dendrobium fimbriatum*

Medium components (mgl)	No.of protocorm +SD	Growth status
½ MS	7.30±0.18	Yellowish, green, less difference
N1	5.40±0.18	Yellow, white, somewhat difference
IBS+0.10AA	6.80±0.10	Light, Yellow, somewhat difference
0.3+0.5NAA	6.70±0.19	More difference

Table 3. Effect of GA3 on the protocorm browsing, differentiation and multiplication of *Dendrobium fimbriatum*

GA3(mgl)	% of browning±SD	% of differentiation± SD	No. of buds per protocorm ± SD
0	40.50±0.72	50.50±1.20	4.62±0.02
0.1	40.32±0.42	55.64±0.66	5.64±0.12
1	40.12±0.38	50.66±0.11	6.62±0.12
2	50.58±0.58	40.20±0.62	7.11±0.18

Conclusion

The conclusion of the present study, we have developed a simple and efficient protocol for rapid micro propagation to get a large number of plantlets from rhizome bud cultures of *Dendrobium fimbriatum*. Could be used for large-scale propagation and *ex situ* conservation of this orchid species.

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