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REGENERATION OF *EULOPHIA DABIA* THROUGH RHIZOME EXPLANTS AND FLOWERING: A STUDY *IN VITRO*

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Abstract

Eulophia dabia D. Don (Hochr.) is a rhizomatous ground growing orchid; its seeds were collected from Mullanpur near Chandigarh and germinated *in vitro* using three different nutrient media (PDA, M, and MS). The seedlings thus obtained after 32 wks of culturing were used as the source for the rhizomatous explants (*ca.* 5-7mm long). The efficacy of these explants was assessed on M (Mitra *et al.,* 1976) medium and its combinations with various growth regulators for regeneration *in vitro*. The basal medium supported shoot bud initiation in 55% cultures in 5.75 ± 0.50 wks and the shoots with 2-3 leaves and roots were formed. BAP at 0.5 mgl^{-1} and 1mgl^{-1} favored regeneration via shoot bud formation; the former combination proved useful for inducing rooting in these while the latter combination proved inhibitory to rooting. NAA at 0.5mgl^{-1} also induced regeneration via shoot bud formation within 6.25 ± 0.50 wks whereas its increased concentration (1mgl^{-1}), however, proved inhibitory. BAP (1mgl^{-1}) in combination with NAA (0.5mgl^{-1}) proved the best and plantlets with 2-3 leaves and 3-4 roots were obtained in 4 wks. TDZ in the medium invariably induced multiple shoot buds formation; its concentration at 0.1 and 1mgl^{-1} also induced regeneration via PLBs formation. *In vitro* flowering was induced in combination containing NAA (0.5mgl^{-1}) and TDZ (0.5, 1mgl^{-1}).

Introduction

EULOPHIA DABIA (D. Don) Hochr. (= *E. campestris* Lindl.) is an Indian orchid species met within an altitude of 300-360 m. It dwells on sandy soils near and along the water embankments and its distribution extends from the plains of North India, southward to Deccan and Eastwards to Sikkim and Bengal. *E. dabia* tubers yield *Salep* which is useful as a tonic and aphrodisiac. The tubers are extensively collected for their rejuvenating and curative properties and are used in Ayurvedic formulations as appetizer, tonic, aphrodisiac and blood purifier and to cure stomachache (cf. Pathak *et al.*, 2010). Chauhan (1990) also indicated its use in curing purulent cough and paralytic strokes. Consequently, its natural populations are succumbing to commercial collection pressures. The situation is further compounded by the destruction of its natural habitats due to rapid urbanization. The present paper reports the *in vitro* regeneration potential of rhizome explants and the aim has been to develop a reproducible micropropagation system for the species.

Materials and Methods

Seed Germination and Rhizome Explant Preparation

Mature seeds from dehisced capsules (*pods*) of *Eulophia dabia* (D. Don) Hochr. were collected on sterilized filter paper and surface sterilized with 30%(v/v) Sodium hypochlorite (0.7%) solution with Teepol as the wetting agent for 30 min and then thoroughly and repeatedly rinsed with sterilized distilled water. Sterilized seeds were sown on PDA (Potato Dextrose Agar), M (Mitra *et al.*, 1976) and MS (Murashige and Skoog, 1962) media with 20 gl^{-1} sucrose and 9gl^{-1} agar in test tubes, each containing 25ml of medium. AC (Activated charcoal) at 0.2% was also used in some of the experiments. Rhizome segments (5-7mm) procured from 32 wks-old axenic seedlings, were inoculated on agar-gelled basal M medium and its various combinations with BAP (0.5, 1mgl^{-1}), NAA (0. 5, 1mgl^{-1}) and TDZ (0.1, 0.5, 1mgl^{-1}) at different concentrations.

Culture Media and Culture Conditions

The pH of nutrient media was adjusted to 5.6 prior to autoclaving at 121 °C at 1kg cm^{-2} for 20 min. The cultures were maintained under a 12-hr photoperiod of 30 μmol m^{-2} s^{-1} light intensity and a temperature of 25 ± 2°C, and observed regularly. The problem of phenolic exudates was overcome by frequent subculturing on fresh nutrient media.

Statistical Analysis

All the experimental manipulations were carried out under aseptic conditions and for each experiment at least 4 replicates were used and experiments were repeated thrice. The data was analyzed statistically using one-way analysis of variance (ANOVA), and the

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data means ± SE of at least three different experiments were represented and compared using Duncan's multiple range test with the level of significance set at 5%.

Results

In the basal medium, the explants (55.00 ± 5.78%) regenerated via shoot buds at the nodal region within 5.75 ± 0.50 wks (Fig.1). These differentiated 2-3 leaves in 10 wks and 1st root within 11wks (Fig.2) respectively. The roots became tuberous in 14 wks (Fig.3) and healthy plantlets were obtained in 16 wks. The regeneration response varied with the chemical stimulus in the medium (Table 1; Figs. 1-14). BAP at 0.5 mgl^{-1} and 1mgl^{-1} favored regeneration via shoot bud formation; the former combination proved useful for inducing rooting in these while the latter combination proved inhibitory to rooting. NAA at 0.5mgl^{-1} also induced regeneration via shoot bud formation within a 6.25 ± 0.50 wks whereas its increased concentration (1mgl^{-1}), however, proved inhibitory.

BAP (0.5mgl^{-1}) in combination with NAA (1mgl^{-1}) promoted either cell proliferations at cut ends or shoot bud formation. The callus was brief, creamish-brown and non-organogenetic (Fig.4); callusing was probably due to position effect of the explants on the donor tissue. In the combination containing BAP (1mgl^{-1}) and NAA (0.5mgl^{-1}), shoot buds (Fig.5) followed accelerated development into plantlets (Fig.6). Plantlets complete with 2-3 leaves and 3-4 roots were obtained in 4 wks and these flowered after 7 months (Fig.7). TDZ in the medium invariably induced multiple shoot buds formation (Figs. 8 and 9); its concentration at 0.1 and 1mgl^{-1} induced regeneration via PLBs formation (Fig.10) whereas at 0.5 mgl^{-1} it induced formation of non-organogenetic callus in some cultures (Fig.13). The PLBs soon differentiated into leafy shoots but root development invariably eluded in combination with TDZ at 0.5 and 1 mgl^{-1}. Incidentally, these shoots on their transfer to medium containing activated charcoal (0.1%), developed the roots and subsequently healthy plantlets (Fig.11). *In vitro* flowering was induced in combination containing TDZ (0.5, 1mgl^{-1}) within 1 year (Figs.12,14).

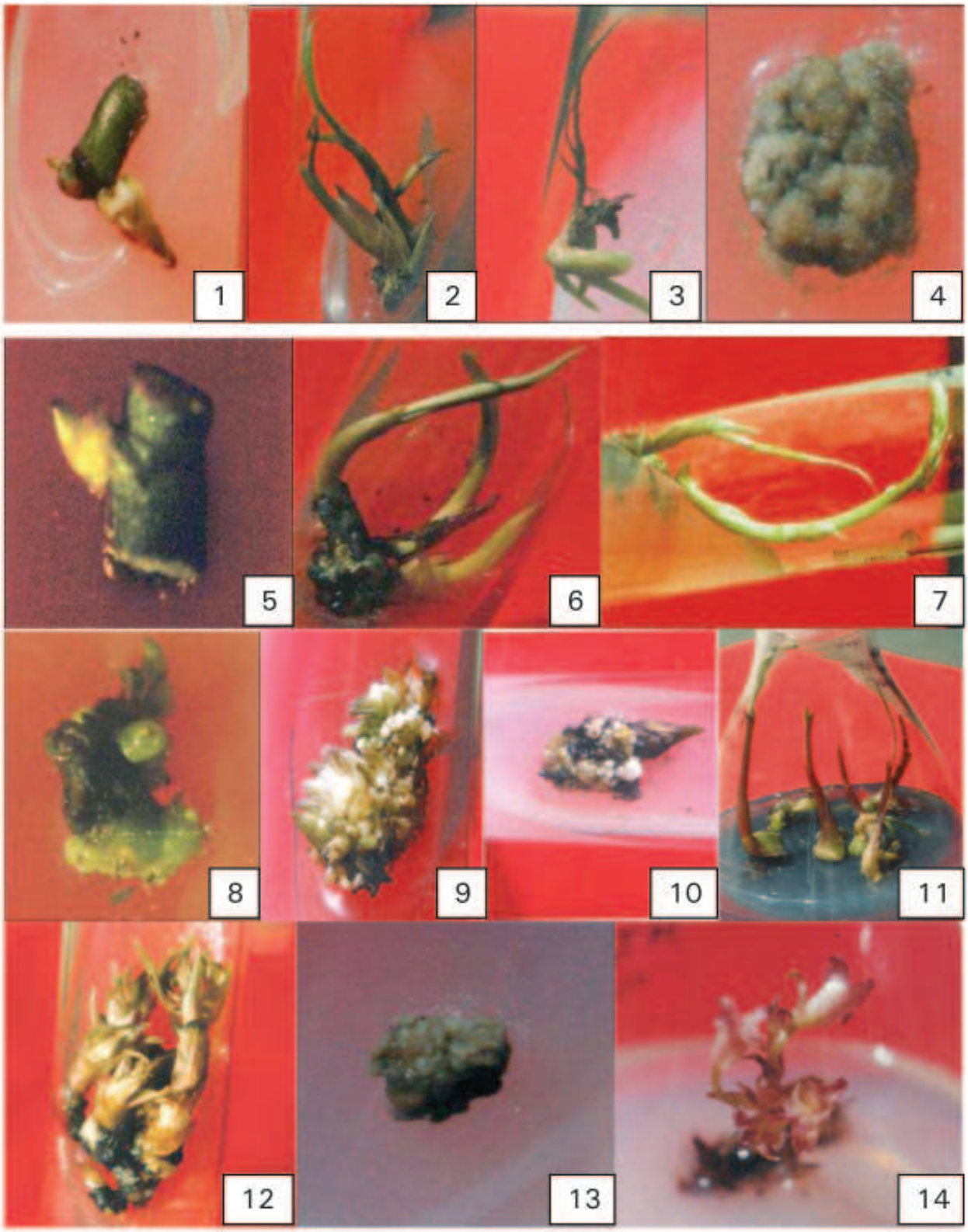
Discussion

Pseudobulbs and other storage organs like rhizomes and tubers are frequently used to propagate orchids *in vivo*, but the technique, often referred to as backbulb culture technique, is a time consuming preposition; it generates only a limited number of propagules and that too only during a favourable season. However, utility of such perennating structures as donor organs for micropropagating orchids is being increasingly realized. Presently, the rhizomes segments were successfully utilized for regenerating *Eulophia dabia* in accord with their similar utility in a number of orchid species (Bapat and Narayanaswamy, 1977; Bhadra and Hossain, 2003; Gayathery and Taha, 2003; Lee *et al.*, 2011; Lu *et al.*, 2001; Martin, 2003; Niimi *et al.*, 1993; Paek and Kozai, 1998; Paek and Yeung, 1991; Sheelavantmath *et al.*, 2000; Shimasaki and Uemoto, 1990; Takahashi and Kondo, 1998; Vij *et al.*, 1989; Yuki and Okubo, 2006). The regeneration response and developmental pathway was, however, markedly influenced by the chemical stimulus. In an earlier study on *E. hormusjii*

Table 1. *In vitro* regeneration through rhizome explants and flowering of *Eulophia dabia* on M (Mitra *et al*, 1976) medium and its combinations with various growth regulators.

Additives	Response (%)	Regeneration response				*In vitro* flowering	Time taken for onset of regeneration (wks)
		PLBs	Shoot Buds	Root	Callus		
-	55.00 ± 5.78^{b}	-	+	+	-	-	5.75 ± 0.50^{c}
$BAP_{(0.5)}$	45.00 ± 5.77^{a}	-	+	+	-	-	5.75 ± 0.50^{c}
$BAP_{(1.0)}$	68.75 ± 12.50^{c}	-	+	-	-	-	4.25 ± 0.50^{b}
$NAA_{(0.5)}$	47.50 ± 5.00^{b}	-	+	+	-	-	6.25 ± 0.50^{c}
$NAA_{(1.0)}$	-	-	-	-	-	-	-
$BAP_{(1.0)} + NAA_{(0.5)}$	96.25 ± 4.79^{d}	-	+	+	-	+	2.50 ± 0.58^{a}
$BAP_{(0.5)} + NAA_{(1.0)}$	93.75 ± 4.79^{d}	-	+	+	+	-	4.25 ± 0.50^{b}
$TDZ_{(0.1)}$	97.50 ± 2.89^{d}	+	+	+	-	-	2.25 ± 0.50^{a}
$TDZ_{(0.5)}$	73.75 ± 2.50^{c}	-	+	-	+	+	3.00 ± 0.00^{a}
$TDZ_{(1.0)}$	40.00 ± 8.12^{a}	+	+	-	-	+	4.50 ± 0.58^{b}

Figures in parentheses indicate the concentration of growth regulators in mgl^{-1}; Entries in column nos. 2and 5 are Mean's: same alphabetical letter in the superscript denotes that the corresponding means are in the same group using Duncan's multiple range test at 5%.



Figs. 1-14. *In vitro* regeneration of *Eulophia dabia* rhizome explant culture; 1, Explant with shoot bud (M); 2, Complete plantlet (M); 3, Tuber formation in 14 weeks (M); 4, Callusing of explant ($M + BAP_{0.5} + NAA_{1.0}$); 5, Shoot bud development ($M + BAP_{1.0} + NAA_{0.5}$); 6, Multiple shoots ($M + BAP_{1.0} + NAA_{0.5}$); 7, *In vitro* flowering ($M + BAP_{1.0} + NAA_{0.5}$); 8, Multiple shoot bud formation at the cut ends of the explant ($M + TDZ_{0.1}$); 9, Multiple shoot buds and PLBs formation ($M + TDZ_{0.1}$); 10, PLBs multiplication ($M + TDZ_{1.0}$); 11, Healthy plantlets ($M + TDZ_{1.0} + AC$); 12, Development of floral buds ($M + TDZ_{1.0}$); 13. Non-organogenetic callus formation ($M + TDZ_{0.5}$); 14, *In vitro* flowering ($M + TDZ_{0.5}$).

(Vij *et al.,* 1989), presence of organic growth supplement (Peptone/Yeast Extract) in the nutrient medium was obligatory for shoot bud development and callusing was invariably eluded. Presently, however, in *E dabia*, the rhizome explants regenerated via PLBs/ shoot buds and non-organogenetic callus was also generated. The formation of non-organogenetic callus similar to our studies was, however, earlier reported in excised rhizomatous segments of *Spathoglottis plicata* (Bapat and Narayanaswamy, 1977). Vij *et al.* (1989) reported that NAA (1mgl^{-1}) in combination with YE and KN (1mgl^{-1}) proved beneficial for development of shoot bud and subsequent development of plantlets. Presently, however, NAA (0.5mgl^{-1}) with BAP (1mgl^{-1}) proved the best combination for regeneration and subsequent plantlet development. Acording to Paek and Yeung (1991), shoot formation in *Cymbidium* species using rhizome segments appears to be regulated by the auxin/cytokinin ratio; higher auxin/ cytokinin ratio in the culture medium generally enhances the rapid growth of the rhizome while a lower auxin/cytokinin ratio promotes shoot formation. The present results in *Eulophia dabia* also conform to this tendency as *Eulophia* is closely related to *Cymbidium* belonging to tribe Cymbidieae. TDZ exhibits strong cytokinin activity, promoting development of multiple shoots and PLBs and eluding rooting at high concentrations (Ernst, 1994; Chang and Chang, 2000).Presently, *In vitro* flowering was induced in combination containing NAA (0.5mgl^{-1}) /or TDZ (0.5, 1mgl^{-1}). Chang and Chang (2003) also reported that it promotes flowering at higher concentrations. Hence, our present results of TDZ in the nutrient medium confirm the earlier findings on TDZ activity. In plant tissue culture, AC is widely used to stimulate rooting of micropropagated shoots since it can adsorb both inhibitory substances and cytokinins in the medium (Luo *et al.*, 2008). The inhibitory effect of TDZ at 0.5 and 1mgl^{-1}on rooting in the presently studied species was counteracted by shifting the plantlets to AC containing medium.

Present studies indicated that M medium containing BAP (1mgl^{-1}) in combination with NAA (0.5mgl^{-1}) proved the best and plantlets with 2-3 leaves and 3-4 roots obtained in 4 wks, flowered after 7 months. All these results suggest that *Eulophia dabia* rhizome explants could be successfully used for its propagation and *ex situ* conservation.

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