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MICROPROPAGATION

In vitro propagation and field establishment of *Eulophia cullenii* (Wight) Bl., a critically endangered orchid of Western Ghats, India through culture of seeds and axenic seedling-derived rhizomes

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Abstract An efficient protocol has been devised for the propagation and field establishment of *Eulophia cullenii* (Wight) Bl., a terrestrial orchid having ornamental potentialities, and is critically endangered in Western Ghats, India. Seeds extracted from 60–90-d-old capsules germinated in ½ MS, ¼ MS, Knudson C, or Mitra liquid medium developed into 1.4–2.5-mm-diameter protocorms in 60 d. Supplementation of organic additives like coconut water, peptone, yeast extract, and casein acid hydrolysate (CH) significantly enhanced protocorm growth. Upon subculture onto agar-gelled Mitra medium fortified with 0.05% CH, 56% of protocorms regenerated into shoots through the formation of linear mini-rhizomes. The regenerated shoots grew vigorously in ½ MS, producing new rhizomes. Mature rhizomes from axenic seedlings produced maximum (13±1.4) shoots/whole rhizome in ½ MS fortified with 44.4 μM 6-benzylaminopurine (BAP), in 120–150 d. Horizontal and longitudinal halves of the rhizome also gave multiple shoots (6–8.5) in the presence of 44.4 μM BAP. Shoots or shoot clumps sub-cultured onto ½ MS basal medium produced roots followed by rhizomes in 60–150 d. Seedlings with mature rhizomes showed 70% establishment in the nursery and added a new rhizome at the end of one growth cycle. An average of 70.6% of the rhizomes originating from seedlings during the second growth cycle sprouted to produce new shoots, when planted in the native localities. Asymbiotic germination and cloning through rhizomes thus can provide a large number of vigorous plants of *E. cullenii* for ornamental exploitation as well as eco-restoration, if rhizome as storage organ is ensured in the propagule.

Keywords Micropropagation · Terrestrial orchid · *Eulophia cullenii* · Asymbiotic germination · Reintroduction

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Introduction

Plant propagation through *in vitro* methods is recognized as a viable alternative method for the multiplication of rare and endangered taxa for conservation and utilization (Wochok 1981; Fay 1994). Reintroduction of plants into their native environment is becoming an increasingly utilized strategy in plant conservation and protected area management, as proven successful in a variety of species (Maunder 1992; Stewart 2008). Seed propagation represents the most efficient method of propagating terrestrial orchids for which both symbiotic and asymbiotic methods are useful (Johnson *et al.* 2007). Asymbiotic seed germination is generally practiced for commercial utilization purposes, as it is the most straightforward method to produce large numbers of propagules. The use of explants from axenic seedlings facilitates further multiplication of popular species. Seedlings cultured symbiotically can serve as both plant material and a source of mycobiont inoculums for reintroduction strategies (Johnson *et al.* 2007; Aggarwal and Zettler 2010).

Eulophia is a pantropical genus with 217 species (Kumar *et al.* 2011), represented by 24 species in India (Misra 2007).The tallest and most robust of these species is *Eulophia flava* (Lindl) J. D. Hook., which grows up to 1.4 m tall. Each inflorescence bears around 25 large, fragrant, bright lemon-yellow flowers making it a species of significant ornamental value (Kumar and Rawat 2009). It is

naturally distributed in China, India, Nepal, Laos, Myanmar, Thailand, and Vietnam (Shrivastava 2004; Kumar *et al.* 2011). In India, *E. flava* is distributed in the lower parts of the Himalayas, Shivalik Hills, Central India, Eastern Ghats, and Western Ghats (Rao *et al.* 2010; Kumar *et al.* 2011) in tropical dry, deciduous forests at altitudes ranging from 120 to 900 m. It is listed as very rare, and in some cases, it has only been re-collected after 50 yr (Kumar and Rawat 2009). The plants collected from Western Ghats region of Kerala and Tamil Nadu are reported to be *Eulophia cullenii* (Wight) Bl. due to their globose pollinia and differently shaped lip and viscidium (Sathish Kumar and Manilal 2004). However, recognition as a separate species was disputed by Govaerts (2003) due to the high degree of similarity to *E. flava*. Nevertheless, *E. cullenii* is an exquisite, very rare, terrestrial orchid (Ravikumar and Ved 2000; CAMP 2001). Rhizomes from the species are used in traditional medicine to treat spider bites (CAMP 2001) as for *E. flava* (Shrivastava 2004). Due to habitat loss, grazing, and overexploitation, natural populations within Kerala and Tamil Nadu are represented by less than 250 individuals, restricted to particular localities, and are highly vulnerable to continuous decline (CAMP 2001). Natural spread of the species is very low due to poor fruit set and seed germination in the disturbed natural habitats. Therefore, an effective conservation strategy is essential not only to enhance their population in the wild but also to ensure sufficient numbers are available for commercial ventures.

E. cullenii perennate *via* condensed underground rhizomes. A rhizome is added every year and therefore a chain of rhizomes (up to seven) was observed during our field studies. Propagation of the species either for reintroduction or utilization is possible through seeds or splitting of rhizomes. However, harvesting of rhizomes for propagation may further deplete natural populations. A viable alternative to propagate the species is through *in vitro* asymbiotic germination of seeds. The available literature provides basic information on *in vitro* propagation and nursery establishment of *Eulophia* spp., such as *Eulophia nuda* (Deepak *et al.* 2012), *Eulophia alta* (Johnson *et al.* 2007), *Eulophia graminea* (Chang *et al.* 2010), and *E. flava* (Sujunya 1996). In all cases, either seed culture was undertaken to raise seedlings, or mericlones have been produced from rhizome explants of field grown plants. However, information on detailed morphogenesis, rhizome formation, and field establishment of seedlings is lacking in *E. cullenii* or its close relative *E. flava*. In the present investigation, seed culture has been carried out to raise seedlings and cultures of seedling-derived rhizomes undertaken to clone vigorous off-types. Pilot-scale reintroduction has also been attempted to assess their performance in native localities as a prerequisite for eco-restoration.

Materials and Methods

Seed culture E. cullenii. mother plants maintained at the Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI), Kerala, India, flower regularly in during March and April. Seed capsules obtained through hand pollinations attained full maturity in 90–120 d. The harvested capsules were washed thoroughly in running tap water using Teepol detergent and surface sterilized by immersing in sodium hypochlorite (3%, *v*/*v*) solution for 15 min followed by washing in sterile water. They were then flamed for 5–10 s after dipping in 70% (*v*/*v*) ethanol. The sterilized capsules were cut open and seeds were transferred to sterile distilled water to prepare a uniform suspension. An equal volume of seed suspension was pipetted into different media (liquid) formulations. The media used were Mitra (Mitra *et al.* 1976), Murashige and Skoog (MS; Murashige and Skoog 1962) with half-or quarter-strength macrosalts, and Knudson-C (Knudson 1946) with Mitra vitamins. In addition, all media were also supplemented with organic additives like casein acid hydrolysate (CH; 0.05%, *w*/*v*), peptone (0.05%, *w*/*v*), yeast extract (YE; 0.05%, *w*/*v*), and coconut water (10%, *v*/*v*). Cultures were maintained in a culture room at 24±2°C with 14:10-h dark/light intervals and agitating (swirling) manually once in a day. Quantitative data, in terms of diameter of protocorms and percentage germination, were made after 60 d. Seed viability were those possessing fully formed embryos, determined using an aliquot of seed suspension.

Protocorm subculture. The protocorms that developed from seeds after 60 d were transferred onto various agar-gelled media: MS, ½ MS, ¼ MS, and Mitra+CH (0.05%, *w*/*v*) for seedling differentiation. Quantitative data on direct shoot regeneration, protocorm proliferation, rhizome formation, rooting, and protocorm survival were taken after 90 d. Rhizomes of different sizes and shapes (mini-rhizomes) which differentiated from protocorms but failed to develop shoots during 6–9 mo culture period were sub-cultured onto fresh media (MS, ½ MS, ¼ MS, Knudson C, and Mitra and supplemented with 0.05% CH) to stimulate shoot differentiation. After shoots and roots had initiated, seedlings were transferred to ½ MS medium for vigorous growth and rhizome development. Observations were made after 120 d, and the number of shoots and rhizomes developed from each mini-rhizome was recorded.

Culture of axenic seedling-derived rhizomes. Seedlings obtained were heterogeneous with white, pale green, and deep green leaves. Thus, healthy large rhizomes from 1 yr old and deep green seedlings were cultured in ½ MS medium supplemented with 6-benzyl aminopurine (BAP) alone or in combination with α-naphthalene acetic acid (NAA) to

clone vigorous off-type plants. Five explant types were used: (1) whole rhizome with three to five nodes, (2) basal half (attached to shoot), (3) upper half (rhizome tip), (4) longitudinal halves, and (5) single node cuttings. After 90 d, explants that had initiated shoots were transferred to fresh medium of the same composition. The number of shoots formed per explant was scored after 60 and 120 d. Clumps of two to three shoots, separated from original explants after 150 d, were sub-cultured onto ½ MS medium devoid of plant growth regulators (PGR) to induce rooting and rhizome formation. Shoots, especially those raised in medium with higher concentrations of BAP, were sub-cultured after 60 d onto fresh medium to induce roots. Data on the number of shoots, roots, and rhizomes produced were collected after 60–120 d.

Hardening and field establishment. Seedlings with roots and rhizomes were planted, in a potting mixture consisting of river sand and vermicompost (5:1). Rhizome-derived plantlets were first hardened by planting in coarse river sand for 30–45 d and then transferred to potting mixture. The rhizomes developed in established plants were re-potted in the potting mixture mentioned above, but it also contained solarized leaf powder and local top-soil. In all cases, the plantlets were maintained in a glasshouse with daily watering. Soil application of NPK (0.5 g/l Greencare 30:10:10; Aristo Chemicals and Aromatics Pvt Ltd, Aluva, India) was provided at 15-d intervals during initial four months of active growth. After 2 yr of growth (two growth cycles) in the mist house, the orchids were planted in three identified natural localities: one in the forest segment of Peppara Wild life Sanctuary, Kerala, India and two in the JNTBGRI campus, as part of the reintroduction studies.

Experimental design and data analysis. All the experiments were completely randomized with two to ten replications. Analysis of variance and mean separation was carried out using Duncan's multiple range test (DMRT) utilizing SPSS software.

Results

Seed culture. Plants reared at the JNTBGRI did not set fruits although it is a natural habitat of the species. However, flowers that were hand-pollinated 1–3 d after opening gave 100% fruit set. The fruits were green and 7×4 cm in size when harvested after 90–120 d and contained 90% seeds with fully formed embryos. The seeds germinated in 2 wk as evidenced by swelling of seeds followed by bursting of seed coat (Fig. 1*a*). The embryos developed into white protocorm in another 2 wk (Fig. 1*b*). An average of 45% of the seeds germinated and developed into protocorms of sizes ranging from 0.1 to 3 mm in 60 d, irrespective of the media used. However, half-strength MS, Mitra, and Knudson-C media supported vigorous growth of protocorms in presence of various organic additives (Fig. 2).

The protocorms showed elongation and chlorophyll development in 30 d upon transfer to agar-gelled media (Fig. 1*c*). They exhibited differential survival and either proliferative or regenerative responses depending on the media used (Fig. 3). Incidentally, MS, ½ MS, or ¼ MS medium resulted in a prolific response through the formation of secondary protocorms or branched mini-rhizomes (Fig. 1*d–e*). Enhanced regeneration of protocorms, i.e., 6–12-mm-long linear mini-rhizomes, occurred in Mitra medium fortified with 0.05% CH. The mini-rhizomes later produced single shoots from their apices (Fig. 1*f*), followed by rooting and rhizome development (Fig. 1*g*). Thus, fully developed seedlings were obtained after a period of 6–8 mo (Fig. 4*a*) if the protocorm directly regenerated into shoots.

The branched mini-rhizomes differentiated from protocorms (Fig. 1*d–e*) produced mainly single shoots, but two to three were observed on rare occasions (Fig. 4*b*) upon sub-culture to different media (Table 1). Among them, ¼ MS+CH emerged as the best with regard to the highest growth index (Table 1) and showed reasonable shoot growth. However, the shoots developed were tubular as their leaves remained unexpanded, even at maturity (Fig. 4*a*).On the contrary, ½ MS supported regeneration of rhizome and shoots having expanded leaves in addition to their vigorous growth (Fig. 4*b*). The latter medium supported single shoot regeneration concomitant with rhizome development in 57% of the mini-rhizomes.

Culture of axenic seedling-derived rhizomes. Rhizomes excised from green and vigorous seedlings (Fig. 4*a–b*) cultured on ½ MS medium exhibited differential shoot multiplication depending on the type of explants and PGRs used (Fig. 5). Shoot initials developed directly from the nodes of explants in 20–45 d, proceeded by medium browning. Multiple shoots developed from nodes (Fig. 6*a–b*) reached 1–5 cm length and a maximum of 3.25±0.14 shoots/explants were obtained in 45–60 d, even in the most favorable medium (supplemented with 44.4 μM BAP). Upon transfer to fresh medium of same composition, the explants produced new shoots. Thus, a whole rhizome explants with three to five nodes gave a maximum of 13± 1.4 shoots in 150 d. BAP (44.4 μM) supported maximum multiplication irrespective of explant type (Fig. 5). However, addition of NAA did not improve the multiplication rate. Multiplication obtained from horizontal halves (upper and basal) together was nearly equal to that of whole tuber explants, when 44.4 μM BAP was used as the PGR. Single node cuttings gave single shoots from 40% of the explants at 13.3–66.6 μM BAP.

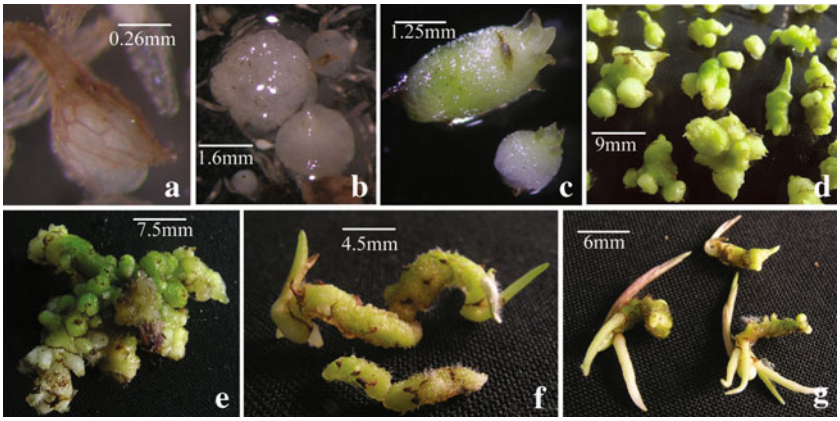

Figure 1. Morphogenesis of seedlings from seeds of *E. cullenii* cultured *in vitro*. (*a*) Seed germination after 10 d. (*b*) Protocorms developed after 60 d. (*c*) Protocorms cultured in agar-gelled Mitra +0.05% CH showing chlorophyll development at 30 d. (*d*, *e*) Proliferation of protocorms into mini-rhizomes of different sizes and shape in ½ MS (*d*) and ½ MS + CH (*e*) at 90 d. (*f*, *g*) Shoots and roots developing from linear mini-rhizomes in Mitra + CH at 90 d.

Multiple shoots produced in presence of 13.3–66.6 µM BAP were devoid of roots. Nevertheless, a few roots were initiated when 1.33–8.88 µM BAP or combinations of BAP and NAA were applied (Fig. 6*c*). Subculture of shoot clumps (two to three shoots) onto ½ MS medium devoid of PGR, however, produced a few additional shoots and new roots (Fig. 6*d*) in 45–60 d. Rhizomes were also initiated in 75% of shoots if cultures were maintained in the same medium for another 60–120 d, or individual shoots were separated and sub-cultured onto fresh medium.

Nursery and field establishment. Establishment of seedlings or mericlones in the nursery was exclusively determined by the presence of rhizomes rather than roots. The seedlings or rhizome-derived plants having sufficient roots, but rhizomes at the initiating stage (Figs. 6*d* and 7*a*) failed to establish in the nursery, whereas seedlings or rhizome-derived plants (Fig. 4) having roots and mature rhizomes showed 70% establishment and survived to the next growth cycle. However, mature rhizomes (Fig. 7*b*), formed either from seedlings or rhizome-derived *in vitro* plants, underwent a dormancy period of 2–4 mo and 87.5% sprouted to produce healthy plants. Each of the established plants produced two to three leaves (Fig. 7*c*) and a new rhizome. After 7–8 mo of growth, aerial shoots of seedlings withered and the underground rhizomes (Fig. 7*d*) entered into a hibernation period for 3–5 mo (November to March). When watering was reinstated during February, the rhizomes produced new sprouts in 1–2 mo. Rhizomes with new sprouts planted in the Peppara Wild Life Sanctuary and the JNTBGRI campus,

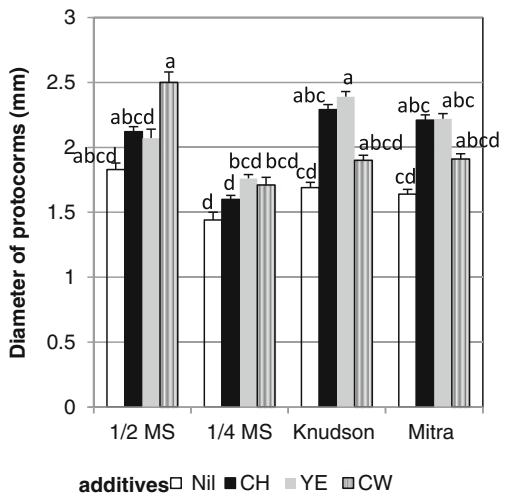

Figure 2. Effect of culture media and organic additives (*CH* casein acid hydrolysate 0.05%, *YE* yeast extract 0.05%, *CW* coconut water 10% on growth of protocorms). Values represent means±SE; n=2–3, each with six to 12 protocorms scored from random fields after 60 d of culture. *Different letters* denote significant difference across all media and additives at P=0.05 based on Duncan's multiple range test.

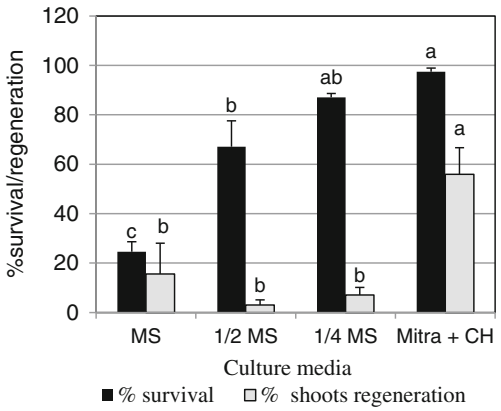

Figure 3. Effect of culture media on survival and shoot regeneration from protocorms. Values represent means±SE; n=3, from a total of 112–452 protocorms for each treatment, collected after 90 d culture. *Different letters* in a *data series* denote significant difference based on DMRT; P=0.05 (CH=0.05%).

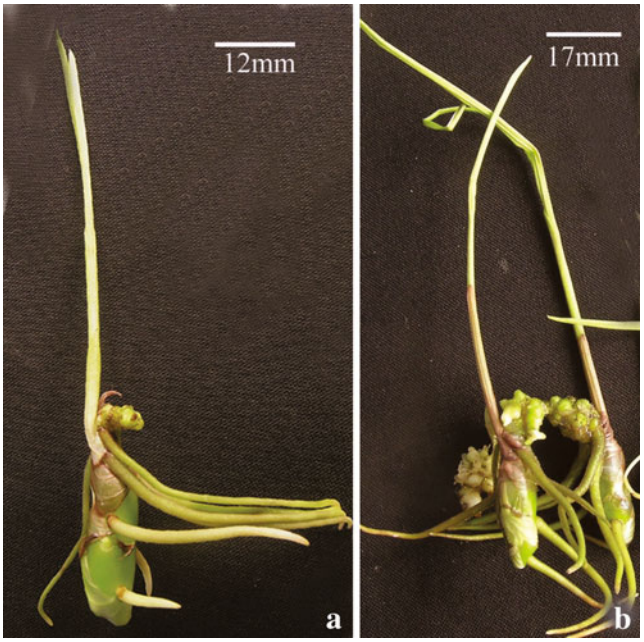

Figure 4. Shoots developed from mini-rhizomes (differentiated from protocorms) after 180 d culture. (*a*) Tubular shoot with rhizome and unexpanded leaves developed from linear mini-rhizome in ¼ MS + CH 0.05%. (*b*) Shoots with rhizomes and expanded leaves developed from branched mini-rhizome in ½ MS.

after receiving summer showers in the month of May, have showed 55.0% and 86.2% establishment, respectively (Fig. 7*e*). The aerial shoots withered, but new sprouts emerged from 63.6% and 76.5% of the dormant rhizomes in the next growth cycle (May–October), respectively, in the two localities.

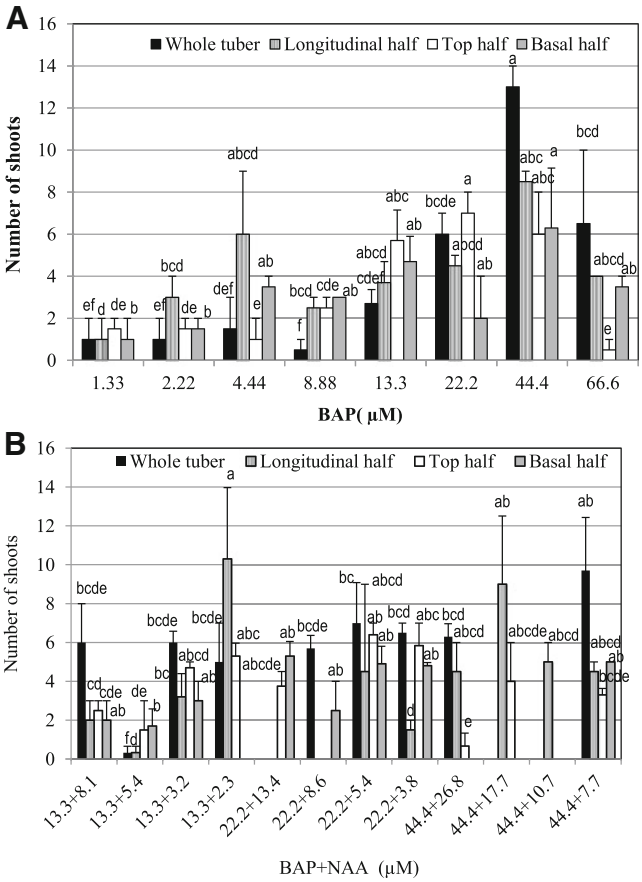

Figure 5. Effect of BAP (*a*) and BAP + NAA (*b*) on shoot regeneration from seedling-derived rhizomes of *E. cullenii*. Data represent means±SE; n=3–6 of data collected after 150 d of inoculation, with one intervening sub-culture (after 90 d) into fresh medium of same composition. Data labels in a *series* followed by the same *letter* do not differ significantly based on DMRT, P=0.05.

Table 1 Effect of different media on differentiation of mini-rhizomes leading to seedling development

Media	No. of shoots/explant (mean±SD; n=11–37[z])	No. of rhizome/explant[y] (mean±SD; n=11–37[z])	Length of shoots (cm) (mean±SD)	Growth index[x] (mean±SD)
MS basal	0.51±0.23 d	0.36±0.09 cde	7.70±0.72 a	1.41±0.72 c
MS + CH	0.48±0.21 d	0.25±0.19 e	7.42±0.52 a	0.89±1.49 c
Half MS	1.03±0.28 abc	0.57±0.13 bc	6.29±0.87 b	3.69±1.66 b
Half MS + CH	0.73±0.11 bcd	0.32±0.07 de	5.65±1.07 bc	1.31±0.52 c
Quarter MS	1.07±0.21 abc	0.53±0.17 bcd	2.97±0.27 e	1.69±0.68 c
Quarter MS + CH	1.26±0.21 a	0.83±0.16 a	5.23±0.77 c	5.47±1.89 a
Knudson C	1.24±0.76 a	0.67±0.32 ab	3.00±1.00 e	2.49±0.7 bc
Knudson C + CH	1.20±0.50 ab	0.36±0.04 de	4.03±0.82 d	1.74±0.42 c
Mitra	0.68±0.31 cd	0.42±0.11 cde	3.18±0.17 de	0.90±0.60 c
Mitra + CH	1.07±0.19 abc	0.44±0.07 cde	4.17±0.56 d	1.96±0.72 c

Observations were made after 120 d of sub-culture. Different *letters* in a *column* denote significant difference at 5% level based on DMRT

[z] The total number is inclusive of protocorms that did not produce shoots

[y] Each shoot produced only a single rhizome

[x] Product of shoots, rhizomes and shoot length

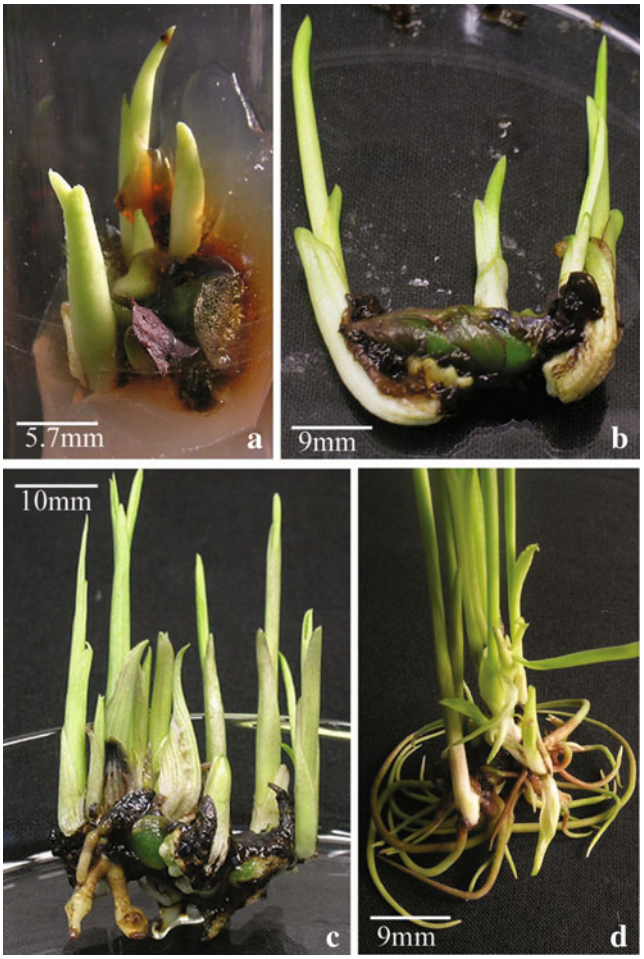

Figure 6. Shoot multiplication from axenic seedling-derived rhizomes cultured in ½ MS medium fortified with BAP and BAP + NAA. (*a*, *b*) Horizontal halves (*a*) and whole tubers (*b*) in 22.2 μM BAP medium, after 60 d. (*c*) Whole tubers in 22.2 μM BAP + 5.4 μM NAA medium, after 120 d. (*d*) Roots developed in clumps of three shoots separated from the plant shown in Fig. *5c* and cultured in ½ MS medium, after 90 d.

Discussion

Terrestrial orchids can be propagated through several *in vitro* procedures (symbiotic/asymbiotic germination, rhizome/tuber segment multiplication) to produce large numbers of healthy plants. Unfortunately, however, these plants are often fragile and survive poorly after transplanting to soil in comparison to plants derived by the sprouting of robust or field-hardy propagules such as rhizomes or root tubers (Medina *et al.* 2009). Propagation techniques can exploit this attribute to ensure the successful survival of plants transplanted to soil. Thus, the propagation of storage organs would be the ideal method for restoration or reintroduction programs as proven effective in *Habenaria bractescens* (Medina *et al.* 2009). The present investigation examined such attributes in the rhizomatous terrestrial orchid *E. cullenii*.

Asymbiotic germination of seeds is the most common method practiced for the propagation of both epiphytic and terrestrial orchids even though symbiotic germination succeeded in some species has been recommended for utilization in restoration program (Stewart and Kane 2006; Aggarwal and Zettler 2010). Asymbiotic seed germination is reported to be successful in some *Eulophia* species, including *E. alta*, *E. graminea*, *Eulophia cucullata*, *Eulophia streptopetala*, and *Eulophia petersii* (McAlister and Van Staden 1998; Johnson *et al.* 2007; Chang *et al.* 2010) using MS, ½ MS, ¼ MS + coconut water, or P723 (Orchid Seed Sowing Medium, *Phyto*Technology Laboratories, Shawnee Mission, KS) as culture media. The present study revealed that ½ MS, ¼ MS, Knudson-C, and Mitra media were equally good at supporting seed germination. However, protocorm growth promoted by complex additives like peptone has been reported for orchid species like *Spathoglottis plicata* (Curtis 1947), *Epidendrum ibaguense* (Hossain 2008), and *Calopogon tuberosus* (Kauth *et al.* 2006). Organic additives (peptone, CH, and YE) that accelerated protocorm growth of *E. cullenii* were a mixture of free amino acids in addition to vitamins and minerals. Studies conducted in terrestrial orchid seeds and protocorms revealed more efficient utilization of amino acids by young protocorms (Curtis 1947; Spoerl 1948; Malmgren 1996). Greater preference for nitrogen from amino acids rather than from ammonium or nitrate salts has also been favored for some epiphytic orchids (Nadarajan *et al.* 2011). The present study also substantiates such a possibility in *E. cullenii*.

Unlike the majority of the orchids where seedlings differentiate directly from protocorms, rhizomatous terrestrial orchids like *Geodorum densiflorum* (Sheelavantmath *et al.* 2000; Bhadra and Hossain 2003) and *E. graminea* (Chang *et al.* 2010) produce mini-rhizomes prior to shoot development. The same pattern of morphogenesis and formation of mini-rhizomes also occurs in *E. cullenii*. These organs are thought to be storage organs as an adaption to support survival and development of seedlings in natural conditions, as suggested in *E. graminea* by Chang *et al.* (2010).

Shoot regeneration occurring from mini-rhizomes was greatly influenced by macro-nutrients in the culture medium used. Quarter-strength MS medium tried in the present study possessed macronutrients nearly equal to the terrestrial Orchid Seed Sowing Medium P723 reported to promote seedling growth rather than germination and early growth in *E. alta* (Johnson *et al.* 2007) and *C. tuberosus* (Kauth *et al.* 2006). The poor growth of protocorms observed at earlier stages (Fig. 1) and enhanced shoot differentiation from mini-rhizomes at later stages in propagation of *E. cullenii*, using ¼ MS medium, substantiate the latter reports. Therefore, separate media are proposed for seed culture (Mitra + CH, peptone or YE), protocorm differentiation (Mitra + CH), and seedling growth (¼ MS + CH or ½ MS) for *E.*

Figure 7. Field establishment of *in vitro* plants. (*a*) Rhizome-derived plants with developing rhizome. (*b*) Dormant rhizome separated from rhizome-derived shoots, at senescence. (*c*) Seedlings established in the nursery. (*d*) Rhizomes developed in the established plants, at the end of second growth cycle (old and newly added rhizome is visible). (*e*) Seedlings at the third growth cycle, in a native field at the JNTBGRI.

cullenii. Differential growth at different developmental stages also suggested use of separate medium for seed germination and seedling growth in *Paphiopedilum* (Tay *et al.* 1988), *C. tuberosus* (Kauth *et al.* 2006), *E. alta* (Johnson *et al.* 2007), and some other epiphytic, terrestrial, and lithophytic species (Nadarajan *et al.* 2011).

Multiplication of orchid species is also possible using different types of explant. Protocorms were effective in a few *Eulophia* species (McAlister and Van Staden 1998); rhizome segments from adult plants in *E. nuda* and *Geodorum purpureum* (Deepak *et al.* 2012; Mohapatra and Rout 2005) and segments of mini-rhizomes differentiated from protocorms were successful for *G. densiflorum* (Sheelavantmath *et al.* 2000). Nevertheless, rhizomes developed in seedlings that had nodes and internodes were equivalent to whole rhizomes from adult plants, which was also a good explant source as proven in the present study. Efficient multiplication achieved from rhizomes in presence of 22.2 to 44.4 μM BAP was in fact useful to clone off-type seedlings in *E. cullenii*. Besides the effect of BAP, the number of nodes in the explants was also an important factor as the total number of shoots obtained from whole rhizomes was equivalent to the sum of shoots from the horizontal halves (upper + basal). Multiplication equal to that of whole rhizome was also evident from symmetrical longitudinal halves that had an equivalent number of nodes (three to five). Earlier studies conducted in *E. nuda* showed that 44.4 μM BAP was the best treatment to obtain maximum multiplication from 1 cm rhizome segments (Deepak *et al.* 2012). However, only single shoots obtained from 40% of the explants suggested that a single node segment (0.5–1 cm) was not ideal for multiplication of *E. cullenii*. Multinode stem segments produced optimal results in another tuberous terrestrial orchid, *H. bractescens* (Medina *et al.* 2009).

Rhizome development in *in vitro* seedlings occurs as part of the normal developmental process rather than being influenced by environmental factors (Kauth *et al.* 2006). However, management of the composition of macro-salts in the culture medium was essential to maximize rhizome formation uniformly in all *E. cullenii* seedlings. Nevertheless, removal of BAP and NAA achieved by sub-culturing in PGR-free medium was sufficient to induce rhizome development in shoots that were previously cloned from rhizome explants in presence of the PGRs. Previous reports also indicated that tuberization or rhizomatization in shoots can be induced by reducing the concentration of BAP in the rooting medium (Medina *et al.* 2009; Deepak *et al.* 2012).

Many terrestrial and wetland orchids form storage organs such as root tubers and rhizomes, which are important for their propagation and survival (Medina *et al.* 2009). Rhizome development only occurs once per year in the growth cycle of *E. cullenii*, with new shoots emerging and actively growing between April and June and assimilatory stage resulting into rhizome development continues up to

September–October in Kerala, India. Therefore, seedlings having fully developed rhizomes and two to three fully expanded leaves planted in the nursery continued to survive leading to rhizome enlargement and/or maturity until entering dormancy. It is as expected in such perennials. New sprouts for next growth cycle emerge from the rhizome only after undergoing a dormancy period of 3 to 4 mo. There were also incidences where the original seedling shoot withered, while developing new shoots from the persisting corns in the same growth cycle, which was also reported in *C. tuberosus* (Medina *et al.* 2009). However, it is unclear why rhizome-derived plants or seedlings with initiated rhizomes, even with profuse roots did not establish in the nursery. It is possible that even though they were photosynthetically active, the roots may not have acquired capacity to absorb essential nutrients, especially nitrogen, from the soil. Alternatively, it may be explained by mycorrhizal symbiosis, when the requirements for amino compounds are most probably satisfied by transport of these substances with a symbiotic fungus (Dijk and Eck 1995). It is particularly true in species like *Eulophia zollingeri* as it is reported to depend on a specific symbiotic fungus for its growth and survival (Yuki and Tomohisha 2008). However, the rhizomes present in the seedlings/rhizome-derived plants seemed to provide some nutrients to sustain them until establishing a symbiotic relationship with a compatible fungus (possibly from top soil taken from native localities, included in the potting mixture), as evident from the establishment (70%) of seedlings with mature rhizomes. The seedlings with rhizomes planted in the native localities producing new rhizomes and resuming growth in the next year also support such possibilities. Thus, it is evident from the results that the presence of a storage organ is sufficient for reintroduction of *E. cullenii* into native localities anticipating that it will establish a mycorrhizal association with compatible fungus in the native field conditions.

In conclusion, the present study demonstrated an efficient micropropagation system for *E. cullenii* using seeds and axenic seedling-derived rhizome as explants. Asymbiotic seed germination and cloning through rhizomes provided large numbers of vigorous seedlings/rhizome-derived clones for ornamental exploitation as well as eco-restoration on ensuring rhizomes as the storage organ in the propagule. However, association with a symbiotic fungus, at least during transplantation from *in vitro* culture, emerged as a possibility yet to be explored for faster growth and enhanced establishment of the species in the field.

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