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Research Article

Ex Vitro Seedling Development from *In Vitro* Rhizome-Like Bodies in *Eulophia promensis* Lindl.: A New Technique for Orchid Propagation

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This communication describes *in vitro* seed germination, embryo differentiation, and *ex vitro* seedling production from *in vitro* rhizome-like bodies of a terrestrial orchid, *Eulophia promensis* Lindl. Effects of two nutrient media, namely, Murashige and Skoog (MS) and Phytotechnology Orchid Seed Sowing medium (P_{723}) supplemented with 6-benzylaminopurine (BAP; 0.5–1.0 mgL^{-1}) and/or α-naphthalene acetic acid (NAA; 0.5–1.0 mgL^{-1}) and activated charcoal (2.0 gL^{-1}), were studied on seed germination and subsequent development of embryos. Maximum seed germination (100%) was recorded in P_{723} medium fortified with 1.0 mgL^{-1} BAP + 2.0 gL^{-1} activated charcoal. The different developmental stages of protocorm morphogenesis were traced out. In subsequent subcultures, the protocorms proliferated profusely and developed rhizome-like bodies (RLBs) with numerous hair-like structures. These RLBs were transferred to pots containing potting mixture composed of humus + coir dust + saw dust (1 : 1 : 1) where ~80% of RLBs survived and produced 1–3 seedlings per RLB. This is the first time report for *in vitro* germination of seeds and *ex vitro* seedling production from *in vitro* raised RLBs in *Eulophia promensis*. This is a time saving and cost effective protocol that could be extended to other economically important, rare, and endangered orchids for propagation and conservation.

1. Introduction

Orchids, the sovereign among ornamentals, attributed outstanding royalty in the world floricultural market and are one of the most important global cut flowers and potted floricultural crops. Orchidaceae is the largest family of the flowering plants consisting of about 30,000 species under ~800 genera. Due to weak reproductive barriers, over 1,06,000 attractive hybrids have already been developed and registered [1]. The world orchid trade exceeded billion dollars and countries of Asia-Pacific regions mainly Thailand, Singapore, and Malaysia dominated the world's floriculture market. In 2012, the global orchid trade among 40 exporting and 60 importing countries around the world was estimated at 504 million US dollars [2]. This figure undoubtedly indicates the necessity of production and improvement of orchids. Nowadays, commercial orchid seedlings are predominantly produced through tissue culture but these seedlings encountered a number of problems including high mortality and slow growth after transplantation from *in vitro* to greenhouse and difficulties in stimulating blooming in adult plants [3].

Eulophia promensis Lindl. (*Eulophia geniculata* King & Pantl.), a rare terrestrial orchid of Bangladesh, has high demand in cut flower trade for its exquisite and perpetual flowers. Besides, it has high therapeutic importance in the traditional system of medicine. Crushed tubers and the extracted juice of *Eulophia promensis* are used as vermifuge [4]. Its tuber yields "salep" which is useful as a tonic and an aphrodisiac [5]. Unfortunately, many orchid species are disappearing at an alarming rate due to continuous destruction of natural habitats, unauthorized trade, and ruthless collections [6]. Furthermore, their high commercial demand has undoubtedly led to an increased emphasis on mass propagation and conservation of important orchids [1, 7]. Therefore, a precise, simple, economical, rapidly multiplying, and highly reproducible protocol is required for conservation

of orchids [8]. The major obstacles for mass propagation of economically important orchids, including *Eulophia promensis*, are (a) nonavailability of efficient and reliable protocols for seed germination, (b) lack of a clear understanding of early seedling growth and development, (c) high mortality of seedlings during transplantation from *in vitro* to *in vivo* conditions, (d) obligate requirement of mycorrhizal association for natural seed germination, (e) selection of suitable explants for micropropagation, and (f) scaling-up and automation of the techniques [6, 8]. Ever since the discovery of asymbiotic germination of orchid seeds by Knudson [9], the technique has been used routinely for large scale propagation of economically important orchids [1, 10–14] but very few studies critically investigated the peculiarities of seed germination and protocorm development [6, 8, 15–17] and no reports are available on regeneration of plants from *in vitro* seed derived rhizome-like bodies in *ex vitro* conditions. Keeping in mind the limitations and importance of mass propagation of orchids, the present studies were undertaken with a view to (i) develop an efficient protocol for germination of seeds, (ii) investigate the mode of morphogenesis of embryo during formation of protocorm and seedling development, and (iii) establish an efficient and easy technique for production of seedlings from *in vitro* seed derived rhizome-like bodies to *ex vitro* conditions in *Eulophia promensis*, an economically important and rare orchid of Bangladesh.

2. Materials and Methods

2.1. Planting Material and Initiation of Aseptic Cultures. Two-three-month-old green capsules of *Eulophia promensis* were collected from the naturally grown populations from the "Kata Pahar" area of the Chittagong University Campus of Bangladesh. These were washed thoroughly under running tap water and Teepol and then treated with 0.04% Bavistin and streptomycin sulphate solution for 20 min and washed three times with sterile distilled water. Subsequently these were subjected to surface sterilization treatment with 0.1% (w/v) $HgCl_2$ solution for 10 minutes with occasional agitation and washed thoroughly with sterile distilled water. Final disinfection was made by dipping the capsules in 70% ethanol for 30 seconds followed by washing with sterile distilled water. The sterilized capsules were flamed and split longitudinally with a sterile surgical blade and the seeds were inoculated on the surface of agar gelled nutrient media.

2.2. Culture Medium and Incubation Conditions. Two nutrient media, namely, Murashige and Skoog (MS) [18] and Phytotechnology Orchid Seed Sowing medium (P_{723}; PhytoTechnology Laboratories, Inc., USA) supplemented with 6-benzylaminopurine (BAP; 0.5–1.0 mgL^{-1}) and α-naphthalene acetic acid (NAA; 0.5–1.0 mgL^{-1}), either individually or in combinations, and two additives such as peptone and activated charcoal (2.0 gL^{-1}), were used for seed germination. The pH of the medium was adjusted to 5.8 prior to gelling with 0.8% agar and media were autoclaved at 121°C for 20 minutes at 15 psi. Culture vessels with inoculated seeds were maintained in a culture room where a cycle of 14/10 h continuous light (60 μmol m^2 s^{-1}) and dark conditions was maintained at 25 ± 2°C. After germination of seeds, protocorms were subcultured at 25-day interval.

2.3. Percent Seed Germination. After two weeks of inoculation, some of the seeds were taken out and dispersed in one drop of water on a glass slide and observed under light microscope. Percentage of germination was calculated employing the following formula:

$$\frac{\text{number of seeds showing swelling of embryos} \times 100}{\text{total number of seeds}}. \quad (1)$$

Once the spherules were formed, observations were recorded at an interval of one week to trace different stages of embryo morphogenesis under stereozoom microscope.

2.4. Transfer of In Vitro Rhizome-Like Bodies to Ex Vitro Conditions. Rhizome-like bodies (RLBs) that developed from the germinated seeds were transplanted to pots containing a potting mixture composed of humus + coir dust + saw dust (1 : 1 : 1) in such a way that the RLBs were fully covered with potting media and kept in shady moist place. Plantlets that developed from RLBs were finally transferred to community pots and kept in orchidarium.

2.5. Experimental Design and Statistical Analysis. A complete randomized block design (CRBD) was applied. The experiments were repeated thrice with maintaining five replicates per treatment for seed germination. For estimating seedlings production 10 RLBs were transplanted in each pot and five replicates were maintained. Means were separated by ANOVA and significant differences were assessed by Duncan's multiple range test at $P = 0.05$ [19]. The statistical analyses were performed using the programme package Statistica ver. 7 (StatSoft, Tulsa, USA).

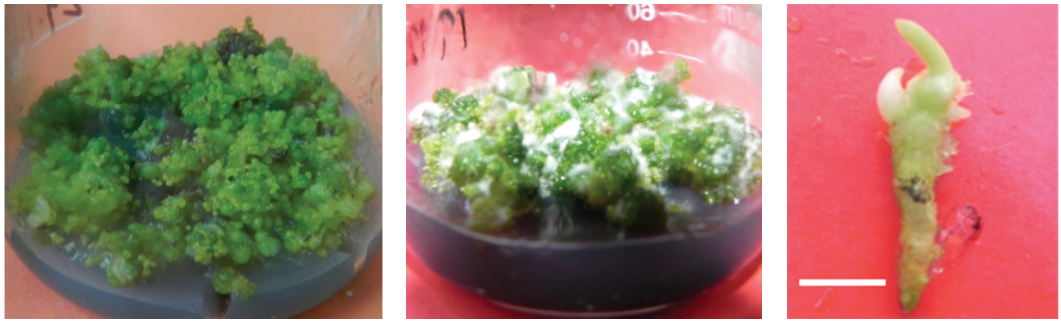
3. Results and Discussion

3.1. Seed Germination. The seeds germinated on all the nutrient media used (Table 1) but germination percentages varied depending on the media composition. Maximum seed germination (100%) was recorded in P_{723} medium when fortified with 1.0 mgL^{-1} BAP + 2.0 gL^{-1} activated charcoal (Figure 1(a)). Species-specific media for seed germination have been reported in orchids [15]. The specificity was reported even within species of the same genus, for example, Mitra et al. [20] medium for *Cymbidium macrorhizon* [21], Nitsch and Nitsch medium for *C. iridioides* [22], and Knudson C for *C. elegans* [11]. The cause of maximum germination of seeds in P_{723} medium compared to MS medium could be due to the fact that P_{723} medium was enriched with peptone. Various complex substitutes including peptone with basal medium have been reported to improve seed germination and subsequent protocorm development in a number of orchids [6, 8, 13, 22, 23]. Peptone contains amino acids, amides, minor elements, and vitamins responsible for

Table 1: Seed germination in *Eulophia promensis*.

Medium	PGRs (mgL^{-1})		Additives ($2.0\ gL^{-1}$)	Time (days)		Seed germination (%) (mean ± SE)
	BAP	NAA		Spherule	Protocorms	
MS	—	—	—	25–30	40–45	73.00 ± 1.20^{cd}
	—	0.5	—	25–30	45–50	67.00 ± 1.30^{cd}
	0.5	—	—	20–25	35–40	$78\ .00 \pm 1.25^{bc}$
	1.0	1.0	—	25–30	40–45	69.00 ± 1.09^{cd}
	—	1.0	AC	20–25	40–45	77.00 ± 1.25^{bc}
	1.0	—	AC	20–25	35–40	85.00 ± 2.30^{ab}
P723	—	—	—	20–25	40–45	95.50 ± 2.30^{b}
	—	0.5	—	25–30	45–50	68.00 ± 2.30^{cd}
	0.5	—	—	20–25	35–40	95.00 ± 1.75^{b}
	1.0	1.0	—	25–30	35–40	70.00 ± 1.69^{cd}
	—	1.0	AC	20–25	40–45	96.50 ± 1.30^{b}
	1.0	—	AC	20–25	30–35	100.50 ± 2.00^{a}

Mean values within a column followed by the same letter were not significantly different at $P = 0.05$.



(a) (b) (c)

Figure 1: Germination of seeds of *Eulophia promensis*: (a) development of protocorms, (b) protocorms proliferated to form rhizome-like bodies (RLBs), and (c) an individual RLB.

enhancement of seed germination and growth of protocorms [24]. Beneficial effects of activated charcoal on seed germination and protocorm development have also been accounted for in orchid tissue culture for its high adsorption affinity to excessive and inhibitory compounds. Activated charcoal is known to adsorb morphogenetically active or toxic substances such as 5-hydroxymethylfurfural, which is produced by the dehydration of sucrose during autoclaving, and inhibitory phenolics and carboxylic compounds produced by the tissues and excessive hormones and vitamins in the media [25–29]. BAP is known to enhance germination frequency in *Cypripedium* spp. [30], *Eulophia dabia*, and *Pachystoma senile* and stimulated protocorm multiplication as well as shoot formation in *Cymbidium pendulum* [31] and *Cattleya aurantiaca* [32].

3.2. Morphogenesis of Embryo and Seedling Development. The protocorms proliferated in germination media but these did not develop seedlings after prolonged culture (>three months). However, in subsequent subcultures these developed long rhizome-like bodies (RLBs) with numerous hairs on the surface and a beak-like structure at their tip region indicating the growing shoot (Figures 1(b) and 1(c)). Germination of orchid seeds followed a peculiar metamorphogenetic pathway; that is, undifferentiated embryos swelled up by absorbing water and nutrients from the media and developed a compact mass of parenchymatous cells called spherule which gradually develop protocorm, an intermediate structure between seed and seedling [33]. At the initial stage of protocorm development, an appendicle, looking like a closed ridge, appeared at the upper part of the protocorms which leads to shoot formation while basal part escorts root development [6, 8, 13, 14]. In *E. promensis* a different type of morphogenesis was observed. In this case, the protocorms became elongated and formed rhizome-like bodies (RLBs) with numerous hairs and some growth appendages on the body surface and a growing tip indicating the development of leafy shoots and the root initials, respectively (Figures 2(a)–2(f)). Formation of RLBs in *in vitro* protocorms has also been reported in a terrestrial orchid, *Geodorum densiflorum* [34]. Each RLB produced 1–3 seedlings within two months of transfer to outside pots containing potting mixture composed

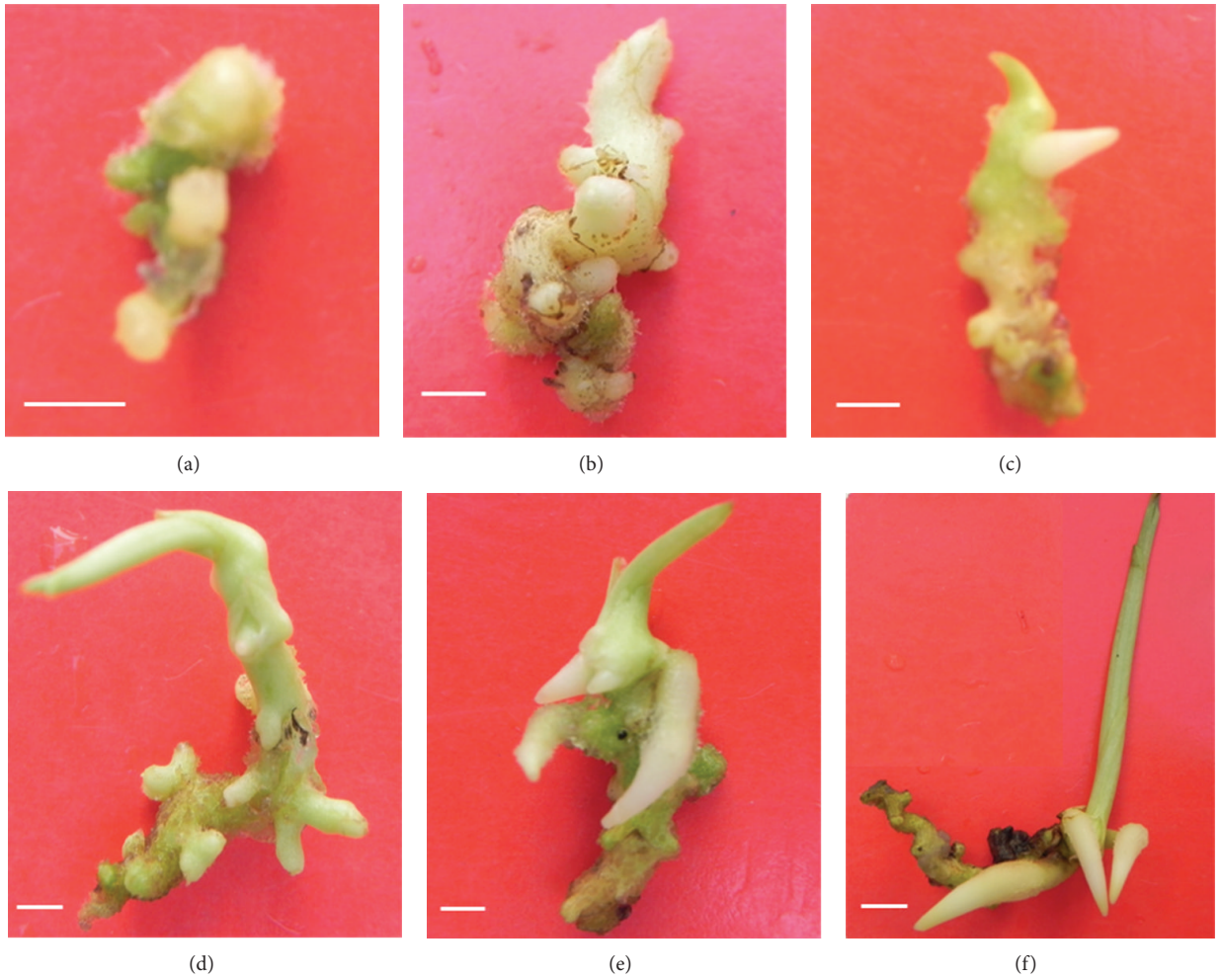


Figure 2: Protocorm morphogenesis and seedling formation in *Eulophia promensis*: (a) proliferation of protocorm, (b) RLB with numerous hairs and appendices, (c) RLB with root and growing shoot, and (d)–(f) development of RLB to seedling.

of humus + coir dust + saw dust (1 : 1 : 1). These were finally transferred to community pots (Figures 3(a)–3(c)). In the potting mixture the seedlings produced 1.0–1.5 cm size oblong-rotund pseudobulbs at the base of the plants and the aboveground part died after six to seven months and the pseudobulbs remained dormant for about six months. In the following year new shoots were regenerated from the underground pseudobulbs (Figures 3(d) and 3(e)). The plants produced in this way flowered after three years from seed germination (Figure 3(f)). In general production of orchid seedlings through *in vitro* seed culture is completed by germination of seeds, development of seedlings, elongation of seedlings, induction of stout root system, and transfer of seedlings to outside environment through successive phase of acclimatization. Plantlets produced *in vitro* often show physiological and anatomical deficiencies, low photosynthesis rate, and incomplete autotrophy and are unable to develop resistance against major and minor microbial pathogens [35]. Moreover, survival of asymbiotically raised orchid seedlings transferred directly to natural habitats is unsuccessful until and unless they developed mycorrhizal associations. When *in vitro* plantlets are transplanted from culture room to greenhouse conditions they may desiccate or wilt rapidly and die as a result of the change in environment, unless substantial precautions are taken to adapt these to new environment. Acclimatization of *in vitro* grown plantlets to *ex vitro* environment by gradual weaning towards ambient relative humidity and light levels facilitates better survival of the young tender plantlets [36, 37]. Therefore, *in vitro* raised plantlets must undergo a period of acclimatization, more specifically, a period of transitional development to correct the anatomical abnormalities and develop physiological performance for survival in *ex vitro* conditions. Thus the novelty of the present study is that the protocol developed for seedling production in *E. promensis* alleviated the above limitations.

4. Conclusions

This is the first report for *in vitro* germination of seeds of *E. promensis* and seedling production from *in vitro* rhizome-like

FIGURE 3: Seedling production in *Eulophia promensis* from *in vitro* RLBs under *ex vitro* conditions: (a) transplantation of RLB to pot, (b) sprouting of RLBs (arrow), (c) young seedlings (1st generation), (d)-(e) sprouting of dormant rhizome (arrow) in the next year, and (f) flowering of three-year-old plants produced from *in vitro* RLBs.

bodies in *ex vitro* conditions. The technique developed for *E. promensis* is a time saving and cost effective protocol that could be extended to other economically important, rare, and endangered orchids for propagation and conservation.

Abbreviations

BAP: 6-Benzylaminopurine
NAA: α-Naphthalene acetic acid
RLBs: Rhizome-like bodies
MS: Murashige and Skoog medium
P_{723}: Phytotechnology Orchid Seed Sowing medium.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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